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Coamplification of Nuclear Pseudogenes and Assessment of Heteroplasmy of Mitochondrial DNA Mutations

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The potential co-amplification of actual mtDNA and nucleus-embedded mtDNA sequences was studied for the mtDNA domains encompassing the major disease-causing mtDNA mutations. By using two different cell lines devoid of mtDNA (ρ° cell lines), it is shown that nucleus-embedded mtDNA sequences readily co-amplified with most of the mtDNA domains encompassing disease-causing mtDNA mutations. The selection of mtDNA primers for specificity on ρ° cells constitutes a simple procedure to avoid such co-amplification. It appears mandatory *prior* to quantify mtDNA mutations, especially when delivering prenatal diagnosis or predictive genetic advise.

INTRODUCTION

Pathological alterations of mitochondrial DNA (mtDNA) include deletions-insertions, depletions and point mutations (1). Most of point mutations (MERRF, MELAS, NARP and Leigh syndrome mutations) are maternally inherited and affected cells usually contain a mixture of mutant and wild-type mtDNA molecules (heteroplasmy). The proportion of mutant mtDNA molecules plays a crucial role in the clinical expression of the disease (1,2,3). Maternal relatives of a patient, or progeny of an affected female might be healthy as long as a threshold value of mutant mtDNA is not reached, but serious troubles might occur when the percentage of mutant molecules raises above this level (3). It is therefore important to accurately estimate the actual proportion of mutant molecules in mtDNA diseases.

On the other hand, ancient mtDNA sequences are known to be present in the human nuclear genome as multiple copies (4). A fortuitous co-amplification of these nuclear copies of mt genes might therefore bias the determination of heteroplasmy level or even confuse the identification of mtDNA mutations. In keeping with this, it was recently shown in Alzheimer's disease patients that the coincidental co-amplification of nuclear copies might lead to confuse various base changes in the silent nuclear copies of cytochrome oxidase (COX) sub-units I and II with actual pathological mutations of mtDNA (5,6).

The aim of this study was to identify the mtDNA domains encompassing disease-causing mtDNA mutations which amplification might be confused with co-amplification of nucleus-embedded mtDNA sequences. It is also shown that the choice of judicious primer pairs using ρ° cell lines (cells devoided of mtDNA) allowed to specifically amplify mtDNA, therefore avoiding the tedious quantification of the potential impact of the nucleus-embedded mtDNA sequences in the context of screening procedures for mtDNA mutations.

PATIENTS AND METHODS

Patients. Three unrelated patients were included in this study. Patient 1 presented with MELAS syndrome. Patients 2 and 3 presented with *diabetes mellitus* and sensorineural deafness. Leukocytes of the three patients were harboring the A3243G mtDNA mutation with various levels of heteroplasmy.

Methods. Cultured ρ° skin fibroblasts from one healthy control and osteosarcoma ρ° cell lines were obtained as described (7). Absence of mtDNA was established by *(i)* the lack of PCR amplification (30 cycles) products using a mtDNA-specific primer pair (nt571-598; nt16220-16193), *(ii)* the absence of detectable cytochrome *c* oxidase activity, and *(iii)* the stability of both phenotype and genotype after 10 passages (cell population doubling).

Total DNA was extracted from patient leukocytes and cultured ρ°

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Abbreviations: COX, cytochrome oxidase; FBSN, familial bilateral striatal necrosis; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy with ragged red fibers; MICM, maternally-inherited cardiomyopathy; MILS, maternally-inherited Leigh syndrome; mtDNA, mitochondrial DNA; NARP, neuropathy, ataxia and retinitis pigmentosa; PCR, polymerase chain reaction; PEO, progressive external ophthalmoplegia.

