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Mitochondrial respiratory dysfunction and mutations in mitochondrial DNA in PINK1 familial Parkinsonism

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Abstract A summary is presented of the cellular function and topology of the protein products of genes whose mutations are associated with familial forms of Parkinsonism, with particular emphasis on mitochondrial involvement. Observations are reviewed which show mitochondrial respiratory depression in the fibroblasts of a patient affected by familial Parkinsomism associated with homozygous PINK1 mutation. The respiratory depression, which was due to loss of mitochondrial cytochrome c, was associated with decreased capacity of respiratory chain oxidative phosphorylation and enhanced cellular level of ROS. Sequence analysis of the overall mtDNA revealed coexistence with the PINK1 mutation of homoplasmic point mutations in the ND5 and ND6 genes of complex I. The presence of these mutations appears to have an impact on the development of the Parkinsonism, which can also occur in the heterozygous PINK1 mutation state.

Keywords Mitochondria · Parkinson disease · PINK1 · Cytochrome c · Complex I · mtDNA oxidative stress

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Introduction

Parkinson disease (PD), one of the most common neurodegenerative disorders, is characterized by loss of dopaminergic neurons in substantia nigra (Dawson and Dawson 2003). Although most cases of PD are sporadic and of unclear origin involvement of toxic-environmental and genetic factors is evident (Farrer 2006). Mutations in more than ten genes have, so far, been identified as responsible for familial forms of Parkinsonism (Fitzgerald and Plun-Favreau 2008, Thomas and Beal 2007). These gene mutations are associated with rare autosomal dominant (ADP) or autosomal recessive Parkinsonism (ARP) (Table 1). Elucidation of the pathogenetic mechanisms of the rare forms of familial Parkinsomism, strategic in designing predictive and therapeutic measures for these, could provide clues in identifying sensible sites and networks, whose acquired dysfunction could, in the course of life, be involved in the development of Parkinson.

There is substantial evidence pointing to a critical role of mitochondrial dysfunction in the pathogenesis of Parkinson. Some of the genes associated with familial Parkinsonisms do in fact impinge, directly or indirectly, on mitochondrial dynamics and function (see Table 1). It has, on the other hand, been known for long time that exposure of animal laboratories to inhibitors of the mitochondrial NADH-ubiquinone oxidoreductase (respiratory chain complex I) reproduces characteristic PD features, like nigrostriatal dopaminergic degeneration and α-synuclein citoplasmatic aggregates. Decreased activity of complex I and oxidative damage of subunits of the complex (Keeney et al. 2006) have been found in substantia nigra, as well as other brain regions (Parker et al 2008), in autoptic samples from PD patients (Büeler 2009; Schapira 2008). Oxidative damage of complex I might be promoted in substantia nigra by the increased

Table 1 Function and cellular localization of the protein products of nuclear genes associated with familial Parkinsonism. Genetic mapping in familial Parkinsonism has identified mutations in more than ten genes (PARK1-13) (Thomas and Beal 2007). [a](Polymeropoulos et al. 1997). [b]The PARK2 gene encodes parkin, a 465 amino acid protein containing an N-terminal ubiquitin like domain (Kitada et al. 1998). Parkin functions as an E3 ubiquitin protein ligase targeting misfolded proteins to the ubiquitin proteasome pathway for degradation. Mutational loss of the E3 ligase activity causes early-onset ARP (Shimura et al, 2000). Parkin can be found associated with the outer mitochondrial membrane suggesting a potential role in modulating mitochondrial dynamics and function. [c](Leroy et al. 1998). [d]PARK6 encodes a serine/threonine protein kinase PINK1 (Valente et al. 2004). Its contains a mitochondrial localization sequence, is found in cytosol as well as in mitochondria (Valente et al, 2004; Silvestri *el al*, 2005). [e]The PARK7 gene encodes a highly conserved protein belonging to the DJ-1/Thi/PfpI protein super family (Bonifati et al. 2003). It presents ubiquitous expression in mammalian tissues. It is found in mitochondria, although a predominant portion of DJ-1 is localized in the cytosol and a small fraction in the nucleus (Zhang et al. 2005; Junn et al. 2009). DJ-1 undergoes self oxidation and may function as a ROS scavenger (Taira et al 2004). [f]The PARK8 gene encodes the Leucine-rich repeat kinase 2, LRRK2 (Zimprich, et al. 2004), which localizes in mitochondria, Golgi apparatus, endoplasmic reticulum and cytoskeleton (West et al., 2005; Gloeckner et al. 2006). [g](Ramirez et al. 2006.). [h]The PARK13 gene encodes the serine protease, HTRA2, with a mitochondrial targeting sequence and localization in the mitochondrial intermembrane space (Strauss et al., 2005). Refer to the OMIM website for latest updates on PD genes (www.ncbi.nlm.nih.gov)

