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Changes of Mitochondria Copy Number in Association with Idiopathic Pulmonary Fibrosis

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Abstract

Background: Idiopathic Pulmonary Fibrosis (IPF) is a fatal lung disease of unknown etiology, where different pathogenetic hypotheses have been suggested. Notwithstanding the unrecognized first trigger that leads to the disease, an imbalance between oxidants and antioxidants seems to accelerate the fibrotic process. The aim of this study was to investigate the mitochondrial DNA (MtDNA) alterations, as oxidative stress marker, in IPF patients in order to explore if it makes sense to carry out an in-depth study with regards to the oxidative stress in IPF, as it plays a key role in the development and progression of this fatal disease.

Methods: 41 IPF patients (age 68.6 ± 5.82; body mass index (BMI) 29.8 ± 2.59) and 20 control subjects (age 66.18 ± 3.72; BMI 27.80 ± 4.64) were enrolled. Patients underwent blood collection. Copy number of MtDNA and nuclear DNA (nDNA) was measured in the blood cells of IPF patients and control subjects by quantitative real-time PCR. The ratio between mitochondrial DNA/nuclear DNA (MtDNA/nDNA) was calculated.

Results: MtDNA/nDNA was significantly higher in IPF patients than in the control group (119.93 ± 79.18 vs 65.97 ± 20.56; $p < 0.05$). The level of MtDNA/nDNA was negatively correlated with FVC% ($R = -0.5$, $p < 0.005$) and baseline DLCO% ($R = -0.35$, $p < 0.05$).

Conclusion: We found an increase of MtDNA/nDNA ratio in IPF subjects that led us to suggest that there is a presence of mitochondrial dysfunction that confirms an important role of the oxidative stress in IPF. Further investigations are required for the prognostic and therapeutic implications that it can have.

ABBREVIATIONS

IPF: Idiopathic Pulmonary Fibrosis; **ROS:** Reactive Oxygen Species; **MtDNA:** Mitochondrial DNA; **nDNA:** Nuclear DNA; **MtDNA/nDNA:** Mitochondrial DNA/Nuclear DNA; **BMI:** Body Mass Index; **FVC:** Forced Vital Capacity; **DLCO:** Carbon Monoxide Diffusing Capacity; **FEV1:** Forced Expiratory Volume in 1 Second; **PaO_2:** Partial Pressure of Arterial Oxygen, **COPD:** Chronic Obstructive Pulmonary Disease; **ACOS:** Asthma-COPD Overlap Syndrome; **OSAS:** Obstruction Sleep Apnea Syndrome; **ATS/ERS/JRS/ALAT:** American Thoracic Society/European Respiratory Society/ Japanese Respiratory Society/Latin American Thoracic Association; **BALF:** Broncho Alveolar Lavage Fluid.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal lung disease of unknown etiology characterized by irreversible change in alveolar structure by fibroblast growth and remodeling of extracellular matrix [1]. The prognosis for IPF patients is poor, with a mean survival of 3–5 years [2]. Although genetic determinants, Environmental exposures, and common insults, such as smoking [3], pollutants, Occupational exposures [4], viral infections [5], and ageing [6] have been identified as risk factors for this disorder, its origin and onset are not fully understood. It has been long believed that pulmonary fibrosis begins with alveolar inflammation and that chronic inflammation modulates fibrogenesis [7]. The role of the inflammatory process in the pathogenesis of IPF is however much-discussed. Selman et al. reported that the clinical degree of inflammation showed no correlation with disease severity or clinical course, and treatment with anti-inflammatory drugs failed to improve the prognosis [8]. On the contrary, Balestro et al. recently highlighted the importance of inflammation among the factors that contribute to determining the rate of disease progression in patients with IPF [9]. As a direct consequence of inflammation, oxidant/antioxidant imbalance in the lower respiratory tract has also been proposed to play a role in the lung injury of IPF [10]. Therefore, recent studies have emphasized the role of oxidative stress as the molecular basis of lung fibrosis. Cantin et al. demonstrated that cells in the bronchoalveolar lavage

