

RESEARCH ARTICLE

Detection of *RET* Mutations in Multiple Endocrine Neoplasia Type 2a and Familial Medullary Thyroid Carcinoma by Denaturing Gradient Gel Electrophoresis

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Germline missense mutations within the coding region of the *RET* proto-oncogene have recently been described in patients with the dominantly inherited cancer syndromes, multiple endocrine neoplasia type 2a (MEN 2a) and familial medullary thyroid carcinoma (FMTC). To date, the sequence variations occur in *RET* exons 10 and 11 and alter highly conserved cysteine residues in the proposed extracellular domain at codons 609, 611, 618, 620, and 634. To expedite rapid screening of populations at risk of MEN 2a or FMTC, we developed a PCR-based denaturing gradient gel electrophoresis (DGGE) strategy that detects polymorphisms occurring at all five Cys codons in both *RET* exons using identical gel conditions. In this report, the screening results from DGGE analysis of 15 distinct MEN 2a and FMTC mutations are shown. Each mutation generated a clearly distinguishable and unique homo- and heteroduplex band pattern. Given the highly efficient, reproducible, and sensitive nature of this approach, DGGE is particularly appropriate for rapid, large-scale screening of patients. Since prior knowledge of the *RET* mutation is unnecessary for analysis, DGGE is potentially valuable for distinguishing germline from seemingly sporadic medullary thyroid cancer as well as identifying novel sequence changes. © 1996 Wiley-Liss, Inc.

KEY WORDS: MEN 2a, FMTC, DGGE, *RET* proto-oncogene

INTRODUCTION

Multiple endocrine neoplasia type 2a (MEN 2A) and familial medullary thyroid carcinoma (FMTC) are dominantly inherited cancer syndromes characterized by the occurrence of medullary thyroid carcinoma (MTC). Patients with MEN 2A also have a predisposition to the development of pheochromocytomas, parathyroid hyperplasia, and Hirschsprung's disease (Decker, 1992; Borst et al., 1995). Recently, germline missense mutations within the coding region of the *RET* proto-oncogene, a putative transmembrane tyrosine kinase, have been described in family members affected with these cancer syndromes (Mulligan et al., 1993; Donis-Keller et al., 1993; Mulligan et al., 1994). To date, all reported mutations occur in *RET* exons 10 and 11 and involve codons specifying highly conserved cysteine residues within the presumed extracellular domain of the receptor. Four of the five cysteines mutated in

these syndromes are located in *RET* exon 10 at codons 609, 611, 618, and 620 (Takahashi et al., 1988; Takahashi et al., 1989). The most frequent events occur at the remaining codon 634 in exon 11, accounting for approximately 84% of patients with MEN 2a and 50% of patients with FMTC (Mulligan et al., 1994).

Identification of the responsible gene allowed us to develop a DNA-based screening strategy for mutation detection to assist in the diagnosis of patients at risk for MEN 2A or FMTC. Previously, testing for hereditary MTC was accomplished by measuring calcitonin levels after the intravenous injection of pentagastrin and/or calcium (Wells et al., 1978). This method of diagnosis is suboptimal for several reasons. First, due to delayed biochem-

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ical penetrance, years of repeated testing may be required before the disease can be safely excluded. Furthermore, borderline results are difficult to interpret and family compliance with screening problematic given the expensive and unpleasant nature of the pentagastrin testing. Finally, biochemical testing of all first-degree relatives in new cases of MTC to distinguish germline from sporadic disease is cumbersome and difficult to accomplish. More recently, various DNA-based techniques have emerged to identify *RET* mutations including direct genomic sequencing, single-stranded conformational polymorphisms (SSCP), and restriction enzyme digestion analysis (Donis-Keller et al., 1993; McMahon et al, 1994; Xue et al, 1994). However, these may not be the most efficient or accurate methods for mutational analysis. Moreover, prior knowledge of the mutation may be necessary limiting test efficacy in prospective screening. Therefore, we have developed a highly reliable denaturing gradient gel electrophoresis (DGGE) strategy to expedite detection of the MEN 2a/FMTC *RET* point mutations in exons 10 and 11. Analysis by DGGE has been successful in our hands as a nonradioactive method for rapid, simultaneous screening of a large number of patients and has replaced other DNA-based approaches to diagnosis as well as biochemical testing in our patients at risk of hereditary MTC.

