ACCUMULATION OF MITOCHONDRIAL DNA COMMON DELETION SINCE THE PREATAXIC STAGE OF MACHADO-JOSEPH DISEASE.

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ABSTRACT

Molecular alterations reflecting pathophysiologic changes thought to occur many years before the clinical onset of Machado-Joseph disease (MJD)/spinocerebellar ataxia type 3 (SCA3), a late onset polyglutamine disorder, remain unidentified. The absence of molecular biomarkers hampers clinical trials, which lack sensitive measures of disease progression, preventing the identification of events occurring prior to clinical onset. Our aim was to analyse the mtDNA content and the amount of the common deletion (m.8482_13460del4977) in a cohort of 16 preataxic MJD mutation carriers, 85 MJD patients and 101 apparently healthy age-matched controls. Relative expression levels of *RPPH1, MT-ND1* and *MT-ND4* genes were assessed by quantitative real-time PCR. The mtDNA content was calculated as the difference between the expression levels of a mitochondrial gene (*MT-ND1*) and a nuclear gene (*RPPH1*); the amount of mtDNA common deletion was calculated as the difference between expression levels of a deleted (*MT-ND4*) and an undeleted (*MT-ND1*) mitochondrial genes. mtDNA content in MJD carriers was similar to that of healthy age-matched controls, whereas the percentage of the common deletion was significantly increased in MJD subjects, and more pronounced in the preclinical stage ($p<0.05$). The *BCL2/BAX* ratio was decreased in preataxic carriers compared to controls, suggesting that the mitochondrial-mediated apoptotic pathway is altered in MJD. Our findings demonstrate for the first time that accumulation of common deletion starts in the preclinical stage. Such early alterations provide support to the current understanding that any therapeutic intervention in MJD should start before the overt clinical phenotype.

INTRODUCTION

Mitochondrial DNA (mtDNA) depletion and an increased number of large deletions, namely the common deletion (m.8482_13460del4977, hereafter named as del4977), previously associated with neurodegeneration, have been observed in Machado-Joseph disease (MJD) cell lines and transgenic (TG) animal models [1–3], as well as in blood samples from MJD patients [1, 4, 5]. MJD, also known as spinocerebellar ataxia type 3 (SCA3; MIM#109150; ORPHA98757), is an autosomal dominant late-onset proteinopathy, which is caused by an abnormal number of coding CAG repeats in the gene encoding for ataxin-3 - *ATXN3* (reviewed in [6]). Initial clinical manifestations of MJD, usually gait ataxia, occur at around the age of 40 (reviewed in [6]) although pathophysiologic changes are thought to start in the preclinical stage, preceding by many years the clinical onset (reviewed in [7]). The knowledge of preclinical alterations is therefore of great importance in testing new therapeutic agents, namely in preventive trials for which biomarkers are completely lacking. Altered conformation of mutant ataxin-3 promotes a toxic gain of function, compromising several cellular mechanisms (reviewed in [8]), including mitochondrial function. Accumulation of reactive oxygen species and defective antioxidant enzyme ability leading to a chronic oxidative stress state [1, 9, 10], compromised mtDNA complex II [11], exacerbation of mitochondrial fission[12] and activation of mtDNA apoptotic pathways by dysregulation of BCL2 family members, namely BCL2 and BAX [13, 14], are mitochondrial-related alterations that have been reported in MJD. Although mtDNA damage and depletion have been reported in blood samples of MJD patients, previous studies have not been conclusive. In 2008, Liu and colleagues described lower levels of mtDNA copy number in 61 MJD patients; age at sampling, however, was not taken into consideration [5]. Yu and colleagues also observed lower mtDNA copy number as well as lower levels of del4977 in 16 MJD patients compared to controls [1]. In a later study, similar levels of mtDNA copy number and del4977, without controlling age at sampling, were

found in 14 MJD patients [4]. Power limitations as well as the non-adjustment of age at sampling as covariate could be the cause of the inconclusive findings.

To our knowledge, no patient-based reports have investigated the correlations between the mtDNA content and/or the amount of the del4977 and clinical features. Also, the behaviour of both mtDNA alterations during the natural history of MJD, including the preclinical stage, remains therefore unknown. Previous work from our group using a MJD TG mouse model in different stages of the disease has shown a decrease in the mtDNA copy number with age that was more pronounced in TG mice than in wild-type controls [2, 3]. The same study has described an accumulation of the 3,867-bp deletion (homolog of the human mtDNA del4977) in the stage prior to disease phenotype establishment. Furthermore, pontine nuclei of TG animals, a brain area affected in MJD, displayed the most pronounced decrease in copy number and the most evident accumulation of the 3,867-bp deletion [3].