	Gene (Product)	Function, cellular localization	Inheritance
PARK 1/4	a-Synuclein[a]	Unknown synaptic function Cytosolic	ADP
PARK 2	Parkin[b]	E3 Ubiquitin ligase Mitochondrial outer membrane	ARP
PARK 3	?		ADP
PARK 5	UCH-L1[c]	Ubiquitin carboxyl-terminal esterase and ligase	ADP
PARK 6	PINK1[d]	Ser-Thr kinase Mitochondrial outer/inner membrane and intermembrane space	ARP
PARK 7	DJ-1[e]	Chaperone, Oxidative stress response Mitochondrial inter membrane space/matrix	ARP
PARK 8	LRRK2[f] (Dardarin)	Mixed lineage kinase Mitocondria, Golgi apparatus, endoplasmic reticulum, cytoskeleton	ADP
PARK 9	ATP13A2[g]	lysosomal type 5 P-type ATPase	ARP
PARK 10	?		ADP
PARK 11	?		ADP
PARK 12	?		Unknown
PARK 13	HTRA2[h]	Pro-apoptotic serine protease Mitochondrial inter membrane space	Unknown

ADP=autosomal dominant Parkinsonism; ARP=autosomal recessive Parkinsonism

level of ROS generated in dopamine metabolism (Chinta and Andersen 2008). Oxidative damage of mitochondria and defects in their autophagic degradation can be particularly detrimental in dopaminergic neurons of substantia nigra, if, like in mice, also in humans these cells have per se a low mitochondrial content (Büeler 2009).

Complex I is the major site of oxygen superoxide production, in mitochondria and some of its 45 subunits are particularly prone to ageing-related oxidative damage (Cadenas and Davies 2000; Murphy 2009). Genetic dysfunction of complex I represents the more abundant cause of primary mitochondrial disease, in particular neurological disorders (Smeitink et al.2001; Papa et al.2009). There is evidence indicating that in mammalian cells the content and functional capacity of complex I is particularly affected, more than the other complexes of the respiratory chain, by the balance between the expression and oligomeric assembly of its subunits and their proteolytic degradation (Iuso et al. 2006; Lazarou et al. 2007; Papa et al. 2009). cAMP-dependent phosphorylation of the CREB transcription factor promotes the expression of the mitochondrial encoded subunits of complex I (De Rasmo et al. 2009). Phosphorylation by PKA of the NDUFS4 subunit of the complex promotes its import into mitochondria (De Rasmo et al. 2008).

In a familial progressive encephalopathy a decrease of complex I content was found, which appeared to be associated with a genetically determined activation of its proteolytic degradation (Papa et al. 2009). In an hereditary spastic paraplegia loss of paraplegin, a putative mitochondrial AAA protease, resulted on the other hand in defective assembly of complex I and increased sensitivity to oxidative stress (Atorino et al. 2003). Thus proteases can play a dual role in the control of the functional level of complex I. Those like paraplegin are essential for subunit maturation and assembly of the complex, their mutational inactivation impairs formation of the mature complex. The second group of proteases, which might include member(s) of the calpain

family (Arrington et al. 2006), appear to be involved in the proteolytic degradation of subunits of the complex. Genetic factors leading to anomalous enhancement of proteolysis can result in a pathological decline of the functional content of complex I.

