
Background: X-linked Charcot-Marie-Tooth type 1 disease has been associated with 280 mutations in the GJB1 [gap junction protein, beta 1, 32kDa (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked)] gene. High-resolution melting analysis with an automated instrument can be used to scan DNA for alterations, but its use in X-linked disorders has not been described.

Methods: A 96-well LightScanner for high resolution melting analysis was used to scan amplicons of the GJB1 gene. All mutations reported in this study had been confirmed previously by sequence analysis. DNA samples were amplified with the double-stranded DNA-binding dye LC Green Plus. Melting curves were analyzed as fluorescence difference plots. The shift and curve shapes of melting profiles were used to distinguish controls from patient samples.

Results: The method detected each of the 23 mutations used in this study. Eighteen known mutations provided validation of the high-resolution melting method and a further 5 mutations were identified in a blind study. Altered fluorescence difference curves for all the mutations were easily distinguished from the wild-type melting profile.

Conclusion: High-resolution melting analysis is a simple, sensitive, and cost-efficient alternative method to scan for gene mutations in the GJB1 gene. The technology has the potential to reduce sequencing burden and would be suitable for mutation screening of exons of large multiexon genes that have been discovered to be associated with Charcot Marie Tooth neuropathy.

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Charcot-Marie-Tooth (CMT) neuropathy is the most common group of hereditary disorders presenting to genetic clinics and affects ~1 in 2500 individuals (1). The CMT syndrome includes many hereditary disorders of peripheral nerves and affects both motor and sensory neurons. Patients suffering from CMT show progressive distal wasting and weakness, pes cavus or foot drop, and loss of deep tendon reflexes. X-linked CMT (CMTX) is the 2nd most common form of demyelinating CMT after Charcot-Marie-Tooth disease type 1A and accounts for 10%–15% of all CMT cases (2). CMTX1 (MIM 302800) is an X-linked dominant trait caused by mutations in the GJB1 [gap junction protein, beta 1, 32kDa (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked)] gene (3). Over 280 mutations have been reported in this gene according to the Inherited Peripheral Neuropathies Mutation Database (www.molgen.ua.ac.be/CMTMutations).

Our laboratory currently uses sequencing to identify gene mutations in the GJB1 gene. High-resolution melting (HRM) curve analysis is a powerful tool for scanning entire amplicons and detecting sequence variations, made possible through the discovery of the saturating double-stranded (ds) DNA dye, LC Green Plus (4), and by advances in instrumentation that enable acquisition of high-resolution fluorescent data (5). LC Green Plus (Idaho Technology) can be used at concentrations that do not inhibit PCR amplification but efficiently saturate PCR products. HRM analysis of PCR products amplified in the presence of LC Green Plus can detect heterozygous and most homozygous sequence variations by the difference in shape and position of the melting curve when compared with a wild-type melt profile (4, 6). The method would therefore be amenable to screening an X-linked dominant disorder with heterozygous females and hemizygous male patients.

Informed consent for DNA studies was obtained from all patients according to protocols approved by the Concord Hospital and the University of Antwerp Ethics Review Committees. To validate the HRM method, we selected 18 known patient samples that were positive for GJB1 mutations (10 males and 8 females) and 4 control individuals (3 females and 1 male). In addition, 10 de-identified DNA samples (6 males, 4 females) with and without GJB1 mutations were selected for blind analysis from the Molecular Genetics Department, University of Antwerp, Belgium. The control and patient samples used in both the validation and blind study were previously confirmed by sequence analysis with primers described by Bergoffen et al. (3). The GenBank sequence NM_000166 was used as the reference sequence for the cDNA. Nucleotide numbering of the A in the ATG translation initiation site was designated +1. There is 1 reported single-nucleotide polymorphism (SNP; rs11551260) in the coding sequence of the GJB1 gene; however, none of the individuals used in this study contained the SNP (c.287 C>G).

