Original Article
Variations in mitochondrial DNA are associated with chronic obstructive pulmonary disease

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Abstract: Individual susceptibility to chronic obstructive pulmonary disease (COPD) can be attributed to a complex interplay between genetic basis and environment. Mitochondrial respiratory function and antioxidant capability are known to be important in determining the ability to cope with the oxidative stress. Oxidant/antioxidant imbalance plays an important role in the development of COPD. However, it is still uncertain whether mitochondrial genes are correlated with COPD susceptibility. To investigate the relationship between mitochondria DNA (mtDNA) and COPD, 60 COPD patients and 60 age-matched volunteers were recruited. Haplogroup classification was carried out by aligning the sequences of the hyper-variable segment (HVS) region and the 10171-10659 region with the revised Cambridge Reference Sequence (rCRS) of mtDNA. The mtDNA copy number was calculated using quantitative real-time PCR. The results showed that peripheral leukocyte mtDNA haplogroups D and M9 might be associated with a decreased risk for COPD (OR=0.353, 95% CI=0.145-0.859, p=0.033, and OR=0.110, 95% CI=0.013-0.911, p=0.038, respectively), whereas haplogroups F and M7 might be associated with an increased risk for COPD (OR=5.800, 95% CI=1.213-27.728, p=0.033, and OR=6.510, 95% CI=1.376-30.792, p=0.019, respectively). Compared with the control group, the mtDNA copy number was significantly increased in the COPD group (159.57±9.96 VS 131.86±9.34, p<0.05). Taken together, these results indicate that peripheral leukocyte mtDNA is related to the development of COPD.

Keywords: Chronic obstructive pulmonary disease, mitochondrial DNA, haplogroups, copy number

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by poorly reversible airflow restriction and abnormal lung inflammation that is induced by noxious particles or gases [1]. Epidemiological surveys show that COPD is the leading cause of death in rural areas and the fourth leading cause of mortality in urban areas in China [2]. In America, 10% of inpatients suffer from COPD, and COPD will become the fourth leading cause of mortality in United States by the year 2030 [3].

Tobacco smoking has been implicated as the leading factor in the etiology of COPD. The reactive oxygen species (ROS) present in cigarette smoke induce direct damage to airway epithelial cells by reacting with antioxidants in the epithelial lining fluid [4]. Additionally, lipophilic components in cigarette smoke may disturb mitochondrial function and increase the generation of mitochondrial ROS by penetrating the airway epithelial cells and entering the systemic circulation [5].

The potential importance of mitochondrial energy metabolism in COPD may best be illustrated by the fact that many antioxidants that are considered to be important in the pathophysiology of COPD are linked to mitochondria [6]. Mitochondria are eukaryotic organelles responsible for energy production. The process of ATP synthesis depends on the action of the electron transfer chain (ETC). Disturbance of the mitochondrial respiratory chain induced by lipophilic components may enhance the leakage of single electrons from the ETC. These electrons can be accepted by oxygen to produce O_2^-, a very potent free radical [7]. Mitochondria also produce approximately 85% of intracellular

Table 1. Grading standards of chronic obstructive pulmonary disease

Grade	Clinical symptoms
I	FEV1 ≥ 80% of predicted value. Patients usually have chronic cough and sputum, but may not show any symptoms; patients may not be aware of pulmonary dysfunction.
II	50%≤FEV1<80% of predicted value. Cough, sputum symptoms get worse.
III	30%≤FEV1<50% of predicted value. Cough, sputum symptoms get worse, accompanied by a typical event of shortness of breath.
IV	FEV1<30% of predicted value or FEV1<50% of predicted value but accompanied with respiratory failure and other serious complications.

