Molecular genetic diagnostic program of multiple endocrine neoplasia type 2A and familial medullary thyroid carcinoma syndromes in Hungary

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Abstract

Medullary thyroid carcinoma (MTC) occurs usually in sporadic form, but about a quarter of the cases are hereditary and appear as part of one of the multiple endocrine neoplasia type 2 (MEN2) syndromes. Mutations in the RET protooncogene are known to be the cause of the MEN2A and familial medullary thyroid carcinoma (FMTC) syndromes in the majority of the families. Direct DNA testing allows prophylactic thyroidectomy to be offered to individuals carrying a mutation in the above codons, and in mutation-negative cases it reduces the yearly screening-related burden on family members at risk of the disease. By DNA sequencing and PCR-restriction fragment length polymorphisms, 65 MTC probands were examined for mutations in residues 609, 611, 618, 620 of exon 10, and in residues 634, 768, 804 of exons 11, 13, and 14 respectively of the RET protooncogene. In our

study, mutations in the above codons were detected in all of the 14 clinically MEN2A and FMTC families. One of these mutations, TGC609 TCC has not been reported previously. Of the 14 probands with the mutation, 25 relatives also had the identified mutation and 18 relatives proved to be non-carriers. Among the 51 probands with clinically sporadic MTC, none was found to carry a mutation in the above positions even if indirect signs of MTC, pheochromocytoma or hyperparathyroidism could be detected in some families. The frequency of the TGC634AGC mutation is unexpectedly high in our samples, which can probably be attributed to a founder effect. We conclude that screening for mutations in these codons is effective in families fulfilling the strict clinical criteria of MEN2A or FMTC.

Journal of Endocrinology (2001) **170**, 661–666

Introduction

Medullary thyroid carcinoma (MTC) arises from parafollicular C-cells and may occur in sporadic (75%) or hereditary (25%) form (Marsh *et al.* 1995). Multiple endocrine neoplasia type 2 (MEN2) refers to autosomal dominantly inherited forms of MTC: multiple endocrine neoplasia type 2A, 2B (MEN2A, MEN2B) and familial MTC (FMTC). Besides MTC, MEN2A is characterised by pheochromocytoma (pheo) and hyperparathyroidism (HPT) in 50% and 20% of the cases respectively. FMTC is characterised by the presence of MTC only. MEN2B, which represents 5% of all MEN2 cases, is defined by the occurrence of MTC, pheochromocytoma (in 50% of the cases) and is associated with mucosal neuromas, ganglioneuromatosis of the gut and a Marfanoid habitus (Thakker & Ponder 1988, Thakker 1998). Approximately 92% of the three variants of MEN2 are related to germline missense mutations of the RET protooncogene (10q11.2, contains 21 exons) that encodes a receptor tyrosine kinase

(Gardner *et al.* 1993, Mole *et al.* 1993, Eng *et al.* 1996). In MEN2 patients, these germline mutations mainly occur on two main functional domains in the RET protein, on the extracellular ligand-binding domain (in MEN2A and in FMTC) and on the intracellular catalytic domain (in MEN2B and in FMTC) (Mulligan *et al.* 1993). The mutations found in 97% of MEN2A families and 86% of FMTC families are clustered together as they affect one of four cysteine codons (609, 611, 618, 620) in exon 10, and one (634) in exon 11 of the extracellular domain of the RET protooncogene (Mulligan *et al.* 1994). The most frequent mutations (80% in MEN2A and 30% in FMTC) occur in codon 634 (Eng *et al.* 1996). These mutations induce constitutive catalytic activity due to the aberrant disulfide homodimerization of RET (Santoro *et al.* 1995, Chappuis-Flament *et al.* 1998).

In the intracellular tyrosine kinase domain of RET, two codons are also routinely screened for mutations in MEN2A and FMTC. One is codon 768 in exon 13: the mutation described so far alters GAG (Glu) to GAC (Asp)

(Eng *et al.* 1995). The other is codon 804 in exon 14: the GTG/TTG (Val/Leu) change was first reported in two unrelated FMTC families by Bolino *et al.* (1995), and the GTG/ATG Val/Met change was described by Fattoruso *et al.* (1998).

