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Frequency of duplications in the D-loop in patients with mitochondrial DNA deletions

Célia H. Tengan *, Claudia Ferreiro-Barros, Marina Cardeal, Moacir A.T. Fireman, Acary S.B. Oliveira, Beatriz H. Kiyomoto, Alberto A. Gabbai

Division of Neurology, Department of Neurology and Neurosurgery, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil

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Abstract

Small duplications (miniduplications) of the D-loop of human mitochondrial DNA (mtDNA) have been described in patients with mtDNA deletions, mtDNA point mutations and in normal aged tissues. The origin of these miniduplications is still unknown but it is hypothesized that they could be formed after oxidative damage. The respiratory chain (RC) is the main source of free radicals in mitochondria and it is believed that a defect in RC increases free radical generation. If miniduplications are originated by oxidative damage, it is expected that they are more abundant in patients with a defect in the RC. We studied the frequency of miniduplications of D-loop in patients with a RC defect due to mtDNA deletions and in controls. We show that four types of miniduplications could be detected with a higher prevalence than in previous studies and that patients with mtDNA deletions did not have higher proportions or increased number of miniduplications, which is against the hypothesis that miniduplications are generated more abundantly in patients with RC defects. We also clearly demonstrate the age-related nature of these miniduplications by a carefully controlled study regarding the age of subjects, which was not considered in other studies on patients with a mitochondrial disease.
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Keywords: Mitochondria; Mitochondrial DNA; Aging; Mitochondrial disease; mtDNA duplication; Free radical

1. Introduction

An approximate 260-bp tandem duplication in the D-loop region of human mitochondrial DNA (mtDNA) was first identified by Brockington et al. [1] and associated with mtDNA deletions in mitochondrial myopathy. The 260-bp duplication is a heteroplasmic length polymorphism found in low abundance and located in the region that controls replication and transcription of mtDNA (D-loop). Because of this location and the finding in 31% of patients with mtDNA deletions (18 out of 58) but not in normal subjects, it was suggested that this duplication could be relevant to deletions formation [1]. Since then, other studies did not confirm a high prevalence of this rearrangement in patients with mtDNA deletions, finding lower proportions that varied from 3% to 12% [2–4]. On the other hand, the 260-bp duplication and several other duplications in the D-loop (miniduplications) were also found in normal aged individuals [5,6], suggesting that these miniduplications in D-loop are early molecular events of the human aging process. More recently, Bouzidi et al. [7] described a 200-bp tandem duplication with high proportion associated with a cytochrome *b* mutation. It was suggested a relationship between miniduplication formation and respiratory chain (RC) deficiency, which was explained by a possible increase in free radical production from the defective RC whether the genetic origin of the deficiency be a deletion or a point mutation in the mtDNA [7].

In this study, we explore the idea that formation of miniduplications could be enhanced by a defective RC in patients with mtDNA deletions. We show that four types of miniduplications could be detected by polymerase chain reaction (PCR) with a higher prevalence than in previous studies and that patients with mtDNA deletions did not have higher proportions or increased number of miniduplications, which is against the hypothesis that miniduplications are generated more abundantly in patients with RC defects. We also clearly demonstrate the age-

* Corresponding author. Disciplina de Neurologia Clínica, Escola Paulista de Medicina, Universidade Federal de São Paulo, R. Pedro de Toledo, 781, setimo andar, 04039-032, São Paulo, SP, Brazil. Tel.: +55-11-5579-9236; fax: +55-11-5081-5005.

E-mail address: chtengan@neuro.epm.br (C.H. Tengan).

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related nature of this rearrangement by a carefully controlled study regarding the age of the subjects, which was not considered in other studies with patients with mtDNA deletions.

2. Materials and methods

2.1. DNA samples

DNA samples were obtained from autopsy material or muscle biopsies specimens performed exclusively for diagnostic purposes. Genomic DNA was isolated with SDS/proteinase K treatment followed by phenol/chloroform extraction. We studied samples divided in two groups: (1) *patients group*: patients with chronic external ophthalmoplegia and single mtDNA deletions (N=24); and (2) *control group*: individuals with no abnormalities on muscle histology (autopsy material or diagnostic muscle biopsies) (N=27). Quantification of total DNA was performed in the VersaFluor fluorometer (BioRad) using the Hoechst dye assay.

All patients had pathogenic deletions and not large-scale duplications as determined by a Southern blot study with different restriction enzymes and two probes according to Tengan et al. [8].

This study was approved by the Ethics Committee of Universidade Federal de São Paulo and is in accordance with the ethical standards of the Helsinki Declaration.

