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Chapter

Genetic Alterations of RET: Possible Implications and Clinical Correlations in Thyroid Carcinogenesis

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Abstract

Thyroid cancers are malignant tumors in the thyroid gland. DNA polymorphisms are playing a decisive role in unscrambling the genomic basis of tumor formation and development in cancer. Thyroid cancer is influenced in a polygenic and low-penetrance manner by *RET* gene polymorphisms and this part of the world (North India) has not recorded any study regarding *RET* alterations in this very cancer. We assessed *RET* G691S (*rs*1799939), L769L (*rs*1800861) and S904S (*rs*1800863) polymorphisms by restriction fragment length polymorphism (RFLP) in order to explain their potential role in the diagnosis and prognosis of Papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). In RET G691S polymorphism, the total dissemination of variant alleles (GA + AA) was 62.9% in cases as related to 44.5% in controls ($P < 0.05$). *RET* L769L variant alleles (TG + GG) was 70% in cases versus 88% in controls ($P < 0.05$). In *RET* S904S, occurrence of variant alleles (CG + GG) was 56% in cases versus 44% in controls ($P < 0.05$). G691S and L769L polymorphism advocate a "Dominant mode of inheritance". The S904S polymorphism approves an "Additive mode of inheritance". In conclusion, there was an over-representation of *RET* G691S/S904S polymorphisms and under-representation of L769L polymorphism in PTC and FTC patients. Additionally, our data suggest that some haplotypes (A T G, G T G and A T C) of *RET* may act as low penetrance alleles for predisposition of thyroid cancer.

Keywords: thyroid cancer, rearranged during transfection, RET, polymorphism, papillary thyroid cancer, follicular thyroid cancer

1. Introduction

Cancer is a large group of diseases that vary in their age of onset, rate of growth, state of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, response to treatment, and prognosis. However, cancer may be a relatively small number of diseases caused by similar molecular defects in cell function

resulting from common types of alterations to a cell's genes as per molecular and cell biological point of view. Ultimately, abnormal gene expression causes cancer which may occur due to mutation, translocation, amplification, deletion, loss of heterozygosity, etc. Cell replication and cell death in a tumor cell population gets imbalanced leading to an expansion of tumor tissue [1, 2].

The most common malignancy of the endocrine system is Thyroid cancer. 2% of all diagnosed cancer cases and majority of endocrine cancer related deaths each year are due to this cancer type [3–5].

The rearranged during transfection (*RET*) proto-oncogene expressed in cells of neural crest origin and encodes a membrane tyrosine-kinase receptor [6]. However, thyroid follicular cells may also express the *RET* tyrosine kinase domain [7]. Somatic *RET* translocations were found in some sporadic and radiation-induced PTCs. Papillary thyroid cancer contains *RET/PTC* chromosomal rearrangement [8]. In this gene rearrangement, a portion of the *RET* gene is fused to one of several genes. *RET/PTC1* and *RET/PTC3* are the most common rearrangement types in which *RET* is fused to *CCDC6* (also known as *H4*) or *NCOA4* (also known as *ELE1* or *RFG*) respectively [9, 10]. *RET* tyrosine kinase domain gets constitutively activated due to *RET* rearrangement that can lead to PTC [11, 12]. 10–20% of sporadic PTC's contain RET/PTC rearrangements. Patients with the history of radiation exposure (50–80%), PTC's from children and young adults (40–70%) have higher frequency of *RET/PTC* rearrangements [13, 14].

Genetic basis of tumor formation and cancer progression is being unraveled by DNA polymorphisms. The human genome as a whole (in which over 3.1 million sequence variations have been mapped), represent 25–35% of the total estimated SNPs [15]. Predisposition to several human cancers is due to polymorphisms. Apart from *RET* rearrangements, the coding sequence of *RET* display polymorphisms in exon 2 (G135A, A45A), in exon 7 (G1296A, A432A), in exon 11 (G2071A, G691S), in exon 13 (T2307G, L769L), in exon 14 (C2508T, S836S), and in exon 15 (C2712G, S904S) [16, 17]. Etiology of sporadic Hirschsprung disease (HSCR) and MTC has been associated with *RET* polymorphisms [18–20]. Silent *RET* polymorphisms; A45A in exon 2 and L769L in exon 13 may represent low-penetrance risk in PTC [20]. Risk of differentiated thyroid cancer with reference to *RET* Polymorphic Haplotypes was also reported by some studies [21]. The mechanisms by which the silent polymorphisms may act in the development of cancer include transcript stability, RNA splicing, and DNA protein binding and protein folding.

The valley of Kashmir is one of the divisions of Jammu and Kashmir State, situated in the Himalayas. Kashmir, regarded worldwide as paradise on earth, with over 07 million populations is heavily burdened with different organ cancers. In Kashmir valley where incidences of almost all types of organ cancers have shown a drastic increase in last couple of decades particularly GIT and lung cancers, the thyroid cancer figures no less in this deadly race. Thyroid cancer is the 8th most common cancer and 7th most common cancer among women in Kashmir valley. The frequency of thyroid cancer has increased from 2.3% in 1995 to 5.4% in 2010 in Kashmir valley [22]. Also, owing to the fact that there is no data on genetic alterations in thyroid cancer available in our population and given the backdrop of a significant presence of thyroid cancer patients, it is the first initiative to study the gene alterations in Thyroid cancer patients of Kashmir Valley. In view of these observations this study was designed to address the disease pathology associated with the thyroid cancer through the Polymorphic analysis of RET gene SNPs—G691S (G2071A), L769L (T2307G) and S904S (C2712G) in order to observe pattern

of association of various SNPs G691S (G2071A), L769L (T2307G) and S904S (C2712G) of RET gene in thyroid cancer.

2. DNA polymorphisms in thyroid cancer

The ability to visualize sequence differences directly in DNA is one of the most important tools underlying the revolution in medical genetics. Polymorphisms are these differences in DNA sequences when studied in the context of a population which may be present in exons (coding regions) or introns (noncoding) regions of genes. Family studies have been undergone by studying the genes of medical importance through visualizing thousands of DNA polymorphisms.

Characteristically, polymorphisms denote sequence variations which confer no deleterious effects and are present in the general population. However, as molecular epidemiological studies were performed and the human genome project was deciphered it became vibrant that some "polymorphisms" were not entirely harmless. Genes for many disorders with a clear pattern of Mendelian inheritance were located and identified by this technique, such as, muscular dystrophies, cystic fibrosis and neurodegenerative disorders and This technique also assists in finding genes that predispose people to diseases in which inheritance patterns are complex, such as diabetes, atherosclerosis, and hypertension. These polymorphisms are crucial in the identification of genes important for susceptibility to common cancers, such as colon cancer, as well as susceptibility to less common childhood tumors, such as retinoblastoma and Wilms' tumor [23]. Over 3.1 million sequence variations have been mapped in the human genome in which, 25–35% of the total estimated SNPs are present [15, 24]. Individual susceptibility is likely due to genetic factors modulating the environmental risk otherwise differentiated thyroid cancer is described by a strong hereditability, hence, the identification of genetic variations is important for understanding the possible mechanisms tangled in thyroid carcinogenesis. In thyroid cancer many single nucleotide polymorphisms have been reported in different genes and functional analysis of many single nucleotide polymorphisms have been carried out. It has been reported that these DNA polymorphisms in various genes predispose a person to higher risk of thyroid cancer and also has a marked effect on various clinicopathological characteristics of thyroid cancer patients [23].

