Rapid, nonradioactive screening for mutations in exons 10, 11, and 16 of the \textit{RET} protooncogene associated with inherited medullary thyroid carcinoma


Germline mutations in exons 10, 11, and 16 of the \textit{RET} protooncogene are associated with the heritable cancer syndromes multiple endocrine neoplasia (MEN) type 2A, familial medullary thyroid carcinoma (FMTC), and MEN type 2B. Nonradioactive mutation analysis with nondenaturing Phastgels® and the Phast System was performed on DNA amplified by the polymerase chain reaction from exons 10, 11, and 16 of the \textit{RET} protooncogene from patients with MEN 2A, MEN 2B, or FMTC. The analysis requires 45–90 min for electrophoresis and 35 min for staining. This assay detected 20 of 21 different mutations that represented 90% of all known mutations associated with these lesions. A rare silent polymorphism within exon 10 was also detected. This form of mutation analysis provides simple, rapid, and highly sensitive nonradioactive detection of mutations known to be associated with MEN 2A, FMTC, and MEN 2B.

INDEXING TERMS: cancer • thyroid disease • heritable disorders • polymerase chain reaction • single-strand conformation polymorphism

About 20% of the cases of medullary thyroid carcinoma (MTC) are inherited in an autosomal dominant fashion as one of three syndromes: multiple endocrine neoplasia (MEN) type 2A or 2B, or familial medullary thyroid carcinoma (FMTC). The inherited form of the disease has been linked to germline point mutations within the \textit{RET} protooncogene. Linkage analysis has localized to chromosome 10 a gene associated with MEN 2A \cite{1, 2}. The \textit{RET} protooncogene was subsequently localized to this same region of chromosome 10 \cite{3}. Thorough investigation of the \textit{RET} protooncogene demonstrated that mutations causing MEN 2A and FMTC appear to be mainly confined to codons for cysteine residues in the extracellular domain within exons 10 and 11 of the gene \cite{4, 5}. Individuals in MEN 2A families carrying the defective gene may have a near-100% risk of medullary thyroid cancer, the penetrance being age-related \cite{12}. Case reports have recently shown that rare germline mutations implicated in FMTC can also occur in exons 13 and 14 \cite{6, 7}. A single-point mutation in exon 16 has been shown to cause MEN 2B in 93% of patients \cite{8–10, 16}.

Before recognition of the gene encoding the defect in these cancer syndromes, diagnosis commonly relied on pentagastrin-induced calcitonin release tests. The advent of direct DNA analysis has provided a highly specific, reliable, and less-invasive alternative to aid diagnosis \cite{11–15}. DNA sequencing, currently the most definitive method used to detect mutations in exons 10 and 11, is, however, cumbersome, time-consuming, and expensive. Restriction endonuclease digestion is useful for the single-point mutation in exon 16 but its utility for analyzing...
exons 10 and 11 is limited because of the multiplicity of point mutations (>20) described for this region [16]. Single nucleotide changes can also be detected by using methods that analyze for conformation polymorphisms, e.g., single-stranded conformation polymorphisms (SSCP). PCR amplicons between 50 and 250 bp are denatured into single strands, snap-cooled, and electrophoresed in a nondenaturing gel. The electrophoresis conditions favor the formation of intrastrand base-pairing. Consequently, the sense and anti-sense single-stranded DNA fold into different conformations, as dictated by their unique primary nucleotide sequence. A single nucleotide change alters these conformations, which can be detected by gel electrophoresis. Generally described methods for SSCP require addition of formamide/KOH/sodium dodecyl sulfate to denature the DNA into single strands; large sequencing gels with or without glycerol to be run in a cold room; \( \text{\textsuperscript{32}} \)P-labeling of the DNA for detection; and 24–76 h to complete the electrophoresis and subsequent autoradiography [17–18].

A rapid, nonradioactive technique for detecting conformation polymorphisms has been previously described by one of us (A.M.) for detecting point mutations within the \( p53 \) gene [19]. Additional recent studies have also shown the utility of Phastgels® and the Phast System® (both from Pharmacia BioTech, Piscataway, NJ) for SSCP analysis [20–22]. We have adapted this technique to detect mutations in exons 10, 11, and 16 associated with inherited MTC (MEN 2A, MEN 2B, or FMTC). To determine the sensitivity of the system, we examined DNA from 21 patients with distinct mutations in codons 609, 611, 618, 620, 634, and 918.

**Materials and Methods**

**Patients.** Whole-blood samples were collected into EDTA or acid–citrate–dextrose preservative from 21 patients. All of the patients had inherited MTC (MEN 2A, MEN 2B, or FMTC) and had known genomic \( RET \) mutations previously analyzed by sequence analysis (by S.N.T. and W.W.N.). DNA was prepared from the peripheral blood leukocytes by standard phenol/chloroform extraction techniques followed by ethanol precipitation.

