CrossMark

ORIGINAL ARTICLE

Screening of *RET* gene mutations in Chinese patients with medullary thyroid carcinoma and their relatives

Junyi Wang[1] · Bin Zhang[2] · Wensheng Liu[2] · Yongxia Zhang[3] · Xuebing Di[4] ·
Yanmei Yang[2] · Dangui Yan[2]

Abstract The rearranged during transfection (*RET*) gene is a proto-oncogene; active mutations frequently occur in medullary thyroid carcinoma (MTC). This study investigated the spectrum of germline *RET* mutations and clinical features in Chinese hereditary MTC patients. A total of 53 family members from 11 different hereditary MTC families were recruited for detection of *RET* exon 8, 10, 11, 13, 14, 15, and 16 mutations, in genomic DNA from peripheral blood leucocytes using polymerase chain reaction (PCR) and direct DNA sequencing. Of the 53 participants, eight different germline RET mutations were detected in 37 individuals. These *RET* mutations were distributed in exons 10, 11, 13, and 16. The most frequent *RET* mutation was localized at exon 11 codon 634 (67.6 %; 25/37) and the most prevalent mutation was C634R (37.8 %; 14/37). The most frequent phenotype was multiple endocrine neoplasia type 2A (MEN2A). The incidences of MTC, pheochromocytoma, and hyperparathyroidism in the MEN2A patients were 100, 36.4 and 18.2 %, respectively. The phenotype of families with Y606C or L790F mutation was categorized as familial medullary thyroid carcinoma. Moreover, one proband was identified with multiple endocrine neoplasia type 2B and carried a de novo mutation of M918T. Two families with C618S/Y mutation were categorized as unclassified multiple endocrine neoplasia type 2. Our results further substantiate that most germline mutations of the *RET* proto-oncogene were localized at codon 634 in Chinese hereditary MTC patients and carriers. *RET* mutation at codon 634 was always associated to the phenotype of MEN2A. Screening of *RET* mutations should be probably limited to exons 10, 11, 13 and 16 initially to be cost-effective in China.

Keywords Medullary thyroid carcinoma · *RET* proto-oncogene · Germline mutation · Cancer · Thyroid

Introduction

Medullary thyroid carcinoma (MTC) is a relatively rare malignant thyroid tumor, accounting for <10 % of all thyroid cancers. Approximately 25 % of MTC represents inherited disease, while the rest is as a sporadic tumor [1–4]. MTC originates from the parafollicular cells (C cells) and produces the hormone calcitonin. MTC is a consistent feature of multiple endocrine neoplasia type 2A (MEN2A) and multiple endocrine neoplasia type 2B (MEN2B), and familial medullary thyroid carcinoma (FMTC), all of which are inherited as autosomal dominant traits. MEN2A is a cancer syndrome characterized as MTC, pheochromocytoma (PHEO), and hyperparathyroidism (HPT), while FMTC is a clinical variant of MEN2A with the presence of MTC only. MEN2B is characterized by MTC, PHEO, mucosal neuromas, ganglioneuromatosis of the gut, and a

✉ Bin Zhang
jzxsya@126.com

1   Key Laboratory of Cancer Prevention and Therapy of Tianjin, Department of Thyroid and Neck Tumor, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin 300060, China

2   Department of Head and Neck Surgery, Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China

3   Department of Otorhinolaryngology, Head and Neck Surgery, Tianjin Medical University General Hospital, Tianjin 300052, China

4   Department of Etiology and Carcinogenesis, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China

Marfanoid habitus. Hereditary MTC occurring with nearly complete penetrance is multifocal and bilateral, and occurs at an earlier age than sporadic ones. Germline mutations of the *RET* proto-oncogene were found to be responsible for development of MEN2 syndromes [5, 6]. Afterwards, a large number of cases with inherited MTC have been investigated for *RET* defects. The *RET* proto-oncogene is localized at chromosome 10q 11.21 and contains 21 exons to encode a transmemebrane receptor tyrosine kinase protein that is important in transferring cell growth and differentiation signals [7]. The RET receptor is primarily expressed in neural crest and urogenital precursor cells during fetal development. A single activating *RET* mutation in one allele could be sufficient to cause neoplastic transformation. To date, several genotype–phenotype correlation studies have identified the association between *RET* mutations and different MEN2 syndrome variants [8–10]. To date, the most common *RET* germline mutations causing MEN2A and FMTC syndromes affect exons 8, 10, 11, 13, 14 and 15 of *RET*, while a single germline *RET* mutation in exon 16 has been found in more than 95 % of MEN2B cases [10]. Thus, a genetic test for *RET* germline mutations is now available for screening and early detection of MTC. In many countries, such a test is suggested for all patients with a family medical history of MTC or MEN2.

