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CASE REPORT

Aberrant synthesis of ATP synthase resulting from a novel deletion in mitochondrial DNA in an African patient with progressive external ophthalmoplegia

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Abstract A young, adult, African male patient presented with progressive proximal muscle weakness, external ophthalmoplegia and ptosis, as well as cardiac conduction abnormalities resembling Kearns–Sayre syndrome (KSS). Magnetic resonance imaging (MRI) of the brain revealed normal basal ganglia but bilateral well-circumscribed lesions in the cerebellar peduncles. Enzyme deficiencies in oxidative phosphorylation (OXPHOS) complexes I, IV and V was measured in muscle tissue. Blue native polyacrylamide gel electrophoresis (BN-PAGE) confirmed decreased protein content and activity of these complexes and revealed the presence of two catalytically active complex V sub-complexes. Upon investigation by molecular genetics, the mitochondrial DNA (mtDNA) copy number was found to be elevated and a novel deletion of 3431 bp was found in 80% of muscle mtDNA between positions 7115 and 10546, flanked by a 5 bp direct repeat sequence. In addition, it could also be concluded that the absence of mtDNA-encoded *ATPase6* and *ATPase8* genes in this patient clearly resulted in aberrant synthesis of ATP synthase.

Abbreviations

BN-PAGE	blue native polyacrylamide gel electrophoresis
ECG	electrocardiogram
EMG	electromyogram
MRI	magnetic resonance imaging
FLAIR	fluid-attenuated inversion-recovery
DWI	diffusion-weighted imaging

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References to electronic databases: Progressive external ophthalmoplegia: OMIM 157640, 609286, 258450, 610131, 609283, 258400. Retinitis pigmentosa: OMIM 600105, 608133, 500004, 612572, 312612, 268025, 268000, 312600, 300389, 180104. Kearns–Sayre syndrome: OMIM 530000. Pearson marrow–pancreas syndrome: OMIM 557000. ATP:creatine *N*-phosphotransferase (creatine kinase): EC 2.7.3.2. NADH:ubiquinone oxidoreductase (complex I): EC 1.6.5.3. Succinate:ubiquinone oxidoreductase (complex II): EC 1.3.5.1. Ubiquinol:ferricytochrome-*c* oxidoreductase (complex III): EC 1.10.2.2. Ferrocytochrome-*c*:oxygen oxidoreductase (complex IV): EC 1.9.3.1. ATP phosphohydrolase (complex V): EC 3.6.1.3. Pyruvate:[dihydrolipoyllysine-residue acetyltransferase]-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor-acetylating; pyruvate dehydrogenase complex): EC 1.2.4.1. Acetyl-CoA:oxaloacetate *C*-acetyltransferase [thioester-hydrolysing, (*pro-S*)-carboxymethyl forming] (citrate synthase): EC 2.3.3.1. Deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (DNA-directed; DNA polymerase gamma): EC 2.7.7.7.

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HbA_{1C}	glycohaemoglobin
mtDNA	mitochondrial DNA
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species

Introduction

Mitochondrial disorders are caused by any one of a great number of mutations in nuclear DNA and mitochondrial DNA (mtDNA). Amongst these, mtDNA deletions are often associated with chronic progressive ophthalmoplegia (OMIM 157640, 609286, 258450, 610131, 609283, 258400), Kearns–Sayre syndrome (OMIM 530000), and Pearson marrow–pancreas syndrome (OMIM 557000; Yamashita et al. 2008; Mancuso et al. 2007). These deletions often occur in an area of the mitochondrial genome that encodes the two mitochondrial genes for ATP synthase, *ATPase6* and *ATPase8* (Mita et al. 1990; Samuels et al. 2004). In our study, clinical, biochemical and molecular genetic findings in an adult African patient with a novel mtDNA deletion in this area of the mitochondrial genome are reported. In addition, the effect of this deletion on the native structure and function of oxidative phosphorylation (OXPHOS) complexes is demonstrated.

