Germ line mutation analysis in families with multiple endocrine neoplasia type 2A or familial medullary thyroid carcinoma

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Abstract
The RET proto-oncogene has been identified as the multiple endocrine neoplasia type 2 disease gene. An association between specific RET mutation and disease phenotype has been reported. We present the phenotype–genotype of 12 Greek families with multiple endocrine neoplasia type 2A (MEN 2A) or familial medullary thyroid carcinoma (FMTC). Seventy members were studied and DNA analysis for RET mutations was performed in fifty-eight of them. Exons 10, 11, 13, 14 and 16 of the RET proto-oncogene were analyzed by single strand conformation polymorphism analysis, direct DNA sequencing and/or restriction enzyme analysis. No mutations of the RET proto-oncogene were identified in 1 of 9 families with MEN 2A and in the 3 families with FMTC. In 7 MEN 2A families, the mutation was demonstrated in codon 634 and in 1 family it was demonstrated in codon 620. There was a low frequency, about 8%, of hyperparathyroidism associated with MEN 2A. The specific causative mutations for parathyroid disease were C634R or C634Y. Among the MEN 2A individuals there was one case with de novo C634R mutation and one case, C634Y, with cutaneous lichen amyloidosis which predated by 24 years the diagnosis of MEN 2A. In 2 children who were MEN 2A gene carriers, microscopic medullary thyroid carcinomas were found. These data show a low frequency of hyperparathyroidism in our cases and provide further evidence that individuals with C634R as well as with C634Y mutations of the RET proto-oncogene could be at risk for parathyroid disease. Cutaneous lichen amyloidosis could be an early feature of MEN 2A. Additionally, direct DNA testing provided an opportunity to resect medullary thyroid carcinoma at an early stage.

Introduction
Multiple endocrine neoplasia type 2 (MEN 2) is an inherited cancer syndrome with three clinically distinct forms, MEN 2A, familial medullary thyroid carcinoma (FMTC), and MEN 2B. All forms are transmitted as an autosomal dominant trait with a high degree of penetrance and variable clinical expression. They are characterized by the development of neuroendocrine neoplasia, with medullary thyroid carcinoma (MTC) as the most common feature of the disease. It has been shown that the RET proto-oncogene, on chromosome 10, is the MEN 2 gene and specific germ line point mutations of the RET gene are responsible for the three forms of MEN 2 (1–3). The RET proto-oncogene encodes one out of many transmembrane receptor tyrosine kinases that are implicated in neural crest tissue development and differentiation. Glial cell line-derived neutropic factor (GDNF) has recently been recognized as a putative ligand for RET (4). Ligand binding normally induces RET receptor dimerization, autophosphorylation on tyrosine residues followed by the downstream signal transduction phosphorylation of cellular proteins (5). It is of interest that within the exons of the RET gene the predominant mutations are restricted to specific positions. In MEN 2A and FMTC a number of different mutations are in cysteines of exons 10 and 11 (6, 7) located in the extracellular cysteine-rich region, a part of the putative ligand binding domain. In contrast, in >95% of MEN 2B patients, a unique mutation involves exon 16 changing the Met to Thr at position 918 (3) lying in the substrate recognition site of the catalytic core of the tyrosine kinase domain (8). However, besides the usual disease phenotype and genotype, some diverse rare cases have also been reported. For example cutaneous lichen amyloidosis (CLA) (9) or Hirschprung’s disease was found in a number of MEN 2 families (10, 11). In some MEN 2-affected families the RET mutations have not yet been detected (7) or have been located at different exons than usual, as occurred in a few families with FMTC in which germ line point mutations were found in exons 13,
14 (12, 13) or in exon 15 in one FMTC and in four MEN 2 families (14, 15). Exons 13, 14 and 15 encode part of the intracellular receptor tyrosine kinase (8). Additional, rare cases of de novo mutations have been described in which RET germ line mutations are found in one affected person and his offspring, but not in his parents (16–18).