MATERIALS AND METHODS

Polymerase Chain Reaction

Genomic DNA representing 15 distinct mutations at Cys codons 609, 611, 618, 620, and 634 were analyzed. Polymerase chain reaction (PCR) amplification of *RET* exon 10 was performed using 1 µg genomic DNA template, 2.2 µM each oligonucleotide primer, 200 µM each dNTP, 1.25 U *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD), 10 mM Tris (pH 8.3), 50 mM KCl, 2.0 mM MgCl₂ in a total volume of 50 µl. Conditions for amplification consisted of 35 cycles at 95°C for 1 min (denaturation), 72°C for 1 min (annealing and extension), followed by a single denaturing and annealing step at 100°C for 10 min using an automated thermocycler (MJ Research, Watertown, MA). Expected product size is 216 bp. The following primers were chosen from intronic sequences flanking the intron/exon boundaries of exon 10 after a melting profile analysis by Melt87 (Lerman and Silverstein 1987), and included a GC-clamp: 5'GCGCCCCCGCCCCCGCCC-CGCCCCCGCGCGCCCCAGGAGGCTG-

AG TG^{3'} (5'primer) and 5'CGTGGTGGTC-CCGGCCGCC^{3'} (3'primer).

For amplification of exon 11, primers 5'-CTCACACACCACCCACCCCA^{3'} (5'primer) and 5'(GC)₁₅GTCCAGCGAGGGCCGGCGG^{3'} (3' primer) were selected from sequences flanking Cys codon 634 following MELT87 analysis. Amplification of the 268 bp fragment was performed using 1 µg genomic DNA, 2.2 µM each primer, 200 µM each dNTP, 1.25 units *Taq* polymerase, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 10% DMSO in a final volume of 50 µl. Thermal cycling conditions consisted of 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 sec, and extension at 72°C for 1 min (MJ Research, Watertown, MD). Following each amplification, samples were heated to 100°C for 10 min and allowed to cool to room temperature to facilitate heteroduplex formation.

Denaturing Gradient Gel Electrophoresis

DGGE analysis was carried out on a 1-mm-thick 9% acrylamide gel (acrylamide:bis-acrylamide 20:1) with a 50–80% linear gradient of denaturants (100% = 7M urea, 40% (v/v) formamide) in 1×TAE buffer (40 mM Tris acetate, 20 mM sodium acetate, 1 mM EDTA at pH 7.8). Gel was overlaid with a 6% polyacrylamide stacker (acrylamide:bis-acrylamide 30:1) containing 1×TAE. Amplified PCR fragments were electrophoresed at 65°C and 17 mA/gel for 18 hr (Hoefer Scientific Instruments, San Francisco, CA) with recirculation of buffer from the lower to upper electrode chamber and ethidium bromide stained (10 min) prior to visualization.

RESULTS

Figure 1 illustrates the DGGE findings of 10 of the reported MEN 2a and FMTC *RET* mutations in exon 10. The analysis includes each of the four cysteine codons within the coding sequence of exon 10 implicated in the pathogenesis of MEN 2a and FMTC. The mutations shown occur at base positions 2021, 2028, 2047, 2048, 2053, and 2054 and involve Cys codons 609, 611, 618, and 620. Shown in Figure 2 are the results of DGGE analysis from five distinct Cys codon 634 mutations involving missense changes at nucleotides 2095, 2096, or 2097. In all cases, DGGE of the PCR-amplified fragment produced clear, sharply distinguishable and unique mutant homo- and heteroduplex band patterns specific for each point mutation. Normal controls showed the expected single banding pattern reflecting the homozygous genotype. Muta-