Patient and methods

Patient

A 22-year-old male patient with progressive proximal muscle weakness, external ophthalmoplegia and ptosis was referred for assessment. The presenting symptom had been ptosis when he was aged 17 years. He had been healthy previously, and there was no family history of any mitochondrial disorder. His initial school performance was satisfactory, and he completed grade 10. However, at the time of the assessment, the results of the Beery–Buktenica developmental test of visual motor integration correlated with that of an 8-year-old child.

He had asymmetrical but bilateral ptosis, with the left side more affected. He experienced progressive external ophthalmoplegia, and complete ophthalmoplegia was present within 15 months after the initial presentation. Retinitis pigmentosa (OMIM 600105, 608133, 500004, 612572, 312612, 268025, 268000, 312600, 300389, 180104) was diagnosed. He had proximal muscle weakness and complained of exercise intolerance. The deep tendon reflexes were diminished. He had dysarthria, a slight tremor and past-pointing, but no ataxia was present initially. Although he did not have ataxia initially, the dysarthria, slight tremor and past pointing were indicative of cerebellar involvement and therefore fulfilled the criteria for Kearns–Sayre syndrome (KSS) (OMIM 530000) as proposed by Moraes et al. (1989).

Gynaecomastia was noted. He had an irregular pulse, but no cardiomegaly or cardiac failure.

Methods

Ethical aspects

For the specialised investigations described in this report, ethics approval was obtained from the University of Pretoria (number 91/98 and amendments), the North-West University (02M02). Informed consent and assent were obtained from the patient.

Enzyme assays

A muscle biopsy was taken from the vastus lateralis muscle for further biochemical and molecular genetics analyses. Mitochondrial respiratory chain enzymes complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3), complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1), complex III (ubiquinol:ferricytochrome-*c* oxidoreductase, EC 1.10.2.2), complex IV (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1), combined activity for complex II and III (succinate-cytochrome c reductase) and pyruvate dehydrogenase complex (PDHc, EC 1.2.4.1) were measured in the muscle, essentially as has been described previously (Rahman et al. 1996; Janssen et al. 2007; Chretien et al. 1995). As frozen muscle tissue was used to prepare 600 *g* supernatants for assay of respiratory chain enzymes, an assay of complex V (EC 3.6.1.3), which requires freshly isolated mitochondria, was not performed. Citrate synthase (CS; EC 2.3.3.1) activity was determined by the method of Shepherd and Garland (1969), and enzyme activities were expressed as a ratio to CS (as mitochondrial marker enzyme) to compensate for mitochondrial enrichment in the sample (Janssen et al. 2007). Values were compared to reference values obtained from muscle samples from 18 healthy controls undergoing orthopaedic surgery.

Blue native polyacrylamide gel electrophoresis followed by in-gel activity staining

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and in-gel activity staining were performed as has been described previously (Van Coster et al. 2001). Briefly, mitochondria were prepared from the patient together with a control muscle specimen. The mitochondria were solubilised, and 50 µg of protein were loaded in duplicate.

The first set of lanes was used for activity staining of complexes I, III (Meulemans et al. 2007) and IV, and the second set was used for activity staining of complexes II and V.

Western blot analysis of mitochondrial proteins resolved by two-dimensional BN-PAGE

After the first dimension (BN-PAGE), a second denaturing electrophoresis [tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)] was performed, for which the BN-PAGE gel was turned 90° and run in a perpendicular second dimension. In this way, the protein subunits within each OXPHOS complex were separated (Devreese et al. 2002). The two-dimensional (2D) gels were electro-blotted onto a nitrocellulose membrane using a tank blotting apparatus from Bio-Rad, as has been described previously (De Vriese et al. 2006). The relative levels of the five OXPHOS complexes were evaluated by immunodetection, using the MS601 MitoProfile® human total OXPHOS complexes detection kit from MitoSciences in a 1/1000 dilution. Detection was done with the enhanced chemiluminescence kit, ECL Plus™ from GE Healthcare. The signals were captured with a ChemiDoc charge coupled device (CCD) camera and processed with Quantity One® software, both from Bio-Rad.

