Screening of six risk exons of the *RET* proto-oncogene in families with medullary thyroid carcinoma in the Czech Republic

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Abstract

Medullary thyroid carcinoma (MTC) occurs as a sporadic form (75%) or as an autosomal dominant inherited familial disorder (25%) called familial MTC (FMTC) or as multiple endocrine neoplasia type 2 (MEN2) syndromes. Germ-line mutations in the rearranged during transfection (*RET*) proto-oncogene in exons 10, 11, 13, 14, 15 and 16 are known to be a cause of most of the familial forms. In this paper we report molecular genetic testing of 106 families with MTC (358 tested persons) from the Czech Republic in which we directly sequenced these six exons of the *RET* proto-oncogene. We detected germ-line mutations in 100% of MEN2B families (4/4 families), 90% of MEN2A families (9/10), 40% of FMTC families (4/10)

and 7% of apparently sporadic MTC (6/82). Eleven different germ-line mutations were revealed. MEN2B was associated with mutation Met918 Thr in exon 16. In one MEN2B family beside this mutation the Tyr791 Phe was also found, which has not yet been reported. MEN2A was restricted to different mutations in exon 11 (codon 634). In FMTC and 'sporadic' MTC families the mutations in exons 10, 11, 13 and 14 were detected. The genotype/phenotype correlations are given. Genetic testing revealed germ-line mutations in 23 index patients, 24 family members and excluded them in 53 relatives.

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Introduction

Medullary thyroid carcinoma (MTC), a rare type of tumours derived from thyroid parafollicular cells (C-cells), accounts for from 5% to 10% of all thyroid malignancies. MTC may occur as a sporadic form (75% of cases) or, less frequently, as a hereditary disorder with an autosomal dominant mode of inheritance (25% of cases). Three clinically hereditary forms are known: familial MTC (FMTC), multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B). FMTC is characterized by the familial occurrence of MTC without other lesions. MEN2A, the most frequent form, is characterized by MTC, pheochromocytoma (Pheo) in 50% of cases, and/or hyperparathyroidism (HPT) in 20% of cases. The most aggressive variant of MTC appears in conjunction with marfanoid habitus, ganglioneuromatosis, bumpy lips, diarrhoea, mucosal neuromas and Pheo (in 50% of cases) in MEN2B syndrome. Because of the autosomal dominant mode of inheritance the offspring of the FMTC and MEN2 patients have a 50% chance of inheriting the disease.

Prospective biochemical screening for early MTC symptoms in individuals at risk is performed by measurement of basal and stimulated serum levels of calcitonin

with a pentagastrin and/or calcium injection. The elevated calcitonin levels are indicative of C-cell hyperplasia or MTC (Telenius-Berg *et al.* 1984). Early diagnosis and surgical intervention can prevent the development of thyroid metastasis and lengthen the survival time of patients after surgery (Gagel *et al.* 1988). However, false positive, as well as false negative, biochemical results sometimes occur. The interpretation of elevated calcitonin levels in children is also complicated (Landsvater *et al.* 1993). Moreover, longterm screening is expensive and the families are subjected to extreme psychological stress.

It is well documented that germ-line activating point mutations in the rearranged during transfection (*RET*) proto-oncogene are associated with the pathogenesis of familial forms of MTC (Donis-Keller *et al.* 1993, Hofstra *et al.* 1994, Mulligan *et al.* 1994). The human *RET* proto-oncogene is located on chromosome 10q11.2, consists of 21 exons and encodes a transmembrane receptor tyrosine kinase that plays a role in the normal development, differentiation and neoplastic growth of neural crest lineages (Ishizaka *et al.* 1989). In MTC patients germ-line mutations mostly occur in two main functional domains of the *RET* protein – in the extracellular ligand-binding domain (in MEN2A and FMTC) and in the intracellular

