Molecular diagnosis of multiple endocrine neoplasia type 2A

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Objective. To identify by means of genetic analyses individuals who are at risk of developing medullary thyroid cancer that is a component of multiple endocrine neoplasia.

Subjects. A three-generation kindred with clinically and biochemically diagnosed medullary thyroid cancer.

Method. Identification of a heterozygote mutation by nucleic acid sequencing and restriction analyses.

Results. A heterozygote T → C (Cys → Arg) mutation at codon 618 in exon 10 of the RET proto-oncogene was identified in 4 family members who had previously been diagnosed with medullary thyroid cancer. The same mutation was also found in one of the proband's pre-symptomatic children who subsequently underwent a pre-emptive thyroidectomy. The genetic diagnosis was confirmed by histology. No mutations were detected in any other family members.

Conclusion. Identification of heterozygote germline mutations in multiple endocrine neoplasia is direct, highly accurate and cost-effective. This study demonstrates that, appropriately used, molecular diagnosis can supersede conventional biochemical methods in the management of patients with inherited cancers.

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Medullary thyroid cancer (MTC) accounts for about 10% of all thyroid tumours. It occurs mainly as a sporadic disease but at least 25% of cases appear to be familial. The familial form of MTC occasionally manifests in isolation but usually occurs as a component of either multiple endocrine neoplasia (MEN) type 2A, comprising MTC, phaeochromocytoma and hyperparathyroidism, or the rarer MEN type 2B characterised by MTC, phaeochromocytoma,

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mucosal neuromas and a marfanoid habitus. The syndromes of MEN 2, including isolated familial medullary thyroid cancer (FMTC), are inherited as autosomal dominant traits which are thought to be fully penetrant.[1] Early diagnosis of affected family members has important implications for clinical management since it allows pre-emptive thyroidectomy that is generally curative. Calcitonin deficiency has no important consequences while replacement of thyroxine is relatively easy.

Until recently, screening for the disease comprised measurement of the calcitonin response to C-cell secretagogues such as pentagastrin or calcium. Although still in use, biochemical analysis has disadvantages such as frequent false-positives, ambiguous results, unpleasant side-effects and the fact that ongoing annual testing is necessary.

In 1987, mapping of the MTC gene to the centromeric region of chromosome 10 opened up a new approach to the identification of gene carriers by linkage analysis.[2,3] While useful, linkage analysis is, however, only 90 - 95% accurate and to be informative requires the availability of multiple family members.[4] In 1993 the RET proto-oncogene was identified as the gene responsible for MEN 2 and FMTC. In MEN 2A, a total of 19 different mutations in this gene has now been detected in the germline of affected families.[5,6] Virtually all of these are missense point mutations at any 1 of 5 cysteine residues (codons 609, 611, 618, 620 and 634) in exons 10 or 11.[7,8] By contrast, in patients with MEN 2B germline mutations occur almost exclusively at codon 918 in exon 16.[5]

Mutations in the RET proto-oncogene are implicated in over 95% of families with MEN 2.[9] Several studies have also demonstrated that all members within a kindred consistently inherit the same RET mutation and that mutations have not been identified in any family members who did not ultimately manifest the disease or in any unrelated members.[10] Failure to identify a specific mutation has been reported in less than 5% of families with MEN 2.[6,8] Therefore the almost invariable occurrence of a limited number of mutations within discrete regions of the RET proto-oncogene has made direct genetic screening for MEN 2 and FMTC a feasible proposition. The initial step is the identification of the exact mutation in the proband or another affected family member. Thereafter all members of the family at risk for the disease can be tested by definitive genetic analysis and, if appropriate, the necessary treatment can be initiated.

In this study we report on 17 members of a South African kindred with clinically and biochemically diagnosed MTC who were assessed by direct DNA analysis.

Subjects and methods

Patients

The proband was a 30-year-old woman who presented in 1993 with a thyroid tumour and an elevated calcitonin response to pentagastrin provocation. Histology confirmed a medullary thyroid carcinoma. Questioning revealed that an uncle had died of a metastatic thyroid tumour and there was similar anecdotal evidence about her paternal grandfather. Subsequently other 'at risk' family members who were agreeable were screened for MEN 2; this included the proband's father, her 5 children, 3 sisters and her cousin. Initially screening was performed by the pentagastrin stimulation test for MTC and by the measurement of urinary catecholamines and serum calcium and phosphate concentrations for phaeochromocytoma and hyperparathyroidism, respectively.

Calcitonin stimulation test

The plasma calcitonin level was measured before and 2, 5, 10 and 15 minutes after intravenous administration of pentagastrin (0.5 µg/kg body weight in 2 ml normal saline) given over a 15-second period.[11] The test was considered positive when there was a 2 - 3-fold peak calcitonin increase above the basal concentration or a peak calcitonin response in excess of 200 ng/l.

