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GERMLINE DINUCLEOTIDE MUTATION IN CODON 883 OF THE *RET* PROTO-ONCOGENE IN MULTIPLE ENDOCRINE NEOPLASIA TYPE 2B WITHOUT CODON 918 MUTATION

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ABSTRACT

The autosomal dominant multiple endocrine neoplasia type 2 syndromes (MEN 2) comprise three clinically distinct entities, MEN 2A, familial medullary thyroid carcinoma and MEN 2B, which share a common clinical feature: medullary thyroid carcinoma (MTC). MEN 2B is considered to have the most aggressive form of MTC. Therefore, early detection of MEN 2B in order to prevent potentially lethal MTC is important. More than 95% of all MEN 2B cases are caused by germline mutation at codon 918 (M918T) in exon 16 of the *RET* proto-oncogene. In this study, we demonstrate the presence of germline codon 883 mutation (A883F) in 2 of 3 unrelated MEN 2B cases without codon 918 mutation. Our data demonstrate a novel etiologic event which may have roles in predisposition to MEN 2B when present in the germline and in the pathogenesis of sporadic MTC when somatic.

Multiple endocrine neoplasia type 2 (MEN 2) comprises three dominantly inherited, clinically distinct cancer syndromes. In patients with familial MTC (FMTC), only the thyroid gland is affected. Patients with MEN 2A develop MTC, pheochromocytoma and primary hyperparathyroidism (1). In contrast, MEN 2B patients have MTC, pheochromocytoma, ganglioneuromas of the digestive tract, mucosal neuromas and skeletal abnormalities (2, 3). The median age of tumor development is at least 10 years earlier in MEN 2B than that of MEN 2A (2, 3).

RET is the susceptibility gene for MEN 2A, MEN 2B and FMTC [reviewed in Eng, 1996 (4)]. The *RET* proto-oncogene, comprising 21 exons, encodes a receptor tyrosine kinase. Germline mutations in MEN 2A and/or FMTC have been found in exons 10, 11, 13 and 14 of *RET* (5). In contrast, a single germline mutation at codon 918 (M918T) in exon 16 has been found in >95% of unrelated MEN 2B cases (5, 6). Interestingly, somatic mutations in exons 10, 11, 13, 15 and 16 have been found in sporadic MTC [reviewed by Eng and Mulligan (7)].

Four unrelated MEN 2B cases have been reported in the literature without germline M918T (5). Of these four, only one MEN 2B family had exons 2-20 analysed and found not to have *RET* germline mutations (8). A preliminary communication reported on the presence of germline mutation at codon 883 (exon 15) in two isolated cases of MEN 2B (9). The aim of this study, therefore, was to determine if mutations within exon 15 accounted for most or all cases of MEN 2B without germline M918T, by studying a sample set of three unrelated MEN 2B patients without M918T.

Materials and Methods

Patients

Three unrelated classic MEN 2B patients [as defined by the International *RET* Mutation Consortium (6)] (2 Australian, 1 German) who did not have the MEN 2B-defining germline mutation M918T were ascertained for this study .

PCR and Mutation Analysis

Constitutional DNA was extracted from peripheral blood leucocytes using standard techniques (10). All samples were obtained with informed consent. Subsequent PCR amplifications were carried out in 1x PCR buffer (Perkin-Elmer Corp., Norwalk, CT), 200uM dNTP, 1uM of each primer [CRT17B and CRT17G (11)], 2.5U *Taq* polymerase (Perkin-Elmer Corp.), and 100-200 ng of genomic DNA template in a 50 ul volume. PCR conditions were 35 cycles of 1 min at 95°C, 1 min at 62°C, 1 min at 72°C followed by 10 min at 72°C. Following PCR, the amplicons were fractionated on 2% low melting point agarose (Bio-Rad Lab., Hercules, CA) and the products visualised with UV transillumination after ethidium bromide staining. These products were further column purified (Wizard PCR Prep, Promega, Madison, WI) and subjected to semi-automated sequencing using the above primers and dye terminator technology as previously described (12, 13).

Overnight digestion of PCR products was performed with an excess of the restriction enzyme *AluI* under the

manufacturer's conditions (New England Biolabs, Inc., Beverly, MA).

Results

Germline DNA samples from three unrelated isolated MEN 2B cases were examined for germline mutations in *RET* within exon 15. Of these three, 2 (1 Australian, 1 German) harbored germline codon 883 mutations (Fig. 1). In each of these, the wildtype GCT triplet sequence at nucleotides 2647-2649, encoding alanine,was altered to TTT (c.2647-2648GC->TT), encoding the mutant phenylalanine (A883F). DNA from both parents of one of the A883F mutation positive individuals was available. Neither carried this mutation. Mutation positive and negative status was confirmed with *AluI* digestion in all cases (data not shown). A883F causes loss of an *AluI* restriction site.

