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ORIGINAL ARTICLE

RET Gene Analysis in Patients with Medullary Thyroid Carcinoma

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SUMMARY

Background: **Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor from the para follicular C cells of the thyroid gland. It occurs either sporadically or as part of an inherited syndrome. It is caused by an autosomal dominant mutation in the RET (Rearranged during Transfection) proto-oncogene.**
Methods: **The studied population consisted of 47 patients diagnosed with MTC in a specific population of northwest Iran along with their three children. Blood samples were collected from all subjects, genomic DNA was extracted and RET exons 10, 11, 13, 14, 15, and 16 were analyzed using PCR and direct sequencing.**
Results: **32 missense mutations were identified in exons 10 (6.25%) and 11 (84.4%). Moreover, two novel mutations in codon 595 in exon 10 (E595D and E595A) and a new mutation in codon 689 exon 11 (S689T) were detected, and a new nucleotide change was found in exon 11 (T675T). Four different polymorphisms were also identified in exons 11, 13, 14, and 15. Based on our data, the frequency profile of RET mutations in the Azari population of Iran with MTC is 61.7%. The most frequent mutation in our population was C364G, whereas in most populations it is C634R.**
Conclusions: **These results underline the importance of the genetic background of family members of any patient with MTC.**
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INTRODUCTION

Thyroid cancer accounts for about 1% of all malignancies. Although it is rare, it is the most frequent endocrine neoplasm [1]. Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor from the parafollicular C cells [2-4]. MTC accounts for 5 - 10% of all thyroid cancers, and its intensity can vary between being an extremely benign tumor or an aggressive variant with a high mortality rate [2-5].

MTC is sporadic in 75% of cases while in 25% of cases it follows a hereditary pattern which is transmitted in an autosomal dominant manner [6,7]. Hereditary MTC is divided into three subtypes, the most common of them being MEN2A, which accounts for 70 - 80% of patients with hereditary MTC. MEN2A is characterized by MTC, pheochromocytoma and primary hyperparathyroidism [8]. The second inherited subtype is MEN2B, which accounts for only 5% of hereditary MTC cases. MEN2B is characterized by clinically aggressive MTC, pheochromocytoma, marfanoid body habitus, mucosal (and other) neuromas, and intestinal tumors (mostly ganglioneuromas); these patients typically do not manifest hyperparathyroidism [9]. The third subtype is familial MTC (FMTC) which accounts for 10 - 20% of hereditary MTC cases. Patients with FMTC develop MTC without any other abnormalities [10]. MTC is caused by a mutation of the RET proto-oncogene [9]. The REarranged during Transfection (RET) proto-oncogene contains 21 exons and is mapped on chromosome 10q11.2. RET encodes a tyrosine kinase receptor that plays a crucial role in regulating cell proliferation, migration, differentiation, and survival through embryogenesis [11,12].

RET proto-oncogene loss of function causes Hirschsprung disease, and its gain of function has a role in a number of cancer syndromes such as MTC [13,14]. The most frequent mutations in the RET proto-oncogene have been found in five cysteine codons: 609, 611, 618, 620 of exon 10, and codon 634 of exon 11. In addition, some other mutations have also been identified in non-cystein codons such as 804 in exon 14, 883 in exon 15, and 918 in exon 16 [15,16]. Activated *RET* germline mutations have been identified as the primary cause of all hereditary MTC syndromes and approximately a quarter to a third of all sporadic MTC cases. Additionally, up to 98% of MEN2 cases have a germline *RET* mutation that leads to the constitutive activation of the RET receptor. Somatic *RET* mutations account for another quarter to half of all sporadic MTCs [9]. The occult or de novo mutation, which occurs frequently among sporadic cases, ranges from 2.5% to 7% [17,18]. Several studies have found that point mutations are the extracellular domain in more than 96% of MEN2A cases and in 86% of patients with FMTC [13,19]. The molecular diagnosis of *RET* mutations has become a crucial tool for the management of MTC. The identification of MTC in a single patient with no family history of MTC or MEN2 poses a dilemma for the clinician. The possibility exists that the affected individuals could be the proband for a new kindred or may represent a de novo mutation that could be transmitted to the offspring [20]. Genetic screening now affords an early identification of carriers of the RET proto-oncogene germline mutations who are likely to develop MTC later in life. These screenings are especially useful for first-degree kindred of MTC patients. In these patients, early prophylactic thyroidectomy must be envisaged to ensure a definitive cure [21].