**PCR.** Exons 10, 11, and 16 of the \( RET \) protooncogene were amplified by using the following primer pairs:

- **exon 10,** forward: 5’ GGG-GCA-GCA-CTG-CTG-GGG-GAC 3’; reverse: 5’ CTG-CTG-GTG-CCC-GGC-GCG-C 3’
- **exon 11,** forward: 5’ CCT-CTG-CCG-TGC-CAA-GCC-TC 3’; reverse: 5’ GAA-GAG-GAC-AGC-GGC-TGC-GAT 3’
- **exon 16,** forward: 5’ AGG-GAT-AGG-GCC-TGG-GCT-TG 3’; reverse: 5’ TAA-CCT-CCA-CCC-CAA-GAG 3’

The PCR reaction mixture consisted of 50 ng of template DNA, 0.5 \( \mu \)mol/L of each primer, 2.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT), 1 × PCR buffer N for exons 10 and 11 (2.0 \( \mu \)mol/L MgCl\(_2\), pH 10.0; Invitrogen, San Diego, CA) or PCR buffer M for exon 16 (1.5 \( \mu \)mol/L MgCl\(_2\), pH 10.0; Invitrogen), and 200 \( \mu \)mol/L dNTPs in a final total volume of 25 \( \mu \)L. The reaction mixture and genomic DNA were mixed in thin-walled microfuge tubes and placed in a Model 9600 Thermocycler (Perkin-Elmer). The DNA was initially denatured at 95 °C for 2 min. The program for exons 10 and 11 consisted of 40 cycles of 95 °C for 60 s, 65 °C for 60 s, and 72 °C for 60 s, with a final elongation step of 72 °C for 10 min. The program for exon 16 consisted of 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, with a final elongation step of 72 °C for 10 min.

**Mutation analysis.** The PCR product (5 \( \mu \)L) was diluted 10-fold in water, heat-denatured at 95 °C for 5 min, and placed without delay on ice for ~15 min. From the diluted product, 4 \( \mu \)L was subjected to electrophoresis at 4 °C (for exons 10 and 16) and 4, 10, and 15 °C (for exon 11) on the Phast System, with use of a 20% precast, nondenaturing polyacrylamide gel (Phastgel). The gel was prerun at 400 V, 5.0 mA, 1.0 W, and 100 Vh. Sample was automatically applied at 25 V, 5.0 mA, 1.0 W, and 2 Vh. Electrophoresis was performed at 400 V, 5.0 mA, 1.0 W, and 350–700 Vh (45–90 min, determined empirically). The DNA products were detected by using the automated Phast System staining apparatus and silver stain kit (35 min).

**Results**

DNA was obtained from 21 individuals with inherited MTC. The germline \( RET \) mutations had been previously characterized by DNA sequence analysis, and samples were selected to represent different point mutations within one of five cysteine codons in exons 10 and 11 of the \( RET \) protooncogene or the T→C transition mutation in exon 16 (see Table 1). PCR primers chosen on the basis of relevant literature [14] and available sequence data were used to amplify exons 10, 11, and 16 of the \( RET \) protooncogene. The Phast System was used for mutation analysis because of the convenience of precast polyacrylamide gels; accurate, flexible temperature control; nonradioactive silver staining; and rapid electrophoresis time. The protocol was further simplified by diluting the PCR products 1:10 in water and using heat denaturation with quick placement of the products on ice rather than denaturing with formamide/KOH/sodium dodecyl sulfate.

Using this method of mutation analysis, we were able to identify 20 of 21 mutations in exons 10, 11, and 16 of the \( RET \) protooncogene, obtaining an overall sensitivity of 95%. Moreover, the banding patterns for each of these mutations were reproducible over repeated runs. All 14 mutations in \( RET \) exon 10 were easily identified in the same electrophoretic conditions, performed at 4 °C (Fig. 1). In general, the mutant bands were as intense as wild-type bands and were well separated from them. Five of six mutations in exon 11 were identified (Fig. 2); however, not all of these were separated at 4 °C. Four of
the five mutations were detected by electrophoresis at 10 °C, although one of these (C634Y) was more distinct at 4 °C. The fifth mutation (C634R) was detected at 15 °C. Additionally, we detected a rare silent polymorphism in codon 623 of exon 10 in one individual (mutation analysis performed at 4 °C). The single known exon 16 mutation associated with MEN 2B and sporadic MTC was identified by the Phastgel system at an electrophoretic temperature of 4 °C (Fig. 3). The total time for mutation analysis of each exon was ~2 h, including electrophoresis and silver staining.

**Table 1. SSCP analysis of RET protooncogene exons 10, 11, and 16 on samples previously characterized by sequence analysis at a reference laboratory.**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Mutation</th>
<th>Exon 10</th>
<th>Exon 11</th>
<th>Exon 10</th>
<th>Exon 11</th>
</tr>
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*Variant; denotes silent polymorphism.