Primers to amplify 4 overlapping amplicons were designed with the Oligo Program Version 6 (Molecular Biology Insights) to provide comprehensive coverage of the GJB1 single-exon 852-bp open reading frame. Primer information, fragment size, fragment coverage, and pathogenic codons are shown in Table 1. Fragments 1, 2, and 4 provided adequate coverage for gene scanning, and fragment 3 was designed to test for mutations close to the
To facilitate heteroduplex formation, samples from hemizygous males were mixed with samples of male wild-type DNA (1:1 w/w) before PCR amplification. Amplifications were performed in 10-μL reactions containing 50 ng DNA, 200 μmol/L dNTP, 1 U Bio-X-Act Taq Polymerase (Bioline), 3 mmol/L MgCl₂, 2X PCR Enhancer (Invitrogen) 0.6X LC Green Plus (Idaho Technology), and 0.25 μmol/L primers. PCR was performed on either a 9700 thermocycler (Applied Biosystems) or Mastercycler (Eppendorf) with an initial denaturation of 95 °C for 5 min, followed by 35–40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 40 s, with a final extension of 68 °C for 5 min. We used LC Green Plus at 0.6X in our reactions and found no difference in the fluorescence detection sensitivity at this concentration compared with the 1X concentration. To facilitate heteroduplex formation, we subjected the samples to a 2-temperature hold profile (95 °C for 5 s followed by 50 °C for 5 min).

We performed melting acquisition on a 96-well LightScanner (Idaho Technology). The plate was heated from 80 °C to 98 °C at 0.1 °C/s with a 300-ms frame interval, 15-ms exposure, and 100% LED power, giving 14 points/°C (5, 7). Melting-curve analysis was performed by use of previously described methods (7) with LightScanner Software (version 1.0.1.524). Melting curves were normalized by selecting linear regions before and after the melting transition. These regions were defined for each curve, with an upper (100%) fluorescence and lower (0%) baseline being common for all curves. To eliminate slight temperature errors between samples, the normalized melting curves were temperature shifted by moving the curves along the X-axis to bring them through a common temperature that facilitates clustering into groups. To avoid false negatives, we performed this procedure at a temperature at which the entire mixture of duplexes had melted. Fluorescent difference curves were generated from normalized temperature-shifted data by selecting a control for comparison and subtracting the fluorescence of the control from all other melting curves. The fluorescence difference between all other curves and the comparison curve was then plotted against temperature.

A total of 18 known GJB1 mutations (mutations 1–18, Fig. 1A–1F) were used to validate the HRM method. Mutation detection sensitivity was 100% for all the known mutations. The control (wild-type) sample melting curves grouped tightly for all fragments, and altered difference curves were easily distinguished for the 18 mutations. HRM analysis also demonstrated detection sensitivity for mutations close to the primer. The mutations located closest to either end of fragment 3 were present in patient 7 (located 3-bp in from the 5’ end of the forward primer) and in patient 16 (located 2-bp from the 3’ end of the reverse primer). In both instances, the mutations gave altered melting curves compared with the control group (Fig. 1C and 1D). The 2 deletion mutations in patients 5 and 7 clearly showed an altered fluorescence curve for the 1-bp (Fig. 1A) and the 18-bp deletion (Fig. 1B and 1C) compared with the wild-type profile. For the blind analysis, the same controls used in the validation experiments were amplified in addition to the 10 deidentified samples. Mutation detection sensitivity was 100%, with 5 of 5 mutations (mutations 19–23, Fig. 1G–1I) being identified. Altered melting curves were observed on fragments 1, 3, and 4 and samples negative for mutations grouped tightly with the known control melting profiles. The melting curves for mutation 23 (509T>A) confirmed localization of the base change to an overlapping region on fragments 3 and 4. Mutations 1 (Fig. 1A), 8, 10 (Fig. 1B and 1C), 18 (Fig. 1F), and 21 (Fig. 1G) are previously unreported novel mutations that further demonstrate the allelic heterogeneity of CMTX1.