Biochemical tests are used to screen for early symptoms of thyroid disease, although a negative result, especially in a younger person, cannot exclude the risk of the disease in an affected family. The finding that mutations in the RET protooncogene are associated with MEN2 has simplified the management of kindreds with this disease and has established the place of its preventive operative therapy (Donis-Keller *et al.* 1993, Mulligan *et al.* 1993). Now it is generally accepted that the most reliable way to prevent this form of MTC is prophylactic thyroidectomy performed in asymptomatic mutation carriers as early as 5 years of age (Lips *et al.* 1994, Wells *et al.* 1994, Lallier *et al.* 1998). It leads to cause-specific survival similar to that of the general population. In the event of a negative result, the family members of the affected proband are relieved of the physical and emotional consequences of the disease and the follow-up procedures.

Our laboratory has established a protocol for quick and effective genetic diagnosis of MEN2A and FMTC in Hungary. As analysis of samples by two different laboratories or by two different methods in the same laboratory are recommended by the International RET Mutation Consortium, our protocol contains PCR-restriction fragment length polymorphisms (RFLPs) with the ability to detect all the possible changes of the most common site of mutations, codon 634 in exon 11 (McMahon *et al.* 1994). In positive cases, sequencing of the area of interest in exon 11 was performed to support the diagnosis. In negative cases, the fragment of exon 10 containing codons 609, 611, 618 620, and the part of exons 13 and 14 containing codons 768 and 804 respectively, were sequenced. If mutation was detected on one strand, the sequencing was performed on the other strand also.

Materials and Methods

Management of MTC

MTC was suspected if fine needle aspiration cytology of a thyroid nodule was pathognomic for MTC or if it was indicated histologically after thyroidectomy. Genetic screening was initiated following a positive histopathological finding (including immunohistochemistry) in the proband.

Among the family members of MTC probands, the initial screening procedures included the history, physical examinations, laboratory tests (basal calcitonin, and plasma calcium and parathyroid hormone (PTH) levels), and diagnostic imaging (cervical and abdominal ultrasonography (US)). MTC or C-cell hyperplasia was suspected on the basis of an increased calcitonin level. The positive

results of biochemical and/or genetic screening of family members initiated extensive diagnostic imaging investigations, including cervical US, cervical and mediastinal computed tomography (CT) and magnetic resonance imaging (MRI), upper abdominal MRI, whole-body metaiodobenzylguanidine (MIBG) scintigraphy and [^{18}F]fluorodeoxyglucose positron emission tomography (PET) to rule out pheo and HPT, and also to aid the surgeon in decision-making as to the extent of lymph node dissection.

The regular follow-up of MTC consisted of the determination of the basal calcitonin level (every 6 months), cervical US (yearly) and chest imaging (chest X-ray yearly, and spiral CT every 2nd or 3rd year). If hypercalcitoninemia and/or general symptoms (diarrhea/flush) were observed during follow-up, all the above mentioned diagnostic imaging examinations were initiated, supplemented by bone scan, liver angiography (Ésik *et al.* 2001) and laparoscopy. Patients from MEN2A families are screened regularly (every year) for HPT (plasma PTH and calcium levels) and pheo (by forms of diagnostic imaging).

RET mutations carriers exceeding 5 years of age were subjected to preventive thyroidectomy if informed consent was available. The treatment that followed the diagnosis of pheo is primarily operation. HPT has not been found among patients with RET mutations.

Genetic counseling

Every proband, and his or her family members known to be at risk following proof of the inherited form of the disease, received written information on the background of the disease and genetic screening. They were also informed during personal consultations, after which they could give informed consent to DNA testing.

After the results of genetic screening were available, the counselor reviewed the findings with the family members and discussed the therapeutic possibilities. The benefits and potential complications of the specific therapies were also explained. Informed consent was obtained before any therapeutic intervention.

