Coexistence of mutations in PINK1 and mitochondrial DNA in early onset parkinsonism

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ABSTRACT

Aims and background: Various genes have been identified for monogenic disorders resembling Parkinson’s disease. The products of some of these genes are associated with mitochondria and have been implicated in cellular protection against oxidative damage. In the present study we analysed fibroblasts from a patient carrying the homozygous mutation p.W437X in the PTEN-induced kinase 1 (PINK1), which manifested a very early onset parkinsonism.

Results: Patient’s fibroblasts did not show variation in the mtDNA copy number or in the expression of the oxidative phosphorylation complexes. Sequence analysis of the patient’s mtDNA presented two new missense mutations in the ND5 (m.12397A>G, p.T21A) and ND6 (m.14319T>C, p.N119D) genes coding for two subunits of complex I. The two mutations were homoplasmic in both the patient and the patient’s mother. Patient’s fibroblasts resulted in enhanced constitutive production of the superoxide anion radical that was abrogated by inhibitor of the complex I. Moreover enzyme kinetic analysis of the NADH:ubiquinone oxidoreductase showed changes in the substrates affinity.

Conclusion: To our knowledge, this is the first report showing co-segregation of a Parkinson’s disease related nuclear gene mutation with mtDNA mutation(s). Our observation might shed light on the clinical heterogeneity of the hereditary cases of Parkinson’s disease, highlighting the hitherto unappreciated impact of coexisting mtDNA mutations in determining the development and the clinical course of the disease.

Parkinson’s disease (PD) is the second most common age related neurodegenerative disorder. Although for most cases of classical PD the aetiology is unknown, a clear genetic component has been determined in a minority of familiar cases. Noteworthy, defects in some of the PD related nuclear genes impinge directly or indirectly on mitochondrial function, causing fatal dysfunction of dopaminergic neurons. Consistently, mutations in mtDNA are reported in sporadic cases of PD and neurons appear more sensitive than other cells to mutations in genes encoding mitochondrial proteins.

PTEN-induced kinase 1 (PINK1) is a serine/threonine kinase that co-localises with mitochondria and provides a protective function, albeit not yet mechanistically defined, against stressors causing cell death. So far, TRAP1 (also known as HSF75) is the only recognised substrate of PINK1. In addition, the protease HtrA2 has also been shown to be regulated by PINK1 but through an indirect phosphorylation mechanism. Both TRAP1 and HtrA2 are involved in the control of apoptosis, given that their PINK1 dependent phosphorylation contributes to an increased resistance of cells to mitochondrial stress.

Mutations disseminated along the PINK1 gene have been found in autosomal recessive forms and some sporadic cases of PD hallmarkied by an early onset. Notably, even if the heterozygous state of subjects carrying PINK1 mutations, in the majority of the cases, does not evolve toward the disease, in some other documented cases it does, albeit with a later onset. This would suggest that the complete or even partial loss of PINK1 function may promote the neurodegenerative disorder in combination with other acquired or inherited defect(s).

The aim of the present study was to characterise the genotype and phenotype of samples from a patient carrying a homozygous defect in PINK1 with a very early onset of the disease. Along with an extensive analysis of the mitochondrial oxidative phosphorylation (OXPHOS) system we sequenced the mtDNA of the patient, finding unreported homoplasmic single nucleotide changes resulting in amino acid substitution of complex I subunits and functional alterations.

PATIENT AND METHODS

Patient

The patient with the homozygous W437X missense mutation in PINK1 received the diagnosis of parkinsonism at 22 years of age. The clinical description of the family’s pedigree, provided in the report first describing the case, was consistent with an autosomal dominant transmission of the disease.

Cell culture

Dermal fibroblasts were isolated from explanted patient’s biopsy specimen and grown in low glucose (5 mM) Dulbecco’s modified Eagle’s medium (DMEM, EuroClone) supplemented with 10% fetal bovine serum, plus 2 mM glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin. Adult normal human dermal fibroblasts (NHDF, Cambrex CC-2611) were used as control. The growth rates of NHDF and patient’s fibroblasts were comparable (doubling time ~24 h). Comparison of the functional features of control and patient’s fibroblasts was made under comparable culture passage number and confluence.