3. Structure and biology of RET receptor

Located on chromosome 10q11.2 near the centromere, the *RET* gene includes 21 exons. *RET* (rearranged during transfection) was first identified by Takahashi et al. in 1985 as a proto-oncogene that can undergo activation by cytogenic rearrangement [25]. *RET* gene was cloned by the same investigators 3 years later [26]. This gene encodes the RET receptor, a plasma membrane-bound tyrosine kinase enzyme. RET gene is expressed by neuroendocrine and neural cells, including parasympathetic, sympathetic and colonic ganglia, cells of the urogenital tract, thyroid C cells, adrenal medullary cells and parathyroid cells derived from branchial arches [27, 28]. The RET protein contains two intracellular tyrosine kinase domains, a transmembrane domain, an extracellular region (four cadherin-like repeats, a calcium binding site, and a cysteine-rich domain) and N terminal signal peptide. The cysteine-rich extracellular domain is central for receptor dimerization,

whereas, the extracellular cadherin-like domains are key for cell-cell signaling. The RET C-terminal tail shows three splicing variants producing three protein isoforms with 9 (RET9; short isoform), 43 (RET43; middle isoform), or 51 (RET51; long

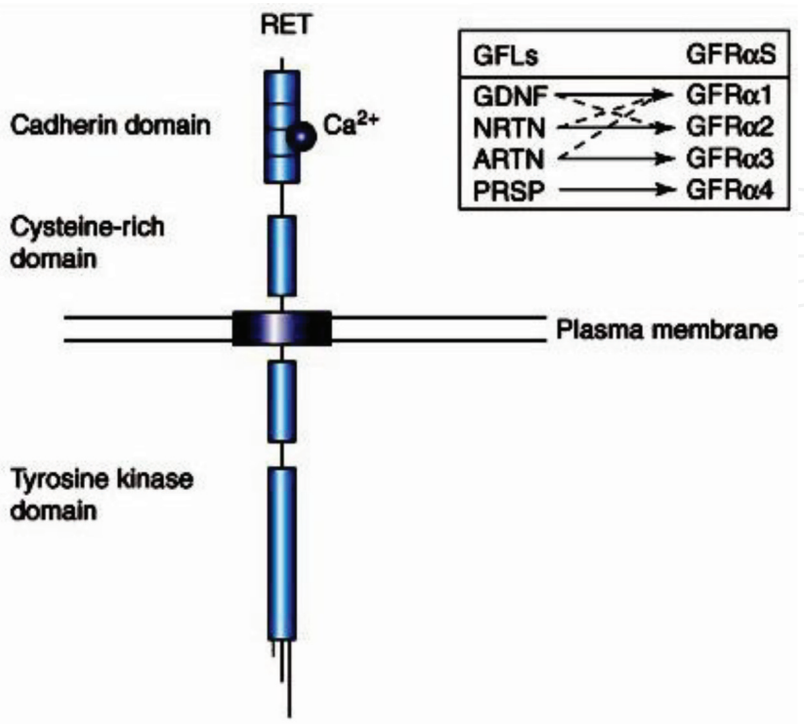

Figure 1.
The RET protein, its functional domains, ligands and co-receptors. Left, *functional domains of the three RET isoforms.* Right, *canonical (unbroken lines) and noncanonical (broken lines) interactions of the RET ligands GDNF, neurturin (NRTN), persephin (PSPN) and artemin (ARTN) with their GFRa co-receptors. Lipid rafts are depicted as a purple box in the plasma membrane.*

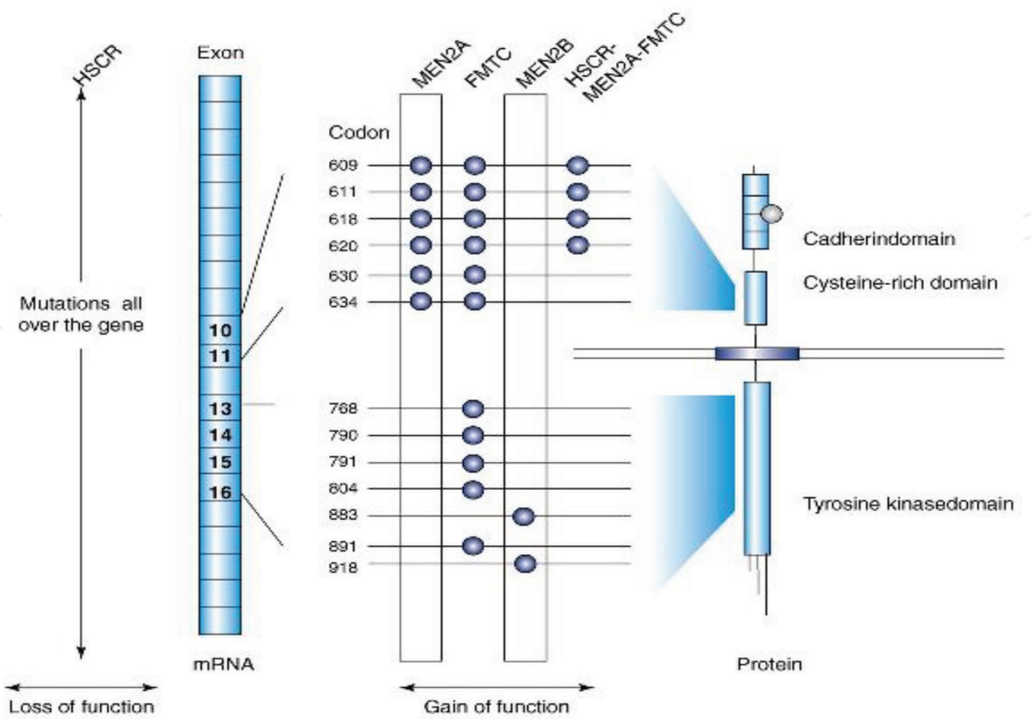

Figure 2.
Germline missense mutations in RET associated with MEN2 and Hirschsprung disease (HSCR). Shown are the structure of the RET mRNA and protein. The codons mutated, the associated clinical entities, and the location of these mutations in relation to the exons and structural domains are indicated.

isoform) distinct amino acids at their C termini [29, 30] (**Figure 1**). Four ligands for the *RET* receptor have been recognized so far [31, 32]. These ligands are *persephin*, *artemin*, *neurturin* and the *glial cell line-derived neurotrophic factor* (*GDNF*) [33, 34]. **Figure 2** summarizes various *RET* mutations along with disease phenotype.

