

Short report

A unique junctional palindromic sequence in mitochondrial DNA from a patient with progressive external ophthalmoplegia

T Saiwaki, K Shiga, R Fukuyama, Y Tsutsumi, S Fushiki

Abstract

A polymerase chain reaction (PCR) based procedure was modified to determine the deletion of mitochondrial DNA (mtDNA). The protocol consists of coamplification both of deleted and wild-type segments of mtDNA using a long PCR technique; evaluation of the deleted portion within the amplified DNA segments by restriction enzyme digestion followed by densitometrical analysis; and direct subcloning into a plasmid vector for DNA sequencing. The procedure revealed a 5.3 kb deletion of mtDNA in the biopsied muscle tissue obtained from a patient clinically diagnosed with progressive external ophthalmoplegia. The 5' and 3' sequences at both sides of the breakpoint comprise a 17 bp palindrome and 5 bp tandem repeats, suggesting that the deletion might occur through slipped mispairing and other novel mechanisms. This improved procedure has the potential to detect deletions occurring in the entire length of mtDNA, and might be useful for clinical screening of progressive external ophthalmoplegia.

(*J Clin Pathol: Mol Pathol* 2000;53:333–335)

Keywords: progressive external ophthalmoplegia; mitochondria; diagnosis; polymerase chain reaction

Progressive external ophthalmoplegia is a mitochondrial disease that affects external ocular and skeletal muscles.¹ Deletions of mitochondrial DNA (mtDNA), a few bases to 10 kb in size, were detected in more than 70% of patients with progressive external ophthalmoplegia.² MtDNA deletions were also found in other mitochondrial diseases,^{3–4} as well as in aged patients and those with Alzheimer's disease.⁵ Although the mechanisms underlying these deletions are not completely understood, their preferential occurrence at the sites flanking direct repeats implies that slipped mispairing might play a role in the deletion event.^{4–6}

Goto and colleagues⁷ reported a polymerase chain reaction (PCR) based protocol that is combined with restriction enzyme digestion. That technique is based on the mobility shift of digested bands in the gel; however, if the deletion generates a digested DNA band that

comigrates with any DNA band observed in the control samples the diagnosis becomes misleading. Therefore, we modified Goto's procedure, thereby enabling us to determine the size of a deletion precisely. Using this procedure, we analysed the junction sequence in the deleted mtDNA of one patient with progressive external ophthalmoplegia.

Materials and methods

A 54 year old woman had manifested a right ptosis since she was 18 years old. The weakness of her proximal extremities gradually developed. The neurological examination showed the right ptosis, external ophthalmoplegia, and mild weakness in facial muscles and proximal extremities. A histopathological examination on the biopsied right biceps muscle demonstrated ragged red fibres, suggesting that she is affected with progressive external ophthalmoplegia. The patient's and other DNA samples were extracted from biopsied muscle tissues by a standard procedure after informed consent, and these samples were subjected to PCR using two different primer sets—P1001/P1004 and P1002/P1003—according to Goto *et al.*⁷ These primer sets amplify a 11.2 kb (long (L); nt 5205–16 425) and 5.3 kb (short (S); nt 16 436–5204) fragment, respectively. Modified PCR was carried out as follows: the PCR mixture consisted of 0.5 U of Taq DNA polymerase (Takara Ex Taq; Takara, Kyoto, Japan), 2.5 mM MgCl₂, 0.4 mM of each dNTP, and 0.2 mM of each primer; the PCR conditions comprised an incubation at 94°C for five minutes for pre-reaction (hot start), followed by 22 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for eight minutes, with a final additional extension at 72°C for 10 minutes. We concluded that a concentration of approximately 2.5 ng/ml target DNA was optimal. PCR products were then electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed. We successfully amplified L (11.2 and 5.3 kb) and S (5.3 kb) fragments by applying our modified PCR procedure (data not shown) to mtDNA isolated from biopsied muscle tissues. We purified two L segment bands from the gel using one step spin columns (GenElute™ agarose spin columns; Supelco, Bellefonte, Pennsylvania,

Department of Pathology and Applied Neurobiology, Research Institute for Neurological Diseases and Geriatrics, Kyoto Prefectural University of Medicine, 465 Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602–8566, Japan
T Saiwaki
R Fukuyama
Y Tsutsumi
S Fushiki