Recent observations suggest interaction of PINK1 and parkin in the proteolytic quality control of unfolded or oxidized mitochondrial proteins, autophagy of damaged mitochondria (Clark et al. 2006; Narendra et al. 2008; McBride 2008) and mitochondrial structural dynamics (Yang et al. 2008). Parkin is an E3 ubiquitin ligase which targets misfolded proteins to the ubiquitin-proteasome degradation system in the cytoplasm (Shimura et al. 2000). PINK1, a serine/threonine protein kinase, with an N-terminal mitochondrial targeting sequence, is essentially localized in mitochondria. It has been reported from time to time that PINK1 is associated with the inner membrane (Clark et al. 2006; Pridgeon et al. 2007; Silvestri et al. 2005), the intermembrane space (Plun-Favreau et al. 2007; Pridgeon et al. 2007; Silvestri et al. 2005) and the outer membrane (Zhou et al. 2008). The catalytic domain of the outer membrane PINK1 faces the cytosolic space where it phosphorylates parkin (Kim et al. 2008). Parkin phosphorylation appears to promote its translocation from cytosol to mitochondria (Kim et al. 2008), where it apparently participates to autophagy of damaged mitochondria (Clark et al. 2006; McBride 2008; Narendra et al. 2008). PINK1 has also been reported to phosphorylate TRAP1 (HSP75) (Pridgeon et al. 2007) and to regulate, through an indirect phosphorylation mechanism, the protease HtrA2 (Plun-Favreau et al. 2007), both these proteins being involved in quality control of oxidized mitochondrial proteins. Since TRAP1 and HtrA2 reside in the intramitochondrial space it is possible that PINK1 can partition between the outer and the inner mitochondrial location in response to the prevailing cellular conditions (see also Mills et al. 2008).

It has, also, been reported that the anti-apoptotic proteolytic activity of HtrA2, downregulated in PINK1-mutated PD patients (Plun-Favreau et al. 2007), is enhanced (Ma et al. 2007) by interaction with the protein product of GRIM-19 gene (cell death regulatory gene induced by interferon and retinoic acid) (Angell et al. 2000). The GRIM-19 has been identified as a subunit of complex I (Fearnley et al. 2001), is constitutively phosphorylated (Palmisano et al. 2007) and is essential for the assembly/function of the complex (Huang et al. 2004). This intriguing PINK1/HtrA2/complex I interaction seems to deserve further study. It has, for example, been found that all-trans retinoic acid increases, by a post-transcriptional mechanism, the mitochondrial level of GRIM-19 in complex I (Papa et al. 2007). It would be of interest to investigate whether this effect of retinoic acid, which resulted, however, in a marked inhibition of the NADH-ubiquinone oxidoreductase activity (Papa et al. 2007), is associated with the reported stimulation by retinoic acid of protein phosphatase activity (Vuocolo et al. 2003; Xiao et al. 2006).

DJ-1, a protein with antioxidant activity, which localizes also in mitochondria (Zhang et al. 2005) can contribute together with PINK1 and parkin to counteract pathogenetic impact of altered mitochondria (Fitzgerald and Plun-Favreau 2008; Xiong et al. 2009).

Loss of PINK1 function and mitochondrial respiratory dysfunction in familial Parkinsonism

Investigations on fibroblast cultures from patients affected by familial neurological disorders proved to be a valuable approach to elucidate the impact of a well defined genotype alteration on house-keeping cell functions (Iuso et al 2006; Papa et al 2009; Scacco et al. 2003). It should, however, be noted that in a disease like PD, which is associated with degeneration of a subtype of neuronal cells, like the nigrostriatal dopaminergic neurons, additional cell-specific factors have to be involved.

The mitochondrial respiratory function has been thoroughly characterized in primary skin fibroblast cultures of a female patient carrying a homozygous W437X PINK1 mutation (Piccoli et al 2008a), resulting in a C-terminus truncated protein with loss of the kinase activity (Sim et al 2006). The clinical phenotype of the patient was characterized by early onset (22 years of age) Parkinson. The mother heterozygous for the mutation, was affected by a later onset Parkinson (53 years of age), the heterozygous father more than 70 years old was exempt from the disease (Criscuolo et al 2006). The patient's fibroblasts exhibited a significant depression of the endogenous respiration as compared to control fibroblasts, which was not relieved by the uncoupler CCCP. The $\Delta\mu H^{+}$–controlled, respiratory activity in the presence of oligomicin, was comparable to the control, this showing that no proton leaks or proton pump slips were introduced in the mitochondrial membrane by the pathological cellular state. The specific enzymatic activities of complex I, III and IV (this measured with added ferrocytochrome c) were normal, so was the polypeptide composition of the three complexes. The patient's fibroblasts exhibited only a small depression of the ATPase activity (complex V), but the polypeptide pattern of the complex was also normal. The cellular ATP level was significantly lower than in controls when fibroblasts were grown in a galactose medium, a condition in which ATP is essentially produced by mitochondrial oxidative phosphorylation (Rossignol et al. 2004). In a glucose medium the ATP level in the patient's fibroblasts was, on the contrary, higher than in the control, evidently due to enhancement of glycolitic ATP production.

