Germline RET Mutations in Exons 10 and 11: An Iranian Survey of 57 Medullary Thyroid Carcinoma Cases

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Summary
The susceptibility gene for hereditary Medullary Thyroid Carcinoma (MTC) is the RET proto-oncogene. The aim of this study was to evaluate the prevalence of common germline RET mutations in exons 10 and 11 among Iranian MTC patients. Fifty-seven non-related MTC patients were examined in this study (Females: Males = 1:2.10; Mean age = 40.0 ± 11.5 years) and the existence of mutations was assessed through the PCR-RFLP technique. The only Multiple Endocrine Neoplasia type 2A (MEN2A) patient displayed a G634W mutation in exon 11. Among 53 apparently sporadic MTC patients, one patient showed a G620R mutation in exon 10 and two other patients displayed G624V mutations in exon 11 of RET proto-oncogene. Neither the only Multiple Endocrine Neoplasia type 2B (MEN2B) patient nor two familial MTC patients was found to carry germline mutations in exons 10 and 11. This study reports, for the first time, the prevalence of common RET mutations among Iranian, apparently sporadic MTC patients, underlining the critical importance of screening for RET mutations in such patients.

Key Words: Medullary thyroid carcinoma, RET proto-oncogene, Multiple endocrine neoplasia, Mutation analysis

Introduction
Medullary Thyroid Carcinoma arises from parafollicular C-cells and may occur in sporadic (75% of cases) or hereditary (25% of cases) forms. The hereditary forms of MTC occur either isolated as Familial MTC (FMTC) or as part of an inherited cancer syndrome called multiple endocrine neoplasia type 2 (MEN2). This syndrome, transmitted in an autosomal dominant fashion, is characterized by MTC, pheochromocytoma and parathyroid hyperplasia (MEN2A, 95% of MEN2 cases) or MTC, pheochromocytoma, mucosal neuromas, ganglioneuromatosis of the gut and a Marfanoid habitus (MEN2B, 5% of MEN2 cases).

The gene responsible for these tumors is the RET proto-oncogene, coding for a tyrosine kinase receptor expressed in neural crest-derived cells. This single-pass transmembrane protein contains four cadherin-like repeats, one calcium-binding site and one cysteine rich domain in its extracellular portion. The intracellular portion of this protein consists of a typical tyrosine kinase domain. Upon ligand binding, RET dimerization is induced and mutual transphosphorylation of tyrosine residues occurs. Phosphotyrosines then propagate the signal by recruiting intracellular proteins that carry SH2 and phosphotyrosine binding domains.

Approximately 92% of hereditary MTC cases are related to germline missense mutations of the RET proto-oncogene. The mutations found in 97% of MEN2A and 86% of FMTC families are clustered together as they affect one of four cysteine codons [609, 611, 618, 620] in exon 10 and one cysteine codon [634] in exon 11 of the extracellular cysteine-rich domain of RET. These

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mutations induce constitutive catalytic activity due to the aberrant disulfide homodimerization of RET6. Germline mutations also occur in the intracellular domain of RET in FMTC: in exon 13 (codons 768, 790 and 791), exon 14 (codon 804 and 844) and exon 15 (codon 891). On the other hand, in 2.5-7% of apparently sporadic MTCs, a germline RET mutation is found9.

Genetic testing for germline mutations in the RET proto-oncogene is now available and the early identification of individuals who carry RET mutations and, hence are susceptible to develop MTC later in life, is possible. This genetic test is especially useful for the first-degree relatives of MTC patients. If the unaffected family members bear no RET mutation, they can be assured and further clinical and biochemical investigation for MTC can be safely abandoned. Thus, prophylactic thyroidectomy should be advised merely for those family members who carry a RET mutation10. The following strategy has been proposed for mutation detection analysis in a MTC patient: Initially, the mutation analysis must focus on exon 11 (codon 634) and if no mutation was detected, exons 10 (codons 609, 611, 618 and 620), 16 (codon 918), and 13-15 must be considered in order for further analysis11.

A few germline RET proto-oncogene mutational analysis studies have been reported in recent years from Asian countries12-14. In Iran, the DNA analysis of MTC patients and their family members is not routinely advised and the genetic background of Iranian MTC patients has not been reported previously. The current study aimed to assess the existence and prevalence of common germline RET mutations in exons 10 and 11 among Iranian MTC patients.