Our aim was to document the behaviour of mtDNA content and the percentage of del4977 in a cohort of 16 preataxic MJD mutation carriers, 85 MJD patients and 101 apparently healthy age-matched controls, to explore its potential as a disease biomarker.

SUBJECTS AND METHODS

The characteristics of MJD subjects and controls used in this study are displayed in Table 1. Sixteen preataxic MJD mutation carriers, 85 MJD patients and 101 apparently healthy controls were enrolled in this study between 2006 and 2016. To the best of our knowledge, MJD subjects and community controls were not related. According to age at blood collection, population controls were assigned in two different sets: CTRL-G1 matched with the younger preataxic carriers, and CTRL-G2 matched with patients. Gender and age were used as matching variables. A variation of ± 3 years was admissible when matching controls with patients or with preataxic carriers. The preataxic carriers were classified as in Maas and colleagues [7]. The average time elapsed from age at blood collection to the predicted age at onset for preataxic carriers was 11±8 (SD) years. The predicted age at onset was calculated by a linear regression model, considering the reported age at onset and the number of CAG repeats in the expanded allele from 90 Azorean MJD patients (Predicted age at onset = 211,794 + (-2,482) x (CAGs)). The age at disease onset was defined as the age of first appearance symptoms, usually gait disturbances, reported by the patient and/or a close relative. Disease duration was defined as the number of years elapsed from the reported onset to the time of blood collection. The number of CAG repeats at the *ATXN3* locus was determined using the protocol described by Bettencourt and colleagues[15]. All participants provided written informed consent; this study is part of a project approved by the Ethics Committee of Hospital do Divino Espírito Santo (Ponta Delgada).

Total DNA was isolated from whole blood by standard protocols and stored at -20°C until needed. DNA quality and concentration were assessed using Nanodrop 2000c (Thermo Fisher Scientific). One region of the nuclear genome (RNase P H1 RNA, *RPPH1*) and two different regions of the mitochondrial genome (NADH dehydrogenase 1, *MT-ND1* and the NADH dehydrogenase 4, *MT-ND4*) were selected. *RPPH1* is a nuclear gene widely recommended as

the standard reference assay for human gDNA copy number quantitation (TaqMan Copy Number Reference Assay RNase P by Applied Biosystems). *MT-ND1* is located outside the del4977 and *MT-ND4* is overlapping the deletion region. This design allows the detection of a highly conserved mtDNA region (*MT-ND1*) as well as a mtDNA region (*MT-ND4*) absent in over 95% of all reported deleted molecules [16]. Quantitative real-time PCR (qPCR) was performed to obtain the relative expression values ($2^{-\Delta Ct}$) for the *RPPH1*, the *MT-ND1* and the *MT-ND4* genes, following the manufacturer instructions (TaqMan® Gene Expression Assays Protocol by Applied Biosystems). *MT-ND1* (FAM™ dye signal)*/RPPH1* (VIC® dye signal) or *MT-ND4* (VIC® dye signal)/*MT-ND1* (FAM™ dye signal) were run simultaneously in a duplex qPCR reaction. Triplicate reactions were performed and raw data were collected in the Applied Biosystems 7900HT Fast Real-Time PCR system. The mtDNA content was calculated as the difference between the expression levels of a mitochondrial gene (*MT-ND1)* and a nuclear gene (*RPPH1)*; the amount of del4977 was calculated as the difference between expression levels of a deleted (*MT-ND4*) and an undeleted (*MT-ND1*) mitochondrial genes and its percentage was calculated according as follows: (expression value of del4977 amount/expression value of mtDNA content) x 100.

BCL2 and *BAX* mRNA levels from a subset of 12 preataxic carriers, 71 MJD patients and 22 controls, previously obtained in Raposo and colleagues [17], were used to calculated the ratio between anti-apoptotic (*BCL2*) and pro-apoptotic (*BAX*) members of the BCL2 family further providing an indication of the activation/inactivation of the intrinsic apoptosis pathway [18].