Analysis of the mtDNA/nDNA ratio

Quantitative polymerase chain reaction (PCR) was performed on DNA extracted from the fibroblast...
samples using iQ SYBR Green Supermix (Biorad, Hercules, California, USA) on a Bio-Rad iCYCLER iQ Real Time PCR Instrument. Primers for mtDNA were 5'-ACGACCTCGA TTGTGGATC-3' (for) and 5'-GCTCTGCCATCTTAACAAACC-3' (rev) (263 bp product, comprising part of the 16S rRNA and of the tRNAleu, from nucleotide position 2981 to 3246); those for the 28S rRNA gene were 5'-TTAAGGTAGCCAAATGCCTCG-3' (for) and 5'-CCTTGGCTGTGGTTTCGC-3' (rev) (102 bp product). To rule out different primers dependent PCR amplification efficiency, absolute quantification was preferred to relative comparison between mitochondrial and nuclear DNA amplicons. To this aim a standard curve based method was applied. PCR products from NHDF DNA extract for 28S and mtDNA were ligated into pGEM-T Easy vector (Promega, Leiden, The Netherlands) and trans- 
formed in DH5α competent cells (Invitrogen, Carlsbad, California, USA). The isolated plasmid DNA was quantified spectrophotometrically and fivefold serial dilutions performed to give final concentrations between 10^7 and 10^3 copies for both 28S rRNA and mtDNA. The resulting CT copy number calibration curves; 110 μg of proteins were loaded for both NHDF and PINK1 samples. CI, CV, CII, CIV, CIL indicate the OXPHOS complexes resolved in their subunit pattern in the 2nd dimension. Representative of three independent assays. (C) Immunoblotting analysis on 50 μg total protein extract from control and patient’s fibroblast lysates with mAbs against the indicated OXPHOS complexes subunits and β-actin. Representative of western blotting repeated twice.

mdDNA sequencing

A set of 43 primer pairs was used to amplify the entire mitochondrial genome. DNA amplification was performed by standard procedures. The overlapping PCR products were purified using GFX PCR purification kit (Amersham- Pharmacia Biotech, Piscataway, New Jersey, USA) and bidirectional sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, UK) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). The sequence of the patient’s mtDNA was compared to the normal “Cambridge sequence” and deviations from it analysed using MitoAnalyzer (http://www.mitomap.org). Genetic alterations were checked against databases of previously reported polymorphisms maintained on the same website.

Enzymatic assay

NHDF:ubiquinone oxidoreductase activity was measured as described. Briefly, ultrasound treated fibroblasts were suspended at a concentration of 0.5–1×10^6 cells/ml in 40 mM potassium phosphate buffer (pH 7.4) and 5 mM MgCl2 supplemented with 3 mM KCN. Complex I activity was assessed following spectrophotometrically (ε_{340nm} = 6.22 mM^-1 cm^-1) the rotenone sensitive oxidation of NADH by decylubiquinone. V_{max} and K_m were determined from Lineweaver–Burk plots by least square analysis of the datasets.

Confocal microscopy imaging of mitochondrial O_2^- production in live cell

Cells cultured at low density on fibronectin coated 35 mm glass bottom dishes were incubated for 20 min at 37°C with 0.5 μM MitoSOX (Molecular Probes, Eugene, Oregon, USA). Stained cells were washed with phosphate buffered saline (PBS) and examined by a Nikon TE 2000 microscope (images collected using a 60× objective (1.4 NA)) coupled to a Radiance 2100 confocal scanning microscopy system (Biorad) using the He-Ne laser beam (543 nm). Confocal planes (18–20) of 0.2 μm in thickness were examined along the z axes. Acquisition, storage and analysis of data were made by LaserSharp and LaserPix software (Biorad) or ImageJ.

Electrophoretic procedures and antibodies

Two dimensional gel analysis (blue native PAGE/SDS-PAGE) of mitoplasts from NHDFs or patient’s fibroblasts was carried out as described. Western blotting: proteins from cell lysate were separated by a 12% PAGE-SDS Laemmli Protocol and blotted on
PVDF membrane by standard procedure; mouse mAbs (Molecular Probes, Eugene, Oregon, USA) were used at appropriate dilutions; staining was performed by enhanced chemiluminescence under not limiting detecting secondary antibodies.

Statistical analysis
Two tailed Student’s t test was applied, with a \( p < 0.05 \), to evaluate the significance of differences measured throughout the datasets reported.

