MITOCHONDRIAL DNA COPY NUMBER IS ASSOCIATED WITH ATTENTION DEFICIT HYPERACTIVITY DISORDER

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SUMMARY

Background: Attention deficit hyperactivity disorder (ADHD) is the most common psychiatric disorder in children. Several hypotheses have been proposed to explain its etiology. Mitochondrial dysfunction (MD) is suggested to be one of the causes of Attention Deficit Hyperactivity Disorder. The objective of the study was to evaluate the relationship between MD and ADHD by investigating mitochondrial DNA (mtDNA) levels from peripheral blood leukocytes, one of the best biomarkers of mitochondrial dysfunction.

Subjects and methods: This study included 56 children aged 6-16 years who were diagnosed with ADHD for the first time and 56 age- and sex-matched children without ADHD. Real-time PCR was performed to determine the relative mtDNA copy number in each study participant.

Results: The mean mtDNA copy number of the case group was 57.623 ± 24.827 and that of the control group was 44.204 ± 18.926 ($p=0.002$). The mtDNA copy number of the case group was higher than that of the control group. Results of ROC curve analysis provided a mtDNA cutoff value of 45.

Conclusion: Significantly higher mtDNA copy number in ADHD group may suggest mitochondrial dysfunction in the etiopathogenesis of ADHD.

Key words: attention deficit hyperactivity disorder - ADHD - mitochondrial dysfunction - mtDNA - oxidative stress

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INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a developmental neuropsychiatric disorder characterized by inattention, hyperactivity, and impulsivity, which begins during early childhood and continues throughout the lifetime of the affected individual (APA 2013, Polanczyk et al. 2007).

Several hypotheses have been proposed to explain the etiology of ADHD. Oxidative stress is suggested to be one of the causes of ADHD and has garnered considerable attention in recent years. Normal oxidation-reduction reactions occurring during cellular metabolism lead to the formation of toxic metabolites called reactive oxygen species (ROS). Organisms produce antioxidants to counteract the harmful effects of the ROS. Oxidative stress occurs when the proportion of antioxidants produced is not sufficient to counteract the harmful effects of ROS (Valko et al. 2006). Oxidative stress damages cellular proteins, lipids, carbohydrates, and nucleic acids (Filomeni & Ciriolo 2006). The brain tissue is highly susceptible to the harmful effects of oxidative stress induced by free radicals because of the moderate levels of antioxidants in the brain despite its high energy requirement (Kim et al. 2015). Various

studies have shown that levels of total oxidant stress (TOS) (Selek et al. 2012); pseudocholinesterase (Archana et al. 2012); nitric oxide synthase, adenosine deaminase, and xanthine oxidase (Ceylan et al. 2012); and nitric oxide and malondialdehyde (Ceylan et al. 2010) are higher in patients with ADHD than in control subjects.

Despite these evidences highlighting the potential role of ROS in ADHD development, mechanisms underlying the contribution of oxidative stress in the pathogenesis of ADHD at the cellular level is unclear. Mitochondria, particularly mitochondrial DNA (mtDNA), which is considered as a potential biomarker of mitochondrial dysfunction (MD) (Malik & Czajka 2013), is highly susceptible to the harmful effects of ROS. MD is suggested to be the most likely mechanism through which oxidative stress contributes to the pathogenesis of ADHD (Burçak & Andican 2004, Eşrefoğlu 2009). Although several studies have shown the relationship of MD with schizophrenia (Altar et al. 2005, Ben-Shachar 2009, Mancuso et al. 2008), bipolar disorder (Andreazza et al. 2010, Cataldo et al. 2010, Sequeira et al. 2012), and major depressive disorder (MDD) (Gardner et al. 2003, Koene et al. 2009, Ryu et al. 2009), limited information is available on the relationship between MD and ADHD (Verma et al. 2016). The present study aims

to investigate the relationship between ADHD and mtDNA copy number, one of the best biomarkers of MD, by comparing mtDNA copy number between the case and control groups, the relationship between mtDNA copy number and ADHD presentations and the association between mtDNA copy number and the severity of ADHD.