catalytic tyrosine kinase domain (in MEN2B and FMTC). Approximately 92% of MEN2 syndromes are related to germ-line missense mutations of the *RET* proto-oncogene (Eng *et al.* 1996). In almost all MEN2A families mutations involve one of the six cysteines in the extracellular domain of *RET* encoded by exon 11 (codons 630 and 634) or exon 10 (codons 609, 611, 618, and 620) (Mulligan *et al.* 1995). The most frequent mutation occurs at codon 634 in exon 11 (Eng *et al.* 1996). The MEN2A mutations result probably in modulation of receptor disulfide dimerization, whereas the MEN2B mutations alter the *RET* catalytic properties (Santoro *et al.* 1995). MEN2B is caused by the mutation Met918 Thr in exon 16 in the tyrosine kinase domain of the *RET* (Hofstra *et al.* 1994). Rare causes of MEN2B are mutations in exon 15 at codon 883 and in exon 16 at codon 922 (Kitamura *et al.* 1995, Smith *et al.* 1997). In FMTC families *RET* mutations affect either one of the cysteine codons in exons 10 or 11 or, less frequently, codons 768, 790, or 791 (exon 13), codon 804 (exon 14), or codon 891 (exon 15) in the tyrosine kinase domain or codon 533 in exon 8 in the extracellular domain (Bolino *et al.* 1995, Eng *et al.* 1995a, Fattoruso *et al.* 1998, Da Silva *et al.* 2003).

Specific mutations in different codons may influence the phenotypic expression. The mutations involving codon 634, the substitution of cysteine for arginine, is significantly predictive for the development of Pheo and parathyroid disease (Mulligan *et al.* 1994, Schuffenecker *et al.* 1994). Some mutations could represent milder variants of the disease, as in exons 13 and 14 (Fitze *et al.* 2002, Lombardo *et al.* 2002). Recently, in some MEN2 families, other clinical features have been described such as Hirschsprung's disease (HSCR) or cutaneous lichen amyloidosis (CLA) (Borst *et al.* 1995, Verga *et al.* 2003).

The identification of the *RET* proto-oncogene mutations responsible for MEN2 syndrome provides the opportunity to find mutation carriers in families at risk and simplifies the management of kindreds with this disease. The prophylactic total thyroidectomy can be performed at a very early stage of the disease. Therefore, the morbidity and mortality of these patients is markedly reduced. The traditional MEN2A and FMTC categories could be, thanks to genetics, updated and reclassified (Machens *et al.* 2001) and some familial cases can be found among apparently sporadic MTC families.

The aim of this study was to determine frequency and position of the germ-line mutations in exons 10, 11, 13, 14, 15 and 16 and to correlate the genotype with disease phenotype in a large cohort of 106 Czech MTC families.

Materials and Methods

MTC families

We collected 106 unrelated MTC families (106 index cases and their relatives, a total of 358 persons tested)

selected from the population of the Czech Republic (10 million). The index patients were clinically and biochemically characterized and classified as sporadic MTC (82 families/235 tested persons), FMTC (10/62), MEN2A (10/50) and MEN2B (4/11). We considered FMTC as families with one index patient and at least one other family member who was operated on on the basis of elevated calcitonin levels and C-cell hyperplasia or where MTC was histologically confirmed. Due to the small family sizes it was not possible to fulfil the strict International *RET* Mutation Consortium criteria for FMTC that suggests at least four cases of MTC per family (Eng *et al.* 1996).

Clinical and biochemical screening

MTC or C-cell hyperplasia was determined on the basis of increased basal and stimulated calcitonin levels and histologically confirmed by pathologists after an operation. The basal and pentagastrin-Ca stimulated calcitonin levels were performed with RIA kit (DSL-1200, Webster, TX, USA). Basal and stimulated calcitonin values below 40 pg/ml and 200 pg/ml respectively were considered normal. All positive probands were screened for the presence of Pheo by measurement of blood pressure, plasma and/or urinary levels of catecholamines using HPLC methods. Screening for HPT was performed by measuring serum calcium and parathyroid hormone levels.

Molecular genetic analysis

Before genetic testing, all patients had given their informed consent in accordance with institutional ethics guidelines and national regulations. Pedigrees were created by the Cyrillic programme, Family Genetix, Oxford, UK. Molecular analyses were carried out on 106 index patients and, in mutation positive cases, their relatives at risk of the disease were also screened. Molecular genetic data were then correlated with phenotype, sex, age at diagnosis, Primary tumor, Regional Lymph Nodes, Distant Metastasis (TNM) classification, tumorous foci, laterality, presence of Pheo and other clinical features were obtained from the patients' anamnesis.