Molecular genetic analyses

DNA was extracted from muscle with the NucleoSpin® kit from Macherey–Nagel. In order to investigate the integrity of mtDNA, we performed Southern blot analysis (Selden 1989). For this, 0.1 µg to 0.5 µg of DNA were digested with *Pvu*II before electrophoresis and membrane transfer. A probe encompassing the complete mtDNA sequence was prepared by polymerase chain reaction (PCR) (Long PCR Enzyme Mix, Fermentas), using an ND1 gene primer set (forward 5′-GTCTCAGGCTT CAACATCG-3′; reverse 5′-GCATTAGGAATGC CATTGCG-3′). The probe was labelled with alpha 32-phosphorus deoxycytidine triphosphate (α-[P^{32}]dCTP; Izotop) using random prime labelling. After autoradiography, the percentage of deleted DNA was determined by densitometry (GeneTools software, Syngene). In addition to Southern blot analysis, PCR was used to identify and verify the general position of the deletion. The mtDNA primer sets described by Taylor et al. (2001) were used for this purpose.

The total DNA extracted from the patient's muscle and the muscle control DNA samples (n=18) were used to determine the relative mtDNA content. Relative mtDNA content was measured by real-time PCR and calculated using nuclear DNA as a normaliser. The primers and probe for the nuclear reference β-globin gene were 5′-GTGCACCTGACTCCTGAGGAGA-3′ (forward), 5′-CTTGATACCAACCTGCCCAG-3′ (reverse) and 5′-FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3′ (probe), synthesised by Metabion International. For mtDNA amplification, an MT-ND2 TaqMan® gene expression assay from Applied Biosystems was used (assay Hs02596874_g1). The PCR was performed with Applied Biosystems's ABI 7300 real-time PCR system in a 25 µl volume. Each reaction mixture contained 12.5 µl TaqMan® universal PCR master mix, No AmpErase® UNG (2×; Applied Biosystems), 0.5 µM forward and reverse primers, 0.2 µM probe for β-globin gene or 1 × dilution of primers/probe mixture for the MT-ND2 gene, and 10 ng of DNA. The PCR conditions were 10 min at 95°C, followed by 40 cycles of denaturation at 95°C and annealing/extension at 60°C for 1 min, with fluorescence measurement during this step. C_T values were calculated with 7300 System Sequence Detection software (version 1.4; Applied Biosystems). All reactions were performed in triplicate. Each assay also included a no-template control, three serial dilution points (in steps of five-fold) of a DNA mixture, and each of the test DNAs (patient and controls). In order to calculate the relative mtDNA content in the patient's DNA, C_T we exported the values obtained from real-time PCR analyses of the patient and control samples to REST© software (Relative Expression Software Tool; Pfaffl et al. 2002). PCR efficiency for each primer set was calculated by serial dilution using the REST software tool. The specific mtDNA fragment between positions 6113 and 11727 that contained the deletion was sequenced with an AB1 3130XL genetic analyser (Applied Biosystems) at Inqaba Biotechnical Industries in Pretoria, South Africa. DNA polymerase gamma (EC 2.7.7.7) subunit 1 gene (*POLG*) was sequenced, essentially as described by Nguyen et al. (2006).

Results

The results of the special clinical investigations revealed a right bundle branch block on the electrocardiogram (ECG). The findings of an ultrasound examination of the heart were within normal limits. The findings of an electromyogram (EMG) and nerve conduction studies were within normal limits. The MRI of the brain revealed normal basal ganglia, but bilateral well-circumscribed lesions in the cerebellar peduncles were demonstrated. Low signal intensity on T1-weighted images and high signal intensity on T2-weighted images were observed (Fig. 1). The intensity was mixed on the fluid-attenuated inversion-recovery (FLAIR) images, and restricted diffusion was found on diffusion-weighted imaging (DWI).

