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Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC

Helen Donis-Keller[1,*], Shenshen Dou[1], David Chi[1,2], Katrin M.Carlson[1], Koji Toshima[3], Terry C.Lairmore[2], James R.Howe[2], Jeffrey F.Moley[1,2,4], Paul Goodfellow[1] and Samuel A.Wells, Jr[2]

[1]Division of Human Molecular Genetics and [2]Division of General Surgery, Department of Surgery, Washington University School of Medicine, 660 S. Euclid, St Louis, MO 63110, USA, [3]Second Department of Surgery, Nippon Medical School, Tokyo 102, Japan and [4]St Louis Veterans Administration Medical Center, St Louis, MO 63106, USA

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Multiple endocrine neoplasia type 2A (MEN 2A) and familial medullary thyroid carcinoma (FMTC) are dominantly inherited conditions which predispose to the development of endocrine neoplasia. Evidence is presented that sequence changes within the coding region of the RET proto-oncogene, a putative transmembrane tyrosine kinase, may be responsible for the development of neoplasia in these inherited disorders. Single strand conformational variants (SSCVs) in exons 7 and 8 of the RET proto-oncogene were identified in eight MEN 2A and four FMTC families. The variants were observed only in the DNA of individuals who were either affected or who had inherited the MEN2A or FMTC allele as determined by haplotyping experiments. The seven variants identified were sequenced directly. All involved point mutations within codons specifying cysteine residues, resulting in nonconservative amino acid changes. Six of the seven mutations are located in exon 7. A single mutation was found in exon 8. Variants were not detected in four MEN 2B families studied for all exon assays available, nor were they detectable in 16 cases of well documented sporadic medullary thyroid carcinoma or pheochromocytoma that were tested for exon 7 variants. Coinheritance of the mutations with disease and the physical and genetic proximity of the RET proto-oncogene provide evidence that RET is responsible for at least two of the three inherited forms of MEN 2. Neither the normal function, nor the ligand of RET are yet known. However, its apparent involvement in the development of these inherited forms of neoplasia as well as in papillary thyroid carcinoma suggest an important developmental or cell regulatory role for the protein.

INTRODUCTION

The RET proto-oncogene encodes a transmembrane protein tyrosine kinase. Other members of this class of molecules include TRK-A, TRK-B, TRK-C, EGFR and PDGFR (1, 2). The normal function of the RET proto-oncogene remains to be established, but it is presumably a receptor for a growth factor or other ligand. However, induction of RET by retinoic acid in neuroblastoma cell lines and its temporal expression suggest a role in neuroendocrine cell development (3–5). The proposed structural domains of the protein consist of an N-terminal leader sequence followed by an extracellular domain that includes a cysteine-rich region, a transmembrane region, and a tyrosine kinase domain that lies within the terminal one-third of the molecule (5, 6). Due to its low or absent expression in most adult tissues, much of what has been learned about the structure and activity of the RET proto-oncogene has come from studies in transformed or malignant cells, where RET is often expressed at high levels (4, 5). In addition, the RET proto-oncogene also undergoes at least three abnormal genomic DNA rearrangements which result in the replacement of different segments of the 5′ portion of the gene and produce chimeric (activated) versions of the gene, each of which retains the tyrosine kinase domain and is expressed (7–9). One such rearranged version has been identified in approximately 25% of cases of human papillary thyroid carcinoma (10–12). The RET proto-oncogene is expressed in medullary thyroid cancers and pheochromocytomas isolated from individuals with the familial and sporadic forms of these cancers (13, 14) and is virtually absent in normal control tissue. It is, therefore, possible that RET may play a role in the development of these malignancies.

Physical and genetic mapping data also focused attention on RET as a candidate gene for the dominantly inherited multiple endocrine neoplasias type 2 (MEN 2A, MEN 2B) and familial medullary carcinoma (FMTC). The genes responsible for these three clinically distinct disorders map to the pericentromeric region of chromosome 10 (15–17). Physical and genetic mapping studies have further refined the interval which includes RET (18, 19). The small size of MEN 2B and FMTC pedigrees available for study has limited genetic mapping experiments and it was unclear as to whether MEN 2A, MEN 2B and FMTC represent allelic mutations or result from mutations in closely linked loci.