Genomic DNA was isolated from peripheral blood leukocytes using phenol-chloroform extraction, precipitated with ethanol and dissolved in TE buffer. At present, the NucleoSpin Blood kit (Macherey-Nagel, Duren, Germany) is used for isolation of DNA. After measurement of the DNA concentration and DNA/protein ratio, the DNA was used for PCR amplification.

Genomic DNA was amplified using PCR. The primers for exons 10, 11, 13, 14, 15 and 16 are given in Table 1. All reactions were performed in 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 160 µM dNTPs, 0.1 µM of each primer, 0.45 U Gold AmpliTaq polymerase (Perkin Elmer, Langen, Germany), 60 ng DNA and

optimized concentration of MgCl_2 (Table 1). The running profile of the amplifications was: initial denaturation at 95 °C for 10 min followed by 40 cycles (denaturation at 95 °C for 30 s, annealing at optimized temperature (Table 1) for 30 sec, elongation at 72 °C for 1 min) and final elongation at 72 °C for 10 min (thermocycler; Biometra, Goettingen, Germany). PCR products were analyzed by 1.5% agarose Tris–borate–EDTA gel electrophoresis. The gel was stained with ethidium bromide and analyzed under u.v. light. A negative control was included in each amplification analysis.

Sequencing of 6 exons in the sense and antisense directions was carried out on the ALF-express gel sequencing machine (Pharmacia, Uppsala, Sweden). For the sequenase reaction the previously purified matrix PCR products (GenElute Gel purification kit, Sigma, St Louis, MO, USA), the fluorescent (Cy5)-labelled primers (Table 1) and the Thermo-sequenase Cycle Sequencing kit (USB, Cleveland, OH, USA) were used.

Results

Detected mutations

We examined 106 unrelated families suffering from MTC and found germ-line mutations in 23 of them (Table 2). We detected 11 different types of mutations in the *RET* proto-oncogene in exons 10, 11, 13, 14 and 16 (in one family a double mutation in exons 13 and 16 was found). No mutation in exon 15 was found. The prevalences of mutations were: 47.8% in exon 11 (eleven families), 17.4% in exon 13 (four families), 13.0% in exon 14 (three families), 17.4% in exon 16 (four families) and 8.7% in exon 10 (two families). Among mutations in exon 11, the most frequent amino acid replacement was Cys634 Arg (63.6%, seven out of 11 families).

MEN2B phenotype was associated with the mutation in exon 16 at a single codon 918. In one MEN2B family, beside this mutation, the mutation in exon 13 at codon 791 was also found. MEN2A phenotype was restricted to mutations in exon 11 at codon 634 but there were different amino acid substitutions: replacements of cysteine with arginine, serine, tryptophan or tyrosine. More varied mutations were connected with the FMTC phenotype, where mutations in exons 11, 13 and 14 were detected. The majority of FMTC mutations (75%) were found in non-cysteine codons. In apparently sporadic MTC, germ-line mutations in exons 10, 11, 13 and 14 were also found (the distribution between cysteine and non-cysteine mutations was equal).

Detection rate

Table 3 shows the detection rate in our series of patients. Among 252 relatives, 24 inherited the mutation (RET+

carriers) and 53 people were wild type *RET* carriers (RET – carriers) and they could therefore be excluded from further clinical screening. Fourteen carriers of mutation had high plasma calcitonin levels and had been operated on and 10 carriers are still without sign of MTC (one with a mutation in the exon 10 and nine with low penetrance in exons 13 and 14).