Materials and Methods

Patients: The study population included 57 index (non-related) MTC patients who had been operated in different university hospitals and institutions of Tehran. The diagnosis of MTC had been confirmed by histopathologic proofs. The patients were invited to participate in the study and the volunteer patients were included in the survey after obtaining an informed consent. The familial forms of disease were categorized as MEN2A, MEN2B and FMTC according to clinical, biochemical and/or histopathologic evidences. The remaining MTC cases were assigned as the apparently sporadic MTC group. After germline RET mutation analysis, the first-degree relatives of MTC patients with positive mutations were also examined for RET mutations.

Mutation analysis: DNA was extracted from peripheral blood samples according to a standard salting-out technique15 mainly in the molecular genetic laboratory of the Endocrine Research Center, Shaheed Beheshti University of Medical Sciences. For amplification of the DNA segment containing RET exon 10, the following primers were used: (10F 5'GCGCCCCAGGAGGCTGATGC3') and (10R 5'CGTGCTGGTGTCGCGGCCG3'). The RET exon 11 was amplified using following primers: (11AF 5'CCTCTGGGCGTGCAAAGCCT3') and (11AR 5'CACCGGAAGAGGACTAGCTG3'). Amplification was carried out in a volume of 50 μl containing 2.5 μl DNA-containing cell extract, 0.2 mmol/l of each dNTP (Boehringer Mannheim Co.), 0.5 μmol/l of each exon 10 primers and 0.25 μmol/l of each exon 11 primer (TIB MOLBIOL Synthesizealabor Co.), and 0.2 U Taq polymerase (Boehringer Mannheim Co.). For exon 10, 40 cycles were performed in an automatic thermocycler (Omnigene & Hybaid Co.) under the following conditions: denaturation at 94°C for 45 s, annealing at 66.5°C for 45 s and extension at 72°C for another 45 s. For exon 11, 28 cycles were performed under similar conditions except that the temperature of annealing phase was 68°C. The PCR products were then precipitated by ethanol17.

The amplified PCR products were digested by each of Taq I, BstU I, Mbo II, Rsa I, Nla IV (England Biolabs) and Cfo I (Roche Molecular Biochemicals) restricting enzymes for exon 10 and by each of Cfo I, Rsa I, Hae III and Dda I restricting enzymes for exon 11, according to the manufacturer's instructions. Table I demonstrates the RFLP patterns created by these restricting enzymes in presence and absence of each RET exon 10 or 11 mutation. The digested samples were separated by electrophoresis through a 10% non-denaturing polyacrylamide gel electrophoresis and detected by UV illumination after ethidium bromide staining.

Results

Altogether, 57 patients were included in this survey. The mean age of MTC patients was 40.0 years with a standard deviation of 11.5 years. The overall female-to-male ratio was 1.2:1. Among these 57 MTC patients, there were 1 case of MEN2A, 1 case of MEN2B and 2 cases of FMTC. The remaining 53 cases were diagnosed as apparently sporadic MTC. In the MEN2A patient, the Cfo I cleavage of exon 11 PCR product demonstrated a
C634W mutation. As demonstrated in Fig. 1, the C634W mutated allele was cut into 170 and 64 bp fragments by Cfo I restriction enzyme. Among apparently sporadic MTC patients, one patient showed a C620R mutation in exon 10 (in BstU I cleavage of exon 10 PCR product) and two other patients displayed C624Y mutations in exon 11 of RET proto-oncogene (in Rsa I cleavage of exon 11 PCR product). None of MEN2B or FMTC patients was found to carry germline mutations in exons 10 and 11 of RET proto-oncogene.

The MEN2A patient with a germline C634W mutation in exon 11 was a 65-year old man. He was known to have MEN2A since 21 years ago. In his family, there were multiple cases of MTC. While his brother was free of MTC, his sister had died when she was 50-years old with MTC and pheochromocytoma. Among his two sons and seven daughters, one son (39-year old) and two daughters (36- and 46-year old) had MTC and pheochromocytoma. Another daughter (28-year old) had MTC without pheochromocytoma. The germline DNA analysis of children showed the same mutation in all affected cases, but the similar mutation was not found in those without any clinical manifestations. The family of 39-year old son was investigated for C634W mutation in exon 11 of RET and his 12-year old son was found to carry the same mutation (his nine year old son and 4-year old daughter did not carry that mutation).

