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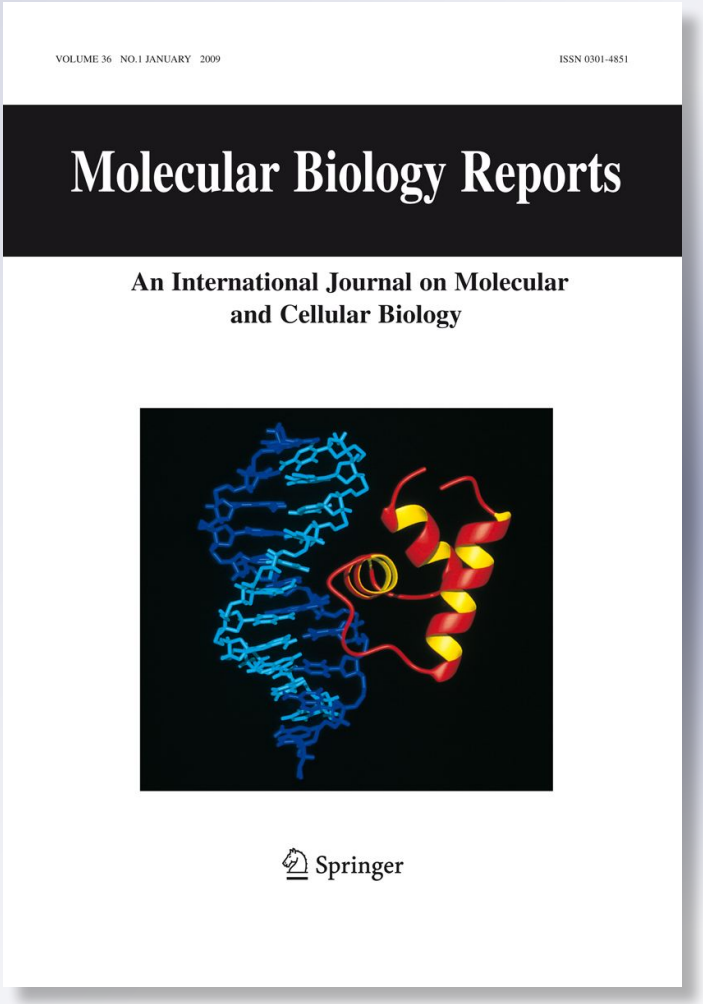

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Diagnosis of mitochondrial disorders applying massive pyrosequencing

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Abstract Mitochondrial disorders are a frequent cause of neurological disability affecting children and adults. Traditionally, molecular diagnosis of mitochondrial diseases was mostly accomplished by the use of Sanger sequencing and PCR–RFLP. However, there are particular drawbacks associated with the use of these methods. Recent multidisciplinary advances have led to new sequencing methods that may overcome these limitations. Our goal was to explore the use of a next generation sequencing platform in the molecular diagnosis of mitochondrial diseases reporting our findings in adult patients that present with a clinical-pathological diagnosis of a mitochondrial encephalomyopathy. Complete genomic sequences of mitochondrial DNA were obtained by 454 massive pyrosequencing from blood samples. The analysis of these sequences allowed us to identify two diagnostic pathogenic mutations and 74 homoplasmic polymorphisms, useful for obtaining high-resolution mitochondrial haplogroups. In summary, molecular diagnosis of mitochondrial disorders could be efficiently done from readily accessible samples, such as blood, with the use of a new sequencing platform.

Keywords Mitochondrial disorders · Genetics · Next generation sequencing

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M. A. Kauffman (✉) · D. González-Morón
Consultorio de Neurogenética. Centro Universitario de Neurología JM Ramos Mejía, Urquiza 609, 1221 Buenos Aires, Argentina
e-mail: marcelokauffman@marcelokauffman.info

M. A. Kauffman · S. Kochen
IBCN Eduardo de Robertis, Facultad de Medicina, UBA-CONICET, Paraguay 2155, 2do piso, 1121 Buenos Aires, Argentina

D. Consalvo · S. Kochen
Centro de Epilepsia, Centro Universitario de Neurología JM Ramos Mejía, Urquiza 609, 1221 Buenos Aires, Argentina