RESULTS
The case of a patient affected by unusually early onset parkinsonism was selected. The patient carried the homozygous mutation p.W437X in PINK1, resulting in the C-terminus truncated form of the wild-type kinase with partial loss of function. Results of an extensive functional analysis showed that, when compared with normal human dermal fibroblasts, those isolated from the patient displayed a significant 30% inhibition of the KCN sensitive endogenous respiration but no detectable change in the specific activity of any of the single mitochondrial respiratory chain complexes. The amount of cytochrome \( c \) was, however, notably reduced in patient’s fibroblasts and the level of constitutively generated hydrogen peroxide was fivefold higher as compared with normal fibroblasts. Mitochondrial dysfunction and intracellular redox state alteration have been reported to affect the rate/efficiency of mtDNA replication. Therefore, we first determined by quantitative PCR the absolute value of the mtDNA copy number per cell. Fig 1A shows that normal and patient’s fibroblasts had a comparable content of mtDNA amounting to around 10\(^3\) mtDNA copy number/cell. Two dimensional blue native/SDS PAGE on mitoplasts and immunoblotting detection on cell lysate of constituent subunits of the oxidative phosphorylation (OXPHOS) complexes showed no detectable

Figure 2 Mutational analysis of the patient’s mtDNA. (A) Human mtDNA map showing the location of the single nucleotide changes (SNCs) found in this study. (B) mtDNA sequence electropherograms highlighting the m.14319T>C and m.12397A>G mutations in the ND6 and ND5 genes, respectively. (C) Prediction of the membrane structure of the wild-type and patient’s mutated ND6 subunit of complex I. Transmembrane protein topology of wild-type (top panel) and N119D mutated (bottom panel). ND6 was attained with a hidden Markov model using the online service at http://cbs.dtu.dk/services/TMHMM/.
NADH was kept constant at 15 mM substrate concentration. In a set of measurements the concentration of panel). The values reported are averages of two measurements at each from 5–200 mM was 200 mM. 

ND5 subunit (in a hydrophilic segment likely exposed to the mutation changes a threonine in alanine at position 21 of the J Med Genet 2008; 599:45:596–602. doi:10.1136/jmg.2008.058628

Figure 3 Measurement of NADH:ubiquinone oxidoreductase enzymatic activity. Enzyme kinetic analysis of control and patient’s NADH:ubiquinone oxidoreductase assayed by double reciprocal plots of the activity measured as a function of UQ (top panel) or NADH (bottom panel). The values reported are averages of two measurements at each substrate concentration. In a set of measurements the concentration of NADH was kept constant at 15 mM and that of decylubiquinone varied from 5–200 μM; in the other set, the concentration of decylubiquinone was 200 μM and that of NADH varied from 3.5–15 μM.

The m.14319T>C mutation changes an asparagine in aspartic acid at position 119 of the ND6 subunit. This mutation is located in the fifth of six predicted transmembrane helices (TMH). A comparison of the hydropathy plot between the ND6 wild-type and the p.119N>D mutant resulted for the latter in a pronounced destabilising effect on the TMH-V (ΔΔG = −0.6) (fig 2C).

Figure 3 shows the enzyme kinetic analysis of the complex I carried out on mitoplasts from cell lysates of normal and patient’s fibroblasts. The results indicate an almost fivefold decrease of the apparent Km for both UQ2 and NADH in the patient’s sample. Conversely, the estimated maximal rates of complex I were practically unchanged when UQ2 was used as limiting substrate, whereas a 26% inhibition of the Vmax was observed in patient’s fibroblasts titrated with NADH.

Figure 4 illustrates the result of a confocal microscopy analysis aimed to detect production and localisation of reactive oxygen species (ROS) in the patient’s fibroblasts. As the seminal ROS, produced at the level of the mitochondrial respiratory chain complexes, is constituted by the superoxide anion O2− we used the mitochondrial specific O2− probe MitoSox. The patient’s fibroblasts displayed a constitutive significant twofold increase of the probe related fluorescence when compared with NHDFs. Magnification of the images of patient’s fibroblasts showed, as expected, a spotted distribution of the fluorescence consistent with the accumulation of the probe in the mitochondria. Notably the MitoSox fluorescent signal was completely abrogated by pre-treatment of the patient’s fibroblasts with the inhibitor diphenyleneiodinium (DPI) at a concentration known to bind irreversibly to the prosthetic flavin moiety of the mitochondrial complex I.