MEN 2A and FMTC are both characterized by the development of bilateral medullary thyroid carcinoma (MTC) but the age of onset for FMTC is usually later (ranging from 40–50 years as compared to the average age of onset of 20–30 years for MEN2A) (20). MEN 2A patients also develop pheochromocytomas and parathyroid hyperplasia which are not apparent with FMTC. MEN 2B, a more complex phenotype, includes diffuse ganglioneuromas of the intestinal tract, mucosal

* To whom correspondence should be addressed

neuromas, and skeletal abnormalities in addition to the presence of bilateral MTC and pheochromocytomas. MEN 2B is invariably recognized within the first decade of life and the MTC tends to take a more biologically aggressive course (21).

Chromosome translocations, deletions, or rearrangements detected by long-range or standard Southern restriction mapping have not been found within or adjacent to the RET locus (Brooks-Wilson et al., unpublished data; Lairmore and Donis-Keller, data not shown). Consistent deletions within other chromosomes such as the short arm of chromosome 1 have, however, been observed in tumors from patients with the MEN 2 syndromes (22, 23). In an effort to evaluate RET as a candidate for MEN 2 we implemented single strand conformation polymorphism (SSCP) analysis of constitutional and tumor DNA. We report the identification of DNA sequence mutations in four codons that predict nonconservative amino acid changes within the proposed extracellular domain of the RET proto-oncogene.

RESULTS

SSCP analysis of RET proto-oncogene genomic sequence

The genomic organization of RET was determined using a PCR and sequence analysis approach. Oligonucleotide primer sequences were devised based on published cDNA sequences (5, 6; GenBank accession numbers X12949 and X15262) and PCR assays developed using cloned genomic sequences. A total of 17 exons and 16 introns were identified encompassing 22 kb of genomic DNA. SSCP PCR assays defining products of 300 bp or less were developed (the complete genomic structure and SSCP methods will be reported elsewhere) (Chi et al., unpublished).

We screened a panel of genomic DNAs from seven independent MEN 2A, MEN 2B and FMTC families including normal controls from the pedigrees with our full set of exon assays and 33 families with the exon 7 and 8 assays only (Table 1). In some cases DNA isolated from MTCs or pheochromocytomas from these individuals was also included (17 total). A panel of 16 sporadic MTC and pheochromocytomas matched with normal DNA from the same individuals was also examined. Several SSCPs in amplified products from exons 4, 8, and 10 were identified. They segregate in both MEN 2 and CEPH reference pedigrees, and as such are believed to represent normal DNA sequence variation in the coding region and intron/exon boundaries of the RET proto-oncogene. Additional and as yet undetected polymorphism may exist.

A total of seven mobility shift variants (SSCVs) were observed in MEN 2 families and not in DNA from normal individuals. Six of these were identified in exon 7 and one in exon 8. The exon 7 PCR assay includes sequence flanking the 120 bp exon (nucleotides 1689−1809 from 5), 15 nucleotides flanking the 5′ and 8 nucleotides flanking the 3′ side of the exon that lie outside the primer sequences. The exon 8 amplification product is 233 bp in length including the 195 bp exon 8A sequence and 18 nucleotides of flanking intronic sequence. Figure 1 shows the mobility shifts for four of the exon 7 SSCVs and the normal SSC pattern. All seven SSCVs were found in DNA from individuals who were diagnosed with MEN 2A or FMTC or who carried the MEN 2 DNA haplotype, but who had not yet expressed symptoms (24, and unpublished results). Thus far 128 normal reference chromosomes have been examined for exon 7 SSCVs and 80 chromosomes for exon 8 SSCVs. None showed variation in the SSC mobility pattern for exon 7 and 8 amplifications.