G. Westergaard · M. Vazquez · E. Mancini
INDEAR, Ocampo y Esmeralda, 2000 Rosario, Argentina

A. L. Taratuto
Departamento Neuropatología, FLENI, Montañeses 2325, 1428 Buenos Aires, Argentina

R. Rey
Centro Universitario de Neurología JM Ramos Mejía, Urquiza 609, 1221 Buenos Aires, Argentina

Introduction

Mitochondrial disorders are a frequent cause of neurological disability affecting children and adults. These pathologies could be caused by mutations in two genomes: the 16,569 bases circular mitochondrial genome (mtDNA) or the nuclear one [1]. There are particular features of mtDNA that distinguish it from the nuclear genome: (a) each cell has hundreds to thousands copies of mtDNA; (b) It is inherited only from the mother; (c) there could be a mixture of mtDNA populations in different proportions, where some harbor mutant alleles and some contain wild type sequences, a phenomenon called heteroplasmy [2]. Determining heteroplasmy levels is of importance in mitochondrial diseases diagnosis [3].

Traditionally, molecular diagnosis of mitochondrial diseases was mostly accomplished by the use of Sanger sequencing and PCR–RFLP [4]. However, there are

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particular drawbacks associated with the use of these methods. Sanger sequencing is comprehensive for detecting mitochondrial variants but it is not sensitive and precise enough to assess heteroplasmy [5]. On the other hand, PCR–RFLP is more sensitive and precise for assessing heteroplasmy but it is not comprehensive [6]. Recent multidisciplinary advances have led to new sequencing methods that may overcome these limitations [7]. Although there have been some reports on the use of this sequencing technology in mitochondrial genetics [8–10], its use in clinical diagnosis is still limited.

Our goal was to explore the use of a next generation sequencing platform in the molecular diagnosis of mitochondrial diseases reporting our findings in two adult patients that presented with a clinical-pathological diagnosis of a mitochondrial encephalomyopathy.

Materials and methods

Study population

Two adult patients with a Walker's criteria [11] definite diagnosis of respiratory chain disorder assisted at Neurogenetics Unit in Hospital Ramos Mejia from Buenos Aires, Argentina. Informed written consent from both patients was obtained. The study was approved by bioethics and institutional review board of Hospital Ramos Mejia.

Mitochondrial DNA sequencing

We purified genomic DNA from blood samples of both patients and additionally from a muscle sample in case 2, using a commercial kit from Promega Corporation following manufacturer instructions. For Roche 454-sequencing, we first amplified by means of three long-range PCR reactions that resulted in overlapping fragments of about 6, 5 Kb the whole mtDNA of both patients using Phusion® high-fidelity DNA polymerase (Thermoscientific, EEUU). PCR products were checked in agarose electrophoresis and thereafter purified by means of a commercial column-based purification system. We conducted Roche 454 FLX sequencing for the 2 cases at INDEAR institute, Rosario, Argentina. Using standard protocols [12], we ''shotgun'' sequenced the three mtDNA PCR fragments for each of the 2 cases using 2/8 of one PicoTiterPlate in one sequencing run.

We sequenced by Sanger method, PCR amplified hypervariable mtDNA regions and flanking regions where pathogenic mutations were identified in both samples in order to validate pirosequencing findings following standard protocols [13].

Bioinformatic analysis

We used the Roche 454 GS Mapper software (version 2.5.3) to assemble and compare the sequencing reads to the mtDNA reference sequence (NC_012920). We obtained and considered for analysis high confidence differences (HCDiffs) and those all differences that were found in more than 4% of reads and were visually validated using the Tablet multiple alignment viewer software [14]. The criteria for HCDiffs were defined by GS Mapper: variants detected in at least three unique (non-duplicate) sequencing reads of high quality with both forward and reverse reads and found in more than 10% of the total unique sequencing reads. Each variant was classified as pathogenic or as benign polymorphism by a search of MITOMAP database [15]. Each variant found in at least 96% of reads was considered homoplasmic. Heteroplasmy was quantified as the result of (n variant reads/n total reads) $\times$ 100. We discarded each indel variant that was associated with a homopolymer (4 to 8 bases) at or within four bases of the variant position since it was probably a well-recognized technical error associated with 454 pyrosequencing [10]. High resolution haplogroups were determined using PHYLOTREE [16].

