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Mitochondria-wide Association Study of Common Variants in Osteoporosis

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Abstract

Many lines of evidence suggest that mitochondrial DNA (mtDNA) variants are involved in the pathogenesis of human complex diseases, especially for age-related disorders. Osteoporosis is a typical age-related complex disease. However, the role of mtDNA variants in the susceptibility of osteoporosis is largely unknown. In this study, we performed a mitochondria-wide association study for osteoporosis in Caucasians. A total of 445 mitochondrial single nucleotide polymorphisms (mtSNPs) were genotyped in a large sample of 2,286 unrelated Caucasian subjects by using the Affymetrix Genome-Wide SNP Array 6.0, and 72 mtSNPs survived the quality control. We first tested for association between single-mtSNP and bone mineral density (BMD), and identified that, a mtSNP within the NADH dehydrogenase 2 gene (*ND2*), *mt4823* C/A polymorphism, was strongly associated with hip BMD ($P = 2.05 \times 10^{-4}$), even after conservative Bonferroni correction. The C allele of *mt4823* was associated with reduced hip BMD and the effect size (β) was estimated to be ~ 0.044 . Another SNP *mt15885* within the Cytochrome b gene (*Cytb*) was found to be associated both with spine ($P = 1.66 \times 10^{-3}$) and hip BMD ($P = 0.023$). The T allele of *mt15885* had a protective effect on spine ($\beta = 0.064$) and hip BMD ($\beta = 0.038$). Next, we classified subjects into the nine common European haplogroups and conducted association analyses. Subjects classified as haplogroup X had significantly lower mean hip BMD values than others ($P = 0.040$). Our results highlighted the importance of mtDNA variants in influencing BMD variation and risk to osteoporosis.

Keywords

mtSNP; haplogroup; osteoporosis; BMD; association

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Conflict of Interest

All the authors declare that they have no conflicts of interest.

Introduction

Mitochondria are the principal generators of cellular ATP by oxidative phosphorylation (OXPHOS) machinery and play a crucial role in energy metabolism, the generation of reactive oxygen species (ROS), and the regulation of apoptosis (Wallace, 2005). Mitochondrion has its own DNA. The human mitochondrial DNA (mtDNA) is a 16.6-kb, double-stranded circular molecule that contains 37 genes. Mitochondrial dysfunction is heavily implicated in the aging process. There is growing evidence that mutations in mtDNA are associated with aging and result in a series of inherited diseases with neurological, skeletal, muscular, or metabolic manifestations (Hiona & Leeuwenburgh, 2008, Trifunovic & Larsson, 2008).

Recently, a number of population genetics studies have suggested a functional role for common mtDNA variants in the human complex diseases (Taylor & Turnbull, 2005), especially for the age-related diseases, such as Alzheimer (Chagnon *et al.*, 1999), Parkinson and Huntington diseases (van der Walt *et al.*, 2003, Schapira, 1999, Pyle *et al.*, 2005). Investigating the relationship between common mtDNA variants and complex diseases has caused much increasing attention.

Osteoporosis is a classic age-related complex disease, which is mainly characterized by low bone mineral density (BMD). BMD starts to decline at the age of ~50 and the trend continues during the rest of life (Ensrud *et al.*, 1995). Therefore, the aging process is associated with bone loss leading to a decrease in BMD and increased susceptibility to osteoporosis. Given the essential role of mitochondria in the aging process, we hypothesize that mtDNA variants may contribute to the development of osteoporosis. Such a hypothesis has been supported by several studies. For example, Trifunovic *et al.* found that increased levels of somatic mtDNA mutations were associated with reduced BMD and osteoporosis in mtDNA mutator mice (Trifunovic *et al.*, 2004). Varanasi *et al.* detected specific mtDNA deletion to be associated with oxidative stress and severe male osteoporosis (Varanasi *et al.*, 1999). However, till now no comprehensive study has been performed at the level of whole mitochondrial genome to identify specific common mtDNA variants influence the risk of osteoporosis.

The aim of the present study was to perform mitochondria-wide association analyses to systematically search for common mtDNA variants in the susceptibility of osteoporosis. To address this, we genotyped a total of 445 mitochondrial single nucleotide polymorphisms (mtSNPs) across the whole mitochondrial genome. Moreover, human mtDNA usually exists in haplogroup forms. In European populations, mtDNA can be assigned to nine common European haplogroups with frequencies of at least 1%. Each of these haplogroups are defined on the basis of specific mtSNPs scattered throughout the mitochondrial genome, which has been reported by Torroni *et al.* (Torroni *et al.*, 1996). Therefore, we further classified subjects into nine common European haplogroups. Association analyses were performed with BMD both at the single-mtSNP level and at the mtDNA haplogroups level in a large sample of 2,286 unrelated Caucasian subjects.