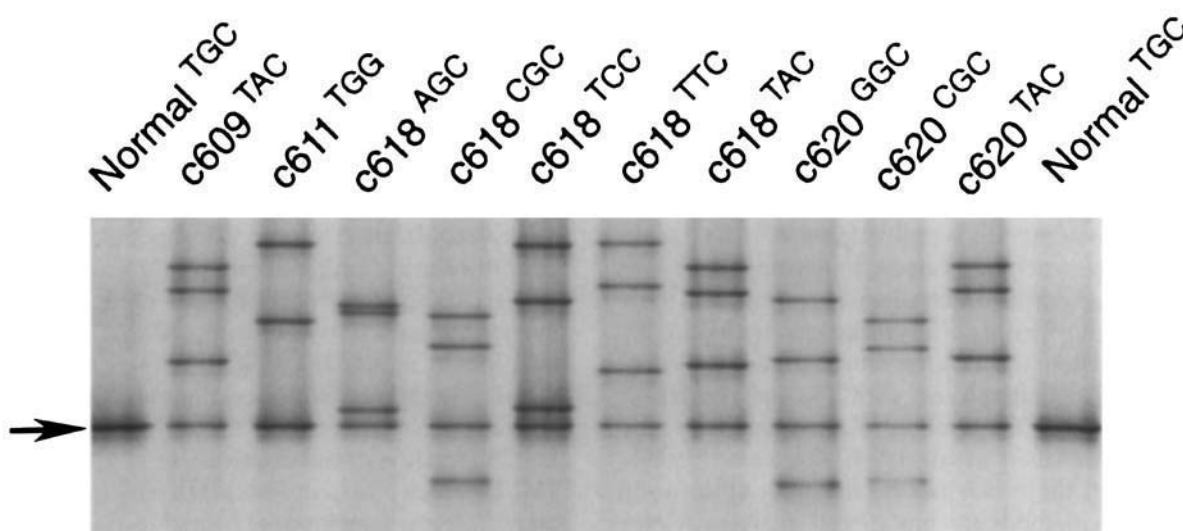


FIGURE 1. DGGE analysis of PCR amplified fragments representing 10 different missense mutations at Cys codons 609, 611, 618, and 620 in exon 10 of the *RET* proto-oncogene and normal sequenced controls. Missense mutation and corresponding codon relative to the full length *RET* coding sequence are as indicated. Unique mutant and homo- and heteroduplex bands were observed in each case. Wild-type homoduplex is indicated by the arrow.

tions involving base pair transversions as well as transitions were successfully delineated. Normal and mutant homoduplex bands were superimposed in the TGC-to-TGG transversion at codons 611 and 634. Therefore, final classification of mutations in these cases relied on differences in the heteroduplex patterns. The DGGE results were consistently and easily reproducible in separate experiments under identical PCR and gel conditions.

The DGGE analysis of *RET* exon 10 in a segment of a large MEN 2A kindred is shown in Figure 3. The mutant heteroduplex banding pattern is clearly present in all affected patients whereas the DGGE wild-type conformation, characterized by a single band, is seen in those unaffected with the disease. Direct DNA sequencing of mutants demonstrated a heterozygous base change of adenine for thymine at nucleotide 2047 resulting in a serine for cysteine substitution at codon 618 (not shown). All affected patients have undergone thyroidectomy with histological evidence of MTC. The normal status of individuals at risk for MTC in this family has been verified biochemically by the pentagastrin provocative testing.

DISCUSSION

The DNA samples analyzed collectively represent 15 of the reported missense mutations involving *RET* exons 10 and 11 in MEN 2A and FMTC. Our findings demonstrate that each point muta-

tion reliably generated a DGGE polymorphism. Furthermore, DGGE of each mutation throughout this region of *RET* displayed a unique mutant homo- and heteroduplex banding pattern accurately reflecting the heterozygous point mutation. Final characterization of the mutations relied on direct comparison with known mutants and overall banding pattern. DGGE is a sensitive and highly reproducible technique that separates double-stranded DNA fragment under denaturing conditions based on differing melting characteristics (Fischer and Lerman, 1983). To ensure detection of base changes in the region of the lowest melting domain, a GC clamp was included in the sequence to be amplified. Given the high degree of resolution and reproducible nature of these results, we believe that all codon 609, 611, 618, 620, and 634 mutations can be identified, as well as novel mutations at other positions in patients with familial or seemingly sporadic MTC or pheochromocytoma.

Other techniques have been used to detect germline *RET* mutations including direct DNA sequencing, restriction enzyme digestion, and SSCP analysis, however, the DGGE strategy confers several advantages in this setting. First, because of the relatively few manipulations, this approach permits rapid turnover of results making it amenable to mass screening of patients. Second, it does not require the use of radioisotopes. Third, the proce-