We have demonstrated a rapid and sensitive method for mutation scanning the GJB1 gene by use of the ds DNA-binding dye LC Green Plus and a 96-well format dedicated melting and detection instrument (LightScanner). GJB1 provided an excellent gene model, enabling us to analyze many different mutations spanning the GJB1 gene by use of the ds DNA-binding dye LC Green Plus and a 96-well format dedicated melting and detection instrument (LightScanner). GJB1 provided an excellent gene model, enabling us to analyze many different mutations spanning the

| Fragment | Primers 5’ to 3’ | Amplicon size, bp | Coverage of ORF, bp | Codon (mutation number)¹  
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<tr>
<td>1</td>
<td>AAG GTG TGA ATG AGG CAG CTC AAG CGG TAG CAT TTT C</td>
<td>346</td>
<td>1–328</td>
<td>18 (1) 22 (2) 28 (3) 35 (4) 73 (5) 94 (6) 26 (19) 49 (20) 82 (21)</td>
</tr>
<tr>
<td>2</td>
<td>CAC CAG CAA CAC ATA GAG GGG TAG AGC AGA TAA AAG</td>
<td>185</td>
<td>289–473</td>
<td>111–116 (7) 132 (8) 141 (9) 151 (10)</td>
</tr>
<tr>
<td>3</td>
<td>AAT GCT AGC GCT TGA GG GAC GTT TTT CTC GGT GGG</td>
<td>256</td>
<td>312–567</td>
<td>111–116 (7) 132 (8) 141 (9) 151 (10) 154 (11) 159 (12) 164 (13) 181 (14) 182 (15) 183 (16) 170 (23)</td>
</tr>
<tr>
<td>4</td>
<td>GGT GTT CCG GCT GTT GCA GGT TGC CTG GTA TGT</td>
<td>458</td>
<td>417–852</td>
<td>141 (9) 151 (10) 154 (11) 159 (12) 164 (13) 181 (14) 182 (15) 183 (16) 205 (17) 226 (18) 213 (22) 170 (23)</td>
</tr>
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¹ Open reading frame.

² Pathogenic codons detected within each of the overlapping amplicons are shown. The numbers in brackets refer to the patient mutation numbers in Figure 1. The reported neutral variant (rs11551260) on amplicon 1 can be present in codon 96.
gene. The HRM results for the 18 known mutations and the detection of the 5 mutations in the blind study were 100% concordant with the results obtained by sequencing. We recognize that HRM analysis will not replace sequencing for confirmation of altered fluorescence melt curves; however, in a gene scanning scenario, samples in the blind study producing a melt curve consistent with known normal controls would not be sequenced, a situation that demonstrates the potential of this technology to reduce sequencing burden. Although the GJB1 gene contained a reported SNP (rs11551260), retrospective sequence analysis of all individuals confirmed the absence of this neutral variant in our study, as evidenced by the tight grouping of control samples in fragment 1 of both the validation (Fig. 1A) and blind (Fig. 1I) study. Accounting for reported SNPs in an amplicon is important in HRM analysis, as is eliminating the amplification of neutral intronic DNA variants through primer design. These neutral variants will produce altered fluorescence difference curves and will require sequence analysis because the specificity of scanning methods is not 100%.

We have shown that HRM analysis requires simple PCR protocols to prepare samples for melting curve analysis, and that the method has the sensitivity required for the detection of deletion gene rearrangements. Other methods relying on heteroduplex formation to detect sequence variation [e.g., dHPLC and temperature gradient capillary electrophoresis (TGCE)] often fail to detect small homozygous insertions and deletions unless sequential analyses are performed and manual processing is undertaken (8). Our findings complement the report for detecting internal tandem duplications (ranging from 6 bp to 102 bp) by this method (9).