Patients and methods

Twelve unrelated Greek families, designated I-XII, were studied. Families I-IX were classified as MEN 2A, family X was classified as FMTC (7). Families XI and XII each had three members with MTC (see Table 1). Although the criteria for the classification require at least four cases of MTC per family (7), we thought it was reasonable for these to be included in the FMTC cases. In these families the MTC appeared in first degree relatives (parent/children) and the presence of pheochromocytoma (PHEO) or hyperparathyroidism (HPT) was excluded by extensive testing of all affected individuals of two generations. Fifty-eight family members were evaluated by medical history, physical examination and biochemical measurements of fasting serum calcium, basal and/or stimulated plasma calcitonin (CT) levels after intravenous infusion of calcium gluconate (2.7 μg Ca²⁺/kg over 10 min), plasma parathyroid hormone (1–84) (PTH), 24-h urinary excretion of catecholamines and metabolites, and DNA analysis. The family histories were also reviewed and we gathered information for an additional 12 affected members in up to 4 generations in 5 MEN 2A families. Among the fifty-eight family members, thirty-three (aged 12–65 years), were patients previously thyroidectomized for MTC. From this MTC group 9 patients had been operated on for PHEO and 1 for HPT. The diagnosis of MTC, PHEO and parathyroid hyperplasia was confirmed by pathological examination after operation. Twenty-five individuals, aged 3 months to 86 years, apparently healthy, were first-degree relatives of the patients. Patients’ spouses and twenty normal subjects served as controls.

DNA analysis for mutations of the RET proto-oncogene

Genomic DNA was extracted by standard methods from peripheral blood leukocytes. Using the polymerase chain reaction (PCR) technique, exons 10, 11, 13, 14 and 16 of the proto-oncogene were amplified using primers and PCR conditions described previously: ref. (19) for exons 10 and 11, and ref. (18) for exons 13 and 16. For exon 14 the following primers were used: F14S, 5’ TGG CTC CTG GAA GAC CCA A 3’ and F14R, 5’ TGC ACG CAC CTT CAT CTC G 3’. In the same PCR conditions as the primers for exons 10 and 11. The amplified exons were scanned for mutations by non-isotopic single strand conformation polymorphism analysis (SSCP) (20). All samples with observed SSCP variants were purified and directly sequenced by the use of fmol DNA cycle sequencing kit (Promega, Madison, USA). Based on the findings of the above sequencing analysis, PCR products were directly digested with the appropriate restriction enzyme.

Figure 1 Representative SSCP analysis. Distinct electrophoresis migration pattern of the single strands are shown (arrow heads) in lanes corresponding to the individuals carrying the mutation 634Cys → Arg (lanes 1, 2, 3, 4, 5, 6 and 8), or 620Cys → Tyr (lanes 10 and 11). Lanes 7, 9 and 12 are non-carriers. Lanes N, control subjects.

Figure 2 HhaI restriction endonuclease digestion of a 264-bp amplified DNA fragment of exon 11. Lanes N, control subjects. Lanes 2, 3, 5, 6, 7, 8, 10 and 13 correspond to heterozygous individuals for the 634Cys → Arg mutation. Lanes 4 and 12 are non-carriers. Lanes M, HindIII digested pBR322 plasmid DNA as a molecular standard.

Figure 3 Partial DNA sequence analysis of exon 11 of the RET gene. The arrow indicates the G to A mutation in codon 634. The DNA sequence from a control subject and from a MEN 2A patient is shown beside the autoradiogram.
Table 1 Summary of cases with MEN 2A or FMTC.