A clue to understand the cause of the respiratory depression was provided by the finding the the respiratory activity of cytochrome c oxidase was severly depressed as compared to control when assayed with ascorbate plus TMPD, a condition in which it depends on the endogenous mitochondrial content of cytochrome c. That the inhibition was indeed due to loss of cytochrome c from mitochondria was directly verified by confocal microscopy of cells treated with a fluorescent antibody of cytochrome c as well as by western immuno blotting of the mitochondrial fraction. Thus the depression of the respiratory activity in the patient's fibroblasts was essentially due to loss of mitochondrial cytochrome c (Piccoli et al. 2008a).

Confocal microscopy analysis with fluorescent probes revealed in patient's fibroblasts cellular accumulation of H_2O_2 with a reticular distribution resembling the mitochondrial network and accumulation of oxygen superoxide in mitochondria. The accumulation of H_2O_2 in patient's fibroblasts was not prevented by apocynin, which specifically inhibits the plasma membrane NADPH oxidase (Johnson et al. 2002), neither by dibutyryl-cAMP. Under condition of depressed functional activity of complex I cAMP has been found to restore the activity of the complex and prevent oxygen free radical production (Piccoli et al. 2006). On the contrary DPI, which binds irreversibly to the prosthetic flavin moiety of redox enzyme including complex I (Majander et al. 1994), prevented completely the accumulation of H_2O_2 in the patient's fibroblasts (Piccoli et al. 2008a).

Impaired activity of the mitochondrial respiratory chain and enhancement of ROS production with unbalance of the intracellular redox homeostasis, have been reported from time to time in sporadic as well as in familial cases of Parkinsomism. Decreased cytochrome *c*/cytochrome *c* oxidase, which can scavenge ROS produced by complex I and III (Lin and Beal 2006), might have contributed to their accumulation in patient's fibroblasts. The PINK1 mutation can be responsible for the decreased mitochondrial cytochrome *c* content. As mentioned in the introduction, defective phosphorylation by the mutated PINK1 kinase of the mitochondrial chaperone TRAP1 (Pridgeon et al. 2007) can abrogate a protective effect exerted by this protein in preventing oxidative-stress-induced cytochrome *c* release and apoptosis (see Figure) (Hoepken et al. 2007; Pridgeon et al. 2007; Wang et al. 2007).

In the patient's fibroblasts the impact of the loss of mitochondrial cytochrome *c*, the consequent deficiency of respiratory activity and ROS accumulation was not such detrimental to impair cell growth and induce apoptosis. Indeed the depressed oxidative phosphorylation was apparently compensated by enhanced ATP production by glycolysis. This will, however, result in vivo in adverse cellular acidosis. The accumulation of ROS did not overwhelm the scavenger capacity of the glutathione system, which showed a normal activity (Piccoli et al 2008a). This is consistent with the slowly progressive Parkinsonism of the patient. The respiratory dysfunction can, however, have at long, in this and other autosomal recessive cases a more adverse influence on the functional activity of the nigrostriatal dopaminergic neurons (Lin and Beal 2006). These cells like other neurons have, in fact, a relatively low threshold for mitochondrial respiratory activity (Burke et al. 2004).

Are PINK1 mutations the only determinants to the clinical outcome of the Parkinson disease?

Mutations disseminated along the *PINK1* gene have been found in autosomal recessive forms and some sporadic cases of PD hallmarked by an early onset (Bonifati et al. 2005; Marongiu et al. 2007; Valente et al. 2004). 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 (Abou-Sleiman et al. 2006). This would suggest that complete or even partial loss of PINK1 function may promote the neurodegenerative disorder in combination with other(s) acquired or inherited defect(s).

