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Inherited mitochondrial variants are not a major cause of age-related hearing impairment in the European population

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ABSTRACT

Mitochondrial DNA (mtDNA) mutations have been implicated in various age-related diseases. To further clarify the role of mtDNA variants in age-related hearing impairment (ARHI), we determined the DNA sequence of the entire mitochondrial genome of 400 individuals using the Affymetrix Human Mitochondrial Resequencing Array. These were the 200 worst hearing and the 200 best hearing from a collection of 947 Belgian samples. We performed association tests with individual mitochondrial variants, comparison of the mutation load, and association with European haplogroups and their interaction with environmental risk factors. We also tested the influence of rare variants on ARHI. None of these tests showed any association with ARHI.

1. Introduction

MtDNA (mitochondrial DNA) is highly susceptible to mutations (Wallace et al., 1987) due to its higher turnover rate compared with nuclear DNA, the lack of histones and a less effective repair system. Wallace and Fan (2010) have divided the mtDNA variants affecting disease susceptibility into three classes: maternally transmitted deleterious mutations, ancient adaptive variants that predispose individuals to disease in different environments and somatic mtDNA mutations accumulating over time. mtDNA mutations can trigger several complicated cascades of reactions, involving deregulation of oxidative phosphorylation, formation of reactive oxygen species (ROS) and apoptosis. Increased ROS production may result in additional acquired mtDNA mutations or clonal expansion of mtDNA mutations (Kokotas et al., 2007). Cells that accumulate large numbers of mitochondrial deletions and mutations exceeding a critical threshold become damaged and bioenergetically deficient, with cell loss or tissue pathology as a consequence (Seidman et al., 2002). This is especially relevant in post-mitotic tissues in which cellular regeneration is impossible. It is therefore not surprising that many studies highlight the importance of mtDNA mutations in age-related complex disorders (Greaves and Taylor, 2006; Reeve et al., 2008; Seidman et al., 1997; Wallace, 1997).

Age-related hearing impairment (ARHI) or presbycusis is the most common sensory impairment in elderly. The prevalence of clinically significant hearing loss is 60% for people aged 71 to 80 (Davis, 1994). ARHI is a complex disorder, influenced by genetic as well as environmental factors, with a heritability between 0.35 and 0.75 (Demeester et al., 2010; Gates et al., 1999; Karlsson et al., 1997; Viljanen et al., 2007). The impact of several environmental factors has been extensively studied and occupational noise exposure, smoking, BMI and solvent exposure have been indicated as the most significant environmental risk factors (Fransen et al., 2008). Moderate alcohol consumption has a protective effect. Genetic association studies have implicated variants in *GRHL2* and *GRM7* in ARHI (Friedman et al., 2009; Van Laer et al., 2008), but these results still have to be corroborated by independent replication studies. Since the inner ear contains post-mitotic cells, it is not surprising that an accumulation of mitochondrial mutations has been implicated in the loss of hair cells underlying ARHI (Seidman et al., 1997).

Patients with ARHI show a highly significant increase in mitochondrial mutations in auditory tissue (Fischel-Ghodsian et al., 1997; Seidman et al., 2000). The acquired mitochondrial mutation that occurs most frequently in humans is the common mtDNA 4977 bp deletion, which deletes 4977 bp between two 13-bp repeats starting at nucleotides 8470 and 13447. Analyses of human temporal bones indicated that the 4977 bp deletion occurred frequently in ARHI patients (Bai et al., 1997; Dai et al., 2004), whereas it was almost

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absent in age-matched control patients without a history of ARHI (Seidman et al., 1996). Several mouse models for aging indicated that accumulation of mtDNA mutations contributed to ARHI (Kujoth et al., 2005; Trifunovic et al., 2004; Zhang et al., 2002). Inserting a point mutation in the proof-reading domain of mitochondrial DNA polymerase gamma, responsible for replication and repair of mtDNA, resulted in a random and progressive accumulation of somatic mutations. These mice show age-related phenotypes including hearing loss. The knock-in mice exhibited degeneration of the hair cells by 9 months of age (Yamasoba et al., 2007).

Ten different European haplogroups, classified using specific mtDNA polymorphisms, are recognized: H, I, J, K, M, T, U, V, W, and X (Torroni et al., 1994, 1996; Mitomap, 2010, www.mitomap.org). Some of these haplotype groups have been associated with ARHI. Manwaring et al. (2007) found that haplogroups U and K associated independently with a higher prevalence of ARHI. A number of the specific polymorphisms that identify haplogroups and sub-haplogroups are non-synonymous variants (Kivisild et al., 2006), possibly affecting the function of the protein complexes. Lehtonen et al. (2003) found slightly deleterious risk haplotypes among patients with sensorineural hearing impairment which may be due to the cumulative effect of rare sequence variants. It has been suggested that rare variants could be more disease predisposing than common variants (Gorlov et al., 2008).

