



Distribution of RET Mutations and Evaluation of Treatment Approaches in Hereditary Medullary Thyroid Carcinoma in Turkey

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Genetic screening of germline RET mutations provides the early diagnosis of hereditary cases. Prophylactic thyroidectomy (PTx) is the only preventive option for mutation carrier family members.

WHAT THIS STUDY ADDS?

Turkish people has a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries i.e. Italy and France. Despite complimentary RET gene testing by the courtesy of Society of Endocrinology and Metabolism of Turkey, the number of the PTx in Turkey is limited and relatively late in the lifespan of the gene carriers. This is mainly due to patient and family incomppliance and incomplete family counselling.

ABSTRACT

Objective: This retrospective multicenter study, centrally conducted and supported by the Society of Endocrinology and Metabolism of Turkey, aimed to evaluate the impact of free RET proto-oncogene testing in medullary thyroid carcinoma (MTC) patients. Surgical timing, adequacy of the treatment, and frequency of prophylactic thyroidectomy (PTx) in mutation carriers were also assessed.

Methods: Genetic testing for MTC and pheochromocytoma was conducted between July 2008 and January 2012 in 512 patients. Application forms and RET mutation analyses of these patients whose blood samples were sent from various centers around Turkey were assessed retrospectively. An evaluation form was sent to the physicians of the eligible 319 patients who had confirmed sporadic MTC, familial MTC (FMTC), multiple endocrine neoplasia type 2 (MEN2), or who were mutation carriers. Physicians were asked to give information about the surgical history, latest calcitonin levels, morbidity, mortality, genetic screening, and PTx among family members. Twenty-five centers responded by filling in the forms of 192 patients.

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ABSTRACT

Results: Among the 319 patients, RET mutation was detected in 71 (22.3%). Cys634Arg mutation was the most prevalent mutation (43.7%), followed by Val804Met in 18 patients (25.4%), and Cys634Tyr in 6 patients (8.5%). Among 192 MTC patients, the diagnosis was sporadic MTC in 146 (76.4%), FMTC in 14 (7.3%), MEN2A in 15 patients (7.9%), and MEN2B in one patient. The number of mutation carriers among 154 apparently sporadic MTC patients was 8 (5.2%). Ten patients were submitted to PTx out of twenty-four mutation carriers at a mean age of 35±19 years.

Conclusion: Turkish people have a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries. Despite free *RET* gene testing, the number of the PTx in Turkey is limited and relatively late in the life span of the carriers. This is mainly due to patient and family incomppliance and incomplete family counselling.

Keywords: Sporadic medullary thyroid carcinoma, hereditary medullary thyroid carcinoma, multiple endocrine neoplasia, RET mutation

Conflict of interest: None declared

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Introduction

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor of the thyroid gland and accounts for 5% of thyroid cancers. The tumor originates from parafollicular 'C' cells and secretes calcitonin (CT). MTC mainly occurs sporadically (70-75%) (1). Hereditary syndromes of germline RET proto-oncogene mutations are the cause of MTC in the remaining 25-30% of patients.

The RET proto-oncogene is located on chromosome 10 (10.q11.2). It is a member of the receptor tyrosine-kinase family and comprises 21 exons. 'C' cells of the thyroid gland, parathyroid glands, adrenal medulla, urogenital system all express RET proto-oncogene (2). Various germline mutations of RET proto-oncogene cause distinct clinical features and influence the course of the disease (3). Multiple endocrine neoplasia type 2 (MEN 2) and familial MTC (FMTC) are both autosomal dominant inherited hereditary cancer syndromes.

MEN2A accounts for 80% of hereditary MTC syndromes; consists of MTC in all patients, pheochromocytoma in 50% and primary hyperparathyroidism in 20-30% of patients (4). Cutaneous lichen amyloidosis (10%) and Hirschprung disease (7%) may also develop in MEN2A patients (5,6). MEN2A prevalence is estimated to be 1 per 50.000 and age of diagnosis is usually 20-30 years. *De novo* mutations may be responsible in up to 5% of MEN2A patients (7). Codon 634 mutations are the most frequent mutations in many of European countries (8,9).