Clinical characteristics of index patients and persons at risk of MTC with positive testing

Table 4 shows the clinical behaviour of mutations in index patients. The female/male ratio is 2.3. The median ages at diagnosis differed with the type of affected exons. Median age at diagnosis in index patients with mutations in exon 16 was 20.5 yrs (14–31 yrs); in exon 10, 28 yrs (21 and 35 yrs); in exon 11, 28 yrs (18–47 yrs); in exon 13, 44 yrs (40 and 48 yrs) and in exon 14, 49 yrs (46–52 yrs). Milder tumour behaviour is connected with mutations in exons 13 and 14. All patients with mutations in exon 16 have the characteristic MEN2B phenotype (tall thin body habitus, long thin arms and legs, thick bumpy lips and mucosal neuromas), diarrhoea and neurofibromatosis. The biochemical and, more recently, the molecular genetic screening carried out on the cohort of relatives of index patients revealed 24 persons with positive biochemical and/or genetic tests, but only 14 of them were operated on until now. Their clinical characteristics are given in Table 5. The female/male ratio is 1.0. The median age at operation is 17 yrs. In comparison with index patients, carriers have lower TNM classification due to prophylactic total thyroidectomy based on biochemical or genetic screening.

Discussion

This is the first comprehensive report of molecular genetic screening of MTC families in the Czech Republic. This set of patients does not overlap with those previously reported in the International *RET* Mutation Consortium and EUROMEN study group (Mulligan *et al.* 1995, Eng *et al.* 1996, Machens *et al.* 2003). Identification of the mutations in the *RET* proto-oncogene confirms the clinical diagnosis and identifies asymptomatic family members with FMTC or MEN2 syndrome. Germ-line mutations were found in 22% of these probands of all MTC families. This result is in agreement with the data stating that up to 25% of MTC cases are inherited (Egawa *et al.* 1998, Klein *et al.* 2001). Genetic testing of our 106 MTC families revealed germ-line mutations in the *RET* proto-oncogene in 23 index patients and in 24 relatives and excluded the germ-line mutation in 53 family members.

In agreement with data from other countries (Mulligan *et al.* 1994, Schuffenecker *et al.* 1994, Frank-Raue *et al.* 1996, Sanchez *et al.* 1999, Machens *et al.* 2001, 2003,

Table 1 Primers and PCR conditions for amplification and sense and antisense sequencing of exons 10, 11, 13, 14, 15 and 16

Exon	PCR amplification	Size of PCR product	Annealing temperature	Concentration of MgCl ₂	Sequencing Cy5-labelled primers
10	10F: 5'-GGG CCT ATG CTT GCG ACA CCA-3' 10R: 5'-CCA GAG GGA GGG AGG GAA GTT T-3'	373 bp	61 °C	2 mM	10F: 5'-GGA CAC TGC CCT GGA AAT A-3' 10R: 5'-CCT TGT TGG GAC CTC AGA TGT G-3'
11*	11F: 5'-GGT CTA GGA GGG GGC AGT AAA TGG-3' 11R: 5'-CAG CGT TGG CAG CCC CTC ACA G-3'	561 bp	63 °C	1.5 mM	11F: 5'-CCT CTG CGG TGC CAA GCC TC-3' 11R: 5'-CAG CGT TGG CAG CCC CTC ACA G-3'
13	13F: 5'-AGA AGC CTC AAG CAG CAT CGT C-3' 13R: 5'-AGG AGC AGT AGG GAA AGG GAG AAA-3'	346 bp	61 °C	1.5 mM	13F: 5'-AGA AGC CTC AAG CAG CAT CGT C-3' 13R: 5'-AGG AGC AGT AGG GAA AGG GAG AAA-3'
14	14F: 5'-CAC GAG CAG CAG GAG GCA GAG A-3' 14R: 5'-GAG TGT GGC ATG GTG GGG GAG TGG-3'	548 bp	65 °C	1.5 mM	14F: 5'-CTG TGT CCA CCC CCT TAC TCA TTG-3' 14R: 5'-GAG TGT GGC ATG GTG GGG GAG TGG-3'
15	15F: 5'-CCC CCG GCC CAG GTC TC-3' 15R: 5'-GCT CCA CTA ATC TTC GGT ATC TTT-3'	354 bp	61 °C	2 mM	15F: 5'-GTC TCA CCA GGC CGC TAC CC-3' 15R: 5'-GCT CCA CTA ATC TTC GGT ATC TTT-3'
16	16F: 5'-GGC CTT CTC CTT TAC CCC TCC TT-3' 16R: 5'-CAG CCA TTT GCC TCA CGA ACC C-3'	336 bp	61 °C	2 mM	16F: 5'-GGC CTT CTC CTT TAC CCC TCC TT-3' 16R: 5'-CAT GTA TAA GGG TGT TTC TG-3'

*Elongase enzyme was used, elongation temperature at 68 °C.