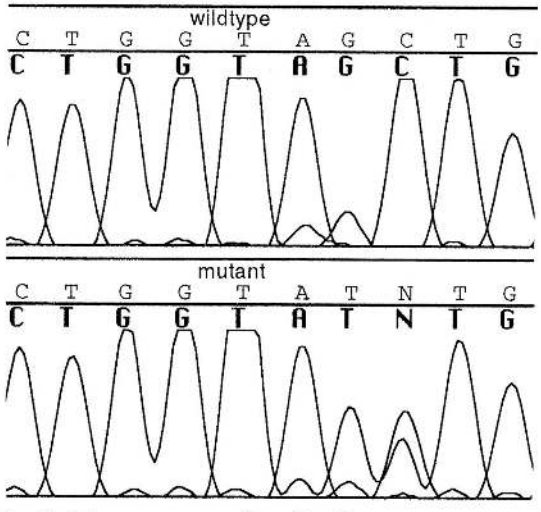

Fig. 1. Chromatogram of nucleotide sequence around *RET* codon 883. Note the wildtype sequence GCT is mutated to TTT in MEN 2B, resulting in A883F.

Discussion

While mutations at several codons are associated with MEN 2A and FMTC, MEN 2B appears more homogeneous, with >95% of the latter carrying germline M918T (5, 6). We now show that germline A883F can be associated with MEN 2B as well. Together with a previous preliminary report (9), a total of 4 MEN 2B cases without germline M918T have germline A883F. Although no functional analysis was performed, the germline A883F found in these MEN 2B cases are likely pathogenic for the following two reasons. First, somatic A883F (mutation present in the tumor only but not in the germline) have been described in at least four of 111 (3.6%) sporadic MTC [reviewed by Eng and Mulligan (7)]. Second, no polymorphisms at codon 883 have been found by the international community studying *RET* after analysis of hundreds of individuals who are unaffected, have MEN 2, Hirschsprung disease or a variety of sporadic tumors [reviewed by Eng and Mulligan (7)]. Parental DNA was available for one of the MEN 2B patients with A883F, clearly identifying this patient as a *de novo* case of MEN 2B. Parental DNA for the second patient with A883F was not available for analysis, however, lack of the MEN 2B phenotype in these individuals is highly suggestive that their A883F offspring is also a *de novo* case of MEN 2B.

The *RET* proto-oncogene encodes a protein receptor tyrosine kinase which comprises an extracellular part with a cysteine-rich domain, a small transmembrane region and an intracellular segment with a tyrosine kinase domain (14, 15). Whereas mutations causing MEN 2A have been found in one of six cysteine codons in the juxtamembrane region of the extracellular domain (5), FMTC mutations may affect intracellular non-cysteine codons as well (5, 16, 17). In contrast, thus far, only one mutation, M918T, has been associated with MEN 2B (5). This same mutation, when present somatically, has also been found in a sizable proportion (mean 40%, with most in the 30-50% range) of somatic MTC (7, 18). Methionine at position 918 is a highly conserved residue lying in the substrate specificity pocket of the central catalytic core of the tyrosine kinase domain (19). The residues which make up the central catalytic core, to some extent, determine the substrate preference of various tyrosine kinases. *In vitro* studies have shown that altering the methionine at RET residue 918 to a threonine (the MEN 2B M918T) causes the RET tyrosine kinase to alter its substrate preference (20-22). Alanine at position 883 also lies in the central catalytic core of the tyrosine kinase domain (19). Interestingly, this amino acid is not highly conserved across various tyrosine kinases but lies in close proximity to a motif which is highly conserved in tyrosine kinases and also plays a role in substrate preference. It is believed that this is one of the motifs that helps separate tyrosine kinases from serine/threonine kinases (19). By extrapolation, A883F, which alters a small neutral amino acid to a large nonpolar hydrophobic residue, could be postulated to stearically alter the three-dimensional structure of the motif region and hence, cause alteration of substrate preference. Although it is interesting that both MEN 2B-associated mutations occur in amino acids that likely determine substrate specificity, it is not known why fewer than 5% of such patients are accounted for by A883F.

In terms of molecular diagnosis, the clinician should consider *RET* codon 918 testing first in cases of MEN 2B and suspected MEN 2B. Should such testing yield a mutation negative result, then the physician should consider analysis of codon 883. This 883GCT->TTT mutation causes loss of the *Alu*I restriction enzyme recognition site, thus providing a straightforward, inexpensive manner for molecular diagnostic purposes.

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