Mean differences of mtDNA content or percentage of del4977 between preataxic carriers and matched controls (CTRL-G1) or patients and matched controls (CTRL-G2) were calculated using the Wilcoxon Signed Ranks test. An ANCOVA test was performed to compare the mtDNA content and the percentage of del4977 between preataxic carriers and patients, using the age at blood collection and the number of CAG repeats in expanded allele (CAG_{exp}) as covariates. In

the group of preataxic carriers and/or patients, partial correlation was used to explore the relationship between the mtDNA content, the percentage of common deletion, the *BCL2/BAX* ratio, the CAG_{exp}, the age at onset and disease duration using age at blood collection as covariate. *BCL2/BAX* ratio was compared between the three biological groups using an ANCOVA, using age at blood collection as covariate. Data were log transformed whenever necessary. All statistical tests were performed in IBM SPSS Statistics 22; a p-value lower than 0.05 was considered as statistically significant.

RESULTS

A slight increase of mtDNA content in preataxic MJD carriers compared to age-matched controls (CTRL-G1) was observed, although not reaching significance (Figure 1). The mtDNA content in patients was similar to that of age-matched controls (CTRL-G2) as well as to that of preataxic carriers ($p>0.05$; Figure 1). When compared to the respective age-matched controls, a significantly higher percentage of del4977 was observed in the preataxic carriers ($Z=-2.689$, $p=0.007$) as well as in the patients ($Z=-3.041$, $p=0.002$; Figure 2). The amount of deletions was higher in MJD subjects: in preataxic carriers it was over three-fold higher than in CTRL-G1; in patients it was nearly two-fold higher than in CTRL-G2 (Figure 2). The amount of deletion was similar in preataxic carriers and patients (data not shown). No significant associations were found between mtDNA content or percentage of del4977 with $(CAG)_{exp}$, age at onset or disease duration.

Evaluation of the activation or inactivation of the intrinsic apoptosis pathway, by using the *BCL2/BAX* ratio, showed lower values in carriers of the *ATXN3* mutation (patients = 0.448±0.024 (SD)) as well as preataxic subjects = 0.373±0.054 (SD)), when compared to controls (0.693±0.034 (SD)) resulting in statistically significant differences (ANCOVA test, $p<0.0005$). This suggests that activation of apoptosis is favoured in MJD. *BCL2/BAX* ratio was similar between preataxic carriers and patients. A significant negative correlation between the percentage of common deletion and *BCL2/BAX* ratio was found in MJD subjects ($N=83$, $r=-0.333$, $p=0.02$), indicating that high levels of common deletion are associated with more pronounced activation of apoptosis. This correlation was not observed in controls ($r=0.066$), which indicates that the presence of mutated ataxin-3 could be causing the significant relationship.

DISCUSSION

In the present study, we investigated the behaviour of the mtDNA content and the percentage of the common deletion in blood samples from 101 MJD subjects, including patients and mutation carriers in the preclinical stage. The mtDNA content in both groups was found to be similar to that of healthy age-matched controls, whereas the percentage of the common deletion was significantly increased in MJD subjects, and more pronounced in the preclinical stage.

The quantification of the mtDNA content by different methodologies creates difficulties in the comparison of results across studies. Our results do not corroborate published reports (with lower number of subjects), which suggested a decrease in mtDNA content in blood samples from MJD patients [1, 5]. It is of note that age at sampling was not always used as covariate in published studies [1]. As previously demonstrated in several studies with human subjects, there is an association between mitochondrial alterations and aging (see, amongst others, [19, 20]), implying that the effect of age needs to be accounted for when looking for associations between such alterations and disease. The increase in the percentage of the common deletion in the presence of mutated ataxin-3 observed by us is in agreement with a previous report that analysed MJD patients [1], as well as with our own data from a MJD TG mouse model [2, 3]. In this TG model the amount of deletions was consistently higher than the observed in wild-type control mice; in particular, the affected brain area analysed (pontine nuclei) presented the highest percentage of deletions [2, 3]. These observations suggest that the accumulation of deletions in blood of MJD subjects could be mimicking alterations occurring primarily in affected tissues.