Whereas current *RET* mutation data are primarily based on observations in Caucasian populations, studies are rare for other ethnicities, such as Chinese. Collection of such data is very important since the genetic background of Chinese populations is different and the natural course of various *RET* mutation carriers may also be different [11]. Thus, in this study, we performed a molecular analysis of MTC patients and their family members to collect such information that could be useful for early detection and screening of Chinese MTC patients by selected to assess RET mutations at exons 8, 10, 11, 13, 14, 15, and 16. Our selection of these mutation spots in this study was also cost-effectively considered although we will miss *RET* mutations in other spots.

Subjects and methods

Subjects

A consecutive series of 74 MTC patients were recruited from the Department of Head and Neck, Cancer Hospital (Beijing, China) between January 2011 and September 2014. MTC was diagnosed histologically according to previously published criteria [12]. Germline mutations of the *RET* proto-oncogene were assessed in all MTC patients. If germline mutations of *RET* were detected, the patient's

relatives were also invited for *RET* screening. The control group consisted of 60 healthy people without a history of thyroid cancer. Genetic counseling was provided for all participants. This study was approved by the Human Research Ethics Committee of the Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences and an informed consent form was obtained from all participants.

Methods

Genomic DNA was extracted from peripheral blood leucocytes and then subjected to PCR and detection of *RET* mutations. The primers of *RET* exon 8, 10, 11, 13, 14, 15, and 16 are shown in Table 1. PCR was performed in 40-μL mixtures of 100–200 ng of genomic DNA, 0.2–0.4 μM of each primer, 1 × PCR buffer with 1–2 mM of Mg^{2+}, and 0.062 U of Taq polymerase (Takara Bio Inc., Dalian, China). The concentrations of Mg^{2+} (Takara Bio Inc.), dNTPs (Takara Bio Inc.) and template DNA were adjusted specifically for detection of each exon. PCR conditions were set for a pre-heating cycle of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58–65 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. After that, 5 μL of each PCR product was separated on 1.5 % agarose using gel electrophoresis and a portion of PCR products from each case was sent to DNA sequence for both sense and antisense orientations using ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequence data were analyzed using a mutation surveyor version 4.0 (SoftGenetics, State College, PA, USA) and visual inspection.Student's *t* test was used to analyze differences in mean values.

Results

Characteristics of study population

According to clinical and molecular features, 63 out of 74 MTC patients were diagnosed as sporadic MTC and 11 patients as hereditary MTC; thus, we also collected data on 42 relatives of these 11 hereditary MTC patients.

Among all 53 family members of these 11 unrelated pedigrees (including 11 probands and 42 family members), *RET* mutations were detected in 37 (69.8 %). Among these carriers, only 30 were diagnosed as hereditary MTC; 7 of them were asymptomatic carriers of *RET* mutations. The mean age at diagnosis in the hereditary MTC patients was significantly higher than that of mutation carriers (34.7 ± 13.8 vs. 11.0 ± 5.7 years, $p < 0.05$), but lower

Table 1 Primers and PCR conditions for amplification of *RET* exons

Exon	Primer sequences	Size of PCR product (bp)	Annealing temperature (°C)
8	5′-TTGGGCACTAGCTGGACG-3′	427	60
	5′-ACCTTCCCAAGTCCAGAGT-3′		
10	5′-CAGAAAGGCACTGTGACCAA-3′	374	58
	5′-GGGACCTCAGATGTGCTGTT-3′		
11	5′-AGCCATGAGGCAGAGCATACG-3′	385	65
	5′-TGGGGAGGGCAGGGGATCTTC-3′		
13	5′-AGCCTCAAGCAGCATCGT-3′	341	65
	5′-GCAGTAGGGAAAGGGAGAAAG-3′		
14	5′-CCCTGGCTCCTGGAAGAC-3′	396	65
	5′-GGCTAGAGTGTGGCATGGTG-3′		
15	5′-GTCTCACCAGGCCGCTAC-3′	378	65
	5′-AGATTTGGGGTGAGGGCTAT-3′		
16	5′-TCTGTGCCCAGGAGTGTCTACA-3′	395	65
	5′-GCCATTTGCCTCACGAACA-3′		

than that of sporadic ones (34.7 ± 13.8 vs. 44.8 ± 10.9 years, $p < 0.01$).