MEN2B is characterized with MTC, pheochromocytoma, mucosal neuromas, ganglioneuromatosis of the gut, and marfanoid habitus. This syndrome is caused by autosomal dominant genetic inheritance and *de novo* mutations equally (10). Prognosis of MEN2B is worse than MEN2A since MTC is more aggressive (11).

FMTC is classically characterized by only hereditary predisposition to MTC for at least three generations (12).

Germline mutations of the RET proto-oncogene are found in 98% of MEN2A, 95% of MEN2B, and in 88% of FMTC

patients (13). Germline RET mutations can also be found in 7-10% of apparently sporadic forms of MTC by routine RET screening (14,15). Hence, only genetic testing could rule out hereditary forms of MTC.

Genetic screening of germline RET mutations is also the means for the early diagnosis of hereditary cases. Prophylactic thyroidectomy (PTx) is the only preventive option for mutation carrier family members. The association between RET mutations (genotype) and the biological behavior of tumor (phenotype) is well documented (3). Current guidelines recommend to consider risk stratification, mainly depending on the type of the mutation, in decisions on the timing of PTx (16). Age, family history, and basal/stimulated serum CT levels are the other factors which may have impact on the timing of PTx. In 2009, American Thyroid Association (ATA) recommended a 4-level risk classification for RET mutation carriers. PTx was recommended in the first year of life for MEN2B (M918T, A883F), before 5 years of age for subjects with level C (634 mutations) and also for level B (609, 611, 618, 620, 630, 631 mutations). The lower risk category (level A) involves distal codons (768, 790, 791, 804, 891). Family history of tumor behavior and family preference for these rare mutations with low genetic penetrance also need to be considered in decisions for surgery. However, there is no consensus on the management of these subjects (6,16,17,18).

This retrospective multicenter study aimed to evaluate the impact of free RET proto-oncogene testing of MTC patients. All data were evaluated in one center. The study was supported by the Society of Endocrinology and Metabolism of Turkey (SEMT). Adequacy of early diagnosis, surgical timing, and frequency of PTx in mutation carriers were also assessed.

Methods

Genetic testing for MTC and pheochromocytoma was conducted between July 2008 and January 2012 in 512 patients. Application forms and RET mutation analyses of these 512 patients whose blood samples were sent from various

centers around Turkey were assessed retrospectively. Patients with pheochromocytoma without detected mutations and RET negative relatives of hereditary MTC patients were excluded. A total of 319 patients with familial/sporadic MTC, MEN2, and mutation carriers from known MEN2/FMTC families were found to be eligible for retrospective analysis. An evaluation form including information on the surgical history, latest CT levels, survival of the patients, and PTx had been prepared and sent to several physicians working in centers around the country. Twenty-five centers responded by filling in the forms of 192 patients.

Patients who were considered to be sporadic cases by their physicians before mutation analysis were categorized as 'apparently sporadic' cases. After the final analysis of the RET mutations and of the evaluation forms, the final diagnoses were categorized as MEN2A, familial/sporadic MTC, and mutation carriers from MEN2A/FMTC families.

The study was approved by the Local Ethics Committee of Ankara University Faculty of Medicine (September 5, 2012).

RET Mutation Analysis

RET mutation analyses were performed in Düzen Laboratory Groups. Genomic DNA from patients was isolated from peripheral blood leukocytes automatically by MagNA Pure LC 2.0 (Roche Applied Science). *RET* gene exons 1-20 were amplified by polymerase chain reaction (PCR) using previously reported primers flanking the intron-exon junctions. PCR amplification of the exons 1-20 was carried out in a 25 µl PCR mix containing 5 ng genomic DNA, 0.1 µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂, and 1.25 U Taq polymerase (19,20). PCR [touchdown (TD) TD-PCR] was carried out. TD-PCR cycling program was: initial denaturation at 95 °C for 15 min followed by 10 cycles of 1 min at 95 °C, 1 min at 62 °C with an 0.5 °C decrease of temperature per cycle and 1 min at 72 °C and an additional 25 cycles of 1 min at 95 °C, 1 min at 57 °C, 1 min at 72 °C and 10 min at 72 °C for final extension. The amplicons were analyzed in ethidium bromide-stained agarose gels and showed a single band with expected size. Then, amplicons were sequenced in an automated sequencer (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The sequence data was analyzed by using SeqScape version 2.7 (Applied Biosystems) and Sequencing Analysis version 5.1 (Applied Biosystems) softwares. The results were interpreted by a professor in medical genetics.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software, version 20.0 (IBM Corp, NY, and USA). Categorical data were compared using the chi-square Fisher exact test. Group data with a normal distribution were compared using the Student t-test or analysis

