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Possible Impact of *RET* Polymorphism and Its Haplotypic Association Modulates the Susceptibility to Thyroid Cancer

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ABSTRACT

Rearranged during Transfection (*RET*) gene polymorphisms act to influence thyroid cancer in a polygenic and low-penetrance manner and no study regarding *RET* alterations in thyroid cancer has undergone from this part of the world (North India). We evaluated *RET* G691S (*rs*1799939), L769L (*rs*1800861), and S904S (*rs*1800863) polymorphisms to elucidate their possible role as risk factors in papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). Polymorphic analysis of *RET* gene was performed by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP). In *RET* G691S polymorphism, the overall distribution of variant alleles (GA + AA) in cases was 62.9% as against 44.5% in controls ($P < 0.05$) whereas frequency of *RET* L769L variant alleles (TG + GG) in cases was 70% versus 88% in controls ($P < 0.05$). In *RET* S904S, frequency of variant alleles (CG + GG) in cases was 56% versus 44% in controls ($P < 0.05$). Interestingly, G691S/L769L variant showed increased risk for the non-smokers ($P < 0.05$). *RET* S904S variant showed association with benign thyroid disease as against those with no history. The over-representation of homozygotes in G691S and L769L polymorphic variants was not observed, which suggest a "Dominant mode of inheritance." The S904S polymorphism heterozygote lies almost in the middle of the two homozygotes confirming an "Additive mode of inheritance." In conclusion, *RET* gene G691S/S904S polymorphisms were over-represented and L769L polymorphism was under-represented in PTC and FTC patients. *RET* polymorphic variants could act synergistically in the development or progression of PTC and FTC. J. Cell. Biochem. 116: 1712–1718, 2015.

KEY WORDS: THYROID CANCER; REARRANGED DURING TRANSFECTION; THYROID STIMULATING HORMONE; BENIGN THYROID DISEASE

Thyroid cancer is the most common malignancy of the endocrine system. It accounts for approximately 2% of all newly diagnosed cancer cases and majority of endocrine cancer-related deaths each year [Hundahl et al., 1998; Sarlis, 2000; Sarlis and Benvenga, 2004]. Thyroid cancer incidence has increased significantly during the past decades [Davies and Welch, 2006] and site among top 10 leading cancer types in females in the United States [Jemal et al., 2011]. Thyroid cancer is the 8th most common cancer in Kashmir valley and its frequency has increased from 2.3% in 1995 to 5.4% in 2010, keeping overall frequency of 3.2% in the vale of Kashmir [Pandith and Siddiqi, 2012]. Genetic polymorphisms are reported to be an important cause of the predisposition to several human cancers including thyroid cancer. The Rearranged during Transfection (*RET*) proto-oncogene encodes a membrane tyrosine-kinase receptor and is expressed in cells originating in the neural crest [Grogan et al., 2010]. However, the *RET* tyrosine kinase domain may also be expressed in thyroid follicular cells [Bunone et al., 2000]. It is not only the high penetrant germline *RET* mutations, which have a key

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role in disease development, but also *RET* polymorphisms exist that are believed to be genetic modifiers and might be associated with an increased relative risk for the development of disorders derived from neural crest cells. The most frequently investigated *RET* polymorphisms in association studies and most frequent in terms of minor allelic frequency are the non-synonymous variant G691S (G2071A) in exon 11 and the synonymous variants L769L (T2307G) in exon 13, S836S (C2508T) in exon 14, and S904S (C2712G) in exon 15. This panel of SNPs are being referred to as disease modifiers owing to the observation that they were found more often in patients with sporadic medullary thyroid cancer (MTC), papillary thyroid cancer (PTC), and follicular thyroid cancer (FTC) [Baumgartner-Parzer et al., 2005; Cebrian et al., 2005; Machens et al., 2012]. It has recently been suggested that silent *RET* polymorphisms A45A in exon 2 and L769L in exon 13 may represent low-penetrance risk alleles for PTC [Lesueur et al., 2002]. *RET* polymorphic haplotypes and risk of PTC and FTC was also described by some studies [Ho et al., 2005]. In case of G691S non-synonymous SNP, the two amino acids, glycine in the wild-type RET protein and serine in the polymorphic *RET* variant, confer different electrochemical and conformational structures to the RET protein, and consequently influence the processing, folding, subcellular localization or function of the protein [Robledo et al., 2003]. The mechanism by which the silent polymorphisms may act in the development of thyroid cancer may include transcript stability, RNA splicing, and DNA protein binding and protein folding [Ho et al., 2005]. Because polymorphisms are comparatively common in the population, they could bestow a much higher attributable risk on the general population as compared with rare mutations in high-penetrance disease susceptibility genes such as *RET*.

Owing to the fact that *RET* gene polymorphisms have not been studied in PTC and FTC patients of India especially northern region and since *RET* gene polymorphisms play an important role in modulation of thyroid cancer, we conducted a population-based case–control study to investigate distribution of *RET* gene polymorphism in G691S (*rs*1799939), L769L (*rs*1800861), and S904S (*rs*1800863) to elucidate the possible role of these polymorphisms as risk factor in thyroid cancer development and to examine its correlation with the various demographic and clinicopathological variables.

