Difference in mitochondrial DNA copy number in peripheral blood cells between probands with autism spectrum disorders and their unaffected siblings

Hee Jeong Yooa, Mira Parkb and Soon Ae Kimc

aDepartment of Neuropsychiatry, Seoul National University Bungdang Hospital, Seongnam, Korea; bDepartment of Preventive Medicine, School of Medicine, Eulji University, Daejeon, Korea; cDepartment of Pharmacology, School of Medicine, Eulji University, Daejeon, Korea

ABSTRACT

Objectives: Several reports suggest that mitochondrial dysfunction is involved in the pathophysiology of autism spectrum disorders (ASD). Therefore, mitochondrial DNA (mtDNA) copy number, a common biomarker for mitochondrial dysfunction, might be associated with ASD phenotypes.

Methods: Relative mtDNA copy number in the peripheral blood cells of 100 Korean ASD patients and their unaffected sib-pairs was measured by quantitative polymerase chain reaction (qPCR).

Results: ASD patients had significantly higher relative mtDNA copy numbers than their unaffected sibs \( (P = .042) \). In addition, there were statistically significant correlations between mtDNA copy number and clinical phenotypes for language and communication in ASD.

Conclusions: Our findings suggest that mitochondrial dysfunction and elevated mtDNA copy number may be a biological subtype of ASD that is related to the phenotype for communication.

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Introduction

Autism spectrum disorder (ASD) is a neuropsychiatric developmental disorder characterised by impaired social communication and a tendency for repetitive, solitary interests and behaviours. The prevalence of ASD has markedly increased over the past 40 years. Approximately 4–10 per 10,000 individuals were diagnosed with ASD in the early 1990s, 20–116 per 10,000 individuals were diagnosed after 2000, and as many as 1 in 68 individuals were diagnosed in recent reports (Elsabbagh et al. 2012). The aetiology of ASD is not clearly known in most cases, mainly because of the phenotypic and genotypic heterogeneity among affected individuals.

Mitochondria are intracellular organelles that generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) for energy metabolism. Mitochondrial dysfunction is an excellent candidate for basic cellular abnormalities that cause disturbances in a wide range of physiological and organ systems, since mitochondria are essential for many basic cellular functions throughout the body (Frye & Rossignol 2011).

Considerable evidence has supported a role for mitochondrial impairment in the pathophysiology of autism (Dhillon et al. 2011; Anitha et al. 2012; Rossignol & Frye 2012; Lee et al. 2013; Goh et al. 2014; Goldenthal et al. 2015). In a previous study using magnetic resonance spectroscopy, the brains of ASD patients showed increased lactate doublets, decreased ATP synthesis and disturbed energy metabolism (Goh et al. 2014). Post-mortem studies of human autism brains have also found decreased protein expression of electron transport chain (ETC) complexes in specific regions of the brain (Gu et al. 2013). Although more studies are needed using a comprehensive set of mitochondrial markers to determine the true prevalence of each mitochondrial disease in children with ASD, several studies have estimated a much higher prevalence of mitochondrial diseases in autism than in the general population.
population of children (Rossignol & Frye 2012; Legido et al. 2013).

Mitochondrial DNA (mtDNA) is very susceptible to oxidative or genotoxic damage, and is associated with higher mutation rates (10–200-fold higher) than nuclear DNA. To compensate for the damage, mitochondria replicate to increase their copy number in response to trans-acting factors encoded by nuclear DNA, possibly as a feedback mechanism to counterbalance the metabolic defects in mitochondria carrying mutated mtDNA and impaired respiratory systems (Yu 2011). Abnormal mtDNA number, one of the most common biomarkers, has been associated with mitochondrial dysfunction and increased oxidative stress (Malik & Czajka 2013). Previous studies have suggested that the mtDNA copy number may increase with mtDNA damage or mitochondrial dysfunction, and may compensate for mitochondrial energy metabolism in patients with ASD (Gu et al. 2013). Although mitochondrial function in disease has often been tested by muscle biopsy, the detection of mitochondrial defects in cells readily available from body fluids such as lymphocytes and platelets would be valuable, assuming that many such defects are not confined to the muscle or brain, tissues in which mitochondrial diseases are most strongly evident. Studies of peripheral blood and lymphoblastoid cell lines in individuals with autism have detected elevated mtDNA copy numbers (Giulivi et al. 2010; Chen et al. 2015).

