Pitfalls in the Denaturing High-Performance Liquid Chromatography Analysis of Mitochondrial DNA Mutation

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Denaturing high-performance liquid chromatography (DHPLC) purification of heteroduplexes has been reported as a method to increase sensitivity of the detection of low-level heteroplasmy by DNA sequencing, and DHPLC profiling has been suggested as a method to allow the correlation of a characteristic chromatographic profile with a specific sequence alteration. Herein we report pitfalls associated with the use of DHPLC for these purposes. We show that the purified heteroduplex fraction does not contain a 50:50 mix of wild-type and mutant DNA in DNA samples containing low-level mutations, and that with a commonly used protocol, DNA sequencing gave false negative results at the 1% mutation level, potentially leading to misdiagnosis. We improved the protocol to detect low levels of mutations and evaluated the sensitivity of DNA sequencing in the detection of mutation in these fractions. We also studied the DHPLC profiles of several mutations in the tRNA^{Leu}(UUR) region of mitochondrial DNA and found a characteristic profile in only one of five mutants tested, whereas four other mutants showed identical chromatographic profiles. (*J Mol Diagn* 2008, 10:102–108; DOI: 10.2353/jmoldx.2008.070081)

DNA sequencing has been considered to be the gold standard in the molecular genetic testing that is widely used in the detection of DNA mutations and the clinical diagnosis of many genetic disorders. However, several drawbacks have been reported in DNA sequencing, including its low sensitivity and the difference in the detection limit between forward and reverse sequencing.¹ DNA sequencing is also prone to laboratory and interpretation errors,^{2,3} which may explain the differences in detection limits among laboratories when standard reference materials with known low heteroplasmy levels were used in an interlaboratory study to examine the ability of a number of mutation detection procedures (including the various DNA sequencing techniques and chemistries) to

detect low heteroplasmy levels of mitochondrial DNA mutations.¹ Most of these procedures were unable to detect mutation levels below 20%. It is now established that the degree of heteroplasmy for some mitochondrial DNA mutations varies considerably among tissues, with muscle specimens and urine epithelial cells reported to carry higher levels of mutation than peripheral blood lymphocytes.^{4–6} While muscle biopsy has become a routine procedure in the diagnostic testing for mitochondrial diseases, it poses a problem in the screening for mitochondrial tRNA^{Leu}(UUR) A3243G mutation in the diabetic population, in which muscle samples are not easily obtainable from patients. Traditionally, blood is the most commonly used specimen in clinical studies, but blood may contain low levels of the A3243G mutation, which can be as low as 3% in some patients with maternally inherited diabetes mellitus and deafness.^{7,8} Therefore, the detection of low-level mutations is important. One of the methods reported to improve the low detection limit involves the use of denaturing high-performance liquid chromatography (DHPLC) to collect elution fractions containing heteroduplex for DNA fragment amplification before DNA sequencing.^{9,10} In this report, we used the published protocols to attempt to detect the A3243G mutation in blood DNA obtained from the mother of a patient affected with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) in which DNA sequencing failed to identify the mutation. We then determined the proportions of wild-type and mutant DNA in a purified heteroduplex fraction collected from her DNA as well as from DNA with known A3243G mutation (0–5%) and evaluated the sensitivity of DNA sequencing in the detection of mutation in a DHPLC-purified heteroduplex fraction. We report an improved protocol to increase the detection limit of DNA sequencing.

The use of DHPLC elution profiles has been suggested as a method allowing the correlation of a characteristic

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chromatographic profile with a specific sequence alteration. When the elution profiles of normal control samples were compared with elution profiles of heterozygous mutant samples for each DNA fragment and DHPLC condition, several laboratories have observed different chromatographic profiles for different mutations as a result of the difference in destabilization at different temperatures of partially double-stranded DNA in the C18 reverse-phase DNA separation column.^{11–16} In particular, in a recent report the two most common nuclear DNA mutations of β -thalassemia *c.316–197C>T* and *c.125_128delTCTT* were identified based on the DHPLC profiles for the disease¹¹ and confirmed by DNA sequencing. The reliable identification of DHPLC peak patterns were also reported in another gene, the MET proto-oncogene.¹³ DHPLC has been widely used in the screening of the mitochondrial genome for mutations.^{9,10,17–19} Using site-directed mutagenesis to generate a panel of five mutations in mitochondrial DNA, we explored whether mutations lying in the tRNA^{Leu}(UUR) region of the mitochondrial genome can be identified by their chromatographic patterns.