The sporadic MTC patients with exon 10 or 11 RET proto-oncogene mutations were also investigated regarding similar RET mutations in their first-degree relatives. None of those analyzed carried mutations detected in related MTC patients.

<table>
<thead>
<tr>
<th>RET mutation</th>
<th>Exon</th>
<th>Base pair change</th>
<th>Restriction enzyme</th>
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Fig 1: Representative analysis of a common exon 11 mutation in RET gene (C634W); While the normal allele is characterized by a single 234 bp fragment after treatment with Cfo I restriction enzyme, the mutated C634W allele is cut into 170 and 64 bp fragments.

Discussion

Among 57 non-related MTC patients examined in this study, the only MEN2A patient and three apparently sporadic MTC patients had mutations in exon 10 or 11 of RET proto-oncogene. Neither MEN2B nor FMTC patients carried exon 10 or 11 RET mutations. It has been reported that mutations found in 97% of MEN2A affect either one of four cysteine codons [609, 611, 618, 620] in exon 10 or one cysteine codon [634] in exon 11 of the extracellular cysteine-rich domain of RET. In this study, the MEN2A patient showed the common C634W mutation in exon 11 of RET and the same mutation was found in his son, three of his daughters and his 12-year old grandson. It is well known that persons with this mutation are at high risk of MTC. Therefore, prophylactic thyroidectomy was highly recommended for the 12-year old grandson of the MEN2A patient.

MEN2B syndrome is most associated with RET mutations in the intracellular tyrosine kinase domain. It has been reported that a single missense mutation, in codon 918 in exon 16, is observed in 95% of patients with MEN2B. As expected, the only MEN2B patient of the present study showed no RET mutation in exons 10 and 11. If the exon 16 would be screened for mutations, our chance to find mutation would have been high. It is widely believed that most (86%) FMTC families demonstrate mutations in either one of four cysteine codons [609, 611, 618, 620] in exon 10 or one cysteine codon [634] in exon 11 of the extracellular cysteine-rich domain of RET. However, germline mutations may also occur in the intracellular domain of RET in FMTC in exons 13, 14 and 15. On the other hand, a more recent study of a large series of FMTC patients, including 148 patients from 47 families, indicated that non-cysteine RET mutations were not infrequent in FMTC patients and its prevalence could be around 60% of FMTC families. Neither exon 10 nor exon 11 mutations were found in our 2 FMTC patients. If we had extended the RET mutation analysis to exons other than 10 and 11, we might be able to find responsible RET mutations.

Three of 53 apparently sporadic MTC patients (5.7%) were found to carry RET exon 10 or 11 mutations. This prevalence rate is within the range (2.5-7%) reported for overall germline RET mutations in such patients. Apparently sporadic MTC patients may carry low-penetrance RET mutations in exons 13-15. If we had examined RET mutations in exons 13-15, the rate of germline RET mutations might be higher than 5.7%. Therefore, the mutational analysis of RET proto-oncogene is highly recommended for Iranian apparently sporadic MTC patients. The information obtained by such analyses can help the clinician discriminate between truly sporadic and hereditary forms of MTC, to provide a more accurate risk assessment for first-degree relatives and to make his prophylactic interventions limited to those who are at prominent risk of MTC.
Clearly, the fact that no RET mutations were found in exons 10 and 11 among FMTC and MEN2B patients and 50 of apparently sporadic MTC patients does not rule out the possibility of RET mutations in other exons. So, the next part of our study is to extend the RET mutation analysis to other relevant exons (exons 13, 14, 15 and 16) and also use other screening methods like Single Strand Conformation Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE), and denaturing High Performance Liquid Chromatography (dHPLC). Direct sequencing analysis will also be performed to find unknown RET mutations.

Conclusion
This study is the first report of the genetic background of Iranian MTC patients. Using the PCR-RFLP technique on 57 non-related Iranian MTC patients, it was demonstrated that the prevalence of germline RET mutations in exons 10 or 11 is prominent among Iranian apparently sporadic MTC patients. The results of this study can be taken to suggest that the apparently sporadic MTC patients should be investigated for likely RET mutations and in the case of positive result, their first-degree relatives should be screened for similar mutations. However, the hereditary forms of MTC require a more extended investigation for RET mutations.

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