Mitochondrial DNA deletions in oculopharyngeal muscular dystrophy

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Abstract The deletions in the mitochondrial DNA from skeletal muscle samples of two oculopharyngeal muscular dystrophy cases were studied using polymerase chain reaction techniques. The 4977 bp 'common deletion' was present in both specimens, exceeding the corresponding values of similarly aged, healthy controls. In the two samples multiple different mitochondrial DNA deletions, some case-specific and present at quite high, although not pathogenetic levels, were observed. The results suggest that mitochondrial DNA deletions, and the 'common deletion' in particular, might be a sensitive and early marker of a generalized mitochondrial suffering, due to a variety of pathological and physiological causes.

Key words: Mitochondrial DNA; Deletion; Oculopharyngeal muscular dystrophy; Mitochondrial suffering

1. Introduction

Oculopharyngeal muscular dystrophy (OPMD) is a late-onset muscle disease associated with progressive ptosis of the eyelids and dysphagia which is inherited as an autosomal dominant trait with complete penetrance and without sex preference [1]. A disease locus has recently been mapped through linkage analysis to a region of less than 5 cM on chromosome 14q11.2-q13 [2]. The markers closest to the locus are two cardiac α and β heavy chain myosin genes which raise some intriguing possibilities about the pathogenetic mechanism. The disease usually manifests itself in the fifth or sixth decade at the level of the levator palpebrae and pharyngeal muscles, later all extraocular and other voluntary muscles may become affected resulting, finally, in undernutrition and, eventually, in death by starvation or by aspiration pneumonia because there is no known therapy by drugs or other means [1].

The frequency of OPMD in France has been estimated to be at least 1 in 200 000 and probably from France (in the first half of the 17th century) people carrying a single OPMD mutation (which has been traced back that far) emigrated to Quebec spreading the disease there [1]. In fact, numerous familial cases of OPMD have been reported in the French Canadian community.

There are several histological changes in the OPMD-affected muscles, but most of them are common to many muscular dystrophies, whereas the real hallmark of the disease is the presence of tubulofilamentous intranuclear inclusions of yet unknown origin [1]. The histological marker, in particular, is very helpful in differentiating OPMD from other myopathies such as the mitochondrial myopathies, especially the oculocraniosomatic disease with ragged red fibers (RRF) or Kearns-Sayre syndrome (KSS) [3] and the autosomal dominant mitochondrial myopathy with PEO and bilateral cataract (ad-PEO) [4], which share with OPMD the involvement of the ocular muscle, or te myasthenia gravis. RRF and mitochondrial morphological alterations are not a feature of OPMD although the presence of bizarre cristae inside enlarged organelles and/or of electron-dense homogeneous material inside the matrix have been reported by some authors [5,6]. These authors, together with other researchers [7], have raised the possibility of a central role of mitochondrial dysfunction in OPMD. The issue has been a controversial one especially because RRF and other mitochondrial morphological alterations as well as defects of the respiratory chain enzymes have been reported occasionally in aging subjects [1,8] and always in KSS patients [1]. However, the quite definitive mapping of the OPMD locus to a nuclear gene has completely ruled out the etiopathological relevance of mitochondria for the disease.

Recently, the analysis of mitochondrial DNA (mtDNA) in ocular myopathies has become a diagnostic criterion. In particular, Rowland and coworkers [9] have studied the mtDNA of an OPMD patient belonging to one of the largest French-Canadian affected pedigrees by means of Southern hybridization without finding deletions of the organelle genome. In contrast, the proportion of deleted molecules detected by Southern hybridization ranged from 45% to 85% [4,10] in patients affected by KSS or ad-PEO.

It had also been suggested that OPMD might be a genetically determined 'senescent myopathy' [11] and since we have previously reported an age-related accumulation of mtDNA deletions in the skeletal muscle of healthy aging human subjects [12], we have analyzed the mtDNA of two unrelated cases of OPMD in order to compare these data with those of similarly aged, healthy subjects.

