

Research Article

A Decade Mutational Screening: Exon 16 of *RET* Proto-Oncogene Mutations in Medullary Thyroid Carcinoma

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Abstract

Objective: Medullary Thyroid Cancer (MTC) is a rare neuroendocrine tumor and accounts for around 5% of all cases of thyroid cancer. Approximately 75% of cases are sporadic, and 25% are hereditary (Multiple Endocrine Neoplasia [MEN] 2A, MEN2B, and Familial [F] MTC). The REarranged during Transfection (*RET*) proto-oncogene is known to be a genetic cause of MTC, and it seems MTC is the most prevention among endocrine cancer. Therefore, the objective of this study was to identify, and quantify the frequency of, germline mutations in exon 16 of the *RET* proto-oncogene.

Methods: A total of 422 participants were assessed. Genomic DNA was extracted from leukocytes using the standard salting out/proteinase K method, and mutation detection was performed using PCR and direct DNA sequencing.

Results: In total, three single nucleotide polymorphisms and one mutation were identified in the study population. As expected, the M918T mutation was found only in MEN2B cases. Cysteine to adenine substitution at position 10:43617610 were found in one FMTC family, with C611Y mutation at exon 10. This had not been previously reported and established in the NCBI BioSystems Database.

Conclusion: The data obtained showed the frequency profile of *RET* mutations in exon 16 among Iranian individuals with MTC and their relatives. Determination of the specific genetic mutation can guide patient management and screening.

Keywords: Medullary Thyroid Carcinoma; Multiple Endocrine Neoplasia type 2B; *RET* proto-oncogene; Exon 16

Abbreviation

RET: REarranged during Transfection; MTC: Medullary Thyroid Carcinoma; sMTC: sporadic MTC; FMTC: Familial MTC; MEN2A: Multiple Endocrine Neoplasia 2A; MEN2B: Multiple Endocrine Neoplasia 2B; PCR: Polymerase Chain Reaction

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence has risen in recent decades. In 2015, SEER has reported which Thyroid cancer represents 3.8% of all new cancer cases in the United State (SEER1). It is classified into four main histological groups: Papillary Thyroid Cancer (PTC), Follicular Thyroid Cancer (FTC), Medullary Thyroid Cancer (MTC), and anaplastic, thyroid carcinoma [1-3].

MTC is a rare neuroendocrine malignancy that accounts for up to 5% of all thyroid cancers, but it's morbidity and mortality remain high if untreated. It occurs in sporadic and familial forms. The familial forms have been subdivided into two distinct syndromes: multiple endocrine neoplasia (MEN 2A; 55% of all cases), (MEN 2B; 5%-10%) syndromes and familial MTC (FMTC; 35%-40%) [4-8].

MTC is generally the first manifestation of MEN2A because of its higher penetrance. Unilateral or bilateral pheochromocytoma (>50% of cases) and hyperparathyroidism (15-30% of cases) are the other

manifestations [8].

MEN2B is characterized by the earlier (usually 10 years earlier than that for MEN2A) occurrence of more aggressive MTC, pheochromocytoma (40-50% of patients), marfanoidfacies, ganglioneuromatosis, and gastrointestinal disorders [8-10].

FMTC refers to an occurrence in which MTC is the only manifestation. In order to diagnose FMTC, it is essential to demonstrate the absence of primary hyperparathyroidism and pheochromocytoma in one or two generations within a family, or provide evidence of an identified *RET* mutation in families with FMTC [11,12].

Germlinemissense, and point mutations on chromosome 10q 11.2 are associated with all variants of the MTC phenotype. *RET* is exactly located on mentioned location with 21 exons, and encodes a plasma membrane-bound tyrosine kinas. The gene is an abbreviation of REarranged during Transfection and was so named after it was found to be rearranged during transfection with DNA from lymphoma cells in 3T3 cell lines [6,13].