4. Polymorphisms and haplotypes in RET

RET polymorphisms are might be associated with an increased relative risk for the development of disorders derived from neural crest cells and are believed to be genetic modifiers. High penetrant germline *RET* mutations have a key role in disease development. Various disease phenotypes have relatively strong association with *RET* polymorphisms. The most common *RET* polymorphisms; the nonsynonymous variant in exon 11 (G691S; G2071A) and the synonymous variants in exon 13 (L767L; T2307G), in exon 14 (S836S; C2508T) and in exon 15 (S904S; C2712G), are being denoted as disease modifiers due to their presence in patients with sporadic MTC and DTC [35–37]. Disease-associated germline mutations might interact with these polymorphisms or other genetic variants, tempering the age of onset or disease phenotype. Polymorphisms could bestow a much higher attributable risk on the general population as compared with rare mutations in high-penetrance *RET* gene. G691S and S904S polymorphisms of *RET* have a transformer effect on the age of onset of MEN2A [38]. Sporadic MTC has been associated with several *RET* polymorphisms [39]. A low-penetrance *RET* haplotype comprising the wild-type allele at IVS1-126 and IVS1-1463 and a 16-basepair intron-1 deletion of these SNPs is strongly associated with and over represented in *sporadic pheochromocytoma* [40]. *Hirschsprung disease* has disease associated polymorphisms linked to it [41, 42]. Two closely located SNPs, rs2435357 and rs2506004, in intron 1 has been observed by two groups as disease-causing candidates on the basis of comparative genomics, functional assays and association studies [43, 44]. G691S and S904S linkage have been proposed previously [45]. G691S/S904S cosegregated together as haplotype ($P < 0.001$) in one of the studies, proposing that these polymorphisms are in linkage disequilibrium with each other [46].

5. RET polymorphisms and haplotypes in differentiated thyroid cancers

Follicular and parafollicular-type C cells were shown to have some interconnections. The MTC cells provide the microenvironment that has the capacity to stimulate the proliferation of follicular cells, resulting in hyperplastic and adenomatous follicles which could ultimately acquire a fully developed neoplastic phenotype. Some patients with Hashimoto thyroiditis had C-cell hyperplasia [47, 48]. *RET* receptor has been shown to express in thyroid follicular component, which may be activated in the existence of precise ligands in the thyroid microenvironment.

Presence of RET ligands in this microenvironment is highly reasonable because C cells express the RET receptor. Follicular cell derived thyroid cancers contain *RET* mRNA which may be activated in the presence of specific ligands [7]. So the role of *RET* gene polymorphism in differentiated thyroid cancers came into existence. Some variants within *RET* could represent low penetrant alleles for the PTC phenotype. A study revealed the toughest association of A45A (G135A) and L769L (T2307G) with PTC [20]. Borrego et al. described the seven most frequent haplotypes in cases and controls and some of which differed in their distribution [19]. The G G C C haplotype is over-represented in both populations of sporadic PTC.

G allele of exon 2 and the G allele of exon 13 are included in the G G C C haplotype. A432A and S836S polymorphisms had strongest association with DTC as per the study conducted by Lesueur et al. Yet, the scale of the detected effect between DTC and *RET* SNPs was modest [20].

The mechanism by which these silent polymorphisms act and develop *RET* related diseases is still a question of debate. *First*, stability of protein synthesis could be disturbed by such genetic sequence variations through influence on RNA splicing, hence, a new cryptic splice acceptor, donor, or enhancer site could be formed which could result in a receptor that did not bind ligand well or in a truncated protein [49]. *RET* A45A polymorphism may result in alternative splicing and produce a mRNA isoforms as per Borrego et al. [49]. They further hypothesized that decreased protein expression on the cell surface could be due to these RNA isoforms, erroneous ligand binding, microRNA binding, change of structure/copies and mRNA stability synthesis of incomplete proteins and also the change in the structure of proteins caused by slowing down of translation [50, 51]. However, homozygosity confers the phenotypically evident changes [49]. *Second*, other nearby mutations could be influenced by these silent polymorphisms. *Third*, the polymorphism may incline to decreased expression of the variant allele, thus leading to low level functional haplo insufficiency. *Fourth*, these polymorphisms may lie in linkage disequilibrium with other sequences that may directly confer low level predisposition to or protection against disease. *Fifth*, slightly decreased efficiency of RET translation may be due to preferential usage of tRNA molecules. The variant(s) is less favored and the wild type would be the favored sequence. *Sixth*, *RET* gene will be susceptible to damage by environmental insults such as radiation by these silent polymorphisms [21]. *Finally*, mRNA conservation in the case of presence of various polymorphic variants in the *RET* gene based on bioinformatics methods is the answer. Codon usage bias refers to differences among organisms in the frequency of occurrence of synonymous codons in mRNA. Faster translation rates and higher accuracy is achieved by optimal codons. If the translation rate changes before the process of beta sheet formation is finished, newly synthesized sequence influences the structure earlier (or later) than usual and may have an effect on the folding of the protein as the folding of the beta sheet occurs slower than the alpha helix formation [52]. Translation or protein folding disorders because of ribosome stalling (pause) may occur if the mutation is a change from optimal to less frequent codon. S904S SNP gives rise to less frequent codons, so ribosome stalling can happen. In the case of SNP L679L where the codon with higher codon usage appears, the sheet may not finish creating the structure when the helix appears [53]. As a consequence, kinase activity and/or specificity get changed. These postulated mechanisms are not mutually exclusive [54]. The mutant Y791F reduces the energy of the wild type by 7% and L769L (T > G) variant the by 17%, concluding that the L769L polymorphism reduces the MFE of small *RET* mRNA [45, 55]. Because of its cosegregation of S904S with G691S, the results obtained could be interpreted as a founder effect without influence as genetic modifier [38].

The G691S SNP occurs close to the residue Y687 in the cytoplasmic tail of the RET amidst transmembrane region. Two scenarios are possible to explain the G691S polymorphism exerting an effect in PTC without activation by *RET* rearrangement. *Firstly*, although *RET* expressed in the parafollicular C-cells and hence might influence the microenvironment of the follicular cells [56]. *Alternatively*, 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 accordingly effect the subcellular localization, folding, processing or function of the protein [7, 57, 58]. So lot of changes in *RET* proto-oncogene at mRNA level or at protein level can be conferred by single nucleotide

polymorphisms that deregulates the *MAP* kinase or Akt pathway, hence predisposing a person to thyroid cancer [18, 45].

6. Patients and controls

Blood samples of 140 cases were collected from thyroid cancer patients attending Department of Nuclear Medicine, at Sher-I-Kashmir Institute of Medical Sciences (SKIMS), besides blood samples were obtained from 180 healthy controls from the Out Patients Departments of SKIMS. The cases and controls gave written pre-informed consent. Questionnaire was used to record demographic and clinicopathological characteristics of each patient. This study was approved by the Ethical committee of the SKIMS.

0.5 ml of peripheral blood was obtained from each subject in EDTA containing vials (200 μl of 0.5 M, pH = 8.0) and stored at −20°C till use.