Inheritance of SSCV8A-1 from exon 8 in an MEN 2A family is shown in Figure 2. In this pedigree we identified a recombination event in the interval between D10S141 and RET (sTCL-2), within the most narrowly defined location for the MEN2A gene (approximately 480 kb between D10S141 and D10S94 (18, 19). The individual (numbered 4 in Fig. 2) who carries the recombinant chromosome has the MEN2A haplotype at RET and other distal loci and has also inherited the SSCV8A-1 marker.

DNA sequence mutations and predicted amino acid changes

The SSCV and the invariant SSC DNA sequences from exon 7 and 8 amplification products were determined by PCR amplification of products excised from SSCP gels followed by direct PCR sequencing. In cases where both SSCV conformers were well resolved, coding and noncoding strands were sequenced. An example of a portion of one of the conformer sequences that reveals a mutation (G→C) is shown in Figure 3. We sequenced several SSC versions of these exons and found that they were identical to the published cDNAs (which were isolated from a monocytic leukemia cell line, THP-1) (5, 6). Results of SSCV sequence analysis are summarized in Figure 4. Each of the seven SSCVs resulted from a sequence difference, and all mutations affected codons specifying cysteine residues. Analysis of two forms of SSCV7-2 (SSV7-2 and SSCV7-2′) proved that subtle mobility differences were attributable to different mutations, both involved cysteine codons (T→C and

Table 1. SSCP analysis of constitutional and tumor DNA

	Numbers Examined Assays			Mutations Detected Assays			Variants Detected Assays		
	7	8A	1−6, 8B-17	7	8A	1−6, 8B-17	4	8B	10
Inherited Disease									
MEN 2A	11	11	5	7	1	0	1	0	3
MEN 2B	17	17	4	0	0	0	1	1	2
FMTC	5	5	2	4	0	0	0	0	0
Tumors									
Sporadic	16	16	1	0	0	0	0	0	0
Familial	29	29	1	5*	1*	0	1*	0	5*

* same mutations or neutral variants as observed in constitutional DNA.
Exon 8 [this designation is based on the cDNA sequence of Takahashi et al. (5)] is approximately 260 base pairs in length. It was divided into two assays in an effort increase sensitivity through analysis of smaller PCR products. Assay 8A corresponds to the more 5′ portion of the exon.

G→A). Mutations were found in three of the four cysteine codons contained within exon 7, and for all three the predicted change is a nonconservative substitution: Cys→Arg, Cys→Tyr, Cys→Ser, Cys→Trp. A single mutation has been observed in exon 8 that predicts substitution of an Arg residue for a Cys. The amino acid sequence for exon 7 shown here is part of a proposed extracellular domain which presumably serves as a binding site for a growth factor or other ligand (5). The mutation in exon 8 also changes a cysteine in the extracellular domain of the protein (Figure 4) in the region immediately adjacent to the proposed transmembrane domain. Both exons 7 and 8 lie upstream of the tyrosine kinase domain coding sequence (which begins at approximately C_{2106}; 5). One individual representative of each of the sequence mutations identified has been tested for additional SSCVs in the remaining exons. No detectable sequence variation has been observed using four SSCP gel conditions. Although we have tested all of the coding sequence as defined in Takahashi et al. (5), studies are currently underway to survey an additional reported 5′ coding sequence that extends the 5′ coding region for the RET proto-oncogene (6) and the putative promoter sequence for the gene for additional variation with the mutation panel shown in Figure 4 (Chi et al., unpublished).

In an attempt to prove that the families that shared the same RET exon 7 mutations (SSCV7-1, SSCV7-2′, SSCV7-3, Fig. 4) are unrelated we determined alleles at the RET microsatellite marker (sTCL-2) (17) which is located approximately 75 kb telomeric to the RET gene (data not shown). In the case of SSCV7-2′, it was found that the RET alleles segregating with the disease and SSV7-2′ differed. Similarly for SSCV7-3, alleles at the RET microsatellite locus that are inherited with MEN 2A differ between K12 and K10 (haplotype data was not available for K21). Haplotype data was available for K17 but not for K57 and K64 that share the SSV7-1 mutation, therefore haplotype analysis was not possible. In addition, historical records indicate that the families that share mutations are not known to be related and are geographically isolated.