Materials and Methods

Sample Preparation

DNA extraction and site-directed mutagenesis were performed as described previously, and primer sequences used in the generation of mutants can be found in our earlier report.²⁰ Plasmid DNA was isolated from the mutant clones using the Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Blood DNA from the healthy mother of a patient diagnosed with MELAS was used in an attempt to detect low-level heteroplasmy by DNA sequencing. The volunteer's blood DNA was obtained under University of California San Diego Institutional Review Board-approved protocol, and informed consent was obtained from the individual.

DHPLC Analysis

Polymerase chain reaction (PCR) primers (Invitrogen, Carlsbad, CA) and conditions used for the amplification of the region of interest in the mitochondrial genome, restriction enzyme digestion of the amplicons with *DdeI*, heteroduplex formation, and DHPLC analysis of the samples have been described previously.²⁰ In DHPLC purification of heteroduplex species, the measurement of heteroduplex proportion in a DNA sample was obtained during DHPLC analysis of the reannealed PCR amplicon as the ratio of heteroduplex peak area to total peak area (%). Peak areas were determined using Navigator software (version 1.6.2, Transgenomic, San Jose, CA). In DHPLC profiling, each mutation was analyzed at temperatures of 50°C, 57°C, 58°C, 59°C, 60°C, and 61°C to determine the optimum temperature for the separation of heteroduplex from homoduplex.

DNA Sequencing

PCR products were purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA (500 ng) and PCR products (50 ng) were sequenced using 50 pmol of the appropriate forward or reverse primer at the Retrogen Sequencing Center (San Diego) using ABI 3730 sequencers.

Detection of mutations in samples with low-level heteroplasmy was performed according to published protocols.^{9,10} Elution fractions containing the heteroduplex peak of 342-bp fragment were collected at the corresponding elution time during the DHPLC analysis. Ten fractions were collected at a flow rate of 0.9 ml/min within a 1-minute time window around the elution time, with a collection time of 6 seconds each. Preliminary study showed that there is a 0.1-minute time delay between the moment a peak was detected at the detector and the time of arrival of the peak at the fraction collector. We found that the elution fraction collected at the time corresponding to the 0.1-minute time window following the peak contained the highest amount of heteroduplexes (data not shown). An aliquot of the fraction was subjected to further PCR amplification using primers 5'-TATACCCACACCCACCCAAG-3' (3200–3219 bp) and 5'-CGGTGATGTAGAGGGTGATG-3' (3507–3526 bp). PCR reactions were performed in 70 μ l of Optimase reaction buffer (Transgenomic) containing collected heteroduplex fraction, 200 μ mol/L each dNTP (Transgenomic), 21 pmol of the forward and reverse primer, and 3.5 units of Optimase DNA polymerase (Transgenomic). PCR was performed using the iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). The conditions for PCR were as follows: 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by a final extension step of 72°C for 5 minutes. The PCR products were sequenced after removal of unincorporated primers and nucleotides using the QIAquick PCR purification kit (Qiagen).

Results

DNA Sequencing and DHPLC Analysis of Blood DNA Sample

Using the published protocols to detect low-level mutations,^{9,10} we analyzed the blood DNA of the asymptomatic mother of a patient with MELAS by performing DNA sequencing on the amplicon generated from the purified heteroduplex fraction. We observed no base change at position 3243 (Figure 1A) despite the fact that the DHPLC analysis of the amplicons generated from the blood DNA even before the first enrichment procedure revealed the presence of heteroduplex species (Figure 2A). This suggested that either DNA sequencing failed to identify a mutation (false negative) or DHPLC analysis falsely identified a mutation (false positive).

