Denaturing gradient gel electrophoresis to diagnose multiple endocrine neoplasia type 2

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Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant cancer syndrome caused by mutations in the RET protooncogene. Others have already demonstrated the value of genetic testing in known MEN 2 kindreds. Previously described approaches to DNA-level diagnosis, particularly of index cases, are tedious. We developed appropriate denaturing gradient gel electrophoresis (DGGE) conditions for analysis of exons 10, 11, and 16 of this gene, where many of the pathogenic mutations map. We screened 16 members of a three-generation MEN 2 kindred by DGGE and found five affected but still asymptomatic patients, ranging in age from 5 to 67 years. We used DGGE to localize the pathogenic mutations and screen at-risk individuals in several other kindreds. DGGE—which requires no radioactive, fluorescent, or chemiluminescent labeling—is ideally suited to the diagnosis of MEN 2 because of the syndrome's dominant genetics and the rarity of clinically silent variants in the RET gene.

INDEXING TERMS: neurocristopathy • thyroid neoplasms • Hirschsprung disease • mutational analysis

Multiple endocrine neoplasia type 2 (MEN 2) is a group of autosomal dominant disorders that include familial medullary carcinoma of the thyroid (MCT), MEN 2A, and MEN 2B.5 MEN 2 is characterized by hyperplasia of the calcitonin-secreting C cells of the thyroid [11]. These hyperplastic C cells frequently undergo malignant transformation to MCT in affected individuals. In familial MCT, no other tumors develop. In the MEN 2A syndrome, pheochromocytomas and hyperparathyroidism also develop in ~50% and 20% of affected individuals, respectively. In the MEN 2B syndrome, MCT and pheochromocytomas develop in patients who have a characteristic body habitus and multiple ganglioneuromata of both the enteric nervous system and mixed peripheral nerves [2, 3].

Genetic tests for MEN 2 have recently been developed to simplify the screening of individuals from affected kindreds whose mutation is known. Most of these feature the use of altered restriction sites to allow the detection of specific pathogenic mutations in the RET protooncogene [4–7]. This approach, while extremely useful when a restriction site is altered, is limited by the fact that some pathogenic mutations do not result in such an alteration [4]. Moreover, assessing new patients for the presence of pathogenic RET mutations requires other means. Investigation of these individuals is a time-consuming and labor-intensive task.

In this study, we report the development of denaturing gradient gel electrophoretic (DGGE) assays for exons 10, 11, and 16 of the RET gene and demonstrate the utility of this method in diagnosing individuals in MEN 2 kindreds. Using DGGE, we localized the RET mutations in the index case of a new MEN 2B kindred and in a previously uncharacterized kindred with familial MCT. We were also able to provide rapid diagnosis of five asymptomatic members of a family with MEN 2A. We anticipate that DGGE methodology will greatly facilitate the identification of familial disease among new index cases of MCT.

Materials and Methods

DNA extraction. Peripheral blood (5 mL) from each patient was collected in EDTA-containing tubes, and the DNA was purified with the QIAGEN (Chatsworth, CA) blood extraction kit as recommended by the manufacturer. DNA was resuspended in Tris-EDTA buffer (10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA) at a concentration of 50–200 mg/L and stored at 4 °C.

PCR amplification. PCR reactions were performed in 50-μL volumes in a Perkin-Elmer (Norwalk, CT) 480 thermal cycler. All reactions were cycled (94 °C for 1 min, Tannealing for 1 min, 72 °C for 1 min) for 35 cycles, followed by a 10-min incubation at 72 °C. PCR primers, annealing temperatures, magnesium...
concentrations, and product lengths are listed in Table 1. Amplification reactions were carried out in 50 μL of 10 mmol/L Tris (pH 8.3 at room temperature) containing 50 mmol/L KCl, 0.1 g/L gelatin, 0.2 mmol/L of each dNTP, and 50 ng of DNA. PCR products were purified from agarose gels with the QIAEX (QIAGEN) gel extraction kit as recommended by the manufacturer.