of variance, and nonparametric data were compared using the Mann-Whitney U or Kruskal-Wallis tests. Values were expressed as mean ± standard deviation or median as appropriate. A p-value <0.05 was considered statistically significant.

Results

Figure 1, organized as a flow chart, depicts the numbers of patients included in the study at each step of the analysis. Between 2008 and 2012, among the 319 patients with hereditary/sporadic MTC whose blood samples were sent for RET analysis, mutation was detected in 71 patients (22.3%). Codon 634 mutation was detected in 39 (54.9%) patients. Cys634Arg was the most prevalent mutation (n=31) and accounted for 43.7% of all mutations. Cys634Tyr and Cys634Gly mutations were detected in 6 patients (8.5%) and in 2 patients (<1%), respectively. Val804Met germline mutation accounted for 25.4% of mutations. Distribution of RET mutations is summarized in Table 1.

Among the 192 MTC patients with available clinical information, 146 patients had sporadic MTC, 15 patients MEN2A, 14 patients FMTC, and one subject had MEN2B. Sixteen patients were mutation carriers from known families. However, so-called FMTC patients may not fulfill the diagnostic criteria yet since three generations of follow-up is mandatory (12).

Distribution of RET proto-oncogene mutation in 191 patients with available clinical information is summarized in Table 2, and distribution of RET mutations according to final diagnosis is summarized in Table 3. One MEN2B patient with Met918Thr mutation was excluded before statistical analyses.

RET mutation was detected in 8 of 154 patients (5.2%) defined as 'apparently sporadic' cases. Among these patients, three had Cys634Arg, three Val804Met, one Cys618Ser, and one subject had Y790Phe mutation.

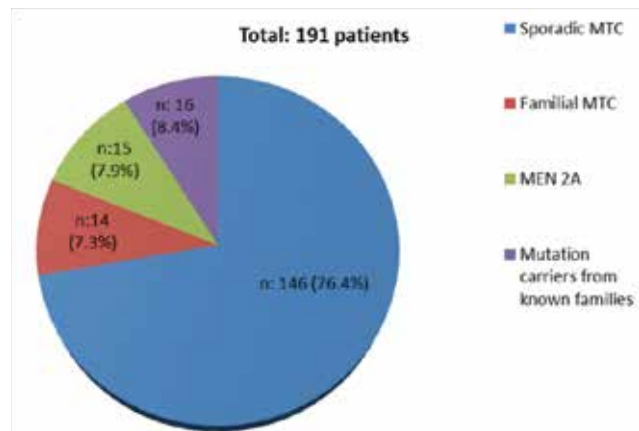


Figure 1. The distribution of diagnosis for 191 medullary thyroid carcinoma patients with available clinical information (multiple endocrine neoplasia type 2 B patient was excluded). MTC: Medullary thyroid carcinoma, MEN2A: multiple endocrine neoplasia type 2 A

Table 1. Distribution of RET proto-oncogene mutations in 71 out of 319 patients with hereditary sporadic medullary thyroid carcinoma or pheochromocytoma who had been tested for RET proto-oncogene

	N	%	Frequency of mutations % (n=71)
Cys634arg	31	9.7	43.7
Val804Met	18	5.6	25.4
Cys634Tyr	6	1.8	8.5
Tyr791Phe	4	1.2	5.6
Cys618Ser	3	<1	4.2
Met918Thr	2	<1	2.8
Cys634Gly	2	<1	2.8
Y790Phe	1	<1	1.4
Tyr790Phe	1	<1	1.4
Leu790Phe	1	<1	1.4
Ser891Ala	1	<1	1.4
D631Y	1	<1	1.4
Total	71	22.3	100.0