If DHPLC analysis gave a false positive result, the purified heteroduplex fraction would presumably contain little or no heteroduplex species. We measured the het-

Figure 1. Determination of mutation by DNA sequencing in blood DNA obtained from the mother of a patient with MELAS. The elution fractions containing the heteroduplex species were collected during DHPLC analysis of the blood DNA and amplified before DNA sequencing. DNA sequencing showed an absence of mutation after one round of DHPLC purification-PCR cycle (A) but the presence of the A3243G mutation after two rounds of DHPLC purification-PCR cycles (B).

eroduplex proportion in the PCR amplicons generated from the purified heteroduplex fraction and observed $27.5 \pm 4.8\%$ ($n = 3$) of heteroduplex in the fraction (Figure 2B). This level was significantly higher than the level in the PCR amplicons generated from the blood DNA ($3.9 \pm 1.2\%$) but was below the expected level of 50% that would exist in a purified heteroduplex peak containing equal amounts of heteroduplex and homoduplex species.

Figure 2. Determination of the heteroduplex proportion by DHPLC in blood DNA obtained from the mother of a patient with MELAS. During the DHPLC purification (A), the heteroduplex fraction collected (shaded area, F1) was used for PCR amplification. The amplicons generated from this first round of purification-PCR cycle were then analyzed by DHPLC (B) to determine the heteroduplex proportion. As this sample showed no mutation detectable by DNA sequencing, we collected the heteroduplex fraction (F2) for additional PCR amplification. This latter amplification (the second round of purification-PCR) generated sufficient amplicons of the heteroduplex (C) for detection by DNA sequencing.

Table 1. Determination of Heteroduplex Proportion in Samples with Low-Level Mutation by DHPLC Analysis

Mutation load (%)	Heteroduplex proportion	
	Before purification-PCR	First purification-PCR cycle
0	2.4 ± 0.1	5.3 ± 0.4
1	3.5 ± 0.3	24.0 ± 0.5
2.5	5.0 ± 0.4	34.6 ± 0.1
5	9.6 ± 0.7	42.2 ± 0.2

PCR products from the A3243G mutant clones were mixed with wild-type samples to produce 0, 1, 2.5, and 5% of A3243G mutation. These samples were subjected to DHPLC analysis, during which the heteroduplex fractions were collected for PCR amplification. The amplicons generated by PCR from this round of purification-PCR cycle were analyzed by DHPLC to determine the heteroduplex proportion. Each measurement represents the mean $\pm$ SD for at least two separate samples.

DHPLC and DNA Sequencing Analysis of Mutation Standards with Low-Level Heteroplasmy

Using DHPLC we studied whether purified heteroduplex fractions obtained from other low levels of heteroplasmic mutation also contained less than 50% heteroduplex by generating samples containing 1, 2.5, and 5% of the A3243G mutation and subjected them to the same purification and PCR procedure. We measured heteroduplex proportions in the DHPLC-purified heteroduplex fractions. These fractions contained a significantly higher amount of heteroduplex species when compared to the amount before purification, as indicated by the increased heteroduplex proportions: $\sim 24\%$ (for 1% mutation), $\sim 35\%$ (for 2.5% mutation), and $\sim 42\%$ (for 5% mutation) (Table 1). Similar to the heteroduplex proportion observed in purified heteroduplex fraction of the blood DNA, all of the heteroduplex levels in the purified heteroduplex fraction of the mutation standards fell short of the theoretically predicted level of 50%.

Analysis of the 0% A3243G mutation standard showed that the background heteroduplex level before DHPLC purification was $\sim 2\%$ (Table 1), whereas the background level in the purified heteroduplex fraction was $\sim 5\%$. The level measured in the purified heteroduplex fraction obtained from the mother's blood DNA was significantly higher than the background level ($27.5 \pm 4.8\%$ versus $5.3 \pm 0.4\%$), suggesting that DHPLC identified a true mutation.