The *RET* protein is composed of three domains: extracellular, transmembrane, and intercellular. *RET* point mutations mostly affect exons 10, 11, and 16, and less common mutations are found in exon 5, 8, 13, 14, and 15 [7]. *RET* point mutations in the extracellular domain involving codons 609, 611, 618, and 620 of exon10, and 634 of exon

Table 1: The established Ensembl nucleotide changes in Exon16 of the *RET* proto-oncogen.

| Residue | Variation ID | Type | Phenotype | Alleles | Residues | Codons | Polyphen* |
|---------|-------------------------------|--------------------|---|---------|----------|----------|-----------|
| 914 | rs375963128 | Synonymous variant | | A/G | P | CCA, CCG | - |
| 918 | rs377767442 | Missense variant | MEN2B | A/G | M, V | ATG, GTG | 0.998 |
| 918 | rs74799832 | Missense variant | MEN2B, Sporadic medullary, pheochromocytoma | T/C | M, T | ATG, ACG | 1 |
| 919 | COSM970 | Missense variant | | C/T | A, V | GCA, GTA | 0.989 |
| 919 | COSM1347819 | Synonymous variant | | A/G | A | GCA, GCG | - |
| 920 | rs527787676 | Missense variant | | A/G | I, V | ATT, GTT | 0.962 |
| 921 | COSM20889 | Missense variant | thyroid | G/A | E, K | GAA, AAA | 0.982 |
| 922 | rs377767432 | Missense variant | | C/A | S, Y | TCC, TAC | 1 |
| 923 | COSM48745 | Missense variant | lung | C/G | L, V | CTT, GTT | 1 |
| 925 | COSM1347821 | Missense variant | Large intestine | A/G | D, G | GAT, GGT | 0.998 |
| 925 | COSM969 | Missense variant | Adrenal gland | G/C | D, H | GAT, CAT | 0.954 |
| 930 | KinMutBase_RET_DNA:g.45941C>T | Missense variant | | C/T | T, M | ACG, ATG | 0.997 |
| 932 | COSM1347822 | Missense variant | Large intestine | G/C | S, T | AGT, ACT | 1 |

*Polyphen: a tool which predicts the variation effect on protein function based on physical and comparative considerations.

11 lead to the MEN2A and FMTC phenotypes [7,14]. Approximately 95% of MEN2B cases occur via the replacement of methionine with threonine in codon 918 [10,4]. Two somatic missense mutations, S922P and T930M, in exon 16 of the *RET* proto-oncogene were detected in two sporadic MTC cases [15]. Table 1 shows the all nucleotide changes in exon 16. The polyphen score of nucleotide changes in codon 922, 923, 932 are as high as M918T. Some of them strictly correlate with the aggressiveness of MTC and is associated with a peculiar clinical phenotype [16].

Genetic testing for germline mutations in the *RET* proto-oncogene is now available and today is the basis for MTC screening procedures, meaning that molecular biology allows early identification of carriers of *RET* proto-oncogene germline mutations that will develop into MTC later in life. Early prophylactic thyroidectomy must be considered in these individuals to ensure definitive cure. In fact, early thyroidectomy may decrease mortality rate of MTC patients [17].

Multiple aggressive primary tumors develop at a very young age, and MTC (primarily MEN2B) tends to metastasize at an early stage, so identification of gene mutation carriers is highly important. Mutational screening can be performed easily at any age, using a blood sample, and it leads to the reduction of MEN2 morbidity and mortality.

The objective of the present study was to identify, and quantify the frequency of, germline mutations in exon 16 of the *RET* proto-oncogene, in order to aid in diagnosis and identify prognostic variables in Iranian MTC patients and their families.

Materials and Methods

Subjects

The Research Institute for Endocrine Sciences of Shahid Beheshti University of Medical sciences is a referral center for molecular testing of germline *RET* mutations in Iran. The individuals referred for molecular investigation were invited to participate in the *RET* mutational screening in exons 2, 3, 5, 8, 10-19 in MTC patients and their relatives study which was approved by the Institutional Review

Board and Ethics Committee of the Research Institute. The current study is a part of mentioned study.