Department of Neurology and Gerontology, Research Institute for Neurological Diseases and Geriatrics, Kyoto Prefectural University of Medicine
K Shiga

Correspondence to:
Dr Fushiki
sfushiki@koto.kpu-m.ac.jp

Accepted for publication
11 April 2000

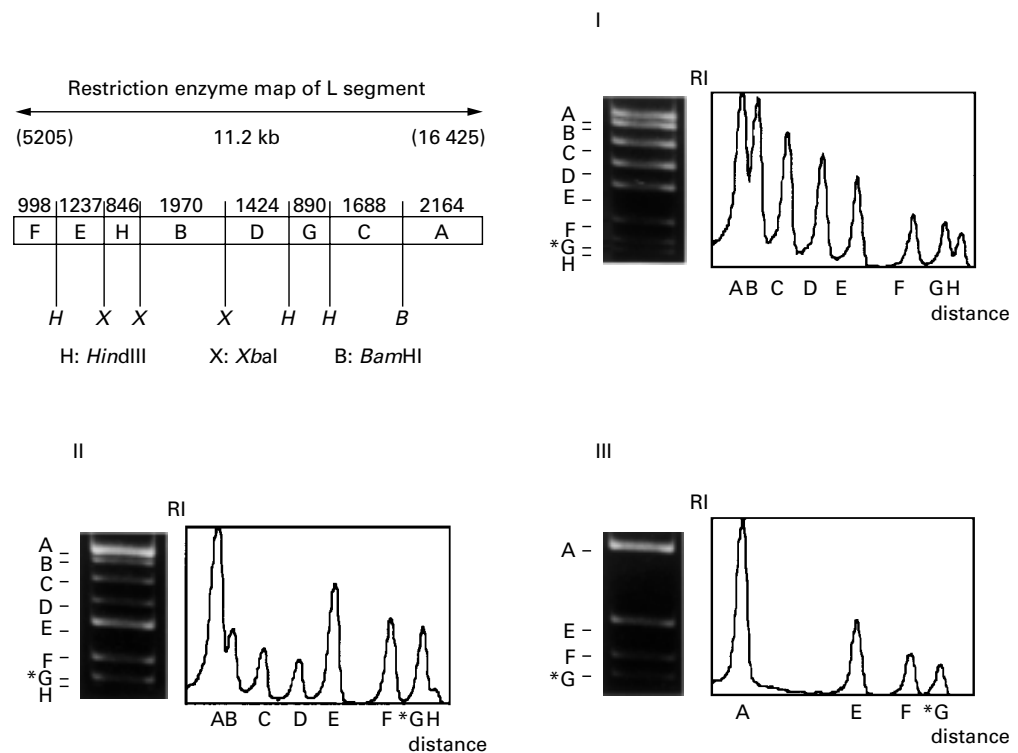


Figure 1 Electrophoretic patterns of the digested bands (photographs at the left side of each graph) of the L segment and its densitometrical analysis (graphs at the right side to the photographs). I, PCR product of an unaffected subject; II, PCR product of the patient with progressive external ophthalmoplegia; III, a purified *5.3 kb segment from the patient with progressive external ophthalmoplegia. Bands A through H correspond to the fragments within the L segment of mitochondrial DNA (left upper panel). *G in panels II and III indicates the DNA segment in question. RI, relative intensity. Distance, migrating distance of DNA segments in gel.

USA), and digested them with a combination of three restriction enzymes: BamHI, XbaI, and HindIII. Electrophoretograms were analysed densitometrically using an NIH image program (Wayne Rasband, NIH image, version 1.60). We revealed that another S fragment could also be analysed with the same enzyme sets. We subcloned the 5.3 kb fragment, which is probably formed de novo by the deletion (see below), into the BamHI/XbaI site of a cloning vector (pBluescript SK; Toyobo, Osaka, Japan) for sequencing. The plasmid was purified with a kit (QIAprep spin miniprep kit; Qiagen GmbH, Hilden, Germany) and subjected to sequencing using the ALF DNA sequencer according to the manufacturer's instructions

(Pharmacia, Uppsala, Sweden). Sequences were compared with the reported human mtDNA sequence (GenBank, J0145)⁸ using DNASIS software (version 3.6; Hitachi, Tokyo, Japan). As controls, we also analysed DNA samples isolated from the muscle tissue of two patients with polymyositis, and blood DNA samples from three healthy subjects and nine patients with neurological disorders.