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TABLE 1

Amplification of Nucleus-Embedded mtDNA Sequences in ρ° Cells

5′ primer	3′ primer	mtDNA-like sequence	Point mutations reported
135–154	433–413	–	
572–592	1150–1130	+	
3130–3149	3301–3272	–	MELAS A3243G LHON G3460A MICM A3260G
3130–3149	3423–3404	+++	
3214–3238	3804–3770	–	
3929–3949	4300–4280	+++	LHON A4136G T4160C T4216C MICM A4269G
4981–5001	5500–5480	–	LHON G5244A
4981–5001	5920–5900	–	PEO T5692C C5703T G5877A
5881–5901	6195–6175	+++	
6136–6156	6450–6430	+++	
6385–6405	6710–6690	+++	
6650–6670	6970–6951	+++	
6910–6930	7230–7210	+++	
7170–7190	7471–7451	+++	LHON G7444A Deafness 57445C
7170–7190	7983–7963	+++	
7561–7581	7983–7963	+++	
7923–7943	8308–8288	+++	
7923–7943	8643–8593	+++	
8199–8221	8385–8345	+++	MERRF A8344G, T8356C MERRF 8356
8199–8221	8382–8357	+++	
8581–8604	9296–9273	+++	NARP/MILS T8993G/T8993C FBSN T8851C
9185–9205	9505–9485	+++	LHON G9438A
9441–9461	9765–9745	+	
9705–9725	10014–9994	+++	LHON G9804A MELAS T9957C MICM T9997C
9705–9725	10700–10680	+++	
10390–10409	10700–10680	–	
11113–11133	11839–11820	+++	LHON G11778A
11720–11740	11839–11820	+	
11720–11740	12386–12366	+	
12005–12029	12865–12841	+	
12005–12029	12386–12366	–	
13801–13821	14736–14716	–	LHON G14459A T14484C
14248–14268	15021–15001	–	Myopathy/diabetes T14709 C
14399–14419	14513–14486	+++	
15052–15072	15480–15460	+++	LHON G15257A
15604–15624	16190–16170	–	Infantile Respiratory deficiency A15923G
15604–15624	16220–16193	–	Myopathy C15990T
15604–15624	16503–16483	–	

–, +, +++: no, low and high amplification of nucleus-embedded mtDNA sequences, respectively. Similar data were obtained on human skin ρ° fibroblasts and osteosarcoma ρ° cells. FBSN: familial bilateral striatal necrosis; LHON: Leber's hereditary optic neuropathy; MELAS: mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF: myoclonus epilepsy with ragged red fibers; MICM: maternally-inherited cardiomyopathy; MILS: maternally-inherited Leigh syndrome; NARP: neuropathy, ataxia and retinis pigmentosa; PEO: progressive external ophthalmoplegia

cell lines and mtDNA domains encompassing disease-causing mtDNA mutations were 30 cycles PCR amplified.

Cytochrome *c* oxidase activity was spectrophotometrically determined as previously reported (8).

RESULTS

In order to assay for the presence of nuclear copies of clinically relevant mtDNA domains in the human genome, we PCR amplified 39 regions of the nuclear DNA from two types of ρ° cell lines (Table I). Based on a 30 cycles PCR assay on total DNA of these mtDNA-less cells, we readily detected an amplification product for 26 of the 38 primer pairs tested. Several clinically relevant domains were found readily amplified. In particular, these included the regions encompassing the MERRF (8344, 8356), MELAS (3243, 8356, 9957), NARP (8993) and LHON (3460, 7444, 9438, 9804, 11778, 15257) disease-causing mtDNA mutations.

We next attempted to illustrate the potential incidence of nuclear copies in quantifying mtDNA heteroplasmy in the case of three patients harboring the A3243G mutation. The level of heteroplasmy was successively determined by using two primer pairs, namely (i) primer A (nt3130-nt3149; forward primer) and B (nt3242-nt3404; backward primer), which co-am-

plified nuclear and mt sequences, and (ii) primer A and C (nt3301-nt3149; backward primer) which selectively amplified mtDNA gene. In the three cases, the variations between the respective level of normal and mutant mtDNA species due to the coamplification of nuclear-embedded mtDNA sequences never exceeded 10%. This ruled out in the case of this particular mutation a major contribution of nuclear-embedded mt pseudogene in the determination of the heteroplasmy level.

DISCUSSION

The above results deal with the potential confusion between actual mtDNA and nucleus-embedded mtDNA sequences. It is shown that these latter readily co-amplified with most of the mtDNA domains encompassing disease-causing mtDNA mutations. It is also shown in the case of three patients with A3243G MELAS mutation that the co-amplification can be negligeable. It should nevertheless be born in mind that the situation might differ (i) depending of the mitochondria content of the cells or tissues studied, (ii) when dealing with other co-amplifying domains for which nuclear-embedded pseudogenes may be presented in a very high copy number and (iii) when looking for traces of mutant mtDNA species.

It therefore appears important to be aware of potential co-amplification of nuclear-embedded mt pseudogenes. Selection of mtDNA primers for specificity on ρ° cells constitutes a simple procedure to avoid such co-amplification. It appears mandatory *prior* to quantify mtDNA mutations, especially when delivering prenatal diagnosis or predictive genetic advise.

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