Mitochondrial DNA

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SHORT COMMUNICATION

Mitochondrial DNA deletions detected by Multiplex Ligation-dependent Probe Amplification

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Abstract

The genetic diagnosis algorithm for mitochondrial (mt) diseases starts looking for deletions and common mutations in mtDNA. MtDNA's special features, such as large and variable genome copies, heteroplasmy, polymorphisms, and its duplication in the nuclear genome as pseudogenes (NUMTs), make it vulnerable to diagnostic misleading interpretations. Multiplex Ligation-dependent Probe Amplification (MLPA) is used to detect copy number variations in nuclear genes and its application on mtDNA has not been widely spread. We report three Kearns Sayre Syndrome patients and one Chronic Progressive External Ophthalmoplegia adult, whose diagnostic mtDNA deletions were detected by MLPA using a very low amount of DNA. This managed to "dilute" the NUMT interference as well as enhance MLPA's efficiency. By this report, we conclude that when MLPA is performed upon a reduced amount of DNA, it can detect effectively mtDNA deletions. We propose MLPA as a possible first step method in the diagnosis of mt diseases.

Keywords

Chronic progressive external ophthalmoplegia, copy number changes, diagnosis, Kearns Sayre Syndrome, mitochondrial disease, NUMTs

History

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Introduction

Mitochondrial diseases are genetic disorders that cause respiratory chain dysfunction. The complexity of the mitochondrial machinery (>1100 proteins) (Pagliarini et al., 2008), its dual genetic regulation (mt and nuclear DNA), and the coexistence of mutated and wild type mitochondrias (heteroplasmy), make their diagnostic approach always a challenge.

From these case reports, we want to stand out the difficulties in the diagnosis of mtDNA-related diseases, especially the ones related to deletions. For the detection of mtDNA deletions Long-range PCR, Southern Blot, and Next-generation sequencing (NGS) are usually used (Cui et al., 2013; Haas et al., 2008).

In 2011, the first mtDNA deletion was detected by MLPA (Kwon et al., 2011). MLPA is a probe-hybridization and PCR-based quantitative method, with the main purpose to detect copy number changes in nuclear genes (Schouten et al., 2002). Four years ago, a MLPA version was developed to detect deletions and common mutations in mtDNA. One of the difficulties of applying MLPA on mtDNA resides in the fact that each cell can have 100–1000 mitochondrias, each of which can bear 2–10 mtDNA copies, and not all copies are homogeneous in their sequence. Another obstacle is mtDNA's high homology with the nuclear genome, more than 95% of mtDNA is duplicated as nuclear pseudogenes (NUMTs) (Malik et al., 2011) which cause their co-amplification in PCR based diagnostic methods.

Tonska et al. (2012) reported the use of MLPA for the detection of mtDNA deletions and concluded that it was a useful method, surpassing the results obtained with Southern Blot, but not those with Long-Range PCR.

Recently, we introduced at IHEM (CCT-CONICET-National University of Cuyo) the SALSA P125-B1 kit (MRC-Holland®, MRC-Holland, Amsterdam, The Netherlands), designed to study mtDNA. We report four patients with mtDNA deletions that were detected by MLPA. We show that by using very low amounts of total DNA and thereby diluting NUMTs, the method was able to detect deletions and determine effectively heteroplasmy levels.

Methods

Patient description

Patients 1, 2, and 3: pediatric patients who met clinical criteria for Kearns Sayre Syndrome. Patients 1 and 2 underwent muscle biopsies that showed ragged red and Cytochrome C oxidase (COX)-deficient fibers, with low COX enzymatic activity.

Patient 4: female adult presenting as a Chronic progressive external ophthalmoplegia. Her muscle biopsy had ragged red and COX-deficient fibers.

Molecular techniques

Molecular techniques were performed according to standard procedures of the participating centers, with the purpose to reach genetic diagnosis, not being designed as an experimental study.

