A duplication of 12 bp in the critical cysteine rich domain of the RET proto-oncogene results in a distinct phenotype of multiple endocrine neoplasia type 2A

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Activating germline mutations in the cysteine-rich domain of the RET proto-oncogene cause endocrine neoplasia type 2A (MEN2A) and familial medullary thyroid carcinoma (FMTC). In virtually all patients with identified mutations one of five cysteines is altered by a missense mutation. In a MEN2A family with 14 affected and 11 unaffected living members, hypercalcemia was diagnosed in eight patients and histological evaluation revealed parathyroid hyperplasia in all cases examined (10/10). No member of this family showed any evidence for the existence of pheochromocytoma. This is the first documentation of a family without pheochromocytoma but with a high incidence of parathyroid disease. Genetic analysis revealed the presence of an unusual heterozygous mutation in exon 11 of the RET proto-oncogene representing a duplication of 12 bp resulting in the insertion of four amino acids between codon 634 (Cys) and 635 (Arg), thus creating an additional cysteine residue.

INTRODUCTION

Activating germline mutations in the cysteine-rich domain of the RET proto-oncogene cause endocrine neoplasia type 2A (MEN2A), an autosomal dominant inherited cancer syndrome affecting cells derived from the neural crest, and thus leading to medullary thyroid carcinoma (MTC), pheochromocytoma (pheo) and parathyroid hyperplasia (primary hyperparathyroidism pHPT) (1,2). Several missense mutations have been found in five cysteine codons encoded in exon 10 (codons 609, 611, 618 and 620) and exon 11 (codon 634) in >92% of families with medullary thyroid carcinoma only (FMTC) or MEN2A (MTC and pheo and/or pHPT).

The RET proto-oncogene encodes a receptor tyrosine kinase which is involved in the normal development of neural crest lineage (3–5). Glial cell-derived neurotrophic factor (GDNF), a member of the transforming growth factor (TGF)-β superfamily, has been demonstrated to be a ligand for the c-RET proto-oncogene (6). However, the physiological relevance of GDNF and the existence of other ligands is an open question. It has been demonstrated that mutated RET (C634W) transfected into NIH-3T3 L1 cells confers the transformed phenotype (7) and that the mutated receptors dimerize through intermolecular disulfide bridges and undergo autophosphorylation at tyrosine residues. The mutation of a single cysteine residue into any other amino acid obviously enables the formation of intermolecular disulfide bridges and changes the conformation to activate the intracellular tyrosine kinase domain without the presence of the ligand. This is believed to be the crucial event in the stimulation of neoplastic growth. The C634W mutation is the most common mutation found in MEN2A families (8,9). The resulting clinical phenotype, however, is very similar when Cys634 is mutated into any other amino acid or when one of the other four cysteines is mutated. It is concluded, therefore, that the disappearance of any of the cysteine residues in the cysteine-rich domain is fundamental to the progression of the disease.

Here we describe a novel class of mutation in the germline of a MEN2A family. A duplication of 12 bp in exon 11 creates an additional cysteine codon in the cysteine-rich domain and results in a distinct clinical phenotype of the MEN2 syndrome.

RESULTS

Since 1987 we have followed a MEN2A family with MTC and pHPT (the pedigree is given in Fig. 1). Fourteen affected and 12 unaffected living members agreed to clinical analyses after informed consent.

Pathological examination after thyroidectomy showed an MTC in nine of the affected members, and C-cell hyperplasia in three children using pathological calcitonin stimulation tests (performed by combined stimulation with pentagastrin and calcium, refs 10,11). Two affected patients refused thyroidectomy.

Pre-operatively increased serum calcium and parathyroid hormone concentrations were present in six patients (III,2; IV,1; IV,9; IV,10; IV,13; V,6). In these as well as in four additional patients with normal serum calcium (V,3; V,4; V,8; VI,1),

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histological examination demonstrated a hyperplasia of the parathyroid glands. Two further patients (III,6; III,10) suffered from hypercalcemia but histological evaluation of the parathyroid glands was not performed. In five patients with normal serum calcium, the parathyroid glands were not examined either because the patients refused thyroidectomy (IV,15; V,9), or they had died without consideration and evaluation of MEN2 (III,9; IV,8) or because the parathyroid disease was not suspected since thyroid operation was performed on an index patient (V,1). One patient (V,8) with normocalcemia but parathyroid hyperplasia at the time of the initial operation now suffers from hypercalcemia with increased parathyroid hormone concentration. We suspect hyperparathyroidism resulting from a parathyroid gland transplanted to the forearm 8 years ago. Two further patients (IV,13 and V,6) had recurrence of hyperparathyroidism after transplantation 2 and 7 years earlier.

