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World J Gastroenterol 2002;8(3):426-430
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

Rapid screening mitochondrial DNA mutation by using denaturing high-performance liquid chromatography

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Received 2002-01-28 **Accepted** 2002-03-14

Abstract

AIM: **To optimize conditions of DHPLC and analyze the effectiveness of various DNA polymerases on DHPLC resolution, and evaluate the sensitivity of DHPLC in the mutation screening of mitochondrial DNA (mtDNA).**

METHODS: **Two fragments of 16s gene of mitochondrial DNA (one of them F2 is a mutant fragment) and an A3243G mutated fragment were used to analyze the UV detection limit and determine the minimum percentage of mutant PCR products for DHPLC and evaluate effects of DNA polymerases on resolution of DHPLC. Under the optimal conditions, we analyzed the mtDNA mutations from muscle tissues of mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and screened blindly for variances in D-loop region of mtDNA from human gastric tumor specimen.**

RESULTS: **Ten A3243G variants were detected in 12 cases of MELAS, no alterations were detected in controls and these results were consistent with the results obtained by analysis of RFLP with ApaI. We also identified 26 D-loop variances in 46 cases of human gastric cancer tissues and 38 alterations in 13 gastric cancer cell lines. The mutation of mtDNA at 80ng PCR products containing a minimum of 5% mutant sequences could be detected by using DHPLC with UV detector. Moreover, Ampli-Taq Gold polymerase was equally as good as the proofreading DNA polymerase (e.g., Pfu) in eliminating the false positive produced by Taq DNA polymerases.**

CONCLUSION: **DHPLC is a powerful, rapid and sensitive mutation screening method for mtDNA. Proofreading DNA polymerase is more suitable for DHPLC analysis than Taq polymerase.**

Liu MR, Pan KF, Li ZF, Wang Y, Deng DJ, Zhang L, Lu YY. Rapid screening of mitochondrial DNA mutation by using denaturing high-performance liquid chromatography. *World J Gastroenterol* 2002;8(3):426-430

INTRODUCTION

A number of methods, such as PCR-SSCP, DGGE and CSGE, have been developed to screen the gene mutation. PCR-SSCP is a common method in mutation detection[1-16]. The low resolution and reproducibility or time-cost limit their application for mutation screening[17-23]. Recently, a more accurate and rapid DNA screening strategy[24-29]-Denaturing High-performance Liquid Chromatography (DHPLC) has been applied to mutation screening in human disease-related gene[30-42] and prenatal diagnosis[43,44].

However, there are few published data on detection of mitochondrial DNA (mtDNA) variation by DHPLC, especially in cancer. In order to detect effectively the mtDNA mutation, the optimizations of DHPLC for mtDNA mutation screening have been evaluated. The evaluation included the ultraviolet (UV) detection limit for PCR products with variant ingredients, minimal detected ratio of variant to wild type in PCR mixture. In addition, small peak was often observed preceding the chromatography profile in both amplimers of nucleus and mitochondrial genes when Taq DNA polymerases were used in PCR amplification. It may result in failure of DHPLC analysis. Therefore, we also evaluated the effect of DNA polymerasess on the resolution of DHPLC in detection of heteroduplex. Basing on those analyses, we identified the mutation of A3243G substitute for patients with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). Finally, the variances of D-loop in mtDNA were blindly screened by DHPLC for patients with gastric carcinoma and gastric cancer cell lines.

MATERIALS AND METHODS

Samples and DNA preparation

The specimen in this study included: (1) 12 cases of MELAS, 72 cases of other mitochondrial encephalomyopathies and 30 cases of controls. (2) 46 paired samples of gastric tumor tissues and non-tumor tissues, and 13 gastric cancer cell lines. (3) A randomly selected fragment (fragment 6: from 2616 to 2884nt) within 16s gene was selected to evaluate effect of 11 brands of DNA polymerases on resolution of DHPLC. (4) Another variant fragment (fragment 2: from 1777 to 2069nt) in 16s gene of gastric cancer cell line MKN45 was used to analyze the UV detection limit of PCR products for DHPLC. A variant sample of MELAS,whose ratio of mutant type (G) to wild type (A) at 3243 allele site was about 60 percent, was employed to determine the minimum percentage of mutant PCR products for DHPLC. The genome DNA was isolated following standard phenol/chloroform and ethanol precipitation extraction procedures.