Results

Case reports

Case 1

A 38 year-old male who, since his adolescence, suffered from recurrent and fluctuating depressive episodes, with symptoms of anxiety that required chronic psychiatry treatment. At age 25, he started to show progressive gait disturbances and limb girdle weakness. Thereafter, progressive bilateral hypoacusis, dysarthria and dysfagia were insidiously noticed. At time of consultation, his gait was severely impaired as a consequence of limb weakness and ataxia and he referred memory and concentration difficulties, showing depressive mood and moderate anxiety. His familial history was remarkable for a diagnosis of bipolar disorder in his twin sister and for a maternal cousin who had died of an epileptic encephalopathy at an early age. He never had seizures. Examination revealed an extensive axial lipomatosis, hypoacusis, atrophy and weakness in the proximal limb muscles, and ataxia. EMG revealed mixed myopathic and neurogenic abnormalities. Serum CK was reiteratively increased in more than 10 times normal values. MRI showed cerebellar and diffuse cortical atrophy without abnormalities in white matter signal. EEGs were normal. In the muscle biopsy, there were abundant COX

negative ragged red fibers. These fibers contained intramitochondrial paracrystalline inclusions. He died at age 40 from sepsis.

Case 2

A 44 year-old woman was assisted in our center presenting hemianopia noticed after a partial seizure. A stroke was initially suspected and antiaggregant treatment was initiated. However, her examination was remarkable for the presence of bilateral ptosis, papillary atrophy and bilateral proximal limb weakness besides the expected left-sided homonymous hemianopia; her family history revealed the antecedent of four brothers that died at an early age and several maternal relatives with ptosis; her MRI showed a hyperintense area on right temporo-occipital region that extended beyond posterior cerebral artery vascular territory. DWI/ADC sequences showed restriction of diffusion. Therefore, we considered a non-atherosclerotic etiology such as MELAS. EMG revealed mixed myopathic and neurogenic abnormalities. VEP showed bilateral prechiasmatic optic nerve dysfunction. EEG revealed bilateral occipital spikes over an organized background. CSF lactic acid levels were mildly elevated. In the muscle biopsy, there were abundant COX negative ragged red fibers. These fibers contained intramitochondrial paracrystalline inclusions. Sanger sequencing showed the 3243 A>G mtDNA mutation in muscle but not in blood.

mtDNA sequences

Roche GS FLX Titanium massive parallel sequencing of the LR-PCR material yielded 227,469 high-quality reads of an average length of 331.51 bases (SD 114.92) encompassing more than 75,000,000 high-quality bases. More than 92% of these reads properly mapped to mtDNA reference sequence which lead to a mean 454 depth of coverage (non-redundant sequences) of more than 350× per sample (367× and 368× for cases 1 and 2, respectively). Figure 1 shows total reads coverage (redundant and non-redundant sequences) plotted at each mtDNA position for each case. We obtained mean total reads coverage of 1,858× (range: 181–12,834) and 2245X (range: 676–19,406) for cases 1 and 2, respectively.

We identified 40 high confidence variants in case 1 sample (see Supplementary Table 1). All of them were homoplasmic but 8344 A>G which was heteroplasmic at 92% level. All of the homoplasmic variants were previously reported as benign polymorphisms in MITOMAP. On the other hand, the 8344 A>G variant is a well-recognized