Table 2 Detected mutations in 23 RET+ families

Family number	Exon	Codon	Base change	Amino acid change	Phenotype	RET+/family members studied
1	10	609	TGC/TAC	Cys/Tyr	"spor.MTC" + HSCR	2/2
2	10	611	TGC/TAC	Cys/Tyr	"spor.MTC"	2/4
3	11	634	TGC/CGC	Cys/Arg	MEN2A	1/3
4	11	634	TGC/CGC	Cys/Arg	MEN2A	5/9
5	11	634	TGC/CGC	Cys/Arg	MEN2A	1/1
6	11	634	TGC/CGC	Cys/Arg	MEN2A	2/3
7	11	634	TGC/CGC	Cys/Arg	MEN2A	3/11
8	11	634	TGC/CGC	Cys/Arg	MEN2A	2/3
9	11	634	TGC/CGC	Cys/Arg	MEN2A	1/4
10	11	634	TGC/AGC	Cys/Ser	"spor.MTC"	1/2
11	11	634	TGC/TGG	Cys/Trp	FMTC	1/1
12	11	634	TGC/TCC	Cys/Ser	MEN2A	1/4
13	11	634	TGC/TAC	Cys/Tyr	MEN2A+HPT	3/10
14	13	768	GAG/GAC	Glu/Asp	FMTC	3/10
15	13	791	TAT/TTT	Tyr/Phe	"spor.MTC"	2/3
16	13	791	TAT/TTT	Tyr/Phe	FMTC	2/7
17	14	804	GTG/ATG	Val/Met	"spor.MTC"	1/3
18	14	804	GTG/ATG	Val/Met	FMTC	6/7
19	14	804	GTG/ATG	Val/Met	"spor.MTC"	1/1
20	16	918	ATG/ACG	Met/Thr	MEN2B	1/2
21	16	918	ATG/ACG	Met/Thr	MEN2B	1/5
22	16	918	ATG/ACG	Met/Thr	MEN2B	1/1
23	13+16	791+918	TAT/TTT+ ATG/ACG	Tyr/Phe+ Met/Thr	MEN2B	3/3

RET+, includes index patients; HSCR, Hirschsprung's disease.

Table 3 Detection rate of the set of Czech MTC families

	Phenotypes				
	spor.MTC	FMTC	MEN2A	MEN2B	Total
Total number of families	82	10	10	4	106
RET+ families (%)	6 (7%)	4 (40%)	9 (90%)	4 (100%)	23 (22%)
Number of index patients	82	10	10	4	106
RET+ index patients	6	4	9	4	23
No mutation found	76	6	1	0	83
Number of relatives	153	52	40	7	252
RET+ carriers	3	9	10	2	24
RET- carriers	6	13	29	5	53
No mutation found	144	30	1	0	175

*RET- carriers, persons excluded from further clinical screening.

Niccoli-Sire *et al.* 2001, Pinales *et al.* 2003) the most frequent mutations were found in exon 11 at codon 634 (47.8%), mainly the replacement Cys634 Arg was present. Nine out of 10 MEN2A families were positive for point mutations exclusively involving codon 634. In one FMTC family the Cys634 Trp mutation was detected, which is usually described as a cause of MEN2A (Hansford & Mulligan 2000). It could be explained by the misclassifi-

cation of MEN2A with low penetrance of Pheo or there could be an influence of *RET* polymorphisms or other modifier genes that protect the FMTC from the development of Pheo (Robledo *et al.* 2003).

