Mutation Scanning of the RET Protooncogene Using High-Resolution Melting Analysis, Rebecca L. Margraf,1,2 Rong Mao,1,2 W. Edward Highsmith,3 Leonard M. Holtegaard,3 and Carl T. Wittwer1,2 (1 ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; 2 Department of Pathology, University of Utah Medical School, Salt Lake City, UT; 3 Molecular Genetics Laboratory, Mayo Clinic, Rochester, MN; * address correspondence to this author at: Advanced Technology Group, ARUP, 500 Chipeta Way, Salt Lake City, UT 84108; fax 801-584-5114, e-mail rebecca.margraf@aruplab.com)

Background: Single-base pair missense mutations in exons 10, 11, 13, 14, 15, and 16 of the RET protooncogene are associated with the autosomal dominant multiple endocrine neoplasia type 2 (MEN2) syndromes: MEN2A, MEN2B, and familial medullary thyroid carcinoma. The current widely used approach for RET mutation detection is sequencing of the exons.

Methods: Because RET mutations are rare and the majority are heterozygous mutations, we investigated RET mutation detection by high-resolution amplicon melting analysis. This mutation scanning technique uses a saturating double-stranded nucleic acid binding dye, LCGreen®, and the high-resolution melter, HR-1™, to detect heterozygous and homozygous sequence variations. Mutant genotypes are distinguished from the wild-type genotype by an altered amplicon melting curve shape or position.

Results: Samples of 26 unique RET mutations, 4 non-pathogenic polymorphisms, or the wild-type genotype were available for this study. The developed RET mutation-scanning assay differentiated RET sequence variations from the wild-type genotype by altered derivative melting curve shape or position. A blinded study of 80 samples (derived from the 35 mutant, polymorphism, or wild-type samples) demonstrated that 100% of RET sequence variations were differentiated from wild-type samples. For exons 11 and 13, the nonpathogenic polymorphisms could be distinguished from the pathogenic RET mutations. Some RET mutations could be directly genotyped by the mutation scanning assay because of unique derivative melting curve shapes.

Conclusion: RET high-resolution amplicon melting analysis is a sensitive, closed-tube assay that can detect RET protooncogene sequence variations.

Mutations in the RET protooncogene (exons 10, 11, and 13–16) cause multiple endocrine neoplasia type 2 (MEN2) syndromes, autosomal dominant disorders that lead to a high lifetime risk of medullary thyroid carcinoma. Detection of RET germline mutations can identify MEN2 patients before disease progression, when thyroidectomy can prevent cancer development and increase survival rates. The gold standard for RET mutation detection is sequencing. Other methods, such as single-strand conformation polymorphism analysis, heteroduplex detection by conformation-sensitive gel electrophoresis, restriction enzyme digestion of PCR products, pyrosequencing, fluorescently labeled hybridization probes, and microarrays, have also been developed to detect RET mutations (1–9). These methods, however, can require additional post-PCR processing of the amplicon to detect mutations, may misidentify nonpathogenic polymorphisms as mutations (false positives), or may target mutation hotspots and thus miss some rare mutations (false negatives) (10). High-resolution melting analysis is a rapid, closed-tube mutation scanning assay that detects sequence variation within the PCR amplicon by use of a saturating double-stranded DNA dye, but does not require post-PCR manipulation of samples or use of expensive labeled probes (11–14). This technique detects mutations anywhere between the primers, in contrast to more localized techniques, such as hybridization probes or restriction enzyme-based assays, that target <30 nucleotides (15).

We obtained deidentified wild-type and RET variant genomic DNA samples from the Mayo Clinic (Rochester, MN), with Institutional Review Board approval, and amplified the samples according to the GenomiPhi™ protocol (Amersham Biosciences). Additional wild-type and RET variant cell lines were from the Coriell Institute. Sample genotypes were confirmed by sequence analysis and by comparison with the RET genomic sequence (GenBank AJ243297). All RET sequence variations tested were the result of a single nucleotide change. The RET variants that alter RET function to cause MEN2 syndromes are mutations, whereas variants that do not cause MEN2 syndromes are polymorphisms (2, 16–20).