fluid (BALF) produced oxidants and myeloperoxidase at higher concentrations in IPF patients than in control patients. They also showed that increased peroxidase activity was involved in the epithelial injury in IPF [11]. Rahman et al., showed an imbalance between oxidative stress and anti-oxidative potency in serum and BALF in IPF patients [12]. The diagnosis of IPF is reached almost always in a late stage because there are no tests for early detection. Nonetheless, the development of radiological examinations, such as high resolution computed tomography, allows for the diagnosis of this disease in a less advanced stage. We do not have the tools that allow for the screening of apparently healthy patients at the onset of IPF. The therapeutic market is now directed toward the development of drugs that act on biological steps recognized in the pathogenesis of this disease. Perhaps the research of early markers for IPF might be directed toward the biology of this disease. Some biological markers have already been investigated in IPF as those behind the epithelial damage and repair, myofibroblast accumulation and matrix deposition, inflammation, oxidative stress, angiogenesis and genes [13,14]. With this study we analyzed a new intriguing oxidative stress marker that is represented by the mitochondrial DNA. Mitochondria are independent organelles present in the eukaryotic cells [15], highly susceptible to be attacked by reactive oxygen species (ROS), due to lack of protective histones, introns and their limited DNA repair capacity [16]. In conditions of oxidative stress, the transcriptional and replication machinery of mitochondrial biogenesis will be up-regulated resulting in increased mitochondrial biogenesis via replication of the mitochondrial genome; therefore in its presence they undergo an adaptive response that causes an increase of MtDNA copy numbers. For their predisposition, MtDNA content or mutation have already been studied in several diseases characterized by an oxidative redox imbalance; contributions with this regard exist for lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), lung cancer, asthma-copd overlap syndrome (ACOS) [17] and obstruction sleep apnea syndrome (OSAS) where an alteration in quantity or quality of mitochondrial genome has been described [18-20]. Recently, our group reported a positive correlation in OSAS patients between MtDNA/nDNA and reactive oxygen metabolites (ROMs) measured in paired plasma. Thus suggesting that this marker of mitochondrial dysfunction might also be used as a marker of oxidative stress [21]. A simple way to measure mitochondrial DNA copy number in a cell is to measure mitochondrial versus nuclear genome ratio, termed MtDNA/nDNA [22]. MtDNA/nDNA is effective as a putative biomarker because it can be measured in as little as 1 pg of genomic DNA [23]. To our knowledge, there are few papers on the role of mitochondria in IPF patients. The aim of this study was therefore to investigate the oxidative stress, through the measurement of MtDNA/nDNA, in IPF patients to give a further contribution to determine if the oxidative stress is really a protagonist in the development of IPF.

MATERIALS AND METHODS

Population

Patients were consecutively recruited for the study from the outpatient facility of the Institute of Respiratory Diseases of the University of Foggia, Italy, between January 2014 and October 2015. We enrolled 41 IPF patients and 20 ages matched normal subjects. IPF was diagnosed according to the criteria of ATS/ERS/JRS/ALAT statement for IPF after evaluation of all clinical, laboratory, functional, imaging and histological data [1]. All patients were treatment naive. This study was conducted in accordance with the amended Declaration of Helsinki. Written informed consent was obtained from all subjects, and the study was approved by our Institutional Ethics Committee of Foggia. All patients underwent blood collection at enrollment.

Lung function

Pulmonary function tests were performed. Forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC) and Carbon Monoxide Diffusing Capacity (DLCO) were measured using a spirometer (Sensormedics, USA). The best value of three maneuvers was expressed as a percentage of the predicted normal value.

Blood collection and DNA extraction

Blood samples were collected in the morning. A total of 3 ml peripheral blood sample was collected in EDTA tubes and then was stored at -80° C. Whole blood DNA was extracted with QIAamp DNA MiniKit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The concentration of extracted DNA was measured at 260 nm with NanoDrop Spectrophotometer (Thermo Scientific NanoDrop, USA) and was adjusted to 10 ng/µl. Extracted DNA was stored at -20°C until further use.

