Efficient Testing of the RET Gene by DHPLC Analysis for MEN 2 Syndrome in a Cohort of Patients

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Abstract. Background: Multiple Endocrine Neoplasia type 2 (MEN 2) is an autosomal dominant inherited syndrome characterized by a strong predisposition for developing endocrine tumors. MEN 2 is caused by germline mutations in the ret proto-oncogene. We investigated the feasibility of using the DHPLC technique in mutation detection of the ret gene in members of MTC families. We compared DHPLC analysis with direct sequencing with regard to sensitivity, reliability, cost and time. Materials and Methods: Exons 10 and 11 were amplified with PCR from forty-three samples in seventeen unrelated Greek families and were analyzed for mutations by DHPLC and DNA sequencing. Results: Eight PCR amplicons showed a distinct non-wild-type DHPLC profile. Sequence analysis confirmed different nucleotide variations: six of them were localized in exon 10 and two in exon 11. Mutations were detected in five out of seventeen families tested (29%). Conclusion: None of the alterations detected by direct sequencing was missed by DHPLC. We conclude that DHPLC is a fast, sensitive, cost-efficient and reliable method for the scanning of ret germline mutations.

Multiple Endocrine Neoplasia type 2 (MEN 2) is an autosomal dominant inherited syndrome characterized by a strong predisposition for developing endocrine tumors. According to the different tissues involved, MEN 2 is classified into three subtypes: MEN 2A, FMTC (familial medullary thyroid carcinoma) and MEN 2B. In all three phenotypic subtypes, medullary thyroid carcinoma (MTC) is the key component and accounts for 20-25% of new cases of medullary thyroid carcinoma. The remaining 75-80% of MTC cases are sporadic (1-3). MEN 2 is caused by germline mutations in the ret proto-oncogene. The major disease-causing mutations in MEN 2A and FMTC are substitutions located in one of five cysteine codons in the extracellular domain of the encoded protein. They include codons 609, 611, 618 and 620 in exon 10 and codon 634 in exon 11 (4-7). Mutations in exons 13 and 14 appear to account for a very small percent (8, 9). In addition, between 1% and 24% of individuals with MTC and no known family history of MTC have disease-causing germline mutations in the ret gene (10, 11). Thus, there are ever increasing requests for genetic screening, since some experts recommend germline ret gene testing for all individuals with MTC (12). Genetic testing for MEN 2 is considered part of the standard management for at-risk family members (13). Genetic identification of asymptomatic carriers of ret mutations allows the accurate diagnosis of the disease at an early stage, when prophylactic surgical treatment can be performed (14).

Many methods for detecting mutations have been described, and strengths and limitations are inherent in each technique. DNA-sequence analysis is considered to be the gold standard for the identification of point mutations. Since the technique of denaturing high performance liquid chromatography (DHPLC) analysis is available in our laboratory (15, 16), we decided to use it for screening patients for ret mutations. In this study, we present the application of DHPLC for screening of mutations in the ret gene in comparison to sequence analysis.

Materials and Methods

Patients. Seventeen unrelated Greek families were studied. In these families the MTC appeared in first degree relatives (parent/children). In total 43 family members were tested and were evaluated by medical history, physical examination and biochemical measurements.

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Key Words: RET, DHPLC, sequencing, MEN 2.
DNA amplification. Genomic DNA was extracted by standard methods from peripheral blood leukocytes. Using the PCR technique, exons 10 and 11 of the ret proto-oncogene were amplified using primers and PCR conditions described previously (17, 18). PCR products were separated by agarose gel electrophoresis and visualised by EtBr staining.