2.2. Detection of miniduplications

Miniduplications of the D-loop region were detected by PCR using 20 pmol of back-to-back primers located at positions 336–357 and 335–305 of mtDNA ($5' \rightarrow 3'$) [4], 10 ng of genomic DNA, 20 nmol of each deoxynucleotide triphosphate (dNTP), 10× PCR buffer, 2.5 units of Taq DNA polymerase (Amersham-Pharmacia Biotech). PCR conditions were: 94 °C for 5 min followed by 40 cycles with 94 °C for 30 s and 60 °C for 30 s. PCR products were separated by electrophoresis through an ethidium bromide stained agarose gel and then visualized under a UV source. A positive control of the reaction was performed using a DNA sample from cybrid cells containing 100% of the 260-bp miniduplication [9], kindly provided by Dr. C.T. Moraes. Positions in mtDNA were numbered according to the published mtDNA sequence [10].

2.3. DNA sequencing

PCR products encompassing miniduplications were gel purified according to GeneClean protocol (Bio101) and subcloned into vector pCRII (Invitrogen). Sequencing reaction was performed according to BigDye Sequencing kit instructions using M13 Universal primer and run in a ABI Prism 377 Automated Sequencer (Applied Biosystems).

2.4. Quantitative analyses

The quantitation of miniduplication was performed by the serial dilution method as previously described [11] with slight modifications in the PCR conditions. Briefly, 1 μl of serial 10-fold dilutions was submitted to a short cycle PCR (40 cycles consisted of 94 °C for 30 s and 60 °C for 30 s). Two parallel PCR reactions were performed using two pairs of primers. The first pair of primers flanked the miniduplication (primers as cited above). The second pair of primers (forward primer from mtDNA position 5472 to 5491 and reverse primer from mtDNA position 5982 to 5960) amplified a 510-bp fragment (control fragment) that is present both in normal and duplicated mtDNA. Ten microliters of the PCR amplification was submitted to electrophoresis through an ethidium bromide-stained agarose gel, visualized and photographed under a UV source. Optical densities (OD) of the bands were measured by scanning nonsaturated positive prints and quantifying the pixel density (freeware package NIH IMAGE 1.57). The OD obtained was plotted as a function of the template dilutions (the points corresponding to the saturation phase of the PCR amplification were excluded). A linear regression analysis was performed to identify the dilution corresponding to the disappearance of each specific template. The ratio between these dilutions (minidup ratio) was used to estimate the amount of miniduplications in each group (patient and control).

2.5. Phenotype–genotype correlation

The results on detection of miniduplications were correlated with clinical and molecular features of patients. The features analysed included age, type of clinical presentation, presence of endocrinopathy, molecular defect (deletion: size, location, proportion, direct repeats, presence of large duplication).

2.6. Statistical analyses

Statistical analyses were performed with Statview 5.01 (SAS Institute Inc.). Comparison of means was performed by the Student's t-test and statistical significance was set to a $P<0.05$.

3. Results and discussion

In order to determine the prevalence of miniduplications, we studied two groups (patients and controls), formed by individuals with similar distribution of age, from 11 to 70 years old in controls and from 11 to 66 years old in patients. PCR was performed with equal amounts of total DNA (10 ng) in both groups and miniduplications were detected in 17 (63%) controls and 14 (58%) patients. The higher frequency of miniduplications detected in our study could be explained by the use of different PCR conditions leading to a more

efficient amplification. We detected four different bands that corresponded to duplications of approximately 150, 190, 260 and 650 bp (Fig. 1). The 190-bp duplication was the same duplication described by Lee et al. [5]. The other duplications had similar sizes to the ones described previously [1,2,4,5,7], but the exact breakpoints were slightly different. Although the 260-bp duplication was the first one to be described [1], it was not the most frequent, in fact it appeared in only two (9%) patients and six (20%) controls (Table 1). The 650-bp miniduplication was the most frequent in both groups, found in 48% of patients and 37% of controls. Some patients and controls presented two or three types of miniduplications at the same time. We detected only one band in seven patients and nine controls, and two or three bands in seven patients and eight controls. The number of bands obtained were not statistically different between both groups, but we observed that one band was obtained in individuals with age around 30 years and two or more bands around 50 years, suggesting an increase in the number of miniduplications with age (Fig. 2). A clear correlation between the presence of miniduplication and age could be observed when we analysed the age of the individuals with and without miniduplications. Miniduplications were detected mostly in patients and controls with higher ages ($P<0.001$), 40 years for patients (mean=40.4, S.E.=4.2) and controls (mean=40.1, S.E.=3.6). Samples without miniduplications had a mean age of 18.1 years (SE=2.6) for controls and 20.7 years (SE=2.7) for patients (Fig. 3). Previous studies on patients with mitochondrial diseases and miniduplications did not consider the age of the patients as a factor for the presence of miniduplications [1–4,7]. However, when the age of the subject is informed, we could observe that they were older than 35 years in those studies: 67 [1], 58 [4], 35 and 42 years [7].

