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Identification of Large Deletion of Mitochondrial DNA in *Kearns-Sayre Syndrome* (KSS)

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Abstract Large-scale deletions of mitochondrial DNA (mtDNA) have been documented in patients with mitochondrial myopathies and seem to be especially frequent in patients with *Kearns-Sayre syndrome* (KSS). About one third of all patients shows a 4,977 bp deletion, known as the "*common deletion*", that removes a segment of DNA that includes several genes encoding for respiratory chain subunits. In this disorder, the population of deleted mtDNA molecules coexists with a population of normal, wild-type full length mtDNAs, a situation known as *heteroplasmy*. We have performed *polymerase chain reaction* (PCR) on paraffin-embedded muscle tissues from two korean KSS patients. The PCR analysis revealed the existence of two amplified fragments, the deleted fragment of 123 bp characteristic for common deletion and the wild-type fragment of 152 bp.

Key words: mitochondrial DNA (mtDNA), PCR, *Kearns-Sayre syndrome* (KSS)

Introduction

Human mtDNA is a double-stranded circle of 16,569 base pairs (bp) containing 37 genes (Fig.1). Of these, 13 encode subunits of various complexes of the respiratory chain: seven subunits of complex I, ond subuint of complex III, three subuints of complex IV, and two subunits of complex V. The remaining 24 genes are required for translation inside of the mitochondrion. Two genes specify ribosomal RNAs (12S and 16S rRNAs), and 22 genes specify transfer RNAs (tRNAs): these are required for incorporation of amino acids into the growing peptide chain as the mtDNA-encoded mRNAs are translated on mitochondrial rebosomes. The tRNA genes are strategically interspersed between the rRNAs and structural genes, and they play an important role in the precise transcription of tRNAs, rRNAs, and mRNAs

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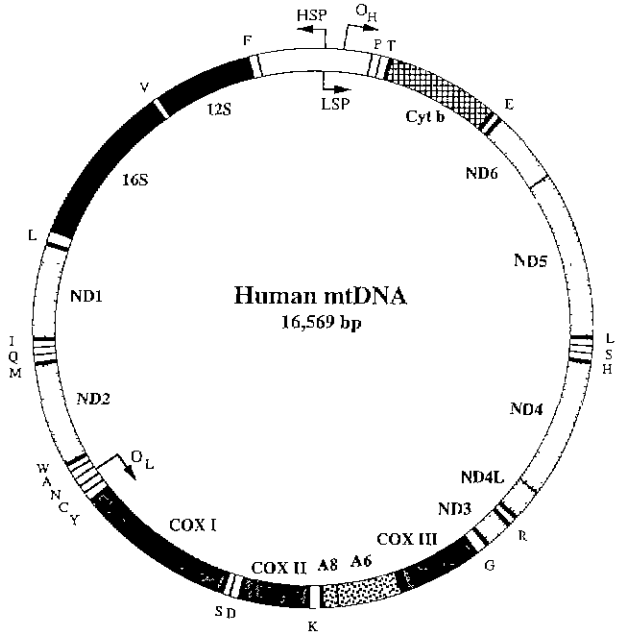

Fig. 1. The human mitochondrial genome. Shown are the structural genes for the 12S and 16S ribosomal RNAs, the subunits of complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), complex III (Cyt b), complex IV (COX I, COX II, and COX III), and complex V (A6 and A8), and 22 tRNAs (1-letter amino acid nomenclature). The origins of heavy-strand (O_H) and light-strand (O_L) replication, and of the promoters for initiation of transcription from the heavy-strand (HSP) and light-strand (LSP), are shown by arrows.