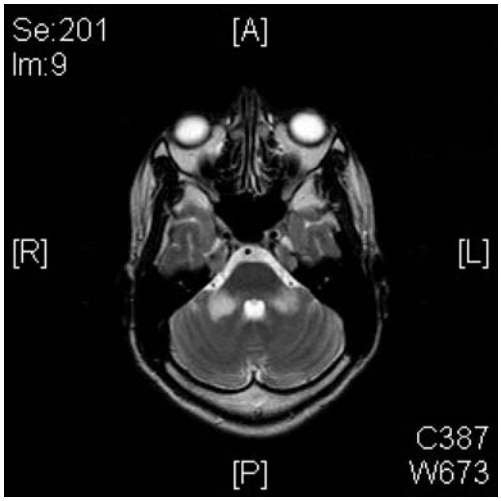

Fig. 1 T2-weighted magnetic resonance image demonstrating the high signal intensity, well-circumscribed lesions in the cerebellar peduncles

Urine analyses revealed the presence of elevated levels of lactic acid, elevated levels of Krebs cycle metabolites, a mild generalised amino-aciduria and glucosuria, but the glycohaemoglobin (HbA_{1C}) and serum glucose levels were within normal limits. Serum lactate was 4.2 mM and the lactate-to-pyruvate ratio was 31.3. Blood creatine kinase (EC 2.7.3.2) activity was initially 278 U/l, but it increased over a period of 18 months to 2,083 U/l. Human immunodeficiency virus (HIV) infection was excluded. The patient did not have any proven endocrinological abnormalities.

Muscle histology revealed ragged red fibres and mild lymphocyte infiltration (results not shown). Biochemical analyses of the muscle biopsy confirmed the suggestion of a mitochondrial disorder and were informative as to the deficiency that existed. From the data from respiratory chain and PDHc enzyme activity, which are summarised in Table 1, it was concluded that there was a combined deficiency of complexes I, II+III, and IV. BN-PAGE analysis of muscle provided additional information on the deficiency (Fig. 2). The OXPHOS complexes were already visible in the gel without additional staining, owing to the coomassie dye, which induced a charge shift of the proteins and which thus revealed the protein content of the complexes (Smet et al. 2005). By comparing the intensities of the OXPHOS protein bands between the lane loaded with the patient's muscle mitochondria and a control lane, we noticed decreased protein content of complexes I and IV and the V holo-complex (Fig. 2, left side of panel A). Consequently, following in-gel activity staining, decreased catalytic activity of those complexes could be demonstrated. The enzyme activities of complexes II and III seemed comparable. Two catalytically active complex V sub-complexes in the patient's sample were observed (Fig. 2, right side of panel A).

Using western blotting following 2D BN/SDS-PAGE, we observed a reduced signal for the complex I subunit (NDUFB8-20 kDa) and the complex IV subunit (COX II, 26 kDa) in the patient. Three different bands originating from the complex V alpha subunit were also detected. The signal intensities of the complex II subunit (Ip, 30 kDa) and the complex III subunit (core 2, 47 kDa) were, however, comparable between patient and control samples (Fig. 2, panel B).

Initial analysis of mtDNA structure (Fig. 3a, b) clearly revealed a deletion of ~3.4 kbp, which was present in 80% of the mtDNA copies of the patient's muscle biopsy according to the densitometry of various DNA dilutions. Further investigations revealed that the deletion occurred in an area between nucleotide positions ~6000 and ~12000 (Fig. 3c, d). It was also observed that the mtDNA copy number was notably higher (219%) than the average of that of controls ($n=8$). In addition, no pathogenic mutations were detected in the *POLG* gene. From sequencing data, the position of the deletion on mtDNA was determined to be present between positions 7115 and 10546, as shown in Fig. 4. This 3,431 bp deletion included five transfer RNA (tRNA) genes, two genes of complex I (*ND4L* and *ND3*), all three genes of complex IV (*CO I-III*) and both mitochondrially encoded genes of complex V (*ATPase6* and *ATPase8*). The deletion occurred immediately after, and on the 3′ end also included, the sequence ACACC.

