The International Journal of Biochemistry & Cell Biology 75 (2016) 117–122

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Short communication

A unique combination of rare mitochondrial ribosomal RNA variants affects the kinetics of complex I assembly

Anna Maria Porcelli[a,b], Maria Antonietta Calvaruso[a], Luisa Iommarini[a], Ivana Kurelac[c], Roberta Zuntini[c], Simona Ferrari[c], Giuseppe Gasparre[c,*]

[a] Dip. Farmacia e Biotecnologie (FABIT), Università di Bologna, via Irnerio 42, 40126 Bologna, Italy
[b] Centro Interdipartimentale di Ricerca Industriale Scienze della Vita e Tecnologie per la Salute, Università di Bologna, 40100 Bologna, Italy
[c] Dip. Scienze Mediche e Chirurgiche (DIMEC), U.O. Genetica Medica, Pol. Universitario S. Orsola-Malpighi, Università di Bologna, via Massarenti 9, 40138 Bologna, Italy

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 8 April 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
Mitochondrial rRNA mutations
Bioenergetics
Ribosomal structure
Mitochondrial protein synthesis

ABSTRACT

Mitochondrial DNA (mtDNA) mutations in respiratory complexes subunits contribute to a large spectrum of human diseases. Nonetheless, ribosomal RNA variants remain largely under-investigated from a functional point of view. We here report a unique combination of two rare mitochondrial rRNA variants detected by serendipity in a subject with chronic granulomatous disease and never reported to co-occur within the same mitochondrial haplotype. *In silico* prediction of the mitochondrial ribosomal structure showed a dramatic rearrangement of the rRNA secondary structure. Functional investigation of cybrids carrying this unique haplotype demonstrated that the co-occurrence of the two rRNA variants determines a slow-down of the mitochondrial protein synthesis, especially in cells with an elevated metabolic rate, which impairs the assembly kinetics of Complex I, induces a bioenergetic defect and stimulates reactive oxygen species production. In conclusion, our results point to a sub-pathogenic role for these two rare mitochondrial rRNA variants, when found in the unique combination here reported in a single individual.

1. Introduction

Mitochondrial DNA (mtDNA) mutations are responsible for a large number of neuromuscular diseases and syndromes with a spectrum of different clinical phenotypes. Among common symptoms of mitochondriopathies, muscular weakness, cardiomyopathy, ocular pathologies and deafness are the most frequently reported (Carelli and Chan, 2014). Nowadays, most mtDNA-related diseases are well characterized from the molecular genetics point of view, and pathogenic mutations are well established, such as those causing Leber's Hereditary Optic Neuropathy (LHON, OMIM #535000) or the MELAS syndrome (Myoclonic Epilepsy with Lactic Acidosis and Stroke-like episodes, OMIM #540000), mapping in mitochondrial Complex I (CI) or tRNAs genes. Moreover, the m.1494C>T and m.1555A>G in *MT-RNR1* are well established pathogenic mutations for aminoglycoside-induced non-syndromic sensorineural hearing loss (OMIM #500008) (Prezant et al., 1993; Zhao et al., 2004). Beside these, a wide number of mutations in both *MT-RNR1* and *MT-RNR2* genes have been reported in association with various pathological phenotypes, but their pathogenic potential is controversial or not clearly established. In fact, only few functional studies have been carried out in order to explain the mechanism by which such mutations may exert a pathogenic phenotype (Zhao et al., 2004) and a scoring systems for the prediction of pathogenicity has been set up (Smith et al., 2014).

Chronic granulomatous disease (CGD) is caused by defects in the subunits of the NADPH oxidase complex, the main cellular source of reactive oxygen species (ROS). The disorder is due to an insufficient production of ROS in the vacuoles of phagocytes, impairing the ability to kill the microbes. Clinical presentations of CGD vary in severity from relatively mild recurring infections to deep abscesses and septicaemia. In the context of a preliminary study in which we explore the potential contribution/association of mtDNA lesions to the degree of severity and variable expressivity of CGD caused

Abbreviations: mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; CI, respiratory complex I; LHON, Leber's Hereditary Optic Neuropathy; MELAS, Myoclonic Epilepsy with Lactic Acidosis and Stroke-like episodes; 2D BN/SDS-PAGE, two dimensional Blue Native sodium-dodecyl sulphate polyacrylamide gel electrophoresis; HmtDB, Human Mitochondrial DataBase; 1D BN-PAGE, first dimension Blue Native sodium-dodecyl sulphate polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

* Corresponding author at: Dip. Scienze Mediche e Chirurgiche—DIMEC, U.O. Genetica Medica—Pad.11, Pol.S.Orsola-Malpighi, via Massarenti 9, 40138, Bologna, Italy.
E-mail address: giuseppe.gasparre@gmail.com (G. Gasparre).

http://dx.doi.org/10.1016/j.biocel.2016.04.007
1357-2725/

by germ line mutations in the *CYBB* gene encoding the gp91Phox subunit of the NADPH oxidase complex, we came across a unique mtDNA haplotype of a subject affected with the disease. Aside of the association with CGD, we decided to investigate in depth these variants due to their peculiar combination found in mtDNA.