The fact that there are no significant differences in mtDNA content, on one hand, but a higher amount of deletions in MJD subjects than in age-matched controls needs to be addressed. Accumulation of mtDNA molecules with deletions could be reflecting oxidative stress,

promoted by the presence of mutated ataxin-3 [1]. Deleted mtDNA molecules, which are smaller than wild-type counterparts, replicate faster [21]; this process could explain the similar levels of mtDNA content found either in preataxic carriers and MJD patients compared to age-matched controls, a result in disagreement with previous findings [1,5]. Furthermore, the cellular imbalance of wild-type/deleted mtDNA molecules could compromise mitochondrial function and lead to the activation of the intrinsic apoptotic pathway, including the tumor protein p53 (p53) signalling cascade. In the presence of mutated ataxin-3, p53-mediated apoptosis could be aberrantly activated by the up-regulation of p53 [22] and/or the inactivation of PNKP (polynucleotide kinase 3'-phosphatase; [23]). Recently, p53 was identified as a novel substrate of ataxin-3. Mutated ataxin-3 abnormally interacts with p53, leading to its up-regulation and to an increased p53-dependent neuronal cell death, as observed in MJD zebrafish and mouse models [22]. Gao and colleagues showed that in the presence of mutated ataxin-3, PNKP 3'-phosphatase activity during DNA repair is abolished, resulting in the accumulation of DNA strand breaks in cell lines and mouse models, as well as SCA3 brain tissues [23]. The modulation of DNA repair pathways by the interaction and stimulation of PNKP enzymatic activity by the wild-type ataxin-3 was described [24]. In contrast, when ataxin-3 is mutated the PNKP is inactivated and/or rescued in polyglutamine aggregates, promoting a chronic activation of the DNA damage-response ATM – p53 signalling pathway [23], which was further confirmed by the increased expression of p53 target genes, including *BAX, PUMA* and *NOXA* ([23]; reviewed in [25]). Several evidences suggesting that DNA repair pathways, including base excision repair, single-strand break repair, mismatch repair and possibly homologous recombination are also active in the mitochondria to preserve the integrity of mtDNA (reviewed in [26]). Moreover, PNKP was recently found in mitochondria (reviewed in [26]).

p53 mediate apoptosis by the regulation of several BCL2 family members, including *BAX* and *BCL2* (reviewed in [27]). In fact, a decreased *BCL2/BAX* ratio in *ATXN3* mutation carriers

compared to controls was found; low levels of *BCL2* and high levels of *BAX* indicate higher apoptotic activity. As an attempt to deal with accumulation of mtDNA deletions, we hypothesise that apoptosis is initiated during the preataxic stage, leading to an upregulation of cell death in subsequent stages of the disease and explaining the observed decline in the percentage of deletions from the preataxic to the ataxic stage. Longitudinal studies will be valuable to test this hypothesis.

Correlations between mtDNA content and/or amount of del4977 and additional clinical features were tested, failing to produce significant results. Because mitochondrial function is one of the several mechanisms involved in MJD pathogenesis (reviewed in [8]), capturing its direct effect on phenotype should be hard to achieve.

Our findings demonstrate for the first time that mtDNA damage, evaluated by quantification of the del4977, is present many years before the MJD clinical onset. Although a small cohort of preataxic carriers has been used, the high percentage of del4977 was also observed in MJD patients, reinforcing the hypothesis that this alteration is related with the presence of mutated ataxin-3. Notwithstanding, a larger number of preataxic carriers should be analysed to confirm this finding. Together with other molecular alterations, the del4977 could aid us to define a timeline of disease-related events occurring prior to clinical onset. Such early alterations also support the current idea that any therapeutic intervention in MJD should start before the overt clinical phenotype.

