Detection of α -Globin Gene Deletions Using Denaturing High-Performance Liquid Chromatography and Multiplex Ligation-Dependent Probe Amplification

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Background: Multiplex ligation-dependent probe amplification (MLPA) has been used to detect deletions and mutations of the α -globin gene for diagnosis of α -thalassemia. MLPA reaction products are usually separated and analyzed by high-voltage capillary gel electrophoresis (CGE). The goal of this study was to find and use a cost-effective method to separate and analyze MLPA products. **Methods:** Blood samples were collected from China. DNA was extracted and amplified by PCR using fluorescently labeled primers. In this study, denaturing high-performance liquid chromatography (DHPLC) was used to separate and analyze the reaction products. And the optimal separation conditions were determined using nondenaturing column

temperature. **Results:** The DHPLC conditions were optimized and have been applied to separate MLPA products and 27 of the MLPA products from 50 to 320 bp were well separated. DHPLC was able to separate up to 37 reaction products that differed by 4–12 base pairs and detected target gene deletions by differences in peak size. Compared with CGE, both the specificity and sensitivity of DHPLC for the 107 DNA samples were 100%. **Conclusions:** DHPLC could be used to test routinely for α -globin gene mutations and deletions. Combined with MLPA, DHPLC is a low-cost, simple to use, accurate technique with practical value. *J. Clin. Lab. Anal.* 25:426–431, 2011.

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INTRODUCTION

In 2002, Schouten et al. (1) first described the multiplex ligation-dependent probe amplification (MLPA) technique. MLPA can be used to quantitate more than 40 different target sequences in a simple, single tube format (1). Deletions in the α -globin and β -globin genes, as well as chromosomal aneuploidies, have been detected using MLPA (2–4). A high incidence of α -thalassemia is found in Southern China. Three

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large deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--SEA$,) in the α -globin gene cluster on chromosome 16 and gene mutation $\alpha^{CS}\alpha$ are the major causes of α -thalassemia in the Chinese population (5). MPLA has been used to detect deletions and mutations that cause α -thalassemia (2,6). Novel large gene deletions have also been discovered using MLPA (7,8). The reaction products of MLPA are usually separated and analyzed by high-voltage capillary gel electrophoresis (CGE). To some extent, the high cost of CGE instruments, reagents, and consumables has restricted the routine application of MLPA.

Denaturing high-performance liquid chromatography (DHPLC) is a technique that has been widely used to detect point mutations under partially denaturing conditions (2,9). DHPLC can also be used to separate double-stranded DNA molecules by size and is less costly than CGE. In this study, DHPLC conditions were optimized in order to separate and analyze MLPA reaction products. After optimization, it was possible to separate 19–37 different MLPA products under nondenaturing conditions and to perform semi-quantitative copy number analysis based on peak height differences. Therefore, rapid and accurate analysis of α -globin gene deletions can be achieved using a combination of MLPA and DHPLC.

MATERIALS AND METHODS

DNA Samples

DNA samples with known genotypes ($-\alpha^{3.7}/\alpha\alpha$, $n = 7$; $--SEA/\alpha\alpha$, $n = 5$; $-\alpha^{4.2}/\alpha\alpha$, $n = 6$; $\alpha^{CS}\alpha/\alpha\alpha$, $n = 2$; $--FJ/\alpha\alpha$, $n = 2$; normal $\alpha\alpha/\alpha\alpha$, $n = 5$) that have been identified in our laboratory by multiplex gap-PCR or MLPA (6,10) were used to develop the DHPLC method. One hundred and seven blood samples were collected from Liuzhou and Nanning in Guangxi, China, for a blinded study. Informed consent was obtained from the subjects and the study was approved by the Beijing Chao-Yang Hospital Ethics Committee. We confirmed that we have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Reagents and Equipment

SALSA MLPA P140 and P140-B2 kits were purchased from MRC-Holland (The Netherlands). The P140 kit includes 37 probes pairs that, after ligation and PCR, differ in size by increments of 6–9 nucleotides. PCR of the ligated probe pairs resulted in amplicons of 130–418 bp. Five probe pairs equal to or less than 92 nucleotides were used to produce control amplicons. The MLPA reaction products were routinely separated and analyzed using an AB3130xl Genetic Analyzer system (Applied Biosystems, Foster City, CA).

Additional control probe pairs of 100 and 105 nucleotides were added into the P140 mixture (P140-B2) and were used to analyze samples when the P140 reagents were consumed.

α -Globin^{xs} probe mixes of (α -Globin^{xs} MPLA Kit; ServiceXS, The Netherlands) were comprised of 35 different probe pairs covering a region of 700 kb, including the α -globin gene cluster on 16p13.3. The size difference between adjacent amplified, ligated probe pairs was 2 bp. After ligation, 19 probe pairs were labeled with FAM and 20 probe pairs were labeled with HEX by means of two separate PCR reactions.