MATERIAL AND METHODS

PATIENTS AND CONTROLS

Blood samples were collected from 140 post-thyroidectomy patients diagnosed with PTC and FTC attending Department of Nuclear Medicine for undergoing radioiodine ablation, at Sher-I-Kashmir Institute of Medical Sciences (SKIMS), besides blood samples were obtained from 180 healthy controls free from any tumor or benign thyroid disease (BTD) from the Out Patients Departments of SKIMS. The power of study was calculated to be 80% using *nMaster2.0* statistical software. A written pre-informed consent was obtained from all cases and controls. Demographic and clinicopathological characteristics of each patient were recorded in a questionnaire. This study was approved by the Institutional Ethical Committee (Protocol No. 0990-0279), Sher-I-Kashmir Institute of Medical Sciences, Soura, Srinagar (IEC-SKIMS).

EXTRACTION OF GENOMIC DNA

DNA was extracted from the blood of PTC and FTC patients by using DNA Extraction kit (Zymo Research Corporation, USA) while as salting out method was used for the extraction of DNA from control blood samples.

POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF RET GENE

To amplify *RET* gene, exons 11, 13, and 15, we used genomic DNA: 250 ng/μl, 10× PCR buffer: 100 mM Tris–HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.1% gelatin; 1% Triton X-100, deoxyribonucleotide triphosphate (Cinnagen Co., Tehran, Iran): 10 mM dATP; 10 mM dCTP; 10 mM dGTP; 10 mM dTTP, primers (Sigma–Aldrich, USA): 10 pM in sterile deionized water and *Taq* DNA polymerase 5 U/μl (Biotools, Madrid, Spain). The set of primers and thermal conditions used to amplify various exons of *RET* gene and the respective restriction enzymes are given in Table I.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

For RFLP, the PCR products were subjected to restriction digestion with *Ban*I, *Taq*I, and *Rsa*I (Thermo Scientific, USA) for *RET* codons 691, 769, and 904, respectively. Restriction digestion mixture consisted of 18.5 μl of distilled water, 2 μl of 10× buffer G, 10 μl of PCR product, and 5 U of restriction enzyme according to manufacturer's protocol. Incubation temperature and time for *Ban*I, *Taq*I, and *Rsa*I was 37°C for 12 h. For *RET* codon 691, the homozygous wild type (GG) has one *Ban*I site and is characterized by 267 and 187 bp fragments while the (AA) homozygote presented a single fragment of 454 bp and heterozygous form (G/A) displayed 454, 267, and 187 bp. For *RET* codon 769, the homozygous wild type (TT) has one *Taq*I site and is characterized by 270 and 190 bp fragments while the (GG) homozygote presented a single fragment of 460 bp and heterozygous form (T/G) displayed 460, 270, and 190 bp.

TABLE I. Primers and Restriction Enzymes Used for Screening the Various SNPs

RET amplicon	Primer sequence	A_T (°C)	Product (bp)	RE[a]	DP[b] (bp)
Exon 11 G691S (G2071A)	F-5′-CAGAGCATACGCAGCCTGTAC-3′ R-5′-GCCTCGTCTGCCCAGCGTTG-3′	60	454	*Ban*I	267 and 187
Exon 13 L769L (T2307G)	F-5′-CCTGTCCACTGATCCCAAAG-3′ R-5′-CACTCAGCCCGTGGACTC-3′	64	460	*Taq*I	190 and 270
Exon 15 S904S (C2712G)	F-5′-GGTCTCACCAGGCCGCTAC-3′ R-5′-TCGGTATCTTTCCTAGGCTTC-3′	62	332	*Rsa*I	224 and 108

[a]RE, restriction enzyme.
[b]DP, digestion product.

In case of *RET* codon 904, the homozygous wild type (CC) presented a single fragment of 332 bp and the (GG) homozygote has one *Rsa*I site and is characterized by 224 and 108 bp fragments while as heterozygous form (C/G) displayed 332, 224, and 108 bp fragments. The DNA fragments were detected and confirmed by comparing with a 100 bp DNA marker ladder (Thermo Scientific) by electrophoresis on 3% agarose gel containing ethidium bromide (0.5 μg/ml) in a mini gel system (Scie-Plas Ltd., Cambridge UK) and visualized in UV illuminator (Flourchem HD2; Cell Bioscience, Inc., CA). For quality control, each PCR reaction used distilled water instead of DNA as a negative control, and more than 10% of the samples were analyzed twice to confirm the reproducibility of results.