7. Genotyping by restriction fragment length polymorphism (PCR-RFLP)

Salting out method was used for the extraction of DNA from blood samples. Automated DNA sequencing of the *RAS* genes revealed one frequent SNP at codon 27 (T81C SNP) of *HRAS*. This SNP along with three other SNPs mentioned above were conducted in our study by PCR-RFLP which is the simple, cheap and common genotyping method.

Restriction enzymes (REs) are called molecular scissors. They recognize and cut specific sequences. The restriction endonuclease type II for SNP detection is selected, such that it recognizes and cleaves one of the polymorphic bases. Upon incubation at optimum temperature and for optimum time with a buffer, the enzyme restricts the DNA, at a specific site. Electrophoresis of the digested products yields fragments of sizes based on the cleavage pattern. If both the alleles harbor the base recognized by the enzyme, fragments of sizes accounting cumulative to the undigested product are obtained (homozygous for that base). If one of the allele harbors a different base, then the single allele is cleaved resulting usually in 3 fragments—the original undigested product, and two digested fragments of sizes cumulatively accounting to the original PCR product (heterozygous). If more than one restriction site is present within the allele, more than two fragments are made from the same allele and the number of fragments depends on number of restriction sites present within the allele. Absence of the base recognized by the enzyme does not result in digestion thereby retaining the original PCR product (Homozygous).

Oligonucleotide Primers and the corresponding Restriction enzymes for codon 691, codon 769, codon 904 of *RET* SNPs along with Annealing temperatures are elucidated **Table 1**. The PCR products were then checked on 2% agarose gel as described earlier.

7.1 Restriction digestion procedure

10 μl of the PCR products were subjected to restriction digestion by *Ban*I, *Taq*I, and *Rsa*I for codons 691,769 and 904 of *RET* SNPs respectively. The reaction conditions were set up according to the supplier of restriction enzymes (**Table 2**). For *RET* codon 691 the homozygous wild type (GG) has one *Ban*I site and is branded by 267 and 187 bp fragments while the (AA) homozygote (variant) offered a single fragment of 454 bp and heterozygous form (G/A) showed 454, 267 and

Amplicon	Primer sequence	A_T (°C)	Product (bp)	RE*	DP** (bp)
HRAS					
Codon 27 (T81C)	F 5′-CAGGAGACCCTGTAGGAGGA-3′ R 5′-GGCACCTGGACGGCGGCGCTAG-3′	60	186	*Dra*III	128 and 58
RET					
Codon 691 (G2071A)	F-5CAGAGCATACGCAGCCTGTAC-3 R-5-GCCTCGTCTGCCCAGCGTTG-3	60	454	*Ban*I	267 and 187
Codon 769 (T2307G)	F-5-CCTGTCCACTGATCCCAAAG-3 R-5-CACTCAGCCCGTGGACTC-3	64	460	*Taq*I	190 and 270
Codon 904 (C2712G)	F-5-GGTCTCACCAGGCCGCTAC-3 R-5-TCGGTATCTTTCCTAGGCTTC-3	62	332	*Rsa*I	224 and 108

*RE, restriction enzyme.
**DP, digestion product.

Table 1.
Conditions and consumables used for screening RET SNPs.

Reagents	Volume (μl)	Incubation
Water	18	Incubation temperature and time for *Ban*I, *Taq*I and *Rsa*I was 37°C for 1–16 h
10× buffer R	2	
PCR product	10	
Restriction enzyme	1	

Table 2.
Composition of RD mixture for codons 691, 769 and 904 of RET.

187 bp. For *RET* codon 769 the homozygous wild type (TT) has one *Taq*I site and is branded by 270 and 190 bp fragments while the (GG) homozygote (variant) offered a single fragment of 460 bp and heterozygous form (T/G) showed 460, 270 and 190 bp. In case of *RET* codon 904 the homozygous wild type (CC) offered a single fragment of 332 bp and the (GG) homozygote (variant) has one *Rsa*I site and is branded by 224 and 108 bp fragments while as heterozygous form (C/G) showed 332, 224 and 108 bp fragments.

The PCR products were visualized on a 3% agarose gel containing 0.5 μg/ml ethidium bromide and photographed.

8. Statistical analysis

Statistical analysis was performed by using SPSS software (V.16.0). Statistical significance was considered when $P \leq 0.05$ [59].

9. Polymorphic analysis of codon G691S, L769L and S904S of *RET* gene

A total of 140 cases (thyroid cancer patients) and 180 normal healthy controls were studied for polymorphic analysis of codon G691S, L769L and S904S of *RET*

gene. **Table 3** shows the risk and demographic factors of study group. Mean age and smoking was not having significant differences among cases and controls. The mean age of the controls and patients were 38 ± 14.6 years and 35 ± 13.4 years respectively. Almost 71% (100 of 140) were <45 years of age and 29% (40 of 140) were ≥45 years of age. Only 19% (26 of 140) of the cases were females and 81% (114 of 140) were males. There was a difference between cases and controls with respect to their gender. Only 11% (16 of 140) of thyroid cancer patients were smokers and 89% (124 of 140) were non-smokers with no significant difference between the groups ($P > 0.05$). Based on the histology 84% (118 of 140) of the included cases were papillary thyroid cancers and 16% (22 of 140) were follicular type.

9.1 Analysis of RET codon G691S, L769L and S904S polymorphism

Table 4 represents the genotype distributions of *RET* codon L691S, L769L and S904S polymorphisms in the cases and controls. There was a significant difference in the genotype distributions between cases and controls in all the three polymorphisms ($P < 0.05$) (**Table 4**).

In *RET* G691S (*rs*1799939), the overall dissemination of variant alleles (GA + AA) in cases was 62.9% as compared to 44.5% in controls ($P < 0.05$; OR = 2.1). **Table 5** shows the Link between *RET* G691S phenotypes and clinicopathological characteristics. For further classification, our study found higher distribution of variant alleles (GA + AA) in female cases as equated to healthy controls (63.2 vs. 47%; $P < 0.05$). Higher frequency of variant genotype was found in cases with no smoking status as compare to non-smoker controls (71.4 vs. 42.9%; $P < 0.05$). No significance was found between G691S polymorphism and any other clinicopathological characteristics (**Table 5**).

In *RET* L769L (*rs*1800861), the overall dissemination of variant alleles (TG + GG) in controls 88% as against 70% in cases ($P < 0.05$; OR = 0.3). **Table 6** represents the connection between *RET* L769L phenotypes and clinicopathological characteristics of patients. For further organization, our study found lower distribution of variant alleles (TG + GG) in <45 years old patients as compared to healthy controls (68 vs. 88%; $P < 0.05$). A significant difference was found between variant alleles (TG + GG) of cases (males—61.5%, females—71.9%) and controls (males—89.5%, females—87%) ($P < 0.05$). Non-smoker controls had higher frequency of variant allele as compared to cases with no smoking status (87 vs. 67.7%; $P < 0.05$). A higher frequency of variant alleles (82.1%) (62%) was found in thyroid cancer patients having no history of benign thyroid disease as compared to patients with history of benign thyroid disease (62%; $P < 0.05$). *RET* gene L769L polymorphism was not found to be associated with any other clinicopathological characteristics (**Table 6**).