Study population

After informed consent, genomic DNA samples were studied from 65 MTC probands and, in mutation positive cases, from their family members also. The study was approved by the Ethical Committee of the National Health Care Scientific Council (ETT), permission number 309/1999 KO, 6078/1999 ETT. The familial forms of the disease were categorized as MEN2A(1), MEN2A(2), MEN2A(3), FMTC, and 'others' according to the criteria of the International RET Mutation Consortium (Eng *et al.* 1996). Histological proof of MTC, pheo and HPT was required. Two of the three families that were categorized as 'other' had three members with

MTC, and pheo or HPT were excluded by extensive testing. They are most probably FMTC families but they do not yet fulfil the criteria of FMTC (at least four cases of MTC per family) because of small family size. We found no families falling into the MEN2A(3) category. Proband from families with only one MTC member and no histological proof of pheo or HPT were regarded as apparently sporadic cases. The patients and family members had been referred to the National Institute of Oncology from all over the country (population 10 million). The population in this region is predominantly Caucasian.

DNA extraction

DNA was isolated from citrate or EDTA anticoagulated peripheral blood samples by a standard salting-out technique mainly at the Molecular Genetic Laboratory of the National Institute of Hematology and Immunology, Budapest; some samples were processed in the National Institute of Oncology, Budapest.

PCR amplification

For amplification of the DNA segment containing codon 634 in exon 11, the following primers were used: primers MENF (5'-CATGAGGCCGAGCATACTCAGCC) (McMahon *et al.* 1994), 2C (5'-GACAGCAGCACC GAGACAGT) (Mulligan *et al.* 1994). The MENF primer contains 2 mismatches, which introduce two control restriction sites for enzymes DdeI and HaeIII.

For amplification of the sequence containing codons 609, 611, 618, 620 in exon 10 we used primers 19S (5'-GCAGCATTTGTTGGGGGACA) (Mulligan *et al.* 1994) and MENR (5'-ACCTCAGATGTGCTGTT). This gives a 187 bp fragment.

For amplification of the exons 13 and 14 DNA segments the following primers were used: primers 13F (5'-CTCTCTGTCTGAACTTGGGC), 13R (5'-TCACCC TGCAGCAGGCCTTA), 14F (5'-TGGCTCCTGGAA GACCCA) and 14R (5'-AGAGCCATATGCACG CAC) (Chieffari *et al.* 1998).

The exon 11 PCR product was separately digested with the following restriction enzymes: Fnu4HI, HhaI, HaeIII, DdeI using the conditions recommended by the manufacturer (New England Biolabs, Beverly, MA, USA). Separation of the digested DNA was performed on 4% agarose gel and stained by ethidium bromide.

DNA sequencing

The required PCR fragments were purified by QIAquick Gel Extraction Kit (QiaGene, Hilden, Germany). For sequencing, an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA, USA) was used on a GeneAmp PCR System 2400 (Perkin Elmer), and the fragments were separated on an

Table 1 Summary of the positive results found by screening 65 unrelated MTC families for mutations in codons 609, 611, 618, 620, 634, 768 and 804 of the RET protooncogene

Informative PCR-RFLP (codon 634)	Mutant codon	New amino acid	No. of families with mutation
	(Normal: TGC)	(Normal: Cys)	
Fnu4HI	634 TAC	Tyr	3
Fnu4HI	TTC	Phe	3
Fnu4HI	TGG	Trp	1
DdeI	AGC	Ser	3
HhaI	CGC	Arg	2
—	609 TCC	Ser	1
	(Normal: GTC)	(Normal: Val)	
—	804 ATG	Met	1

ABI 373 DNA Sequencer. The sequencing was performed at the Biological Research Center, Szeged, Hungary.

Results and Discussion

This is the first time that genetic diagnosis for MEN2A and FMTC has been available in Hungary. We examined 65 unrelated patients suffering MTC. Hirschsprung disease was excluded in all families. Examining codons 609, 611, 618 620, 634, 768 and 804 of the RET protooncogene, mutations were detected among these probands in 14 cases (21.5%). This result is in agreement with the data stating that about 25% of MTCs are inherited (Marsh *et al.* 1995). In the affected families, 18 people proved to be free of the typical mutation of the family, and 25 relatives inherited the mutation. Among these 25 relatives, only 6 had high (above 80 ng/ml) plasma calcitonin levels (normal value: <10 ng/ml), which is an unquestionable sign of the affected state. The others had 10–80 ng/ml plasma calcitonin values at least once in their lives, although they temporarily presented normal values; in these cases genetic diagnosis was vital.