STATISTICAL ANALYSIS

Statistical analysis was performed using independent *t*-test and paired *t*-test for continuous variables; Pearson's χ^2 test, Fisher's exact test or χ^2 test (trend) for discrete variables. An exact test for Hardy–Weinberg equilibrium was performed to examine the statistical significance of the differences in allele frequency and genotype distribution between the cases and controls. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using logistic regression analysis. ORs with 95% CIs were used as estimates of the relative risk or degree of association between certain genotypes or other related risk factors of thyroid cancer. *P*-value was calculated by Pearson's method. Since the expectation maximization algorithm (EM) does not accurately estimate haplotypes frequencies below 1%; haplotype frequency <1% in both groups were not considered. All reported *P* values were based on two-sided tests. Statistical tests were performed using the software SPSS 16.0 (SPSS, Inc., Chicago, IL).

RESULTS

A total of 140 thyroid cancer cases (PTC-118 and FTC-22) and 180 healthy controls were studied for polymorphic analysis of G691S (*rs*1799939), L769L (*rs*1800861), and S904S (*rs*1800863) SNPs in *RET* gene. There were no significant differences among cases and controls in terms of mean age and smoking. The mean age of the patients and controls were 35 ± 13 years and 38 ± 14 years, respectively. BTDs were found in 84 of 140 (60%) patients out of which 52.3% (44 of 84) patients were having thyroid adenomas, 34.5% (29 of 84) and 13.2% (11 of 84) were having thyroiditis and multinodular goitre, respectively. The demographic and risk factors of study subjects are summarized in Table II. The three polymorphisms have significant difference in the genotype distributions between cases and controls ($P < 0.05$) as shown in Table III.

In *RET* G691S (*rs*1799939) polymorphism, the overall distribution of variant alleles (GA + AA) in cases was 62.9% as against 44.5% in controls with a significant association ($P < 0.05$) and an O.R (95% CI) of 2.1 (1.0–3.9). Association between *RET* G691S phenotypes and clinicopathological characteristics is shown in Table IV. For different groups of pathological classification of thyroid cancer, our study found higher distribution of variant genotypes (GA + AA) in female thyroid cancer cases as compared to healthy controls (63.2% vs. 42.0%) ($P < 0.05$). Cases with no smoking status had higher frequency of variant genotype than non-smoker controls (71.4% vs. 42.9%) ($P < 0.05$) (Table IV).

TABLE II. Frequency Distribution Analysis of Selected Demographic and Risk Factors in Thyroid Cancer Cases and Controls

Characteristics	Cases (n = 140 (%))	Controls (n = 180 (%))	χ^2-Value	P-value
Age group				
<45	100 (71)	130 (72)	0.025	>0.05
≥45	40 (29)	50 (28)		
Sex				
Female	114 (81)	104 (58)	20.2	<0.05
Male	26 (19)	76 (42)		
Dwelling				
Rural	112 (80)	98 (54.4)	22.8	<0.05
Urban	28 (20)	82 (45.6)		
Smoking				
Never	124 (89)	140 (77.8)	6.3	<0.05
Ever	16 (11)	40 (22.2)		
Benign thyroid disease				
Yes	84 (60)			
No	56 (40)			
TSH levels				
Elevated	100 (71)			
Normal	40 (29)			
Histological types				
Papillary	118 (84)			
Follicular	22 (16)			
Tumor grade				
WDTC	134 (96)			
PDTC	06 (04)			
Stage, <45 years				
Stage I	94 (67)			
Stage II	06 (4.3)			
Stage, ≥45 years				
Stage I and II	36 (25.7)			
Stage III and above	04 (03)			
Vascular/capsular invasion				
Yes	68 (48.5)			
No	72 (51.5)			
Lymph node metastasis				
Yes	52 (37)			
No	88 (63)			

TSH, thyroid-stimulating hormone; WDTC, well-differentiated thyroid cancer; PDTC, poorly differentiated thyroid cancer.

In *RET* L769L (*rs*1800861) polymorphism, the overall distribution of variant alleles (TG + GG) in cases was 70% as against 88% in controls, which is significant ($P < 0.05$) with an O.R (95% CI) of 0.3 (0.17–0.6). Association between *RET* L769L phenotypes and clinicopathological characteristics is shown in Table IV. For further classification, our study found lower distribution of variant alleles (TG + GG) in thyroid cancer cases <45 years of age as compared to healthy controls (68% vs. 88%) ($P < 0.05$). Cases with no smoking status had lower frequency of variant genotype than non-smoker controls (67.7% vs. 87.0%; $P < 0.05$). A lower frequency of variant alleles (62%) was found in PTC and FTC patients having history of BTD when compared to patients having no history of BTD (82.1%) and the difference was significant ($P < 0.05$) (Table IV).

