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Mitochondrial DNA haplogroups modify the risk of osteoarthritis by altering mitochondrial function and intracellular mitochondrial signals

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ABSTRACT

Haplogroup G predisposes one to an increased risk of osteoarthritis (OA) occurrence, while haplogroup B4 is a protective factor against OA onset. However, the underlying mechanism is not known. Here, by using trans-mitochondrial technology, we demonstrate that the activity levels of mitochondrial respiratory chain complex I and III are higher in G cybrids than in haplogroup B4. Increased mitochondrial oxidative phosphorylation (OXPHOS) promotes mitochondrial-related ATP generation in G cybrids, thereby shifting the ATP generation from glycolysis to OXPHOS. Furthermore, we found that lower glycolysis in G cybrids decreased cell viability under hypoxia (1% O_2) compared with B4 cybrids. In contrast, G cybrids have a lower NAD^+/NADH ratio and less generation of reactive oxygen species (ROS) under both hypoxic (1% O_2) and normoxic (20% O_2) conditions than B4 cybrids, indicating that mitochondrial-mediated signaling pathways (retrograde signaling) differ between these cybrids. Gene expression profiling of G and B4 cybrids using next-generation sequencing technology showed that 404 of 575 differentially expressed genes (DEGs) between G and B4 cybrids are enriched in 17 pathways, of which 11 pathways participate in OA. Quantitative reverse transcription PCR (qRT-PCR) analyses confirmed that G cybrids had lower glycolysis activity than B4 cybrids. In addition, we confirmed that the rheumatoid arthritis pathway was over-activated in G cybrids, although the remaining 9 pathways were not further tested by qRT-PCR. In conclusion, our findings indicate that mtDNA haplogroup G may increase the risk of OA by shifting the metabolic profile from glycolysis to OXPHOS and by over-activating OA-related signaling pathways.

1. Introduction

Mitochondria are the "cellular powerhouses," which generate most of a cell's ATP through oxidative phosphorylation (OXPHOS). Human mitochondrial DNA (mtDNA) is predominantly maternally inherited and encodes 13 essential polypeptides of the OXPHOS system, as well as 2 rRNAs and 22 tRNAs for mitochondrial translation. Evolutionarily, the genetic background of a specific mtDNA (mtDNA haplogroup) is defined by a set of single nucleotide polymorphisms (SNPs), which are genetically and statistically associated under the selective forces of environmental factors and random drift [1]. Currently, it is well accepted that those past adaptive mtDNA haplogroups might now be either advantageous or detrimental [2].

Different mtDNA haplogroups are reported to be protective or risk factors in a number of degenerative conditions such as aging [3], diabetes [4], Alzheimer's disease [5], Parkinson's disease [6], and schizophrenia [7]. Studies from different research groups have attributed the causes to differences in mtDNA content [8], mtRNA level [8], mitochondrial translational products [9], respiratory chain complex assembly

Abbreviations: ACSS2, acyl-CoA synthetase short-chain family member 2; ALDH1A3, aldehyde dehydrogenase family 1 member A3; AP-1, transcription factor AP-1; BNGE, blue native PAGE; BPGM, 2,3-bisphosphoglycerate mutase; Cybrid, cytoplasmic hybrid; 2-DG, 2-deoxyglucose; DDM, *n*-dodecyl β-D-maltoside; DEG, differentially expressed gene; ECM, extracellular matrix; GMCSF2, granulocyte-macrophage colony-stimulating factor; G6PC3, glucose-6-phosphatase 3; HBSS, Hank's buffered salt solution; HIF, hypoxia-inducible factor; IL6, interleukin 6; LTB, lymphotoxin beta; MMP, matrix metallopeptidase; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; OA, osteoarthritis; OXPHOS, oxidative phosphorylation; qRT-PCR, quantitative reverse transcription PCR; ROS, reactive oxygen species; RPKM, reads per kilobase per million reads; TWSG1, twisted gastrulation homolog 1.

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[10], ATP generation [11], reactive oxygen species (ROS) production [12], mitochondrial membrane potential [9], mitochondrial matrix pH [13], and intracellular calcium dynamics [13] in different sets of haplogroups, e.g., haplogroup H vs. J, haplogroup H vs. UK, haplogroup B4 vs. E, and haplogroup M vs. N. Furthermore, recent studies on mitochondrial-mediated retrograde signaling indicated that the mtDNA haplogroup can affect the expression levels of nuclear genes related to specific diseases such as age-related macular degeneration [11,14]. Together, these results suggest that the mtDNA haplogroup plays a role in the pathogenesis of degenerative disease.

Osteoarthritis (OA) is one of the most common age-related degenerative diseases characterized by the degradation of articular cartilage, which is caused by dysfunctional chondrocytes. As the only cell type in cartilage, chondrocytes are more glycolytic and sensitive to the level of hypoxia inducible factor (HIF)-1α because most chondrocytes occur in a low-oxygen environment [15,16]. Till recently, mitochondrial dysfunction was found to be affecting different pathways such as chondrocyte oxidative stress and apoptosis, decreased chondrocyte biosynthesis, up-regulated chondrocyte inflammation and matrix catabolism, and accelerated cartilage matrix calcification [17–19]. Therefore, it is no surprise that the mtDNA haplogroup plays a role in the progression of knee/hip OA in different geographic populations. However, as most of these studies are purely descriptive, there remains a yawning gap in the understanding of the association of mtDNA haplogroup with OA occurrence. In an earlier study, we showed that mtDNA haplogroups G has a higher risk of OA occurrence, while haplogroup B4 has a lower risk [1]. Here, by using trans-mitochondrial technology, we aimed to elucidate the role of mtDNA haplogroup in the occurrence of OA by characterizing the alternation of mitochondrial function, including ATP production, ROS level, NAD^+/NADH ratio, and mitochondrial-mediated retrograde signaling in haplogroups B4 and G.