Results and discussion

Eight consecutive bands were seen in the gel after amplified DNA was digested with the three restriction enzymes (fig 1). Densitometric scanning of the electrophoretogram of unaffected subjects showed a gradual decrease of the eight peaks, from A through H (fig 1, panel I), whereas that of the patient showed a unique densitometrical pattern (fig 1, panel II). The intensities of the four bands B, C, D, and H were weaker than the normal control, suggesting that these bands are deleted (fig 1, panel II). The 5.3 kb band was purified from the gel, digested with enzymes, and analysed densitometrically. The result indicated that the bands B, C, D, and H were missing (fig 1, panel III), whereas the band G was not (fig 1, panel II). Enzyme digestion of the S segments of the unaffected subjects yielded three fragments, of 1328, 1760, and 2250 bp in size, and these bands appeared to be the same size as the bands from the patient's S segment (data not shown).

The band *G was subcloned and sequenced. Figure 2 demonstrates the position of the

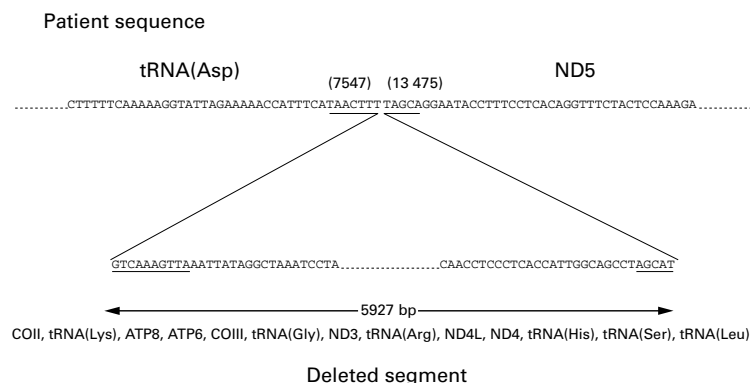


Figure 2 Possible location of the breakpoint (nt 7547–13 475), length of the deleted segment (5927 bp), sequences flanking the breakpoint, and genes encoded within the deletion. Underlined nucleotides at nt 7547 and 13 475 indicate the 17 bp palindrome and 5 bp tandem repeats.

breakpoint (7458 and 13 427)⁸, the size of the deleted segment (5927 bp), and the sequences flanking the breakpoint. Sequences of 7458 and 13 427 bp in size comprise a 17 bp palindrome (TAACTTTGTCAAAGTTA) and 5 bp tandem repeats with the insertion of a T inbetween the repeat (TAGCATTAGCA) (fig 2). As a result, portions of the genes encoding transfer RNA (tRNA(Asp)) and ND5, and the entire genes for COI, COII, ATPase 6 and 8, COIII, ND3, ND4L, and ND4 are deleted in the mtDNA of this patient (fig 2; tRNA to ND5). All healthy and diseased controls subjects showed no evidence of mtDNA deletion.

With our simplified procedure we were able to determine a deletion of mtDNA from a patient with progressive external ophthalmoplegia who started to show the disease phenotype at an early age. Several procedures have been used previously to determine deletions in mtDNA.⁴ Goto and colleagues⁷ reported the use of a PCR technique to amplify two portions of mtDNA—an 11.2 kb segment L (nt 5205–16 425) and a 5.3 kb segment S (nt 16 436–5204), which cover the entire length of mtDNA, with the exception of 11 bp within the D loop region, in combination with restriction enzyme digestion. In theory, if there is a deletion in the L segment, after the restriction enzyme digestion, several normal bands within the deleted region should disappear and an extra band, consisting of the 5' and 3' flanking remnants, should appear on the gel. When this band occasionally migrates to a position similar to one of the normal bands, it is impossible to distinguish the deletion or the breakpoint position and sequence. Densitometric analysis of the electrophoretograms is an alternative way to overcome this problem because the staining intensity of a particular band stained with ethidium bromide in an agarose gel is proportional to its amount. In fact, the eight bands derived from the wild-type 11.2 kb DNA showed a gradual decrement in terms of the peak height from A through to H, whereas the DNA from the patient showed low peaks of bands B, C, D and H, indicating that these bands are deleted. Because the G segment is located inbetween the C and D segments (fig 1), the band pattern in this patient implies two possibilities: (1) the BamHI/XbaI digestion of the L segment gave rise to a new band (*G in fig 1), which comigrates with the normal band G; or (2) the G segment might be spared from the deletion. We subcloned the band *G of the patient into a pBluescript vector whose multiple cloning sites include those for the restriction enzymes used in our study. We were able to determine the sequences flanking both deletion junctions, and concluded that the apparent band G derived from a deleted mtDNA is a

newly formed segment, and does not result from a skipped deletion. By evaluating the digestion pattern with the same three restriction enzymes, we also confirmed that the other 5.3 kb S segment is not deleted.

