Nonradioactive Characterization of Low-Level Heteroplasmic Mitochondrial DNA Mutations by SSCP-PCR Enrichment

BioTechniques 20:430-432 (March 1996)

ABSTRACT

Mitochondrial DNA (mtDNA) mutations have been implicated in an increasing number of human diseases. Many of these mutations are heteroplasmic and are only present at low levels in readily accessible human tissue such as blood. The technique of single-stranded conformational polymorphism (SSCP) allows the detection of mtDNA variants from peripheral blood, but characterization of these variants by automated sequencing is hampered by the low level of heteroplasmy. We have therefore developed a technique for the enrichment of mtDNA mutations that allows reliable sequence data to be obtained even if the variant mtDNA represents only 1% of the total mtDNA. The procedure involves the excision, purification and subsequent PCR amplification of selected DNA fragments from SSCP gels. The techniques can be applied to other heterogeneous mutations such as mosaic mutations in skin biopsies or somatic oncogene mutations in tumor tissue.

INTRODUCTION

A variety of mitochondrial DNA (mtDNA) mutations have been associated with human disease (9). The search for such mutations is confounded by the significant amount of normal polymorphism within mtDNA. However, severely or moderately deleterious mtDNA defects are characteristically heteroplasmic (i.e., both mutant and wild type are present in a single cell), and interest therefore centers on the identification of such defects (6).

The ratio of wild-type to mutant mtDNA can vary considerably from patient to patient and within specific tissues from the same individual (5). In adults, the highest level of mutant mtDNA is usually observed in muscle and brain. Since these tissues are poorly accessible, recent work has focused on the detection of pathogenic mtDNA defects in peripheral blood samples. Unfortunately, the level of heteroplasmy in peripheral leukocytes may be low (<10%), and mutation screening techniques such as restriction fragment length polymorphisms (RFLPs) or direct DNA sequencing may fail to detect the minor mtDNA population. A method is therefore required to enrich heteroplasmic mtDNA variants from peripheral blood samples.

We have previously reported the use of polymerase chain reaction single-stranded conformational polymorphism analysis (PCR-SSCP) for the detection of pathogenic heteroplasmic mtDNA mutations, including those associated with mitochondrial encephalopathies and diabetes (8). The use of precast polyacrylamide gels and a microprocessor-controlled electrophoresis system allows the rapid and reliable detection of low-level heteroplasmic mutations (≥1%) (7). SSCP analysis is not specific for individual mutations, since the same band pattern may be produced by different DNA mutations; sequencing of SSCPs is required. Here we report a nonradioactive method for mutation detection based on SSCP-PCR enrichment followed by automated fluorescence-based DNA sequencing. The technique is based on a method previously reported for germline mutation analysis (1). The unique aspect of this report relates to its application in the characterization of low-level heteroplasmic mutations in mtDNA.

MATERIALS AND METHODS

mtDNA was extracted from peripheral blood samples (using a Nucleon™ DNA extraction kit; Scotlab, Glasgow, Scotland, UK) obtained from two subjects with myoclonic epilepsy and red-ragged fibers (the MERRF syndrome) on muscle biopsy. These subjects had a point mutation in the mitochondrial $tRNA^{lys}$ gene at nucleotide 8344, and the levels of heteroplasmy in peripheral blood samples were 80% and 14% (Figure 1).

A 249-bp region containing this gene was amplified in a 30-cycle PCR, employing a forward primer spanning nucleotides 8196–8215 and a reverse primer spanning nucleotides 8445–8426 (30 s each at 95°, 55° and 72°C and a 10-min final extension step at 72°C).

For SSCP analysis, 2 µL of PCR product were added to 2 µL of distilled water and mixed with 4 µL of denaturing buffer (95% formamide, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heated to 95°C for 5 min and immediately placed in an ice/water bath and maintained at the temperature required for subsequent electrophoresis. Samples were run on the PhastSystem™ (Pharmacia Biotech, Uppsala, Sweden) using 20% precast polyacrylamide gels and native buffer strips at 11°C and 250 V, 2.5 mA, for 550 Vh. Duplicate samples were run on each half of the same gel; only one-half of which was stained using the Pharmacia Biotech silver-staining protocol.