MLPA (IHEM – CCT – CONICET and National University of Cuyo – Mendoza, Argentina):

Performed on total DNA obtained from leukocytes and muscle from patients 1, 2, and 4. For patient 3, only leukocytes were available. The P125-B1 SALSA MLPA kit (MRC-Holland®, MRC-Holland, Amsterdam, The Netherlands) contains 32 probes

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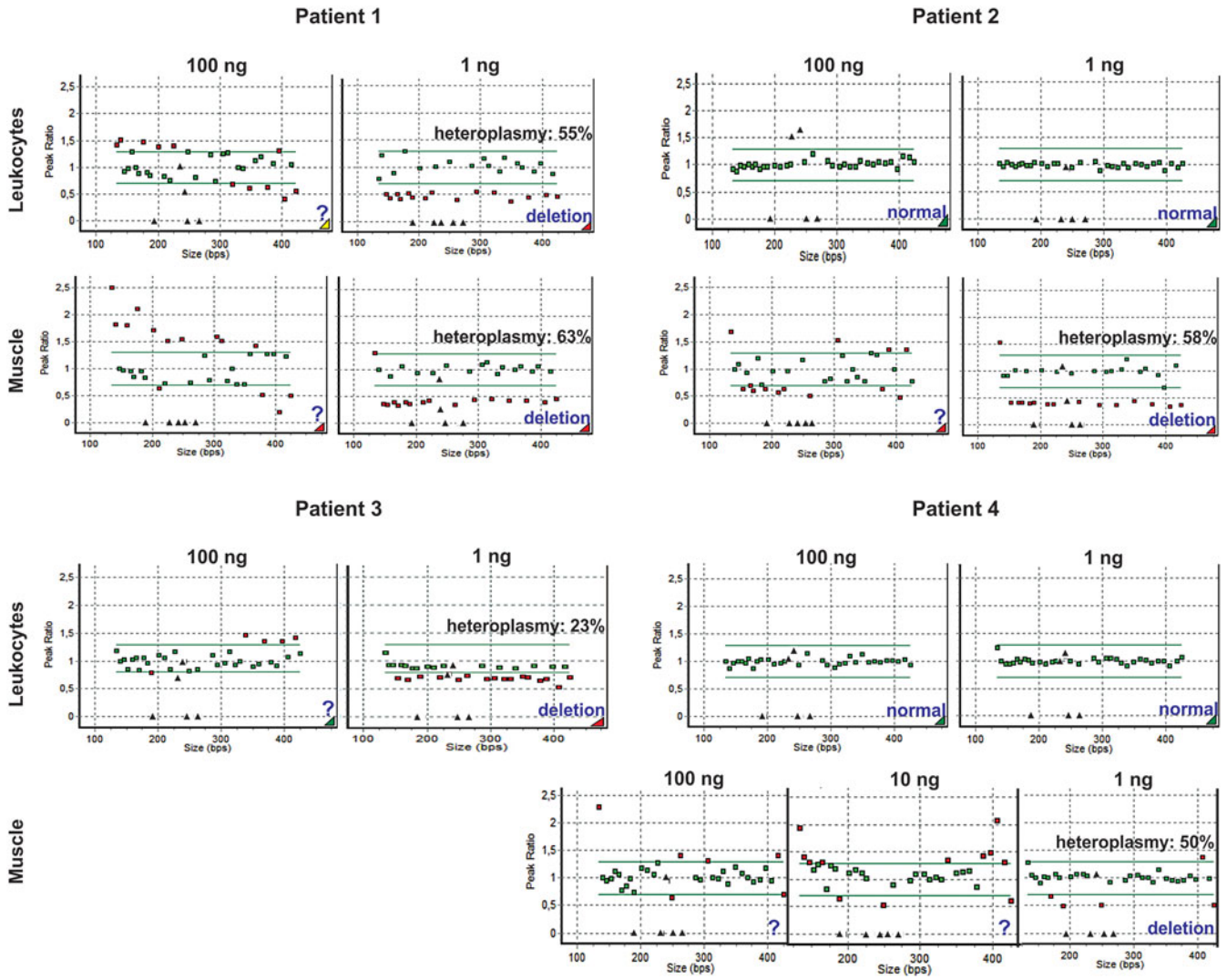

Figure 1. MLPA Ratio charts analyzed by Gene Marker v. 1.75. Boxes inbetween the cut off lines correspond to ratios for regions which are present in a normal copy number. Boxes below and over cut off lines correspond to ratios for regions which are deleted or duplicated in the analyzed sample. Triangles represent ratios for sequence mutated regions. In many charts, amplification signal of probes for two mutation specific regions is observable. These probes (14 484 T > C and 8344A > G) can give a low signal in wild-type mtDNA and should have a 10-fold augmentation to consider the presence of a mutation, which is not seen in these patients. When using 100 ng of DNA template (left panels from each patient), there was in many cases a broad dispersion of the data, making the ratio charts inconclusive or misleading to a normal result (panels with a question mark). The analyses performed with 1 ng of total DNA template (right panels from each patient) showed in all the muscle samples and the leukocyte samples from patients 1 and 3 two distinct ratio populations, one in a normal copy number and the other in a deletion area. 10 ng of DNA were also used in patient 4's muscle sample. In some assays, the smallest amplified region (first left ratio) seems to be duplicated. This fragment corresponds to a sequence located in the D-loop, where the origin of replication of the heavy mtDNA strand begins, making it a three-strand structure, which could be explaining this finding. The heteroplasmy levels were calculated as = mean ratio of normal copy regions − the mean ratio of the deleted regions.