2. Materials and methods

2.1. Genetic analyses

A 4-year-old boy was referred to our genetics unit for a suspected CGD. He was the only child born to non-consanguineous parents, with no relevant family history. Since two years of age, the patient presented with recurrent respiratory infections, recurrent episodes of *S. aureus* infected dermatitis in different body districts, blepharitis, chorioretinitis and retinal detachment of the right eye. At the time of diagnosis, he was admitted to hospital with lobar pneumonia not responsive to antibiotic treatment, persistent mediastinal lymphadenopathy and a hepatic abscess. Genetic analysis of the *CYBB* gene showed the presence of the maternally inherited Val104Leu amino acid change, never described before and predicted to be damaging by PolyPhen-2. The patient was treated with allogeneic bone marrow transplantation and completely recovered. This study was prospectively reviewed and approved by a duly constituted ethics committee. The mtDNA sequences, obtained as previously reported (Guerra et al., 2011), were analyzed by applying the classifier tool available through the Human Mitochondrial DataBase (HmtDB) (www.hmtdb.uniba.it; (Rubino et al., 2012)). An alignment with the revised version of the Cambridge Reference Sequence (rCRS) was performed. The subject mtDNA sequence obtained, belonging to haplogroup T2, was deposited in public HmtDB with identifier PA_EU_IT_0275. Variant sites were compared with the most up-to-date human mtDNA haplogroup classification as annotated in PhyloTree (van Oven and Kayser, 2009). *In silico* rRNA structural prediction was performed using RNA structure, version 5.3 (Reuter and Mathews, 2010).

2.2. Cybrids generation

Platelets from the subject bearing the two homoplasmic variants m.1452T>C/*MT-RNR1* and m.2397C>T/*MT-RNR2* were used as mitochondria donors and the mtDNA-deprived human osteosarcoma 143B.TK$^-$ cell line (Rho0 cells) as acceptor (King and Attardi, 1996). Multiple clones of two different control cybrid lines were generated from mitochondria obtained from two healthy control subjects (CC1 and CC2) and from the subject (CP1-4). Cells were grown in Dulbecco's modified Eagle medium (DMEM high glucose) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin, in an incubator with a humidified atmosphere of 5% CO2 at 37 °C.

2.3. In vitro *mitochondrial translation assay*

Translation of mtDNA encoded proteins was assayed by ^{35}S-methionine labelling (Chomyn, 1996). Briefly, cells were incubated for 15 min with methionine/cysteine-free DMEM, which was then replaced with 1 ml of methionine-free DMEM supplemented with 96 μg/ml cysteine and 100 μg/ml emetine dihydrochloride. Labelling was performed for 60 min after addition of 166 μCi/ml [^{35S}]-methionine. Protein samples (30 μg) were separated on 10–20% SDS–PAGE gels, which were then stained with Coomassie Brilliant Blue and scanned.

2.4. Western blotting

Mitochondria-enriched fractions were obtained by subcellular fractionation (5–10 × 10^6 cells) in presence of digitonin (50 μg/ml) (Ghelli et al., 2013). Mitochondrial proteins (40 μg) were solubilized using 2% *n*-dodecyl ß-D-maltoside (DDM), and separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The latter was incubated overnight at 4 °C with antibodies against ND1 (1:500, a kind gift from A. Lombes), NDUFA9 (1:1000, Mitosciences), NDUFS3 (1:1000, Mitosciences), NDUFV1 (1:1000, Abcam), NDUFB8 (1:1000, Invitrogen), CORE2 (1:1000, Mitosciences), COXIV (1:1000, Mitosciences) and SDHA (1:10000, Mitosciences). Primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000, Jackson ImmunoResearch). Chemiluminescence signals were acquired with Gel Logic 1500 molecular imaging apparatus (Kodak).

2.5. First dimension blue native-PAGE (1D BN-PAGE)

Mitochondrial enriched fractions were suspended in 750 mM aminocaproic acid, 50 mM Bis-Tris (pH = 7) buffer and solubilized by adding DDM/protein ratio of 2.5 (g/g). Aliquots of supernatants (100 μg protein) were separated by 4–15% 1D BN-PAGE, transferred onto nitrocellulose membrane and immunoreactivity for different subunits of respiratory complexes was detected in western blotting as described above (Ghelli et al., 2013).

2.6. Cell viability assay in galactose medium

Cells were seeded in 24-well plates (3 × 10^4 cells/well) in DMEM high glucose medium. After 24 h cells were incubated with glucose-free DMEM supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin, 5 mM galactose and 5 mM Na-pyruvate. Cell viability was measured by the detection of sulforhodamine B absorbance at 570 nm (Porcelli et al., 2009).

2.7. Mitochondrial ATP synthesis

The rate of mitochondrial ATP synthesis was measured in digitonin-permeabilized cells as previously described (Ghelli et al., 2013). The chemiluminescence signal was calibrated with an internal ATP standard after the addition of 10 μM oligomycin. The rates of the ATP synthesis were normalized to protein contents and citrate synthase (CS) activity.

2.8. Hydrogen peroxide production

To determine the H_2O_2 production, cells were incubated with 2 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) as detailed elsewhere (Iommarini et al., 2014). Briefly, 4 × 10^4 cells were seeded in 24 wells plates in DMEM high glucose. After 48 h, medium was removed, cells were washed twice in PBS and incubated with 1X Hank's Balanced Salt Solution (HBSS) without phenol red supplemented with 5.5 mM glucose or 5 mM galactose. Fluorescence was detected at 535 nm using a multilabel counter Victor^3 (Perkin Elmer) at multiple time points.

2.9. Two-dimensional blue native-PAGE (2D BN-PAGE)

Lanes excised from 1D BN-PAGE gradient 4–15% were treated with denaturing buffer containing 1% SDS and 0.1% β-mercaptoethanol for 90 min and then separated on 10% SDS-PAGE