MTC was confirmed by pathological reports. If any participant was found to be a carrier of a known mutation in exons 10, 11, 13-16 of *RET*, their relatives were invited to contribute to this study. If there was no history of a first- or second-degree relative with MTC or pheochromocytoma, MTC was considered to be of a sporadic (s) type. Over the last decade to date, 242 people with MTC have been referred. They included four MEN2B, nine MEN2A, 39 FMTC, six pheochromocytoma, and 184 apparent cases of sMTC.

In all participants, data relating to family history, mode of presentation, treatment, pathology, and screening of family members were collected.

DNA Extraction and PCR

Blood samples were used to identify *RET* germline mutations. Genomic DNA was extracted from peripheral whole blood leucocytes using a standard salting out proteinase K method, and was then amplified using the polymerase Chain Reaction (PCR); The PCR reaction was performed using a Ready mix (ZellBio, Germany) in a 25µL mixture containing 100ng DNA, 200µM dNTP, 0.8U Taq DNA polymerase, 8mM Tris-HCl (pH 9.0), 24mM KCl, 1.2mM MgCl₂, 1µl (10 pm/µ) of each specific primer, and 22µl distilled water. Two oligonucleotide primers for exon 16, 16F (5'- GTGCCAGGAGTGTCTACCA -3') and 16R (5'- CAGGACCACAGGAGGGTAAC -3'), were used and 30 cycles (of 92°C, 57°C, and 72°C) were performed.

The 560-base pair PCR product was electrophoresed on an 8% polyacrylamide gel. Mutation analysis was followed by direct nucleotide sequencing of the PCR products via an ABI 3100 Genetic Analyzer and Big Dye Terminator v3.1 Cycle Sequencing Kit, which were used as per the manufacturer's instructions (Applied Biosystems, California, USA).

Data analysis

The sequencing result files were analyzed and aligned to the *RET* reference sequence with Chromas version 2.33 software and NCBI

Table 2: Demographic features grouped by MTC phenotypes in Exon16 of the *RET* proto-oncogen.

| MTC phenotype | Frequency (n) | frequency | Age (mean \pm SD) | Female (n) | Male (n) | Female:male ratio | Hyperthyroidism (n) | Hypothyroidism (n) | Goiter (n) |
|------------------|---------------|-----------|---------------------|------------|----------|-------------------|---------------------|--------------------|------------|
| sMTC | 184 | 75.8% | 42.85 \pm 13.11 | 110 | 74 | 1.5:1 | 5 | 12 | 5 |
| FMTC | 39 | 16.1% | 31.89 \pm 13.32 | 21 | 18 | 1.2:1 | 1 | 3 | - |
| MEN2A | 9 | 3.8% | 40.67 \pm 9.76 | 5 | 4 | 1.2:1 | 1 | - | - |
| MEN2B | 4 | 1.7% | 14.00 \pm 2.94 | 2 | 2 | 1:1 | - | - | - |
| pheochromocytoma | 6 | 2.5% | 19.50 \pm 6.62 | 2 | 4 | 0.5:1 | - | - | - |

RET: Rearranged during Transfection; MTC: Medullary Thyroid Carcinoma; sMTC: sporadic MTC; FMTC: Familial MTC; MEN2A: Multiple Endocrine Neoplasia 2A; MEN2B: Multiple Endocrine Neoplasia 2B.

Blast, a web-based tool. PolyPhen-2 version 2.2.2 software was used for predicting percentage of protein damaging (Adzhubei et al. 2010).

Results

Analysis of germline DNA mutations of the *RET* gene and after excluding some samples due to poor quality DNA was conducted in 242 Iranian people with MTC and 180 relatives, giving a total of 422 participants. The patients included 58 with hereditary MTC and 184 with apparently sMTC (Table 2). As expected, the majority of patients were apparently affected by sMTC (75.8%), and the phenotype that was least observed was MEN2B (1.7%). The mean age of diagnosis for MEN2B cases was younger than for the other MTC types (14.00 \pm 2.94).

Although the female patients are more than males, there was no significant gender difference among MTC patients (139 vs. 104, $P > 0.05$).

RET sequencing revealed a mutation at codon 918 (exon 16) in four MEN2B out of 242 MTC cases, and this mutation leads to substitution of a methionine residue by threonine. The diagnosis of MEN2B patients was made on the basis of the pathological data and was then confirmed by the molecular data. Total thyroidectomy was performed in all participants.