that hybridize to different mtDNA sequences and five mutation-specific probes: m.3243A > G, m.3460G > A, m.8344A > G, m.11 778G > A, and m.14 484T > C. The assays were accomplished using different amounts of total DNA: 100 ng and 1 ng of template DNA, previously treated with 1 µl of 0.5 mg/ml RNAse (Biodynamics®, Biodynamics S.R.L., Buenos Aires, Argentina). 10 ng of DNA was used in patient 4's muscle sample. After probe hybridization and ligation, PCR was performed and the amplification products were run on an ABI3130 DNA analyzer (Applied Biosystems®, Applied Biosystems, Foster City, CA). As control samples, DNA from a pool of leukocytes from 10 healthy individuals was used. Peak plots of patient and control samples were visualized and normalized using GeneMarker software v. 1.75 (Softgenetics®, State College, PA) in order to establish a dosage ratio of mutated mtDNAs.

To identify copy number variations, a ratio analysis between the patient and the control sample was carried out. An altered ratio was considered according to biological criteria when the ratio data was grouped into two distinguishable populations, if not possible, standard cut off values for nuclear MLPA analyses were used: 1.3 and 0.7.

Long-range PCR (Columbia University – New York, NY: patients 1 and 2/ Ramos Mejía Hospital – Buenos Aires, Argentina: patient 4): Performed on skeletal muscle DNA from patients 1, 2, and 4. DNA was extracted using a standard phenol–chloroform method. Three sets of primers were used: 8286F/13 705R, 8286F/15 591R, and F10 360/R151. The first two were used for patient 1, the second for patient 2, and the last for patient 4. The cycling conditions were 30 cycles of 94 °C 15 s and 68 °C 6 min, with an initial hold of 94 °C for 2 min and a final extension at 68 °C for 11 min. Amplified products were separated in a 1% agarose gel by conventional electrophoresis.

Southern blot (Columbia University – New York, NY): Carried out on DNA extracted from patient 1's muscle tissue, following the protocol previously described (Zeviani et al., 1990). The level