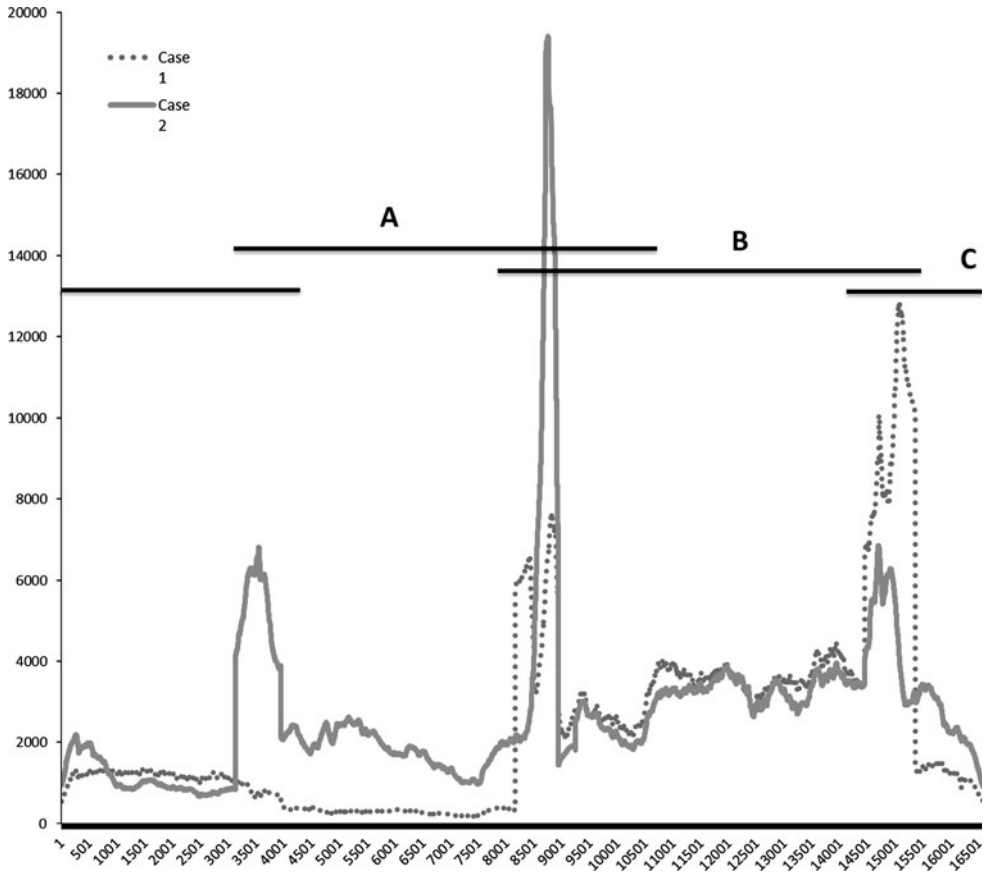

Fig. 1 Total read coverage (redundant and non-redundant) is plotted at each mtDNA position (1 to 16569) and graphed for each case (different line style for each case). The shape of the traces shows coverage variability both between cases and along the same mtDNA. The *black horizontal lines* (*A*, *B* and *C*) above the graph represent the three mtDNA PCR fragments used for 454 sequencing. Greater coverage was noted in the regions in which the PCR fragments overlap compared to coverage in non-overlapping regions

disease causing mutation. The validity of 21 of the 40 variants identified was investigated by Sanger sequencing. An absolute concordance between both sequencing methods was found. The phylogenetic analysis suggested that most mutations in the patient's mitochondrial genome seem to be W3haplogroup-specific.

The goal of analyzing mitochondrial genome in case 2 was to assess the sensitivity of 454 pyrosequencing for the detection of low levels of heteroplasmy in blood samples. First, we analyzed high confidence difference variants and found 35 variants that did not include 3243 A>G mutation (see Supplementary Table 1). However, a manual analysis of the multiple alignments led us to believe that this variant was indeed present but in very low levels of heteroplasmy (5%). See Fig. 2. As our motivation was to evaluate the use of this platform in clinical diagnosis, we applied an algorithm that could be suitable for use in a diagnostic scenario. We explored the all differences file selecting those variants present in at least 5% of reads and excluding technical errors identified by a manual visual analysis of multiple alignments (we discarded any variant that was present into reads harboring more than five variants). Thereafter, only variants that were reported as disease-causing in MITOMAP were considered for subsequent Sanger sequencing in other tissue (muscle in this case). Applying this algorithm, there were only two variants selected for subsequent analysis: 3243 A>G and 3093 C>G at 5 and 6% heteroplasmy levels, respectively. Sanger sequencing identified both variant in muscle tissue. We did not quantify heteroplasmic levels of these variants in muscle. Therefore, this algorithm proved to be sensitive and specific for a molecular diagnosis of this MELAS case. The validity of 15 of the 35 variants identified was investigated by Sanger sequencing. An absolute concordance between both sequencing methods was found. The phylogenetic analysis suggested that most mutations in the patient's mitochondrial genome seem to be R0 haplogroup-specific.