Screen for phaeochromocytoma and parathyroid disease

Phaeochromocytoma was screened for by the measurement of urinary noradrenaline, adrenaline and dopamine levels using reverse-phase chromatography with electrochemical detection.[12] This has been shown to be highly sensitive.[13] Serum calcium (corrected for albumin) and phosphate were used to screen for the presence of hyperparathyroidism. In the absence of abnormal levels, parathyroid hormone level was not determined.

DNA analysis

The details of the procedure are given in the Appendix at the end of the article. In essence, genomic DNA was obtained from 17 family members, and exons 10 and 11 of the RET proto-oncogene were amplified by the polymerase chain reaction (PCR). The amplified segments were sequenced in both directions with the ABI Prism 310 Automated Genetic Analyzer. The sequence results were confirmed with a specific restriction endonuclease (Cfo I) to distinguish between the mutated and wild-type (normal) genes. Initially the mutation was sought in the index case of the kindred and compared with an unrelated reference subject. Subsequently DNA from the 16 remaining family members was analysed.

Results

Calcitonin stimulation

An abnormal calcitonin response to pentagastrin was observed in the proband's father, a sister and a cousin (Fig. 1: I-2, II-3 and II-1, respectively), all of whom immediately underwent thyroidectomies on the basis of these results. Medullary thyroid carcinomas were confirmed histologically in each of these individuals. All other family members on whom the test was performed screened negative at that time. Pentagastrin stimulation tests were continued annually, however. Within 3 years an exaggerated calcitonin response was detected in the proband's 11-year-old asymptomatic daughter and a 3-year-old son demonstrated an ambiguous elevated basal level.

Catecholamines and calcium levels

None of the family members diagnosed with MTC had elevated urinary catecholamine or serum calcium and phosphate concentrations at the time of the original diagnosis. After 3.5 years the proband's father was found to have a raised adrenaline level and was subsequently diagnosed with a phaeochromocytoma.

Direct mutation analysis

PCR amplification of exons 10 and 11 resulted in products of 183 and 219 bp, respectively. Single-strand nucleic acid sequencing did not reveal any mutation in exon 11. In exon 10, however, a heterozygote T → C (Cys → Arg) transition at codon 618 was detected in the proband. The same mutation was subsequently detected in the proband's father, sister and cousin as well as her pre-symptomatic daughter. Confirmation of this mutation was obtained by sequencing of the complementary DNA strand. The mutation was not found in the unrelated control or in any of the 12 remaining members of the kindred, including the 3-year-old child with ambiguous calcitonin levels on pentagastrin testing in whom thyroidectomy was being considered.

Indirect mutation analysis

The heterozygote Cfo I restriction site created by the Cys_{618} → Arg (TGC → CGC) transition was observed as 3 bands (183 bp, 120 bp and 63 bp) on electrophoresis in all 5 individuals in whom the heterozygote point mutation was found by sequencing (Fig. 1). This finding reaffirmed the presence of the mutation.

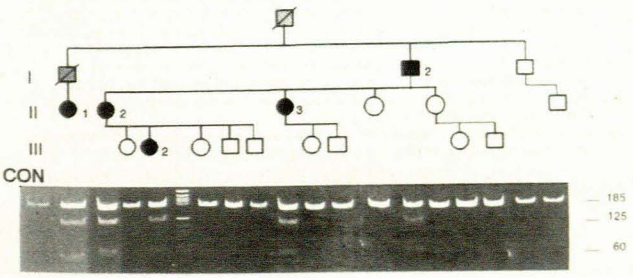

Fig. 1. Confirmation of RET proto-oncogene codon 618 (Cys → Arg) mutation by the presence of a heterozygote Cfo I restriction site (seen as 183 bp, 120 bp and 63 bp) in the proband (II-2) and her father (I-2), sister (II-3), cousin (II-1) and daughter (III-2) and (▨ = died of unknown thyroid disease; ▩ = died of medullary thyroid cancer; ■ or ● = RET codon 618 mutation; □ or ○ = RET codon 618 mutation absent; CON = control).

Discussion

Direct nucleic acid sequencing revealed a T → C (Cys → Arg) transition at codon 618 in exon 10 of the RET proto-oncogene in the kindred studied. The association between this specific mutation and MTC in this family was clearly evident in the presence of the same mutation in all 4 members with histologically confirmed MTC who had previously had thyroidectomies on the basis of an elevated calcitonin response to pentagastrin. This high degree of mutation specificity within a kindred is a valuable tool as it permits presymptomatic, definitive diagnostic genetic testing on other members of the family who may be at risk.[10,14] In some instances, particularly in children, such tests can allow presymptomatic diagnosis which has life-protecting potential.