The first case involving the MEN2B phenotype was that of a 14-year-old male who underwent total thyroidectomy at the age of 11 years. Unfortunately, we did not have sufficient clinical history with regard to his disease, and he died due to distant metastasis to the brain after referral to the institute.

The second case of MEN2B was that of a female who had had symptoms of chronic constipation and an inability to cry tears since birth. At the age of 14 years, she noticed swelling in her neck, upon which she underwent a total thyroidectomy, lymphadenectomy, and unilateral adrenalectomy. She remains alive, 7 years after surgery. Her family does not have any symptoms, and genetic screening (of her parents and one of her siblings) was negative. Therefore, it appears that this is a *de novo* index case.

The third case of MEN2B was that of a 13-year-old female with diarrhea, development of mucosal neuromas on the lips (bumpy lips), and kidney stones. She underwent total thyroidectomy, and is alive and well 12 years after surgery. The result of the pathological examination indicated in Figure 1.

The fourth MEN2B case was a 18-year-old male with Constipation and marfanoid facies. He underwent total thyroidectomy. His brother does not have any symptoms and his genetic screening was negative.

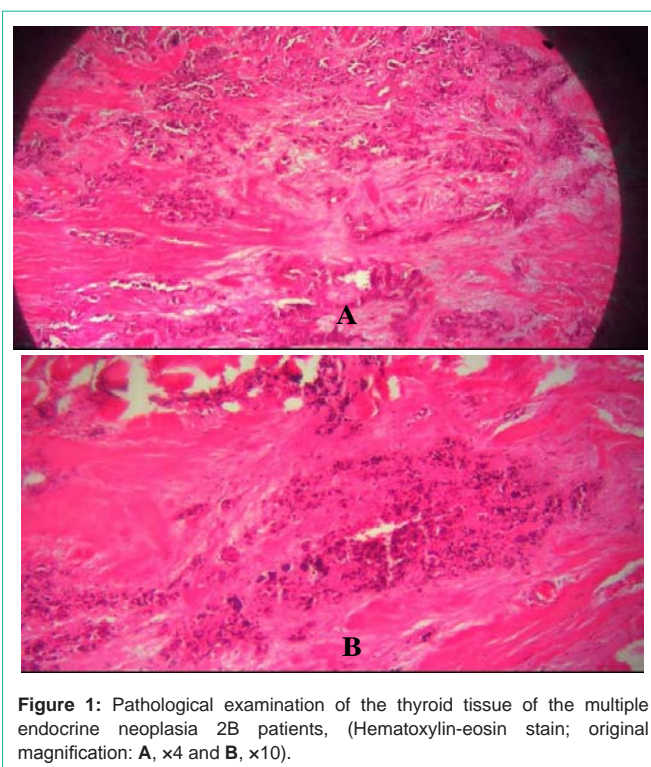


Figure 1: Pathological examination of the thyroid tissue of the multiple endocrine neoplasia 2B patients, (Hematoxylin-eosin stain; original magnification: **A**, $\times 4$ and **B**, $\times 10$).

In addition, three Single Nucleotide Polymorphisms (SNPs) were detected in the intronic region of intron 16: adenine to thymine substitution (rs3026772) at position 10:43617518, a guanine to cysteine substitution (rs3026774) at position 10:43617707, and a novel intron variant, a cysteine to adenine substitution at position 10:43617610, were found. Table 3 summarizes the molecular data.

Discussion

Mutational screening of exon 16 in the *RET* proto-oncogene showed M918T in 4 MEN2B patients and three different SNPs in the intronic region. We found the same missense mutation in the tyrosine kinase domain in all of the MEN2B cases.

The methionine at codon 918 is highly conserved and its mutation causes constitutive activation of tyrosine kinase activity, resulting in an increase in phosphorylation [18]. An association between M918T and MEN2B families has well demonstrated in other studies; Exon 16 mutation has been reported in 95% of MEN2B cases [19-21]. B Cosci and et al. described a new amino acid change at codon 918 that leads to substitution of a methionine residue by valine (ATG-GTG), although this was found at a low prevalence (1/103 families) [22].