2. Materials and methods

2.1. Human subjects and tissues

Patient 1 (O.R.) was a 61 year old man with a clinically defined form of familial dominant OPMD. A skeletal muscle biopsy was obtained from his left biceps muscle and it showed myopathy aspecific signs. Staining for cytochrome *c* oxidase (Cox) and succinate dehydrogenase (SDH) activities showed rare RRFs-Cox-negative fibers in the sections. The morphological examination did not demonstrate the frequent, but not disease-specific, rimmed vacuoles. The biochemical

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assays carried out (according to [13]) on the muscle homogenate were all in the normal range.

Patient 2 (G.F.) was a 63 year old man with a clinically diagnosed autosomal dominant form of OPMD. A skeletal muscle biopsy was obtained from his left deltoid muscle and it showed a tendency to type I fiber hypotrophy. Staining for Cox and SDH activities did not show any RRF or any Cox-negative fibers.

2.2. DNA isolation, polymerase chain reaction (PCR) and DNA sequencing

Total mtDNA was extracted according to described procedures [12]. The analysis of the patient and control mtDNAs was performed by means of PCR. Fragments of mtDNA from both patients and healthy controls were amplified using conditions previously established [12]. Nucleotide positions of primers refer to the L-strand sequence of mtDNA with the 5′-end preceding the 3′-end. The primer pair ND1 For (3007–3023) - 3.5 Rev (3538–3520) amplified a 531 bp fragment from total mtDNA; the primer pair ATP For (8282–8305) - 13 Rev bis (13928–13905) amplified only the 669 bp fragment from 4977 bp deleted mtDNA (mtDNA^{4977}) since the 5646 bp fragment from wild-type mtDNA was too long to be produced with a short elongation time. The primer pairs 8.1 For (8150–8166) - 16.1 Rev (16159–16141), ATP For - 16.1 Rev and 8.3 For (8361–8380) - 16.1 Rev coamplified products of various lengths from different coexisting deleted species of mtDNA. The double-stranded PCR products from deleted mtDNAs were separated by electrophoresis on 1% Sea-Plaque (FMC) agarose gels in 1×TAE (40 mM Tris-acetate pH 7.8, 1 mM EDTA) buffer and detected by ethidium bromide staining under UV light. The bands were excised and treated with the Qiaex gel extraction kit (Qiagen). Sequencing reactions were performed with these templates according to [14] using 0.5 pmol 5′ [γ-^{32}P]ATP-labeled primer in 10 μl reaction mixtures containing: 30 mM Tris-HCl pH 9.0, 4 mM $MgCl_2$, 10 μM of each dNTP, double-stranded PCR-amplified DNA in water, 0.1 mM ddGTP or 0.3 mM ddATP or 0.4 mM ddTTP or 0.2 mM ddCTP and 0.5 units Taq DNA polymerase (Boehringer Mannheim). The reactions were overlaid with 10 μl of mineral oil, incubated in a thermal cycler for 20 cycles (30 s at 94°C, 30 s at 55°C and 60 s at 72°C), followed by 10 cycles (30 s at 94°C and 60 s at 72°C) and terminated by the addition of 5 μl formamide dye solution.

The quantitative dosages were performed as described in [12] for total mtDNA and the various species of deleted mtDNA.

3. Results

The mtDNA from the skeletal muscle of patient 1 with OPMD had not revealed large rearrangements when tested by means of Southern blot hybridization (data not shown) and we analyzed it with PCR. We looked for the 4977 bp deletion, also known as the 'common deletion' because of its frequency in cases of mitochondrial pathology, in the mtDNAs of patient 1 as well as of a similarly aged, healthy control (58 years old). The amplification with the primer pair ATP For-13 Rev bis of the same amount of mtDNA from both samples suggested a quite large presence of the deleted species in the patient mtDNA by comparing the intensities of the ethidium bromide-stained PCR products (Fig. 1).