Table 2. Primer sequences

Primer name	Primer sequences neal	Annealing temperature	Length of PCR products
HVS-F	5'-CTCCACCATTAGCACCCAAA-3'	56°C	1230 bp
HVS-R	5'-GATGTGAGCCCGTCTAAACA-3'		
C-R	5'-ATCCACCCCTTACGAG-3'	50°C	519 bp
C-F	5'-CAGGCGGCAAAGACTA-3'		
B-F	5'-AGCAAACCACAGTTTCAT-3'	48°C	118 bp
B-R	5'-GCTTTACAGTGGGCTCTA-3'		
ND1-F	5'-CCTAATGCTTACCGAACGA-3'	52°C	152 bp
ND1-R	5'-GGGTGATGGTAGATGTGGC-3'		
β-globin-F	5'-CTGCTGGTGGTCTACCCTTG-3'	60°C	134 bp
β-globin-R	5'-AGGCCATCACTAAAGGCACC-3'		

ROS, which can promote cellular differentiation or induce apoptosis [8].

Unlike any other organelle, mitochondria have their own DNA. Human mtDNA encodes 13 respiratory chain subunits that are essential for the mitochondrial surface oxidative phosphorylation system (OXPHOS) [9]. mtDNA is particularly susceptible to oxidative damage and mutation because of the high rate of ROS production, the lack of introns and protective histones, and the limited DNA-repair capacity in mitochondria [10]. Chronic oxidative stress induced by cigarette smoke can lead to mtDNA damage, including point mutations, insertions, and deletions [11]. The accumulation of oxidative damage and the resulting sequence variations in mtDNA ultimately lead to faulty OXPHOS [12]. As such, the oxidative stress induced by cigarette smoking may play a substantial role in the pathogenesis of smoking-related diseases.

Prior studies have demonstrated that the non-pathogenic mutations that define the various mtDNA haplogroups are associated with susceptibility to metabolic and degenerative diseases in humans [13]. These mtDNA mutations also determine differences in OXPHOS perfor-

mance and ROS production. Furthermore, the mtDNA copy number is affected by oxidative stress, and the increased number of mtDNA copies has been found to be positively associated with an increased risk for lung cancer among heavy smokers [14].

Because cigarette smoking is the primary risk factor for COPD, we hypothesized that mtDNA was correlated with the development of COPD. To test the hypothesis, we performed a case-control study to investigate the association between mtDNA (including mtDNA haplotypes and mtDNA copy number) and COPD. From these results, we hoped to provide new experimental evidence to further elucidate the pathogenesis of COPD.

Materials and methods

Study participants

The study was approved by Research Ethics Committee of Shiyan Taihe Hospital. Written informed consent was obtained from all participants. A total of 60 COPD patients and 60 age-matched volunteers were recruited from August 2015 to August 2016. All subjects were unrelated for at least three generations. Inclusion criteria for the COPD patients were consistent with the COPD treatment guidelines formulated by Respiratory Diseases Branch of the Chinese Medical Association. Patients were excluded if they had been diagnosed with tuberculosis, asthma, cardiac disease, lung cancer, or other diseases. Exclusion criteria for the control group were a history of lung disease, cardiomyopathy, breast cancer, or liver disease. According to clinical symptoms and lung function

Table 3. Characteristics of the study population

	Controls (n=60)	Cases (n=60)	p value
Gender (male %)	80	78.33	0.930
Age (years)	73.55±8.96	70.93±9.12	0.116
BMI (kg/m^2)	21.64±1.83	20.33±1.53*	<0.001
FEV1/FVC (%)	88.67±3.90	52.23±10.31*	<0.001

*p<0.05 compared with control group.

at the time of hospital admission, patients with COPD could be divided into four grades (**Table 1**) [1]. Peripheral blood samples were drawn into heparinized tubes, and separation of the leukocytes was completed within 2 hours. Genomic DNA was isolated from the peripheral leukocytes using a QIAamp DNA Mini Kit (QIAGEN, Germany).

mtDNA haplogrouping

According to the nucleotide sequence in Genebank, the primers HVS-F and HVS-R were designed to amplify the hyper-variable segment (HVS) region (**Table 2**). In addition to the HVS region, the 10171-10659 region was considered as an auxiliary sequence for haplogroup classification because it contains substantial valuable classification information. Primers C-F and C-R were designed to amplify the 10171-10659 region. We obtained the mutation sites by aligning sequences of HVS and 10171-10659 regions with the revised Cambridge Reference Sequence (rCRS) of human mtDNA. According to the relationship between mtDNA haplogroups and phylogenetic tree for Han Chinese proposed in 2002 [15], specimens were classified into possible haplogroups. In addition to the sequencing, PCR-restriction fragment length polymorphism (PCR-RFLP) was considered as an adjunct experiment.