In *RET* S904S (*rs*1800863), the overall dissemination of variant alleles (CG + GG) in controls was 44% as against 56% in cases ($P < 0.05$; OR = 1.6). **Table 7** lists the connection between *RET* S904S phenotypes and clinicopathological characteristics of study cases and controls. For further arrangement, our study found lower distribution of variant alleles (CG + GG) in controls of ≥45 years of age as compared to matched thyroid cancer cases (28 vs. 45%; $P < 0.05$). We found a higher distribution of variant alleles (CG + GG) in male thyroid cancer patients as compared to healthy controls (69 vs. 57%; $P < 0.05$). A lower frequency of variant alleles was found in thyroid cancer patients having no history of benign thyroid disease when compared to patients having benign thyroid disease (43 vs. 63%; $P < 0.05$). No association was found between any other clinicopathological characteristic and *RET* gene L769L polymorphism (**Table 7**).

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				
WD	134 (96)			
PD	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; WD, well differentiated thyroid cancer; PD, poorly differentiated thyroid cancer.

Table 3.
Details of thyroid cancer cases and controls for the study.

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

TSH, thyroid stimulating hormone; WD, well differentiated thyroid cancer; PD, poorly differentiated thyroid cancer; BTD, benign thyroid disease.

Table 4.
Genotype frequencies of cases and controls in RET polymorphisms.

9.2 Mode of inheritance in genetic association studies of RET polymorphisms

The risk of thyroid cancer with respect to gender and smoking status with *RET* G691S (G2071A), L769L (T2307G) and S904S (C2712G) polymorphisms. Recessive, dominant, co-dominant and additive inheritance models were used to assess Adjusted ORs. The inheritance model matching individual SNP data shall have lowest P-value. For *RET* G691S and L769L polymorphism dominant inheritance model is appropriate while as additive inheritance model is appropriate for *RET* S904S polymorphism. The results are presented in **Tables 8–10**.

9.3 Association between RET haplotypes and thyroid cancer risk

In order to evaluate the combined effect of the three polymorphisms on thyroid cancer risk Haplotype analyses were conducted. Among both cases and controls all haplotypes have frequencies >5%. G2071/2307G/ C2712 (GGC) was the most

	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)	<0.05
Age group								
<45	100 (71)	30 (30)	70 (70)	130 (72)	62 (48)	68 (52)	2.1 (1.2–3.6)	**<0.05**
≥45	40 (29)	22 (55)	18 (45)	50 (28)	38 (76)	12 (24)	2.6 (3.6–6.2)	**<0.05**
Sex								
Female	114 (81)	42 (36.8)	72 (63.2)	104 (58)	60 (58)	44 (42)	2.3 (1.3–3.9)	**<0.05**
Male	26 (19)	10 (38.5)	16 (61.5)	76 (42)	40 (53)	36 (47)	1.8 (0.72–4.4)	>0.05
Dwelling								
Rural	112 (80)	44 (39.3)	68 (60.7)	98 (54.4)	56 (57.1)	42 (42.9)	2.0 (1.2–3.4)	**<0.05**
Urban	28 (20)	08 (28.5)	20 (71.5)	82 (45.6)	44 (53.6)	38 (46.4)	2.9 (1.1–7.2)	**<0.05**
Smoking								
Never	124 (89)	42 (28.6)	82 (71.4)	140 (77.8)	80 (57.1)	60 (42.9)	2.6 (1.5–4.5)	**<0.05**
Ever	16 (11)	10 (62.5)	06 (37.5)	40 (22.2)	20 (50)	20 (50)	0.6 (0.1–2.8)	>0.05
BTD								
Yes	84 (60)	30 (35.7)	54 (64.2)					
No	56 (40)	22 (39.2)	34 (60.8)				1.2 (0.8–1.9)	>0.05
TSH levels								
Elevated	100 (71)	40 (40)	60 (60)					
Normal	40 (29)	12 (30)	28 (70)				0.6 (0.2–1.5)	>0.05
Histological types								
Papillary	118(84)	44(37.2)	74(62.8)					
Follicular	22(16)	08(36.3)	14(63.7)				1.0 (0.3–3.2)	>0.05
Tumor grade								
WD	134 (96)	50 (37.3)	84 (62.7)					
PD	06 (04)	02 (33.3)	04 (66.7)				0.8 (0.06–9.7)	>0.05
Stage, <45 years								
Stage I	94 (67)	28 (29.7)	66 (70.3)					
Stage II	06 (4.3)	02 (33.3)	04 (66.7)				1.2 (0.1–15.3)	>0.05
Stage, 45 years								
Stage I and II	36 (25.7)	20 (55.5)	16 (44.5)					
Stage III and above	04 (03)	02 (50)	02 (50)				0.8 (0.09–6.3)	>0.05
Vascular/ capsular invasion								
Yes	68 (48.5)	22 (32.3)	46 (67.7)					
No	72 (51.5)	30 (41.6)	42 (58.4)				1.5 (0.75–3)	>0.05
Lymph node metastasis								

	Cases n (%)	GG n (%)	GA + AA n (%)	Controls n (%)	GG n (%)	GA + AA n (%)	OR (95% CI)	P value
Yes	52 (37)	18 (34.6)	34 (65.4)					
No	88 (63)	34 (38.6)	54 (61.4)				1.2 (0.5–2.7)	>0.05

TSH, thyroid stimulating hormone; WD, well differentiated thyroid cancer; PD, poorly differentiated thyroid cancer; BTD, benign thyroid disease.

Table 5.
Clinicopathological characteristics vs. RET G691S (G2071A) genotypes.

	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)	**<0.05**
Age group								
<45	100 (71)	32 (32)	68 (68)	130 (72)	16 (12)	114 (88)	0.3 (0.14–0.5)	**<0.05**
≥45	40 (29)	10 (25)	30 (75)	50 (28)	06 (12)	44 (88)	0.4 (0.13–1.2)	>0.05
Sex								
Female	114 (81)	32 (28.0)	82 (71.9)	104 (58)	14 (13)	90 (87)	0.4 (0.18–0.85)	**<0.05**
Male	26 (19)	10 (38.4)	16 (61.5)	76 (42)	08 (10.5)	68 (89.5)	0.2 (0.07–0.6)	**<0.05**
Dwelling								
Rural	112 (80)	36 (32.1)	76 (67.8)	98 (54)	10 (10)	88 (90)	0.23 (0.1–0.5)	**<0.05**
Urban	28 (20)	06 (21.4)	22 (78.5)	82 (46)	12 (15)	70(85)	0.6 (0.2–1.8)	>0.05
Smoking								
Never	124 (89)	40 (32.2)	84 (67.7)	140 (78)	18 (13)	122 (87)	0.3 (0.16–0.55)	**<0.05**
Ever	16 (11)	02 (12.5)	14 (87.5)	40 (22)	04 (10)	36 (90)	1.1 (0.16–6.2)	>0.05
BTD								
Yes	84 (60)	32 (38)	52 (62)				0.35(0.15–0.78)	**<0.05**
No	56 (40)	10 (17.8)	46 (82.1)					
TSH levels								
Elevated	100 (71)	30 (30)	70 (70)				0.25(0.1–0.62)	>0.05
Normal	40 (29)	12 (30)	28 (70)					
Histological types								
Papillary	118 (84)	36 (30.5)	82 (69.4)				0.85 (0.3–2.2)	>0.05
Follicular	22 (16)	06 (27.2)	16 (72.7)					
Tumor grade								
WD	134 (96)	40 (29.8)	94 (70.14)				1.17 (0.19–6.5)	>0.05
PD	06 (04)	02 (33.3)	04 (66.6)					
Stage, <45 years								
Stage I	94 (67)	28 (29.8)	66 (70.2)				4.7 (0.8–26.8)	>0.05