Quantitative real-time PCR

Mitochondrial DNA copy number was measured by qPCR method using an Applied Biosystems 7300 real-time PCR System (PE Applied Biosystems). MtDNA was measured by quantification of a unique mitochondrial fragment relative to a single copy region of beta-2-microglobulin nuclear gene (β2M) [23]. Primers, probes (IDT, Integrated DNA Technologies, USA) and gene accession numbers are listed in Table 1. Mitochondrial DNA and β2M probes were labelled at 5' end with 6 FAM and MAX fluorescent dyes respectively and both probes contained BHQ-1 as a quencher dye at 3' end. The PCR mix was: 1x TaqMan® Universal PCR Master Mix (PE Applied Biosystems), 200 nM of each primer, 125 nM of TaqMan Probe, 50 ng of total DNA extract in a 20 µl PCR reaction. Quantitative real-time PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C and 60 s of annealing/extension at 60°C. The data presented are the means of 3 measurements. The positive controls (extracted from normal healthy persons) and negative control (DDW + master mix) were added for every PCR run. Standard curves obtained from serial dilutions of PCR-amplified target sequences were used for the quantification of MtDNA and nuclear genome (nDNA), and then the ratio of MtDNA/nDNA was calculated.

Statistical analysis

Descriptive statistics (i.e., means, standard deviations, percentages) were applied to summarize the continuous and categorical variables. All variables analyzed were normally distributed so Student's T-test was used to compare the mean values between the IPF group and the control group. Correlation

between MtDNA/nDNA, clinical and functional data were assessed using the Spearman's rank correlation test, a P value < 0.05 was considered significant.

RESULTS

Demographic, clinical and functional data of patients affected by IPF and healthy controls enrolled in the study are reported in Table (2). IPF patients showed a higher level of MtDNA/nDNA ratio (119.93 ± 79.18 vs 65.97 ± 20.56; p< 0.05) than the control group (Figure 1). The level of MtDNA/nDNA was negatively correlated with FVC% in all subjects (R = -0.55, p< 0.0005). In particular, for IPF patients we found an important negative correlation between MtDNA/nDNA and FVC% (R = -0.5, p< 0.005), and also between MtDNA/nDNA and DLCO% (R = -0.35, p< 0.05; (Figure 2 and Figure 3). In addition, MtDNA/nDNA values had no significant correlation with 6MWT (six minutes walking test) distance-mt in IPF patients. Others clinical parameters such as age, BMI were not associated with MtDNA directly.

DISCUSSION

The main result of the study was to report, for the first time, an alteration of MtDNA copy number in IPF patients as a consequence of increased oxidative stress. Our data also showed that the MtDNA/nDNA ratios correlated with a decrease in FVC and in DLCO.

Oxidative stress is the result of a disorder of the redox balance of the cell, resulting in excessive oxidation of intracellular proteins. It is known that chronic oxidative stress can cause damage to proteins, lipids and DNA molecules within the cell and it is considered to play a role in many common diseases such as diabetes and its complications, cancer [24], neurodegenerative disorders and IPF [25]. In the same way, oxidative stress induces mitochondrial dysfunctions even if its molecular mechanisms are not well understood [26]. Indeed, mitochondria are particularly sensitive to oxidative stress, because they have few repair mechanisms, and in its presence they undergo an adaptive response that causes an increase of content of MtDNA as a result of increased mitochondrial biogenesis [27]. To better explore the role of oxidative stress in IPF we studied a new effective oxidative stress marker that is the abundance of mitochondrial DNA. A common method for measuring mitochondrial DNA copy number is to quantify a mitochondrial encoded gene relative to a nuclear encoded gene to determine the mitochondrial genome to nuclear genome ratio which we have termed MtDNA/nDNA [22]. Earlier studies measuring MtDNA/nDNA utilized hybridization [28], but in the last decade real-time quantitative PCR has become a straight forward detection method [23,29], because it requires less sample and it is a technique which is fast and widely available. Under conditions of oxidative stress there is an accumulation of excess free radicals, as mitochondrial DNA is located close to the source of ROS production, the DNA itself can become damaged resulting in accumulation of deletions and mutations [26]. Therefore, in a condition of oxidative stress the transcriptional and replication machinery of mitochondrial biogenesis via replication of the mitochondrial genome is potentiated, so the increased abundance of MtDNA results as an adaptive response and can be detectable in vivo in blood. Recently, our group analyzed mitochondrial DNA balance in OSAS [21] and ACOS showing an increase of MtDNA/nDNA in the