There are two tandem 9 bp (base pair) repeats in the small non-coding region between COX II and tRNAlys under normal circumstances. However, one of the two repeats may be lost during DNA replication due to slip chain mismatch. This kind of deletion is the diagnostic marker of B haplogroup. Primers B-F and B-R were designed to amplify this small non-coding region.

mtDNA copy number

Quantitative real-time PCR was used to analyze the mtDNA copy number. The beta globin

(β-globin) and ND1 genes were considered to be the reference genes for the nuclear genome and mitochondrial genome, respectively. The primers for β-globin and ND1 genes (**Table 2**) that were used for fluorescence quantitative PCR were designed and synthesized by Sangon Biotech.

In order to obtain the recombinant vectors PGM-β-globin and PGM-ND1, DNA fragments of β-globin and ND1 were amplified and inserted into the plasmid PGM-T. The recombinant vectors were used to transform competent E. coli DH5α cells to produce the recombinant bacteria PGM-β-globin-DH5α and PGM-ND1-DH5α. The recombinant plasmids were extracted from these recombinant bacteria and identified by digestion and sequencing. The copy number of recombinant plasmid was calculated from DNA concentration and molecular weight of plasmid, and the calculation formula is $6.02 \times 10^{14} \times$ plasmid concentration (ng/μL)/(base number of recombinant plasmid× 660). ND1 and β-globin standards were obtained by serial dilutions (10^8-10^3 copies). PCR amplifications of the samples and standards were run simultaneously on an ABI Stepone-Plus fluorescence quantitative PCR instrument. The copy number of ND1 and β-globin in the samples was calculated according to standard curves. The mtDNA copy number per diploid nuclear genome was calculated according to the formula 2×ND1/β-globin.

Statistical analysis

The nucleotide sequences of HVS and 10171-10659 were edited, aligned and compared with rCRS using Vector NTI Advance 11 software. Owing to the complexity and variability, length polymorphisms of the poly-A and poly-C stretches in 16180-16188 and 303-315 regions were disregarded in the analyses. The characteristics of all subjects were analyzed by Pearson X^2 test or Fisher exact test. To assess the relationship between COPD and mtDNA haplogroups, single factor analysis of variance was performed to calculate the adjusted odds ratios (OR) and 95% confidence intervals (CI). The significance of differences in the mtDNA copy numbers between COPD and healthy controls was determined using a Student's t-test with two-tailed *p*-values. All statistical analyses were conducted using Statistical Package for Social Science, version 22.0 for Windows (SPSS,

Table 4. Distribution of mtDNA haplogroups among controls and cases

mtDNA Haplogroup	Controls (n=60)	Cases (n=60)	*p* value	Continuously corrected *p* value	OR (95% CI)
D	20 (33.33%)	9 (15.00%)	0.019	0.033*	0.353 (0.145-0.859)
B	5 (8.33%)	6 (10.00%)	0.752	1.000	1.222 (0.352-4.244)
M7	2 (3.33%)	11 (18.33%)	0.008	0.019*	6.510 (1.376-30.792)
M10	3 (5.00%)	2 (3.33%)	0.648	1.000	0.655 (0.106-4.069)
F	2 (3.33%)	10 (16.67%)	0.015	0.033*	5.800 (1.213-27.728)
A	5 (8.33%)	10 (16.67%)	0.168	0.270	2.200 (0.704-6.877)
N9	4 (6.67%)	3 (5.00%)	0.697	1.000	0.737 (0.158-3.443)
M9	8 (13.33%)	1 (1.67%)	0.015	0.038*	0.110 (0.013-0.911)
G	4 (6.67%)	2 (3.33%)	0.402	0.675	0.483 (0.083-2.741)
M8	7 (11.67%)	6 (10.00%)	0.769	1.000	0.841 (0.265-2.669)

*p<0.05 compared with the control group.

Inc., Chicago, IL, USA). The values were represented as the means ± SD, where p<0.05 was considered statistically significant.

