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Mitochondrial impairment observed in fibroblasts from South African Parkinson's disease patients with *parkin* mutations

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ABSTRACT

Parkinson's disease (PD), defined as a neurodegenerative disorder, is characterized by the loss of dopaminergic neurons in the substantia nigra in the midbrain. Loss-of-function mutations in the *parkin* gene are a major cause of autosomal recessive, early-onset PD. Parkin has been implicated in the maintenance of healthy mitochondria, although previous studies show conflicting findings regarding mitochondrial abnormalities in fibroblasts from patients harboring *parkin*-null mutations. The aim of the present study was to determine whether South African PD patients with *parkin* mutations exhibit evidence for mitochondrial dysfunction. Fibroblasts were cultured from skin biopsies obtained from three patients with homozygous *parkin*-null mutations, two heterozygous mutation carriers and two wild-type controls. Muscle biopsies were obtained from two of the patients. The muscle fibers showed subtle abnormalities such as slightly swollen mitochondria in focal areas of the fibers and some folding of the sarcolemma. Although no differences in the degree of mitochondrial network branching were found in the fibroblasts, ultrastructural abnormalities were observed including the presence of electron-dense vacuoles. Moreover, decreased ATP levels which are consistent with mitochondrial dysfunction were observed in the patients' fibroblasts compared to controls. Remarkably, these defects did not manifest in one patient, which may be due to possible compensatory mechanisms. These results suggest that *parkin*-null patients exhibit features of mitochondrial dysfunction. Involvement of mitochondria as a key role player in PD pathogenesis will have important implications for the design of new and more effective therapies.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder with global incidence rates of 1–2% in individuals over the age of 65 and 4% over the age of 80 years [1]. Pathologically, PD is defined as the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta in the midbrain and clinical motor symptoms include bradykinesia, postural instability, rigidity and resting tremor. Although PD has been associated with various environmental factors, a number of genes have been found to cause familial PD, with both autosomal dominant and autosomal recessive patterns [2]. To date, the genes involved in autosomal dominant forms of PD include *SNCA*, *LRRK2*, *EIF4G1* and *VPS35*, and those that are implicated in autosomal recessive PD, which result in a loss-of-function, include *parkin*, *PINK1*, *DJ-1* and *ATP13A2* [3].

Parkin mutations are the predominant cause of autosomal recessive PD, and are involved in the early-onset form of the disorder in which individuals typically under the age of 50 are affected

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[4]. *Parkin* encodes an E3 ubiquitin ligase protein, which forms part of the ubiquitin–proteasome system (UPS) whereby cytosolic, secretory and membrane proteins undergo degradation [5]. These unwanted proteins are tagged for removal by the parkin protein, which has also been involved in the removal of dysfunctional mitochondria from the cell via the process of mitophagy (selective degradation of mitochondria). Absence of parkin is thought to lead to the accumulation of critical substrates, resulting in the initiatory events that induce changes in mitochondrial structure and function [6].

Animal knockout models of *parkin* show several changes in the mitochondria in comparison to the wild-type controls. A study on a *Drosophila* knockout model revealed that mitochondrial defects were a common characteristic of the pathology [7]. Severe morphological features were observed, including irregular and dispersed myofibrillar arrangements and swollen and malformed mitochondria with disintegration of cristae. In mice knockout models, a decrease in the number of proteins involved in mitochondrial function and oxidative stress, lowered respiratory capacity of striated muscle mitochondria, as well as decreased serum antioxidant capacity were observed [8]. Human studies performed on leukocytes, fibroblasts and post mortem brain tissue obtained from PD patients with *parkin*-null mutations have further implicated structural and functional mitochondrial impairment [9–13]. In particular, human fibroblasts have been used as a primary cell model of PD [14], as expression levels of parkin are at relevant quantities in these cells.

Studies on fibroblasts have noted ultrastructural changes in the mitochondria such as larger mitochondrial mass [9] as well as grossly swollen mitochondria with disintegrated cristae [12]. However, studies on the mitochondrial network in fibroblasts have been controversial. Findings include no differences in the network of patient fibroblasts compared to controls [9], or an increase in mitochondrial branching in patient cells, indicating a possible increase in fusion [11]. Other studies have observed a fragmented mitochondrial network, suggesting that impaired mitochondrial fusion is present in *parkin*-null mutants [12].