Seven different mutations of the RET protooncogene were identified – in 12 families codon 634 was affected, in 1 family codon 609 and in 1 family codon 804 was affected. (Table 1).

Of the 65 probands and their families, 14 families belonged to the familial forms of MTC (MEN2A, FMTC or 'other'). In these groups, evidence of two or more individuals affected by MTC could be documented, or a single individual had both MTC and pheo and the diagnoses were confirmed by histology. All the 14 families which were proved to be affected by a mutation in the above codons showed clinical signs of the familial forms of MTC, i.e. among the clinically familial cases mutation was found in 100% (14/14) of families. Of the 14 families, 4 fell into the MEN2A(1) group, 7 into the MEN2A(2)

Table 2 Clinical data of the 14 MEN2 families and their mutations of the RET protooncogene

Family (No. of individuals tested)	MTC				No. of pheo cases in the family (proof)	No. of HPT cases in the family (proof)	Mutation of TGC/Cys ²
	Age of proband at diagnosis (in decades)	Multiple tumorous foci in proband	hCT among relatives	No. of MTC ¹ cases in the family			
MEN2A(1)							
I (2)	4	y	—	5	4 (h)	1 (h), (1 ks, 1 hCa)	TAC/Tyr
II (5)	5	y	2	6	3 (h)	1 (h), (1 ks, 1 hCa)	TAC/Tyr
III (4)	4	y	—	4	2 (h)	1 (h), (1 hCa)	AGC/Ser
IV (9)	4	y	4	3	3 (h)	1 (h), (2 hPTH)	TTC/Phe
MEN2A(2)							
V (4)	6	y	1	2	2 (h)	—	AGC/Ser
VI (2)	5	y	1	1	1 (h)	(1 hCa)	AGC/Ser
VII (3)	4	y	—	1	1 (h)	—	CGC/Arg
VIII (1)	3	y	—	1	1 (h)	—	CGC/Arg
IX (5)	4	y	—	3	1 (h), (1 M)	(1 hPTH)	TTC/Phe
X (4)	2	y	—	3	2 (h)	—	TGG/Trp
XI (4)	5	y	—	3	2 (h)	—	609 TCC/Ser
Other							
XII (4)	5	y	—	3	—	—	TTC/Phe
XIII (3)	3	y	—	3	—	—	TAC/Tyr
XIV (7)	5	ns	4	2	—	—	804 GTG→ATG/ Val→Met

¹and pathologically proven C-cell hyperplasia.
²in codon 634 of the RET protooncogene, unless otherwise stated.
MEN2A(1), families with MTC, pheo and HPT; MEN2A(2), families with MTC and pheo in at least one individual; objective evidence against the presence of HPT in all affected at-risk individuals; Other, families with 3 or 2 individuals with MTC but no individuals with pheo or HPT on biochemical screening or families in which screening results could not be confirmed.
h, histology; ks, kidney stone; M, bilateral chromaffin cell hyperplasia detected by means of MIBG; hCT, hypercalcaioninemia; hCa, hypercalcemia; hPTH, raised level of parathyroid hormone; ns, not stated; y, yes.

group and 3 into the ‘other’ category (Table 2). Our results correlate with the findings of the International RET Mutation Consortium, as it could find mutations in the above codons in 97% of MEN2A(1), in 99% of MEN2A(2), in 100% of MEN2A(3), and 85% of the ‘other’ group (Eng *et al.* 1996). Among the remaining 51 sporadic cases (one MTC patient in the family), no proband proved to be positive for mutations in the examined codons of the RET protooncogene. It is worth noting that from these 51 sporadic cases indirect signs of pheo or HPT or C-cell hyperplasia were present in many families (Table 3). These signs in the probands were: young age at the time of the diagnosis of MTC (3 probands within the 2nd and 3rd decades of their lives), multifocality of the primary tumor (6 probands), bilateral chromaffin cell hyperplasia detected by means of MIBG (1 proband), and 4 probands with possible signs of HPT, such as elevated level of PTH, kidney stone-related renal insufficiency and kidney stone. However, as the incidence of kidney stone is relatively high in the general population, kidney stone without hPTH and hCa is not a