An ABI GeneAmp PCR System 2400, AB3130xl Genetic Analyzer, and WAVE System 4500 DHPLC were used in this study.

DNA Extraction

DNA was extracted in our laboratory from peripheral blood samples using a routine method (10).

MLPA Reactions Using P140 and P140-B2 Kits

Denaturation and hybridization were carried out as follows. Mixtures of DNA (1 μ l, 0.05–0.1 μ g/ μ l) were denatured at 98°C for 5 min in TE buffer (4 μ l). Then the HBA probe mix (1.5 μ l) and SALSA MLPA buffer (1.5 μ l) were added. The reactants (8 μ l) were gently mixed and incubated at 95°C for 1 min, followed by hybridization at 60°C for 16–18 hr.

After denaturation and hybridization, the temperature of the hybridization mixture was decreased from 60 to 54°C and ligase-65 mix (32 μ l) was added to the hybridization mixture (8 μ l). This was mixed well, incubated at 54°C for 15 min, and then heated at 98°C for 5 min to deactivate the ligase enzyme. The ligation reaction products were stable for about 1 week at 4°C.

The ligation reaction products were amplified by PCR. DNA polymerase (10 μ l) was added to a mixture of SALSA PCR buffer (4 μ l), ultrapure water (26 μ l), and ligation reaction products (10 μ l). The reactants (50 μ l total volume) were mixed and then subjected to 40 PCR cycles of 30 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C. After the final PCR cycle, the reaction mixture was held for 4 min at 72°C. The reaction products were stable for about 1 week at 4°C and then separated and analyzed using an AB3130xl Genetic Analyzer as per the manufacturer's instructions.

MLPA Reactions Using α -Globin^{xs} Kit

Denaturation, hybridization, and ligation were carried out, as described above, for MPLA reactions using P140 and P140-B2 kits. Ligation reaction products were then amplified by PCR using either HEX or FAM

fluorescently labeled primers. For fluorescent HEX labeling, the ligation reaction products (5 μ l) were added to a mixture of SALSA PCR buffer (2 μ l), ultrapure water (15.75 μ l), SALSA enzyme dilution buffer (1.0 μ l), HEX-PCR primer mix (+dNTPs, 1.0 μ l), and SALSA polymerase (0.25 μ l). The reactants (25 μ l total volume) were mixed, denatured at 94°C for 5 min, and then subjected to 33 PCR cycles of 30 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C. After the final PCR cycle, the reaction mixture was held for 20 min at 72°C. For fluorescent FAM labeling, the ligation reaction products (5 μ l) were added to a mixture of SALSA PCR buffer (2 μ l), ultrapure water (14.75 μ l), SALSA enzyme dilution buffer (1.0 μ l), FAM-PCR primer mix (+dNTPs, 2.0 μ l), and SALSA polymerase (0.25 μ l). The reactants (25 μ l total volume) were mixed, denatured at 94°C for 5 min, and then subjected to 35 PCR cycles of 30 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C. After the final PCR cycle, the reaction mixture was held for 20 min at 72°C. The PCR products were stable for about 1 week at 4°C. The reaction products were then separated and analyzed using an AB3130xl System as per the manufacturer's instructions.

Separation and Analysis of MLPA Reaction Products by DHPLC

MLPA reaction products (10 μ l) were injected into the WAVE System 4500 and analyzed automatically. The temperature of the column was set to 50°C. Details of the conditions are described in the Results section.

RESULTS

Optimized DHPLC Conditions for MLPA Product Analysis

Universal linear conditions set automatically by the DHPLC instrument were initially used to separate the MLPA reaction products. However, the results obtained

were not ideal owing to poor resolution of most of the product peaks. Several alternate conditions were tested to improve the separation. The optimized gradient conditions are shown in Figure 1. Buffer A was 0.1 M triethylammonium acetate, pH 7; Buffer B was 0.1 M triethylammonium acetate, 25% (v/v) acetonitrile, pH 7. The column temperature was 50°C and the flow rate was 0.9 ml/min. Twenty-seven MLPA products from 50 to 320 bp were well separated using the optimized conditions.