Functional studies on the respiratory capacity of the mitochondrial electron transport chain complexes have shown a significant decrease in the activity of complex I in both fibroblasts and leukocytes from patients with *parkin* mutations in comparison to age-matched controls [15]. Impaired mitochondrial respiration usually results in ATP depletion which causes increased levels of reactive oxygen species (ROS), and eventually cell death. Studies on fibroblasts from *parkin*-null patients have shown varying results regarding the levels of ATP, with some showing significant reduction [9,11] and others showing a significant increase in ATP levels [12].

It is clear that further studies on fibroblasts harboring *parkin*-null mutations are needed, as previous studies have produced conflicting findings. In the present study, mitochondrial functional and structural analysis was performed on fibroblasts from three PD patients, all with *parkin*-null mutations. Muscle biopsies were assessed for mitochondrial defects. Furthermore, two heterozygous carriers of the *parkin*-null mutations were included for comparison with the homozygous patients.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Health Research Ethics Committee at Stellenbosch University, South Africa (Protocol number 2002/C059) and all study participants provided informed written consent.

2.2. Study participants

For the present study, three PD patients, each with two *parkin* null mutations were selected, one sporadic patient with Mixed Ancestry ethnicity (P1) and two White Afrikaner siblings (P2 and P3). The patients were examined by a movement disorder specialist (JC), and all met the UK Parkinson's Disease Society Brain Bank Research criteria for diagnosis of PD [16]. The exonic deletion mutations had been identified in these patients in previous studies using multiplex ligation-dependent probe amplification (MLPA) and cDNA sequencing; P1 has homozygous deletions of exons 3 and 4, whereas P2 and P3 have homozygous deletions of exon 4 [17–19]. Also included in the study were unaffected family members of the patients who are heterozygous carriers of the mutations. These included P1het (the mother of patient P1) and P2het (the sibling of sisters P2 and P3). In addition, two wild-type controls, WTC1 and WTC2, were included who did not harbor mutations in parkin and had no history of neurological disease. Phenotypic and genotypic data of the PD patients, heterozygous carriers and controls are summarized in Supplementary Table 1.

2.3. Fibroblast culture from skin biopsies

Fibroblasts were obtained from all three PD patients, both carriers and both WT controls, from a skin punch biopsy taken from the inner upper arm. The 2 mm × 2 mm skin piece was suspended in a solution of Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Switzerland) with 10% fetal calf serum and 1% penicillin (1000 U/ml) streptomycin (1000 U/ml). The cells were grown in 25 cm^2 culture flasks at 37 °C, 5% CO_2. For all experiments the passage number was kept to below 12.

2.4. Muscle biopsies

Muscle biopsies were obtained from patients P1 and P2 but not from P3 as she exhibited severe tardive dyskinesias on the day of the procedure. Muscle biopsies were not requested from the WT controls or the carriers. For the biopsies an area of skin overlying the vastus lateralis muscle was sterilely prepared with 5% povidone iodine and 0.5% chlorhexidine. Thereafter, 2% lignocaine hydrochloride local anesthetic was injected intra- and subcutaneously in a 4 cm^2 area. A 1 cm longitudinal incision was made through the skin and fascia, and three percutaneous muscle biopsies were extracted using a 5 mm Bergström biopsy needle. A piece of muscle tissue was dissected with a scalpel and immediately fixed in gluteraldehyde buffer for electron microscopy studies. The remainder of the muscle tissue was snap frozen and stored in liquid nitrogen until sectioning.

2.5. Live cell imaging and analysis of mitochondrial morphology

Mitochondrial morphology was assessed by fluorescence microscopy. Human fibroblasts were seeded in Lab-Tek 8-well coverglasses (Nunc, 155411, Thermo Scientific). Mitochondria were stained with 200 nM Mitotracker Red (Invitrogen, Life Technologies, USA) and nuclei counterstained with Hoechst 33342 (Invitrogen, Life Technologies, USA). Image acquisition was performed on an Olympus IX-81 microscope coupled to an MT-20 Xenon-arc burner (Olympus Biosystems GMBH) and equipped with a F-view-II cooled CCD camera (Soft Imaging systems) and an environmental chamber (Solent Scientific). Images were acquired using 360 nm and 572 nm excitation and a UBG triple bandpass emission filter cube (Chroma). Z-stacking was performed with an increment of 0.26–0.3 μm between image frames, using an Olympus Plan Apo N60x/1.4 oil immersion objective. A total of 7–12 image frames