



Haplotype Frequency of G691S/S904S in the *RET* Proto-Oncogene in Patients with Medullary Thyroid Carcinoma

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Abstract

Background: Medullary thyroid carcinoma (MTC) occurs in both sporadic (75%) and hereditary (25%) forms. The missense mutations of the *REarranged during Transfection (RET)* proto-oncogene in MTC development have been well demonstrated. The aim of this study was to investigate frequency of G691S/S904S haplotype in MTC patients and their relatives.

Methods: In this research 293 participants were studied, including 181 patients (102 female, 79 male) and 112 their relatives (58 female, 54 male). Genomic DNA was extracted from peripheral blood leucocytes using the standard Salting Out/Proteinase K method. Nucleotide change detection was performed using PCR and direct DNA sequencing methods.

Results: According to DNA sequencing results, 159 individuals (104 patients, 55 relatives) had both G691S (rs1799939) missense mutation in exon11 and S904S (rs1800863) synonymous mutation in exon 15 of *RET* proto-oncogene. The allele frequency of G691S/S904S haplotype was 21.15% in patients and 10.75% in their relatives.

Conclusion: The obtained data showed the frequency of G691S/S904S *RET* gene haplotype among Iranian MTC patients and their relatives. The G691S and S904S nucleotide changes were in complete linkage disequilibrium, so the results were grouped together and referred to as G691S/S904S haplotype. Further analysis is need to demonstrate the association between this haplotype and MTC development.

Keywords: Thyroid cancer, Medullary, *RET* proto-oncogene, G691S/S904S haplotype

Introduction

Thyroid cancer is the most common endocrine neoplasia and accounts for 1% of all human cancers (1). Medullary thyroid carcinoma (MTC) is a rare neoplasm of the calcitonin-secreting thyroid cells and accounts for 5–10% of all thyroid cancers. MTC can occurs as sporadically (75%) or as a part of the autosomal dominantly inherited forms (25%), (2, 3). The inherited type of MTC can be divided in three clinically distinct forms: Multiple Endocrine Neoplasia type2A (MEN2A), Multiple Endocrine Neoplasia type2B (MEN2B), and Familial MTC (FMTC) (4). MEN2A is

defined by MTC, bilateral pheochromocytoma, and multiple tumors of the parathyroid glands (parathyroid adenomas, primary hyperparathyroidism or HPT), within a single patient or family. MEN2B is characterized by the early development of an aggressive form of MTC in all affected individuals (typically during the first year of life), pheochromocytoma, the absence of hyperparathyroidism, and visible physical stigmata such as raised bumps on the lips and tongue, gangliogliomas of the intestine, marfanoid body habitus with skeletal deformations. FMTC is characterized

by a strong predisposition to MTC in families with a very low incidence of other endocrinopathies related to MEN2 (1, 5).

The molecular pathology of inherited MTCs is constitutive of *RET* (*REarranged during Transfection*) proto-oncogene. The *RET* gene is located on chromosome 10q11.2, consisting of 21 exons and encodes a tyrosine kinase receptor. This complex plays a critical role in cell proliferation and differentiation of tissues derived from neural crest cells, such as C-cells of thyroid gland (5, 6).

RET is a tyrosine kinase protein and includes three domains; a large extracellular domain containing a cysteine-rich region and a series of cadherin homology domains, a trans-membrane domain, and an intracellular tyrosine kinase domain, required for *RET* phosphorylation and downstream signaling (5, 7). This protein is activated by binding a soluble ligand of the glial cell-line-derived neurotrophic factor (GDNF) family and requires a co-receptor of the GDNF family receptors α (*GFR α*) (8).

The majority of MEN2A families are associated with one of the six point mutations in conserved cysteine residues in exon10 (codons 609, 611, 618, and 620) or exon11 (codons 630, 634) in the extracellular domain of the *RET* (2, 5, 8-10, 12). In FMTC cases, *RET* mutations are mainly detected in the same six codons as for MEN2A and also in exon13 (codon 768), or in exon15 (codon 891), in the intracellular region of *RET* (5, 8, 12). Mutation of codon 918 in *RET* exon16 in the tyrosine kinase domain, accounts for the vast majority of patients with MEN2B (5, 8).