[6,9]. Three clinical presentations, *Kearns-Sayre syndrome* (KSS), *Chronic Progressive External Ophthalmoplegia* (CPEO) and *Pearson's pancreas-bone marrow syndrome*, have been associated with deleted mtDNAs in muscle and other tissues. Among them, KSS is commonly defined as a multisystem disorder with *progressive external ophthalmoplegia, pigmentary retinopathy*, onset before age 20 and other systemic signs and symptoms. Muscle morphology is characterized by "*ragged red fibers*" that are a hallmark of mitochondrial

proliferation. Biochemically, this syndrome often shows reduced respiratory chain enzyme activity, particularly of cytochrome c oxidase [12]. Almost all patients with KSS were found to harbor deletions of mtDNA that differ in size, location and relative amount among patients. Approximately one third of the deletions described for KSS seem to be identical, extending over 4.9 kb within the region 8470-13460 base pairs. The deletion junction has been sequenced and found to be bridged by direct repeats within the regions 8470-8482 in the *ATPase 8* gene and 13447-13459 in the *NAD5* gene. This kind of deletion is known as the "common deletion", due to the frequency with which it occurs [7]. In this disorder, the population of deleted mtDNA molecules coexists with a population of normal, wild-type full length mtDNAs, a situation known as "heteroplasmy". In an attempt to study the molecular nature of korean KSS patients, we did PCR (*polymerase chain reaction*) on DNAs from the paraffin-embedded tissues of two KSS patients.

Materials and Methods

Pathological analysis

The diagnosis of KSS patients was made by clinical presentations, neurological examinations combined with enzyme histochemistry and characteristic electron microscopic findings (data not shown)

DNA extraction from paraffin-embedded muscle tissues

Total DNA was extracted from paraffin blocks with xylene by a modified method of Love et. al. (1993). Small amounts of tissues were cut from paraffin blocks using a sterile scalpel blade and incubated with 400 μl xylene and 500 μl mineral oil for 2 h at room temperature under gentle agitation. After centrifugation (5 min, 12,000 × *g*), the paraffin-containing supernatant was discarded, and the extraction was repeated with 400 μl xylene. In order to remove remaining xylene, the pellet was extracted with 400 μl ethanol. After solubilization in 140 μl PCR-TE buffer (10 mM EDTA, 200 mM Tris-HCl, pH 8.0), 20 μl 10% SDS and 80 μl 1% proteinase K were added and incubated overnight at 55℃. DNA was further purified by phenol/chloroform extraction, and the resulting pellets were resuspended in 50 μl PCR-TE buffer and used for PCR.

Preparation of control DNA

A plasmid standard DNA (pCII) for the common deletion of mtDNA was a gift of Prof. Eric A. Schon, Columbia University, New York, containing a 505 bp PCR-amplified mtDNA fragment, lacking the 4,977 bp common-deletion, as shown schematically in Fig. 2. A control DNA (for wild-type mtDNA) was extracted according to the method of Tengan and Moraes (1996) from a postmortem skeletal muscle sample of a 79-year-old healthy individual.

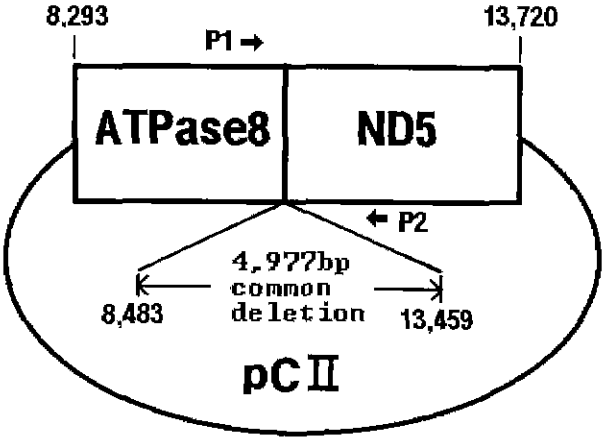

Fig. 2. Schematic representation of the control plasmid DNA (pCII). The shaded boxes denote a 505 bp PCR-amplified mtDNA fragment with nucleotide numbers denoting the first and the last nucleotide present in this fragment, lacking the 4,977 bp "common deletion". *ATPase 8* denotes the gene for ATP synthase, and *ND5* for the subunit of NADH-coenzyme Q reductase. P1 and P2 denote the primers and these primers will amplify only a 123 bp fragment characteristic for common deletion. The remaining circle denotes the DNA fragment of pUC 19 plasmid.