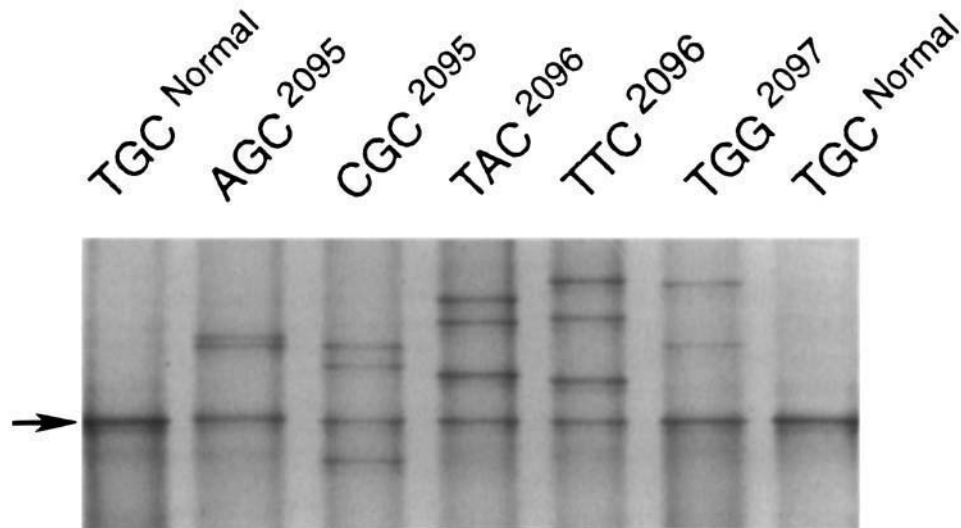


FIGURE 2. DGGE gel analysis of PCR-amplified fragments representing five different missense mutations at Cys codon 634 (nucleotides 2095, 2096, and 2097) in exon 11 of the *RET* proto-oncogene and normal sequenced and clinical controls. Missense mutation and corresponding basepair position relative to the *RET* coding sequence are as indicated. Nucleotides are numbered from the transcription start site as defined by Kwok et al. (1993) and Itoh et al. (1992). Unique mutant homo- and heteroduplex bands were observed in each case. Wild-type homoduplex is indicated by the arrow.

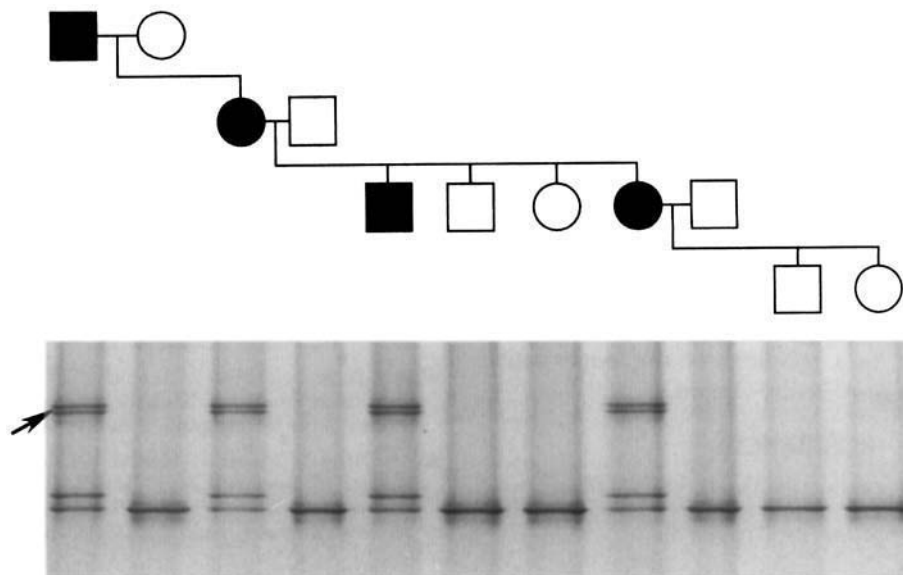


FIGURE 3. Pedigree and DGGE gel analysis of *RET* exon 10 from a family with MEN 2a. Direct DNA sequencing (not shown) revealed a thymine to adenine base substitution at nucleotide 2047 (Cys codon 618). Closed symbols indicate patients with MEN 2a. Unaffected individuals and spouses are indicated by the open symbols. All affected patients have histological evidence of MTC. Arrow, heteroduplexes generated from the mutant allele.

...dure simultaneously tests for all possible mutations at each of the five codons using identical gel conditions making it highly efficient and cost-effective. The DGGE genotype can easily be identified

in comparison with known mutant controls. Until greater experience is gained with our method, all mutations are confirmed by direct sequencing prior to diagnosis and treatment to ensure that the vari-

ations observed are not benign polymorphisms. We anticipate that this method will readily identify novel *RET* germline mutations in patients with familial disease or seemingly sporadic MTC. It has been estimated that missense changes in one of these five cysteines accounts for 97% of mutations in patients with MEN 2a and 86% of those observed in FMTC (Mulligan et al., 1994). Therefore, the DGGE approach described will not encompass all possible MEN 2a/FMTC mutations. Optimization of the DGGE conditions for *RET* exon 13, another potential site in which FMTC mutations occur (Eng et al., 1995) is underway. We are currently testing the efficacy of the DGGE diagnostic strategy in our patients at risk for MEN 2a or FMTC in a prospective ongoing clinical study. To date, discordancy between the DGGE results and findings at thyroid surgery has not been found (Decker et al., 1995). Based on our experience, DGGE is a convenient, inexpensive, highly sensitive and reliable nonradioactive method which can be employed for rapid large-scale screening of patients at risk for *RET* MEN 2a and FMTC mutations.

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