RET mutations in the hereditary MTC families

In these 11 hereditary MTC families, there were eight different heterozygote missense germline point mutations identified in *RET* exons 10, 11, 13, and 16. We did not find any *RET* mutations in exons 8, 14, and 15. In addition, each of these mutation carriers harbored only a single point mutation in *RET*. We also did not have any evidence of double mutations or deletion/insertions in *RET* in this cohort of patients. The *RET* mutations identified were Y606C(Tyr-Cys), C618Y(Cys-Tyr), C618S(Cys-Ser), C634R(Cys-Arg), C634W(Cys-Trp), C634Y(Cys-Tyr), L790F(Leu-Phe), M918T(Met-Thr). Among mutation carriers, a total of 25/37 (67.6 %) individuals were detected with codon 634 mutations and the most prevalent mutation was C634R (37.8 %; 14/37). Meanwhile, there were no *RET* mutations detected in the control group.

Clinical characteristics of patients and association between the phenotype and genotype

The clinical features and molecular data of these 11 hereditary MTC pedigrees are summarized in Table 2. In brief, 25 familial members from I to VI pedigrees carried a *RET* proto-oncogene mutation at codon 634. Among them, 22 showed the clinical MEN2A phenotype and had MTC, while 8 (36.4 %) with PHEO and 4 (18.2 %) had HPT. To date, the other three carriers had no obvious clinical

symptoms and were diagnosed as carriers of *RET* proto-oncogene mutation.

Among the 22 MEN2A patients, only one patient (4.5 %) had MTC in combination with PHEO and HPT, and similarly, only one patient (4.5 %) had MTC solely, 20 other patients (91.0 %) had MTC with PHEO or HPT. Among these MTC patients, the mean age at diagnosis was 30.8 ± 9.1 years, 19 (86.4 %) cases were multifocal and 12 (54.5 %) cases had lymph node metastasis at initial diagnosis. Among the eight patients with PHEO, three (37.5 %) were bilateral, and five (62.5 %) were unilateral. Two patients who had bilateral involvement were among the four patients with HPT.

Furthermore, family VII and VIII had *RET* Y606C and L790F mutations, respectively. Each of these two families had three MTC patients in two generations, and they did not show other endocrine disorders. Based on clinical data and the less rigid definition of FMTC of the American Thyroid Association (ATA) [15], these patients were categorized as FMTC. Although the mutations were different, there is something in common among the patients. The age of all of these MTC patients at diagnosis was over 40 years, and none of them had lymph node metastasis.

In family IX, the proband was a 13-year-old female who had bilateral MTC in combination with evident mucosal neuromas on the lip and tongue, eyelid neuromas, reduced eyesight, and marfanoid habitus. This female was diagnosed histologically with Hirschsprung's disease at the age of 3 years. There was no evidence of adrenal abnormality. The patient carries the *RET* M918T germline mutation; however, the same mutation was not detected in her

Table 2 Clinical characteristics of the 11 MEN2 families and their *RET* mutation

Family	Age at diagnosis/gender of proband	No. of *RET*+/ members tested	MTC (multifocal)	LNM	PHEO	HPT	Age range at diagnosis[a]	Age range of asymptomatic carriers	*RET* mutation codon/amino acid substitution	MEN2 subtype
I	44/M	8/9	5 (4)	4	2	1	22–44	7–15	C634R	MEN2A
II	17/F	6/7	6 (5)	2	2	1	13–55	–	C634R	MEN2A
III	42/F	2/5	2 (2)	1	1	–	15–42	–	C634W	MEN2A
IV	25/M	5/5	5 (4)	2	2	1	25–35	–	C634Y	MEN2A
V	25/F	1/4	1 (1)	1	1	–	25	–	C634Y	MEN2A
VI	26/M	3/3	3 (3)	2	–	1	22–55	–	C634W	MEN2A
VII	47/F	6/9	3 (2)	0	–	–	47–53	15–54	Y606C	FMTC
VIII	43/F	3/3	3 (1)	0	–	–	43–50	–	L790F	FMTC
IX	13/M	1/3	1 (1)[b]	1	–	–	13	–	M918T	MEN2B
X	39/M	1/3	1 (1)	1	–	–	39	–	C618S	Unclassified
XI	22/M	1/2	1 (1)	1	–	–	22	–	C618Y	Unclassified

LNM lymph node metastasis, *MTC* medullary thyroid carcinoma, *PHEO* pheochromocytoma, *HPT* hyperparathyroidism, *MEN2* multiple endocrine neoplasia type 2

[a] All patients' initial symptom were MTC

[b] The patient also had Hirschsprung's disease, marfanoid habitus and mucosal neuromas

parents. This patient had the very aggressive tumor and had multifocal MTC with extensive lymph node metastasis.