Using published protocols,^{9,10} we were able to detect the A3243G mutation standards at the 2.5 and 5% levels by DNA sequencing after PCR amplification of the DHPLC-purified heteroduplex fraction (Figure 3A). However, the 1% mutation could not be reliably detected by DNA sequencing due to the variable background levels, similar to our observation with the mother's blood DNA. This suggested that the absence of the A3243G mutation observed in both the mother's blood DNA (Figure 1A) and the 1% mutation standard (Figure 3A) was due to the failure of DNA sequencing to detect the mutation although the heteroduplex species was present at $\sim 27\%$

Figure 3. Determination of low-level mutation by DNA sequencing. **A:** The elution fractions containing the heteroduplex species were collected during DHPLC analysis from samples containing 0, 1, 2.5, and 5% of the A3243G mutation and amplified before DNA sequencing. PCR amplification of these purified heteroduplex fractions significantly increased the detection limit of sequencing from the commonly reported 20 to 2.5%. The detection of PCR products amplified from the heteroduplex fraction obtained from the 1% mutation standard remained ambiguous due to interference from the background levels of DNA sequencing. **B:** For the sample with 1% mutation, the amplified heteroduplex fraction was further purified by DHPLC for additional PCR amplification to improve the sensitivity.

(Figure 2B) in the purified heteroduplex fraction obtained from the mother's DNA and at ~24% (Table 1) in that obtained from the 1% A3243G mutation standard. Both levels were below the expected level of 50%.

We investigated the underlying reasons for reduced mutant DNA species (<50%) in the purified heteroduplex fractions and speculated that "contamination" of heteroduplex fraction by coeluting homoduplex might be the cause of this problem. We amplified six consecutive DNA fractions collected within a 0.6-minute time window (9.9–10.5 minutes), including the heteroduplex DNA elution time (10.1 minute), with a collection time of 6 seconds each (Figure 4A). These amplicons were then analyzed by DHPLC at 59°C. Figure 4B shows that homoduplex DNA species was observed after PCR amplification in the fraction collected at 9.9 minutes (0.6 minutes before the desired elution time). Fraction 6 contained the highest

Figure 4. **A:** Collection of eluted fractions (9.9 to 10.5 minutes) during DHPLC analysis. DNA containing 1% A3243G mutation was amplified by PCR, digested by restriction enzyme, and analyzed by DHPLC as described in Materials and Methods. Six consecutive DNA fractions were collected within a 0.6-minute time window around the heteroduplex DNA elution time, with a collection time of 6 seconds each. **B:** DHPLC analysis of PCR products generated from the DNA in these collected fractions. The amplicons were analyzed by DHPLC at 59°C.

amount of homoduplex DNA, as expected. Fractions 2 and 3 contained the highest amount of heteroduplex DNA species, 15 and 20%, respectively, after PCR amplification of the collected fraction. This suggested that the homoduplex DNA species coeluted with the heteroduplex DNA species, leading to our observation of a reduced amount of mutant DNA in the collected heteroduplex fraction.

Improved Protocol for the Detection of Low-Level Heteroplasmy

The detection limit of mutations in DNA sequencing is approximately 20%.¹ We recently derived a mathematical formula that relates DNA mutant load to heteroduplex proportion in a heteroduplex analysis, $x \approx 50 \pm \sqrt{2500 - 50y}$, where x = level of heteroplasmy (%) and y = heteroduplex proportion (%).²⁰ According to this formula, a DNA sample with a 20% mutation level would contain 32% heteroduplexes after denaturation and re-annealing. Hence, only PCR products with a heteroduplex proportion above the threshold level of 32% would allow the detection of these mitochondrial mutations by

DNA sequencing. This could explain why DNA sequencing did not detect a 1% mutation level in a sample with ~24% heteroduplexes after the DHPLC purification-PCR cycle (Table 1), although it did detect 2.5 and 5% mutation, which contained ~35 and ~42% heteroduplexes, respectively.