The mtDNAs are maternally inherited (Giles et al. 1980), and the mitochondrial heteroplasmy level in each offspring is often very different from that in the mother. Considering heteroplasmy and shared maternal origin in sib pairs, we compared mtDNA copy numbers in the peripheral blood of probands with ASD to that of their unaffected sib pairs, and examined clinical data to identify related phenotypes.

**Materials and methods**

In total, 100 probands with ASD and their unaffected sibling pairs were recruited through university hospitals to participate in studies conducted by the Korean Autism Genetic Study Consortium. All probands and their siblings were initially screened for pervasive developmental disorders by child psychiatrists, using the Diagnostic and Statistical Manual of Mental Disorders (DSM fourth edition, text revision; DSM-IV-TR), the Korean version of the Social Communication Disorders (DSM fourth edition, text revision; DSM-IV) and the Korean version of the Autism Diagnostic Observation Schedule (ADOS; Yoo & Kwak 2007) and the Autism Diagnostic Interview—Revised (ADI-R; Yoo 2007b). For re-examination of the diagnosis of ASD based on DSM-5, we excluded subjects who did not meet one of the diagnostic threshold criteria of autism in all three domains of pervasive developmental disorders (PDD) to exclude the prior diagnosis of PDD not otherwise specified (PDD NOS) in DSM-IV-TR. As the second edition of ADOS (ADOS-2) (Lord et al. 2012) provides diagnostic algorithms for ASD based on the DSM-5, we re-analysed the data with the revised algorithms and applied the cutoff scores of ADOS-2. As a result, 11.9% of the individuals were excluded from our database, and only those probands satisfying all of the requirements for ASD were ultimately included in this study. The siblings were assessed using the Kiddie Schedule for Affective Disorders and Schizophrenia (Present and Lifetime version) to screen for the presence of concurrent psychiatric diagnoses or possible differential diagnoses. Subjects were excluded if they showed clinically significant neurological diseases, serious medical conditions or chromosomal anomalies, including Down syndrome, fragile X syndrome, Prader-Willi syndrome and neurofibromatosis, which were detected by fluorescence in situ hybridisation. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki Declaration and its later amendments, or with comparable ethical standards. The Institutional Review Boards of the participating institutions approved the study, and written informed consent was obtained from the parents of all subjects.

Each blood sample (2 ml) from the 200 participants was collected in EDTA-containing tubes and stored at −70 °C. Genomic DNA was extracted by the G-spin Genomic DNA Extraction Kit (Intron, Daejeon, Korea) using 500 μl of the blood sample at Eulji University, Korea. The quantity and purity of the DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Korea), and all DNA samples had OD 260/OD 280 values of 1.7–2.0. Total DNA samples were also stored at −70 °C. The mtDNA copy number was evaluated by the ratio of mtDNA to nuclear DNA. In this study, mtDNA copy numbers of three mitochondrial genes, the cytochrome b (CYTB), NADH dehydrogenase 1 (ND1), and NADH dehydrogenase 4 (ND4) genes, were normalised against the single-copy nuclear pyruvate kinase (PK) gene. Changes in mtDNA
copy number were evaluated by quantitative polymerase chain reaction (qPCR) with primers reported in Giulivi et al. (2010). The primers used to amplify human PK were as follows: forward 5'-AGC CCA AAT GCC CTT GAA G-3'; reverse 5'-AGA GAC AGA ATG CCA GTG ACG TT-3'. Primers for CYTB were as follows: forward 5'-CAC GAT TCT TTA CCT TTC ACT TCA TC-3'; reverse 5'- TGA TCC CGT TTC GTG CAA G-3'. ND1 primers were as follows: forward 5'- CCC TAA AAC CCG CCA CAT CT-3'; reverse 5'-CCC TAA AAC CCG CCA CAT CT-3'. ND4 primers were as follows: forward 5'-CCA TTC TCC TCC TAT CCC TCA AC-3' reverse 5'-CAC AAT CTT ATG TTT TGG TTA AAC TAT ATT T-3' (Giulivi et al. 2010). The assay was performed using IQ SYBR Green supermix (BioRad Laboratories, Korea) with the CFX96 Real-Time Detection System (BioRad Laboratories). qPCR was performed under the following conditions: denaturation at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. All assays were carried out in triplicate using 20 ng DNA per 10 µl reaction. The acceptable standard deviation (SD) of the triplicate threshold cycle (Ct) values was set at 0.7. If the result was out of the acceptable range, then the run was repeated using the same sample. The relative mtDNA copy number was calculated using the equation $2^{-\Delta \Delta Ct} (\Delta Ct = \text{Ct}_{\text{mtDNA CYTB}} - \text{Ct}_{\text{PK}})$.