All variant samples were heterozygous for mutations or polymorphisms unless otherwise stated. The wild-type nucleotide sequence for each of the analyzed RET codons is listed in Fig. 1, with the nucleotide change for each variant highlighted in red (bold in the text). In addition to the wild-type and RET mutant genotypes, samples with polymorphisms (exon 15, codon 904 TCC>TCG; exon 11, codon 631 GAC>GAT; exon 14, codon 836 AGC>AGT; and exon 13, codon 769 CCT>CTG) were available for study (2, 16, 19).

With Primer3 software (21), we designed primers for the 6 RET exons to create amplicons that included all known pathogenic mutations and, if possible, to exclude polymorphisms from analysis (Table 1) (16, 17, 21). For each RET exon, sample DNA (~50 ng total) was amplified with the LightCycler® FastStart DNA Master Hybridization Probe Kit (Roche Diagnostics Corp.) in a final PCR reaction volume of 10 µL. The PCR reaction contained 1× FastStart master hybridization mixture, 2 mM MgCl₂, 1 µM of each primer (1.4 µM of each primer for exon 16), 0.01 U/µL uracil-DNA glycosylase (Roche Molecular), and 1× LCGreen® PLUS (Idaho Technology). Thermocycling was performed on a LightCycler (Roche) with the following conditions: initial uracil-DNA glycosylase step (50 °C for 10 min) and polymerase activation (95 °C for 10 min), followed by 40 PCR cycles (denaturation at 95 °C for 1 s, annealing at 62 °C for 1 s, and extension at 72 °C for 1 s, annealing at 62 °C for 1 s, and extension at 72 °C for 30 s).
Fig. 1. High-resolution melting analysis data for RET exons 10, 11, and 13–16.

The black curves are wild-type samples. The colored curves are the variant samples, which are identified for each exon by codon number and wild-type sequence followed by the variant sequence, with the variant nucleotide in red. Every colored curve represents an individual, unique, variant sample. All RET sequence variations are heterozygous except where noted (exon 13). Each exon has the derivative plot (−dF/dT vs temperature) in the top panel and the fluorescence difference plot (fluorescence vs temperature) in the bottom panel. (A), exon 10. Sample curves are colored based on the sequence of the mutation. The mutations were in codons 609, 611, 618, or 620 as indicated. (B), exon 11. The codon 631 polymorphism is displayed as well as all possible heterozygous mutant samples for codon 634. (C), exon 13. Samples heterozygous and homozygous for the codon 769 polymorphism are displayed, as well as the pathogenic mutation at codon 768. (D), exon 14. Data for 2 unique codon 804 mutations and a codon 836 polymorphism are shown. (E), exon 15. Data for a codon 904 polymorphism sample are shown. (F), exon 16. Data for a codon 918 mutation sample are shown.
4 s) with transition rates of 20 °C/s. An exception was exon 14, for which we used 45 PCR cycles and 65 °C for the annealing temperature. Amplion heteroduplexes were obtained by heating samples to 95 °C and then rapidly cooling to 40 °C in the LightCycler.

High-resolution melting analysis was performed on the HR-1™ instrument (Idaho Technology). The LightCycler capillaries were transferred into the HR-1 and heated at a rate of 0.3 °C/s during acquisition between 70 and 96 °C. High-resolution melting data were analyzed with custom software written in LabView (National Instruments) as described previously (11). Briefly, high-resolution data were normalized, temperature-shifted (except exon 14), and then converted to a derivative plot (−dF/dT vs temperature) or fluorescence difference plot (fluorescence vs temperature) for analysis (11, 13). Melting temperatures (Tms) were derived at the greatest −dF/dT value of the derivative curve data. The normalized melting curves, derivative plots, or fluorescence difference plots could be used to distinguish RET sequence variations from the wild-type samples (Fig. 1 and data not shown). For example, the Tms of the variants for exons 15 and 14 are close to wild type in a derivative plot, but demonstrate clearer distinction of sequence variation in the fluorescence difference plot (Fig. 1) (11–14). After variants were detected by mutation scanning, they were conventionally genotyped by sequencing (13, 14). However, some RET mutations could be distinguished, without sequencing, by the shape and Tm shift of derivative curves (Fig. 1).