PCR conditions and quantification of PCR products

Fragment containing A3243G mutatioo in mtDNA of MELAS was amplified by using one pair of oligonucleotide primers. Fragments of D-loop were amplified by using four pairs of overlapping primers desribed by Levin *et al*[45]. Another pair of primers was used to amplify fragment 6 (F6) within 16s gene for assessing effect of DNA polymerases on DHPLC (Table 1). 20μL standard PCR reactive system contain genome DNA 15ng, forward and reverse primer 125nmol·L^{-1} each, 1×buffer (Mg^{2+} 1.6μmol·L^{-1}), dNTP 37.5μmol·L^{-1}, Pfu DNA polymerase 1.5units (or Taq DNA polymrases 1 unit). PCR was performed with 32 cycles consisting of a denaturing step of 94℃ for 35s, primer annealing for 50s and an elongation step of 72℃ for 90s. The final step at 72℃ was extended to 10min. Annealing temperature of each fragment was showed in table 1. In addition, one mutated fragment (F2), harboring in mitochondrial 16s

gene of gastric cancer cell line MKN45, was used to analyze the UV detection limit of PCR products for DHPLC and amplified by PCR protocol for 19, 21, 24, 27, 30 cycles at annealing temperature of 53℃ with Pfu polymerase.

Table 1 Primer sequence, PCR Conditions and DHPLC oven temperature used in this study

Fragments	Size (bp)	Primer Sequence	Annealing temp. (℃)	DHPLC temp. (℃)	DHPLC start gradient
A3243G	494	F: cctccctgtaggaaaggaca R: gcctaggttgaggttgacca	59	58	55% B[a]
D-loop F1	505	F: gctggaagatct ttaactccaccattagcacc R: ctacgcgtcgac gcgaggagagtagcactcttg	65	58	58%B
D-loop F2	515	F: gctggaagatct aaatcaatatcccgcacaag R: ctacgcgtcgac ttaagtgctgtggccagaag	65	58	58%B
D-loop F3	494	F: gctggaagatct caccctattaaccactcacg R: ctacgcgtcgac tgagattagtagtatgggag	58	57	58%B
D-loop F4	585	F: gctggaagatct acaaagaaccctaacaccagc R: ctacgcgtcgac acttgggttaatcgtgtgacc	65	58	59%B
16s-F2	292	F: atatagtaccgcaagggaaaga R: gggttctgtgggcaaat	53	56	49%B
16s-G6	268	F: aatagggacctgtatgaatgg R: tagttcgctttgactggtg	53	60	51 %B

[a]Eluent buffer B was 0.1mmol·L^{-1} TAEE, 25% acetonitrile, pH7.0.

The PCR products of mutant fragment (F2) amplified with 19-30 cycles were electrophoresed by 2% agarose gel. The agarose gel was exposed to Koda Image Station 440CF, and the resulting signal qualified by using the Koda 1d Image Analysis software. Values were normalized against pUC18 message. A variant sample which ratio of mutant type (G) to wild type (A) at 3243 allele site was about 60 percent and a normal blood sample were amplified respectively. Both PCR products were mixed according to ratio of mutant: wild type to be 50%, 40%, 30%, 20%, 10%, 5% and 0%.

DHPLC analysis

PCR products from gastric cancer cell lines were mixed with about 30 percent of PCR ingredients of normal blood in order to detect homozygous mutation in cell lines. Prior to DHPLC analysis, all fragments were heated to 95℃ for 3min, followed by slow cooling to 45℃ over 50min to form a mixture of hetero- and homoduplex. The melting temperature and optimal gradient for each fragment (Table 1) can be obtained with WAVEMAKER4.0 software with some empirical optimization. Aliqots of 3μL PCR products were automatically loaded on the DNASep column and eluted on a linear acetonitrile gradient in a 0.1mol·L^{-1} triethylamine acetate buffer (pH 7.0) with a constant flow rate of 0.9mL·min^{-1}. Elution of DNA from the column was detected by absorbing at 260nm.