	Cases n (%)	TT n (%)	TG + GG n (%)	Controls n (%)	TT n (%)	TG + GG n (%)	OR (95% CI)	P value
Stage II	06 (4.3)	04 (66.6)	02 (33.3)					
Stage, 45 years								
Stage I and II	36 (25.7)	08 (22.2)	28 (77.8)				3.5 (0.42–28.7)	>0.05
Stage III and above	04 (03)	02 (50)	02 (50)					
Vascular/ capsular invasion								
Yes	68 (48.5)	22 (32.3)	46 (67.7)				0.8 (0.4–1.6)	>0.05
No	72 (51.5)	20 (27.8)	52 (72.2)					
Lymph node metastasis								
Yes	52 (37)	18 (34.6)	34 (65.4)				0.7 (0.32–1.4)	>0.05
No	88 (63)	24 (27.2)	64 (72.8)					

TSH, thyroid stimulating hormone; WD, well differentiated thyroid cancer; PD, poorly differentiated thyroid cancer; BTD, benign thyroid disease.

Table 6.
Clinicopathological characteristics vs. RET L769L (T2307G) genotypes.

	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)	**<0.05**
Age group								
<45	100 (71)	40 (64.5)	60 (35.5)	130 (72)	66 (51)	64 (49)	1.5 (0.9–2.9)	>0.05
≥45	40 (29)	22 (55)	18 (45)	50 (28)	36 (72)	14 (28)	2.1 (0.84–5.0)	**<0.05**
Sex								
Female	114 (81)	54 (47)	60 (53)	104 (58)	58 (56)	46 (44)	1.4 (0.8–2.4)	>0.05
Male	26 (19)	08 (31)	18 (69)	76 (42)	44 (43)	32 (57)	3.1 (1.2–8.0)	**<0.05**
Dwelling								
Rural	112 (80)	46 (41)	66(59)	98 (54)	54 (55)	44 (45)	1.7 (0.96–2.9)	>0.05
Urban	28 (20)	16 (57)	12(43)	82 (46)	48 (58.5)	34 (41.5)	1.05 (0.4–2.4)	>0.05
Smoking								
Never	124 (89)	56 (45)	68 (55)	140 (78)	78 (56)	62 (44)	1.5 (0.9–2.4)	>0.05
Ever	16 (11)	06 (37.5)	10 (62.5)	40 (22)	24 (60)	16 (40)	2.5 (0.75–8.2)	>0.05
BTD								
Yes	84 (60)	30 (36)	54 (64)					
No	56 (40)	32 (57)	24 (43)				2.4 (1.2–4.8)	**<0.05**
TSH levels								
Elevated	100 (71)	40 (40)	60 (60)					
Normal	40 (29)	22 (55)	18 (45)				1.8 (0.7–3.7)	>0.05
Histological types								
Papillary	118 (84)	56 (47)	62 (53)					

	Cases n (%)	CC n (%)	CG + GG n (%)	Controls n (%)	CC n (%)	CG + GG n (%)	OR (95% CI)	P-value
Follicular	22 (16)	06 (27)	16 (73)				0.4 (0.15–1.08)	>0.05
Tumor grade								
WD	134 (96)	60 (45)	74 (55)					
PD	06 (04)	02 (33)	04 (67)				0.6 (0.10–3.4)	>0.05
Stage, < 45 years								
Stage I	94 (67)	40 (42.5)	54 (57.5)					
Stage II	06 (4.3)	02 (33)	04 (67)				0.7 (0.12–4.0)	>0.05
Stage, 45 years								
Stage I and II	36 (25.7)	18 (50)	18 (50)					
Stage III and above	04 (03)	02 (50)	02 (50)				1.0 (0.12–7.9)	>0.05
Vascular/ capsular invasion								
Yes	68 (48.5)	32 (47)	36 (53)					
No	72 (51.5)	30 (41.7)	42 (58.3)				0.8 (0.4–1.56)	>0.05
Lymph node metastasis								
Yes	52 (37)	28 (54)	24 (46)					
No	88 (63)	34 (39)	54 (61)				0.5 (0.25–1.0)	>0.05

TSH, thyroid stimulating hormone; WD, well differentiated thyroid cancer; PD, poorly differentiated thyroid cancer; BTD, benign thyroid disease.

Table 7.
Clinicopathological characteristics vs. RET S904S (C2712G) genotypes.

Genotypes and alleles (patients vs. controls)	Cases (n = 140)	Controls (n = 180)	OR (95% CI)	P value
Recessive model (AA vs. GA + GG)				
GA + GG	116	164	1.0 **(ref.)**	
AA	24	16	2.12 (1.0–4.7)	0.027
Dominant model (AA+ GA vs. GG)				
GG	52	100	1.0 **(ref.)**	
AA + GA	88	80	2.11 (1.3–3.3)	**0.001**
Co-dominant model (GA vs. AA + GG)				
AA + GG	76	116	1.0 **(ref.)**	
GA	64	64	1.5 (1.0–2.3)	0.066
Additive model (AA vs. GG)				
GG	52	100	1.0 **(ref.)**	
AA	24	16	2.8 (1.4–5.7)	0.003

Table 8.
G691S (G2071A) polymorphism association with thyroid cancer.

Genotypes and alleles (patients vs. controls)	Cases (n = 140)	Controls (n = 180)	OR (95% CI)	P value
Recessive model (GG vs. TG + TT)				
TG + TT	112	132	1.0 **(ref.)**	
GG	28	48	0.7 (0.4–1.2)	0.17
Dominant model (GG + TG vs. TT)				
TT	42	22	1.0 **(ref.)**	
GG + TG	98	158	0.32 (0.2–0.6)	**0.000**
Co-dominant model (TG vs. GG + TT)				
GG + TT	70	70	1.0 **(ref.)**	
TG	70	110	0.63 (0.4–1.0)	0.047
Additive model (GG vs. TT)				
TT	42	22	1.0 **(ref.)**	
GG	28	48	0.3 (0.15–0.6)	0.001

Table 9.
Genetic model for L769L (T2307G) polymorphism.

common haplotype, with frequencies of 24% in cases and 33% in controls. The complete dissemination of various haplotypes between cases and controls presented a clear difference ($P < 0.0001$). Haplotype pattern for the three SNPs is shown in **Table 11**. Haplotypes with frequency <1% was omitted from the study. After stratified by gender, age and smoking status the haplotype frequencies were estimated from the genotyping data. In our study, the most common haplotype is taken

Genotypes and alleles (patients vs. controls)	Cases (n = 140)	Controls (n = 180)	OR (95% CI)	P-value
Recessive model (GG vs. CG + CC)				
CG + CC	126	172	1.0 **(ref.)**	
GG	14	08	2.4 **(0.97–5.8)**	0.051
Dominant model (GG + CG vs. CC)				
CC	62	102	1.0 **(ref.)**	
GG + CG	78	78	1.64 (1.0–2.6)	0.028
Co-dominant model (CG vs. GG + CC)				
GG + CC	76	110	1.0 **(ref.)**	
CG	64	70	1.3 (0.8–2.0)	0.22
Additive model (GG vs. CC)				
CC	62	102	1.0 **(ref.)**	
GG	14	08	2.8 (1.1–7.2)	**0.021**

Table 10.
Genetic model for S904S (C2712G) polymorphism.