<table>
<thead>
<tr>
<th>Family members</th>
<th>Sex</th>
<th>Age (years) at diagnosis of MTC</th>
<th>PHEO</th>
<th>HPT</th>
<th>Gene carriers or non carriers</th>
<th>Mutations</th>
</tr>
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NT, not tested for mutation; ?, not enough information about the age at diagnosis; M*, patient with CLA.
Results

PCR-SSCP analysis in 33 samples from clinically affected MEN 2A patients exhibited double bands of exons 10 or 11 of the RET proto-oncogene with altered migration relative to those amplified from normal control DNA (Fig. 1). In addition, similar abnormal bands were found in 5 children at risk in which biochemical tests for the presence of MTC were within normal limits or borderline (not informative). In 3 patients from family VII with MEN 2A and in 6 patients from families X, XI and XII screening of exons 10, 11, 13, 14 and 16 failed to detect any abnormal band shifts. Direct sequencing analysis of exons 10 or 11 revealed heterozygosity for a missense mutation in all samples with the altered bands. In 7 of 9 MEN 2A families base substitution was located at codon 634 in exon 11. In 6 families it was a T to C transition leading to the replacement of Cys634 by Arg, creating a new HhaI restriction site (Fig. 2), and in the remaining 2 families it was a G to A transition (Fig. 3) causing conversion of Cys634 to Tyr and introducing an additional RsaI site. In family VI the transition G to A changing Cys620 to Tyr, could not be recognized by any restriction enzyme. In five families five children were identified as MEN 2A gene carriers and in two of them (aged 14 and 10 years) who underwent thyroidectomy, pathological examination revealed microscopic MTC in one and microscopic MTC plus parathyroid hyperplasia in the other. The remaining three children, under 6 years old, have not yet been operated on. Twenty unaffected family members have been excluded from further screening. In family II (Table 1) the affected individual (II-4), had most likely inherited the mutation from her father (II-1). The father had been operated on for MTC and PHEO and died early from MTC metastases, so his DNA was not available. Interestingly the mutation was not found in the father’s parents nor in his brother and sister, and since there was no doubt regarding paternity this case showed a de novo RET mutation of the father’s germ line transmitted to his daughter. All the remaining patients’ samples, from families VII, X, XI and XII, were sequenced and only the wild type sequences were detected in exons 10, 11, 13, 14 and 16. One patient with C634Y had experienced unilateral pruritis for 24 years with extensive lichenoid skin lesions over the upper back. To our knowledge this case is the nineteenth MEN 2A/CLA variant (7). Disease phenotype and mutations are summarized in Table 1. The data show that mutations C634R, C634Y or C620Y are associated with PHEO. The mutations C634R or C634Y could be associated with parathyroid disease, but in our patients we found very low frequency of HPT, about 8% (2 cases among 26 living affected members). The frequency of PHEO was about 50%. In one patient HPT was defined preoperatively with high serum calcium and high PTH. Hyperparathyroidism was confirmed postoperatively and parathyroid hyperplasia involving all glands found by pathology (at 15 years of age). The other patient was a ten-year-old girl with borderline high serum calcium and PTH and the diagnosis of parathyroid hyperplasia was based on histological examination postoperatively. For the other living affected subjects the tests for HPT have been negative up to now.