For the first time, *RET* polymorphisms G691S and S904S were described in 1994 (13, 14). Recent studies have found G691S in exon11, S904S single nucleotide polymorphisms in exon15 of the *RET* pathway to be associated with the risk of developing sporadic MTC (sMTC) (7). It seems, two of these *RET* SNPs (G691S and S904S) may modify the age at onset of MTC tumor in family members (5, 15) but Fabienne Lesueur et al. have rejected this hypothesis (7).

In the present study, we report the frequency of G691S/S904S haplotype in Iranian MTC patients and their relatives.

Material and Methods

Samples

Over the last ten years, one hundred ninety MTC patients, were referred to Cellular and molecular research center of Shahid Beheshti University of Medical Sciences in order to *RET* genetic screening. The volunteer individuals were included in the survey after obtaining an informed consent. This study has been approved by the Ethics Committee of Cellular and Molecular Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences.

Histopathology of biopsy/FNA was used to confirm the diagnosis of MTC. A questionnaire was used to collect information about age, sex, and history of neoplasia. If any mutation was found in patients, their first-degree relatives were invited for screening. So one hundred twenty one relatives participated in our study.

The patients constituted 84 males and 106 females, with mean \pm SD age 37.33 \pm 14.38 years. 121 first-degree relatives from 31 index cases comprised 57 males and 64 females, with mean \pm SD age 29.09 \pm 16.34 years.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leucocytes using standard Salting out/proteinase K method and stored at -20°C. The DNA was quantitated on Spectrophotometer and the 260/280 nm absorbance noted.

PCR assay

For identification of *RET* variant 691 (rs1799939, codon 691 of exon 11, GGT>AGT, Gly>Ser) and 904 (rs1800863, codon 904 of exon 15, TCC>TCG, Ser>Ser), DNA samples were amplified using the polymerase chain reaction (PCR) and the specific oligonucleotides primers (Table1). The exon11 running profile was constituted initial denaturation at 96 °C for 1min and , followed by 30 cycles with 96 °C for 45s, annealing at 60 °C for 1min dependent on the primer sequences, and extension at 72°C for 45s, with a final extension at 72 °C for 10 min.

Table 1: Sequences of oligonucleotide primers used for PCR amplification of exons 11, 15 of the *RET*

Exon	Primers	Tm (°C)	Amplicon (bp)
11	F: atacgcagcctgtaccagt	59.6 C	497
	R: cacagactgtccccacacag	60.2 C	
15	F: ggtctcaccaggccgctac	63.1 C	334
	R: tcggtatctttcctagcttc	57.6 C	

PCR reaction for exon 15 was constituted initial denaturation at 94°C for 3min and, followed by 35 cycles of 94°C for 30s, annealing starting at 56°C for 1min, 72°C for 1min, and a final extension at 72°C for 10min. The PCR products were electrophoresed on 8% polyacrylamide gel. The amplicons were confirmed for the presence or absence of mutations by direct DNA sequencing method (ABI 3100 Genetic Analyzer and Big Dye Terminator v3.1 Cycle Sequencing Kit®, Applied Biosystems, California, USA).

Bioinformatics and Statistical analysis

To detect the *RET* mutations and SNPs, sequences were analyzed by Chromas 2.33 software and were also compared with the reference *RET* gene sequence using web based tool NCBI Blast. Statistical analysis was performed by SPSS 20 software.

Results

Among 311 participants, 18 DNA samples did not have enough quality, so were excluded from the study. We analyzed germline DNA mutations of *RET* gene in 181 Iranian MTC patients and 112 relatives, with a total of 293 members. The patients included, 33 FMTC, 6 MEN2A, 2 MEN2B, 1 pheochromocytoma and 145 apparently sMTC cases (Table 2). The frequency of females in our population was a little higher than males, but this ratio was not significant (160 vs. 133, $P > 0.05$). Specifically, we found 2 germline polymorphisms of *RET* gene at codon691 (GGT>AGT, exon 11, rs1799939) that causes Glycine to Serine amino acid substitution, and codon 904 (TCC>TCG, exon 15, rs1800863) that does not lead to an amino acid alteration. Furthermore, the allele frequencies of each of these SNPs were similar in all

patients and relatives (21.5% and 10.75%, respectively).