To improve the detection limit of DNA sequencing, we increased the proportion of heteroduplex species in the sample to above 32%. This was achieved by further purifying the heteroduplex species from the PCR product generated from DHPLC-purified heteroduplex fraction. We injected the PCR product into DHPLC and collected another heteroduplex fraction for PCR amplification of the DNA (second DHPLC purification-PCR cycle). This additional cycle increased the heteroduplex proportion from $24.0 \pm 0.5\%$ to $48.4 \pm 0.6\%$ and allowed the detection of the 1% mutation by DNA sequencing (Figure 3B). Similarly, DNA sequencing analysis of the mother's blood DNA after a second DHPLC purification-PCR cycle also allowed identification of the A3243G mutation (Figure 1B). In this case, the heteroduplex proportion was increased to $47.5 \pm 2.1\%$.

DHPLC Profiling of *tRNA^{Leu}(UUR)* Mutations

We generated six mutant clones for DHPLC analysis.²⁰ Five of them—A3243G, C3256T, A3260G, T3271C, and T3291C—are the more common mutations found in the *tRNA^{Leu}(UUR)* region. These mutations are known to cause MELAS or maternal myopathy and cardiomyopathy. Further information on the mutations can be found at MITOMAP: A Human Mitochondrial Genome Database (<http://www.mitomap.org>, 2007). These mutations are located between 3243 and 3291 bp of the mitochondrial genome (51–110 bp of the 342-bp *DdeI* restriction fragment), with 5–20 bp between the mutant sites. The sixth mutant clone was T3308C, which lies in the NADH dehydrogenase subunit 1 region and has a similar chromatographic profile to T3271C (data not shown). We predicted the DHPLC temperatures for detection of these mutations using Navigator software (version 1.6.2).²¹ The recommended temperature was 58°C, but we analyzed the samples using six different temperatures: 50°C, 57°C, 58°C, 59°C, 60°C, and 61°C.

We prepared 40% mutants by mixing the PCR products of the wild type with each of the different mutations separately. Comparing the DHPLC profiles of the mutants at these six temperatures, we observed that DHPLC profiles for all of the mutants were distinct from the wild type at more than one temperature, with the mutations usually detected at three or four different temperatures within the range of 57°C to 60°C (data not shown). DHPLC profiles for these mutants are available from the Mitochondrial DNA Analysis website of the University of California San Diego (<http://mmdc.ucsd.edu/phenotype.php>, last accessed October 2007). The separation of the heteroduplex and homoduplex peaks was best resolved at 59°C for all mutations (data not shown).

We then analyzed the differences in the DHPLC profiles at 59°C among these mutants according to peak

Figure 5. DHPLC profiles of both mutant and wild-type DNA at the optimized DHPLC temperature. Samples containing 40% mutation of C3256T, T3271C, A3243G, T3291C, and A3260G were prepared by mixing the PCR products of respective mutations with wild-type DNA to produce samples. These samples were subjected to heteroduplex formation and analyzed by DHPLC at 59°C. DHPLC profiles for T3271C, A3243G, T3291C, and A3260G were not distinctively different from each other, whereas C3256T displayed characteristic peak shapes. Heteroduplex peaks are boxed. The last panel represents the DHPLC profile for wild-type DNA. Shown is the representative diagram based on three separate analyses.

shape. Characterization by retention time was not possible, because all of the mutations lie in the same restriction enzyme fragment and elute at the same time. Moreover, the retention time varied slightly after each hot wash of the column, making it an unreliable parameter. The peak shapes in the DHPLC chromatograms for A3243G, A3260G, T3271C, and T3291C were not distinctively different from each other, whereas C3256T had characteristic peak shapes (Figure 5). Melt profiling, which examines DHPLC profiles at the temperatures around the optimum, might discriminate mutation profiles that are similar at the optimal temperature.¹³ We examined the melt profiles of the four mutants with similar DHPLC profiles (A3243G, A3260G, T3271C, and T3291C) at 59°C but found no notable differences among them over the temperature range of 57°C to 61°C (data not shown).

We further analyzed the DHPLC profiles of the C3256T mutant at 59°C using 0 to 100% of the mutants by mixing the mutants with wild-type samples accordingly. As shown in Figure 6, the mutant and wild-type homoduplex peaks were partially resolved, but the two heteroduplex peaks coeluted. In the sample containing 50% mutant, the wild-type and mutant homoduplex peak sizes were similar.