In the FMTC families our detection rate is only 40%, probably due to the application of milder clinical-genetic criteria for this group. In unsolved families, the disease could still be a part of an inherited syndrome where the

Table 4 Clinical features of RET+ index patients

	Phenotype	Codon change	Sex	Age at diagnosis	Years after operation	TNM	Tumorous foci	Laterality	Pheo
Family No									
1	"spor.MTC"+HSCR	Cys609Tyr(G/A)	F	21	4	T4N0M0	M	b	—
2	"spor.MTC"	Cys611Tyr(G/A)	F	35	10	T3N0M0	U	u	susp.
3	MEN2A	Cys634Arg(T/C)	M	18	17	T4N2Mx	M	b	11 yrs A*
4	MEN2A	Cys634Arg(T/C)	M	47	17	T3N0M1h	M	u	5 yrs B*
5	MEN2A°	Cys634Arg(T/C)	F	25	1	T3N2M0	M	b	—
6	MEN2A	Cys634Arg(T/C)	F	24	18	T4N2M1hpp	M	b	11 yrs B
7	MEN2A	Cys634Arg(T/C)	M	28	25?	T4N0M0	M	b	S*
8	MEN2A	Cys634Arg(T/C)	M	19	12	T3N2M0	M	b	11 yrs A
9	MEN2A	Cys634Arg(T/C)	M	38	18	T4N0M0	M	b	8 yrs B
10	"spor.MTC"	Cys634Ser(T/A)	F	31	3	T2N0M0	U	u	—
11	FMTC	Cys634Trp(C/G)	F	31	19	T4N0M0	M	b	—
12	MEN2A	Cys634Ser(G/C)	F	36	7	T1N0M0	U	u	S
13	MEN2A+HPT	Cys634Tyr(G/A)	F	26	18	T3N0M0	U	b	susp.
14	FMTC	Glu768Asp(G/C)	F	48	19	T1N0M0	U	u	—
15	"spor.MTC"	Tyr791Phe(A/T)	F	40	10	T4N2M0	U	u	—
16	FMTC°	Tyr791Phe(A/T)	F	—	—	—	—	—	—
17	"spor.MTC"	Val804Met(G/A)	F	49	8	T2N0M0	U	u	—
18	FMTC	Val804Met(G/A)	M	46	7	T2N2M0	U	u	—
19	"spor.MTC"	Val804Met(G/A)	F	52	6	T3N0M0	M	u	—
20	MEN2B	Met918Thr(T/C)	F	16	1	T2N2M0	M	b	—
21	MEN2B	Met918Thr(T/C)	F	25	6	T3N2M1h	M	b	4 yrs A
22	MEN2B	Met918Thr(T/C)	M	31	20?	T4N2M1pp	M	b	10 yrs A
23	MEN2B	Tyr791Phe(A/T) + Met918Thr(T/C)	F	14	18	T2N0M0	U	u	17 yrs A

°, Pheo was diagnosed in index patient's mother, who died due to Pheo; °°, this patient was diagnosed abroad; *, how many years after (A), before (B) or at the same time as (S) total thyroidectomy operation that Pheo was operated on; M, multifoci; U, unifoci; b, bilateral; u, unilateral; †, dead; susp., suspected Pheo (higher levels of catecholamines).

mutation may occur in the other exons of the *RET* proto-oncogene (Pigny *et al.* 1999, Da Silva *et al.* 2003); the inheritance of some mutations lying in other genes also cannot be excluded (Gil *et al.* 2002). FMTC families with mild clinical behaviour have non-cysteine mutations in exons 13 and 14 (Fitze *et al.* 2002, Lombardo *et al.* 2002).

A single point mutation Met918 Thr was identified in all patients with the MEN2B syndrome. Besides this mutation the second germ-line mutation in exon 13 was detected in one MEN2B family. The clinical relevance of this double mutation is not clear. Usually, mutations in exon 13 are connected with less aggressive forms of MTC. Therefore, this second mutation could be responsible for postponing the development of Pheo. This patient was operated on for Pheo 17 years after the total thyroidectomy. This double mutation in MEN2B family has not been observed previously but the double mutation in exon 14 (codons 804 and 806) causing MEN2B phenotype has been published (Kitamura *et al.* 1995, Menko *et al.* 2002).