So far, no mutation analysis has been done for patients with MTC in the Azari population of north-west Iran. Therefore, the aim of this study was to determine the allele frequency of predominant RET germline mutations in exons 10, 11, 13 - 16 among MTC patients in this specific population of north-west Iran.

MATERIALS AND METHODS

Patients

This is a descriptive cross sectional study. The studied population consisted of (50) individuals, including 47 patients whose diagnosis of MTC was confirmed by histopathologic documents and the other three were chosen for this study through the examination of their three first-degree relatives. These patients were selected among 4,488 registered cases who were diagnosed for one of the four common thyroid cancers by the different hospitals in Tabriz (center of Azarbaijan province, Iran) between 2004 and 2014. The sample size was calculated based on other similar studies, and it included all survivors whose diagnosis of MTC was confirmed in this specific population during the last 10 years. We selected the patients among all patients with different kinds of thyroid cancer between 2004 and 2014. The including criteria was having MTC that was confirmed by histopathological documents in Azari population and the exclusion criteria was having other kinds of cancer. Among these 47 cases, 24 had positive familial history of MTC and the other 23 patients were apparent sporadic cases. There were 19 males and 28 females and their mean age at diagnosis was 36.74 years. After germline RET mutation analysis, the first-degree relatives of MTC patients with positive mutations were also examined for the RET mutation. This study was approved by the Institutional Review Board and Ethics Committee of Tabriz University of Medical Sciences, Iran. All patients signed an informed consent form before their blood was collected.

RET genetic analysis

Blood samples were collected in EDTA from all 50 subjects and DNA was extracted from peripheral leucocyte samples according to a standard salting-out/proteinase K method, and an aliquot of DNA for each individual was stored at -20°C. Genomic DNA of each case was then amplified by using PCR. The primers for exons 10, 11, 13, 14, 15, and 16 are given in Table 1.

All PCR reactions were performed in 10 µL containing 5 µL master mix, 1 µL of each primer, 1 µl betain, and 2 µl DNA. The running profile of the amplifications was: initial denaturation at 95°C for 6 minutes followed by 30 cycles (denaturation at 95°C for 30 seconds, annealing at optimized temperature (Table 1) for 30 seconds, extension at 72°C for 26 seconds) and a final extension at 72°C for 5 minutes (Peqlab thermocycler, Germany).

PCR products were analyzed by 1.5% agarose Tris-bo-

Table 1. **Primers and annealing temperatures.**

Name	Primer	Annealing Temperature
Exon10-Forward	AGGCTGAGTGGGCTACGTCTG	59°C
Exon10-Reverse	GTTGAGACCTCTGTGGGGCT	
Exon11-Forward	ATGAGGCAGAGCATACGCAGCC	66°C
Exon11-Reverse	CTTGAAGGCATCCACGGAGACC	
Exon13-Forward	AACTTGGGCAAGGCGATGCA	58°C
Exon13-Reverse	AGAACAGGGCTGTATGGAGC	
Exon14-Forward	AAGACCCAAGCTGCCTGA	57.8°C
Exon14-Reverse	GCTGGGTGCAGAGCCATAT	
Exon15-Forward	CATGGCCTGACGACTCGTGC	63°C
Exon15-Reverse	CCTGGGAGCCCCGCCTCATC	
Exon16-Forward	CTGAAAGCTCAGGGATAGGG	56°C
Exon16-Reverse	TAACCTCCACCCCAAGAGAG	