The upstream deletion breakpoint is located at the middle of the 17 bp palindromic sequence and this sequence is not identical to the 13 bp direct repeats.^{4,9} The palindromic sequence has not been reported before, but it is not clear how this unique sequence plays a role in the deletion event. By contrast, the downstream deleted sequence is half of a 5 bp tandem repeat and this is exactly the same sequence as that reported by Hammans *et al.*⁹ A tandem repeat has been assumed to cause a microdeletion through a skipped mispairing mechanism.^{4,6} Furthermore, the deleted segment that we found in our study includes both the reported AT rich and polypyrimidine stretches that might give rise to a unique three dimensional structure.⁴ These upstream and downstream sequences within the mtDNA probably predispose this part of the DNA to the deletion event. Progressive external ophthalmoplegia is caused not only by deletions in mtDNA,² but also by point mutations at nt 3243 and other positions.¹⁰ However, because more than 70% of patients with progressive external ophthalmoplegia possess mtDNA deletions,² our modified procedure would facilitate the clinical diagnosis of progressive external ophthalmoplegia.

We gratefully acknowledge Drs Makino, Nagakane, Ohshima, and Nakajima for introducing the patient with progressive external ophthalmoplegia for our study.

- Wallace DC. Diseases of the mitochondrial DNA. *Annu Rev Biochem* 1992;**61**:1175–212.
- Goto Y, Koga Y, Horai S, *et al.* Chronic progressive external ophthalmoplegia: a correlative study of mitochondrial DNA deletion and their phenotypic expression in muscle biopsies. *J Neurol Sci* 1990;**100**:63–9.
- Blumenthal DT, Shanske S, Schochet SS, *et al.* Myoclonus epilepsy with ragged red fibers and multiple mtDNA deletions. *Neurology* 1998;**50**:524–5.
- Schon EA, Rizzuto R, Moraes CT, *et al.* A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. *Science* 1989;**244**:346–9.
- Corral-Debrinski M, Horton T, Lott MT, *et al.* Marked changes in mitochondrial DNA deletions in Alzheimer brains. *Genomics* 1994;**23**:471–6.
- Shoffner JM, Lott MT, Voljavec AS, *et al.* Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. *Proc Natl Acad Sci U S A* 1989;**86**:7952–6.
- Goto Y, Nishino I, Horai S, *et al.* Deletion of DNA fragments encompassing the deletion junction of mitochondrial genome. *Biochem Biophys Res Commun* 1996;**222**:215–19.
- Anderson S, Bankier AT, Barrell BG, *et al.* Sequence and organization of the human mitochondrial genome. *Nature* 1981;**290**:457–65.
- Hammans SR, Sweeney MG, Holt IJ, *et al.* Evidence for intramitochondrial complementation between deleted and normal mitochondrial DNA in some patients with mitochondrial myopathy. *J Neurol Sci* 1992;**107**:87–92.
- Laforêt P, Lombès A, Eymard B, *et al.* Chronic progressive external ophthalmoplegia with ragged-red fibers: clinical, morphological and genetic investigations in 43 patients. *Neuromusc Disord* 1995;**5**:399–413.



A unique junctional palindromic sequence in mitochondrial DNA from a patient with progressive external ophthalmoplegia

T Saiwaki, K Shiga, R Fukuyama, et al.

Mol Pathol 2000 53: 333-335

doi: 10.1136/mp.53.6.333

Updated information and services can be found at:

<http://mp.bmj.com/content/53/6/333.full.html>

These include:

References

This article cites 10 articles, 3 of which can be accessed free at:

<http://mp.bmj.com/content/53/6/333.full.html#ref-list-1>

Article cited in:

<http://mp.bmj.com/content/53/6/333.full.html#related-urls>

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:

<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:

<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:

<http://group.bmj.com/subscribe/>