Testing the OPMD patient mtDNA for the presence of other deletions by means of a different primer pair (8.1 For-16.1 Rev) three different products coamplified. The identities of these products were assessed through primer shift PCR and sequencing reactions [15]. We identified three deletions, of 7240 bp, 7436 bp and 7679 bp (Table 1). The 7436 bp deletion (nt 8630–16065) in this OPMD patient did not exactly coincide with the same size deletion described in various tissues [16–18] of aging subjects. Patient 1's mtDNA^{7436} was not delimited by any direct repeat, although its 3′ end was still located very close to that 'hot-spot' region (16068–16079) where the 3′ ends of many other described mtDNA deletions map [4,19]. Also the 7240 bp and the 7679 bp deletions were not reported before, they were not enclosed by direct repeats and were located, respectively, mtDNA^{7679} between positions 8388 and 16066 and mtDNA^{7240} between positions 8698 and 15937.

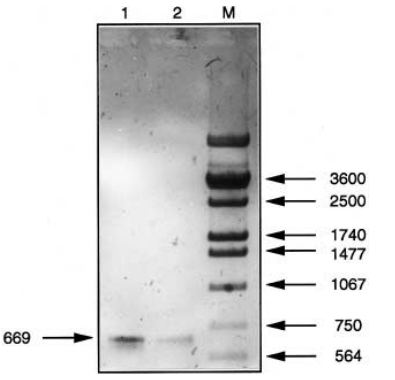

Fig. 1. PCR analysis of mtDNA from OPMD patient 1 (lane 1) and a similarly aged (58 years) healthy control (lane 2). The amplification was carried out using the same amount of mtDNA template. The PCR product of 669 bp derives from the 4977 bp deleted mtDNA molecules. Lane M contains molecular size markers (bp).

We then measured the levels of the mtDNA^{4977} and of the other deleted species in this OPMD patient with the method described in [12]. The results are reported in Table 2. The 'common deletion' is the most represented one with a 4.5% level, which is 64-fold higher than the value in the similarly aged control (0.07%). It is followed by the 7679 bp deletion (3.2%), whereas the other two deletions show lower levels (0.002% for the mtDNA^{7436} and 0.1% for the mtDNA^{7240}).

The mtDNA of OPMD patient 2 was analyzed by means of PCR for the presence of deletions. Three deleted species were identified, encompassing, respectively, 4977 bp, 7436 bp and 7138 bp (Fig. 2). The 4977 bp deletion was the 'common' one already described. The 7436 bp deletion of this OPMD patient showed the same breakpoints reported by other authors in aging healthy subjects, spanning from position 8637 through

Table 1
Nucleotide sequences of the deletion breakpoints in OPMD patient 1

Deletion size (bp)	Sequence		Direct repeat
4977	8470 ACCTCCCTCACCA(AAG.....	13447 AACCTCCCTCACCA)TTG	13 bp
7679	8387 ACTACCG(TAT.....	16067 ATTGA)CTC	None
7436	8629 ACCTCCA(AAT.....	16066 TATTG)ACT	None
7240	8697 ACAAATG(ATA.....	15938 GAAAA)CCT	None

Table 2
MtDNA deletion levels in OPMD patients

Sample	OPMD patient 1 (61 years)	OPMD patient 2 (63 years)	Control[a] (58 years)	Control[a] (65 years)
% mtDNA^{4977}	4.5	2.7	0.07	0.06
fold increase compared to controls	64	45	1	1
% mtDNA^{7436}	0.002	0.00007	N.D.	–
% mtDNA^{7240}	0.1	–	–	–
% mtDNA^{7679}	3.2	–	–	–
% mtDNA^{7138}	–	0.06	–	–

– = not present; N.D. = not detemined.
[a]Values are from [12].

position 16072 and being enclosed by two 12 nt direct repeats at nt 8637–8648 and 16073–16084. The 7138 bp deletion spanned from nt positions 8605 through 15741 and was not delimited by direct repeats.