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 20.0 for Windows) and Prism 7. The difference in the distribution of characteristics between patients and their siblings was evaluated using the chi-square test for categorical variables (gender) and the Student’s t-test for continuous variables (age). The age and sex of the participants, as well as the batch used to measure mtDNA copy number, were included as covariates in matched logistic regression in order to rule out confounding effects. To correct the P values after multiple comparisons, we use Bonferroni’s method. Pearson correlation analyses were performed to evaluate relationships between mtDNA copy number in the CYTB region, and data were analysed by the diagnostic algorithm using ADI-R and ADOS and data from other behavioural tests.

**Results**

In total, 100 probands with ASD and 100 matched, unaffected siblings were included in this study (Supplementary data 1, available online). The average age of the patients and unaffected siblings was 98.44 ± 49.17 and 91.84 ± 62.54 months, respectively (P = .41). Although only 17.0% of the patients were female, 39% of the unaffected sibs were females, causing a sex ratio difference between ASD patients and unaffected sibs ($\chi^2 = 12.0, P < .001$).

The mtDNA copy number was higher in ASD patients than in unaffected sibs (Table 1). Based on the paired t-test, there were statistically significant differences in the mtDNA copy numbers of ASD patients and unaffected sibs in three mitochondrial gene regions ($ND1, P = .009$; $ND4, P = .016$; $CYTB, P = .003$). In addition, there were statistically significant differences in mtDNA copy number ($ND1, \chi^2 = 4.719, P = .030$; $ND4, \chi^2 = 4.239, P = .020$; $CYTB, \chi^2 = 6.354, P = .012$) based on matched logistic regression analysis with covariates (sex and age). After Bonferroni’s correction, the copy number of CYTB showed a significant difference in both the paired t-test and the conditional logistic analysis (adjusted P values 0.009 and 0.036, respectively).

There was a statistically significant correlation between mtDNA copy number in the CYTB region and the summary scores of the language and communication domains of ADOS in ASD patients ($r = -0.284, P = .008$; Pearson’s correlation) (Supplementary data 2, available online). However, there were no significant correlations between mtDNA copy number and clinical phenotypes of ADOS and ADI-R in the unaffected sibs group and in whole sample.

**Discussion**

It is known that neurons, as high energy-requiring cells, require large quantities of ATP and maintain high

| Gene | Group    | Mean   | Standard deviation | P value (adjusted P value) | Analysis of Maximum Likelihood Estimates |
|------|----------|--------|--------------------|---------------------------|------------------------------------------|
|      |          |        |                    |                           | $\chi^2$ | df | P value (adjusted P value) |
| ND1  | Patients | 79.92  | 29.12              | .009 (0.027)              | 4.719  | 1  | .030 (0.090)               |
|      | Unaffected siblings | 69.97  | 33.42              |                           |         |    |                           |
| ND4  | Patients | 105.02 | 40.15              | .016 (0.048)              | 4.239  | 1  | .020 (0.060)               |
|      | Unaffected siblings | 92.24  | 45.45              |                           |         |    |                           |
| CYTB | Patients | 132.61 | 47.91              | .003 (0.009)              | 6.354  | 1  | .012 (0.036)               |
|      | Unaffected siblings | 113.46 | 57.49              |                           |         |    |                           |
mtDNA copy numbers (Trounce 2000). Regulation of mtDNA copy number is essential for cells to maintain their energy requirements. Thus, increased mtDNA copy number may be induced by a compensatory mechanism for mitochondrial dysfunction. Tang et al. (2013) suggested that mitochondrial function and intracellular redox status are compromised in the pyramidal neurons of ASD brains, and that mitochondrial dysfunction is a cause of ASD symptoms that appear during early childhood.