For each RET exon, heterozygous variant samples (mutations or polymorphisms) melted at a lower temperature or had a broader melting transition than the wild-type samples (Fig. 1). The Tm shift and the shape of the derivative melting curve determined the genotype for some of the variants. Because the derivative melting curves overlapped, however, other unique mutations were not distinguishable (Fig. 1, A and B). The derivative melting curves for heterozygous TAC and TTC mutations in both exons 10 and 11 (wild-type sequence, TGC) were nearly identical. In addition, the exon 10 melting curves segregated by mutation sequence, not by the codon of mutation. For example, the derivative curves for all exon 10 samples of TCC mutant sequence at codons 609, 618, or 620 were nearly identical, as were the derivative curves for all exon 10 samples of TTC mutant sequence at codons 611, 618, or 620. Such findings are expected from the nearest-neighbor thermodynamics of mismatches (22, 23).

Derivative plots of exon 13 generated 2 peaks, indicating 2 melting domains within the amplicon (11). The lower melting domain contained the mutations, and the higher melting domain was identical for all samples. A homozygous polymorphism at codon 769 was available for testing in our assay. The more stable homoduplex polymorphism sample (GC base pair) was distinguished by a higher Tm than the less stable wild-type sample (AT base pair) (11, 12). Samples with pathogenic mutations in RET exon 15 were not available for this study; therefore, only cell lines with the wild-type sequence or the codon 904 polymorphism were tested (Fig. 1E). A sample that is heterozygous for both an RET mutation and the polymorphism should be detected as sequence variation because of the greater destabilization of 2 mismatches (11).

The common mutation in exon 16 had a 0.5 °C temperature shift from wild type, whereas the exon 14 variant samples had only a small temperature shift (~0.1 °C) from wild type in derivative plots. All of the RET mutations and polymorphisms, including those in exon 14, were more clearly distinguishable from the wild-type samples when we used the fluorescence difference plot for the analysis (Fig. 1).

We performed a blind study to determine the accuracy of the developed mutation scanning assay to detect and genotype RET mutations. Eighty samples, derived from the 5 wild types and 30 variant RET samples, were blinded and genotyped for exons 10, 11, 13, 14, and 16. Derivative curves of the blinded samples were compared with the wild-type controls for each exon. An additional mutant control was included for exons 10 and 14 to help distinguish sequence variations from wild type. All RET variants were correctly differentiated from wild type. In addition, the polymorphisms in exons 11 and 13 were correctly distinguished from the mutant genotypes. Although the exon 14 codon 836 polymorphism was distinguished from the codon 804 mutation, the other tested mutation, 804(GTG>ATG), was not always distinguishable from the polymorphism in the blinded study because of a similar derivative melting curve shape and Tm. The same results were obtained when difference plots were used instead of derivative plots (data not shown). Some, but not all, genotypes could be distinguished from others in the blinded study. Differentiation of genotypes was not possible when the derivative melting curves overlapped, as seen for some mutations in RET exons 10, 11, and 14 (Fig. 1).