Table 2: The frequency of patients with medullary thyroid carcinoma

Description	Frequency (n)
Total patients	293
sMTC	192
MEN2A	6
MEN2B	2
FMTC	33
Pheochromocytoma	1

As the G691S and S904S variants were in complete linkage disequilibrium, so the results were grouped together and referred to as G691S/S904S haplotype (Fig.1). The analysis of G691S/S904S *RET* gene haplotype showed that 104 of 181 (57.45%) MTC patients and 55 of 112 (49.1%) relatives had this haplotype. In particular, 82 MTCs and 47 relatives were heterozygous and 22 MTCs and 8 relatives were homozygous for the G691S/S904S haplotype (Table 3). There was not significant correlation between age of diagnosis and the presence of G691S/S904S haplotype.

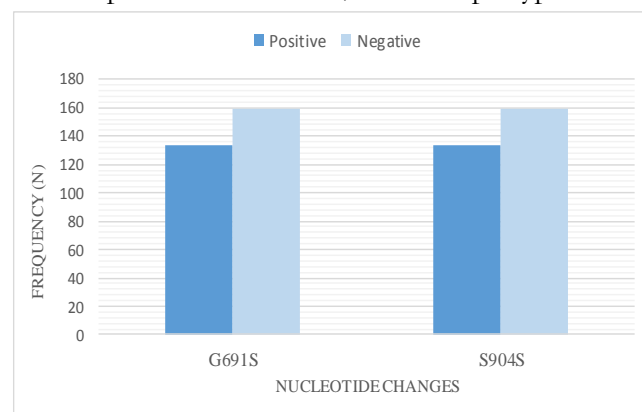
**Fig. 1:** The frequency of G691S and S904S variants in the population

Table 3: The frequency distribution of G691S and S904S variants among MTC patients and their relatives

Exon	Codon	Nucleotide Change	AA Change	Genotype frequency (n)			Phenotype (n)				Relative (n)
				Heterozygote	Homozygote	Pheo	sMTC	MEN2B	MEN2A	FMTC	
11	691	(GGT>AGT)	Gly>Ser	129	30	1	78	-	5	20	55
15	904	(TCC>TCG)	Ser>Ser	129	30	1	78	-	5	20	55

Discussion

In this study, we found two variants of *RET* gene, G691S (GGT>AGT, exon 11, rs1799939) and S904S (TCC>TCG, exon 15, rs1800863) that are cosegregated together as a haplotype, suggesting that these polymorphisms are in linkage disequilibrium with each other.

The assessment of the occurrence of two polymorphic changes G691S, and S904S in our population is similar to the study performed by Lucieli Ceolin et al. (16). Unfortunately, in our study we did not have control group to compared with healthy population, but some studies have demonstrated that the *RET* variant G691S is more frequent in MTC patients than in the general population (16, 17). It has also been postulated that such variants are relatively common in the population which may confer a much higher attributable risk in the general population than mutations in high penetrance cancer susceptibility genes (5, 11).

As our study shows this haplotype presents in all type of MTC disease. Some studies support our data that both G691S and S904S SNPs are associated with sporadic MTC and MEN2A (5, 7, 18). Two studies have shown that this haplotype may modify the age at onset of MTC tumor in family members (5, 15), however, this hypothesis is controversial (7).

The *RET*-G691S polymorphism has been suggested as a genetic modifier in MEN2A (15). Overall, these data strengthen the potential role of the *RET*-G691S polymorphism in MTC.

One of the probable mechanisms is that the G691S polymorphism seems to enhance *RET* oncogenicity of other distinct mutations affecting *RET* (18-20), on the other hands, bases exchange in the DNA molecule could create a new alternative splicing site, leading to the synthesis of a truncated protein, erroneous ligand binding, micro-RNA binding, change of structure and mRNA stability as well as a number of copies, and also the change in the secondary structure of proteins (16). The *RET* gene mutations are a critical factor in patient management, e.g. to decide on total or partial thyroidectomy or the age at which the child should be subject to surgery (6).

RET gene mutations are very important in MTC patient management, especially in decision making for total or partial thyroidectomy or the age at which the child should be subject to surgery. In the case of inherited mutations, all family members will be offered genetic screening of their blood DNA. Those who test negative for germline mutations will be reassured; those who test positive for germline mutations will be screened for calcitonin levels and will be offered a prophylactic thyroidectomy depending on the complete clinical and biochemical picture.

Conclusion

This data showed the frequency of G691S/S904S haplotype among Iranian MTC patients and their

family, for the first time. Even though G691S/S904S haplotype are not considered as oncogenic mutations at this time, its functional role should be investigated. Further researches on this issue can contribute toward clarifying the prevalence of this haplotype and its probable modifying effect on the phenotypic characteristic of MTC.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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