Table 3: The distribution of nucleotide changes among MTC patients and their relatives in Exon16 of the *RET* proto-oncogene.

| | Base change | AA change | Genotype distribution (n) | | | Phenotype (n) | | | | | Patients/relative NO. |
|-------------|-------------|-----------|---------------------------|--------------|------------|------------------|------|-------|-------|------|-----------------------|
| | | | Wild type | Heterozygous | Homozygous | Pheochromocytoma | sMTC | MEN2B | MEN2A | FMTC | |
| rs377767442 | (ATG>ACG) | Met>Thr | 418 | 4 | - | - | - | 4 | - | - | 4/0 |
| Rs3026772 | (A>T) | - | 401 | 21 | - | - | 10 | - | 2 | 3 | 15/6 |
| Rs3026774 | (G>C) | - | 413 | 9 | - | - | 2 | - | 1 | 1 | 4/5 |
| | (C>A) | - | 415 | 6 | 1 | 1 | - | - | - | 3 | 4/3 |

RET: REarranged during Transfection; MTC: Medullary Thyroid Carcinoma; sMTC: sporadic MTC; FMTC: Familial MTC; MEN2A: Multiple Endocrine Neoplasia 2A; MEN2B: Multiple Endocrine Neoplasia 2B.

In some studies, mutations of codon 883 in exon 15 (A883F) have also been shown to occur in 2-3% of MEN2B cases; it has been identified as the highest-risk mutation, and is sometimes found without M918T mutation. However, A883F mutation appears to be a less aggressive form of MTC than that which is usually observed in patients with RET M918T mutation [19,21,23,24].

Some previous studies have suggested a high incidence of M918T in tumor tissues of Sporadic cases, as well as the involvement of other mutations, such as G911D, G921K, S922P, and T930M in exon 16 [25-27]. However, No mutation in sMTC cases was found in the present study, because the tumor tissues of the participants were not available. The difference in frequency of exon 16 and the variety of mutations in different populations could be due to ethnic and environmental factors, and/or differences in detection methods and type of sample.

In the present study, The M918T mutation was only detected in MEN2B proband, which is in accordance with the findings of other investigations. Its absence in their families may suggest that this mutation occurred as *de novo*, but it may also be due to the fact that MEN2B cases seldom reproduce. Detection of other mutations, such as those found in exons 10 and 11 of this proto-oncogene, in probands and their families revealed that these mutations are heritable [19,28,29].

The early occurrence of MTC means that the mean age of diagnosis of MEN2B cases who complained of their disease was 14.00 ± 2.94years, which is lower than the large survey of MEN2 pedigree carried out in a Chinese population (20.0 ± 8.1), so the life expectancy of MEN2B patients is reduced. It also shows that MTC in MENB is diagnosed early in Iran [30,31].

In addition to M918T, which is characteristic of the MEN2B phenotype, three SNPs in the intronic region were detected. A cysteine to adenine substitution at position 10:43617610 were found in one FMTC family, with a C611Y mutation at exon 10. This had not previously been reported and established in the NCBI BioSystems Database. With the exception of this family, it was found in only four unrelated cases. In addition, rs3026772 (A>T) and rs3026774 (G>C), which have been previously reported, were also detected [29].

The participants with 918 mutations are at the highest risk of MTC development. Age of onset in this group is the first year of life, and aggressive MTC occurs in 100% of cases of MEN 2B. Molecular analysis of the *RET* gene makes it possible to perform a prophylactic thyroidectomy in children [32,33].

In four MEN2B cases, the first clinical signs of MEN 2B, in the

gastrointestinal system, and then aggressive MTC, appeared. The inability to cry, which is found in children with MEN2B, was detected (in one case), in accordance with previous studies [33,34].