DISCUSSION

The onset of the clinical manifestation of the parkinsonian phenotype in the patient in this study was at age 22 years. In the seven previously described cases, homozygotes for the same PINK1 mutation, it was in the fourth to fifth decade. Although not necessary for the development of the disease, PINK1 heterozygous mutations in PD affected patients have also been reported. The patient’s parents recapitulated the heterogeneous outcome of the heterozygosis state. Indeed, the patient’s father examined at age 79 years did not show any sign of neurological disorder, whereas the mother was affected by the disease albeit at 53 years of age. These elements would imply the involvement of other hits contributing, along with the PINK1 mutation, to the development and severity of the disease.

The main outcome of this study is the finding of two new SNCs in the mtDNA from the PD affected patient. Both mutations are missense and fall into two of the seven mitochondrial encoded subunits (ND) of the complex I. Complex I is the entry port of reducing equivalents into the mitochondrial respiratory chain catalysing the proton pumping linked transfer of electrons from NADH to ubiquinone. It contains an FMN molecule and seven iron–sulphur clusters which are embodied in an extra-membrane peripheral arm. Electrons are transferred from NADH via FMN to the metal redox pathway and thereby to ubiquinone which is located in the membrane embedded part of the complex. More than one molecule of ubiquinone are thought to be bound to complex I, which exchange reducing equivalents before reducing a ubiquinone molecule in equilibrium with the membranous pool. This electron transfer is prone to diversion leading to direct one-electron reduction of O2− with formation of O2 thereby change in their compositional pattern and content between patient and control fibroblasts (fig 1B,C).

Next we decided to sequence the mtDNA of the patient’s fibroblasts. A number of single nucleotide changes (SNCs) were detected disseminated through the entire mtDNA (fig 2A). All but two were already reported as known polymorphisms (http:\www.mitomap.org\). The two unknown SNCs were m.12397A>G and m.14319T>C in the ND5 and ND6 genes, respectively. Notably, both were homoplasmic (fig 2B) and confirmed in a different batch of the originally isolated pool of fibroblasts from the patient’s biopsy and in DNA extracted from a patient’s blood sample. Both the ND5 and ND6 mutations were confirmed in the homoplasmic state in the mtDNA of a patient’s fibroblasts and in DNA extracted from a patient’s biopsy and DNA extracted from a patient’s blood sample. Both the ND5 and ND6 mutations were confirmed in the homoplasmic state in the mtDNA of a patient’s mother blood sample. Conversely, the same analysis carried out on a patient’s father blood sample resulted in a normal ND5 and ND6 genotype. Collectively these results demonstrate that the homoplasmic mtDNA mutations found in the patient coexisted with the PINK1 mutation at birth and were inherited by the mother, which carried the same homoplasmic mtDNA mutations.

ND5 and ND6 are two of the seven mtDNA encoded, membrane embedded, subunits constituting NADH:ubiquinone oxidoreductase (complex I). The m.12397A>G missense mutation changes a threonine in alanine at position 21 of the ND5 subunit (in a hydrophilic segment likely exposed to the inter-membrane mitochondrial space).
accounting for most of the constitutive ROS generated by the mitochondrial respiratory chain. The sites in complex I of electron detour have been proposed, by time, to be one of the iron–sulphur centres or the reduced or semi-reduced radical species of FMN or UQ10. All these redox active centres have the potential to react with O2 and it is likely, depending on physiological conditions or specific pathological modifications of the complex I, that each of them may contribute to ROS generating activity. The function of each ND subunit in the membrane domain of complex I is incompletely understood. However, biochemical studies suggest the involvement of ND5 and ND6 in the proton pumping activity and/or in the binding of ubiquinone.

The m.12397A>G missense mutation causes an amino acid substitution (p.21T>A) in the N-terminus of ND5. Interestingly, a mutation leading to p.8T>A in the same subunit has been described in two patients affected by PD. The m.14319T>C mutation causes substitution of a polar to charged amino acid (p.119N>D) in the TMH-V of the ND6 subunit. ND6 is a hot spot for mutations associated with the Leber's hereditary optic neuropathy (LHON). A mutation in a nearby position (p.117N>D) has been reported in one case of idiopathic LHON. Moreover, structural prediction by homology based modelling and computational chemistry indicate large conformational changes of the TMH-V as a consequence of distally located LHON linked mutations in the same subunit.