Table 1 Respiratory chain and PDHc enzyme activities in the patient's muscle. Activities are expressed against several reference enzymes as shown: mU, nmol/min; UCS, μmol/min citrate synthase activity; PDHc, pyruvate dehydrogenase complex

Sample	Complex I			Complex II		Complex III			Complex II+III			Complex IV		PDHc	
	mU/UCS	CI/CII	CI/CIV	mU/UCS	CII/CIV	mU/UCS	CIII/CII	CIII/CIV	mU/UCS	CII+III/CII	CII+III/CIV	mU/UCS	CIV/CII	mU/mg	mU/UCS
Patient	150[a]	1.9[a]	12.0	79.2	6.3	392	4.95	31.2	75[a]	0.94[a]	5.94	13[a]	0.2[a]	42.6	101
Control range ($n=18$)	219– 372	1.9–4.5	2.1–7.1	72.8–149	0.78–2.1	327–787	2.97–9.32	5.04–14.5	117–234	1.14–2.2	1.28–3.67	43.1–124	0.5–1.3	4.0–96.5	7.8–104

[a] Indicates patient's values equal to or lower than the lowest control value

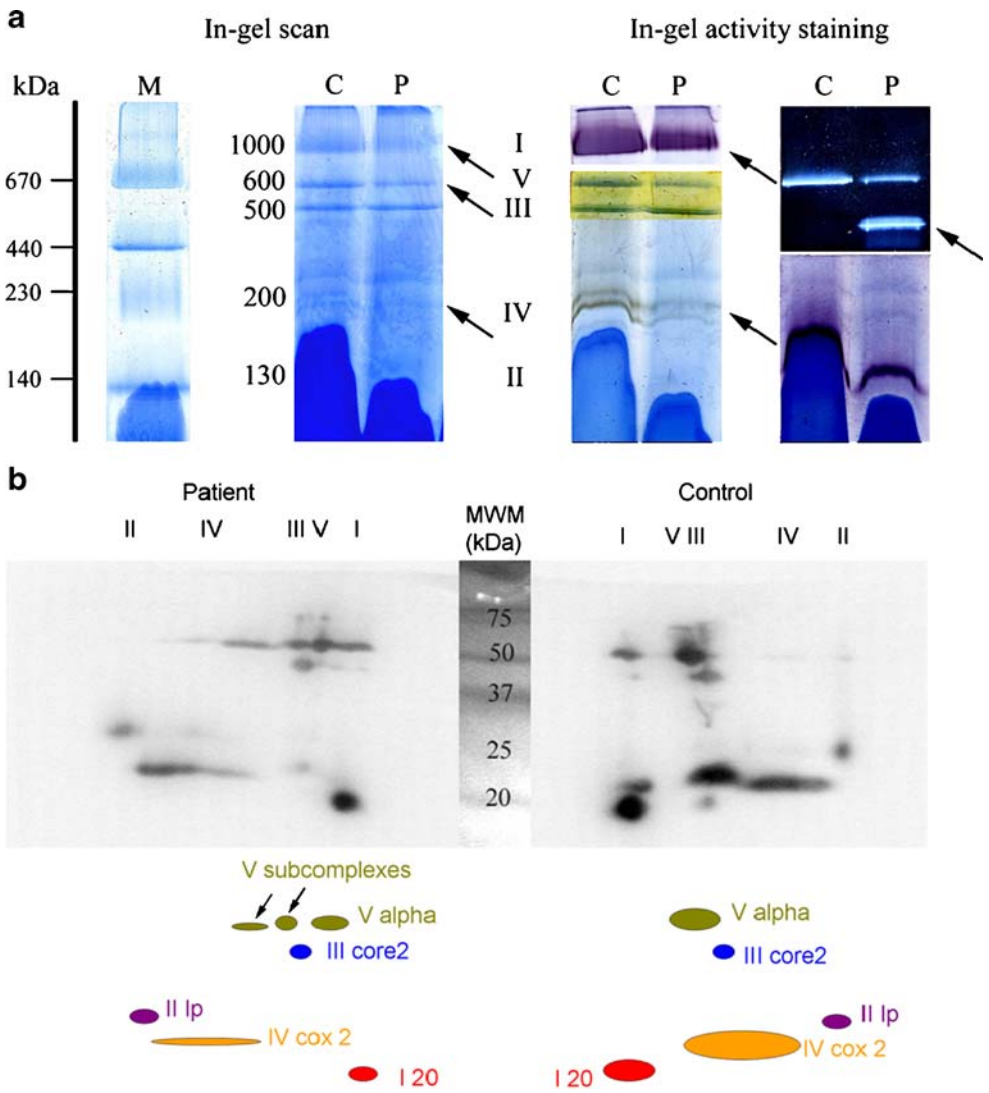

Fig. 2 BN-PAGE and 2D BN-PAGE western blotting. Panel A. Legend: (M) native molecular weight marker, (C) control muscle, (P) patient muscle. Left side: scan of the gel following BN-PAGE; arrows mark decreased amounts of OXPHOS complex proteins (I, IV and V) in the patient compared to those in the control sample. Right side: in-gel activity of the patient's muscle, indicating decreased activity of complexes I and IV, whilst the activities of complexes III and II are comparable to those of the control sample. Note the presence of catalytically active complex V sub-complexes, which are suggestive of disturbed intra-mitochondrial protein synthesis. Panel B. Immunostaining of OXPHOS proteins from patient and control samples following 2D BN-PAGE, using