DNA sequencing

PCR products were purified by 4% PAGE gel. Direct sequencing of the PCR products was performed by use of the fluorescent terminators on an ABI Prism 377 sequencer (PE Biosystems, USA). The sequence data were checked with MITOMAP Human mtDNA "Cambridge" Sequences (http://www.gen.emory.edu/MITOMAP/mitoseq.html).

RESULTS

Optimization of DHPLC for mtDNA mutation detection

The UV detection limit was analyzed for PCR products with variants. The concentration of PCR products of 16s gene containing a variance from gastric cancer cell line MKN45 was about 8, 20, 40, 85 and 110ng·μL^{-1} after amplification with 19, 21, 24, 27, 30 cycles respectively. The UV detection limit was about 80ng for the PCR products whose mutated ingredients were efficiently detected by DHPLC.

The minimal ratio of heteroduplex detected by DHPLC was studied. To investigate the sensitivity of DHPLC in detection of mtDNA variants, we used PCR products from the mutated MELAS sample to mix with normal blood amplimers based on ratio of mutant to wild type to be 50%, 40%, 30%, 20%, 10%, 5%, 1% and 0%. Our results showed that heteroduplexes were sensitively detected by DHPLC, when the ratio (mutant:wild) range from 5% to 50% in the mixture of PCR products (Figure 1). Moreover, the chromatography profiles of DHPLC were similar when mutant products in mixture were about 10-40%. It suggested that homozygous mutation could be easily identified if 20-30% wild PCR products were added.

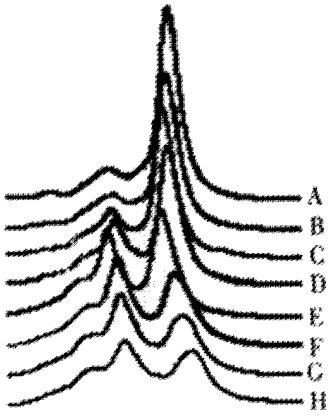

Figure 1 The minimal ratio of hetero-: homoduplex in mixture were identified by DHPLC. The composition of mutant type in PCR product mixture are 0% (A, wild type), 1% (B), 5% (C), 10% (D), 20% (E), 30% (F), 40% (G) and 50% (H) respectively. Chromatogram B has no difference to chromatogram A. Heteroduplex peak start to change in sample containing 5% variant (C). The heteroduplex can be obviously discerned in sample with 10% variant (D). Chromatogram E to H is almost similar. It indicates that about 5% mutant composition in PCR product mixture can be detected by DHPLC.

The effect of DNA polymerases on DHPLC was evaluated. A small peak was often observed preceding the main peak in most of fragments amplified by Taq DNA polymerases. In order to understand whether this was an universality gor DHPLC resolution induced by Taq polymerase, we chose 6 brands of Taq, 1 type of Taq Gold and 4 kinds proofreading DNA polymerases to amplify a randomly selected fragment (F6) for DHPLC analysis. Our findings displayed that all in this study gave a broadened peak proceeding the main peak, but Taq Gold and proofreading DNA polymerases (such as Pfu and Vent) had no or tiny peak before the main peak (Figure 2). To exclude the results that were induced by polymerase buffer, we further amplified the fragment using Taq polymerase to match with Taq Gold and proofreading polymerase buffer, or Taq Gold and proofreading polymerase with Taq polymerase buffer. The results indicated that the particular small peak was only related to Taq DNA polymerase itself.