Exon 11 products for DGGE were prepared in two rounds of PCR. The first round used the clamped forward and unclamped reverse primer designed by Donis-Keller et al. [8]. Purified product from this amplification was reamplified by using the double-clamp primer and an internal reverse primer that lies 5’ to a neutral polymorphism in this exon.

Restriction digests. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Digests were carried out in 20-μL volumes of the buffers supplied by the manufacturers. Digests included 100–500 ng of purified PCR product and 0.5–2 U of enzyme. Incubations were for 2–16 h at 37 °C except for BstUl, which was incubated at 60 °C.

DNA sequencing. Direct sequencing of PCR products was carried out with the Taq sequencing (US Biochemicals, Cleveland, OH) kit and use of 32P as recommended by the manufacturer. Electrophoresis of sequencing ladders was carried out in 8% polyacrylamide gels containing 7 mol/L urea with 1× TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, and 2 mmol/L EDTA, pH 8.3 at 25 °C) at a constant power of 70 W.

DGGE. Gels were 8% polyacrylamide (acylamide:bisacrylamide 19:1 by wt) in 0.5× TBE and were used within 3 h of casting. DGGE was performed in a Bio-Rad (Hercules, CA) Miniprotein apparatus at 45 V for 3–5 h. The 100% denaturant was 7 mol/L urea + 10 mol/L (40% by vol) formamide. Intermediate denaturants were prepared by diluting 100% denaturant with 8% acrylamide in 0.5× TBE. In all experiments, we used 1.5-mm spacers, which resulted in a gel volume of ~9 mL. Gradients were prepared bottom-up by gravity flow in a 20-μL gradient maker with 5 mL of each acrylamide solution. Details of the conditions used for each exon of RET are summarized in Results. Samples were heated to 95 °C for 5 min and allowed to cool to room temperature for 15 min before loading. Samples were visualized by staining in ethidium bromide and using ultraviolet transillumination.

Results

We undertook DNA-level analysis of the RET gene in members of known MEN 2 kindreds and in patients in whom the existence of familial disease was uncertain. Because ~90–95% [12] of the mutations causing MEN 2 had previously been found in exons 10, 11, 13, and 16 of the RET gene [8, 10, 13–15], we limited our analysis to these four exons. Full discussion of the molecular and clinical features of MEN 2 in this patient population will be presented elsewhere.

After extracting DNA from the peripheral blood from each patient and at-risk individual, we carried out PCR amplification and direct sequencing of the PCR products for each kindred’s index case and for each sporadic patient. Both strands of the PCR products were sequenced. As we developed the DGGE assays, we used these assays as the initial screen for their respective exons. The presence and the absence of mutations were confirmed by restriction digestion. Additional members of known kindreds were screened by DGGE or restriction digestion. We confirmed RET genotypes for all individuals by repeating the genotyping with complementary methods.

Development of DGGE assays for RET gene mutations

Because of the substantial effort required to seek mutations in the RET gene by direct sequencing, we sought to develop a reliable method with which we could find mutations more rapidly. DGGE, which detects differences in the melting behav-
ior of PCR products as they migrate through a gradient of formamide and urea in a polyacrylamide gel matrix [16], 
appeared to be an appropriate technique for this use [11]. Because 
affected individuals are heterozygotes, amplification of the 
mutated exon of the RET gene, denaturation, and annealing will 
result in four products: wild-type and mutant homoduplexes, 
and two heteroduplexes (wild-type coding strand and mutant 
coding strand). In particular, the heteroduplexes will have 
nonhomologous “bubbles” present at the site of the mutation. The 
bubbles in the heteroduplexes result in their having a lower 
melting temperature than the homoduplexes. Consequently, an 
affected individual will display two to four bands on DGGE, one 
for each DNA species. In contrast, normal subjects will display 
only a single band, as their PCR products contain only wild-type 
homoduplex DNA. This difference in pattern is dramatic and 
evident.