Separation and Analysis of MLPA P140 Kit Reaction Products Using DHPLC

DHPLC analysis of MLPA reaction products from five DNA samples with known genotypes is shown in Figure 2. Although not all the peaks are as well-resolved by DHPLC in comparison to AB3130xl analysis (6), 37 peaks representing all P140 reaction amplicons can be identified. Peaks 1–27 are well-resolved by DHPLC. A comparison of normal and $-\alpha^{3.7}/\alpha\alpha$ DNA shows that 9 peaks (2, 5, 6, 10, 11, 15, 18, 20, and 29) were reduced from the $-\alpha^{3.7}/\alpha\alpha$ reaction products. This is consistent with results from AB3130xl analysis (6). Eighteen peaks are also reduced when $--SEA/\alpha\alpha$ DNA is compared with normal DNA (2, 4, 5, 6, 9, 10, 11, 12, 14, 15, 16, 18, 20, 23, 26, 29, 33, and 36). The $-\alpha^{4.2}/\alpha\alpha$ DNA results were slightly different from the $-\alpha^{3.7}/\alpha\alpha$ DNA results. Peaks 12, 14, 16, and 33 were reduced in the $-\alpha^{4.2}/\alpha\alpha$ DNA sample results. Peaks 5, 11, and 18 were nearly 50% reduced, as the probe sites for peaks 2 and 6 are found in both the α_1 and α_2 genes. This is consistent with the deletion of one α gene. Peaks 10, 15, and 20 were still present normally, but peaks 12, 14, and 16 were reduced, indicating that the deleted gene was α_1 instead of α_2 . These results are consistent with AB3130xl results with $-\alpha^{4.2}/\alpha\alpha$ DNA (6). A specific peak located between peak 1 and 2 showing up indicates the DNA sample containing $\alpha^{CS}\alpha$ mutation.

Fig. 1. Optimized gradient profile and gradient set up for optimized DHPLC analysis of α -globin MLPA reaction products using a WAVE 4500 system. DHPLC, denaturing high-performance liquid chromatography; MLPA, multiplex ligation-dependent probe amplification.

Fig. 2. DHPLC analysis of MLPA reaction products from five DNA samples with known genotypes of $\alpha\alpha/\alpha\alpha$ (normal), $-\alpha^{3.7}/\alpha\alpha$, $-\alpha^{4.2}/\alpha\alpha$, $--SEA/\alpha\alpha$, and $\alpha^{CS}/\alpha\alpha$ using SALSA MLPA P140 kits under the optimized conditions. Each DNA sample was approximately 50 ng. Peaks indicated by arrows were approximately 40–50% smaller than the corresponding peaks in normal DNA. DHPLC, denaturing high-performance liquid chromatography; MLPA, multiplex ligation-dependent probe amplification.

Fig. 3. CGE analysis patterns of MLPA reaction products from DNA samples with known genotypes of $\alpha\alpha/\alpha\alpha$ (normal), $\alpha^{CS}/\alpha\alpha$, and $--SEA/\alpha\alpha$ using MLPA P140 kits. MLPA, multiplex ligation-dependent probe amplification; CGE, capillary gel electrophoresis.

Separation and Analysis of MLPA P140 Kit Reaction Products Using AB3130xl

AB3130xl analysis of the MLPA P140 kit reaction products for $\alpha\alpha/\alpha\alpha$, $\alpha^{CS}/\alpha\alpha$ and $--SEA/\alpha\alpha$ DNA are shown in Figure 3. AB3130xl analysis shows same results for these samples as DHPLC analysis but with a higher resolution.

Separation and Analysis of α -Globin^{XS} MLPA Kit Reaction Products Using AB3130xl

AB3130xl analysis of the FAM-labeled MLPA reaction products for normal and $--FJ/\alpha\alpha$ DNA are shown

in Figure 4. $--FJ/\alpha\alpha$ is a large gene deletion of 210 kb that covers all α -globin gene clusters and was found in a family from the Fujian province (8). Compared with normal DNA, seven peaks were approximately 50% lower (1, 2, 5, 7, 9, 12, and 16); peak 4 was not significantly lower than in normal DNA.

Separation and Analysis of α -Globin^{XS} MLPA Kit Reaction Products Using DHPLC

DHPLC analysis of MLPA reaction products from two DNA sequences are shown in Figure 5. Nineteen normal DNA reaction products peaks were clearly

identified and are consistent with those observed in Figure 4 [trace (a)]. When compared with normal DNA, --FJ/ $\alpha\alpha$ DNA had 7 lower height peaks (1, 2, 4, 5, 9, 12, and 16). This is consistent with AB3130xl analysis (Fig. 4). Interestingly, peaks 3 and 4 were well separated by DHPLC but not by AB3130xl. For normal DNA, peak 5 was larger than peak 6, whereas for --FJ/ $\alpha\alpha$ DNA, peak 5 was significantly smaller than peak 6. Similarly, for normal DNA, peak 16 was larger than peak 17, whereas for --FJ/ $\alpha\alpha$ DNA, peak 16 was smaller

than peak 17. These results indicate that changes in the seven peaks associated with --FJ/ $\alpha\alpha$ DNA detected by AB3130xl analysis could also be detected by DHPLC. Therefore, DHPLC analysis of MLPA reaction products is able to detect and describe large gene deletions.