We confirmed that the genetic screening is very useful in 'sporadic' MTC families. The germ-line mutations (in

exons 10, 11, 13, 14) were detected in 7% of these families (Eng *et al.* 1995b, Wohllk *et al.* 1996, Scurini *et al.* 1998). They could have been misclassified due to the small family number and poor family data or due to the occurrence of de novo germ-line mutations. In one apparently sporadic MTC family we found an asymptomatic carrier (the patient's mother) with germline mutation Cys609 Tyr. The relatively small number of our samples does not allow us to draw widespread conclusions on phenotype/genotype correlations. But, according to the literature, the presence of mutations at codon 634 is associated with a higher risk of Pheo (Eng *et al.* 1996, Yip *et al.* 2003). However, no specific mutation was associated with this predisposition. Age at diagnosis was related to a specific exon and unrelated to a specific nucleotide and amino acid exchange within each codon according to Machens (Machens *et al.* 2001, Machens *et al.* 2003). We confirmed that exons 13 and 14 present a less aggressive disease, where age at diagnosis ranges between 40 and 50 yrs of age, whereas mutations in exons 10, 11 and 16 are linked with a much younger age at diagnosis (20–30 yrs). Hirschsprung's disease (loss of the *RET* function) was identified in one MTC family where the mutation in exon

Table 5 Clinical features of RET+ relatives who underwent the total thyroidectomy due to the positive biochemical and/or genetic tests

	Phenotype	Codon	Sex	Age at operation	Years after operation	TNM	Tumorous foci	Laterality	Pheo
Family No									
2	"spor.MTC"	Cys611Tyr(G/A)	F	15	1	T1N0M0	U	u	—
4	MEN2A	Cys634Arg(T/C)	M	9	4	T2N0M0	M	u	—
4	MEN2A	Cys634Arg(T/C)	F	21	17	T2N0M0	M	u	susp.
4	MEN2A	Cys634Arg(T/C)	F	22	18	T3N2M1hh, colon	M	b	5 yrs A°
4	MEN2A	Cys634Arg(T/C)	M	7	0	T1N0M0	U	u	—
6	MEN2A	Cys634Arg(T/C)	M	8	13	T1N0M0	U	u	susp.
7	MEN2A	Cys634Arg(T/C)	F	9	21	T1N0M0	U	u	susp.
7	MEN2A	Cys634Arg(T/C)	F	22	26	T3N0M0	U	u	15 yrs A
8	MEN2A	Cys634Arg(T/C)	M	43	11	T2N0M0	U	u	susp.
13	MEN2A+HPT	Cys634Tyr(G/A)	M	19	5	T2N0M0	M	u	—
13	MEN2A+HPT	Cys634Tyr(G/A)	F	25	2	T1N0M1h	U	u	susp.
14	FMTC	Glu768Asp(G/C)	F	51	18	T1N0M0	U	u	—
23	MEN2B	Tyr791Phe(A/T)+ Met918Thr(T/C)	M	7	5	T1N1M0	U	u	—
23	MEN2B	Tyr791Phe(A/T)+ Met918Thr(T/C)	M	5	2	T1N0M0	U	u	—

Ten other RET+ relatives are still without sign of MTC (one has mutation in exon 10 and will be operated on this year. The other nine relatives have less aggressive mutations in exons 13 and 14 still with normal levels of calcitonin and without ultrasound findings). M, multifoci; U, unifoci; b, bilateral; u, unilateral; °, how many years after (A) total thyroidectomy operation Pheo was operated on; susp., suspected Pheo (higher levels of catecholamines).

10 at codon 609 was detected. Hirschsprung's disease mostly segregates with codons 620 and 618 in exon 10 (Pasini *et al.* 2002). In one MEN2A family with a mutation in exon 11 at codon 634 HPT was detected. It represents the occurrence of HPT in 10% of our MEN2A families, which corresponds to the literature (Karga *et al.* 1998).

It is evident that biochemical and genetic screening in family relatives helped to decrease the TNM stage and changed MTC characteristics to unifocal and unilateral (Frilling *et al.* 1995, Pacini *et al.* 1995, Sanso *et al.* 2002) but it did not influence the development of Pheo.

Our laboratory is now responsible for the MTC molecular genetic diagnostic programme in the Czech Republic. The programme covers all Czech MTC patients and their families at present. It was confirmed that genetic screening is a valuable tool for identification of predisposed individuals in families at risk.

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