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COMPETING INTERESTS

The authors declare that they have no competing interests

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FIGURES

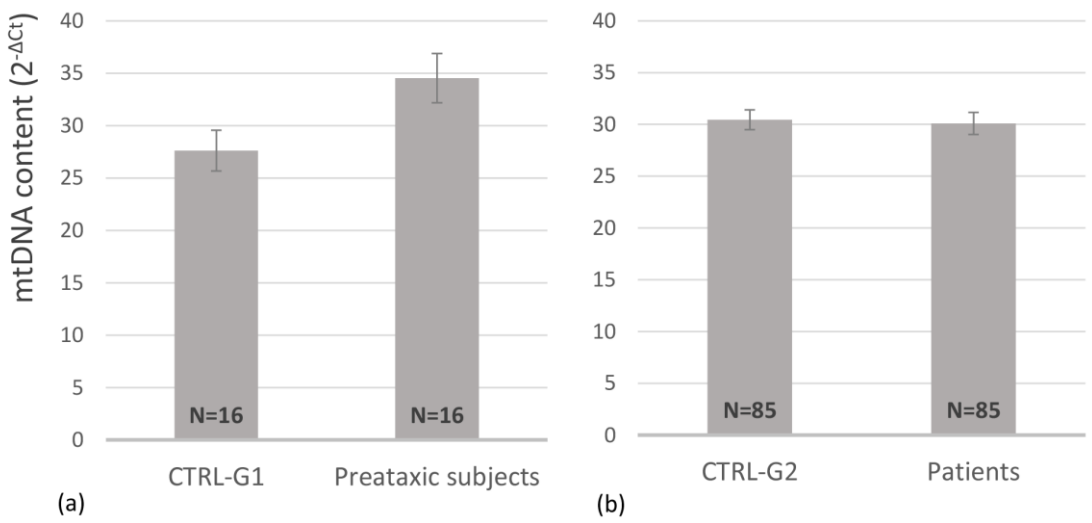

Figure 1. qPCR relative quantification of mtDNA content in blood from preataxic subjects, patients and controls. Comparisons were performed between (a) preataxic carriers and age-matched controls (CTRL-G1) and (b) patients and age-matched controls (CTRL-G2). Mean differences between CTRL-G1 and preataxic carriers and CTRL-G2 and patients were tested using the Wilcoxon Signed Ranks test.

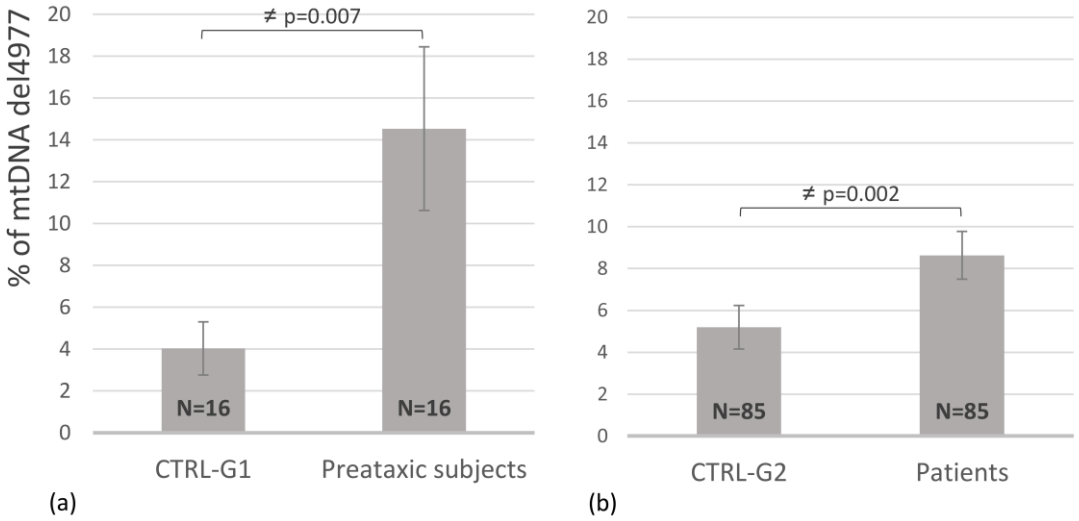

Figure 2. qPCR relative quantification of percentage of del4977 in blood from preataxic subjects, patients and controls. Comparisons were performed between (a) preataxic carriers and age-matched controls (CTRL-G1) and (b) patients and age-matched controls (CTRL-G2). Mean differences between CTRL-G1 and preataxic carriers and CTRL-G2 and patients were tested using the Wilcoxon Signed Ranks test. ≠differences were statistically significant at a $p<0.05$.

TABLES

Table 1. Gender and age at blood sampling of preataxic subjects, MJD patients and controls analysed in the present study. Clinical and genetic main features were described for preataxic subjects and patients.

	Preataxic subjects	***CTRL-G1***	***Patients***	***CTRL-G2***
	N=16	N=16	N=85	N=85[1]
Gender (male|female)	5|11	8|8	45|40	40|45
Age at sampling (years)	30±6* (22-43)	30±6 (22-43)	46±13* (17-81)	46±13 (18-77)
CAG_{normal}	20±4 (14-28)		22±5 (14-29)	
CAG_{exp}	68±2* (65-74)		71±3* (63-79)	
Predicted age at onset (years)	41±8 (26-51)			
Time to predicted age at onset[#] (years)	11±8 (27-(-4))			
Age at onset (years)			36±11 (13-71)	
Disease duration (years)			10±8 (0-34)	

Age at sampling, age at onset and disease duration are presented as mean ± standard deviation (minimum-maximum); [1]one control has a difference of 4 years to the matched patient. *p<0.05 (means were compared by the independent-samples T-Test). [#]Time elapsed from age at blood collection to the predicted age at onset.