The main proposed mechanism for the generation of duplications in the D-loop is by slipped strand mispairing during replication [12–14]. Analysis of nucleotide sequences at the breakpoint region demonstrated that duplications occurred in regions with polycytosine stretches and perfect direct repeats varying from 5 to 11 nucleotides (Fig. 1).

Table 1
Detection of four types of miniduplications in patients and controls

	150 bp (%)	190 bp (%)	260 bp (%)	650 bp (%)	Total (%)
Patient	5 (24)	4 (19)	2 (9)	10 (48)	21 (100)
Control	4 (13)	9 (30)	6 (20)	11 (37)	30 (100)
Total	9	13	8	21	49

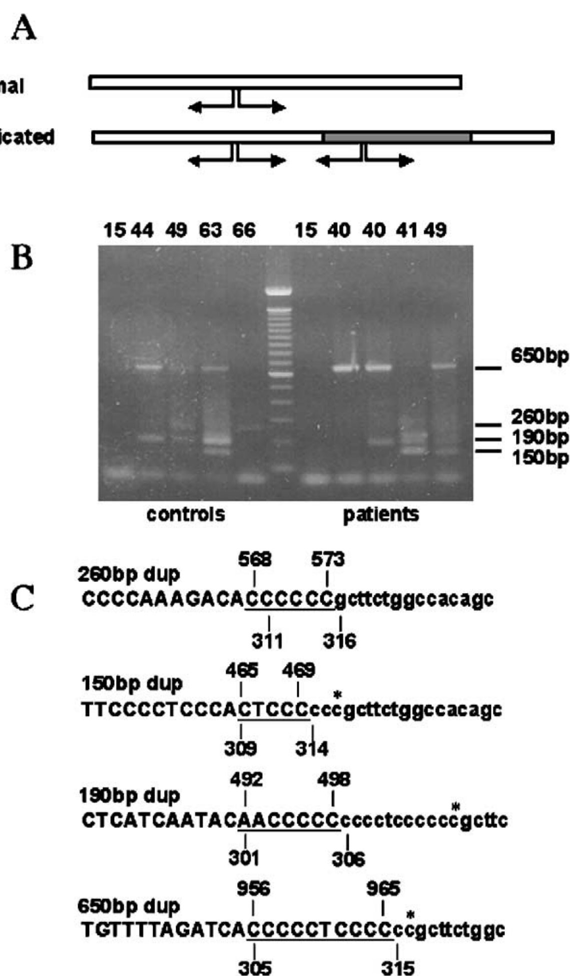

Fig. 1. Detection of four types of miniduplications of the D-loop. PCR was performed with back-to-back primers located at positions 336–357 and 335–305 of mtDNA (5′→3′). This pair of primers can only amplify a fragment if there is a duplicated region (A). Miniduplications in the D-loop of mtDNA were observed in 17 (63%) controls and 14 (58%) patients. Four different bands could be detected after electrophoresis through a 1% agarose gel: 150, 190, 260 and 650 bp (B). The ages of the individuals are showed at the top of the gel. Panel C shows the breakpoint region of each type of miniduplication. Nucleotide positions are indicated above and below the sequence and direct repeats are underlined. There was an insertion of a cytosine (*) just before the guanine at nucleotide position 316.

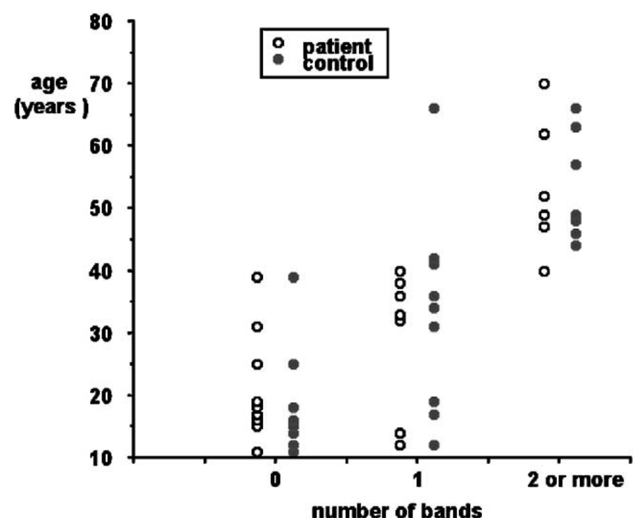

Fig. 2. Number of bands detected in patient and control groups. The primers used for the amplification of miniduplications amplified one, two or three bands in the same patient. The graph demonstrates that the number of bands detected was not increased in the patients but two or more bands were detected in patients and controls with more than 40 years of age.