**Discussion**

The genetic mutations within the RET protooncogene predisposing to MTC provide a dramatic example of the impact molecular analysis can have on patient management and diagnosis. Unlike biochemical assays, methodologies based on DNA analysis require a single blood test with only the mild side effects of venipuncture. Also, unlike provocative biochemical assays, the test can provide diagnostic information before development of C-cell hyperplasia or foci of MTC. Because a mutation in the RET protooncogene remains consistent within a family, once a mutation is identified, at-risk members need be screened only once, as opposed to annually for biochemical screening. Family members with a mutation would...
benefit from total thyroidectomy. Family members without a mutation (and their descendants) require no further testing.

Despite their high degree of clinical utility, DNA analyses frequently can be laborious, time intensive, and expensive and can require considerable technical skill. The rapid, nonradioactive screening technique presented here, performed on the Phast System, eliminates many of these impediments to common clinical utility, and can detect germline mutations in the RET protooncogene with a sensitivity of ~90%. The use of dilution in water and boiling to denature the PCR products before electrophoresis produced a banding pattern that was generally very easy to interpret. Whether this gentle denaturing actually produces single-stranded DNA or heteroduplex/homoduplex formation is unknown at present. In our experience, the mutant bands detected in exons 10 and 16 were generally quite well-resolved and distinct compared with those in exon 11, and all of the former yielded to analysis under our standard conditions at 4 °C. Analysis of exon 11 frequently required the additional maneuver of altered temperature. Using three different downstream primers for exon 11 did nothing to resolve the bands more distinctively or to detect the one mutation that our standard conditions did not detect. The undetected exon 11 mutation accounts for ~5% of the codon 634 mutations reported by the International RET Mutation Consortium [16]. The basis for the relative resistance to this analysis of the exon 11 PCR product remains unclear. Nonetheless, all but one of these mutations was resolved with this method.

Any patient with MTC, whether suspected to be inherited or sporadic, should be screened for germline point mutations in both exon 10 and 11 of the RET protooncogene by one of the methods for direct DNA analysis [11–15]. In our mutation analysis method (Fig. 4), exon 11 would be screened initially for mutations at an electrophoretic temperature of 10 °C, given that 87% of the mutations associated with MEN 2A involve codon 634 in exon 11 [16]. If no abnormal bands are detected, then electrophoresis at 4 °C and 15 °C would be performed. Finally, mutations within exon 10 would be analyzed at an electrophoretic temperature of 4 °C. MEN 2B patients who need exon 16 analysis are generally identified ahead of time by the unique clinical features of this disease. A presumptive positive diagnosis can be made if any bands of altered electrophoretic mobility are noted. Because this is a screening technique, DNA sequence analysis of the initial proband is needed to confirm that the mutation did indeed affect one of the five cysteine codons. Once the presence of the mutation is confirmed, the method could be used to screen the additional family members at relatively low cost. If no abnormal bands are present, 20 of 21 mutations can be confidently ruled out. We hope to improve the assay to allow us to detect all of the mutations associated with the disease. Currently, for complete confidence, DNA sequence analysis is recommended for all initial probands with a negative screen.

These results are similar to those of another study that successfully used SSCP and the Phast System to detect 19 known mutations/polymorphisms in the insulin receptor, GLUT-4, and transthyretin genes [20]. Several other authors describe the utility of various SSCP techniques, both radioactive and nonradioactive, for analysis of the RET protooncogene [23–27]. However, in only one of these papers do the authors begin to address the sensitivity of their SSCP method, with less-comprehensive analysis than that presented here (15 mutations in these exons) for these syndromes [26]. In addition, none of the other methods described is as easy to perform as this mutation analysis technique performed with the Phast System.

One limitation of any screening assay based on conformation polymorphisms in predicting a disease phenotype is that point mutations that are silent polymorphisms or polymorphisms having no functional consequences for
the molecule are also detected. However, reports of silent polymorphisms within exons 10, 11, and 16 are very rare [28]. In addition, because banding patterns by our methodology are so reproducible and so much less complex than those in many radioactive-based SSCP techniques, we believe the patterns generated by new mutations would probably be distinct and could be selected for further characterization, with ultimate confirmation by sequence analysis. In fact, using this SSCP method, we successfully predicted (and later confirmed by sequence analysis) previously uncharacterized mutations in at least three families.

In summary, this inexpensive, rapid, nonradioactive mutation analysis has advantages over other DNA-based assays by being simple to perform, requiring no radiolabeling or organic denaturants, and providing rapid turnaround. The whole procedure, after PCR, can be completed in <2 h. Mutation analysis with the Phast System provides a simple, rapid technique that may have general applicability for analyzing discrete point mutations within the human genome with better sensitivity than other SSCP methods.

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References