Table 2. Distribution of results of the RET proto-oncogene testing in 191 patients with available clinical information

	N	%	Frequency in mutations % (n=45)
No mutation	146	76.4	-
Cys634Arg	15	8.0	33.5
Cys634Tyr	7	3.6	16.0
Val804Met	15	8.0	33.5
Cys618Ser	2	1.0	4.0
Y790Phe	3	1.5	7.0
Tyr791Phe	2	1.0	4.0
D631Y	1	0.5	2.0
Total	191	100.0	100.0

Table 3. Distribution of RET mutations according to final diagnosis

Diagnosis	Cys634Arg	Cys634Tyr	Val804Met	Cys618Ser	Tyr791Phe	Y790Phe	D631Y
MEN2A	9	2	1	2	1	-	-
FMTC	2	-	11	-	-	1	-
Mutation carrier	4	5	3	-	1	2	1

MEN2A: multiple endocrine neoplasia 2 A, FMTC: familial medullary thyroid cancer

Mean age at diagnosis was similar in the two genders (44.2±14 years in females vs. 44.6±13.6 years in males, p=0.7), but was significantly lower in the patients diagnosed with genetic testing compared to those diagnosed with classical clinical features (31±18.2 years vs. 46.5±12.7 years, p<0.01). Mean age at diagnosis was also not statistically different between sporadic MTCs (47.6±1.05 years) and FMTCs (41.3±2.9 years) (p>0.05). MEN2A patients were younger (35.6±2.78 years) than sporadic and FMTCs at presentation (p<0.01) (a summary of preoperative clinical data is given in Table 4).

Mean follow-up period was 40±27 months in the whole group. The stage of tumor at diagnosis and preoperative CT levels are summarized in Table 4. Preoperative CT level was significantly higher in sporadic cases compared with the MEN2A and FMTCs (p<0.05) (Table 4).

Follow-up data was available for 138 patients. On their most recent follow-up visit, 80 patients (58%) were in full remission, 21 patients (15%) had locoregional disease (17 operable, 4 inoperable) and 29 (21%) had distant metastases. Seven sporadic and one MEN2A patient (6%) died due to distant metastases.

Sorafenib was the most preferred chemotherapeutic drug in metastatic patients (n=14). Conventional chemotherapeutics were chosen for 9 metastatic patients.

The mean number of surgical procedures was not statistically different between sporadic (1.67±1.05) and hereditary cases (1.31±1.5) (p=0.09).

Table 4. Preoperative clinical data of patients*

	Sporadic MTC	MEN2A	FMTC	p
Age (Mean, years)	47.6±1.05	35.6±2.78	41.3±2.9	p<0.01
Stage at diagnosis				
Stage I	41	6	8	NS
Stage II	7	2	0	NS
Stage III	17	4	3	NS
Stage IV	26	1	2	p<0.01
CT (mean, pg/mL)	1072±1102	704±594	254±569	p<0.05

MTC: Medullary thyroid carcinoma, CT: calcitonin, FMTC: familial medullary thyroid carcinoma, MEN2A: multiple endocrine neoplasia type 2 A, NS: non significant
*Tumor stage at the time of diagnosis was available for 117 patients

Table 5. Mutations and age of the patients with known RET mutation who had not yet undergone thyroid surgery

Patient	Age (years)	RET mutation	Patient	Age (years)	RET mutation
1-H.K.	29	Val804Met	8-A.K.	7	Cys634Tyr
2-A.K.	27	Val804Met	9-K.K.	57	Cys634Tyr
3-B.A.	17	Tyr791Phe	10-E.P.	4	Cys634Arg
4-M.B.	34	Val804Met	11-I.B.	47	Tyr791Phe
5-Ü.Y.	33	Tyr791Phe	12-M.K.	53	Cys634Tyr
6-S.E.	23	Cys634Arg	13-N.K.	56	Cys634Tyr
7-H.E.	46	Cys634Arg	14-D.Ö.	5	Cys634Arg