A potential mechanism for regulating mtDNA copy number has been proposed in several studies. In general, changes in mtDNA copy number may be attributed to the combined effect of mutations in nuclear and mitochondrial DNA, in response to endogenous or exogenous oxidative stress (Yu 2011). Multiple bioenergetics or metabolic genes and their genetic polymorphisms have been related to mitochondria disease, which might affect the mental function in ASD via mitochondrial dysfunction (Smith et al. 2012). In addition, studies have shown that copy number variations in autistic patients commonly encompass genes that are important for mitochondrial function, ion transport, and synaptic structure and function. mtDNA replication and transcription are tightly regulated by the expression of several genes, including mitochondrial transcription factor A (TFAM) and polymerase γ A (POLGA; Parisi & Clayton 1991; Tomaska et al. 2001; Kucej & Butow 2007). Genetic mutations in other key genes such as mtDNA polymerase γ (POLG), a nuclear-encoded mtDNA polymerase catalytic subunit, are also associated with mtDNA content (Chang et al. 2009; Singh et al. 2009). Furthermore, other nuclear DNA-encoded trans-acting factors responsible for mitochondrial biogenesis and mtDNA maintenance are associated with changes in mtDNA copy number (Yin et al. 2004; Jornayvaz & Shulman 2010). In addition to genetic factors, several environmental factors such as chemical exposure might cause increased mtDNA copy numbers via oxidative stress. Oxidative stress has been well established as a factor that affects mtDNA copy number. mtDNA is highly susceptible to a wide range of damage after exposure to reactive oxygen species (ROS) because mtDNA lacks introns and histones, and because of the decreased level of repair. Furthermore, lipid peroxidation may cause the damage to mtDNA to last longer than damage to nuclear DNA because of subsequent ROS formation (Lin & Beal 2006). Although there is a debate about whether increased oxidative stress is a primary or a secondary event in ASD, the occurrence of increased oxidative stress in ASD patients is consistent in many studies (Kaur et al. 2014).

Gu et al. (2013) analysed the mtDNA copy number in the brain tissues of nine autistic children and nine unaffected individual controls, and found increased copy numbers of three mitochondrial genes (ND1, ND4, CYTB) in autistic patients, which indicated higher mtDNA copy numbers in autism. However, this finding could not be reproduced (Tang et al. 2013). The complex phenotypes of ASD, the diversity of ASD, and the limited sample size in these studies may account for the inconsistent results.

Peripheral blood cells can be collected using non-invasive techniques, and it is relatively easy to collect samples to study mtDNA. The mtDNA copy number in peripheral blood might be an easy indicator of mitochondrial function in brain tissues, because mtDNA copy number in the peripheral blood strongly correlates with that in brain tissues (Kazachkova et al. 2013; Feng et al. 2013). Giulivi et al. (2010) suggested that autism patients were more likely to have mitochondrial dysfunction, mtDNA over-replication and mtDNA deletions than typically developing children. Recently, Chen et al. (2015) confirmed that elevated mitochondrial DNA copy number in the peripheral blood was associated with autism in a relatively large case–control study. However, they did not report any significant associations between relative mtDNA copy number and clinical phenotypes in childhood autism, such as paternal age, maternal age, age of onset, duration of illness, CARS score, ABC score and family training status. In the present study, we did not select unrelated normal controls but rather used ASD affected–unaffected sib pairs with consideration of the potential effects of maternal mitochondria origin, and used ADI-R and ADOS as diagnosis and phenotype-assessment tools. These study design differences may have helped to reveal significant differences in the mtDNA copy number of peripheral blood cells between controls and patients, with controlled age and sex covariates. In addition, we found a negative correlation between mtDNA copy number and the language and communication domain score of ADOS in ASD patients. Because ADOS scores were measured across four modules that depended on the language level of the subjects, the limitation of this analysis was that the scores do not quantitatively reflect communication ability; however, we suggest that our results provide a baseline measurement of the relation between symptom severity and mtDNA copy number.

Although there are several limitations in this study, such as the lack of tissue specificity that results from using peripheral blood and the relatively small sample size, our findings suggest that mitochondrial dysfunction and elevated mtDNA copy number may be a
biological subtype of ASD that is related to the phenotype for communication.

Disclosure statement
None to declare.

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