We quantified the deletions with the same PCR kinetic method [12], obtaining the results shown in Table 2. Also in this case the mtDNA^{4977} shows the highest percentage of 2.7%, which is 45-fold higher than the value of the similarly aged (65 years old) control (0.06%). In contrast, the other two deletions are quite poorly represented reaching 0.06% for the mtDNA^{7138} and 0.00007% for the mtDNA^{7436}.

We demonstrated the presence in the skeletal muscle of various, similarly aged, healthy controls of different mtDNA deletions besides the 'common deletion'. However, their levels were too low to be quantified in a reliable way while being synchronously coamplified by a single primer pair (results not shown).

4. Discussion

In conclusion, both our OPMD patients did not present Southern-detectable levels of deleted mtDNA in their skeletal muscle. In fact, only the PCR technique could find various species of deleted mtDNA in the two analyzed OPMD cases. The 'common deletion' was present in both patients whereas other deletions were case-specific (see Table 2) and were usually not enclosed by direct repeats. This last condition has

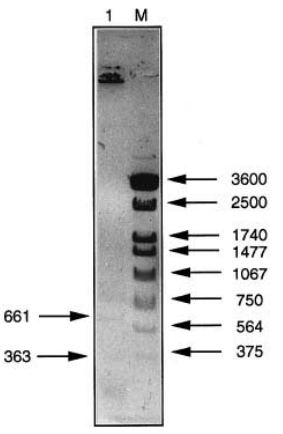

Fig. 2. PCR analysis of mtDNA from OPMD patient 2. The amplification was performed with the primer pair 8.3 For-16.1 Rev and two faint bands, of 661 bp and 363 bp, are visible in lane 1. The 661 bp product was excised, cycle-sequenced and demonstrated to derive from a 7138 bp long deletion. The 363 bp product, treated with the same procedure, was demonstrated to derive from the already described deletion of 7436 bp. Lane M contains molecular size markers (bp).

been already described in aging healthy subjects [20] as well as patients [21,22] suffering from various kinds of pathology.

We have quantified the level of the 4977 bp deletion and of the other deletions by means of our quantitative method demonstrating a quite important increase (45–64-fold) of the 'common deletion' with respect to the values of the similarly aged controls (Table 2). An explanation for this suggests that the 'common deletion' might be a marker of generalized mitochondrial suffering in the skeletal muscles affected by the disease. In fact, some recent papers have shown that various pathologies, sporadic or inherited in a mendelian way and affecting the skeletal muscle, are associated with an increased level of mtDNA large deletions including the 'common deletion'. Among such pathologies are: sporadic inclusion body myositis [23], chronic hypoxia [24], polymyalgia rheumatica [25] and myotonic dystrophy [26,27], where the high sensitivity of the PCR has allowed the detection of deleted mtDNA molecules with a higher frequency than in the control counterparts. In most of the cited studies, however, no quantitative dosage of the deleted mtDNA has ever been performed.

In contrasty, in other pathologies where there is a direct involvement of the mitochondrial genome through a probable nuclear gene controlling its integrity as in Wolfram syndrome [28], ad-PEO and cataract [4] and familial mitochondrial myopathy [29,30], the mtDNA deletion level increases reaching high percentages (56–80%), detectable by Southern blot and affecting the bioenergetic capacity of the organelle. This is suggested by the deficit of various respiratory chain complexes [28] or the decrease of a specific subunit of the ATP synthase complex [30], demonstrated in some pathologies inherited as mendelian traits. A defect of the bioenergetic capacity, in contrast, is generally not present in the OPMD cases, which do not show defects of the respiratory complexes, supporting our idea of the mtDNA deletions as a sensitive and early marker of a generalized mitochondrial suffering [31,32].

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