Materials and Methods

Subjects

The study was approved by the required Institutional Review Board or Research Administration Dept. of the involved institutions. Signed informed-consent documents were obtained from all subjects before they entered the study. The sample consisted of 2,286 unrelated healthy subjects. All of the subjects were US Caucasians of Northern European origin living in the Midwest. Subjects with chronic diseases and conditions that might

potentially affect bone mass, structure, or metabolism were excluded from the study to minimize the influence of known environmental and therapeutic factors on bone variation. The exclusion criteria have been detailed in our earlier publications (Deng *et al.*, 2002).

For all subjects, measurement of anthropometric variables was performed and a structured questionnaire addressing medical history and lifestyles was administered. BMD measurements at the hip and spine were obtained using dual-energy X-ray absorptiometry (DEXA, Hologic QDR4500) under the protocol suggested by the manufacturer (Hologic Inc., Bedford, MA, USA). The machines were calibrated daily. The coefficients of variation (CV) values of the DEXA measurements for hip and spine BMDs were approximately 1.40%.

Genotyping and quality control

Genomic DNA was extracted from whole human blood using a commercial isolation kit (Gentra systems, Minneapolis, MN, USA) following the standard protocol. Genotyping was carried out using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA), which includes 445 mtSNPs throughout the mitochondrial genome, according to the Affymetrix protocol. Briefly, approximately 250 ng of genomic DNA was digested with restriction enzyme NspI and StyI. Digested DNA was adaptor-ligated and PCR-amplified for each sample. Fragment PCR products were then labeled with biotin, denatured, and hybridized to the arrays. Arrays were then washed and stained using Phycoerythrin on Affymetrix Fluidics Station, and scanned using the GeneChip Scanner 3000 7G to quantitate fluorescence intensities. Data management and analyses were conducted using the Genotyping Command Console. SNPs were identified using Birdsuite (version 1.5.2; <http://www.broad.mit.edu/mpg/birdsuite/analysis.html>)

In order to ensure a high quality of the genotyping data, quality control procedures were conducted as follows. First, only samples with a minimum call rate of 95% were included. After repeated experiments, all samples ($n = 2,286$) met this criteria and the final mean call rate reached a high level of 98.93%. Second, out of the initial 445 mtSNPs, we discarded mtSNPs: 1) with a call rate $< 95\%$ ($n = 25$); 2) with genotyping concordance rate $< 95\%$ (obtained by duplicate samples, $n = 6$); 3) those having a minor allele frequency (MAF) < 0.01 ($n = 342$). Such performance was consistent with the HapMap data (In HapMap data, 366 of 445 mtSNPs had a MAF less than 0.01). Because most of mtSNPs were observed as rare SNPs (MAF < 0.01) and were therefore excluded, only 72 common mtSNPs passed our quality control for the subsequent analyses. The basic characteristics of these mtSNPs are summarized in Supplementary Table 1.

Statistical analyses

Before association analyses, principal component analysis implemented in EIGENSTRAT (Price *et al.*, 2006) was used to correct for potential population stratification that may lead to spurious association results using all the nuclear SNPs in the SNP Array 6.0. The first ten principal components emerging from the EIGENSTRAT analyses, along with sex, age, weight, height, BMI, and physical activity, were used as covariates to adjust the raw BMD values at hip and spine. The definition of the physical activity phenotype has been detailed in our previous study (MH *et al.*, 2009). We classified individuals as regular versus non-exercisers. After the adjustment by the above covariates, the residuals were used for association analyses.

Single-mtSNP Analysis—All association analyses were performed in R v.2.11. For single-mtSNP variants, linear regression model was used to assess the difference in subjects carrying different mtSNP allele. A raw P value of < 0.05 in our study was considered

nominally significant. A conservative significance threshold for a single test was set at a P value of $< 3.47 \times 10^{-4}$ ($0.05/144_{(72 \text{ mtSNPs that passed our quality control check and 2 tested phenotypes})}$), which takes into account Bonferroni correction.