In addition, we also found other types of *RET* germline mutations that were clearly associated with MEN2. For example, in families X and XI the index cases harbored the codon 618 mutation. In the two MTC patients, there was no evidence of adrenal or parathyroid abnormality. Moreover, their relatives undergoing genetic screening were not found to have any *RET* mutations. Therefore, these two families were categorized as unclassified MEN2. The two patients had multifocal MTC with lymph node metastasis at initial diagnosis.

Discussion

In this study, we analyzed *RET* mutations in 116 participants (74 MTC patients and 42 family members) and observed 37 individuals from 11 families with eight different *RET* mutations. These 37 *RET* mutations were distributed in *RET* exons 10, 11, 13, and 16. Most frequent *RET* mutations were at codon 634 in MTC patients and the most prevalent mutation was C634R among the carriers. The mutation at codon 634 was always associated to MEN2A phenotype. Moreover, one proband identified with MEN2B syndrome showed the characteristic phenotype and *de novo* mutation of M918T with very aggressive tumor. The phenotype of families with *RET* L790F or Y606C mutation was categorized as FMTC. The family

with *RET* C618S/Y was categorized as unclassified MEN2. Hereditary MTC patients were diagnosed at an earlier age than the sporadic ones. These data clearly indicate that *RET* mutations, especially at codon 634, are useful in screening and early detection of Chinese hereditary MTC.

Currently data on the distribution and rate of *RET* mutations are very limited in the Chinese population. A previous cross-sectional study that evaluated 119 family members from MEN2A pedigrees showed that *RET* mutations were restricted to codon 634 in Chinese families with MEN2A [13]. In our current study, the most common clinical subtype of MEN2 is MEN2A. Indeed, *RET* mutations in all of MEN2A patients were localized at codon 634, which is consistent with the previous study. The most frequent substitution at the codon 634 is to replace a cysteine with arginine change, followed by Cys634Tyr and Cys634Trp. This phenomenon was similar to studies on *RET* mutations in other races, such as Caucasian populations where Cys634Arg is the most prevalent [14, 15]. The typical age of onset of MEN2A is in the third or fourth decade of life [12], the risk of developing unilateral or bilateral PHEO is as high as 57, and 15–30 % of *RET* mutation carriers will develop HPT [16]. In this study, incidence of PHEO and HPT were similar to the previous reports. In addition, according to previous reports, individuals with *RET* mutation in codon 634 were at high risk of developing PHEO, but not related to any specific base mutation. The development of HPT was closely related to *RET* mutation in codon 634, but not to in codon 609, 611,

618, 620, 790 or 791 [17, 18]. The findings of this study are in agreement with these reports, i.e., PHEO occurred with *RET* codon 634 mutation in different genotypes and two patients with HPT carried the C634R or C634W mutation.

Furthermore, FMTC is characterized as a simple MTC without other endocrine neoplasms. MTC in these patients tends to be the least aggressive, with a later age of onset and earlier stage at diagnosis. Genetically, FMTC is most commonly associated with *RET* mutations in exon 10 codon 618, and 620 or exon 13 codon 768 [10]. In the cohort used for this study, the germline *RET* mutations affecting codons 606 and 790 were associated with FMTC; the *RET* Y606C mutation affected six members of family VII. Among them, the index patient and her mother and sister suffered from MTC but they all lacked other endocrine neoplasms. The mean age at diagnosis of these patients was 50 years (range 47–53 years) and the tumors were at early stage without regional lymph node metastasis. To date, *RET* codon 606 mutation has been rarely reported. The *RET* Y606C mutation has been reported in only a single patient with MTC and this mutation was not found in any relatives of the patient [19]. Y606C variant was able to lead to an increase in activation of the downstream signaling of *RET* receptor and thus should be the cause of MTC development [19, 20]. However, although we classified the family as FMTC, persistent follow-up will be needed to evaluate the risk of PHEO in the patients and other family members. In family VIII, the mean age at diagnosis of these patients was 46 years (range 43–50 years) and the tumors were early stage without regional lymph node metastasis. *RET* codon 790 mutations differs from other common *RET* mutations, such as exon 10 and 11, as they do not affect the extracellular domain of the protein but, rather, the intracellular tyrosine kinase 1 domain, mutation of which increases the transforming activity, possibly by altering the substrate specificity or the ATP-binding capacity of *RET* to modify the kinase activity [21]. *RET* mutations in codon 790 and 791 have been described in 5 of 181 German MEN2A/FMTC families as rare spots leading to hereditary MTC and PHEO [21]. After the analysis of the phenotypic expression, Fitze [22] suggested that *RET* codon 790/791 mutations might be associated with low penetrance and might generate a mild thyroid disease phenotype compared to that of the more common MEN2 mutations.