The observed changes in the Km of the substrates for the patient’s NADH:ubiquinone oxidoreductase activity would argue in favour of subtle alterations in the catalytic features of the enzyme. This, without compromising the overall electron transfer throughout the mitochondrial respiratory chain, may cause nevertheless substantial enhanced production of ROS as shown here by the DPI sensitive MitoSox oxidising activity of the patient’s fibroblast mitochondria. It would be tempting to speculate that the higher affinity to ubiquinone of the patient’s complex I predisposes to a longer occupancy of the reduced quinone or of the semiquinol radical species in the substrate binding site. This in turn might cause enhancement of the

**Figure 4** Confocal microscopy analysis for mitochondrial superoxide anion generation. Control and patient’s fibroblasts (NHDF and PINK1, respectively) were treated with 5 µM of MitoSox as indicated in the text. When indicated the patient’s fibroblasts were pre-treated for 2 h with 100 µM diphenyleneiodinium (DPI) (DPI treatment of NHDF was ineffective on the probe fluorescence). Bar: 20 μm. The histogram on the right indicates the fluorescence intensity values (SEM) from 10 randomly selected fields and normalised to the value of the NHDF sample. Representative of four independent analyses. Statistical significance was assessed by Student’s t test and the p value indicated.

**Figure 5** Working model for the combined effect of PINK1 and mtDNA mutations in the PD progress. The symbols −/−, +/-, +/- refer to the homozygosis, heterozygosis and wild-type conditions of the mutated PINK1 gene, respectively: “−” and “−” refer to the absence or presence of inherited/acquired mtDNA mutation(s). The double arrow delimitates the range of age where most of the idiopathic forms of Parkinson’s disease start to be clinically manifest. See text for further detail of the model.
reduction potential in redox centres located upstream of the final reducing equivalent acceptor. Alternatively, the increased electron transfer efficiency of complex I might increase the \( \frac{QH_2}{Q} \) ratio of the membranous ubiquinone pool. In either cases, the direct single electron reduction of \( O_2 \) can take place.\(^{19,20}\)

It is well established that exposure of rats to the complex I inhibitors rotenone and 1-methyl-4-phenylpyridinium (MPP\(^+\)) causes oxidative stress and reproduces features of PD, including selective nigrostriatal dopaminergic degeneration and \( \alpha \)-synuclein positive cytoplasmic inclusions.\(^{20,21}\) Moreover, it is worth mentioning that the anti-apoptotic proteolytic activity of HtrA2, shown to be downregulated in PINK1-mutated PD patients,\(^6\) is influenced by the interaction with GRIM19,\(^{20}\) a bona fide subunit of complex I,\(^{11}\) thereby establishing an intriguing linkage between PINK1 and complex I dysfunctions.

The results here presented suggest that the heterozygosis for PINK1 mutation when accompanied with germlinal or somatic mutation(s) in the mtDNA, possibly associated with enhanced mitochondrial ROS production, could predispose to the development of the disease once a threshold level for compensatory mechanisms in dopaminergic neurons has been overcome\(^{22}\) (fig 5). This might have been the case of the patient’s mother in our study. If the heterozygosis for PINK1 mutation is, or remains, the only altered genetic carrier in the patient, this would probably not result in PD as in the case of the patient’s father. In the homozygous condition a defective PINK1 linked protection to oxidative damages by ROS, which are physiologically produced during ageing, may accelerate a vicious cycle causing overwhelming mitochondrial dysfunction.\(^{23}\) This, once it has reached a critical threshold, may start making the PD phenotype manifest. In the case of the patient in the present study the unfortunate coincidence of the homozygous condition of the PINK1 mutation with the mtDNA mutation, homoplasmically inherited by the mother, may have dramatically anticipated the onset of the disease.

The present observations underscore the importance of performing mutagenic analysis of the mtDNA in patients carrying hetero- or homozgyous PD linked genetic defects. This would help not only in achieving a better understanding of the pathogenesis of this devastating neurodegenerative disease, but also in preventing its progress by earlier diagnosis.

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