specific antibodies against subunits of the five OXPHOS complexes. MWM: molecular weight marker. The signals from the complex I subunit (NDUFB8, 20 kDa) and the COX II subunit are reduced in the patient in comparison with those of the control. The signals of the other OXPHOS subunits, complex III core 2 and complex II Ip, are comparable to those of the control. Notice the presence of two additional signals from the complex V alpha subunit, indicating the presence of two sub-complexes of complex V

Discussion

A patient of African origin with a novel deletion in his mtDNA has been described. The patient presented with external ophthalmoplegia, ptosis and retinitis pigmentosa resembling KSS. The MRI findings comprising normal basal ganglia and bilateral well-circumscribed lesions in the cerebellar peduncles (Fig. 1) might not comply entirely with the classical description of MRI findings in KSS, but there is limited correlation between the MRI findings and the neurological deficits in KSS (Chu et al. 1999; Lerman-Sagie et al. 2005). Bilateral lesions of high signal intensity on T2-weighted images of the globus pallidus and subcortical cerebral white matter are characteristic of KSS (Lerman-Sagie et al. 2005). Other common MRI findings include cerebral and cerebellar atrophy, bilateral lesions of high signal intensity in the thalamus, substantia nigra and brain stem (Leutner et al. 1994; Wray et al. 1995; Saneto et al. 2008). On the other hand, according to Barragan-Campos et al. (2005), basal ganglia involvement might not always be