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.

Table 11.
Haplotype frequencies and its association with thyroid cancer.

as the reference group and haplotype-specific ORs are estimated by the haplotype-based logistic regression method [60]. The frequencies for the estimated 3-marker haplotypes among patients and controls are shown in **Table 11**.

10. Conclusion

Thyroid tumors signify a proper model for the study of epithelial neoplastic transformation. At the genomic level, thyroid cancers gather a number of changes and it has been projected that genomic instability has a vital role in the development of thyroid neoplasms [61].

With erudite genetic tools producing a treasure of information, we have added improved vision into the mechanisms driving thyroid tumor development. Recognition of these features is crucial to the management of patients with TC. New therapeutics is being designed based on our improved understanding of this disease course.

In Kashmiri population we studied genetic alterations of *RET* genes in thyroid cancer patients. Following are the major findings of our study;

- In *RET* G691S (*rs*1799939), the total distribution of variant alleles (CA + AA) in controls was 44.5% as against 62.9% in controls which revealed a 2-fold higher risk of variant allele (TC + CC) in cases against the controls ($P < 0.05$).

- In *RET* L769L (*rs*1800861), the total distribution of variant alleles (TG + GG) in controls was 88% as against 70% in cases ($P < 0.05$; OR = 0.3).

- In *RET* S904S (*rs*1800863), the total distribution of variant alleles (CG + GG) in controls was 44% as against 56% in cases ($P < 0.05$; OR = 1.6).

- We found higher distribution of variant alleles (CG + GG) in TC cases of ≥45 years of age and male gender (45 and 69%) as compared to matched healthy controls (28 and 57%) ($P < 0.05$). Association was also observed with BTD ($P < 0.05$).

- *RET* G691S and L769L polymorphisms follow "*Dominant inheritance model*" while as "*Additive inheritance model*" is appropriate for analysis of *RET* S904S polymorphism.

- In *RET* codon G691S, L769L and S904S polymorphism 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).

In conclusion, our study shows that among different polymorphisms predisposing to thyroid tumors, *RET* gene polymorphism is among the prominent ones. Our results support the earlier reports of the G691S/S904S polymorphism in *RET* gene as a marked predisposing factor for the risk of developing thyroid tumors in our population with G691S variant showing an increased risk for the non-smokers but this needs to be authenticated in a large sample study in the future to determine the course of thyroid cancer. Further we conclude L769L polymorphism to be protective in our series of thyroid cancer patients. Apart from it 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 thyroid cancer.

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References

[1] Corner J, Bailey C. Cancer Nursing Care in Context. Oxford: Blackwell Publishing; 2001

[2] Yarbro C, Frogge M, Goodman M. Cancer Nursing: Principles and Practice. 6th ed. Boston, MA: Jones and Bartlett Publishers; 2005

[3] Sarlis NJ, Benvenga S. Molecular signaling in thyroid cancer. Cancer Treatment and Research. 2004;**122**: 237-264

[4] Sarlis NJ. Expression patterns of cellular growth-controlling genes in non-medullary thyroid cancer: Basic aspects. Reviews in Endocrine and Metabolic Disorders. 2000;**1**:183-196

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

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

[7] Bunone G, Uggeri M, Mondellini P, et al. RET receptor expression in thyroid follicular epithelial cell-derived tumors. Cancer Research. 2000;**60**:2845-2849

[8] Santoro M et al. Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. The Journal of Clinical Investigation. 1992;**89**:1517-1522

[9] Grieco M, Santoro M, et al. PTC is a novel rearranged form of the ret protooncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell. 1990;**60**:557-563

[10] Santoro M et al. Molecular characterization of RET/PTC3: A novel rearranged version of the RET proto-oncogene in a human thyroid papillary carcinoma. Oncogene. 1994;**9**:509-516

[11] Eng C. RET proto-oncogene in the development of human cancer. Journal of Clinical Oncology. 1999;**17**:380-393

[12] Tallini G, Asa SL. RET oncogene activation in papillary thyroid carcinoma. Advances in Anatomic Pathology. 2001;**8**:345-354

[13] Fenton CL, Lukes Y, Nicholson D, et al. The RET/PTC mutations are common in sporadic papillary thyroid carcinoma of children and young adults. The Journal of Clinical Endocrinology and Metabolism. 2000;**85**:1170-1175

[14] Nikiforov YE. Molecular analysis of thyroid tumors. Modern Pathology. 2011;**24**:S34-S43

[15] Frazer KA et al. A second generation human haplotype map of over 3.1 million SNPs. Nature. 2007;**449**:851-861

[16] Stephens LA, Powell NG, Grubb SJ, Jeremiah JA, et al. Investigation of loss of heterozygosity and SNP frequencies in the RET gene in papillary thyroid carcinoma. Thyroid. 2005;**15**:100-105

[17] Adjadj E, Schlumberger M, de Vathaire F. Germ-line DNA polymorphisms and susceptibility to differentiated thyroid cancer. The Lancet Oncology. 2009;**10**:181-190

[18] Fitze G, Schreiber M, Kuhlisch E, Schackert HK, et al. Association of RET protooncogene codon 45 polymorphism with Hirschsprung disease. American Journal of Human Genetics. 1999;**65**: 1469-1473

[19] Borrego S, Ruiz A, Saez ME, Gimm O, Gao X, Lopez-Alonso M, et al. RET genotypes comprising specific

haplotypes of polymorphic variants predispose to isolated Hirschsprung disease. Journal of Medical Genetics. 2000;**37**:572-578

[20] Lesueur F, Corbex M, McKay JD, et al. Specific haplotypes of the RET proto-oncogene are over-represented in patients with sporadic papillary thyroid carcinoma. Journal of Medical Genetics. 2002;**39**:260-265

[21] Ho T, Li G, Zhao C, Wei Q, Sturgis EM. RET polymorphisms and haplotypes and risk of differentiated thyroid cancer. Laryngoscope. 2005;**115**: 1035-1041

[22] Pandith AA, Siddiqi MA. Burden of cancers in the valley of Kashmir: 5 year epidemiological study reveals a different scenario. Tumor Biology. 2012;**33**: 1629-1637