Results

Subject characteristics

In this study, 60 unrelated COPD patients and 60 unrelated controls were recruited from Northwest Hubei, China. Descriptive characteristics of the study population are presented in **Table 3**. The variables mainly include gender, age, lung function (FEV1/FVC) and body mass index (BMI). There was no difference in gender composition between the COPD and control groups (p>0.05). For BMI and FEV1/FVC, there were extremely significant differences between the two groups (p<0.01).

mtDNA haplogroups

Subjects in the COPD and control groups were classified into 10 major mtDNA haplogroups: A, B, D, F, G, M7, M8, M9, M10 and N9 (**Table 4**). Compared with the control group, haplogroups F and M7 were significantly more prevalent (OR=5.800, 95% CI=1.213-27.728, p=0.033, and OR=6.510, 95% CI=1.376-30.792, p= 0.019, respectively) in the COPD group, whereas haplogroups D and M9 were significantly lower in the COPD group (OR=0.353, 95% CI=0.145-0.859, p=0.033, and OR=0.110, 95% CI=0.013-0.911, p=0.038, respectively). Therefore, we speculated that haplogroups F and M7 might be risk factors for COPD. In contrast, haplogroups D, and M9 might be associated with a reduced risk for COPD.

mtDNA copy number

The mtDNA copy number in the control group was significantly lower than that in the COPD group (131.86±9.34 VS 159.57±9.96, p<0.05) (**Figure 1**). We hypothesized that the mtDNA copy number in the peripheral leukocytes increased with the occurrence and development of COPD. According to the grading standards presented in **Table 1**, the COPD patients included in this study could be divided into 3 grades (grade I, II and III). The average mtDNA copy numbers in these three grades were 125.46 ±12.25, 165.57±21.43 and 184.34±15.37, respectively (**Figure 2**). Our analysis showed that the difference between grade I and III was significant (p<0.05), but there were no statistically significant differences between grade I and II or between grade II and III.

Discussion

In our research, a case-control study was used to explore the relationship between peripheral leukocyte mtDNA and COPD in a Han Chinese population. Through strict case selection and a scientifically rigorous experimental process, we found that haplogroups F and M7 might be risk factors for COPD, whereas haplogroups D and M9 might be associated with a reduced risk for COPD. The peripheral leukocyte mtDNA copy number in the COPD group, which increased with the severity of disease, was higher than that in control group. Our results confirmed once again that mtDNA is related to the occurrence and progression of COPD. Due to the small sample size, caution needs to be exercised in interpreting these results.
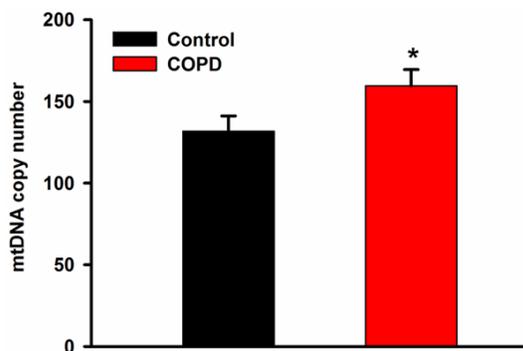
Figure 1. mtDNA copy numbers in the control group and COPD group. The average copy numbers in the two groups were 131.86±9.34 and 159.57±9.96, respectively. *p<0.05 compared with the control group.
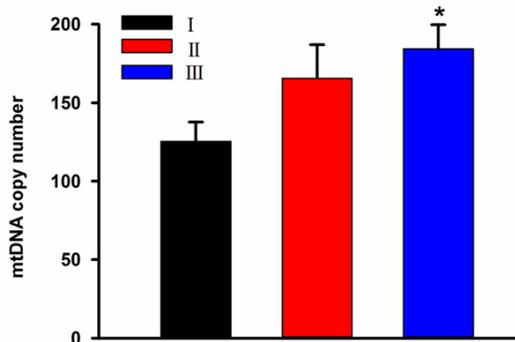
Figure 2. mtDNA copy numbers in various grades of COPD. The average copy numbers in grade I, II and III disease were 125.46±12.25, 165.57±21.43 and 184.34±15.37, respectively. *p<0.05 compared with grade I.