In *RET* S904S (*rs*1800863) polymorphism, the overall distribution of variant alleles (CG + GG) in cases was 56% as against 44% in

TABLE III. Genotype Frequencies of RET Gene Polymorphisms in Cases and Controls

SNP	Cases (n = 140 (%))	Controls (n = 180 (%))	OR (95% CI)	*P*-value
G691S (G2071A)				
Genotype				
GG	52 (37.1)	100 (55.5)	1.0 (ref.)	<0.05
GA	64 (45.7)	64 (35.5)	1.9 (1.1–3.2)	
AA	24 (17.2)	16 (09)	2.9 (1.2–6.6)	
Allele type				
G	168 (60)	264 (73.3)	1.0 (ref.)	<0.05
A	112 (40)	96 (26.7)	1.8 (1.2–2.5)	
L769L (T2307G)				
Genotype				
TT	42 (30)	22 (12)	1.0 (ref.)	<0.05
TG	70 (50)	110 (61)	0.32 (0.17–0.57)	
GG	28 (20)	48 (27)	0.30 (0.15–0.6)	
Allele type				
T	154 (55)	154 (42)	1.0 (ref.)	<0.05
G	126 (45)	206 (58)	0.61 (0.44–0.82)	
S904S (C2712G)				
Genotype				
CC	62 (44)	102 (56)	1.0 (ref.)	<0.05
CG	64 (46)	70 (40)	1.5 (0.93–2.4)	
GG	14 (10)	08 (04)	2.8 (1.1–7.0)	
Allele type				
C	188 (67)	274 (76)	1.0 (ref.)	<0.05
G	92 (33)	86 (24)	1.5 (1.0–2.1)	

TABLE IV. Association Between RET G691S (G2071A), L769L (T2307A), and S904S (C2712G)

G691S (G2071A)	Cases, n (%)	GG, n (%)	GA + AA, n (%)	Controls, n (%)	GG, n (%)	GA + AA, n (%)	OR (95% CI)	*P*-value
Overall genotype	n = 140	52 (37.1)	88 (62.9)	n = 180	100 (55.5)	80 (44.5)	2.1 (1.3–3.2)	0.001
Sex								
Female	114 (81)	42 (36.8)	72 (63.2)	104 (58)	60 (58)	44 (42)	2.3 (1.3–3.9)	0.002
Male	26 (19)	10 (38.5)	16 (61.5)	76 (42)	40 (53)	36 (47)	1.8 (0.72–4.4)	0.2
Smoking status								
Never	124 (89)	42 (28.6)	82 (71.4)	140 (77.8)	80 (57.1)	60 (42.9)	2.6 (1.5–4.5)	0.0001
Ever	16 (11)	10 (62.5)	06 (37.5)	40 (22.2)	20 (50)	20 (50)	0.6 (0.1–2.8)	0.4
L769L (T2307A)	Cases, n (%)	TT, n (%)	TG + GG, n (%)	Controls, n (%)	TT, n (%)	TG + GG, n (%)	OR (95%CI)	*P*-value
Overall genotype	n = 140	42 (30)	98 (70)	n = 180	22 (12)	158 (88)	0.3 (0.17–0.6)	0.0001
Age group								
<45	100 (71)	32 (32)	68 (68)	130 (72)	16 (12)	114 (88)	0.3 (0.14–0.5)	0.0004
≥45	40 (29)	10 (25)	30 (75)	50 (28)	06 (12)	44 (88)	0.4 (0.13–1.2)	0.1
Dwelling								
Rural	112 (80)	36 (32.1)	76 (67.8)	98 (54)	10 (10)	88 (90)	0.23 (0.1–0.5)	0.0002
Urban	28 (20)	06 (21.4)	22 (78.5)	82 (46)	12 (15)	70 (85)	0.6 (0.2–1.8)	0.4
Smoking								
Never	124 (89)	40 (32.2)	84 (67.7)	140 (78)	18 (13)	122 (87)	0.3 (0.16–0.55)	0.0002
Ever	16 (11)	02 (12.5)	14 (87.5)	40 (22)	04 (10)	36 (90)	1.1 (0.16–6.2)	0.8
BTD								
Yes	84 (60)	32 (38)	52 (62)				0.35 (0.15–0.78)	0.01
No	56 (40)	10 (17.8)	46 (82.1)					
S904S (C 2712 G)	Cases, n (%)	CC, n (%)	CG + GG, n (%)	Controls, n (%)	CC, n (%)	CG + GG, n (%)	OR (95%CI)	*P*-value
Overall genotype	n = 140	62 (44)	78 (56)	n = 180	102 (56)	78 (44)	1.6 (1.0–2.4)	0.02
Age group								
<45	100 (71)	40 (64.5)	60 (35.5)	130 (72)	66 (51)	64 (49)	1.5 (0.9–2.9)	0.1
≥45	40 (29)	22 (55)	18 (45)	50 (28)	36 (72)	14 (28)	2.1 (0.84–5.0)	0.09
Sex								
Female	114 (81)	54 (47)	60 (53)	104 (58)	58 (56)	46 (44)	1.4 (0.8–2.4)	0.2
Male	26 (19)	08 (31)	18 (69)	76 (42)	44 (43)	32 (57)	3.1 (1.2–8.0)	0.02
BTD								
Yes	84 (60)	30 (36)	54 (64)				2.4 (1.2–4.8)	0.01
No	56 (40)	32 (57)	24 (43)					

Genotypes and clinicopathologic characteristics.
BTD, benign thyroid disease.