strong pathognomic symptom. In the relatives of the MTC probands, indirect signs of pheo (sudden death at young age in 2 families), HPT (kidney stones and kidney stone-related renal insufficiency in 1 family) and MTC (hypercalcaioninemia in 11 families – in many families more than one member) were also found. On the whole, 20 of the 51 families (39%) with one MTC proband showed at least one sign suspicious for an inherited form of MTC. In these families, the disease could still be part of an inherited syndrome where the mutation occurs in other codons of the RET protooncogene. Inheritance of mutations in other genes also cannot be excluded. No difference in the mean age at diagnosis of the MTC of the probands could be detected between the sporadic and inherited groups of families: in clinically sporadic cases it is about 53 years and in the clinically MEN2 cases it is about 54 years. In all available cases for examination, the inheritance of the mutation was established, and no *de novo* mutation was found in the families with affected individual(s). The small number of our samples does not allow us to draw widespread conclusions for phenotype/genotype

Table 3 Clinical data of the families of the 51 apparently sporadic MTC probands

MTC					
Family	Age of proband at diagnosis (in decades)	Multiple tumorous foci in proband	hCT among relatives	No. of pheo cases in the family (proof)	No. of HPT cases in the family (proof)
1	2	—	—	1 (sd)	—
2	3	—	3	—	—
3	3	—	2	—	—
4	5	y	—	—	—
5	5	y	3	—	—
6	4	y	—	—	1 (pb: hPTH)
7	6	y	—	—	—
8	5	y	—	—	—
9	4	y	—	1 (sd)	—
10	5	—	—	1 (pb: M)	—
11	5	—	2	—	1 (pb: sri)
12	4	—	1	—	1 (pb: sri)
13	6	—	3	—	—
14	5	—	1	—	—
15	4	—	1	—	—
16	4	—	4	—	—
17	5	—	1	—	—
18	5	—	2	—	—
19	5	—	—	—	4 (ks), 1 (sri)
20	5	—	—	—	1 (pb: ks)

In the families of the other 31 clinically sporadic MTC cases there is no sign of pheo, HPT or more MTC. The age of these probands at diagnosis varies from the 4th to the 8th decade.

hCT, hypercalcitoninemia; y, yes; sd, sudden death at young age; pb, proband; hPTH, raised level of parathyroid hormone; M, bilateral chromaffin cell hyperplasia detected by means of MIBG; ks, kidney stone; hCa, hypercalcemia; sri, stone-related renal insufficiency.

correlations, but the TGC634TAC (Tyr) is found more frequently in families with HPT than without it.

The most common mutations in our study are the TGC634TAC, TTC and AGC (21% each), followed by the CGC (14%) mutation of codon 634 (see Table 1). In the International RET Mutation Consortium analysis, the most frequent alterations were CGC (52.1%) and TAC (26.0%) and the least frequent was AGC (1.8%). The surprisingly high frequency of the AGC mutation in our population may be caused by a founder effect as the three families carrying this mutation – although they do not know about one another – live within a 30 km area in the North-East part of Hungary. Further investigation of these pedigrees is in progress.

The TGC609TCC (Cys→Ser) mutation identified in family XI was not observed previously (The Human Gene Mutation Database, Cardiff). The family has a history of MTC and pheo and it does not differ substantially from the history of the other MEN2A(2) families.

Our laboratory established a MEN2A molecular genetic diagnostic program in Hungary. The program will cover all of the Hungarian MEN2 patients and their relatives in the near future. According to our results, mutations in the above codons can be found exclusively in those families that fall strictly into the categories defined by the the

International RET Mutation Consortium, e.g. where histological proof of at least two MTC, and/or pheo and HPT is available in the family.

Acknowledgements

This work was funded by the National Scientific Research Fund (OTKA) grant number T 29809.

IK is a recipient of a scholarship from the Hungarian Soros Foundation. We thank Györgyi Demeter for the skilful technical help and the Laboratory of Molecular Biology of the National Institute of Oncology, Budapest and the Laboratory of Molecular Biology of the National Institute of Hematology and Immunology, Budapest, for the DNA preparation.

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Received 8 May 2001

Accepted 30 May 2001