Because HRM can scan an entire amplicon for sequence variation, this method is ideal for screening the complete open reading frame of a gene. Although the coding region of the GJB1 gene is only a single-exon gene, it allowed us to test and validate HRM as a gene-scanning method for an X-linked disorder, in which all heterozygous female and hemizygous male mutations were detected. In contrast, many of the genes reported for CMT are large (>15 exons) e.g., DNM2 (dynamin 2) and MFN2 (mitofusin 2), and would be suited to this method of mutation scanning. We have estimated the cost of reagents to be $0.60 per sample per amplicon, which is one tenth of our current sequencing cost. HRM analysis of single exons of large multiexon genes is a simple, sensitive, and cost-efficient gene-scanning method that would clearly reduce the sequencing burden.

Fig. 1. The subtractive fluorescent difference plots of wild-type and patient mutations for the validation study (A–F) and blind analysis (G–I) of the GJB1 gene using HRM analysis.

The numbers correspond to the patient mutation. Each mutation is annotated by its location in the open reading frame followed by the base change. The amplicon on which mutation lies is indicated by fragment number.
Background: The impact of blood sampling in sitting vs supine positions on measurements of plasma metanephrines for diagnosis of pheochromocytoma is unknown.

Methods: We compared plasma concentrations of free metanephrines in samples from patients with primary hypertension obtained after supine rest with those obtained in the sitting position without preceding rest. We also assessed the effects on diagnostic test performance retrospectively in patients with and without pheochromocytoma, and we calculated cost-effectiveness for pheochromocytoma testing.

Results: Upper reference limits of plasma free metanephrines were higher in samples obtained from seated patients without preceding rest than from supine patients with preceding rest. Application of these higher upper reference limits to samples from supine patients with pheochromocytoma decreased the diagnostic sensitivity from 99% to 96%. In patients without pheochromocytoma, adjusting the plasma concentration for the effects of sitting while preserving the 99% sensitivity by use of the supine upper reference limits increased the number of false-positive test results from 9% to 25%.

Conclusions: To preserve high diagnostic sensitivity we recommend the use of upper reference limits determined from blood samples collected in the supine position. Under these conditions, negative test results for blood samples obtained with patients sitting are as effective for ruling out pheochromocytoma as negative results from samples obtained after supine rest. Repeat testing with samples obtained in the supine position offers a cost-effective approach for dealing with the increased numbers of false-positive results expected after initial sampling in the sitting position.

Measurements of plasma free metanephrines (normetanephrine and metanephrine) provide a particularly sensitive test for the diagnosis of pheochromocytoma (1–4). Suboptimal specificity in some studies may be attributable to blood sampling conditions (5). We therefore examined diagnostic test performance for samples obtained from patients who were either supine or sitting.

Study participants were 60 patients [38 females, mean (SD) age 45 (13.3) years, range 22–78 years; blood pressure 154 (15)/96 (8) mmHg] with primary hypertension (off medication for 2–3 weeks) and normal renal function. A first blood sample was drawn through an antecubital cannula immediately after patients sat down and a second blood sample after 30 min of supine rest. Plasma samples were assayed for concentrations of metanephrines (6).

Gaussian distributions of metanephrines were obtained after logarithmic transformation of the data. The antilogarithm of the mean ± 2 SD of the transformed data of the 60 patients was used as an estimate of the 97.5th percentile, which was adopted as the upper reference limit.

The data obtained from sitting and supine conditions were used in a retrospective analysis of a dataset of samples drawn after at least 20 min of supine rest from patients with (n = 228) and without pheochromocytoma (n = 644) (1). We used the upper reference limits of samples obtained from the 60 hypertensives in the sitting and supine positions to recalculate all diagnostic indices in the retrospective dataset. To further assess the potential influences of posture on diagnostic specificity, we adjusted the test results in the 644 patients without pheochromocytoma for the influence of sitting. We did not do so for the pheochromocytoma patients because of the likely negligible influence of posture on the already considerably increased plasma concentrations of metanephrines in these patients.

The distributions of the 4 variables from sampling in supine and sitting positions were tested for normality using the Shapiro–Wilk test (SPSS v12). The effect of the