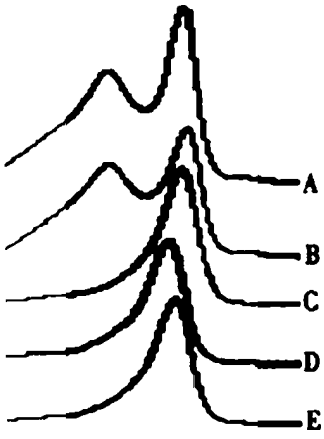

Figure 2 The universality of a broad small peak proceeding the main peak induced by Taq DNA polymerase. An extra small peak is observed preceding the eluted chromatography profile of A and B but not in chromatogram of C, D and E. Chromatogram A is a representative of products amplified by Taq DNA

polymerase. B is typical chromatography profile when Taq DNA polymerase matched with Pfu DNA polymerase buffer in PCR reaction. C and D respectively comes from amplimers amplified by Ampli-Taq Gold and Vent DNA polymerase. E is one of chromatograms coming from PCR amplification by using Pfu DNA polymerase.

Mutation screening of mtDNA

Patients with MELAS have been suggested to associate with mutation of A3243G in $tRNA^{Leu1}$[46,47]. 10 variant cases out of 12 MELAS patients were successfully identified by DHPLC. No alterations were detected in 72 cases with other mitochondrial encephalomyopathies and 30 controls at this allele site. These results were completely consistent with results obtained by restriction endonuclease Apa I.

Basing on above results, we further screened blindly the variants of non-coding D-loop region of mtDNA in gastric cancer using 4 pairs of overlapping primers. In the primary scanning, 28 heteroduplexes were identified in gastric tumor tissues and 38 variances were distinguished in cell lines. The typical chromatograms of heteroduplexes in each fragment were showed in Figure 3. To prove the results, all of positive samples were detected repeatedly. 26 heteroduplexes in tumor group were confirmed, 2 cases were checked to be negative. The two samples were distinguished to be positive due to change of retention time in contrast with other samples, but their chromatograms were similar with wild type. The detected variant frequency of mtDNA by DHPLC was listed in table 2. Ten variant fragments were randomly chosen for direct DNA sequencing, and all of mutated fragments were confirmed.

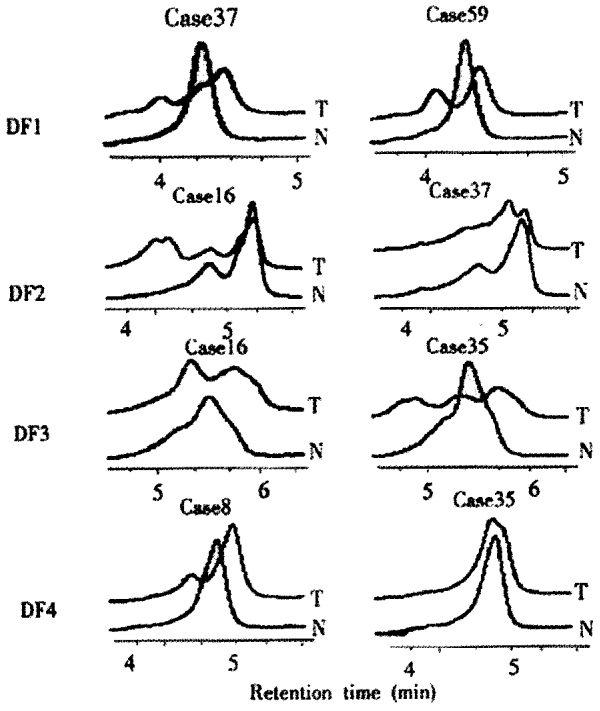

Figure 3 The representative DHPLC chromatograms of variant fragments detected blindly for patients with gastric cancinoma in D-loop of mtDNA. The chromatogram of tumor tissue is visibly different to that of its counterpart non-tumor tissue. The variant cases are listed on the top of each DHPLC chromatogram. DF1, DF2, DF3 and DF4 represent the tested fragments of D-loop respectively. T: tumor tissues; N: non-tumor tissues.