In this kindred screening positively identified the proband's 11-year-old daughter as a carrier of the mutation and therefore at risk of developing MTC. This child was clinically asymptomatic, although she had recently manifested an elevated calcitonin response to pentagastrin provocation. The positive identification of the MTC-associated mutation provided unequivocal support for the diagnosis and allowed pre-emptive thyroidectomy without delay. Histological examination of the resected thyroid revealed hyperplasia of the C-cells of the medulla and a small medullary carcinoma. Earlier genetic analysis of this child, had it been available, would have allowed surgery prior to the development of potentially life-threatening changes in the thyroid. In the case of the proband's 3-year-old son, a recent pentagastrin stimulation test was ambiguous in that there was no apparent calcitonin response but the basal level was elevated 2-fold above normal. The absence of the mutation in this child served to eliminate ongoing anxiety and the possibility of an inappropriate thyroidectomy. The 8 remaining family members who were at risk because of a parent with the defective gene were all found to have the normal nucleic acid sequence for codon 618. These individuals can now be considered to carry only the normal risk of developing a tumour of the medullary thyroid and require no further special monitoring for the disease.[10,14]

The absence of phaeochromocytoma and hyperparathyroidism on clinical and laboratory investigation at initial presentation of this family suggested a diagnosis of FMTC, in accordance with the criteria set down by the International RET Mutation Consortium which specified at least 4 confirmed cases of MTC and no phaeochromocytoma or parathyroid disease in patients or living relatives. The isolated familial form of MTC appears to be rare, having been reported in only 23 of 361 (6.4%) families worldwide; mutations at codons 618, as seen in this study, are the most frequent.[9] Recently, more than 3 years after diagnosis, the proband's father presented with a phaeochromocytoma. The diagnosis in this family was consequently revised to that of MEN 2A, supporting a suggestion[15] that the entity of true isolated FMTC may not exist. All patients with a germline mutation of the RET proto-oncogene should therefore be monitored regularly for both phaeochromocytoma and hyperparathyroidism.

As shown in this study and by others[16-18] there are several important advantages to the use of genetic analysis in the diagnosis of MEN 2 and the familial forms of MTC. Firstly, because penetrance is 100% a genetic diagnosis allows early thyroidectomy to be undertaken, before the age of 5 - 6 years, avoiding the need for repeated biochemical testing in genetically normal individuals. Besides the improved clinical outcome and the greater sense of security, the elimination of unnecessary screening is extremely cost-effective in the long run. Genetic testing requires only a

single blood sample on one occasion. Given the clinical implications of the results, every precaution needs to be taken to eliminate the possibility of error during the pre-analytical, analytical and reporting stages of the analysis. Some laboratories go so far as to recommend resampling and repeat analysis of each pedigree prior to release of results.[19] With regard to technical aspects, all mutations should be confirmed by sequencing of the reverse DNA strand. In addition, wherever possible, restriction analysis should be used to confirm the presence or absence of the mutation.

This study, together with a recently published report on MEN 2A in a black South African family,[20] demonstrates the clinical utility and cost-effectiveness of genetic screening in selected monogenic, neoplastic disorders. Selected laboratories in this country are now capable of doing this, with a consequent substantial reduction in costs. Biochemical screening of family members of a patient with an apparent sporadic tumour may reveal affected members.[21] It has been suggested that all patients with MTC be tested for RET proto-oncogene mutations and, if positive, other family members followed up.[19,22] This principle can be more generally applied to other cancers in which sporadic and familial cancers cannot be distinguished on clinical or histological grounds.

Appendix

Genomic DNA was extracted by means of a salting out procedure[23] for amplification by PCR of exon 10 and part of exon 11 of the RET proto-oncogene. Briefly, 1 µg DNA was amplified in a 50 µl reaction containing 1 × reaction buffer (pH 8.3), 1.5 mmol/l $MgCl_2$, 200 µmol/l dNTPs, 0.2 µmol/l of each primer and 1.25 U Taq polymerase. Genomic DNA was denatured for 5 minutes at 95°C prior to 30 cycles through 95°C, 63°C, or 61°C (for exon 10 or 11, respectively) and 72°C for 30 seconds at each temperature followed by a 7-minute 72°C extension.

Sequences selected for the oligonucleotide primers were:

RET10F	5' GGA GGC TGA GTG GGC TAC GT 3'
RET10R	5' GAG GTG GTG GTG GTC CCG GC 3'
RET11F	5' CCT CTG CGG TGC CAA GCC TC 3'
RET11R	5' AGC TGA CCG GGA AGG CCT GG 3'

obtained from Genbank by accession numbers U11521, U11523, U11504 and MS 7464, respectively.

Direct bidirectional cycle sequencing of the PCR products was performed with the Perkin Elmer Taq FS Dyedeoxy Terminator Sequencing Kit followed by analysis on an Applied Biosystems Prism 310 Automated Genetic Analyzer. Heterozygote mutation analysis was carried out with the Sequence Navigator Software (Applied Biosystems Version 1.0.1).

The presence of a heterozygote mutation in exon 10 was verified by restriction digestion of the PCR product with the endonuclease Cfo I. Restriction products were size-separated by electrophoresis on a 12% polyacrylamide gel and stained with ethidium bromide.

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