Discussion

Mutations that cause activation of RET have been well characterized and several groups have studied the disease phenotype–genotype (5). We have studied 12 Greek families with MEN 2A or FMTC in order to provide additional information for the disease genotype–phenotype characteristics. Mutational analysis of the RET gene at the ‘hot’ sites for MEN 2A and FMTC by three different approaches demonstrated the presence of heterozygous germ line missense mutations in eight out of nine MEN 2A families. In addition, among individuals at risk in the nine families, non-symptomatic MEN 2A gene carriers were identified and it was of great importance to operate on the two children based on the direct DNA test, regardless of the plasma level of CT. Both had a microscopic MTC, and there is agreement that once the specific RET mutation is identified direct genetic analysis for RET mutations is the test of choice for the identification of asymptomatic individuals at risk for hereditary MTC (7). The nature of the mutations in our MEN 2A families was similar to those described in previous studies (7). It is interesting to report that in one family an apparently de novo MEN 2A mutation was identified at codon 634 and since a few such cases have been described this case could be added to the database of de novo RET mutations associated with MEN 2A. On the other hand, in several families RET mutations could not be identified. In 4 of 12 families, screening of exons 10, 11, 13, 14 and 16 was negative for RET mutations. One case was a classic MEN 2A family and three were classified as FMTC (7). The possibility of false negative results was excluded because analysis involved three different techniques. In these cases extensive analysis of the RET gene is under way to exclude the possibility of mutations in other regions of the RET gene. So far, negative results of DNA analysis on patients with apparent sporadic MTC cannot exclude the possibility of hereditary disease. Linkage analysis using highly polymorphic dinucleotide (CA)n-repeat markers could be an accurate alternative approach for the identification of gene carriers (21). Although the small number of our families is not sufficient for statistical analysis, the high frequency of the absence of common RET mutations may reflect different hereditary factors in our population. All the mutations detected in our cases, as well as in most other studies, are concentrated in cysteine residues of the RET receptor, immediately adjacent to the transmembrane domain, affecting the configuration of the putative ligand-binding domain in MEN 2A and FMTC. These mutations result in ligand-independent
receptor dimerization and auto-phosphorylation converting the mutated RET allele to a dominant transforming gene (22). Various environmental agents may act as mutagens at a different RET region. There is accumulating evidence that the disease phenotype is dependent on the nature or the location of RET abnormalities suggesting that different mutations have tissue-specific effects which reflect a true biological variation. Such examples are the PTC/RET oncogene in papillary thyroid carcinoma (23) or the mutations M918T and A883F in MEN 2B, the more severe form of MEN 2. Many groups (24–26) have found a strong correlation between mutations in codon 634 and the presence of PHEO, but there are some discrepancies with those groups who suggested that the specific change TGC→CGC at codon 634, is consistent with a higher frequency of HPT. In our cases, among the 26 living subjects, HPT was found at a low frequency, about 8% of parathyroid disease compared with 15–30% in other studies. An explanation for the low frequency of HPT could be that among the affected individuals a subgroup consists of still young patients although the pathological examination did not show any parathyroid abnormality. Recently, in a series of patients carrying mutations at codon 634 an age-related and mutation-specific HPT penetrance was found (27). For example the penetrance was 14% by age 30 and rose to 81% by age 70. In our study, among a subgroup of 10 patients over 40 years of age no one has (until now) clinical or laboratory findings of HPT. It is also possible that early thyroidec- tomy with removal of some parathyroid glands had altered the natural course of the parathyroid disease. On the other hand, patients within the same family or in different families sharing identical predisposing mutations may develop distinct clinical phenotypes suggesting that the main function of the RET onco-protein is modified by other factors. Because of our geographical differences one could speculate that high exposure to abundant sunshine in Greece and consequently to high doses of vitamin D, a potent inhibitor of PTH gene transcription (28), may modify the appearance of HPT in our MEN 2A patients. The specific mutation, C634Y, may also account for the presence of a sensory neuron lesion in a subset of MEN 2A cases (6) and in accordance with this, in our patient with the rare variant of MEN 2A/CLA, the specific causative mutation was a change of Cys634 to Tyr. The RET proto-oncogene is expressed in the developing nervous and excretory system and it has been suggested that it plays a critical role in the signaling mechanism that mediates neurogenesis and kidney organogenesis (29). As a result, several neurocristopathies appeared as variants of the MEN 2 disease spectrum. Mutations in codon Cys618 or Cys620, not often associated with MEN 2A, can also predispose to Hirschprung’s disease and this could be a potential risk for the offspring of family VI. We conclude that the mutations C634R or C634Y of the RET proto-oncogene in MEN 2A could be associated with parathyroid disease. The low frequency of HPT in Greek patients with MEN 2A may reflect ethnic differences which contribute to the phenotypic variation. We also reaffirmed that CLA could be the earliest feature of MEN 2A as noted previously (30). Our results also indicate that an early thyroidec- tomy based on direct DNA analysis could prevent spread of local MTC.

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References


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