A significant number of mutation carriers (n=14) had not been operated yet although RET mutation carrier status was known at least for a year. Three out of fourteen subjects had elevated basal CT levels at the time of the study (Table 5). PTx or total thyroidectomy with central lymph node dissection was performed only in 10 out of 24 patients (41.6%). Mean age at thyroidectomy was 35±19 (12-60) years. Our study population included only five pediatric cases, and three of them had not undergone PTx although they had Codon 634 mutation.

Discussion

This retrospective multicenter study aimed to evaluate the impact of complimentary RET proto-oncogene testing in MTC patients. The timing of the surgical intervention, adequacy of diagnosis, treatment in familial/sporadic MTCs, and frequency of PTx for mutation carriers were also assessed.

More than 145 germline mutations of RET proto-oncogene have been identified during the past 20 years, and it has been shown that common mutations are localized in eight exons (exons 5, 8, 10, 11, 13, 14, 15, 16) (21). Previously, the approach for the screening of family members of this autosomal dominant inherited disorder consisted of repeated analyses of basal and/or stimulated CT measurements. In 1993, two different groups identified RET proto-oncogene mutations as the cause of hereditary cases and MEN2 syndromes, and RET genetic testing has since become the method of screening gradually (22,23). The prognosis of MTC is not favorable compared to differentiated thyroid carcinomas. Tumor stage at the time of diagnosis is the most important prognostic factor (24). Curability of metastatic disease is quite difficult. When possible, early diagnosis and treatment is the cornerstone of the management.

In asymptomatic carriers, mutation analysis facilitates early diagnosis and treatment of the disease. The ATA guidelines recommend the use of a four-level risk classification for RET mutations (16). Risk categories are important to decide the timing of PTx for mutation carriers. Although the biological behavior of the disease is usually comparable within the families, it can occasionally be variable within the same family (25,26).

It is well-known that early PTx reduces cancer mortality to lower than 5% in MEN2A patients (27). The primary goal of early prophylactic surgery is to prevent lymph node

metastases. Unfortunately, cervical lymph node metastases are found in 70% of patients when the nodule becomes palpable (28). On the other hand, surgery at early ages is associated with increased morbidity (29). Thus, annual follow-up of stimulated CT has been suggested to guide the timing and extent of surgery. Elisei et al (30) achieved cure in RET mutation carriers who were treated early after stimulated CT levels were detected to be elevated. However, in a recent study, it has been reported that basal and stimulated CT levels failed to detect MTC before surgery in three of the 31 (10%) mutation carriers (31). European Multiple Endocrine Neoplasia (EUROMEN) study also demonstrated that 75% (12/16) of codon 634 mutations developed MTC before the age of five. Thus, early diagnosis with RET proto-oncogene testing, risk stratification, and stimulated CT levels are the mainstay for the timing of PTx in hereditary cases.

Our results in this retrospective analysis showed that, although free genetic testing is available with the courtesy of SEMT and conducted centrally by the society itself, the number of PTx is still low in Turkey and the timing of surgical intervention is late. Current data showed that prophylactic/total thyroidectomy was performed only in 10 patients out of 24 mutation carriers identified and mean age of hereditary cases at surgery was relatively late [35±19 years (12-60)]. Fourteen of the known mutation carriers had not yet undergone surgery at the time of the study due to patient and family incompliance and/or incomplete family counselling. Unfortunately, people deny genetic diseases, parents still have hesitations about surgical interventions for their children, especially during younger ages, and healthcare professionals may not be able to overcome these problems.

MEN2A is frequently caused by the mutations in codons 634, 620, 618, 611, 609. Mutations at codon 634 of exon 11 account for 85% of cases and approximately half of them are cysteine to arginine amino acid substitution (Cys634Arg) (32). In FMTC, more than 85% of patients have mutations in exons 10 and 11 (22). Other rare mutations were described in exon 13 (codons 768, 790, 791), exon 14 (codons 804,844) and exon 15 (codon 891).