Approximately 95 % of MEN2B cases were associated with *RET* exon 16 codon 918 (M918T) mutation that affects the intracellular domain of *RET* protein [10]. We found this mutation in a 13-year-old female from family IX, and this young patient had already been characterized as MEN2B and had the very aggressive tumor, but the same mutation was not detected in her parents, suggesting that this mutation occurred de novo. Indeed, approximately 75 % of MEN2B patients have de novo *RET* mutations and 95 % of them have germline M918T mutation [23]. Previously, Zhou et al. [13] also reported five Chinese probands identified in MEN2B and all of them showed de novo mutation of M918T.

In addition, our current data showed a *RET* mutation in exon 10 codon 618 affecting cysteine-rich extracellular domain. *RET* codon 618 mutation is usually described in the literature as the cause of MEN2A [24], but in the Netherlands, the highest rate of *RET* mutation in FMTC has been related to codon 618 [25]. The penetrance of PHEO and HPT to *RET* codon 618 mutation carriers is significantly lower than *RET* codon 634 mutations carriers [24]. However, in family X and XI, the only two index patients with MTC showed to have *RET* codon 618 mutation but without other mutations. Two daughters of the proband in family X and the father of the proband in family XI did not carry the same mutation. The small number in the current study does not allow us to draw a conclusion on phenotype/genotype correlations. But our present study showed that clinical characteristics of patients with *RET* codon 618 mutation did not show any "less aggressiveness", although a previous study demonstrated that MTC caused by *RET* codon 618 mutation is generally less aggressive than *RET* codon 634 mutation [12].

Clinically, to date, surgery is still the curable therapy for MTC patients; thus, genetic testing could diagnose the disease early for thyroidectomy to prevent or cure MTC. In our current study, a total of 6 asymptomatic carriers were detected by genetic screening. Combined with clinical examination, three were suspected to be MTC (two for positive neck ultrasound and one for increased calcitonin), and they were all confirmed by postoperative pathology. However, three other asymptomatic carriers did not accept further clinical examination, because their parents worried that the result might bring a spiritual and psychological burden to the families. As a result, prophylactic thyroidectomy was not carried out during the period of our study. In China, prophylactic surgery still faces difficulties.

Although the exome sequencing of *RET* is feasible technically, it may not be financially cost-effective. Based on our current data and other previous studies, in Chinese HMTC families, the germline mutation spectrum of the *RET* proto-oncogene is limited. Screening of *RET* mutations should be probably limited to exons 10, 11, 13 and 16 initially and could be cost-effective, and then extended to the other hotspot exons if necessary, such as 8 and 15 exons and beyond.

Although MEN2 is an autosomal dominant hereditary syndrome with low prevalence, China is a country of diversity with a large population, and thus the number of MEN2 patients may be relatively higher than other countries [13]. However, reports for the genetic characteristics

of *RET* in Chinese MEN2 patients are rare. This study is an example for comprehensive survey of patients with MTC in China. Further studies using a larger sample size are needed to comprehensively identify *RET* mutations in MTC patients and their families. More importantly, we have to plan to pursue the goal of prophylactic surgery for MTC patients and *RET* mutation carriers in the near future with the launching of genetic counseling and health education in China.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest in this work.