DHPLC analysis. The WAVE DNA Fragment Analysis System (Transgenomic, Inc., Omaha, USA) and associated WAVE-Maker™ software were used. Unpurified PCR products were denatured at 96°C for 10 minutes and then slowly cooled to room temperature. An aliquot (4 - 8 µl) of the PCR product was injected into a DNASep column (Transgenomic, Inc.). Each fragment was tested under at least three different partially denaturing temperatures. The optimum conditions were determined empirically, based upon the suggested fragment melting profile generated by the WAVE-Maker™ software and the software described by Jones et al. (19). The column mobile phase for sample elution consisted of the following mixture: Buffer A, 0.1 M triethylammonium acetate (TEAA) and Buffer B, 0.1 M TEAA with 25% acetonitrile. Samples were eluted in a linear gradient of Buffer B over a 4.5-minute period at a constant flow rate of 0.9 ml/minute. The starting gradient was determined by the WAVE-Maker™ software and varied among fragments, depending upon the DNA sequence and fragment size. The chromatograms of each fragment were compared to those of the wild-type.

Sequence analysis. Briefly, purification of the PCR products was performed using the Rapid PCR purification kit (Marligen Bioscience Inc., Ijamsville, USA). Automated cycle sequencing for both strands was performed using the Big-Dye DyeDeoxy terminator cycle sequencing kit from Applied Biosystems. Analysis of the sequencing reactions was carried out on the ABI Prism® 310 Genetic Analyzer (Applied Biosystems), (Warrington, UK). Sequences obtained were aligned with normal sequences, using Sequencer PC software from Gene Codes Inc. (Ann Arbor, USA), and examined for the presence of mutations. Wild-type sequences for ret DNA were obtained from Genbank using AJ243297 accession number.

Results

Forty-three samples were examined by sequence analysis and DHPLC at the same time. Exons 10 and 11 were amplified from these samples and examined by DHPLC at three different temperatures (Table I). The conditions for mutation analysis of exons 10 and 11 were determined on the basis of the data obtained by the simulation of the melting behaviour of the wild-type sequence using the WAVE-Maker™ software and the software described by Jones et al. DHPLC analysis of exon 10 detected all variations in the three selected sets of conditions (63, 64 and 65°C), whereas analysis of exon 11 detected all variations only at two temperatures (64 and 65°C) (Figure 1). Eight of the amplicons tested showed a distinct elution profile. Sequence analysis confirmed the following nucleotide variations: six of them were localized in exon 10 (codon 618 Cys to Arg; codon 620 Cys to Tyr and codon 620 Cys to Arg) and two in exon 11 (codon 634 Cys to Tyr; codon 634 Cys to Arg). Mutations were detected in five out of seventeen families tested (29%). MTC cases in other families were either sporadic or belonged to the small percent of MEN 2 cases that have mutations in other exons. Table I summarizes the sequence analysis and DHPLC results from the forty-three samples examined. Every sequence alteration revealed by sequencing could easily be detected by DHPLC. Examples of variant DHPLC chromatograms and their confirmatory sequences are shown in Figure 2. The elution profiles for the normal control samples showed a single peak. In contrast, each of the confirmed positive samples produced an elution profile with multiple peaks.

It has been suggested that different sequence variations within the same fragment produce different DHPLC elution profiles (20,21). Therefore, the comparison of the elution profiles could allow the prediction of the variation. Our results (Figure 3) show the elution profiles from two different sequence variations, Ex 10 /TGC→CGC (codon 620) and Ex 10 /TGC→CGC (codon 618). By overlapping the two elution profiles, we did not observe any differences between them. These results suggest that the strategy of diagnosis based on characteristic elution profiles and shapes of the DNA DHPLC peaks is not reliable.

Table I. Ret mutation detection by DHPLC and sequence analysis.