Table 1: Primers/probes sequences for MtDNA/nDNA determination using real-time qPCR; mitochondrial DNA and β2M probes were labelled at 5'end with 6 FAM and MAX fluorescent dyes respectively, both probes contained BHQ-1 as a quencher dye at 3'end.

Gene accession number	Primer/probe	Sequence	Product size (bp)
Human mitochondrial genome NC_012920	Mito F	TTAAACACATCTCTGCCAAACC	150
	Mito R	AGATTAGTAGTATGGGAGTGGGA	
	Mito P	AA CCC TAA CAC CAG CCT AAC CAG A	
Human β2M accession number M17987	β2M F	CTTTCTGGCTGGATTGGTATCT	100
	β2M R	CAGAATAGGCTGCTGTTCCTAC	
	β2M P	AG TAG GAA GGG CTT GTT CCT GCT G	

Table 2: Characteristics of patients with idiopathic pulmonary fibrosis and controls.

	IPF Patients	Control	p
n	41	20	
Male	29	6	0.001
Age (median), yr	68.6 ± 5.82	66.18 ± 3.72	0.08
BMI	29.8 ± 2.59	27.80 ± 4.64	ns
Spirometry			
FVC (% predicted)	72,33 ± 10,80	103.25 ± 14.05	0.001
FEV_1 (% predicted)	76,94 ± 14,50	90.97 ± 6.63	0.04
% DLCO (% predicted)	54,47 ± 17,02	-	-
Arterial blood gases			
PaO_2 (mm Hg)	72,52 ± 12,06	98.90 ± 18.0	ns
**Values are mean ± SD			

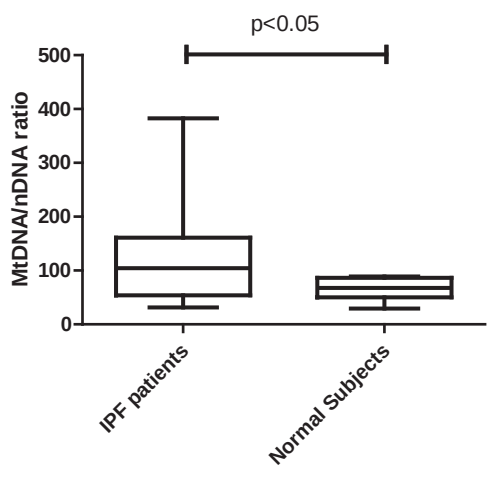

Figure 1 MtDNA/nDNA ratio in 41 IPF subjects and 20 controls. The values are represented with arithmetic mean and standard deviation. Student's T-test was used to compare the mean values between the IPF group and the control group. The two groups differ significantly ($p< 0.05$).

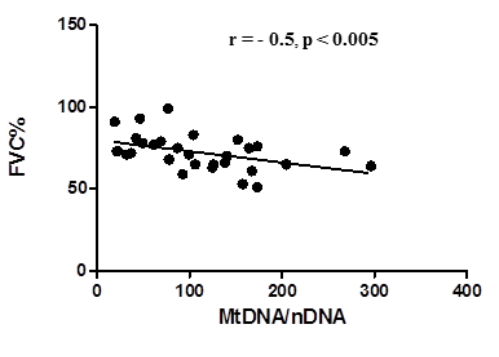

Figure 2 MtDNA/nDNA ratio correlates negatively with FVC% in IPF patients: R = -0.5, $p< 0.005$. Correlation between MtDNA/nDNA and FVC% was assessed using the Spearman's rank correlation test (P value <0.05 was considered significant).