In conclusion, so far hypercalcemia has developed in eight out of 14 living affected family members. Furthermore, if a histological examination of the parathyroid glands was performed hyperparathyroidism was always detected (10/10).

None of the 14 living nor three deceased members with available medical files had clinical signs of pheochromocytoma. For two patients, we were informed by their family doctors that 24 h urine collections had given normal results. In our institution, 12 patients were screened on a regular basis for manifestation of pheochromocytoma. In multiple 24 h urine collections (medium 8, range 2–16), epinephrine, norepinephrine, metanephrine, normetanephrine and vanilmandelic acid were measured, as described before (12). So far, with a median age of 41 years (range 14–75) and between 1 and 13 years after operation of MTC (median 9 years) none of these patients have biochemical evidence for the existence of a pheochromocytoma.
After informed consent, genomic DNA was analyzed for a mutation in the \textit{RET} proto-oncogene in 11 affected and 10 unaffected members to verify the diagnosis of the familial form of these tumors. Amplification by polymerase chain reaction of the DNA fragment representing the critical part of exon 11 of the \textit{RET} proto-oncogene did not result in a single sharp band of 156 bp as routinely seen in normal probands or in patients heterozygous for point mutations in codon 634. Rather, the DNA of these patients produced an additional band >10 bp larger than the wild-type band (Fig. 1A). The single-stranded conformation polymorphism (SSCP) pattern obtained from this amplified DNA differed markedly from any other pattern obtained for any of the point mutations we have found in codon 634 in FMTC or MEN2A patients (Fig. 1A).

The two DNA bands obtained were separated on a preparative agarose gel and subjected to DNA sequencing analysis. The result revealed a duplication of 12 bp in exon 11. After the first base of codon 635, CGC for arginine, the previous 12 bp are repeated and then the normal sequence is continued (Figs 2, 3). The insertion of the duplicated sequence results in a new histidine codon (CAC) at the 5' breakpoint and creates an additional cysteine residue at the 3' end of the insertion. The codons for glutamine and leucine in the middle of the duplicated part are conserved. Exons 10 and 13 of the \textit{RET} proto-oncogene were also screened for mutations, but no abnormalities were detected.

**DISCUSSION**

We have demonstrated a novel type of mutation in the \textit{RET} proto-oncogene causing the MEN2A syndrome. The mutation duplicates four codons in exon 11 and creates an additional cysteine residue. The result for the molecular mechanism stimulating neoplastic growth seems to be similar, as if a cysteine residue is lost by a missense mutation. We have published a similar type of mutation in the \textit{RET} proto-oncogene in the germline of a patient with a complete MEN2A phenotype: MTC, pheochromocytoma and pHPT (13). In this patient, an in-frame duplication of 9 bp was detected including codon 634.

According to the data collected by the international \textit{RET} mutation consortium (14), codon 634 in exon 11 of the \textit{RET} proto-oncogene is by far the most common codon mutated in MEN2A families (85%). The remaining 15% had mutations in the cysteine codons 609, 611, 618 or 620 in exon 10. Very few non-cysteine mutations have been found. There is a statistically
significant association reported between the presence of mutations at codon 634 and the presence of pheo and HPT. However, no strict relationship between any mutation in codon 634 and specific features exists. Any mutation in 634 can cause all three features or various combinations of these within different families. A significant number of MTC-only families has been found expressing the mutation C634Y (seven of 27). For nine families with mutations in exon 11, MTC and HPT without pheo are reported. However, these families are considerably small and not all of the affected members had parathyroid disease (C. Eng and F. Raue, personal communication) in contrast to the family described in the present study, with a positive histological finding for hyperplasia of the parathyroid glands in every patient studied and a high incidence of hypercalcemia. During a follow-up of 384 patient years (88 after thyroid operation), neither clinical nor biochemical data gave any evidence of adrenal disease. Although we can only report one family with this unusual duplication type of mutation in the RET proto-oncogene, we speculate that this distinct pattern of tissue involvement is related to the mutation.

The role of the mutated ret proto-oncogene must be different in C-cells of the thyroid, medullary adrenal cells and parathyroid cells reflected by the absence of pheos and the high incidence of pHPT. The mutation described in this study obviously causes a stronger effect on parathyroid cells than most other mutations and has no clinically significant effect on adrenal cells. It is intriguing to speculate how this unusual mutation is associated with the clinical presentation of a MEN2A family without any pheochromocytoma but with an unusual extent of parathyroid disease. Expression experiments in cell lines are underway to characterize the molecular mechanism of this ret protein mutation in detail.

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