Polymerase chain reaction (PCR)

PCR amplifications were carried out in a volume of 50 μl using 20 μM of each dNTP, 0.5 μl *Vent* polymerase (2,000 units/ml, New England Biolabs), 2 mM $MgSO_4$ and 30 pM of each primer overlaid with 35 μl wax. After a single predenaturation step (5 min, at 95℃), PCR was done by 1 min denaturation at 94℃, 1 min annealing at 56℃, and 1 min elongation at 72℃ for 30 cycles in a Perkin-Elmer PCR-Thermal Cycler. PCR products were electrophoresed through a 2% analytical agarose gel (FMC, USA) and the amplified fragments were visualized by ethidium bromide staining.

Results and Discussion

To detect and distinguish both deleted-mtDNA and wt-mtDNA in KSS patients, we used the following three primers : Forward primer P1 (nt.8421-8440, 5'-CACTATTCCTCA TCACCCAA-3'), Reverse primer P2 (nt.13520-13501, 5'-ATGTGGTCTTTGGAGTAGAA-3') and Reverse ptimer P3 (nt.8573-8557, 5'-CCTAGGATTGTGGGGGC-3') (numbering of mtDNA sequence according to Anderson *et al.*, 1981). As shown schematically in Fig. 3 (A,B), P1 and P2 flank the deleted region, whereas P3 corresponds to a mtDNA region inside the deletion. Therefore the primer set, P1 and P2 only amplify deleted-mtDNAs and the other set, P1 and P3 only amplify wt-mtDNAs. First we did PCR with the primer P1 and P2 to detect the common deletion (deleted-mtDNA molecules). Generally the Southern blot analysis

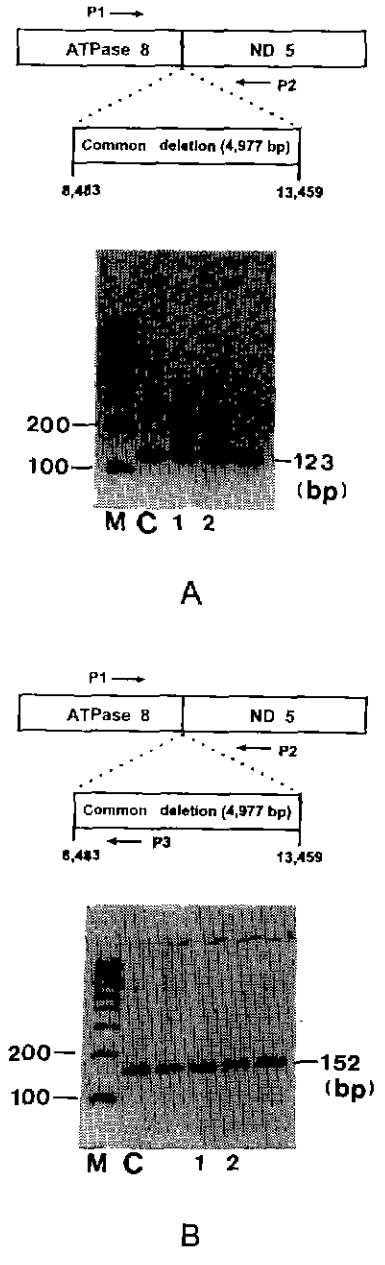

Fig. 3 (A,B) Schematic presentation of PCR stratagy and agarose gel electrophoretic analysis of PCR products. P1, P2 and P3 denote PCR primers used in this study. The shaded upper boxes in A and B denote the linearized mitochondrial DNA fragments, showing the genes *ATPase 8* and *ND5*. The intervening 4,977 bp mtDNA fragment found in normal wild-type mtDNA is shown as shaded lower box with nucleotide numbers denoting the first and the last nucleotide present in the deleted fragment (in upper parts of A and B). In the lower parts, the 2 % agarose gel electrophoretic analysis is shown. Lane M indicates the 100bp-ladder M.W. marker DNA; lane C (in A), PCR products of control plasmid DNA (for common deletion) and lane C (in B), PCR products of control DNA (for wild-type). Lane 1 and 2 (in A) indicate the 123bp-amplified PCR products (common deletion) of two patients, respectively, and lane 1 and 2 (in B), 152bp-PCR products (wild-type) of these patients.