Haplogroup Analysis—According to the published references and the Mitomap database (Herrnstadt *et al.*, 2002, Macaulay *et al.*, 1999, Torroni *et al.*, 1996), we selected 10 specific mtSNPs to define the most common European haplogroups including H, I, J, K, T, U, V, W, and X (Table 2). Genotypes of these 10 mtSNPs were combined to construct mitochondrial haplogroups. Haplogroups that could not be assigned to one of these nine major haplogroups by the mtSNP combination were designated as “others”. For haplogroup association analysis, we compared BMD difference of each haplogroup with all other haplogroups pooled into one group. This is conceptually the same as the binary SNP allele comparison, which was accomplished by linear regression model. Multiple testing was also adjusted by Bonferroni correction, which yielded a new significance level of 5.56×10^{-3} ($0.05/9$ comparisons).

Results

The basic characteristics of the study subjects are presented in Table 1. We tested for association with hip and spine BMD for all the 72 mtSNPs that passed our quality control criteria as well as for the nine previously defined European haplogroups. For single-mtSNP analysis, we summarized the major association results with hip and spine BMD in Table 3 ($P < 0.05$). Three mtSNPs showed at least nominally significant level of association with hip BMD. The most strongly associated mtSNP was *mt4823*, with a P value of 2.05×10^{-4} , which remained significant even after adjustment for multiple testing by Bonferroni correction (significance threshold: $P < 3.47 \times 10^{-4}$). The effect size (β) was estimated to be ~ 0.044 . Subjects with C allele at *mt4823* had significantly lower mean hip BMD value than those with A allele (decreased by 29 mg/cm^2 , corresponding to a difference of about 0.2 BMD z-score units). For spine BMD, four mtSNPs were detected as nominally significant with P values less than 0.05 (Table 3). The most significant mtSNP was *mt15885*, with a P value of 1.66×10^{-3} . The T allele of *mt15885* was associated with increased spine BMD values and the β was estimated to be ~ 0.064 . Interestingly, *mt15885* was also found to be associated with hip BMD with a P value of 0.023, and the effect is in the same direction as for the spine BMD ($\beta = 0.038$ for T allele). However, the P values became non-significant after Bonferroni correction.

For haplogroup analysis, all nine common European haplogroups were observed in our sample, and the frequency of each haplogroup did not differ significantly from those previous studies of similar North American populations (Torroni *et al.*, 1994, van der Walt *et al.*, 2003) (Table 4). Haplogroup X was identified as nominally significant for association with hip BMD ($P = 0.040$). Subjects carrying the haplogroup X had lower mean hip BMD values than others, and the β was estimated to be ~ 0.042 . A similar finding was revealed for haplogroup X with spine BMD ($P = 0.097$). However, the association did not achieve a nominally significant P value.

Discussion

This study represents the first attempt to perform a mitochondria-wide association study examining common mtSNPs and haplogroups for association with osteoporosis. Previous studies have tested subsets of mtDNA variants for association with a variety of traits (Chinnery *et al.*, 2005, Poulton *et al.*, 2002, Saxena *et al.*, 2006, Autere *et al.*, 2004). However these studies did not analyze association with osteoporosis nor did they test DNA variants at the mitochondrial genome-wide scale. Recently, a growing body of evidence had

implicated a potential role of mtDNA in the development of osteoporosis (Papiha *et al.*, 1998, Trifunovic *et al.*, 2004, Varanasi *et al.*, 1999). In this study, we identified a significant association between *mt4823* C/A polymorphism and hip BMD, which remained significant even after conservative Bonferroni correction. Another SNP *mt15885* T/C polymorphism was also found to be nominally associated both with spine and hip BMD. We further classified individuals into the nine common European haplogroups to test for association of these haplotypes with BMD. Haplogroup X achieved nominally significant association with hip BMD. Overall, our results suggested that mitochondrial genetic variants may play a role in modifying an individual's risk of osteoporosis.

The *mt4823* C/A polymorphism is located in the NADH dehydrogenase 2 gene (*ND2*). *ND2* gene polymorphisms have been reported to be associated with longevity, hypertension, and effects of coffee, alcohol, and cigarette consumption (Catri *et al.*, 2009, Kokaze *et al.*, 2009, Kokaze *et al.*, 2007). Our study first suggested the potential link between this gene and osteoporosis, although the detailed mechanism is still unclear. One possible explanation for the mechanism could be that, *ND2* gene belongs to complex I of the mitochondrion. Complex I is the first and the largest enzyme in the mitochondrial respiratory chain system, and is central to energy production in the cell. Malfunction of complex I has been found to be associated with a number of age-related complex diseases, such as Parkinson disease and ageing (van der Walt *et al.*, 2003). It is conceivable that osteoporosis risk might be caused by the malfunction of complex I. The performance of complex I is possibly mediated through the strongly associated SNP *mt4823* or some other SNPs in high linkage disequilibrium (LD). Individuals who inherit such genotypes may have inadequate capability for energy metabolism and thus be at greater risk for developing osteoporosis. However, the detailed pathological mechanisms await further functional analyses.