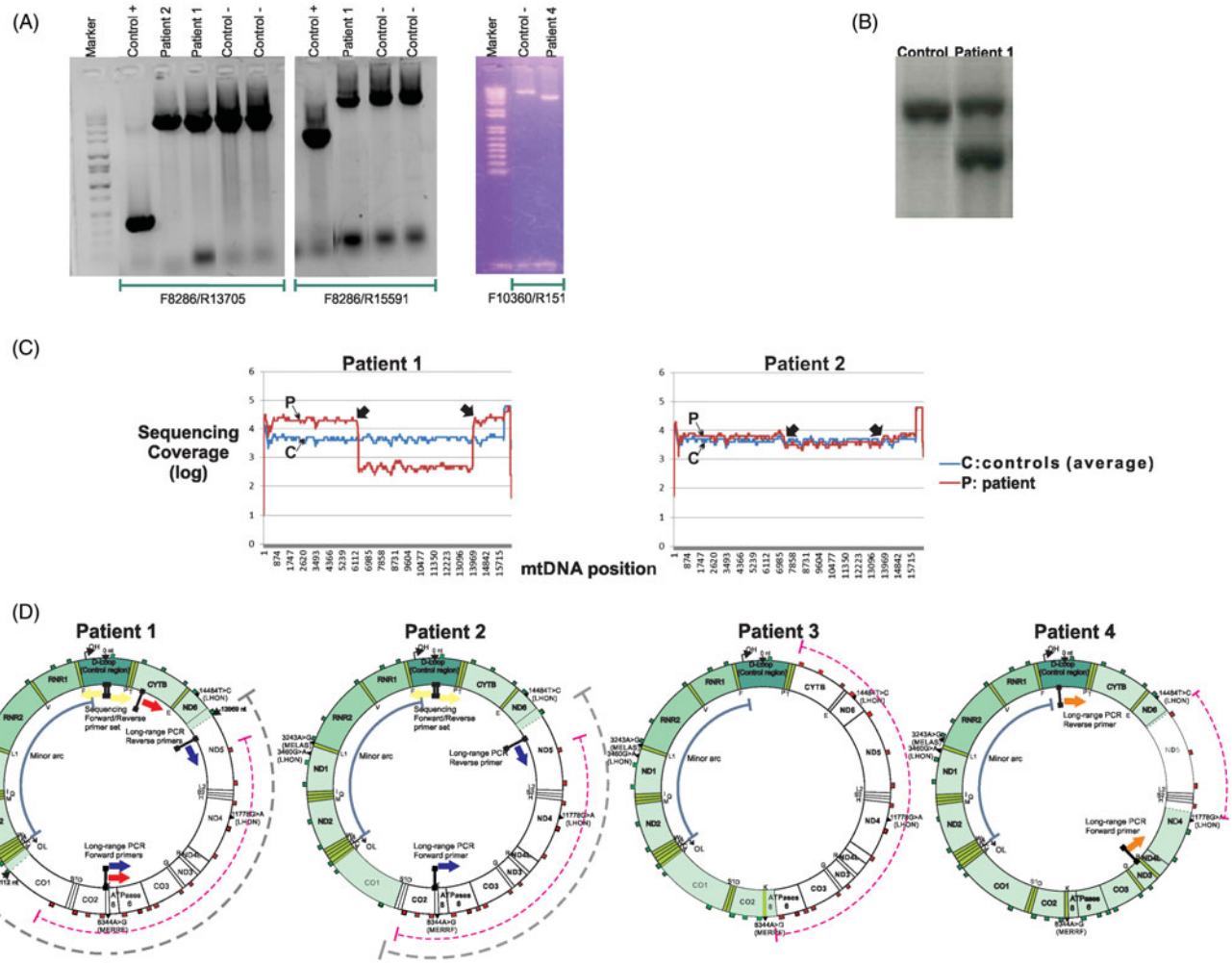

Figure 2. (A) Long-range PCR in muscle samples from patients 1, 2, and 3. Patients 1 and 2: two sets of primers were used: 8286F/13 705R and 8286F/15 591R for patient 1 and the first set of primers for patient 2. Control is a healthy sample, Control + corresponds to a mt deletion sample previously detected by long-range PCR. In both amplification strategies, patient 1's lanes show the same pattern as a healthy control, suggesting no deletion. Patient 2 also showed a normal pattern with the applied amplification strategy. Patient 4: F10,360/R151 was the used primer set. In a patient's sample, the amplified product is lighter than the control, indicating a deletion. (B) Southern blot patient 1 done in muscle mtDNA. The patient's lane shows two bands at different heights, which reveals the presence of a shorter and larger mtDNA fragment, suggesting a mtDNA deletion in heteroplasmic proportions. Image J analysis (Santa Cruz Biotechnology, Santa Cruz, CA) of these images revealed a 52% heteroplasmy level. (C) Next-generation sequencing patients 1 and 2: *y* axis: log. sequencing coverage, *x* axis: mtDNA nt position. P lines represent patients' sample results, C lines represent average controls' samples results. Patient 1 evidenced a large mtDNA deletion from nucleotide 6112 nt to 13 969 nt (thick arrows). Patient 2's analysis was interpreted as normal, but thick arrows point out inflection points where patient 2's coverage line crosses down and up controls' coverage line, suggesting a probable deletion between these points. (D) Mitochondrial DNA maps from the four patients: deletions detected by MLPA and NGS. The circular images represent the 16 569 base-pair mtDNA. They are divided into different genes following proportions found in the NIH gene database (http://www.ncbi.nlm.nih.gov/gene). Boxes around mtDNA: MLPA probes. Triangles: mutation specific MLPA probes. OH: origin of replication heavy strand. OL: origin of replication of light strand. Narrow genes encode tRNAs for different amino acids, letters next to them stand for the amino acid that corresponds to each tRNA. Genes in normal copy amount are filled in with different shades according to their function as protein encoding, rRNA encoding, tRNA encoding genes, or non-coding region (D-loop). Genes in deleted areas are colorless. Sets of arrows represent the primer pairs used for long-range PCR and NGS. Fine dotted lines frame the deletions found with MLPA. Thick dotted lines enclose the deletions detected by NGS.

of heteroplasmy was inferred using Image J Version 1.48 s (Santa Cruz Biotechnology, Santa Cruz, CA).

mtDNA next-generation sequencing (Columbia University – New York, NY): mtDNA obtained from patient 1 and 2's muscle tissues was sequenced using Illumina technology. Four sets of PCR primers were used to amplify mtDNA covering the following overlapping fragments in a counterclockwise direction: from nucleotide (nt) position 16 331 to 3729, from 3646 nt to 9158 nt, from 8753 nt to 16 566 nt and from 10 nt to 16 494 nt.