is done first to study large deletion of mtDNA, and then PCR follows, but we did PCR on the assumption that our patients have common deletion. After PCR, the 10 ul of each sample was electrophoresed in a 2 % agarose gel and we could find the 123 bp fragments characteristic of the common deletion in all of two patients (in lane 1 and 2 in Fig. 3-A). In the case of the plasmid standard (C), also the 123 bp fragment is shown, since this plasmid contains a short 505 bp mtDNA fragment, lacking the 4,977 bp "common deletion". This result is in accordance with the suggestion that most patients fulfilling the clinical and morphological criteria of KSS will have deletions in mtDNA, especially "common deletion" in this study. Taken together with the previous studies in other laboratories, this result indicates that this 123 bp fragment is a deletion junction fragment harbouring an authentic 4,977 bp "common deletion" [2,7,13]. The distance between the two primers P1 and P2, approximately 5.1 kb, is too long to be amplified under our PCR conditions. With the primer P1 and P3, PCR was done further to detect wt-mtDNA molecules in these patients. Expectedly, we could find the 152 bp fragments characteristic of wt-mtDNA in all of patients (in lane 1 and 2 in Fig.3-B), since the primer set, P1 and P3 only amplify wt-mtDNAs. Taken together, our PCR results indicate that the wt-mtDNAs and deleted-mtDNAs (common deletion) coexist in these two KSS patients. Therefore to detect and distinguish both deleted-mtDNA and wt-mtDNA simultaneously and also, in order to ascertain that an amplified fragment was not due to artefact of PCR (i.e., misannealing of primers, or non-specific amplification), we are currently doing the so called "*3-primer PCR*", in which we use the pimers P1, P2 and P3 simultaneously. The second interesting finding in this study was that we could identify deletion by PCR in DNAs extracted from formalin-fixed, paraffin-embedded muscle tissues, although PCR inhibitors may be present in our DNA. The existence of an as-yet-unidentified PCR inhibitor which is intrinsic to paraffin-embedded tissue has been inferred from the results of many studies [4,5] It was reported that although PCR may be successfully performed directly on pieces of tissue simply scraped off a paraffin block or a stained histological tissue section, only standardized DNA extraction allows reproducible quantitative preparation of DNA and thus "diluting out" of PCR inhibitors. In our hands, the tissues were extracted twice with xylene to remove paraffin and further, with ethanol to remove remaining xylene before phenol/chloroform extraction. Most laboratories woking on mitochondrial disease prefer to use fresh frozen tissues than paraffin blocks, because the DNA in paraffin blocks is partially degraded into smaller fragments due to the extensive and possibly deleterious interaction between fixative and nucleic acids. Therefore, determination of mutations of mtDNA in paraffin-embedded tissues by PCR-based methods is possible, if the amplified fragments are small. In our study,

we could amplify the size of 123 and 152 bp. Further systematic analysis is necessary to identify the PCR inhibitors present in paraffin-embedded tissues which can adversely affect amplification. Our results is in accordance with the findings that PCR allows detailed genetic analysis of formalin-fixed paraffin-sections and the archieves of paraffin-embedded tissue stored in most histopathology departments represent a valuable repository of genetic material for future investigation [4,5]. Experimental evidence suggests that mtDNA deletions impair oxidative phosphorylation by preventing translation of mtDNA-encoded polypeptides [10]. Because deleted mitochondrial genomes coexist with wild-type ones, the percentage of deleted-mtDNAs have to reach a certain threshold before ATP production is impaired and a cellular phenotype is observed. Therefore, we need to establish the threshold necessary for the deleted-mtDNAs to trigger a biochemical dysfunction in muscle [6]. It is also noteworthy to mention that De Coo *et.al* (1997) could do a PCR test for KSS on DNA from blood samples. They concluded that a high percentage of diagnostic questions regarding mtDNA deletion syndromes could be answered by performing a simple PCR test on blood DNA, thereby saving the patient a muscle biopsy.

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