2. Materials and methods

2.1. Generation of cell lines and culture conditions

Trans-mitochondrial cybrids were obtained by fusion of mtDNA-less ρ0 human osteosarcoma 143B cells with platelets of haplogroup G ($n = 2$) and B4 ($n = 2$), as described previously [20]. The transformant cybrid clones were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Waltham, MA, USA) containing 10% cosmic calf serum (Gibco, Carlsbad, CA, USA).

2.2. mtDNA sequencing and genotyping

Forty healthy volunteers were recruited from The First Affiliated Hospital of Wenzhou Medical University. Informed consent was obtained from all subjects under protocols approved by the Ethics Committee of the Wenzhou Medical University. To obtain platelets used to generate cybrids G and B4, genotyping of mtDNA haplogroups G and B4 was performed according to our published protocols [1]. Briefly, subject with haplogroup-diagnostic variants of mt.4833A > G + mt.16363 T > C + mt.16278C > T + mt.10400C > T + mt.10398A > G and mt.16189 T > C + mt.16217 T > C were considered as G and B4, respectively. For the mtDNA sequencing of cybrids G and B4, genomic DNA was extracted using a sodium dodecyl sulfate (SDS) lysis protocol, as described previously [21]. The entire mtDNA genome was Sanger sequenced on an ABI 3730XL system (Applied Biosystems, Foster City, CA, USA) using 24 previously reported pairs of mtDNA primers [22]. Detailed mitochondrial DNA haplotype was annotated using http://www.phylotree.org based on the entire mtDNA genome.

2.3. mtDNA copy number quantification

Genomic DNA was extracted using a SDS lysis protocol as described earlier [21]. The ratio of mtDNA versus nuclear DNA (nDNA) was generated to represent the relative amount of mtDNA copy number. Real-time PCR reactions were performed on a on a Step-One Plus Real-Time PCR System (Applied Biosystems) using SYBR® Green qPCR Mastermix (Takara, Dalian, China). The primers were as follows: mtDNA ND2, forward, 5′-CCTATCACCCTTGCCATCAT-3′, and reverse, 5′-GAGGCTGTTGCTTGTGTGAC-3′; 18 s nDNA, forward, 5′-TAGAGGGA CAAGTGGCGTTC-3′, and reverse, 5′-CGCTGAGCCAGTCAGTGT-3′. PCR efficiencies of these primers were 90–110%.

2.4. Quantitative reverse transcriptase PCR analysis

RNA was extracted from G and B4 cybrids using TRIzol as described previously [23]. Total RNA (5 μg) was reverse-transcribed into cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). The primers used to determine the mRNA levels of 13 mtDNA-encoded oxidative phosphorylation subunits were as described previously [11]. For nuclear genes, the primers used are listed in Table S1. In all cases, the expression levels of mRNA were normalized by 18S RNA, and real-time PCR was performed as described above. Data obtained from qRT-PCR were analyzed by the ΔΔCT method.

2.5. Mitochondrial protein preparation, blue native PAGE, and respiratory complex enzymatic activity assay

Mitochondria from cultured cells were isolated as previously described [24]. Mitochondrial membrane proteins were extracted from mitochondria using DDM (Sigma, St. Louis, MO, USA) at a ratio of 2.5 g detergent/g protein for the isolation of individual OXPHOS complexes. Protein (60 μg) containing 0.5% Blue G-250 (Sigma) and 5% glycerol were run on a 3–11% gradient BNGE as previously described [25]. Immunoblotting of OXPHOS complexes was performed as described in another study [26], and blotting was done with anti-NDUFA13 (Grim19) (1:1000; MitoSciences, Eugene, OR, USA), anti-SDHA (1:1000; MitoSciences), anti-Core2 (1:1000; MitoSciences,), anti-COXI (1:1,1000; MitoSciences), and anti-ATP synthase subunit alpha (1:1,1000; MitoSciences) antibodies. The enzyme activity of four mitochondrial respiratory chain complexes was measured in the mitochondria of cybrids as described previously [26]. The activity of each enzyme was normalized against that of citrate synthase, a mitochondrial matrix marker enzyme.

2.6. ATP measurements

ATP was measured using an ATP measurement kit (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were grown in 6-well plates to approximately 80% confluence. Approximately 1×10^6cells were washed with cold phosphate-buffered saline (PBS) buffer and then boiled in 100 μL boiling buffer (100 mM Tris, 4 mM EDTA, adjusted to pH 7.75 with acetic acid) for 90 s. Supernatants were retrieved by centrifugation at 10,000 × *g* for 1 min. ATP content was determined by measuring the luminescence of supernatants mixed with luciferase assay buffer using a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). ATP luminescence was normalized by protein concentration. To measure the generation of respiratory complex I-related ATP, a parallel set of cells was incubated with 200 nM rotenone (Sigma) for 24 h before measuring the ATP.

2.7. Measurement of NAD^+/NADH, lactate, and mitochondrial ROS

The NAD^+/NADH ratio was determined in cells cultured with or without 200 nM rotenone for 24 h using a fluorescent NAD^+/NADH detection kit (Cell Technology Inc., Mountain View, CA, USA) [27]. The extracellular lactate level was measured using a fluorimetric-based lactate assay kit (Amplite, Foster City, CA USA) according to the manufacturer's instructions. Briefly, culture medium was filtered through a 10-kDa