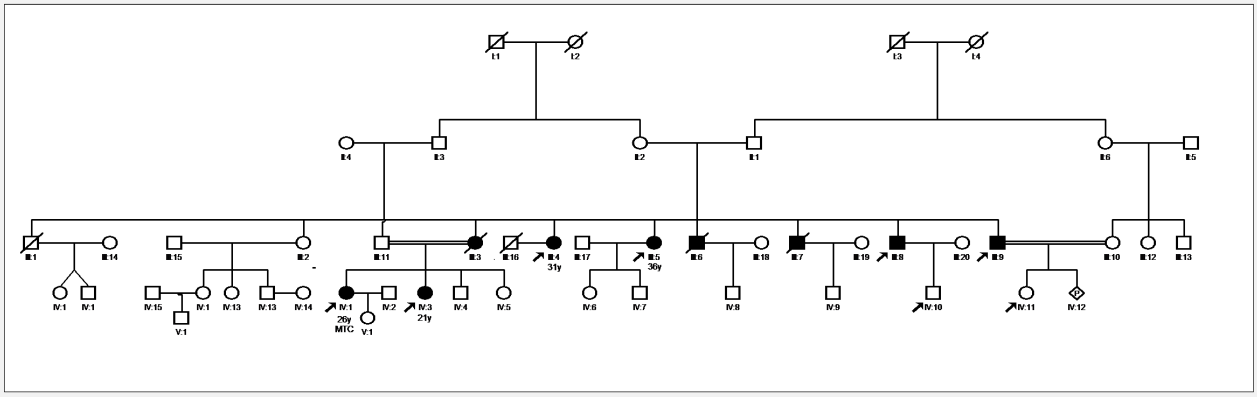

Figure 1. Pedigree of one family.

rate-EDTA gel electrophoresis. The gel was stained with safe stain and analyzed under UV light. A negative control was included in each amplification analysis. Then all purified PCR products were sequenced to the 6 exons in the sense and antisense by Macrogen, South Korea.

RESULTS

We examined 47 patients suffering from MTC and 3 of their first-degree relatives. Among them, 23 individuals had a sporadic form of MTC and the others were divided into 5 families (pedigree of each family investigated, as example Figure 1). Genetic analyses of 6 hot spot exons revealed germline RET nucleotide substitutions in all 50 individuals, which included all of the patients and 3 of their children.

In FMTC patients, the majority of nucleotide changes were located on exon 11 (37 out of 53 nucleotide changes). Four families (22 patients and 2 children) had one of the following mutations: C634R, C634Y, C634G and C634W in codon 634. Fourteen of them also displayed a G691S polymorphism, and a child of one of the patients (IV 11, Figure 1) had T675T change in exon 11. Among 23 cases, 14 (3 families) had L769L polymorphism in exon 13. In one family, made up of two sisters, exon 10 had a C620S mutation. We did not

find any nucleotide changes in exons 14, 15, and 16 in FMTC patients and their relatives.
All 23 sporadic cases showed L769L polymorphisms in exon 13. G691S, C634G, and C634Y polymorphisms were also found in exon 11 of 6, 2, and 1 patients, respectively. One individual had S689T in exon 11. Two cases had a changed codon 595 in exon 10 (E595A and E595D). A S836S polymorphism was found in exon 14 of 2 patients and only one case of sporadic MTC had a S904S polymorphism in exon 15. We did not find any mutations in exon 16 of Ret gene.