Ethical approval Ethical approval was given by the Human Research Ethics Committee of the Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, with the following Approval Number: 11-05/440.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Pelizzo MR, Boschin IM, Bernante P et al (2007) Natural history, diagnosis, treatment and outcome of medullary thyroid cancer: 37 years experience on 157 patients. Eur J Surg Oncol 33:493–497

2. Baloch ZW, LiVolsi VA (2001) Prognostic factors in well-differentiated follicular-derived carcinoma and medullary thyroid carcinoma. Thyroid 11:637–645

3. Paszko Z, Sromek M, Czetwertynska M et al (2007) The occurrence and the type of germline mutations in the *RET* gene in patients with medullary thyroid carcinoma and their unaffected kindred's from central Poland. Cancer Investig 25:742–749

4. Bergant D, Hocevar M, Besic N et al (2006) Hereditary medullary thyroid cancer in Slovenia–genotype–phenotype correlations. Wien Klin Wochenschr 118:411–416

5. McKusick VA, Amberger JS (1993) The morbid anatomy of the human genome: chromosomal location of mutations causing disease. J Med Genet 30:1–26

6. McKusick VA, Amberger JS (1994) The morbid anatomy of the human genome: chromosomal location of mutations causing disease (update 1 December 1993). J Med Genet 31:265–279

7. Elisei R, Romei C, Cosci B et al (2007) RET genetic screening in patients with medullary thyroid cancer and their relatives: experience with 807 individuals at one center. J Clin Endocrinol Metab 92:4725–4729

8. Mulligan LM, Kwok JB, Healey CS et al (1993) Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature 363:458–460

9. Machens A, Gimm O, Hinze R et al (2001) Genotype–phenotype correlations in hereditary medullary thyroid carcinoma: oncological features and biochemical properties. J Clin Endocrinol Metab 86:1104–1109

10. Eng C, Clayton D, Schuffenecker I et al (1996) The relationship between specific *RET* proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. Int RET Mutat Consort Anal JAMA 276:1575–1579

11. Chang CF, Yang WS, Su YN et al (2009) Mutational spectrum of multiple endocrine neoplasia type 2 and sporadic medullary thyroid carcinoma in taiwan. J Formos Med Assoc 108:402–408

12. American Thyroid Association Guidelines Task F, Kloos RT, Eng C et al (2009) Medullary thyroid cancer: management guidelines of the American Thyroid Association. Thyroid 19:565–612

13. Zhou Y, Zhao Y, Cui B et al (2007) *RET* proto-oncogene mutations are restricted to codons 634 and 918 in mainland Chinese families with MEN2A and MEN2B. Clin Endocrinol (Oxf) 67:570–576

14. Mulligan LM, Eng C, Healey CS et al (1994) Specific mutations of the *RET* proto-oncogene are related to disease phenotype in MEN 2A and FMTC. Nat Genet 6:70–74

15. Punales MK, Graf H, Gross JL et al (2003) *RET* codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. J Clin Endocrinol Metab 88:2644–2649

16. Brandi ML, Gagel RF, Angeli A et al (2001) Guidelines for diagnosis and therapy of MEN type 1 and type 2. J Clin Endocrinol Metab 86:5658–5671

17. Karga HJ, Karayianni MK, Linos DA et al (1998) Germ line mutation analysis in families with multiple endocrine neoplasia type 2A or familial medullary thyroid carcinoma. Eur J Endocrinol 139:410–415

18. Magalhaes PKR, Antonini SRR, de Paula FJA et al (2011) Primary hyperparathyroidism as the first clinical manifestation of multiple endocrine neoplasia type 2A in a 5-year-old child. Thyroid 21:547–550

19. Ahmed SA, Snow-Bailey K, Highsmith WE et al (2005) Nine novel germline gene variants in the *RET* proto-oncogene identified in twelve unrelated cases. J Mol Diagn 7:283–288

20. Ercolino T, Lombardi A, Becherini L et al (2008) The Y606C *RET* mutation causes a receptor gain of function. Clin Endocrinol (Oxf) 69:253–258

21. Berndt I, Reuter M, Saller B et al (1998) A new hot spot for mutations in the ret protooncogene causing familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A. J Clin Endocrinol Metab 83:770–774

22. Fitze G, Schierz M, Bredow J et al (2002) Various penetrance of familial medullary thyroid carcinoma in patients with *RET* protooncogene codon 790/791 germline mutations. Ann Surg 236:570–575

23. American Thyroid Association Guidelines Task F, Wells SA, Asa SL et al (2015) Revised american thyroid association guidelines for the management of medullary thyroid carcinoma. Thyroid 25:567–610

24. Quayle FJ, Fialkowski EA, Benveniste R et al (2007) Pheochromocytoma penetrance varies by *RET* mutation in MEN 2A. Surgery 142:800–805

25. Landsvater RM, Jansen RP, Hofstra RM et al (1996) Mutation analysis of the *RET* proto-oncogene in Dutch families with MEN 2A, MEN 2B and FMTC: two novel mutations and one *de novo* mutation for MEN 2A. Hum Genet 97:11–14