[23] Kleiman DA, Buitrago D, Crowley MJ, Housman D. Human DNA polymorphism. The New England Journal of Medicine. 1995;**332**:318-320

[24] The International Hap Map Consortium. The International Hap Map project. Nature. 2003;**426**:789-796

[25] Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell. 1985;**42**:581-588

[26] Takahashi M, Buma Y, et al. Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene. 1988;**3**:571-578

[27] Pachnis V, Mankoo B, Costantini F. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development. 1993;**119**:1005-1017

[28] Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, Asai J. Spatial and temporal expression of the retproto-oncogene product in embryonic, infant and adult rat tissues. Oncogene. 1995;**10**:191-198

[29] Tahira T, Ishizaka Y, Itoh F, Sugimura T, Nagao M. Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene. 1990;**5**:97-102

[30] Myers SM, Eng C, Ponder BA, Mulligan LM. Characterization of RET proto-oncogene splicing variants and polyadenylation sites: A novel C-terminus for RET. Oncogene. 1995;**11**: 2039-2045

[31] Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, et al. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell. 1996;**85**: 1113-1124

[32] Treanor JJ, Goodman L, et al. Characterization of a multicomponent receptor for GDNF. Nature. 1996;**382**: 80-83

[33] Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, et al. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFR alpha3–RET receptor complex. Neuron. 1998;**21**: 1291-1302

[34] Milbrandt J, de Sauvage FJ, Fahrner TJ, et al. Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron. 1998;**20**:245-253

[35] Baumgartner-Parzer SM, Lang R, Wagner L, et al. Polymorphisms in exon 13 and intron 14 of the RET protooncogene: Genetic modifiers of medullary thyroid carcinoma? Journal of Clinical Endocrinology and Metabolism. 2005;**90**:6232-6236

[36] Cebrian A, Lesueur F, Martin S, et al. Polymorphisms in the initiators of RET (rearranged during transfection)

signaling pathway and susceptibility to sporadic medullary thyroid carcinoma. Journal of Clinical Endocrinology and Metabolism. 2005; **90**:6268-6274

[37] Machens A, Frank-Raue K, Lorenz K, Rondot S, Raue F, Dralle H. Clinical relevance of RET variants G691S, L769L, S836S and S904S to sporadic medullary thyroid cancer. Clinical Endocrinology. 2012;**76**:691-697

[38] Robledo M et al. Polymorphisms G691S/S904S of RET as genetic modifiers of MEN 2A. Cancer Research. 2003;**63**:1814-1817

[39] Elisei R, Cosci B, Romei C, Bottici V, Sculli M, Lari R, et al. RET exon 11 (G691S) polymorphism is significantly more frequent in sporadic medullary thyroid carcinoma than in the general population. The Journal of Clinical Endocrinology and Metabolism. 2004; **89**:3579-3584

[40] McWhinney SR et al. Intronic single nucleotide polymorphisms in the RET protoonco gene are associated with a subset of apparently sporadic pheochromocytoma and may modulate age of onset. The Journal of Clinical Endocrinology and Metabolism. 2003; **88**(10):4911-4916

[41] Fitze G et al. Functional haplotypes of the RET proto-oncogene promoter are associated with Hirschsprung disease (HSCR). Human Molecular Genetics. 2003;**12**:3207-3214

[42] Garcia-Barcelo M et al. TTF-1 and RET promoter SNPs: Regulation of RET transcription in Hirschsprung's disease. Human Molecular Genetics. 2005;**14**: 191-204

[43] Burzynski GM et al. Identifying candidate Hirschsprung disease associated RET variants. American Journal of Human Genetics. 2005;**76**: 850-858

[44] Plaza-Menacho I, Burzynski GM, de Groot JW, et al. Activated ras and ret oncogenes induce over-expression of cmet (hepatocyte growth factor receptor) in human thyroid epithelialcells. Oncogene. 1997;**14**: 2417-2423

[45] Borrego S, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antiñolo G, et al. Specific polymorphisms in the RET proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. Journal of Medical Genetics. 1999;**36**:771-774

[46] Gil L, Azanedo M, et al. Genetic analysis of RET, GFR alpha 1 and GDNF genes in Spanish families with multiple endocrine neoplasia type 2A. International Journal of Cancer. 2002; **99**:299-304

[47] Tsui-Pierchala BA, Milbrandt J, Johnson EM. NGF utilizes c-Ret via a novel GFL-independent, inter-RTK signaling mechanism to maintain the trophic status of mature sympathetic neurons. Neuron. 2002;**33**:261-273

[48] Sipple JH. The association of pheochromocytoma with carcinoma of the thyroid gland. The American Journal of Medicine. 1961;**31**:163-166

[49] Borrego S, Eng C, Sánchez B, Sáez ME, Navarro E, Antiñolo G. Molecular analysis of RET and GDNF genes in a family with multiple endocrine neoplasia type 2A and Hirschsprung disease. The Journal of Clinical Endocrinology and Metabolism. 1998; **83**:3361-3364

[50] Parmley JL, Hurt LD. How do synonymous mutations affect fitness? Bioassay. 2007;**29**:515-519

[51] Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, et al. A "silent" polymorphisms in the

MDR1 gene changes substrate specificity. Science. 2007;**26**:525-528

[52] Muñoz V, Thompson PA, Hofrichter J, Eaton WA. Folding dynamics and mechanism of β-hairpin formation. Nature. 1997;**390**:196-199

[53] Nakamura Y, Gojobori T, Ikemura T. Codon usage tabulated fromthe international DNA sequence databases. Nucleic Acids Research. 2007;**26**: 334-340

[54] Sromek M, Czetwertyńska M, et al. The frequency of selected polymorphic variants of the RET gene in patients with medullary thyroid carcinoma and in the general population of Central Poland. Endocrine Pathology. 2010;**21**: 178-185

[55] Ceolin L, Siqueira DR, et al. Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness. European Journal of Endocrinology. 2012;**11**:1-29

[56] Fabien N, Paulin C, Santoro M, Berger N, et al. Detection of RET oncogene activation in human papillary thyroid carcinomas by in situ hybridisation. British Journal of Cancer. 1992;**66**:1094-1098

[57] Fluge O, Haugen DR, Akslen LA, Marstad A, et al. Expression and alternative splicing of c-ret RNA in papillary thyroid carcinomas. Oncogene. 2001;**20**:885-892

[58] Leviev I, Negro F, James RW. Two alleles of the human paraoxonase gene produce different amounts of mRNA. An explanation for differences in serum concentrations of paraoxonase associated with the (Leu-Met54) polymorphism. Arteriosclerosis, Thrombosis, and Vascular Biology. 1997;**17**:2935-2939

[59] Rao J, Scott A. On chi-squared tests for multiway contingency tables with cell proportions estimated from survey data. The Annals of Statistics. 1984;**12**: 46-60

[60] Akaike H. Information measures and model selection. Bulletin of the International Statistical Institute. 1983; **50**:277-290

[61] Sobrinho-Simoes M et al. Molecular pathology of well-differentiated thyroid carcinomas. Virchows Archiv. 2005; **447**:787-793