Discussion

Theoretically, a heteroduplex fraction should contain 50:50 of wild-type and mutant DNA. We observed that an equal mix of wild-type and mutant DNA did not exist in purified heteroduplex fractions obtained from DNA samples with low-level mutation. Despite the fact that the heteroduplex and homoduplex were well resolved (Figure 5), collection of heteroduplexes from samples with low levels of mutation was always affected by the homoduplex species that coeluted. This effect became obvious when highly sensitive PCR was used to amplify the DNA in the collected fraction. The effect of coelution can

Figure 6. DHPLC profiles of 0 to 100% C3256T mutations at the optimized DHPLC temperature. Varying amounts of PCR products generated from mutant and wild-type DNA were mixed to produce samples of different mutant loads ranging from 0 to 100%, and these samples were subjected to heteroduplex formation and analyzed by DHPLC at 59°C. The heteroduplex species (eluted at 9.6 minutes, dotted line) resulting from the mutation at C3256T were well separated from the homoduplex species (eluted after 10 minutes). The two homoduplex peaks (eluted at 10.1 and 10.5 minutes) were also well separated. Shown is a representative diagram based on three separate analyses.

be detrimental as the coeluting homoduplex lowers the heteroduplex proportion observed in the purified heteroduplex fraction and, therefore, interferes with the resolution of DNA sequencing of the fraction. This effect was seen in the analysis of the mother's DNA and the 1% mixture of the A3243G mutation standard in which DNA sequencing failed to identify the A3243G mutation when the common protocol, including one round of DHPLC purification-PCR cycle, was performed. The mutation was only detectable after a second round of DHPLC purification-PCR cycle. Hence, in samples with low heteroduplex proportion (<2.5% mutation), it is important to perform two rounds of DHPLC purification-PCR cycles before DNA sequencing to confirm or exclude a possible mutation as the first round of DHPLC purification-PCR cycle may give a false negative result. This is crucial in the diagnosis of diseases associated with low-level heteroplasmy such as maternally inherited diabetes mellitus and deafness. It is unlikely that the reduced amount of mutant in the heteroduplex fraction is due to differential amplification of wild-type DNA. In a study by Ruano and Kidd,²² PCR products generated from a pooled sample containing both wild-type and mutant DNA were shown to be representative for every template present even when a trace element accounts for only 1 in 20 elements in the pool. We recently showed that the amplification of only 1% of mutant DNA follows the mathematical model that predicts the proportion of mutant DNA in a mixture of wild-type and mutant DNA.²⁰ In the recent article published by Wulfert et al,⁹ amplification of a purified heteroduplex fraction was shown (Figure 4 in the reference). Although the authors did not discuss the asymmetrical peaks of heteroduplex and homoduplex species, a higher amount of homoduplex species was observed in the purified heteroduplex fraction. This was further confirmed in their DNA sequencing data which demonstrated higher levels of 198C in the purified heteroduplex fraction

(198C&T mixture). It is likely that the coelution effect we describe here also contributed to the observation of Wulfert and colleagues.⁹

DHPLC profiling of four of the five mutants (A3243G, A3260G, T3271C, and T3291C) showed no characteristic peak shapes or retention times that could allow correlation with a specific mutation. Similarly, analysis of the melt profiles at 57°C to 61°C did not reveal any difference among these four mutations. Therefore, contrary to reports detailing DHPLC analysis of nuclear DNA mutations,^{11–14} we observed no characteristic DHPLC signatures for four common mitochondrial DNA mutations. DNA sequencing is necessary for the identification of the mutation even when the profiles show very similar characteristic patterns. The presence of the characteristic peak was, however, observed in the C3256T mutation. This is due to the separation of the two homoduplex peaks of 3256C and 3256T. Homoduplexes with the AT base pair have less retention than those with the GC base pair as a result of the difference in thermal stabilities of typical Watson-Crick and mismatched base pairs.²³ Therefore, it is likely that any mutation that involves a purine to pyrimidine transversion would result in a shorter retention time as observed in our DHPLC analysis of C3256T mutant DNA.

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