DISCUSSION

In modern molecular medicine, genetic screening is an increasingly valuable tool that is currently applied to diagnosing inherited tumor syndromes [22]. This is the first study of molecular genetic screening of MTC patients (familial and sporadic) in the Azari population of north-west Iran.
Identification of the mutations in the RET proto-oncogene confirms the clinical diagnosis and identifies asymptomatic family members with FMTC or MEN2 syndrome [23]. Since sporadic MTC (isolated, non-familial MTC) seems to be the presenting clinical feature for some MEN2 patients, RET genotyping is often performed for patients with sporadic MTC [24]. In this study, by screening exons 10, 11, and 13 - 16 of the RET proto-oncogene we found 32 germline mutations in the predominant codons of exons 10 and 11, and 63 polymorphisms in exons 11, 13, 14, and 15 among 47 individuals with MTC and their 3 children. These mutations occurred in the cysteine codon 620 in exon 10 (6.25%) and cysteine codon 634 in exon 11 (84.4%). The majority of RET mutations (27 out of 32, 84.4%) were located in exon 11 (9 C634G, 7 C634Y, 7 C634R, 4 C634W), followed by 2 mutations in exon 10 (C620S). We also found two new mutations in codon 595 in exon 10 (Glu595Asp, Glu595Ala), one new mutation in codon 689 in exon 11 (T689S), and a new nucleotide change in codon 675 (Thr675Thr) in exon 11 which have not yet been reported.
Mutations of the extracellular RET cysteine-rich domain at codon 634, 609, 611, and 620 resulted in ligand-independent dimerization of receptor molecules, enhanced phosphorylation of intracellular substrates, and cell transformation. Germline mutations in codons 609, 611, 618, 620, 634, and 768 have been discovered predominantly in MEN2 and FMTC [23].
The mutations at codon 634, accounted for 84.4% of all mutations found in the Azari patients with MTC from our study in north-west Iran. This mutation is known as a common mutation in Caucasians [26]. Among four different types of nucleotide substitutions found in this codon, changes from Cys to Gly (9 of 27) were the most common, followed by Cys to Tyr (7 of 27), Cys to Arg (7 of 27), and Cys to Trp (4 of 27). Our data, like other available literature on Caucasians, indicates the common alteration form as Cys634Gly, which may represent a founder effect. It has been reported that the Cys634Arg mutation (the second most common mutation in our study) is the most common mutation in MTC patients in many populations, and is related to parathyroid diseases [27]. However, in other studies of different Iranian populations carried out by Alvandi et al. and Mehdi Hedayati et al., the most frequent mutation was Cys634Arg (five mutations in 55 patients) and Cys634Gly (eleven mutations in 151 patients) [28,29]. These different results in comparison with our study may be related to different genetic backgrounds of the researched populations.
Valente et al. and Fernandez et al. illustrated that C634Y is the most frequent RET mutation in MTC families in Brazili and Spanish populations, respectively [30,25]. Two independent studies by Schuffenecker et al. and Berard et al. showed that the most frequent mutations of RET proto-oncogene in French hereditary MEN2 and their first-degree relatives are C634R and C634Y [31,32]. In contrast, the common mutation in FMTC in Sardinia was observed in codon 804 (V804M) and the mutation in codon 634 had the lowest frequency [33]. Eng et al. showed a high prevalence of RET mutations in the hereditary type of MTC that is found in codons 634 (C634R), 918 (M918T), 768, and 804 in American population [16]. Another study by Zhou et al. in China proved that the highest frequency of the RET mutation in patients with hereditary MTC occurred in codons 634 (C634Y) and 918 (M918T) in MEN2A and MEN2B, respectively [34]. However, the most frequent RET proto-oncogene mutations in Saudi families with MEN2A and FMTC [35] along with a Netherlands population with FMTC, were found in codon 618 [16]. In Portugal, Czech Republic, and Italy, population mutations in codon 918 (M918T) were the most common mutations in the sporadic type of MTC [32,36-39]. However, we did not find any M918T germline mutations in our studied population. Romei et al. showed that the most frequent RET mutations in Italian patients with MTC is V804M (19.6%) followed by C634R (13.6%) [40]. A study by Mahesh et al. illustrated that C634S is the most common mutation in a large Indian family with MTC [41]. A study conducted in China by Qi XP showed C634Y and C634R are the most common mutations in Chinese families with MEN2 [42]. Our study shows that the frequency profile of RET mutations in the Azari population of Iran with MTC is 61.7%. Based our data, the most common mutation of the RET proto-oncogene, from a sample of an Azari population in Iran with MTC, is located in exon 11. These results underline the importance of genetic background in the distribution of RET mutations and should be taken into account in the genetic evaluation of MTC patients.

CONCLUSION

Since this is the first study of RET proto-oncogene in the Azari population of Iran, more studies should be done in this specific population along with the examination of other RET exons.
Furthermore, a transforming activity and functional effect(s) of three new RET mutations (E959D, E595A and S689T) and a new nucleotide change (T675T) need to be elucidated.

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Declaration of Interest:
There is no conflict of interest.

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