In conclusion, the rarity of the MEN 2B syndrome can lead to delayed diagnosis and to cases being missed. Therefore, early diagnosis and treatment is essential, especially in the first 6–12 months of life. Although MEN2B is often associated with typical physical features, this syndrome may not be properly recognized in some people, and may be followed as a sporadic case. Therefore, a molecular genetic test should be performed in all suspicious cases of MEN. This study analyzed the blood samples for detecting germline mutations in MTC patients and their first degree relatives. Additional study would be done on tissues samples for detecting the somatic mutations in sporadic cases.

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References

- SEER Cancer Statistics Factsheets: Thyroid Cancer. National Cancer Institute.
- Morris LG, Myssiorek D. Improved detection does not fully explain the rising incidence of well-differentiated thyroid cancer: a population-based analysis. *Am J Surg.* 2010; 200: 454-461.
- Czene K, Lichtenstein P, Hemminki K. Environmental and heritable causes of cancer among 9.6 million individuals in the Swedish Family-Cancer Database. *Int J Cancer.* 2002; 99: 260-266.
- Sheikholeslami S, Zarif Yeganeh M, Hoghooghi Rad L, Golab Ghadaksaz H, Hedayati M. Haplotype Frequency of G691S/S904S in the RET Proto-Onco-gene in Patients with Medullary Thyroid Carcinoma. *Iran J Public Health.* 2014; 43: 235-240.
- Griebeler ML, Gharib H, Thompson GB. Medullary thyroid carcinoma. *Endocr Pract.* 2013; 19: 703-711.
- Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, *ret*, by DNA rearrangement. *Cell.* 1985; 42: 581-588.
- Öberg K. The genetics of neuroendocrine tumors. *Semin Oncol.* 2013; 40: 37-44.
- Falchetti A, Marini F, Luzi E, Tonelli F, Brandi ML. Multiple endocrine neoplasms. *Best Pract Res Clin Rheumatol.* 2008; 22: 149-163.
- King SK, Southwell BR, Hutson JM. An association of multiple endocrine neoplasia 2B, a RET mutation; constipation; and low substance P-nerve fiber density in colonic circular muscle. *J Pediatr Surg.* 2006; 41: 437-442.
- Majidi M, Haghpanah V, Hedayati M, Khashayar P, Mohajeri-Tehrani MR, Larijani B. A family presenting with multiple endocrine neoplasia type 2B: A case report. *J Med Case Rep.* 2011; 5: 587.