In conclusion, RET protooncogene mutation scanning by high-resolution melting analysis was 100% accurate in distinguishing sequence variation from homozygous wild-type samples in the blinded study. Genotyping of

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers*</th>
<th>Amplicon size, bp</th>
<th>Codons*</th>
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<tr>
<td>10</td>
<td>GGCAGCATTTGGTGAGGAC</td>
<td>146</td>
<td>603, 609, 611</td>
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<tr>
<td></td>
<td>TGGTGTCCTCCGCGCGCAC</td>
<td></td>
<td>618, 620</td>
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<tr>
<td>11</td>
<td>GGGCTGCAAGCCTCAC</td>
<td>114</td>
<td>630, 634</td>
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<tr>
<td></td>
<td>GAGCAAGACCGACAGAGAC</td>
<td></td>
<td>640</td>
</tr>
<tr>
<td>13</td>
<td>ATCTTTGCAACTGCTCTTG</td>
<td>207</td>
<td>768, 778</td>
</tr>
<tr>
<td></td>
<td>GAAGGGGCGCTAGTGGAGC</td>
<td></td>
<td>790, 791</td>
</tr>
<tr>
<td>14</td>
<td>CAGGAGCCCCCTCTTCGCG</td>
<td>235</td>
<td>804, 844</td>
</tr>
<tr>
<td></td>
<td>TCTGGGCAACTGCTGATCG</td>
<td></td>
<td>852</td>
</tr>
<tr>
<td>15</td>
<td>GCTGGACAGCTGGCTCTATT</td>
<td>187</td>
<td>883, 891, 904</td>
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<td></td>
<td>CTGGGACCCCCCCCTCAT</td>
<td></td>
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</tr>
<tr>
<td>16</td>
<td>ATAGGTTGCCTGGCTCTCC</td>
<td>154</td>
<td>918, 922</td>
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<tr>
<td></td>
<td>ACACATCACTTTGGTGAGT</td>
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* Primers are listed 5' to 3', with the forward primer above the reverse primer.

RET codons that contain pathogenic mutations within each amplicon are listed. Nonpathogenic polymorphisms can be present in codons 631, 769, 836, and 904.
the unique RET mutations was not possible when the derivative curves overlapped. Although not all pathogenic RET mutations were available for analysis, a recent systematic study of high-resolution melting detection of heterozygous point mutations within a PCR amplicon found a sensitivity and specificity of 100% for amplicons <400 bp in size (15). High-resolution melting analysis for mutation scanning is a rapid (1–2 min after PCR), cost-effective assay that requires no processing or separation steps. As applied to RET mutation scanning, accuracy of heterozygote detection appears to be 100%, and some (but not all) sequence variations can be distinguished from each other. Because samples are immediately available for further processing after high-resolution melting analysis, the detected variant samples can be sequenced for confirmation of genotype.

We thank Idaho Technology for providing the dye, LCGreen PLUS. Aspects of high-resolution melting analysis are licensed from the University of Utah to Idaho Technology. Dr. Carl T. Wittwer holds equity interest in Idaho Technology. We thank Dr. Karl Voelkerding and Maria Erali for helpful discussions and review of the manuscript and Dr. Yuan-Pei Xin for technical assistance.

References


Detection of Biological Threat Agents by Real-Time PCR: Comparison of Assay Performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler Platforms, Deanna R. Christensen, Laurie J. Hartman, Bonnie M. Loveless, Melissa S. Frye, Michelle A. Shipley, Deanna L. Bridge, Michelle J. Richards, Rebecca S. Kaplan, Jeffrey Garrison, Carson D. Baldwin, David A. Kulesh, and David A. Norwood* (United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; * address correspondence to this author at: United States Army Medical Research Institute of Infectious Diseases, 1425 Porter St., Fort Detrick, MD 21702; fax 301-619-2492, e-mail david.norwood@amedd.army.mil)

Background: Rapid detection of biological threat agents is critical for timely therapeutic administration. Fluorogenic PCR provides a rapid, sensitive, and specific tool for molecular identification of these agents. We compared the performance of assays for 7 biological threat agents on the Idaho Technology, Inc. R.A.P.I.D.®, the Roche LightCycler®, and the Cepheid Smart Cycler®.

Methods: Real-time PCR primers and dual-labeled fluorogenic probes were designed to detect Bacillus anthracis, Brucella species, Clostridium botulinum, Coxiella burnetii, Francisella tularensis, Staphylococcus aureus, and Yersinia pestis. DNA amplification assays were optimized by use of Idaho Technology buffers and