<table>
<thead>
<tr>
<th>RET Exon</th>
<th>Temp 1</th>
<th>MPT Temp 2</th>
<th>Temp 3</th>
<th>Sequence Alteration</th>
<th># Cases</th>
<th>DHPLC</th>
</tr>
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<tbody>
<tr>
<td>Exon 10</td>
<td>63°C</td>
<td>64°C</td>
<td>65°C</td>
<td>None</td>
<td>37</td>
<td>HM</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TGC→CGC (618)</td>
<td>4</td>
<td>HT</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TGC→TAC (620)</td>
<td>1</td>
<td>HT</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TGC→CGC (620)</td>
<td>1</td>
<td>HT</td>
</tr>
<tr>
<td>Exon 11</td>
<td>63°C</td>
<td>64°C</td>
<td>65°C</td>
<td>None</td>
<td>41</td>
<td>HM</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TGC→TAC (634)</td>
<td>1</td>
<td>HT</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TGC→CGC (634)</td>
<td>1</td>
<td>HT</td>
</tr>
</tbody>
</table>

MPT, mobile phase temperature; HM, homozygote; HT, heterozygote; (618), codon 618; (+), detected; (−), not detected.
Figure 1. Chromatograms produced by DHPLC analysis of ret mutations (A: C618R, B: C634Y) at three column temperatures. A, In exon 10, temperatures between 63 and 65°C give resolution of heteroduplex and homoduplex strands. B, In exon 11, only temperatures between 64 and 65°C give resolution of heteroduplexes and homoduplexes.

Figure 2. DHPLC elution profiles for two ret mutations detected in two patients with MEN 2. A, Exon 10 elution profiles (65°C) for a normal individual and a patient carrying the C618R mutation. The DHPLC profiles are superimposed to illustrate differences. The direct sequencing result illustrates the corresponding TGC→CGC (618) substitution. B, Exon 11 elution profiles (64°C) for a normal individual and a patient carrying the C634Y mutation. The direct sequencing result illustrates the corresponding TGC→TAC (634) substitution.
However, in large families when the mutation is identified, the DHPLC elution profiles are very conclusive. Figure 4A shows DHPLC elution profiles of the PCR products of family IV (Figure 4B) carrying the Ex 10/TGC→CGC (codon 618) ret mutation. Although there are some slight differences, these different PCR products seem to show the same retention pattern on DHPLC.

### Discussion

On the basis of these results, a two-tiered molecular diagnostic strategy was applied. Our current testing strategy entails that we first analyze both exon 10 and exon 11 by DHPLC. PCR fragments encoding a sequence variant are further analyzed by DNA double-stranded sequencing. This two-tiered approach, combining DHPLC with direct sequence analysis, has a number of advantages over our original sequencing protocol. In contrast to the very cumbersome evaluation of sequence data, the evaluation of results by DHPLC is quite effortless because the discrimination between single and multiple peaks in the elution profiles is simple. Another advantage of DHPLC compared with other screening methods is the fact that time-consuming tasks, like gel preparations and handling, are unnecessary.

The sensitivity of the method is dependent on the temperature at which the analysis is completed and, to partially circumvent the operator’s experience, software has been developed for predicting the optimal temperature for DHPLC analysis. For the analysis of ret mutations, we have relied on the software-based predictions and we also selected overlapping temperatures to increase the chance of heteroduplex detection. The choice of operating temperature for DHPLC is critical, since in exon 11 at 63°C not all three mutations were detected. In this exon, a 1°C change from 64°C to lower temperatures resulted in a shift...
in retention time and had a significant effect on the separation of hetero and homoduplexes (Figure 1).

Under the conditions used in the present study, DHPLC detected 100% of the mutations detected by sequencing. No nucleotide variation was detected by sequencing in any of the samples for which the elution profile was the same as that of the wild-type on DHPLC analysis. However, as with any quick screening method, positive results need to be further characterized by sequencing.

**Conclusion**

In this study, using DHPLC analysis for the *ret* gene for the first time in Greece, we evaluated DHPLC for its potential as a screening method to reduce the need for sequencing of our caseload. We showed that this screening method is sensitive, efficient and accurate, since 100% of mutations identified by sequence analysis were successfully detected by DHPLC.

**Acknowledgements**

This work was supported by the Stavros Niarchos Foundation For Charity and ‘HYGEIA’ Diagnostic and Therapeutic Center of Athens, Greece.

**References**