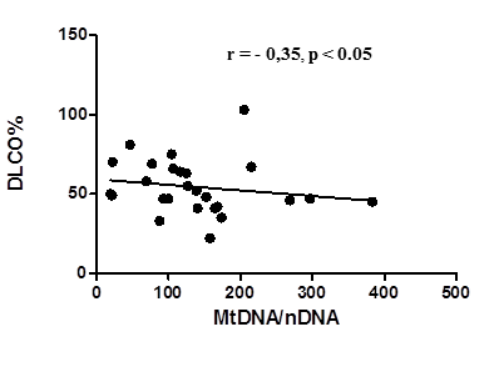

Figure 3 MtDNA/nDNA ratio correlates negatively with DLCO% in IPF patients: R = -0.35, $p< 0.05$. Correlation between MtDNA/nDNA and DLCO% was assessed using the Spearman's rank correlation test (P value <0.05 was considered significant).

blood of subjects affected by these pathologies [31]. In this study we have demonstrated, for the first time, that there is an increase of mitochondrial DNA, as an imbalance of MtDNA/nDNA, in IPF patients. Our results indicate that an increased abundance of mitochondrial DNA is present in the blood of individuals with IPF suggesting an increased prevalence of oxidative stress and is in line with Daniil et al. who reported an increase of the oxidative stress level in IPF [32] and Naik et al. who suggested that the oxidative stress, mediated by ROS and NADPH oxidase (NOX) activity, promotes pulmonary fibrosis [33]. Also, Zhou et al., previously demonstrated that after an inflammatory stimulus, the accumulation of damaged mitochondria precipitates in an increase in mtROS production [34]. In this study we also observed that MtDNA/nDNA was negatively correlated with FVC and DLCO, two clinically important and sensitive markers of IPF progression. Also, Matsuzawa et al., previously reported that serum oxidative stress values increased with disease progression in IPF patients as it was significantly correlated with a reduction in FVC and DLCO [25]. These results lead us to suggest that a positive feedback may exist between pulmonary fibrosis and oxidative stress: increased oxidative stress accelerates pulmonary fibrosis following alveolar cell injury, and progression of pulmonary fibrosis may potentially increase oxidative stress over time. This study is limited by the small number of enrolled patients. However, this is a preliminary study that we are planning to extend in the future on a larger population of IPF patients. In addition, in this preliminary study we didn't analyze other markers of inflammation and oxidative stress. In consideration of the importance of the oxidative characterization of MtDNA/nDNA, through the correlation of this marker with other validated markers of oxidative stress such as 8-isoprostane, ROS and the hydroperoxyl radical (HO_2), we have planned a new study on this topic.

CONCLUSION

We reported, for the first time, an increase of MtDNA/nDNA in IPF subjects as an expression of the mitochondrial dysfunction. The MtDNA/nDNA may be considered an easy and potential marker of the oxidative stress. This study highlights the fact that it makes sense to study oxidative stress in IPF as it plays a key role in the development and progression of the disease and might be a new target of biological therapeutic strategies. Furthermore, from our point of view, this research field could open interesting sceneries to better understand the pathophysiology of others pulmonary diseases and non-pulmonary diseases in which oxidative stress is always involved, because if we can control the oxidative stress pathway we may be able to create therapeutics for a wide range of chronic diseases.

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Author contributions: GEC and GC designed the study; DL, GC, MM, and GP contributed to the clinical and laboratory work for the study; GEC take responsibility for the integrity of the data in the study and the accuracy of the data analysis; GEC and GC analyzed the data and wrote paper; GEC, DL, MM, MPFB contributed to critical review and final approval of the manuscript. All authors read and approved the final manuscript.

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