TABLE V. Inheritence Models for RET Gene Polymorphisms

SNP	Genotypes and alleles (patients vs. controls)	Cases (n = 140)	Controls (n = 180)	OR (95% CI)	*P*-value
G691S (G2071A)	Dominant model (AA + GA vs. GG)				
	GG	52	100	1.0 (ref.)	0.001
	AA + GA	88	80	2.11 (1.3–3.3)	
L769L (T2307G)	Dominant model (GG + TG vs. TT)				
	TT	42	22	1.0 (ref.)	0.000
	GG + TG	98	158	0.32 (0.2–0.6)	
S904S (C2712G)	Additive model (GG vs. CC)				
	CC	62	102	1.0 (ref.)	
	GG	14	08	2.8 (1.1–7.2)	0.021

controls with a significant association ($P < 0.05$) having OR (95% C.I) of 1.6 (1.0–2.4). Association between *RET* S904S phenotypes and clinicopathological characteristics is shown in Table IV. For further stratification, our study found higher distribution of variant genotypes (CG + GG) in thyroid cancer cases ≥45 years of age as compared to matched healthy controls (45% vs. 28%) and this difference between two groups was observed to be statistically significant ($P < 0.05$). Higher distribution of variant alleles (CG + GG) in male thyroid cancer patients as compared to healthy controls (69% vs. 57%) was found ($P < 0.05$). A higher frequency of variant alleles (64%) was found in thyroid cancer patients having BTD when compared to patients having no history of BTD (43%) and the difference was significant ($P < 0.05$) (Table IV). We did not find any other clinicopathological characteristic showing any significance with *RET* gene G691S, L769L, and S904S polymorphisms.

Adjusted ORs were assessed using different inheritance models. We found *Dominant inheritance model* appropriate for analysis of *RET* G691S and L769L polymorphism while as *Additive inheritance model* appropriate for analysis of *RET* S904S polymorphism. The results of the single SNP association analysis are shown in Table V. Haplotype analyses were conducted to evaluate the combined effect of the three polymorphisms on thyroid cancer risk. All haplotypes have frequencies >5% among both cases and controls. The most common haplotype was the G2071/2307G/C2712 (GGC) haplotype, with frequencies of 24% in cases and 33% in controls. The overall distribution of different haplotypes between cases and controls showed a marked difference ($P < 0.0001$). Table VI shows the haplotype pattern for the three SNPs. Haplotypes of frequency <1% was excluded from the analysis. Haplotype frequencies were estimated from the genotyping data after stratified by gender, age, and smoking status. Table VI demonstrates the frequencies for the estimated 3-marker haplotypes among patients and controls.

DISCUSSION

Pathogenesis of thyroid cancer involves multiple genetic alterations among which *RET* and *BRAF* are most predominant. While BRAF inhibitors show promise in mouse models by reducing their proliferative index, RET being targeted by receptor tyrosine kinase inhibitors is currently in early phase trials [Nehs et al., 2010; Chakravarty et al., 2011; Huyck and Agulnik, 2011].

RET polymorphisms act to influence thyroid disease in a complex, polygenic, and low-penetrance manner [Borrego et al., 1999, 2000]. Incidence of *RET* rearrangements are observed in 2.5–60% of sporadic PTC tumors [Grieco et al., 1990; Bongarzone et al., 1996; Cinti et al., 2000]. *RET* polymorphisms have a relatively strong association with various disease phenotypes.

So far, only a few studies have evaluated the association between *RET* SNPs and thyroid cancers arising from the follicular cells [Lesueur et al., 2002; Ho et al., 2005; Stephens et al., 2005]. Since the role of these polymorphisms in relation to PTC and FTC risk has not been reported from this region particularly in the backdrop of the fact that scenario of cancers is different from the most parts of the world [Pandith and Siddiqi, 2012] therefore, we investigated the influence of *RET* variants, isolated or in combination, on risk and clinical presentation of thyroid cancer cases and controls.

In case of G691S SNP (G2071A), we found significant difference in the distribution of three genotypes G/G, G/A, and A/A as 37.1%,

TABLE VI. Haplotype Frequencies Estimation and Haplotype Association With Disease (Adjusted by Gender + Age + Smoking Status)

G2071A	T2307G	C2712G	Total frequency	Cases	Controls	Cumulative frequency	OR (95% CI)	*P*-value
G	G	C	0.3061	0.2498	0.3337	0.3061	1.00 (ref.)	–
G	T	C	0.2122	0.2004	0.2321	0.5183	0.94 (0.48–1.84)	0.86
A	G	C	0.1224	0.1084	0.1503	0.6407	1.26 (0.55–2.87)	0.59
A	T	G	0.0999	0.1442	0.0657	0.7406	**0.24 (0.10–0.57)**	**0.0012**
A	T	C	0.0843	0.1129	0.0506	0.8249	**0.38 (0.17–0.83)**	**0.016**
G	T	G	0.0817	0.0925	0.0738	0.9066	**0.23 (0.08–0.64)**	**0.0051**
G	G	G	0.075	0.0573	0.0938	0.9816	1.59 (0.59–4.31)	0.36
A	G	G	0.0184	0.0345	0	1	0.05 (0.00–1.37)	0.076

The OR (95% CI) of thyroid cancer associated with each haplotype was estimated by comparison with the common reference haplotype. ref., reference. Bold signifies the statistical significance.