Tumor DNA analysis

SSCVs present in germline DNA were also found in MTC and pheochromocytomas from familial cases. No variants were detected in well documented cases of sporadic MTC or pheochromocytomas. In one case (MEN 2A K57-1400) we detected a mutation in germline DNA as well as in a pheochromocytoma. This individual had multifocal MTC several years prior to diagnosis of pheochromocytoma. No other relatives in this person's family have apparently had MTC or pheochromocytoma (three siblings were biochemically screened and detailed family histories taken). We must consider this a *de novo* MEN2A mutation.

A novel SSCV in exon 7 was identified in an adrenal cortical tumor (data not shown). DNA sequence analysis of the excised SSCV band did not, however, detect a difference from the normal. We expect that SSCV7-6 is a mutation within intronic sequences, immediately adjacent to one of the primers used for PCR amplification of exon 7. Direct sequencing of the SSCV band did not allow us to examine those bases. Additional

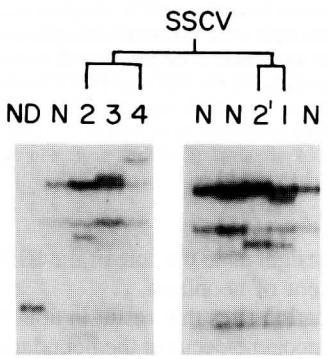

Figure 1. Five SSCVs within the coding region of the RET proto-oncogene detected in MEN 2A and FMTC-affected family members. Genomic DNAs from lymphoblastoid cell lines or pheochromocytomas were amplified by PCR using oligonucleotide primers flanking exon 7 of the RET proto-oncogene. PCR products uniformly labeled with ^{32}P were resolved by electrophoresis at 4°C through 5% polyacrylamide gels containing 5% glycerol and 0.5×TBE. ND), non-denatured control sample, N designates SSC pattern of the normal exon 7 sequence. From left to right the Ns are genomic DNA from MEN 2A K5-4041, sporadic pheochromocytoma PH7, MEN 2A K3-2339, and sporadic pheochromocytoma PH18. Five SSCV variant forms 1, 2, 2′, 3, 4, present only in MEN 2A and FMTC-affected individuals, are shown in the lanes as designated. These individuals also display an SSC pattern reflecting the normal exon sequence. 2) MEN 2A K9-6002, 3) MEN 2A K12-8060, 4) FMTC K22-1002, 2′) variant pattern nearly indistinguishable from SSCV-2 but shown to contain a different DNA mutation (Fig. 4) from a pheochromocytoma of MEN 2A K2-8001, 1) variant pattern 1 from a pheochromocytoma of MEN 2A-affected individual MEN 2A K57-1400.