Discussion

This study shows the utility of GS-FLX 454 massive pyrosequencing platform in the molecular diagnosis of human mitochondrial diseases. This methodology demonstrated to have enough sensitivity to detect mtDNA mutations at low levels of heteroplasmy in a readily accessible tissue such as blood. This advantage over Sanger sequencing without compromise in accuracy supports a future use of new sequencing technologies in routine molecular diagnosis of mitochondrial disorders. A few recent works analyzed different next generation sequencing technologies in mitochondrial genetics [8, 9]. The goal of these studies was to assess technical validity of these methods for sequencing mitochondrial genomes. They mostly analyzed normal samples instead of pathological ones, with the exception of Zaragoza et al. [10] study which explored 454 pyrosequencing in mitochondrial cardiomyopathies diagnosis. Therefore, our report further supports 454 pyrosequencing uses in mitochondrial medicine molecular diagnosis.

Among the limitations of our work, we must mention that we excluded indel variants associated with homopolymers according to the low accuracy of 454 pyrosequencing for this type of variants observed by Zaragoza et al. [10]. However, we acknowledge that some of them could be real and erroneously discarded by us.

The clinical phenotype of our patients shows some noteworthy features. The first manifestations of disease in

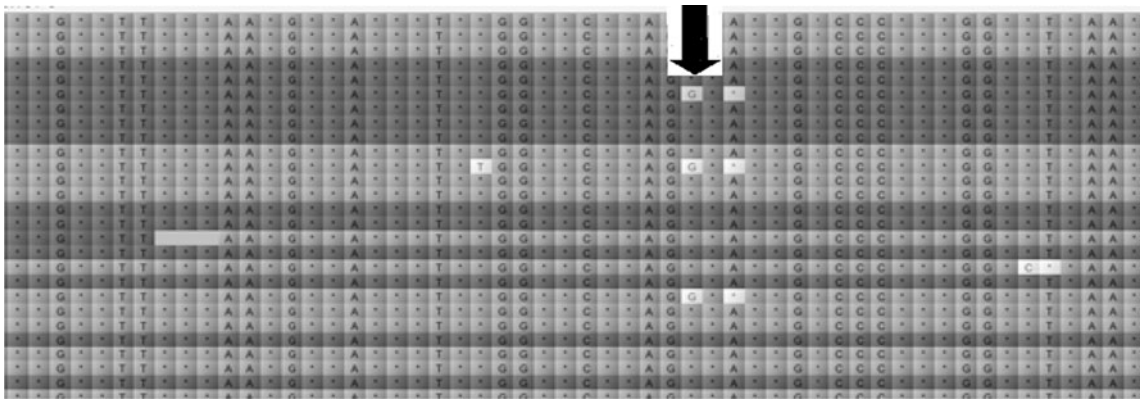

Fig. 2 Multiple alignment showing mtDNA 3243 A>G variant at low heteroplasmy levels. Capture of multiple alignment visualized by Tablet software. *Arrow* point out to 3243 A>G substitution

patient 1 were psychiatric disturbances. Mitochondria have been implicated in the pathophysiology of several neuropsychiatric disorders such as depression, anxiety, schizophrenia, autism, and Alzheimer's dementia [17]. However, the report of patients harboring mtDNA mutations presenting with psychiatric symptoms has been exceptional [18]. The 8344 A>G mutation in the $tRNA^{Lys}$ gene typically produces MERRF syndrome which characteristically includes myoclonus epilepsy, ataxia and myopathy with ragged red fibers [19]. However, Molnar et al. [20] reported on three patients carrying the 8344 A>G mutation who presented with mood disorders without suffering from epilepsy in a similar way to our patient. On the other hand, patient 2 presented with typical MELAS symptoms [21] but with an infrequent compromise of optic nerve. Although optic nerve could be affected in mitochondrial disorders, its affection in typical MELAS patients carrying 3243 A>G mutation is exceptional [22]. Furthermore, the 3093 C>G transversion discovered in patient 2 is another unusual finding in MELAS. So far, Hsieh et al. [23] have reported the only MELAS patient that carried the same two mutations found in our case which presented with an atypical MELAS syndrome.

In summary, molecular diagnosis of mitochondrial disorders could be efficiently done from readily accessible samples, such as blood, with the use of a new sequencing platform.

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Conflict of interest The authors declare that they have no competing interests.

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