There is a very strong physiological link between the mitochondria and the human respiratory system. The lungs extract oxygen which is essential for mitochondria to generate ATP. Under physiological conditions, oxygen-based radicals are byproducts of the OXPHOS process. To ensure an appropriate defense against these byproducts, the mitochondria have both enzymatic and non-enzymatic antioxidant systems. However, among COPD patients and smokers, the increased exogenous and endogenous ROS leads to oxidant/antioxidant imbalance [16]. Even in respiratory diseases that are not associated with smoking, such as pulmonary fibrosis, the oxidant/antioxidant balance may be disturbed by other factors [17].

Studies that have investigated the role of mtDNA in respiratory disease, especially cancer

and COPD, have been accumulating in recent years. However, until now, the relationship between mtDNA and COPD was not very clear, and the clinical application of mtDNA remains to be explored. To elucidate the potential role of mtDNA including mtDNA haplotypes and copy number in the development of COPD, we examined the haplotype and copy number of mtDNA in the peripheral blood leukocytes from COPD patients. The results suggest that only the distributions of haplogroups F, M7, D and M9 between COPD patients and controls were significantly different. Specifically, under the same conditions, mtDNA haplotypes F and M7 will increase the risk of COPD, whereas haplogroups D and M9 will decrease the risk of COPD. In addition, we found that the mtDNA copy number was higher in the COPD patients than that in the healthy controls, and this number increased with the severity of disease. Oxidative stress is known to cause mtDNA damage which in turn leads to abnormal OXPHOS. However, the mtDNA copy number will increase to compensate for this damage [18, 19]. From this point of view, both increases in the mtDNA copy number and the incidence of COPD are closely related to the accumulation of oxidative stress, and the mtDNA copy number can be used to assess the severity of COPD.

Several mtDNA haplogroups have been found to participate in human longevity, carcinogenesis, lung cancer, Leber's hereditary optic neuropathy (LHON), and other metabolic and degenerative diseases. Haplotype D is defined by the specific variation C5178A in mitochondrial NADH dehydrogenase subunit 2 (ND2). Prior studies have shown that the protective effect of a Leu→Met substitution at amino acid 237 (L237M) of ND2 (C5178A) not only contributes to human longevity [13] but also decreases the risk of lung cancer and COPD [20, 21], which supports our findings that mtDNA haplotype D can reduce the risk of COPD. It has been proposed that methionine residues constitute an important antioxidant defense mechanism [22]. According to the predicted model of the human ND2 molecule [23], the Leu→Met substitution at amino acid 237 of ND2 is exposed at the surface of complex I and may play an important role in the protective effect against oxidative damage to mitochondria by serving as an efficient oxidant scavenger.

Haplotype M9 is another kind of mtDNA haplotype. Although haplotype M9 does not corre-

late with lung cancer [20], it has been shown to be associated with resistance to COPD [21]. In this study, haplotype M9 was also identified less often in COPD cases than healthy controls, and the difference reached the level of significance. The possible reason for the different roles of haplotype M9 in susceptibility to COPD and lung cancer may be the role of chronic hypoxia in the development of COPD. Chronic hypoxia is observed not only in patients with severe COPD but also after exercise in patients with moderate COPD [24]. Hypoxia is involved in muscle dysfunction, anorexia, hormonal derangement, and other COPD processes [25]. Thus, chronic hypoxia may be responsible for the different roles of haplotype M9 in lung cancer and COPD. However, the profound role of haplotype M9 in the resistance for COPD should be investigated further.

It has been reported that the haplotype F is related to a decreased risk for lung cancer and COPD [20, 21]. However, haplotype F was found to be a risk factor for COPD in this study. One hypothesis for this phenomenon is the small sample size. Although the sample size of this study was less than others, a significant difference in the frequencies of the haplotype F between COPD patients and healthy controls was observed. According to phylogenetic classification, Haplotype F can be divided into sub-haplotypes F1-4 in East Asians [26]. The correlation between these sub-haplotypes and COPD susceptibility needs to be further explored. The other possible explanation for this phenomenon is that COPD risk is not influenced solely by mtDNA variations. The nuclear background in which the mtDNA haplogroups are classified may also contribute to the individuals' susceptibility to COPD. Investigating the protective effect against oxidative damage to the mitochondria, the generation of endogenous ROS under various conditions in the different mtDNA haplogroups with the same nuclear background, and investigating the different nuclear background with the same mtDNA haplogroups, may help us, at least in part, to explore the fundamental mechanisms.