A limitation of our study is the lack of replication for our original findings. However, only the latest SNP arrays contain the mitochondrial SNPs, it is difficult for us to seek the *in silico* replication from the published studies. This study represents the best we can do under current conditions. In addition, association studies cannot establish cause and effect. It is hard to tell whether the SNP is the causal variant, or just in high LD with the true functional variants. To address the above limitations, our future research will be directed to the following aspects. First, we would like to perform sequencing analysis to validate our findings and identify truly causal variants. Second, further cellular and molecular studies will be performed, e.g., transfection of cell lines with allelic constructs and testing activities of the different alleles, aiming to reveal and clarify the potential mechanisms.

In conclusion, we identified two polymorphisms, *mt4823* C/A and *mt15885* T/C, and one haplogroup X as potential susceptibility variants for osteoporosis. Our findings highlight the importance of mtDNA variants in osteoporosis risk and open a new avenue for exploring the pathogenesis of osteoporosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Basic characteristics of the study subjects

	Total sample
Number	2,286
Gender (Males/Females)	558/1,727
Age (years)	51.37 (13.76)
Weight (kg)	75.27 (17.54)
Height (cm)	166.35 (8.47)
BMI (kg/m ²)	27.14 (5.75)
Regular/non-exercisers	1635/651
hip BMD (g/cm ²)	0.96 (0.15)
Spine BMD (g/cm ²)	1.02 (0.15)

Note: Data are shown as mean (standard deviation, SD.).

Table 2

Classification of common European haplogroups

Haplogroup	4217C/T	7029C/T	8252A/G	10399G/A	11720C/T	12309G/A	13709A/G	14471C/T	15219G/A	16393T/C
H	---	C	---	A	---	---	---	---	---	---
I	---	T	A	G	---	---	G	---	---	---
J	---	T	---	G	---	---	A	---	---	---
K	---	T	---	G	---	G	G	---	---	---
T	C	T	G	A	---	---	G	---	---	---
U	---	T	---	A	---	G	---	---	---	---
V	T	T	G	A	C	---	---	---	A	C
W	---	T	A	A	---	A	G	---	---	---
X	---	T	---	A	---	A	---	C	---	---

Table 3

Major association results of mtSNPs with BMDs at hip and spine ($P < 0.05$)

SNP	Alleles	Gene	Amino change	MAF	Hip BMD		Spine BMD	
					β	P value	β	P value
<i>mt4823</i>	C/A	ND2	Val -> Val	0.042	-0.044	2.05×10^{-4}	-0.002	0.841
<i>mt15885</i>	T/C	Cytb	Ala -> Val	0.020	0.038	0.023	0.064	1.66×10^{-3}
<i>mt4716</i>	G/A	ND2	Gly -> Gly	0.032	0.030	0.037	0.001	0.861
<i>mt3198</i>	C/T	16S rRNA	-	0.099	-0.003	0.475	-0.022	0.019
<i>mt3012</i>	A/G	16S rRNA	-	0.231	0.001	0.652	0.016	0.026
<i>mt13618</i>	G/A	ND5	Ala -> Thr	0.093	-0.004	0.335	-0.022	0.027

Table 4
Association results of the nine common European haplogroups with hip and spine BMD

Haplogroup	Frequency (%)	Hip BMD		Spine BMD	
		P value	β	P value	β
H	43.70	0.701	-0.002	0.458	-0.005
I	2.71	0.795	-0.004	0.659	-0.008
J	8.57	0.739	-0.003	0.509	0.007
K	5.91	0.365	0.011	0.519	0.008
T	9.89	0.944	-0.001	0.796	-0.003
U	16.40	0.439	-0.005	0.176	-0.011
V	5.16	0.620	0.006	0.656	-0.006
W	2.27	0.145	0.024	0.849	0.003
X	1.05	0.040	-0.042	0.097	-0.046
Others	4.34	-	-	-	-