Mutant SSCP bands were visualized on the silver-stained portion of the gels, and corresponding acrylamide slices were taken from the paired, unstained gel half using a sterile scalpel blade (equal-sized slices of acrylamide from adjacent lanes of the same gel that contained no DNA were used as nega-

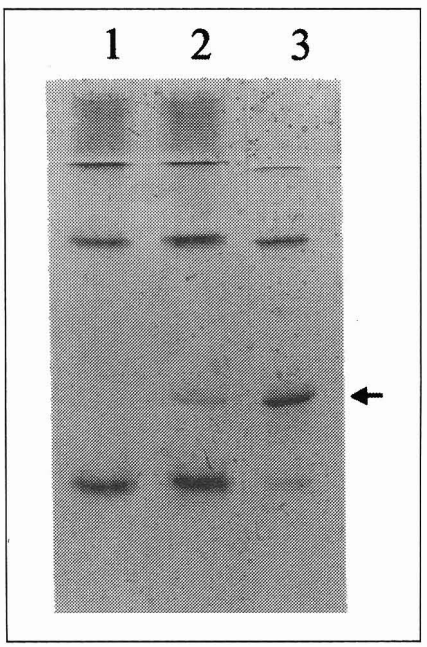

Figure 1. SSCP analysis of the mitochondrial $tRNA^{lys}$ gene using the PhastSystem. Lane 1: wild type. Lanes 2 and 3: MERRF patients 1 and 2 having wild-type to mutant percentage ratios of 14:86 and 80:20, respectively (ratios determined by densitometry). Arrow indicates position of variant SSCP band.

tive controls). Sterile deionized water (25 µL) was added to each acrylamide slice in a sterile tube and incubated for 1 h at 37°C. After centrifugation at 13 000× *g* for 2 min, 5 µL of the supernatant were added to a PCR to give a final volume of 25 µL. The PCR products were purified using the Wizard™ PCR Prep kit (Promega, Southhampton, England, UK) and 300 ng of DNA used in an Applied Biosystems Taq DyeDeoxy™ Terminator Cycle sequencing reaction (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Warrington, England, UK). Samples were run on a Model 373A Automated DNA Sequencing System (PE/ABI).

To assess the sensitivity of SSCP analysis and automated fluorescence-based DNA sequencing in the detection of low-level heteroplasmic mutations, the PCR product obtained from the subject known to have 80% mutation (Figure 1, lane 3) was diluted with various amounts of normal control PCR product (Figure 1, lane 1). Ratios of mutant:normal PCR product ranging from 40% mutant:60% normal to 0% mutant:100% normal were produced (Figure 2). Mutant and normal PCR products were adjusted to the same concentration before mixing.

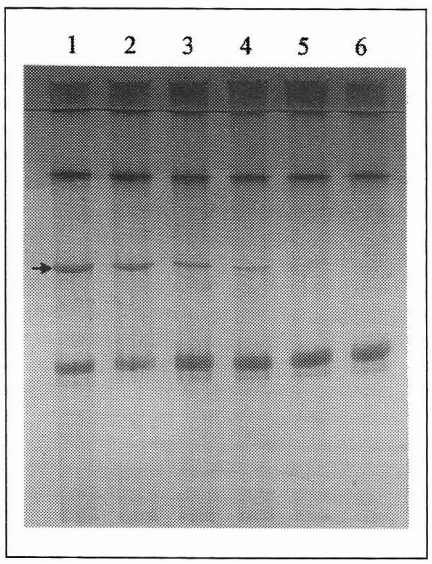

Figure 2. The sensitivity of mitochondrial heteroplasmic mutation detection by SSCP analysis using the PhastSystem and silver staining. PCR products from normal and a known mutation were mixed to give final normal to mutation percentage ratios of lane 1, 60:40, lane 2, 80:20, lane 3, 90:10, lane 4, 95:5, lane 5, 99:1; and lane 6, 100:0. Arrow indicates position of variant SSCP band.

The percentage of heteroplasmy in samples was estimated by scanning the silver-stained SSCP gel (Figure 1) with a Model GS-670 Imaging Densitometer (Bio-Rad, Hemel Hempstead, Herts, England, UK). Data were analyzed using Bio-Rad Molecular Analyst software.