Thus far, we have found appropriate DGGE conditions for 
the detection of mutations in exons 10, 11, and 16, as shown in 
Fig. 1. We used the primer program [17] to estimate the melting 
behavior of each PCR product by processively calculating the 
annealing temperature of 20-bp windows. This analysis revealed 
that residue 634 is contained within an unusually high melting-
point region of the RET gene. This fact required that we use a 
61/62-base GC clamp and a higher gel temperature to detect 
mutations at this site. We used a two-step amplification protocol 
to incorporate this “double” GC clamp.

APPLICATION OF DGGE TO A THREE-GENERATION KINDRED 
We used the exon 10 DGGE assay to test 16 members of a 
family carrying the C620R mutation, as shown in Fig. 2. Five of 
the individuals diagnosed with MEN 2 in this family were 
asymptomatic on presentation; that is, they were first diagnosed 
on the basis of the DGGE results. These results were in perfect 
concordance with those obtained by BrUI digestion (data not 
shown), which cleaves mutant but not wild-type exon 10. One of 
these five asymptomatic individuals was in fact an obligate 
affected individual, her son being clinically affected with 
Hirschsprung disease and MCT.

Exclusion of disease is also important. Excluded individuals 
need not undergo further assessment for the development of any 
MEN 2-associated tumors, and their progeny are not at risk of 
developing MEN 2.

MOLECULAR PATHOLOGY OF THE RET GENE
The evidence is compelling that mutations of the RET protoon-
cogene cause MEN 2. Genetic studies had demonstrated that 
the autosomal dominant locus causing MEN 2 was closely 
linked to RET on chromosome 10q [18–20]. A limited set of 
germline mutations in RET have been found in ~95% of MEN 2 
kindreds, and several of these mutations have been found in 
sporadic MCTs. Documentation of new RET mutations and de 
novo MEN 2 [21–23] also provides compelling evidence estab-
lishing RET mutation as the molecular lesion underlying MEN 2. Most cases of MEN 2A and familial MTC are caused by 
substitution of cysteine by another amino acid at one of five sites 
ne the transmembrane segment of the affected protein 
[7, 15, 24, 25]. MEN 2B kindreds and one kindred with familial 
MTC carry germline missense mutations at two sites within 
RET's kinase domain [10, 13, 14, 20, 26]. Mutations at these 
seven sites account for nearly all MEN 2 cases [12, 27].

RET mutations also cause an autosomal dominant form of 
Hirschsprung disease, in which a segment of bowel lacks 
inervation; this presents clinically as intestinal obstruction in 
infancy. Mutations in Hirschsprung disease span the entire RET
gene and include nonsense, frameshift, and deletion mutations [9, 28–30]. Rarely, Hirschsprung disease and MEN 2A are both present in a single kindred. These families have been found to carry mutations characteristic of MEN 2A [31].

**CLINICAL FEATURES OF MEN 2 AND SCREENING ISSUES**

The tumors classified as MEN 2 may occur either as components of the syndrome or as sporadic neoplasms—and the clinician must be able to distinguish between these. Various features, including pathological findings and age of onset, help to distinguish familial from sporadic disease but are far from absolute criteria for doing so [32–36]. The increase of stimulated calcitonin concentrations [37] is limited by age-dependent sensitivity [34] and the occurrence of C cell hyperplasia in individuals for reasons unrelated to MEN 2 [5, 38, 39].

Genetic methods overcome most limitations of the combined use of clinical and biochemical methods for diagnosing MEN 2. In the case of members of known MEN 2 kindreds, one must determine only whether the pathogenic mutation is present in the at-risk individual. A search for pathogenic mutations need be performed only once for each kindred. Thus far, ~95% of families with MEN 2 have had one of several characteristic mutations in exon 10, 11, 13, or 16 of the RET gene. Availability of an improved assay for these mutations would simplify genetic diagnosis of the familial disease. We have achieved this by establishing the conditions for DGGE of exons 10, 11, and 16.