Many mtDNA variants still await their identification (DiMauro and Andreu, 2000; Montoya et al., 2009), including variants or haplogroups associated with ARHI. To detect putative causative variants, the entire mtDNA must be sequenced. In the past, whole mitochondrial genome screening strategies were limited by the lack of a high-throughput platform for mutation detection (Maitra et al., 2004). The Genechip Human Mitochondrial Resequencing Array 2.0 (Mitochip 2.0) (Affymetrix, Santa Clara, CA, USA) allows a rapid high-throughput analysis of the entire mitochondrial DNA (Zhou et al., 2006). Previous results have indicated that the Mitochip 2.0 platform may be a valuable tool for the identification of new mtDNA mutations in the entire mitochondrial genome (Leveque et al., 2007; Van Eijsden et al., 2006).

To further clarify the role of mtDNA variants in ARHI, we sequenced mtDNA extracted from blood from the 200 worst hearing individuals and the 200 best hearing individuals from a collection of 947 Belgian samples using the Mitochip 2.0 platform. We performed the following analyses: association between single mitochondrial variants and the phenotype, between an individual's mutation load and the phenotype, association with European haplogroups and the interaction between these haplogroups and environmental risk factors.

2. Materials and methods

2.1. Subject samples

A set of 947 independent samples from Belgian volunteers aged 53–67 years, collected as part of a larger study and described previously (Van Eyken et al., 2006), was used for this study. These samples were collected through population registries. Based on the ISO 7029 standard, z_{high}-scores were calculated for the best hearing ear as described by Fransen et al. (2004). Phenotypic outliers for z_{high} were excluded. The 200 best and the 200 worst hearing subjects from this sample collection were selected for the discovery set.

The replication set represents Caucasian volunteers aged 53–67 years originating from nine different centers in seven European countries (described in Van Eyken et al., 2007a, 2007b). Using the z_{high}-scores, the 34% best and the 34% worst hearing 2078 subjects were selected.

2.2. Human resequencing array 2.0

The 400 genomic DNA samples of the discovery set were resequenced using the Genechip Human Mitochondrial Resequencing Array 2.0 (Affymetrix, Santa Clara, CA, USA) covering the entire mitochondrial genome. Two long fragments A and B (Supplementary Table 1) were amplified for each DNA sample by long range PCR with the Expand Long Template PCR system (Roche, Diagnostics, Basel, Switzerland) and further prepared as directed in the Affymetrix genechip customseq resequencing array protocol version 2.1 (http://www.affymetrix.com/support/technical/manuals.affx).

Base calling was performed by a first analysis of the raw Mitochip 2.0 data using the Affymetrix Genechip DNA analysis software (GCOS; GeneChip Operating Software and GSEQ 4.1; GeneChip Sequence Analysis Software 4.1; Model Type = 0, Call Quality Threshold = 3) (Affymetrix, Santa Clara, California, USA) to identify mitochondrial variants.

The rCRS (revised Cambridge Reference Sequence; http://www.mitomap.org/MITOMAP; Andrews et al., 1999) was utilized as the reference sequence for the detection of single nucleotide changes, deletions and insertions. We corrected the reference sequence for known rare variants (mitochondrial DNA positions 750, 1438, 4769, 8860, and 15326) in the rCRS. In all further data-analyses we considered the rCRS allele of these positions as the minor allele.

2.3. Validation Mitochip 2.0

For validation of the Mitochip 2.0 five randomly selected regions throughout the mitochondrial genome, measuring approximately 500 bp, were sequenced (Supplementary Table 2). Four of them were sequenced using conventional Sanger sequencing on the ABI PRISM® 3130xl Genetic analyzer (Applied Biosystems, California, USA). A fifth amplicon covering a region between rCRS mitochondrial position 15,000 and position 15,500 was sequenced by Polymorphic DNA Technologies (California, USA).

2.4. Replication

Mitochondrial variant, mtDNA position 3720, representing European sub-haplogroups U2e was genotyped by Kbioscience (Kbioscience, Hoddesdon, UK) in the replication set.

2.5. Statistical analysis

Across all analyses, hearing was considered a quantitative trait, with the individual z_{high}-scores as ARHI phenotype. We only analyzed variants in the coding region; the D-loop (mtDNA positions 16,024–576) was omitted. Associations between individual variants and the ARHI phenotype were tested using the Mann–Whitney test. Allele frequencies of multi-allelic sites were collapsed into one common allele versus all minor alleles.

Association between the individual mutation load and the z_{high}-score was tested using linear regression. This analysis was performed using 3 distinct sets of variants 1) all variants, 2) only rare variants (MAF < 0.05) and 3) only singletons (N = 1).

To weigh the mutation load, conservation scores were obtained via MegAlign (DNAstar, Wisconson, USA) by comparing the human mitochondrial sequence to those of *Mus musculus* (DQ106412 and V00711), *Rattus norvegicus* (X14848), *Gallus gallus* (AP003580), *Danio rerio* (AC024175), *Xenopus laevis* (M10217), *Caris lupus* (EU442884) and *Bubalus bubalis* (AF547270). We assigned a conservation score between 1 and 6 to each polymorphic site. To calculate an individual's weighted mutation load, conservation scores were summed across all mutations of that individual. Association between the weighted mutation load and the z_{high}-score was tested using linear regression.