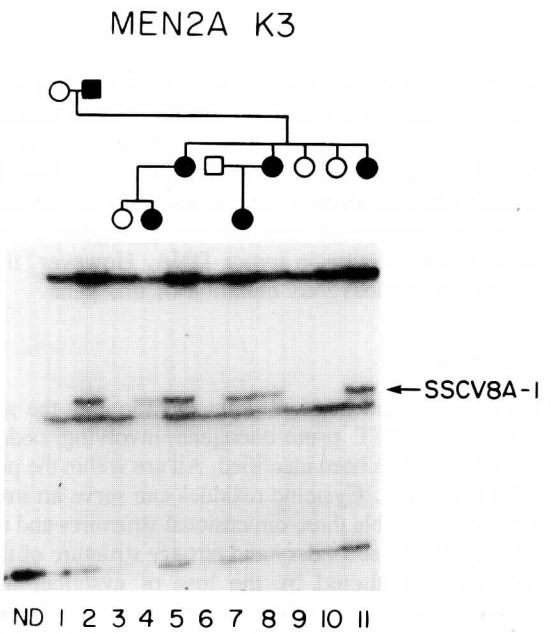

Figure 2. Inheritance of SSCV8A-1 in MEN 2A family K3. Genomic DNAs from lymphoblastoid cell lines were amplified by PCR using oligonucleotide primers flanking approximately the first half of exon 8 (assay 8A) of the RET proto-oncogene. PCR products uniformly labeled with ^{32}P were resolved by electrophoresis at 4°C through 5% polyacrylamide gels containing 5% glycerol and 0.5×TBE. ND), non-denatured control sample. Lanes 1–11 designate MEN 2A kindred 3 family members. The pedigree structure is shown at the top of the figure, filled in symbols indicate MEN 2A-affected or carrying the MEN 2A DNA haplotype. Individuals 2, 5, 7, 8, and 11 have been diagnosed with MEN 2A. Individual 4 has a crossover between the closest genetic markers to MEN 2A (D10S41 and RET) but carries the MEN 2A haplotype at the RET locus and distal to it. Arrow indicates detectable mobility shift band, SSCV8A-1.

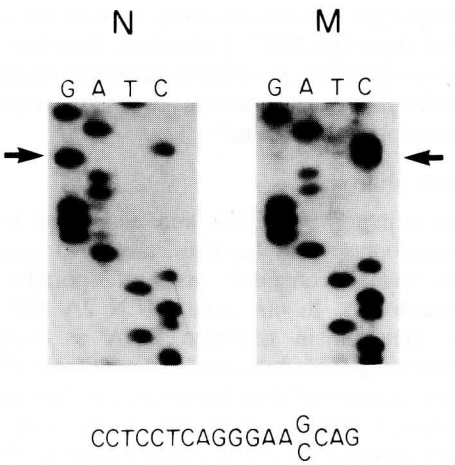

Figure 3. Genomic DNA sequence of normal and mutant alleles from a portion of the exon 7 coding region of the RET proto-oncogene. Genomic DNAs from lymphoblastoid cell line DNAs were PCR amplified, electrophoresed through SSCP gels, and electrophoretic variant bands excised and sequenced directly (see Fig. 1, the uppermost band was sequenced from SSCV-4). N, M indicate normal and mutant sequences and lanes G, A, T, C sequencing ladder reaction products. Arrows show position of point mutation. The SSCV7-4 sequence from FMTC-affected individual K22-1002 (the uppermost band shown in lane numbered 4 in Fig. 1) reveals a G→C mutation in the noncoding strand. This mutation in the third base of the codon predicts an amino acid change, Cys→Trp (also see Fig. 4).

EXON 7		2 3 4
Normal		AAC-TGC-TTC-CCT-GAG-GAG-GAG-AAG-TGC-TTC-TGC-GAG
		Asn---Cys--Phe--Pro---Glu---Glu---Glu---Lys---Cys---Phe--Cys--Glu
SSV7-1		TTC-CGC-GAG
MEN 2A K17-4520		Phe--Arg---Glu
MEN 2A K57-1400		
FMTC K64-0004		
SSV7-2		AAG-CGC-TTC
MEN 2A K261-02		Lys---Arg---Phe
SSV7-2'		TTC-TAC-GAG
MEN 2A K2-8001		Phe--Tyr---Glu
MEN 2A K9-6002		
SSV7-3		AAG-AGC-TTC
MEN 2A K12-8060		Lys---Ser----Phe
MEN 2A K10-2454		
FMTC K21-001		
SSV7-4	AAC-TGG-TTC	
FMTC K22-1002	Asn--Trp---Phe	
SSV7-5		AAG-TAC-TTC
FMTC K34-0010		Lys---Tyr---Phe
EXON 8		
		1 2
Normal		CTG-TGC-GAC-GAG-CTG-TGC-CGC-ACG
		Leu--Cys---Asp--Glu---Leu--Cys--Arg---Thr
SSV8A-1		CTG-CGC-CGC
MEN 2A K3-2623		Leu--Arg---Arg