**DGGE COMPARED WITH OTHER APPROACHES TO GENETIC DIAGNOSIS OF MEN 2**

DGGE is ideally suited for diagnosis of MEN 2 for several reasons. First, very few neutral polymorphisms are known in these exons [40, 41]. Second, the dominant genetics of the syndrome means that virtually all affected individuals are heterozygous, which allows formation of heteroduplexes on PCR amplification of DNA from affected individuals. The heteroduplexes have early melting nonhomologous bubbles that are readily apparent on DGGE. The presence of these heteroduplexes greatly simplifies the distinction of affected individuals, who display multiple bands on DGGE, from normal subjects, who display a single band on DGGE. Moreover, DGGE does not require the use of fluorescent, chemiluminescent, or radioactive labeling for performance of the assay. These features, together with the technical ease of performing an established assay, make this an attractive method for use in clinical settings.

The sensitivity of DGGE in detecting single-base substitutions is ~95% when performed with GC-clamping and PCR products <500 bp long [11]. Because the method cannot detect mutations in the highest melting temperature domain of the molecule, GC-clamping introduces a high melting-point segment of DNA at the end of a PCR primer so as to place amplified mutations in a context that maximizes their chance of detection [16]. In the case of RET exon 11, the standard GC clamp did not allow the detection of codon 634 mutations, but these were revealed by extending the clamp length. In a recent report, Attie et al. used DGGE of the RET gene to uncover a 7-bp deletion leading to Hirschsprung disease [28], but this application required distinguishing between molecules that differed over the length of the microdeletion rather than at a single base. Here, we have shown that DGGE with GC clamping is sufficiently robust to detect the single-base substitutions that result in MEN 2. Every RET mutation we have tested thus far has been detectable by DGGE, and every individual tested has had the results of DGGE confirmed by either restriction digestion or sequencing of the PCR product. Two of our index cases have had their status as MEN 2 patients established initially by DGGE, one with the C620F mutation and another with M918T.

A major limitation of DGGE is that neutral polymorphisms will yield false-positive results. Thus, DGGE is inappropriate for seeking exon 13 mutations, because the known pathogenic mutation and a common neutral polymorphism [14, 41] occur at consecutive residues in this exon; this mutation is a rare cause of MEN 2, however. Another known polymorphism in exon 11 [40] of the RET gene is sufficiently distant from codon 634 that a PCR product excluding the polymorphic site can be prepared and analyzed.

Single-strand conformational polymorphism (SSCP) is an alternative strategy for seeking mutations in defined regions of a gene. SSCP conditions for all of the RET exons have recently been published [41]. This method depends on the difference between the snap-back conformations of mutant and wild-type DNA molecules subjected to heat denaturation and rapid cooling. SSCP reportedly has a sensitivity of ~80% and is easy to perform. However, subtle differences in technique can have a substantial impact on its results, and labeling of samples is required [11]. Although DGGE conditions may be more difficult to establish, once found, they are easily reproduced.

Alternative methods of mutation analysis are less attractive in screening for MEN 2. Restriction digestion, chemical mismatch cleavage, and direct sequencing all require additional manipulations after PCR and before electrophoretic analysis. Each manipulation increases the opportunity for human error or technical failure. In addition, some pathogenic mutations of RET do not result in alteration of restriction sites. The accumulation of heteroduplexes over the course of PCR amplification results in reduced digestion, and distinguishing heterozygosity from partial digestion is sometimes difficult. Finally, restriction digestion is ill-suited to seeking mutations in new index cases, because a large number of reactions must be used to screen for all pathogenic mutations detectable by this approach.

In summary, we have developed DGGE assays for exons 10, 11, and 16 of the RET gene. Mutations in these three exons are found in >90% of MEN 2 cases. DGGE is particularly well suited to the diagnosis of this syndrome because of the dominant genetics involved and low prevalence of neutral polymorphism, and because of the sensitivity and ease of performance and interpretation of the assay. These methods allow detection of most currently known pathogenic mutations in a simple, rapid assay without the use of radioactive, fluorescent, or chemiluminescent labels. They are suitable for both investigating members of known kindreds and seeking pathogenic mutations in new index cases.
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