Results

MLPA analysis: Different DNA amounts were used (Figure 1). When using the standard 100 ng, ratio charts were not conclusive in muscle samples from all patients and leukocytes from patients 1 and 3. A broad dispersion was observed; many ratios above 1.3 indicated duplicated regions and others below 0.7 seemed to be deleted. Patients 2 and 4 showed a normal result with 100 ng of DNA in leukocytes. After literature analysis, we concluded that two obstacles were interfering: (1) the hybridization of mitochondrial MLPA probes on NUMTs and (2) the ratio MLPA probe/mtDNA was very low because we were using the same amount of probes as with nuclear MLPA assays on a genome that has 500–5000 more copies. To overcome these drawbacks, we considered using 100 times less starting DNA, i.e. 1 ng, being that at this extremely low level of DNA, nuclear regions cannot effectively hybridize to specific probes (Schouten et al., 2002). 10 ng was also tried in patient 4's muscle assay, three consecutive

regions appeared deleted, but many others seemed duplicated and the result was not conclusive.

Patient 1: A deletion from *MT-CO1* to *MT-ND5* genes was detected in muscle and leukocytes. The mean ratio value of the patient's samples is indicative of the heteroplasmy level. In muscle, a 63% heteroplasmy level was inferred, and in leukocytes 55%.

Patient 2: A deletion from *MT-CO2* to *MT-ND5* genes was found in muscle in a 58% heteroplasmy level. In leukocytes' DNA, no deletion was detected.

Patient 3: A large mtDNA deletion was evidenced in leukocytes' DNA from *MT-ATP6* to *MT-CYTB* genes in a 23% heteroplasmy level.

Patient 4: A small deletion in a 50% heteroplasmy level was found in muscle comprising *MT-ND4* and *MT-ND5* genes. No deletion came up in leukocytes.

Deletions detected by MLPA are also represented in Figure 2(D) as fine dotted lines.

Long-range PCR: Detected the deletion only in patient 4 (Figure 2A). Patients 1 and 2 showed normal results because the chosen primer sets were placed within the deletions (see Figure 2D).

Southern blot: Performed on patient 1's muscle DNA (Figure 2B). A deletion was found, in a 52% heteroplasmy level.

NGS analyses: Carried out on muscle DNA from patients 1 and 2 (Figure 2C).

Patient 1: A deletion was detected from 6112 nt to 13 969 nt, confirming Southern blot's and MLPA's results.

Patient 2: No deletion was found. However, the sequencing coverage line of the patient inverted its position with respect to the control's coverage line at the same nt positions where MLPA detected the deletion breakpoints. The difference between coverages was not significant, and, therefore, the result was not read as a deletion. Probably, NUMT interference was the reason for this result, since mtDNA enrichment was performed from a series of amplicons and not the recently used single amplicon (Cui et al., 2013). See thick dotted lines in Figure 2(D).

Discussion

mtDNA is analyzed as a first approach for genetic diagnosis of mt diseases (Chi, 2015). This comes along with many obstacles due to the special features of this genome: heteroplasmy, polymorphic feature, and homology with the nuclear genome. Sophisticated strategies have been proposed to bypass the interference of nuclear pseudogenes (Cui et al., 2013).

MLPA has been poorly used to study mtDNA. To our knowledge, only two papers report the use of it (Kwon et al., 2011; Tonska et al., 2012). It seems that NUMTs were not taken into account before. Nowadays, NGS has swept with traditional genetic tools and can effectively study mtDNA, including deletions (Cui et al., 2013), but in developing countries it is still not an option as a first step study because of high cost and low availability.

By this report, we propose that diluting the DNA sample is enough for a sensitive method such as MLPA, to detect mtDNA deletions in a cost-effective manner, making it a possible first step diagnosis tool.

Conclusions

We propose that MLPA applied to low amounts of DNA is a cost-effective method applicable in the diagnosis of mt diseases because of (1) its affordable cost and friendly methodology; (2) its outcome that does not depend on ''choosing the right primer sets'' as in long-range PCR or sequencing; (3) its accurate localization of the deletion in a unique step; (4) its sensitivity to show heteroplasmy levels; (5) its capability to detect mtDNA point mutations in the same assay.

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Declaration of interest

The authors report that they have no conflicts of interests. The authors did not count with any funding source for the execution of the study nor preparation of the manuscript.

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