SUBJECTS AND METHODS

Subjects

This cross-sectional, and case-control study was conducted in accordance with the Helsinki Declaration and was ethically approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Atatürk University No:B.30.2.ATA.0.01.00/56, Date:28/04/2016).

This cross-sectional, and case-control study was conducted in accordance with the Helsinki Declaration and was ethically approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Atatürk University. Study participants were included on a voluntary basis after informing them and their families about the study and after obtaining written informed consent from the parents of participants who agreed to participate in the study.

The study included 112 children aged 6–16 years who were admitted to the Medical Faculty Research Hospital, Atatürk University, Erzurum, Turkey. Case group included 56 children diagnosed with ADHD for the first time who were admitted to the outpatient clinic of the Child and Adolescent Psychiatry Department, Medical Faculty Research Hospital, Atatürk University. Control group included 56 age- and sex-matched children without any previous history of or without active psychiatric disorders who visited the pediatric outpatient clinic of the Atatürk University Medical School.

Methods

Children diagnosed with ADHD based on clinical examination and semi-structured interview - Kiddie Schedule for Affective Disorders and Schizophrenia Present and Lifetime Version (K-SADS-PL) Turkish adaptation, according to Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria who did not have any comorbid disorder, except oppositional and defiant disorder, and who did not receive any previous medication for ADHD were included in the case group. Children without any active psychiatric disorder according to K-SADS-PL and without any history of psychiatric disorder and treatment were included in the control group. Participants included in the case and control groups filled a sociodemographic data form prepared specifically for this study. The DSM-5-based clinical interviews were performed for determining ADHD Predominantly Inattentive, Predominantly Hyperactive-Impulsive among the children included in the case group. The parents and teachers of the case group were asked to rate their behavior

according to Conners' rating scales to determine the severity of ADHD. All the participants were clinically evaluated and showed normal cognitive development based on the evaluation of their academic skills. Children with acute and/or chronic medical illnesses, major birth and delivery complications, history of trauma, and smoking habit were excluded from both the study groups.

The mtDNA copy number of each study participant was determined by performing real-time polymerase chain reaction (PCR). For this, 2 ml venous blood sample was collected in an EDTA tube from each participant. Total genomic DNA was isolated from peripheral blood leukocytes using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and concentration of the isolated DNA was determined using MaestroNano spectrophotometer (Maestrogen, Las Vegas, NV, USA).

MT-ND1 (mitochondrially encoded NADH dehydrogenase 1) (human) and HBB (hemoglobin subunit beta) (human) primers (Qiagen, Hilden, Germany) were used to calculate relative mtDNA copy number. PCR was performed in a 30-μL reaction mixture containing 17.5 μL 2×SYBR Green Fluor qPCR master mix and 1 μL of each primer. The concentration of primers were 10 μM. DNA concentration in each sample was adjusted to 20 ng·μL⁻¹, and 2 μL DNA was added to each well. All case and controls run on the same plate at the same time with the same gene. Amplification was performed using Rotor-Gene-Q real-time PCR instrument by using the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds. Melting curves for the primers showed specificity. Fluorescence emitted by the SYBR Green dye was recorded using the real-time PCR instrument, and Ct values of the initial concentration of each sample were calculated automatically by the PCR instrument during amplification. Acceptable standard deviation for Ct values was assumed to be 0.05.

Sequences of primers used for determining mtDNA copy number are as follows: forward primer for the NADH dehydrogenase subunit 1 (*ND1*) gene on mtDNA, 5-TTAGTTGCTTGGTTGTGTATTC-3; reverse primer for *ND1* on mtDNA, 5-GAAAAAGGTAAAAAAGCTC TTCAAGC-3; forward primer for the beta-globin (*HBB*) gene on nuclear DNA (nDNA), 5-GGAGA TGCCTCAGAACTGC-3; and reverse primer for *HBB* on nDNA, 5-AGGTTGGAGGTCGG AAAGTT-3.