Mitochondrial dysfunction and intracellular redox state alteration, as those described above in the patient carrying the W437X PINK1 mutation, have been reported to affect the rate/efficiency of mtDNA replication (Lee and Wei 2005) as well as its genetic stability. The mitochondrial DNA was, thus, analysed in the patient with the W437X PINK1 homozygous mutation (Piccoli et al. 2008b). Quantitative PCR showed that normal and patient's fibroblasts had a comparable content of mtDNA amounting to around 10^3 mtDNA copy-number/cell. Sequencing of mtDNA of the patient's fibroblasts unveiled a number of single nucleotide changes (SNCs) disseminated through the entire mtDNA. All but two were already reported as known polymorphisms [http:\\www.mitomap.org\]. These two SNCs were m.12397A>G and m.14319 T>C in the ND5 and ND6 genes respectively. Notably both were homoplasmic and confirmed in a different batch of the originally isolated pool of fibroblasts from the patient's biopsy and in the DNA extracted from a patient's blood sample. Both the ND5 and ND6 mutations were confirmed in the homoplasmic state in the mtDNA of the patient's mother blood-sample. Conversely, the same analysis carried out on the patient's father blood-sample showed a normal ND5 and ND6 genotype. Collectively these results demonstrate that the homoplasmic mtDNA mutations found in the patient cohexisted 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 of NADH:ubiquinone oxidoreductase (Complex I) (Brandt 2006). 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). The m.14319 T>C mutation changes an asparagine in aspartic acid at position 119 of the ND6 subunit. Various ND6 mutations leading to aminoacid changes have been found, associated with mitochondrial diseases (Carelli et al. 1999; Chinnery et al. 2001) and tumour cell metastasis (Ishikawa et al. 2008). Some of these mutations result in