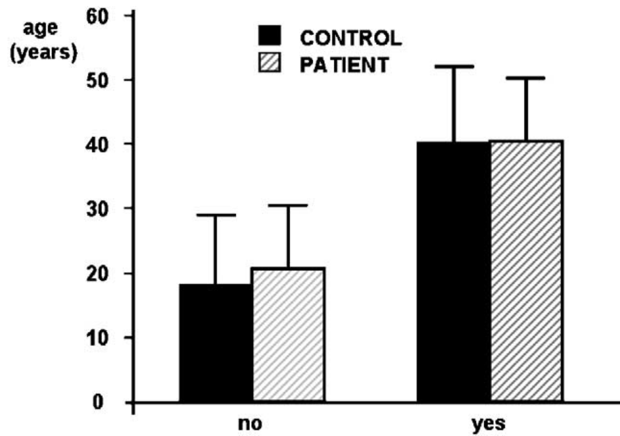

Fig. 3. Correlation of age and the presence of miniduplication. Miniduplications were detected mostly in patients and controls with higher ages (P<0.001), 40 years for patients (mean=40.4, S.E.=4.2) and controls (mean=40.1, S.E.=3.6). Samples without miniduplications had a mean age of 18.1 years (S.E.=2.6) for controls and 20.7 years (S.E.=2.7) for patients. No difference could be detected between patients and controls.

Another interesting feature was that one of the ends of the duplicated segment occurred in the same region in all four types, around nucleotide positions 306–316. This region corresponds to CSB2 (conserve sequence block II) and may represent a hot spot for this type of rearrangement.

Because of the hypothesis that these duplications could arise from oxidative damage favoured by a defective RC [7], we could expect that the appearance of miniduplications would be earlier in patients with mtDNA deletions. However, we did not confirm this expectation since the mean age of patients with duplications were the same in both groups. Furthermore, we could not find increased variety nor quantity of miniduplications detected in the patients group. Quantitation was performed in a total of eight patients and eight controls with similar ages. After obtaining the minidup ratio, the comparison between patients and controls showed a slight tendency of higher values for the patient group but this difference was not statistically significant (Fig. 4). Minidup ratio indicates the level of miniduplications in relation to a control amplification and was used to compare the amount of miniduplications in the patient and control groups. Miniduplications could not be detected by Southern blot (data not shown), demonstrating that they were present in very low levels in skeletal muscle, only detectable by PCR.

Brockington et al. [1] suggested that the presence of the duplication in the D-loop could increase the likelihood of slip replication and hence deletion. In that study, all the patients with miniduplications had deletions flanked by direct repeats and in one without flanking repeats the duplication was not present. The presence of miniduplication was not more frequent in patients and our results did not demonstrate an association between presence of direct repeats and presence of miniduplication. Among 12 patients with deletions flanked by direct repeats, 7 did not present miniduplications of the D-loop. There was also one patient with a miniduplication but no direct repeats. We could not find any other features of the mtDNA deletion that could be associated with the presence of miniduplications, such as location, proportion, presence of a large duplication and any correlation with other clinical features (Table 2).

Our study demonstrated that miniduplications of the D-loop of mtDNA are age-related rearrangements but are not increased in patients with a RC defect. The first age-related mtDNA mutation described and one of the most studied is the 4977 bp deletion of mtDNA [15]. This deletion can be present at high percentages in patients with mitochondrial myopathies [16], but it is also found in very low levels (less than 1%) in aged postmitotic tissues [11]. Other mtDNA deletions [17,18] and more recently several point mutations in D-loop [19] were also detected in aged tissues. The main mechanisms proposed for the appearance of these mutations are due to oxidative damage from free radicals generated in the RC [20]. This hypothesis is supported by the following evidences: increased production of 8-OH dG with age, increase in the levels of mtDNA mutations with age, in-