RESULTS AND DISCUSSION

SSCP analysis, using the PhastSystem with silver staining, clearly distinguished the 8344-bp mutation to a level of 5% heteroplasmy. The sample having 1% heteroplasmy was just visible on direct inspection of the gel (Figure 2). This sensitivity is greater than standard PCR-RFLP analysis on ethidium bromide-stained agarose gels, using mismatch primers to create a unique restriction site in mutant PCR products (10), which could only detect the mutation in samples with heteroplasmy >30% (results not shown). The sensitivity of PCR-RFLP analysis can be enhanced, by using radioactive labeling of PCR products, to a level similar to that seen in our silver-stained PhastGels® (3).

Direct automated sequencing of the heteroplasmic samples only demonstrated the A to G base substitution in the samples with a level of heteroplasmy >20%. Generally, base substitution peaks in samples with between 20% and 30% mutant present could not be clearly distinguished above the level of background. However, a small underlying G peak could have been identified in a patient sample with 14% mutant present (Figure 3B). A comparable level on the limit of detection, between 10% and 25%, was also observed during the sequence analysis of heterogeneous viral populations (4).

The level of background, seen as secondary peaks, and the variable peak intensities characteristic of automated fluorescence-based DNA sequencing of PCR products make it almost impossible to detect unknown heteroplasmic mutations (Figure 3). Despite this, we and others (2) have found that the patterns of the sequencing traces in different samples are faithfully reproduced every time the same gene region

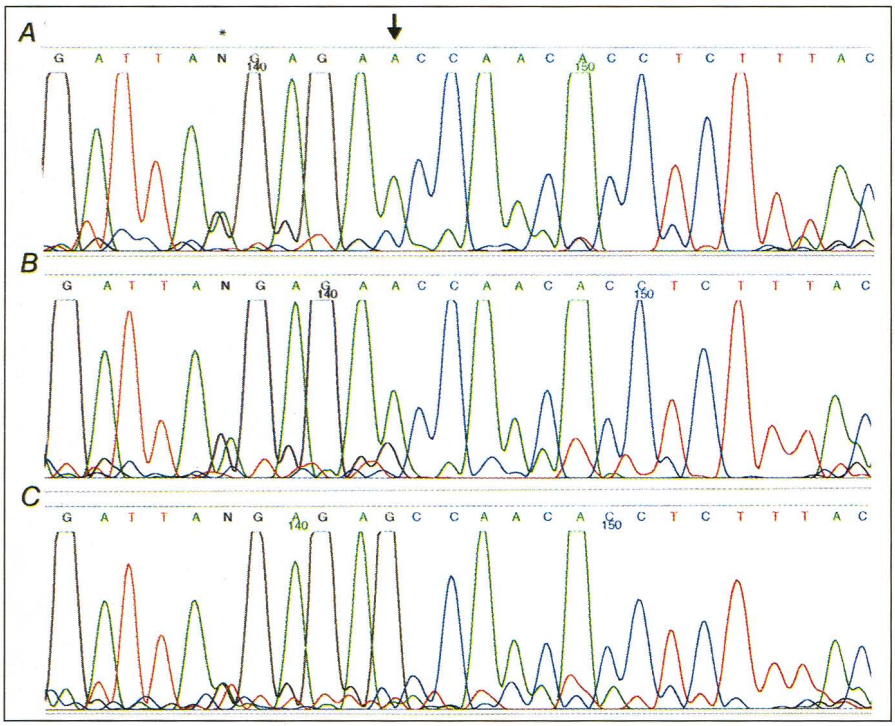

Figure 3. Comparison of sequence traces derived from a portion of the mtDNA $tRNA^{lys}$ gene. (A) Wild-type sequence; (B) MERRF sample (patient 1, Figure 1, lane 2) having 14% heteroplasmy for the A to G base substitution at nucleotide 8344; and (C) sequence of the same MERRF sample after SSCP-PCR enrichment of the mutation. Arrow indicates the position of the A to G base substitution at nucleotide 8344. [Note that in (B) the mutant G peak is lower than the A peak and cannot be distinguished from baseline background. After SSCP-PCR enrichment of the mutant SSCP band from the gel, the A to G transition can be clearly seen (C)]. Asterisk indicates an ambiguous 'N' that is called in all sequences of this DNA region. Although this was spurious, it serves to emphasize the reproducibility of the cycle reaction of double-stranded PCR products using dye-labeled chain terminators.

is examined (Figure 3).