Blinded Study of 107 DNA Samples

One hundred and seven blinded DNA samples that included examples of several different α -thalassemia deletions were analyzed by MLPA, and reaction products separated on DHPLC and AB3130xl systems to test the specificity and sensitivity of DHPLC analysis. For all samples (--SEA/ $\alpha\alpha$, $n = 18$; --SEA/--SEA, $n = 1$; $-\alpha^{3.7}/\alpha\alpha$, $n = 24$; $-\alpha^{3.7}/$ --SEA, $n = 6$; $-\alpha^{4.2}/\alpha\alpha$, $n = 10$; $-\alpha^{4.2}/$ --SEA, $n = 4$; $\alpha^{CS}/\alpha\alpha$, $n = 3$; $\alpha\alpha/\alpha\alpha$, $n = 41$), the results of DHPLC and AB3130xl analysis were consistent. Therefore, compared with CGE, both the specificity and sensitivity of DHPLC for the 107 DNA samples were 100%.

DISCUSSION

DHPLC has been widely used to quickly detect known and unknown mutations, or SNPs, under partially denaturing conditions and to analyze oligonucleotide sequences under completely denaturing conditions (11–13). DHPLC has many advantages over other analytical methods for the analysis of MLPA reaction products. For example, the technique is highly efficient, with a high throughput and low cost (less than 10 Yuan RMB per sample). The technique also generates no

Fig. 4. CGE analysis of normal DNA MLPA products labeled by FAM (A), and a known large deletion carrier's (--FJ/aa) DNA (B). DNAs were analyzed using the α -globin^{XS} MLPA kit. Each of the DNAs was approximately 50 ng. The peaks indicated by arrows were approximately 40–50% lower in height when compared with their neighbors in the two traces. MLPA, multiplex ligation-dependent probe amplification; CGE, capillary gel electrophoresis.

Fig. 5. DHPLC analysis patterns of the MLPA products from a normal DNA (A), and a known large deletion (--FJ/aa) carrier's DNA (B) using the α -globin^{XS} MLPA kit. The peaks indicated by arrows were reduced in height by approximately 40–50% when compared with their neighbors in the two traces. DHPLC, denaturing high-performance liquid chromatography; MLPA, multiplex ligation-dependent probe amplification.

radioactive waste, is highly automated, and generates simple results that can be interpreted visually. In this study, DHPLC was used to separate and analyze MLPA reaction products of more than 20 different lengths under nondenaturing conditions to identify α -globin gene deletions. Conditions were optimized to separate multiplex PCR amplicons using DHPLC at 50°C. Based on DHPLC peak height analysis, a semi-quantitative determination of copy number was possible. The reaction product peaks obtained with DHPLC were smaller than those obtained with an AB3130xl system. However, this did not prevent the detection of three deletions. Therefore, the rapid and accurate diagnosis of α -globin gene deletions could be achieved using single-tube MLPA reactions with DHPLC analysis.

The ability of DHPLC to resolve reaction product peaks was not as great as AB3130xl, especially for the peaks from longer amplicons. As shown in Figure 3, all 37 peaks of reaction products from MLPA 140 kit on AB3130xl were well separated and with high resolution, whereas peak 28–37 on the DHPLC pattern can be separated but with low resolution, as shown in Figure 2. However, it was still possible to use DHPLC to rapidly and accurately diagnose three deletions found commonly in Southern China based on the DHPLC patterns of the shorter amplicons. Larger deletions than --SEA, such as --FIL, --THAI, --DUTCH2, rely on peaks 28–33 for identification. For this reason, DHPLC may not be able to detect these larger deletions until a DHPLC method with improved larger fragment resolving power is developed.

In our laboratory, DHPLC has also been used to detect five common aneuploidies using a SALSA MLPA P095 Aneuploidy kit (MRC-Holland). The resolving power of DHPLC was not as high as with the AB3130xl system with only the first 28 peaks being well resolved. However, the pattern and heights of the first peaks were distinctive enough to allow accurate diagnoses of the five aneuploidies (results not shown). Furthermore, some peaks were separated more clearly by DHPLC than by the AB3130xl system.

In conclusion, DHPLC can be used to separate and analyze a large numbers of target genes generated by MLPA reaction. DHPLC has been shown to be a viable

alternative to using CGE analysis platforms, such as the AB3130xl. In combination with MLPA, DHPLC is a low cost, simple to use, accurate technique with high practical value.

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