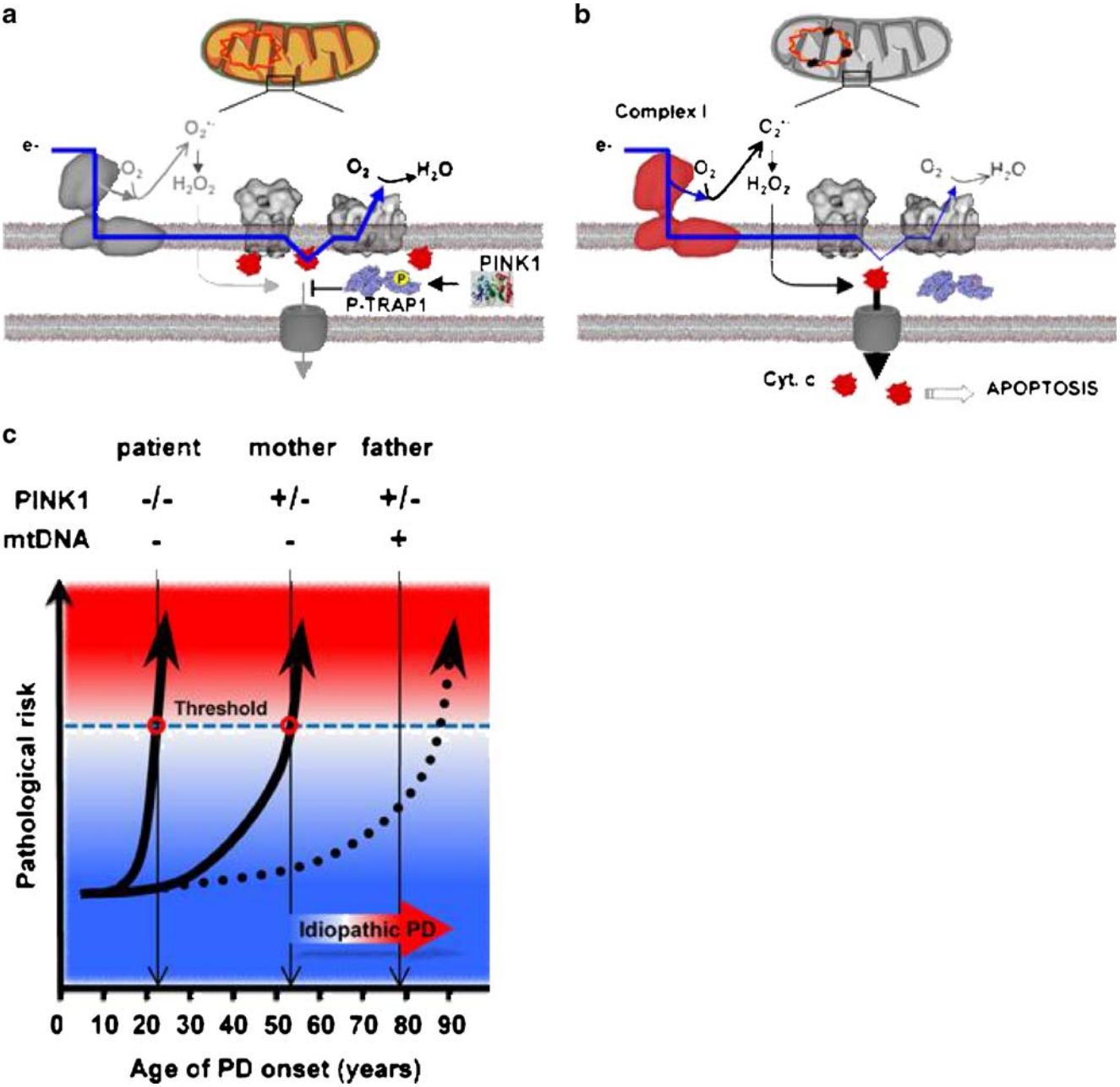

Fig. 1 Combined effect of PINK1 and mtDNA mutations in PINK1 familial Parkinsonism. **a**, **b** scheme of the impact of homozygous PINK1 mutation(s) and homoplasmic mtDNA mutations on loss of mitochondrial cytochrome c release, depression of mitochondrial respiratory activity, and ROS production. **a** and **b** illustrate the normal and pathological condition respectively. Thick and pale lines refer to active and inactive pathways respectively. See text for further details. **c** Scheme showing progression and onset of PD as a function of combination of PINK1 and mtDNA mutations in the patient's family described in the text. 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 homoplasmic mtDNA mutation(s). The dotted arrow indicates the range of age where most of the idiopathic forms of PD start to be clinically manifest. See text for further details

changes in the kinetics parameters of complex I (Carelli et al. 1999; Chinnery et al. 2001;) and/or production of oxygen free radicals (Gonzalo et al. 2005; Ishikawa et al. 2008; see also Papa et al. 2009). Site directed mutagenesis in the NuoJ subunit (counterpart of the mammalian ND6) of E. coli complex I resulted in suppression of the proton pumping activity of the complex (Kao et al. 2005). Similar site-directed mutagenesis indicated that the NuoJ subunit also delineates the binding site of ubiquinone (Pätsi et al. 2008). In both cases the assembly of complex I and its oxidoreductase activity were unaffected by the mutations as, in fact, found in the mitochondria from the patient of our study. Enzyme kinetic analysis of the NADH-ubiquinone oxidoreductase activity of complex I in normal and patient's fibroblasts showed, however, an almost fivefold decrease of the apparent Km for both ubiquinone and NADH in the patient's sample (Piccoli et al. 2008b). The higher affinity for ubiquinone of the patient's complex I could predispose to a longer occupancy of the reduced quinone or of the semiquinone radical species at the catalytic site(s), with altered reduction potential of the upstream redox centers and enhanced oxygen superoxide production.

Conclusion

The present observations, albeit limited to a single familial case of Parkinsonism, can be paradigmatic of the extreme variability observed in the onset and progression of this neurodegenerative disease and underscore its multi-factorial aetiology. Combination of inherited and/or somatically acquired genetic alterations might result in different clinical patterns related to the severity of their impact on the mitochondrial function.

The condition of heterozygosis for PINK1 mutation (and likely for other PD-related gene disorders) when accompanied with germinal or somatic mutation(s) in the mtDNA, could predispose to the development of the disease. This might have been the case of the heterozygous patient's mother in the present study which was affected at 53 years of age. If the heterozygosis for PINK1 mutation is, or remains, the only altered genetic defect in the carrier, this would probably not result in PD as in the case of the patient's father at 79 years (Fig. 1). In the homozygous condition a defective PINK1-linked protection to oxidative damages by ROS, whose production increases during ageing, may accelerate a vicious cycle causing overwhelming mitochondrial dysfunction (Papa and Skulachev 1997). This, once reached a critical threshold, may start making the PD phenotype manifest. In the case of the patient in the present study the unfortunate coexistence of the homozygosis condition of the PINK1 mutation with the mtDNA mutation, homoplasmically inherited by the mother, may have dramatically anticipated the onset of the disease as a consequence of impaired quality control processes of damaged mitochondria. All this underscores the importance of carrying out mutational analysis of the mtDNA in patients carrying hetero- or homoyzygous PD-linked genetic defects. This would help not only in better understanding the pathogenesis of this devastating neurodegenerative disease but also in preventing its progress by earlier diagnosis.

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