Figure 4. DNA sequence mutations and predicted amino acid changes in RET proto-oncogene coding regions. Genomic DNAs from lymphoblastoid cell line or tumors (MTC or pheochromocytomas) were PCR amplified, electrophoresed through SSCP gels, and electrophoretic variant bands excised and sequenced directly (also see Fig. 1 and Materials and Methods). A portion of the 3′ region of exon 7, which includes 3 of the 4 cystines encoded in the normal sequence and the 5′ portion of exon 8 which includes 2 of the 4 cysteines encoded in the normal sequence (numbered above the codons) are shown above the mutant forms. Positions of predicted amino acid changes Cys→Arg, Tyr, Ser, Trp are underlined. Mutant sequences of variants and representative individuals for the 8 MEN 2A families (K2, K3, K9, K10, K12, K17, K57, K261) and 4 FMTC families (K21, K22, K34, K64) are listed beneath the SSCV identified and the mutant sequence found is indicated to the right of the individual's identification number. Inheritance studies confirming the segregation of the SSCVs with disease and/or MEN2 allele were MEN2A families (K2, K3, K9, K17) and FMTC families (K22, K34) (Fig. 3 for K3, data not shown). The DNA sequence of the portion of exon 7 shown in Figure 4 begins with nucleotide A_{1759} and at C_{1615} for exon 8 as numbered in the published cDNA sequence (GenBank accession number X12949; 5).

sequencing studies using primers distal to or internal to, but in the reverse orientation to the amplification primers should serve to identify this mutation. Unexpectedly one of the MTC tumors from an MEN 2A-affected individual appeared to have lost the normal SSCs (data not shown). Studies are underway to determine whether a chromosome deletion has truely occurred, and if so, the extent of the deletion in tumor DNA. However, this case could represent homozygous mutation of this gene.

DISCUSSION

Twelve ostensibly independent point mutations in the genomic sequence of the RET proto-oncogene involving codons for cysteine residues have been identified. All are within the proposed extracellular domain. Cysteine residues can serve an important role in preserving stable three-dimensional structures and as such, it is possible that the secondary and tertiary structure of the RET proto-oncogene is altered by the loss of cysteine, and that alteration of the secondary structure could affect ligand binding. Coinheritance of the sequence changes with the MEN 2A and FMTC phenotypes in these families suggests that the mutations confer a predisposition to malignancy, specifically in neural crest derived tissues. The observation that mutations involving the third and fourth cysteine residues in exon 7 occur in unrelated, geographically isolated MEN 2A and FMTC families, and that nearby microsatellite RET alleles differ in the cases for which data were available, argues against linkage disequilibrium as an explanation for coinheritance of the mutations and the disease. The absence of these sequence changes in normal chromosomes provides further evidence that RET mutations predispose to the development of MEN 2A and FMTC. Biological assays testing the mutant forms in cell culture and transgenic mouse lines should provide further insight as to the role of the RET proto-oncogene in the development of neoplasia.

Two recombination sites that result in abnormal activation of the RET proto-oncogene occur at the boundaries between exons 6 and 7 and exons 8 and 9 (Chi et al., unpublished). One of the first rearrangements to be described involved the first codon of exon 7 such that part of the coding sequences for the extracellular domain in addition to the transmembrane domain and the tyrosine kinase portion of the RET molecule are preserved (5, 7). Two of the other activated forms of RET, including one identified from a human papillary thyroid carcinoma (PTC ret), are characterized by the same recombination site with the result that the extracellular and transmembrane domains are removed (10–12). While the presence of the new 5′ portion of these fusion proteins may confer new properties to the RET protein, it may be a sign that the loss of a cysteine residue as a result of deletion leads to the malignant phenotype.