Table 2 The detected frequency of variance of mtDNA by using DHPLC

Fragments / Samples	A3243G			D-loop	
	MELAS	Other Encephal[a]	Normal blood	Tumor	Cell line
Positive / total fragments	10 / 12	0 / 72	0 / 30	26 / 184	38 / 52
Percent (%)	83.3	0	0	14.1	73.1

DISCUSSION

Mutation detection by DHPLC is performed at a temperature sufficient to partially denature the DNA heteroduplexes. The different retention times of hetero- and homoduplex on the DNASep matrix allow for high sensitivity and rapid detection[48,49]. In generally, the heteroduplex profile is easily distinguished from homoduplex peak.

Although the present data have showed that DHPLC is a convenient and sensitive method in mutation screening[17,22,24-26,28]. It may be a challenge to mutation detection of mtDNA because of complicated heteroplasmy in mitochondria[50,51] and various types of variances in mtDNA molecule. Due to hundreds to thousands mtDNA copies in a cell[52], the ratio of a special mutant to normal mtDNA is relatively low. However, a number of various variants may co-existence in a sample. For these reasons, sensitive and rapid detecting method is needed to distinguish different types of variances in large sets of mtDNA copies. We applied DHPLC to screen the mtDNA mutation in this study. Our data showed that DHPLC was a powerful screening strategy for mtDNA mutation. Firstly, about 80ng of PCR products with variant that could be identified by DHPLC were enough to UV detection limit. The heteroduplex peak was not visible when total PCR products were less than 50ng each injection. But the homoduplex peak could be satisfactorily detected. Next, about 5 percent of mutant products in PCR mixture were effectively discerned. These results indicated that DHPLC was sensitive to distinguish those minimal special variants from a large normal mtDNA. Finally, the results were reproducible.

In detection of A3243G mutation for MELAS patients, we proved our collaborators research. Ten variant samples identified by using restriction endonuclease ApaI were all distinguished by DHPLC. Heteroduplexes of tumor tissues obtained by DHPLC were also visibly different to that of non-tumor counterpart, when DHPLC was used to screen blindly the variances of D-loop of mtDNA in gastric cancer. These results also demonstrated that DHPLC, as same as research for nuclear DNA[25-27,29,34], was a sensitive method for mtDNA mutation screening.

As mutation detection of nucleus genes, several factors must be addressed when DHPLC is employed in mtDNA screening. These factors include primer design, PCR optimization, choice of optimal melting temperature or reverse-phase gradient[29,49,53] and DNA polymerase in this study. We find Taq polymerase may influence identification of mutation for DHPLC screening. Taq DNA polymerase tends to add a deoxyribonucleotide, preferentially dATP, to the 3'-hydroxyl terminus of a blunt-ended substrate, and the high-sensitivity of the WAVE system makes it capable of registering these A-tailed products to form an extra peak. In particular, the GC-rich small fragment with high Tm is easy to be influenced. Because the selected temperature of column oven is high, the retention time of heteroduplex will be reduced and the extra small peak is prone to form a false heteroduplex peak.

The concentration of dNTP in PCR reaction is factor of DHPLC resolution for heteroduplex detection. DNA molecules bind with the DNASep cartridge via the triethylammonium acetate (TEAA). The dNTP contends with DNA in interaction with TEAA and decrease DNASep cartridge's binding sites to TEAA-DNA complex. Therefore, ability of cartridge to bind with DNA and resolution of DHPLC will be reduced. In order to obtain improved resolution of DHPLC, it is necessary to elute the cartridge by 75% acetonitrile regularly.

In conclusion, DHPLC is a powerful screening method for mtDNA mutation because of its high-throughput, automation, sensitivity and high reproducibility.

ACKNOWLEDGMENT

We thank Dr. Z.X. Wan and J.J.Zhang for kindly providing of

mtDNA from MELAS and other known mutated samples. We also thank Dr. W.G. Liu, Assistant professor, Mayo Clinic, Rochester USA, for comments on the manuscript. This work was supported by the National Key Basic Science Research Program, No. G1998051203, and National Science Foundation of China (NSFC), No. 39602526.

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Edited by Pagliarini R