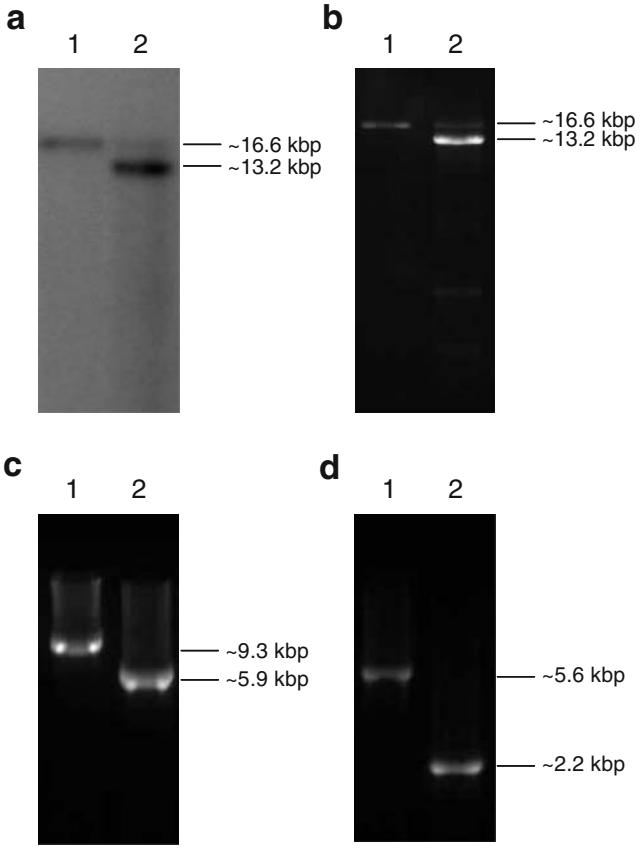

Fig. 3 Southern blot and PCR analysis of mtDNA. DNA of a healthy control (1) and the patient (2) was pre-treated with *Pvu*II and subjected to Southern blot analysis (a). PCR and ethidium bromide-containing gel electrophoresis using several primers were used to identify the region of deletion (b–d). The primers used were complementary to the following positions on the mtDNA genome: 37–58 (forward) and 16537–16558 (reverse), which amplified the complete mtDNA genome (b); 6113–6133 (forward) and 15431–15409 (reverse) in (c); 6113–6133 (forward) and 11748–11727 (reverse) in (d)