45.7%, and 17.2% in cases and 55.5%, 35.5%, and 9%, respectively, in control group, which is in accordance with most of the published work in other ethnic groups [Robledo et al., 2003; Lonn et al., 2007]. The frequency of the rare "A" allele (serine) observed in cases was 40% versus 26.7% in controls ($P < 0.05$) and this observation is supported by previous studies conducted in different populations [Stephens et al., 2005; Lonn et al., 2007; Fugazzola et al., 2008]. Hence, our results seem to indicate that the G691S infrequent "A" allele is more frequent in PTC and FTC patients and may be associated with a predisposition to develop thyroid cancer. The G691S polymorphism has been reported to be more frequent in patients with sMTC when compared with normal subjects [Elisei et al., 2004b; Costa et al., 2005] suggesting a possible role of this variant in *RET* activation. In addition, we observed higher frequency of variant alleles (GA + AA) in PTC and FTC cases with no smoking status as compared to controls (71.4% vs. 42.9%), which is in agreement with many studies [Ericsson and Lindgrade, 1991; Fisher et al., 1997]. A mechanism by which cigarette smoking might reduce the risk of thyroid cancer is by lowering the endogenous levels of TSH [Ericsson and Lindgrade, 1991; Fisher et al., 1997]. Although not all studies have reported this effect [Karakaya et al., 1987], it has been suggested that increased levels of TSH are associated with an increased risk of thyroid cancer [Ron, 1996] Thus, an agent, which lowers TSH would then potentially protect against the disease. In case of L769L SNP (T2307G), the frequency of the "G" and "T" allele observed in cases was 45% and 55% compared to 58% and 42% in controls, respectively ($P < 0.05$), which follows the studies in other ethnic groups [Lonn et al., 2007]. The frequency of the "G" allele found in our study almost coincides with study conducted by Lesueur et al. [2002] and Stephens et al. [2005] in PTC and FTC but in contrary with study conducted by Ho et al. [2005] who have reported higher frequency of "G" allele in PTC and FTC cases. Hence, our results seem to indicate that in L769L (T2307G) SNP, frequent "T" allele is more common in PTC and FTC patients thus showing a protective role of variant alleles in thyroid cancer risk. Further, we observed lower frequency of variant alleles (TG + GG) in cases with age group of <45 years as compared to controls (68% vs. 88%) ($P \leq 0.05$), which is in contrast to the study conducted by Fugazzola et al. [2008]. The frequency of variant alleles is more in patients having no history of BTD ($P < 0.005$) thus depicting a protective role in predisposing a person to BTD, which is a major risk factor for thyroid cancer and this finding is in consistency with study conducted by Ho et al. [2005]. In case of S904S SNP (C2712G), we found a significant difference in the distribution of genotypes C/C, C/G, and G/G as 44%, 46%, 10% in cases and 56%, 40%, 04% in controls, respectively, which is in coherence with several studies conducted by Lesueur et al. [2002] and Elisei et al. [2004a] who have reported a higher frequency of S904S polymorphism in patients with medullary carcinoma but in contrast with the study conducted by Ho et al. [2005]. Our study observed higher frequency of variant alleles (CG + GG) of S904S in thyroid cancer patients ≥45 years of age as compared to controls of same age group (45% vs. 28%), which is in discordance with a study conducted by Fugazzola et al. [2008]. No study other than our study has separately correlated various clinicopathological characteristics with S904S polymorphism. Although, linkage between G691S and S904S has been suggested previously [Borrego et al., 1999; Gil et al., 2002].

In G691S (*rs*1799939) and L769L (*rs*1800861) polymorphic variants, we found a difference between frequency of variant alleles (homozygous mutant and heterozygous mutant) and wild allele in thyroid cancer cases and controls. It is interesting that a single copy of an allele appears to mediate this effect (i.e., over-representation of homozygotes is not observed), which might suggest a "*Dominant mode of inheritance*." The S904S (*rs*1800863) polymorphism heterozygote lies almost in the middle of the two homozygotes, with homozygous variant allele (C/C) having the maximum susceptibility of being diseased and homozygous wild allele (G/G) having the least, so, there is "additivity" in this polymorphism, hence, following an "*Additive mode of inheritance*."