The number of cycles required for obtaining the threshold level of fluorescence signal is called "threshold cycle" or Ct value. ΔCt values of mtDNA and nDNA were calculated for each sample by using the PCR program. Difference between the ΔCt values of the mtDNA (*ND1*) and nDNA (*HBB*) was calculated using the following formula: ΔΔCt = mtDNA Ct value - nDNA Ct value. Relative mtDNA copy number was determined using the ratio of the amount of mtDNA to that of nDNA by using the following formula (Livak & Schmittgen 2001), $2^{-\Delta\Delta C_t}$. The $2^{-\Delta\Delta C_t}$ method has been

used in previous studies for assessing mtDNA copy number (Burch, Rhim & Nyalwidhe 2016, McGarry et al. 2017, Perkins et al. 2016, Xu & Li 2016).

K-SADS-PL Turkish Adaptation

Kauffman et al. developed the K-SADS-PL form according to DSM-3-R and DSM-4 diagnostic criteria for assessing the psychopathology of children and adolescents aged 6–18 years (Kaufman et al. 1997). The K-SADS-PL is a semi-structured interview form whose validity and reliability in Turkish children and adolescents was determined by Gökler et al. (2004). The K-SADS-PL form can be used to diagnose several psychiatric disorders, except special learning disorder, developmental disorders, and negative symptomatic schizophrenia.

Conners' Parent Rating Scale

Conners' Parent Rating Scale (CPRS-R:S) is a four-point Likert scale (0 = never, 1 = occasionally, 2 = often, and 3 = very often) that is used by parents. In the present study, the parents of the study participants were asked to rate the observed behavior of their children according to this scale. The scale includes inattention/cognitive problem, hyperactivity, ADHD index, and oppositional subscales. In the present study, subscale scores and total score were evaluated using a 27-item short version of the CPRS-R:S (Kaner et al. 2013).

Conners' Teacher Rating Scale

Questions in Conners' Teacher Rating Scale (CTRS-R:S) were answered by the teachers of the study participants according to the observed behaviors of the children in school. Like the CPRS, the CTRS is a four-point Likert scale. The CTRS includes inattention/cognitive problem, hyperactivity, ADHD index, and oppositional subscales. In the present study, subscale scores and total score were evaluated using a 28-item short form of the scale (Kaner et al. 2013).

Statistical Analyses

Statistical analyses were performed using Statistical Package for Social Sciences Statistics (SPSS) 23.0 program (SPSS Inc., Chicago, IL, USA). When alpha and beta errors were 5% and 20%, respectively, the calculated power of the study was 89%. Parametric and non-parametric statistical methods were used based on the distribution of data.

Difference in mtDNA copy number between the case and control groups was assessed using Student's *t*-test in independent groups. The Student's *t*-test and one-way ANOVA were used to investigate other differences among the case and control groups. Post hoc analysis (Tukey and Tamhane's *T*2 tests) was performed to compare multiple groups. Pearson's chi-square and Fisher exact tests were used to compare categorical

variables. Pearson's correlation analysis was used to evaluate the relationship between mtDNA copy number and numerical variables. Linear regression analysis was performed to exclude covariate factors that may affect mtDNA copy number for ADHD development. ROC analysis was performed to calculate the cutoff value of mtDNA copy number for ADHD diagnosis. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

Demographic Data and Clinical Variables

Of the included participants, 62.5% (*n*=70) were boys and 37.5% (*n*=40) were girls, with a mean age of 9.88 ± 2.60 years. Both the case and control groups included 56 children each. Classification of the children in the case group according to the DSM-5 ADHD presentations showed that 7 children (12.5%) were predominantly inattentive, 4 children (7.2%) were predominantly hyperactive-impulsive, and 45 children (80.3%) were both inattentive and hyperactive-impulsive. Moreover, 19.6% (*n*=11) children showed comorbid oppositional defiant disorder (ODD).