Haplotype M7 was also found to be a risk factors for COPD in this study. Haplotype M7 is defined by a specific variation at np T9824C [15]. Previous studies have demonstrated that M7b1'2, a sub-haplotype of M7, was found to

increase the penetrance of LHON [27], and haplogroup M7 was a risk factor for mild acute mountain sickness (AMS), lung cancer and COPD [28]. In the present study, haplotype M7 was found to be present in 3.33% of the controls, but was present in the COPD cases at a frequency of 18.33%. The relationship between mtDNA deletion, mtDNA haplotype, and cigarette smoking indicated that haplotype M7 may better tolerate the damage by external ROS caused by cigarette smoking [20].

Mitochondria play important roles in cellular metabolism and energy production and act as a primary source of reactive oxygen species (ROS) [8, 29]. The quantity and quality of mtDNA seriously affect mitochondrial function, which remains relatively stable under physiological conditions. Alterations in mtDNA copy number have been considered to be one of the factors involved in the pathogenesis of OXPHOS disorders [30, 31]. Thus, the levels of antioxidants and pro-oxidants may play a role in the regulation of mtDNA copy number. Recently, it has been reported that an increased mtDNA copy number is associated with future development of lung cancer [14, 32], which supports our findings that the mtDNA copy number in the COPD patients was significantly higher than that in the healthy controls. In addition, we also found that the mtDNA copy number increased even further with increases in the severity of the illness. However, these results contradict another case-control study that showed that a decreased mtDNA copy number was associated with an increased risk of COPD [33]. There is also a study showing that the median level of mtDNA copy number may be associated with a higher risk of lung cancer than the high level among current smokers [34].

The biological mechanism for an association between the increased mtDNA copy number and development of COPD is not completely understood. A positive association between the relative mtDNA copy number and markers of oxidative stress including 8-hydroxyguanosine and thiobarbituric acid-reactive substances was observed in humans [18]. An increased mtDNA copy number was also associated with lower levels of antioxidants in plasma [18]. Fibroblasts that are exposed to mild oxidative stress show an increase in mitochondrial mass through a cell-cycle independent pathway [31].

The high copy number of mtDNA in aging cells is thought to be the result of a compensatory response to the cumulative exposures to oxidative stress and accumulation of mtDNA mutations [35]. An increased mtDNA copy number in peripheral blood may reflect the increased levels of oxidative damage. Additionally, nuclear anomalies of some key players, such as p53 and mtDNA polymerase γ (POLG), are also associated with changes in mtDNA content in several cancer types [36, 37]. Thus, the mtDNA copy number in peripheral blood may reflect the biological processes that are relevant to COPD.

There are several limitations to our population-based case-control study design. First, due to some objective factors, patients with very severe COPD were not included in this study. Further verification with more comprehensive grouping as well as biological studies are needed to confirm the relationship between the mtDNA copy number and the severity of COPD. Second, although several mtDNA haplogroups were found to be associated with COPD susceptibility, the study did not analyze the pathophysiological consequences of the mtDNA variants that defined the mtDNA haplogroups. Nevertheless, prior studies have revealed that mtDNA variations determine differences in OX-PHOS performance and ROS production. Third, because the distribution of mtDNA haplogroups varies spatially in China, large-scale studies are required to clarify the association of mtDNA variation with the risk of COPD in other populations.

In summary, we conducted a case-control study to investigate the relationship between mtDNA and development of COPD. Our findings suggested that mtDNA haplotypes F and M7 may be risk factors for COPD, whereas haplotypes D and M9 may decrease the risk of COPD. In addition, the study also revealed that mtDNA copy number of eripheral leukocytes in COPD group is significantly decreased compared with the control group. Furthermore, the mtDNA copy number may be used as the assessment of severity of COPD.

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Disclosure of conflict of interest

None.

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