The effect of a single polymorphism is unlikely to be substantial in studies of complex diseases. Thus, the approach based on combining multiple polymorphisms or genetic variants that interact in the same pathway may amplify the effect of single variants and enhance the predictive power of polymorphisms analysis for multifactorial disease [Joseph and Nicole, 2004; Beuten et al., 2009; Schottenfeld, 1986]. To confirm whether *RET* polymorphic variants could act synergistically in the development or progression of thyroid cancer, we analyzed if some variants of this gene, or a combination of them, might predispose to PTC and FTC. For this reason, we looked for an association of haplotype(s) of *RET* variants (G691S/L769L/S904S) in PTC and FTC cases and healthy controls from Kashmiri population, matched for sex, age, and smoking status. We found the most over-represented haplotype is A T G followed by G T G and A T C depending upon their Akaike information criteria (*P*-value), which is in coherence with a study conducted by Lesueur et al. [2002], who observed that G T C G haplotype in A45A/L769L/S836S/S904S variants is over-represented in French PTC patients but in disagreement with the study conducted by Ho et al. [2005], who compared *RET* polymorphism haplotype frequencies in A45A/A432A/S691S/L769L/S836S/S904S but did not demonstrate a statistically significant association between control subjects and thyroid cancer (PTC and FTC) cases. However, they found G A G T C C and G G A T C G haplotypes as predisposing alleles for the development of BTD.

CONCLUSION

In conclusion, we found G691S/S904S polymorphisms to be over-represented and L769L polymorphism to be under-represented in PTC and FTC patients. Further, G691S/S904S polymorphisms were more pronounced in females bearing a significant association ($P < 0.05$). Interestingly, G691S variant shows increased risk for the non-smoker cases. Our data suggest that some specific haplotypes (A T G, G T G, and A T C) of *RET* are over-represented and may act as low penetrance alleles in the predisposition to PTC and FTC. These correlations need to be authenticated in a large sample study in the future to determine the course of thyroid cancer.

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REFERENCES

Baumgartner-Parzer SM, Lang R, Wagner L, Heinze G, Niederle B, Kaserer K, Waldhäusl W, Vierhapper H. 2005. Polymorphisms in exon 13 and intron 14 of the RET protooncogene: Genetic modifiers of medullary thyroid carcinoma. J Clin Endocrinol Metab 90:6232–6236.

Beuten J, Gelfond JA, Franke JL, Weldon KS, Crandall AC, Johnson-Pais TL, Thompson IM, Leach RJ. 2009. Single and multigenic analysis of the association between variants in 12 steroid hormone metabolism genes and risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 18:1869–1880.

Bongarzone I, Fugazzola L, Vigneri P, Mariani L, Mondellini P, Pacini F, Basolo F, Pinchera A, Pilotti S, Pierotti MA. 1996. Age-related activation of the tyrosine kinase receptor protooncogenes RET and NTRK1 in papillary thyroid carcinoma. J Clin Endocrinol Metab 81:2006–2009.

Borrego S, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antiñolo G, Eng C. 1999. Specific polymorphisms in the RET proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. J Med Genet 36:771–774.

Borrego S, Ruiz A, Saez ME, Gimm O, Gao X, López-Alonso M, Hernández A, Wright FA, Antiñolo G, Eng C. 2000. RET genotypes comprising specific haplotypes of polymorphic variants predispose to isolated Hirschsprung disease. J Med Genet 37:572–578.

Bunone G, Uggeri M, Mondellini P, Pierotti MA, Bongarzone I. 2000. RET receptor expression in thyroid follicular epithelial cell-derived tumors. Cancer Res 60:2845–2849.

Cebrian A, Lesueur F, Martin S, Leyland J, Ahmed S, Luccarini C, Smith PL, Luben R, Whittaker J, Pharoah PD, Dunning AM, Ponder BA. 2005. Polymorphisms in the initiators of RET (rearranged during transfection) signaling pathway and susceptibility to sporadic medullary thyroid carcinoma. J Clin Endocrinol Metab 90:6268–6274.

Chakravarty D, Santos E, Ryder M, Knauf JA, Liao XH, West BL, Bollag G, Kolesnick R, Thin TH, Rosen N, Zanzonico P, Larson SM, Refetoff S, Ghossein R, Fagin JA. 2011. Small-molecule MAPK inhibitors restore radioiodine incorporation in mouse thyroid cancers with conditional BRAF activation. J Clin Invest 121:4700–4711.

Cinti R, Yin L, Ilc K, Berger N, Basolo F, Cuccato S, Giannini R, Torre G, Miccoli P, Amati P, Romeo G, Corvi R. 2000. RET rearrangements in papillary thyroid carcinomas and adenomas detected by interphase FISH. Cytogenet Cell Genet 88:56–61.

Costa P, Domingues R, Sobrinho LG, Bugalho MJ. 2005. RET polymorphisms and sporadic medullary thyroid carcinoma in a Portuguese population. Endocrine 27:239–243.

Davies L, Welch HG. 2006. Increasing incidence of thyroid cancer in the United States, 1973–2002. J Am Med Assoc 295:2164–2167.

Elisei R, Cosci B, Romei C, Agate L, Piampiani P, Miccoli P, Berti P, Basolo F, Ugolini C, Ciampi R, Nikiforov Y, Pinchera A. 2004a. Identification of a novel point mutation in the RET gene (Ala883Thr) which is associated with medullary thyroid carcinoma phenotype only in homozygous condition. J Clin Endocrinol Metab 89:5823–5827.