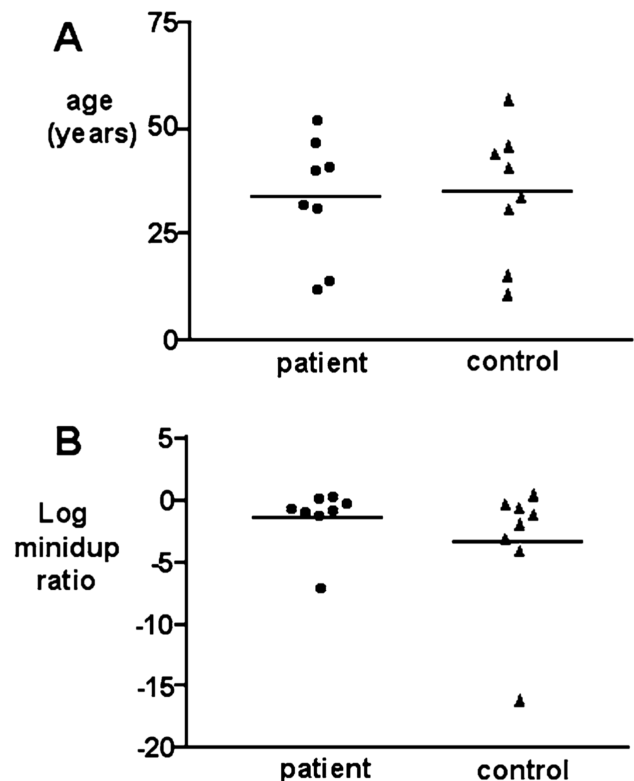

Fig. 4. Quantitative analysis of miniduplications in patients and controls. (A) The amount of miniduplications was estimated by the minidup ratio, as described in Materials and methods. We studied two groups, eight patients and eight age-matched controls with similar age distributions, ranging between 11 and 57 years. (B) Because minidup ratio is related to age in an exponential manner, the values were expressed as log minidup ratio to normalize the data and to better visualize the distribution of the results. The graph demonstrates that there was a slight tendency of increased values in the patient group but there was not statistical significant difference between both groups. Horizontal lines represent the mean values.

Table 2
Presence of miniduplications compared to features of the deletion and presence of a large-scale duplication of mtDNA

Patient	Presence of miniduplication	Location of the deletion	Direct repeats (number of bases)	Percentage of deleted mtDNA	Deletion size (bp)	Presence of a large duplication
1	no	6310–15611	4	60	9300	yes
2	yes	10951–15372	10	83	4420	no
3	yes	8482–13460	13	99	4977	no
4	no	8467–13444	6	82	4977	no
5	no	8482–13460	13	65	4977	no
6	no	7448–13475	no	53	6027	no
7	no	8468–13446	10	72	4978	no
8	no	8482–13460	13	68	4977	no
9	no	8817–15610	5	69	6792	no
10	no	12112–14422	10	89	2309	no
11	yes	9514–13052	no	54	3537	no
12	yes	8476–14812	7	81	6335	no
13	yes	6320–11272	6	19	4952	no
14	yes	8477–13592	10	25	5115	no

creased susceptibility of mtDNA compared to nuclear DNA, proximity of mtDNA to a continuous source of free radicals (RC), decrease of RC activity with age [21–24]. Bouzidi et al. [7] proposes that single-strand breaks, due to free radical damage combined with mispairing could explain the generation of miniduplications at the level of repeated sequences, which are relatively close to each other in the mtDNA D-loop. It is supposed that a defect of RC would increase the leakage of reactive oxygen species from the electron transfer chain. Free radicals induce secondary mtDNA mutations exacerbating mitochondrial respiratory defects, creating a vicious cycle [22]. Although, very attractive, the vicious cycle theory is an unproven hypothesis and it is still not determined whether age-related mtDNA mutations are generated by reactive oxygen species-mediated damage [25]. Based on this theory, we would expect an increase in age-related mtDNA mutations in patients with RC defects caused by high proportions of mtDNA deletions, specially considering that high production of lipid peroxides and 8-OHdG were found in cybrids harbouring high levels of deleted mtDNA [26]. Our results do not support this theory and are in agreement with our previous observation that mtDNA age-related deletions were not increased in patients with mitochondrial diseases caused by mtDNA point mutations [27]. Although it is clear that age-related mtDNA mutations accumulate during life, their contribution to the aging process is still not confirmed. Pathogenicity of miniduplications in the D-loop was not demonstrated in a study of cells homoplasmic for the 260-bp duplication [9]. Moreover, similar insertions in mtDNA control region were also found in other animals, such as rabbit [12] and monkey [28], suggesting that errors in mtDNA replication, leading to insertions, can occur during normal life.

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