Since MEN2A and FMTC have distinctly separable clinical manifestations it was surprising to find that mutations in the same codons resulting in the same amino acid substitutions are found

in both diseases. It is conceivable that other genes could modify the age-of-onset for these disorders resulting in the absence of pheochromocytoma which usually appears about ten years after the onset of MTC. Alternatively, it is possible that due to the small size of some of these kindreds, they are MEN 2A families that have been mistakenly designated FMTC.

If we assume that the RET proto-oncogene is the causative agent in MEN 2A, we have not yet found all of the mutations that would predict disease. Mutations in exons 7 or 8 were not found for 3 MEN 2A families tested and presumably these lie within other exons not yet examined, or they are not detectable by SSCP analysis. We have not yet documented mutations in RET for 17 MEN 2B families tested thus far for exons 7 and 8 and four MEN 2B families tested with our full panel of SSCP assays. Because of the more dramatic phenotype and early age-of-onset, one might expect a contiguous gene syndrome that could be revealed as a deletion. This has not been observed thus far. However the SSCP analysis approach may not reveal such events. It is possible that a truncated protein or the absence of expression from one allele could produce the phenotype. The expression of the RET proto-oncogene early in development of neuroendocrine derived cells and the induction of expression by retinoic acid in neuroblastoma cells provides an intriguing link to the mucosal neuroma and ganglioneuroma manifestations of MEN 2B.

Providing that the RET proto-oncogene is definitively implicated as the causative agent for the MEN 2 syndromes by functional assays, the current SSCV assays and sequencing can be used to distinguish sporadic MTC and pheochromocytomas from spontaneous mutations to the MEN 2 phenotype since germline mutations would not be predicted in the sporadic cases. In addition, the mutations shown in Figure 4 create six new restriction sites which could facilitate rapid, nonradioisotopic detection of mutant alleles in the at-risk population.

MATERIALS AND METHODS

Subjects and tumor DNA samples

MEN 2A, MEN 2B and FMTC pedigree resources available for this study and diagnostic procedures have been described elsewhere (15, 17, 24). The panel of sporadic MTC and pheochromocytomas and preparation of genomic DNAs have been described previously (23). Reference pedigrees available from the CEPH and preparation of DNA from lymphoblastoid cell lines has been reported (24).

SSCP assays

Oligonucleotide primers flanking RET proto-oncogene intron/exon boundaries were chosen using a Primer Choice program made available to us by E.Lander (Whitehead Institute, Cambridge MA). The previously published RET cDNA sequence on which we based our investigation of the genomic structure is available (5; GenBank X12949). The complete genomic structure, sequences of the intron/exon boundaries for all exons, and primer sequences used to amplify exons 1–6 and 8B-17 will be reported elsewhere (Chi et al., unpublished) and can be obtained from the authors. Oligonucleotide primer sequences used to amplify exon 7 are 7F $^{5'}$GCGCCCCAGGAGGCTGAGTG$^{3'}$ and 7R $^{5'}$CGTGGTGGTCCCGGCCGCC$^{3'}$ and for exon 8, 8AF $^{5'}$CCTCTGCGGTGCCAAGCCTC$^{3'}$ and 8AR $^{5'}$CACCGGAAGAGGAGTAGCTG$^{3'}$. These sequences have been submitted to GenBank and assigned the accession numbers L18747, L18748 and L18749. PCR products were uniformly labeled and amplified using the following conditions: In a total reaction volume of 5μl containing 50 ng of genomic DNA, 50mM KCl, 10mM Tris–HCl (pH8.3), 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 0.5U Taq polymerase (AmpliTaq, Perkin Elmer-Cetus Co.), 200μM of each dNTP, 2 μM each of exon 7 primers 7F and 7R, 1.5 μCi α-^{32}P dCTP, 25 cycles of PCR denaturation, annealing and extension were performed at 94°C (30 sec), 68°C (30 sec), 72°C (1min) using an automatic thermocycler (Biometra, Emertson Instruments, Inc. Ontario, Canada). The expected product size is approximately 182 bp. For exon 8 the reaction conditions were essentially the same as for the exon 7 assay with the following modifications: 10 mM Tris–HCl pH8.4, 1.2 mM $MgCl_2$, 0.1% w/v gelatin, 94°C (1 min), 65°C (1 min), 72°C (1 min) for 25 cycles. The expected product size is 233 bp. Reaction mixtures were diluted 1:4 with using 5 μl quenching buffer (9.5% formamide, 2 mM EDTA, 0.005% bromophenol blue, 0.005% xylene cyanol) and 10μl dH_2O. SSCP analysis was done essentially as described by Orita et al. (25). Samples were treated at 95°C for 5 min and directly loaded or placed on ice and loaded on SSCP gels. Two sets of SSCP gel conditions were routinely used to identify SSCPs for all exons examined and in some cases 2 additional gel conditions were used essentially as described (25). SSCVs for the exons were detected by electrophoresis at 35 watts for 3.5 hrs at 4°C through a 5% polyacrylamide 2% N, N′-methylenebisacryamide/acrylamide gels containing 5% glycerol and 0.5×TBE (45 mM Tris-borate, 1 mM EDTA). Gels were vacuum-blotted to Whatman 3MM filter paper and exposed to XAR5 X-ray film (Eastman Kodak, Rochester, NY), at −80°C for 3 hrs, or at room temperature overnight.