Elisei R, Cosci B, Romei C, Bottici V, Sculli M, Lari R, Barale R, Pacini F, Pinchera A. 2004b. RET exon 11 (G691S) polymorphism is significantly more frequent in sporadic medullary thyroid carcinoma than in the general population. J Clin Endocrinol Metab 89:3579–3584.

Ericsson UB, Lindgrade F. 1991. Effects of cigarette smoking on thyroid function and the prevalence of goitre, thyrotoxicosis and autoimmune thyroiditis J Intern Med 229:67–71.

Fisher C, Mannino DM, Herman WH, Frumkin H. 1997. Cigarette smoking and thyroid hormone levels in males. Int J Epidemiol 26:972–977.

Fugazzola L, Muzza M, Mian C, Cordella D, Barollo S, Alberti L, Cirello V, Dazzi D, Girelli M, Opocher G, Beck-Peccoz P, Persani L. 2008. RET genotypes in sporadic medullary thyroid cancer: Studies in a large Italian series. Clin Endocrinol 69:418–425.

Gil L, Azanedo M, Pollán M, Cristobal E, Arribas B, García-Albert L, García-Sáiz A, Maestro ML, Torres A, Menárguez J, Rojas JM. 2002. Genetic analysis of RET, GFR alpha 1 and GDNF genes in Spanish families with multiple endocrine neoplasia type 2A. Int J Cancer 99:299–304.

Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G. 1990. PTC is a novel rearranged form of the ret protooncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell 60:557–563.

Grogan RH, Mitmaker EJ, Clark OH. 2010. The evolution of biomarkers in thyroid cancer-from mass screening to a personalized biosignature. Cancers 2:885–912.

Ho T, Li G, Zhao C, Wei Q, Sturgis EM. 2005. RET polymorphisms and haplotypes and risk of differentiated thyroid cancer. Laryngoscope 115:1035–1041.

Hundahl SA, Fleming ID, Fremgen AM, Menck HR. 1998. A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985–1995. Cancer 83:2638–2648.

Huyck T, Agulnik M. 2011. Molecular therapies in thyroid cancer. Pharmaceuticals 4:91–100.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA Cancer J Clin 61:69–90.

Joseph KB, Nicole AL. 2004. Methods and criteria for model selection. J Am Statist Assoc 99:279–290.

Karakaya A, Tuncel N, Alptuna G, Koçer Z, Erbay G. 1987. Influence of cigarette smoking on thyroid hormone levels. Hum Toxicol 6:507–509.

Lesueur F, Corbex M, McKay JD, Lima J, Soares P, Griseri P, Burgess J, Ceccherini I, Landolfi S, Papotti M, Amorim A, Goldgar DE, Romeo G. 2002. Specific haplotypes of the RET proto-oncogene are over-represented in patients with sporadic papillary thyroid carcinoma. J Med Genet 39:260–265.

Lonn S, Bhatti P, Alexander BH, Pineda MA, Doody MM, Struewing JP, Sigurdson AJ. 2007. Papillary thyroid cancer and polymorphic variants in TSHR- and RET-related genes: A nested case–control study within a cohort of US radiologic technologists. Cancer Epidemiol Biomarkers Prev 16:174–177.

Machens A, Frank-Raue K, Lorenz K, Rondot S, Raue F, Dralle H. 2012. Clinical relevance of RET variants G691S, L769L, S836S and S904S to sporadic medullary thyroid cancer. Clin Endocrinol 76:691–697.

Nehs MA, Nagarkatti S, Nucera C, Hodin RA, Parangi S. 2010. Thyroidectomy with neoadjuvant PLX4720 extends survival and decreases tumor burden in an orthotopic mouse model of anaplastic thyroid cancer. Surgery 148:1154–1162.

Pandith AA, Siddiqi MA. 2012. Burden of cancers in the valley of Kashmir: 5 Year epidemiological study reveals a different scenario. Tumor Biol 33:1629–1637.

Robledo M, Gil L, Pollán M, Cebrián A, Ruíz S, Azañedo M, Benitez J, Menárguez J, Rojas JM. 2003. Polymorphisms G691S/S904S of RET as genetic modifiers of MEN 2A. Cancer Res 63:1814–1817.

Ron E. 1996. Thyroid cancer. In: Schottenfeld D, Fraumeni JF, Jr, editors. Cancer epidemidology prevention, 2nd ed. New York: Oxford University Press. pp 1000–1021.

Sarlis NJ. 2000. Expression patterns of cellular growth-controlling genes in non-medullary thyroid cancer: Basic aspects. Rev Endocr Metab Disord 1:183–196.

Sarlis NJ, Benvenga S. 2004. Molecular signaling in thyroid cancer. Cancer Treat Res 122:237–264.

Schottenfeld D. 1986. Genetic and environmental factors in human carcinogenesis. J Chronic Dis 39:1021–1030.

Stephens LA, Powell NG, Grubb SJ, Jeremiah SJ, Bethel JA, Demidchik EP, Bogdanova TI, Tronko MD, Thomas GA. 2005. Investigation of loss of heterozygosity and SNP frequencies in the RET gene in papillary thyroid carcinoma. Thyroid 15:100–105.