11. Diaz RE, Wohllk N. Multiple endocrine neoplasia: the Chilean experience. *Clinics (Sao Paulo)*. 2012; 67: 7-11.
12. Moraitis AG, Martucci VL, Pacak K. Genetics, Diagnosis, and Management of Medullary Thyroid Carcinoma and Pheochromocytoma/Paraganglioma. *Endocr Pract*. 2014: 1-34.
13. Brauckhoff M, Machens A, Hess S, Lorenz K, Gimm O, Brauckhoff K, et al. Premonitory symptoms preceding metastatic medullary thyroid cancer in MEN 2B: An exploratory analysis. *Surgery*. 2008; 144: 1044-1050.
14. Ferreira CV, Siqueira DR, Ceolin L, Maia AL. Advanced medullary thyroid cancer: pathophysiology and management. *Cancer Manag Res*. 2013; 5: 57-66.
15. Jindrichova S, Kodet R, Krskova L, Vlcek P, Bendlova B. The newly detected mutations in the RET proto-oncogene in exon 16 as a cause of sporadic medullary thyroid carcinoma. *J Mol Med (Berl)*. 2003; 81: 819-823.
16. Flícek P, Ahmed I, Amode MR, Barrell D, Beal K, Brent S, et al. Ensembl 2013. *Nucleic Acids Res*. 2013; 41: 48-55.
17. Puñales MK, Graf H, Gross JL, Maia AL. RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. *J Clin Endocrinol Metab*. 2003; 88: 2644-2649.
18. Eng C, Mulligan LM, Healey CS, Houghton C, Frilling A, Raue F, et al. Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. *Cancer Res*. 1996; 56: 2167-2170.
19. Raue F, Frank-Raue K. Genotype-phenotype correlation in multiple endocrine neoplasia type 2. *Clinics (Sao Paulo)*. 2012; 67: 69-75.
20. Jindrichová S, Vcelák J, Vlcek P, Neradilová M, Nemeč J, Bendlová B. Screening of six risk exons of the RET proto-oncogene in families with medullary thyroid carcinoma in the Czech Republic. *J Endocrinol*. 2004; 183: 257-265.
21. Smith DP, Houghton C, Ponder BA. Germline mutation of RET codon 883 in two cases of de novo MEN 2B. *Oncogene*. 1997; 15: 1213-1217.
22. Cosci B, Vivaldi A, Romei C, Gemignani F, Landi S, Ciampi R, et al. In silico and in vitro analysis of rare germline allelic variants of RET oncogene associated with medullary thyroid cancer. *Endocr Relat Cancer*. 2011; 18: 603-612.
23. Jasim S, Ying AK, Waguespack SG, Rich TA, Grubbs EG, Jimenez C, et al. Multiple endocrine neoplasia type 2B with a RET proto-oncogene A883F mutation displays a more indolent form of medullary thyroid carcinoma compared with a RET M918T mutation. *Thyroid*. 2011; 21: 189-192.
24. Elisei R, Cosci B, Romei C, Agate L, Piampiani P, Miccoli P, et al. Identification of a novel point mutation in the RET gene (Ala883Thr), which is associated with medullary thyroid carcinoma phenotype only in homozygous condition. *J Clin Endocrinol Metab*. 2004; 89: 5823-5827.
25. Dvorakova S, Vaclavikova E, Sykorova V, Vcelak J, Novak Z, Duskova J, et al. Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinomas. *Mol Cell Endocrinol*. 2008; 284: 21-27.
26. Rapa I, Saggiorato E, Giachino D, Palestini N, Orlandi F, Papotti M, et al. Mammalian target of rapamycin pathway activation is associated to RET mutation status in medullary thyroid carcinoma. *J Clin Endocrinol Metab*. 2011; 96: 2146-2153.
27. Mian C, Pennelli G, Barollo S, Cavedon E, Nacamulli D, Vianello F, et al. Combined RET and Ki-67 assessment in sporadic medullary thyroid carcinoma: a useful tool for patient risk stratification. *Eur J Endocrinol*. 2011; 164: 971-976.
28. Alvandi E, Akrami SM, Chiani M, Hedayati M, Nayer BN, Tehrani MR, et al. Molecular analysis of the RET proto-oncogene key exons in patients with medullary thyroid carcinoma: a comprehensive study of the Iranian population. *Thyroid*. 2011; 21: 373-382.
29. Hedayati M, Zarif Yeganeh M, Sheikhol Eslami S, Rezghi Barez S, Hoghooghi Rad L, Azizi F. Predominant RET Germline Mutations in Exons 10, 11, and 16 in Iranian Patients with Hereditary Medullary Thyroid Carcinoma. *J Thyroid Res*. 2011; 264248.
30. Unruh A, Fitze G, Janig U, Bielack S, Lochbuhler H, Coerdts W. Medullary thyroid carcinoma in a 2-month-old male with multiple endocrine neoplasia 2B and symptoms of pseudo-Hirschsprung disease: a case report. *J Pediatr Surg*. 2007; 42: 1623-1626.
31. Zhou Y, Zhao Y, Cui B, Gu L, Zhu S, Li J, et al. RET proto-oncogene mutations are restricted to codons 634 and 918 in mainland Chinese families with MEN2A and MEN2B. *Clin Endocrinol (Oxf)*. 2007; 67: 570-576.
32. Torre M, Martucciello G, Ceccherini I, Lerone M, Aicardi M, Gambini C, et al. Diagnostic and therapeutic approach to multiple endocrine neoplasia type 2B in pediatric patients. *Pediatr Surg Int*. 2002; 18: 378-383.
33. Frank-Raue K, Raue F. Multiple endocrine neoplasia type 2 (MEN 2). *Eur J Cancer*. 2009; 45: 267-273.
34. Lee NC, Norton JA. Multiple endocrine neoplasia type 2B--genetic basis and clinical expression. *Surg Oncol*. 2000; 9: 111-118.