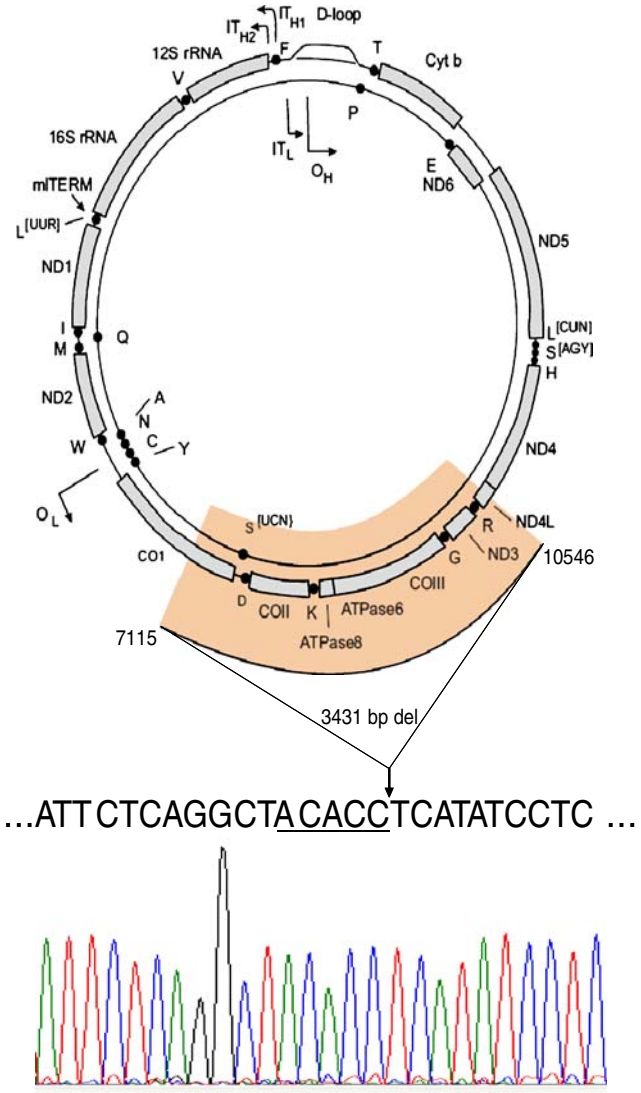

Fig. 4 Position of deletion in the patient's mtDNA. An electropherogram on the bottom indicates the position of the 3431 bp deletion, with the flanking repeat sequence underlined (the repeat sequence occurs at the 3′ end of the deletion as well). The position of the deletion on mtDNA and the genes involved are highlighted in the top part of the figure (adapted from Taanman 1999). Nucleotide positions are according to the Revised Cambridge Reference Sequence (GenBank J01415.2)

present. The white matter lesions can affect both the deep cerebral and cerebellar white matter (Lerman-Sagie et al. 2005). Heidenreich et al. (2006) described involvement of the cerebellar peduncles in their study of patients with chronic progressive external ophthalmoplegia (CPEO), including a patient with KSS. Bianchi et al. (2007) classify the MRI changes found in KSS as the syndromic type II pattern. The rest of the spectrum constitutes the type I pattern, which includes the more non-specific changes, and type III, the more leukodystrophy-like pattern.

A combined deficiency of OXPHOS complexes I, II+III, and IV was identified from the initial enzyme analyses. From our initial diagnostic analyses, which included a very simple but valuable PCR analysis to screen for mtDNA macro-structural changes, an mtDNA deletion was clearly present in this case, which required further investigation. The additional results obtained following BN-PAGE revealed reduced protein content and activities of complex I, IV and V. These findings were in agreement with the genes deleted from mtDNA in this patient. Both the protein content and activity of complex III, which, apart from absent tRNA genes, is not affected by the deletion, as well as nuclear-encoded complex II, were comparable to those of the control sample. Additionally, notable aspects of complex V synthesis and activity were demonstrated in the patient's sample. It was observed that the activity of complex V was reduced in the patient but that a catalytically active sub-complex of complex V existed. Some activity remained in the holo-complex, which might be attributed to the 20% wild-type mtDNA present in the patient. This finding points to disturbed intra-mitochondrial

protein synthesis, which can be attributed to the absence of the two mtDNA genes for complex V, *ATPase6 and ATPase8* (Carrozzo et al. 2006; Smet et al. 2009).

Several characteristics of the deletion should be noted. The position of this relatively small deletion (3,431 bp), present in 80% of mtDNA copies, has not previously been reported in the literature. The deletion break points are flanked precisely by direct repeats, which identify it as a class I deletion according to the classification by Mita et al. (1990). Deletions of this class are the most common and occur in ~60% of all known cases (Samuels et al. 2004). The reported deletion is located in the mtDNA region, where a high deletion frequency occurs. The 5′ end of the deletion (7115) falls within the 5′-end distribution range (5835 to 12112) that is common for most deletions, as reported by Mita et al. (1990). It also falls within the distribution range of around 8 kbp to 9 kbp (7832 bp to 8653 bp median values) for 5′ ends of mtDNA deletions, as reported by Samuels et al. (2004). However, the 3′ end of the reported deletion (10546) is not situated within the common 3′-end distribution range, which is mostly found in the region between 12661 and 15945. Samuels et al. (2004) reported that the distribution of 3′-end deletion has two peaks, which is different from the unimodular distribution for 5′ ends, consisting of a broad peak, centred on the median values of 13958 to 14643, and a sharp peak, at position 16000 to 16100.

The associated increased mtDNA replication observed is known to occur in patients with mtDNA deletions (Bai and Wong 2005). Although increased oxidative stress or oxidative damage was not measured in the tissue, mtDNA overexpression may occur through the reactive oxygen species (ROS)-mediated induction of mtDNA transcription and replication factors (Miranda et al. 1999; Lee and Wei 2005).

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