Excision and re-amplification by PCR of the mutant SSCP band from the patient sample with 14% heteroplasmy (Figure 1, lane 2) enabled the A to G base substitution to be clearly seen on the automated sequence data as a novel peak with no evidence of a secondary signal (Figure 3C). In fact, as long as the variant SSCP band was just visible on the silver-stained gel, it was possible to excise, re-amplify and obtain good sequence data for the sample. This procedure was successful up to the limit of 1% heteroplasmy.

The SSCP-PCR enrichment process that we describe will be of significant value for the detection of other known heteroplasmic mtDNA mutations, especially those occurring at low levels in peripheral blood samples, e.g., the 3243-bp mtDNA mutation associated with diabetes and deafness. As the entire mitochondrial sequence is known, it will be possible to use this procedure to screen for novel heteroplasmic mutations in other mtDNA genes. The technique could also be adapted to identify other heterogeneous mutations in chromosomal genes, e.g., mosaic mutations from skin biopsies or oncogene mutations in mixed populations of normal and cancer cells. Such defects can be present in very small amounts, and automated sequencing may fail to identify low-level heterozygous base substitutions or deletions.

In conclusion, the method of SSCP-PCR enrichment of low-level heteroplasmic mutations will enable the identification and characterization of novel and potentially pathogenic mtDNA defects.

REFERENCES

1. **Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens et al.** 1991. Identification and characterisation of the familial adenomatous polyposis coli gene. Cell *66*:589-600.
2. **Kwok, P-Y., C. Carlson, T.D. Yager, W. Ankener and D.A. Nickerson.** 1994. Comparative analysis of human DNA variations by fluorescence-based sequencing of PCR products. Genomics *23*:138-144.
3. **Larsson, N-G., M. Tulinius, E. Holme, A. Oldfors, O. Andersen, J. Wahlstrom and J. Aasly.** 1992. Segregation and manifestations of the mtDNA $tRNA^{Lys}$ A to $G^{(8344)}$ mutation of myoclonus epilepsy and ragged-red fibres (MERRF) syndrome. Am. J. Hum. Genet. *51*:1201-1212.
4. **Leitner, T., E. Halapi, G. Scarlatti, P. Rossi, J. Albert, E.-M. Fenyo and M. Uhlen.** 1993. Analysis of heterogeneous viral populations by direct DNA sequencing. BioTechniques *15*:120-127.
5. **Lertrit, P., A.S. Noer., E. Byrne and S. Marzuki.** 1992. Tissue segregation of a heteroplasmic mtDNA mutation in MERRF (myoclonic epilepsy with ragged red fibres) encephalomyopathy. Human Genet. *90*:251-254.
6. **Marzuki, S., P. Lertrit, A.S. Noer, R.M.I. Kapsa, H. Sudoyo, E. Byrne and D. Thyagarajan.** 1992. The need for a joint effort in the construction of a reference data base for normal sequence variants of human mtDNA. Am. J. Hum. Genet. *50*:1337-1340.
7. **Thomas, A.W., R. Morgan, A. Majid, A. Rees and J.C. Alcolado.** 1995. Detection of mitochondrial DNA mutations in patients with diabetes mellitus. Diabetologia *38*:376-377.
8. **Thomas, A.W., R. Morgan, M.G. Sweeney, A. Rees and J. Alcolado.** 1994. The detection of mitochondrial DNA mutations using single stranded conformational polymorphism (SSCP) analysis and heteroduplex analysis. Human Genet. *94*:621-623.
9. **Wallace, D.C.** 1992. Diseases of mitochondrial DNA. Annu. Rev. Biochem. *61*:1175-1212.
10. **Zeviani, M., P. Amati, N. Bresolin, C. Antozzi, G. Piccolo, A. Toscano and S. DiDonato.** 1991. Rapid detection of the A to G $^{(8344)}$ mutation of mtDNA in Italian families with myoclonus epilepsy and ragged-red fibres (MERRF). Am. J. Hum. Genet. *48*:203-211.

We thank Joyce Hoy for technical assistance with the automated DNA sequencing. Patient samples were kindly provided by Professor A.E. Harding, Neurology Institute, Queen's Square, London. This work was funded with a grant from the British Diabetic Association. Address correspondence to John C. Alcolado, Dept. of Medicine, Univ. of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales, UK.

Received 30 May 1995; accepted 26 September 1995.

Emma J. Sherratt, Andrew W. Thomas, James W. Gagg and John C. Alcolado
University of Wales College of Medicine, Heath Park
Cardiff, Wales, UK