DNA sequencing of SSCV conformers

SSCV bands were excised from dried SSCP gels and the DNA eluted in 100 μl ddH_2O overnight at room temperature or heated to 50–65°C for 15–30 min. The products were reamplified using 10 μl of the eluted DNA as template in a total reaction volume of 50 μl containing 10 mM Tris–HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 0.1 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer (used in the exon 7 and 8A SSCV assays) and 1.5 U AmpliTaq (Perkin-Elmer-Cetus Co.).The following PCR cycle protocol was used: 94°C (30 sec), 68°C (30 sec), 72°C (1 min), and a total of 30 cycles. Amplified PCR products were purified using 1% low melt agarose gels (Seaplaque, FMC, Rockland, ME).

Sequencing reactions were performed according to the method of J.Kere (personal comm.). Briefly, sequencing primers were end-labeled using 0.1 μM primer, 18 mM Tris–HCl (pH7.5), 2.5 mM $MgCl_2$, 1 mM DTT, 35 μCi γ-^{32}P-dATP and 5 U polynucleotide kinase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 45 min then heat denatured at 95°C for 10 min. Sequencing reactions were done using 1 μl of template in low-melt agarose per 10 μl reaction. Reactions were carried out with the appropriate PCR buffer, 2.5 μM each dNTP, and one of the following: 25μM ddGTP, 250 μM ddATP, 400μM ddTTP or 250 μM ddCTP, 0.25pMole end-labeled sequencing primer, 1 U Taq polymerase (AmpliTaq, Perkin-Elmer-Cetus Co.) at appropriate annealing temperature for 20–30 cycles.

Note added in proof

A six basepair deletion in exon 8 that removes the coding sequence for cysteine one (Fig. 4) has been observed for one RET allele in a sporadic MTC tumor. The normal tissue from this individual shows no deletion. This finding suggests that RET also plays a role in non-familial medullary thyroid carcinoma.

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ABBREVIATIONS

EGFR, epidermal growth factor receptor
FMTC, familial medullary thyroid carcinoma
MEN2A, (no space between MEN and 2A) multiple endocrine neoplasia type 2A
MEN 2A, multiple endocrine neoplasia type 2A (disease)
MEN2B, (no space between MEN and 2B), multiple endocrine neoplasia type 2B gene
MEN 2B, multiple endocrine neoplasia type 2B (disease)
PCR, polymerase chain reaction
PDGFR, platelet derived growth factor receptor
RET, ret proto-oncogene
SSCV, single strand conformational variant
SSCP, single strand conformational polymorphism
TRK-A, -B, -C, (A, B, and C are clarifiers), tyrosine receptor kinase

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