This is the largest mutation analysis ever performed in Turkish population and most common RET proto-oncogene

mutation reported was codon 634 mutation (54.9%). Val804 mutation was also an important mutation, accounting for 25.4% of RET mutations in Turkish population, especially in FMTC patients (79%) although some of these patients did not fulfill the diagnostic criteria yet. Codon 804 mutation is considered to be a low risk in ATA 2009 guidelines, and individual-, patient-, and family-based management is suggested. In our series, 11 patients with FMTC had Val804Met mutation and they underwent surgery at a mean age of 47.2 ± 10.9 . Two patients had local recurrence, one had metastatic disease, and despite relatively late age of surgery, the remaining eight subjects were in remission. Surgical technique could of course be questioned in the local recurrence. Thus, our results are concordant with ATA guidelines, where distal codons (768, 790, 791, 804, 891) were categorized as lower risk mutations with low genetic penetrance.

EUROMEN multicenter study showed that the most prevalent RET mutation in the European population was Cys634 with 67.6% frequency (6). The multicenter ItaMEN network analysis demonstrated that codon 634 at exon 11 was the most affected codon (34.8%) followed by Val804Met mutation (19.6%) among Italian MEN2 syndromes (33). Val804 mutations were threefold frequent in Italian and French when compared to German families. These data are concordant with the results in our series since Val804Met mutations are also prevalent in Turkey (25.4%). Sánchez et al (34) reported that the most frequent RET mutation in MEN2A Spanish families was C634Y, occurring in 73% (22/30) of cases and this finding was attributed to founder effect. A recent German study also demonstrated that RET mutations were distributed fairly similarly among German, French, and Italian families. The mutations in codon 634 were the most prevalent (36%), and codon 790 mutations were also frequent in Germany compared to Italy and France (13% vs. 4% and 4%) (35). In our series, codon 790 mutations were very rare, accounting for 1.4% of all mutations. Other studies from China, India, and Korea also demonstrated that Codon 634 mutations accounted for 60-80 % of hereditary cases. Interestingly, Val 804 mutation was not reported in China (36) and Korea (37) and had a lower frequency in a small Indian study (38) compared to current and other European studies (7% vs. 25.4%) (33,35).

Another important finding in accordance with previous findings of our group and of the others is that genetic testing revealed eight mutation carriers (hereditary cases) (5.2%) among 154 clinically diagnosed as sporadic MTCs (13,14). This data underlines the necessity of genetic screening for all MTC patients (15,16). On the other hand, frequency of mutation carriers among apparently sporadic cases was lower than in the previous TURKMEN study (10.7%), presumably due to increased awareness and genetic testing among physicians in our country. Current guidelines recommend genetic testing for all MTC cases

and if positive, clinical screening for pheochromocytoma and primary hyperparathyroidism (16,21). In the rare situation when the clinical criteria for hereditary syndromes are present but RET mutation analyses are negative, clinical screening of siblings is also recommended. As a cost-effective approach, ATA and ETA recommend to test MEN2-specific exons (i.e. exons 10,11) principally and if found negative, continue testing for exons 13, 14, and 15 subsequently (16,21).

The mean number of surgical procedures were higher in our sporadic cases compared to familial cases, but no statistically significant difference was observed. These findings may reflect delayed timing of diagnosis and small number of PTx for hereditary cases.

This study shows that the Turkish people has a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries such as Italy and France. Despite complementary *RET* gene testing made possible by the courtesy of SEMT, the number of the PTx in Turkey is limited and the surgical intervention is performed at a relatively late stage in the lifespan of the gene carriers. This is mainly due to patient and family incomppliance and incomplete family counselling. Healthcare professionals seem to be unsuccessful to overcome these problems for the moment. Physicians and health authorities should be aware of this situation. Legal measures could be considered for the families who refuse healthcare for their children.

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Ethics

Ethics Committee Approval: It was taken (Ethical Committee of Ankara University Faculty of Medicine, September 5, 2012), Informed Consent: It was taken.

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