Comparison of the sociodemographic characteristics of the children in the case and control groups showed that 64.3% (*n*=36) children with ADHD were boys and 35.7% (*n*=20) children with ADHD were girls. The male to female ratio was 1.8:1. In the control group, 60.7% (*n*=34) children were boys and 39.3% (*n*=22) children were girls. No significant difference was observed between the two groups with respect to sex (*p*=0.696). The mean age of children in the case group was 9.80 ± 2.42 years and that of children in the control group was 9.96 ± 2.78 years, indicating no statistically significant difference between the two groups with respect to age (*p*=0.745).

Association Between mtDNA Copy Number and Demographic Characteristics of the Study Participants

Amplification curves of the genes for calculating ΔC_t values of the two groups are shown in Figures 1 and 2. The calculated mean ΔC_t values of mtDNA (*ND1*) and nDNA (*HBB*) for the case group were 11.98 ± 0.59 and 17.67 ± 0.32 , respectively, and those for the control group were 12.44 ± 0.84 and 17.73 ± 0.55 , respectively. The mean $\Delta \Delta C_t$ value obtained by subtracting the ΔC_t value of nDNA from that of mtDNA was -5.70 ± 0.71 for the case group and -5.29 ± 0.80 for the control group.

The mtDNA copy number was calculated for each study participant. No relationship and correlation was observed between mtDNA copy number and sex and age of the study participants (*p*>0.05). Moreover, no statistically significant association was observed between mtDNA copy number and other sociodemographic characteristics of the study participants.

*The red line shows the threshold. Each color shows one participant's amplification curve (The case group)

Figure 1. Amplification Curves of the Cases Studied HBB Gene

*The red line shows the threshold. Each color shows one participant's amplification curve (The control group)

Figure 2. Amplification Curves of the Controls Studied ND1 Gene

Association Between mtDNA Copy Number and Clinical Variables

The calculation of the mtDNA copy number of the children in the case and control groups showed that the mean mtDNA copy number of the children in the case group was 57.623 ± 24.827 and that of the children in the control group was 44.204 ± 18.926 . This difference was statistically significant, indicating that mtDNA copy number of children with ADHD was significantly higher than that of normal healthy children ($p=0.002$). The mean mtDNA copy number of the children in the case group was 1.30-times higher than that of the children in the control group (95% confidence interval: 1.11–1.53).

Comparison of the children in the case group according to the ADHD presentation types showed that the mean mtDNA copy number of the children who were predominantly inattentive was 60.939 ± 16.881 , that of the children who were both inattentive and hyperactive-impulsive was 58.558 ± 26.368 , and that of the children who were predominantly hyperactive-impulsive was 41.308 ± 11.803 . However, no statistically significant difference was observed among the children showing different ADHD presentations ($p=0.391$). Evaluation of comorbidities showed that the mean mtDNA copy number of children with comorbid ODD was 49.427 ± 24.781 , whereas that of children without any comorbidity was 59.627 ± 24.698 . However, this difference was not statistically significant

($p=0.225$). Table 1 shows the distribution of mtDNA copy number according to the groups, ADHD presentations, and comorbidities. Assessment of the relationship between mtDNA copy number and CPRS and CTRS showed no significant correlation. In Figure 3, this significant difference in the mtDNA copy number can be seen in the form of box-plot chart.

Multiple Linear Regression and, ROC Curve

Multiple regression analysis was performed to exclude confounding factors that affected mtDNA copy number. Factors that were significantly correlated with mtDNA copy number were added to the regression analysis. Results of the multiple regression analysis showed a significant correlation between mtDNA copy number and the study groups. Moreover, we found that mtDNA copy number was higher in the case group than the control group, and was not significantly correlated with other factors. Results of the multiple regression analysis of mtDNA copy number are given in Table 2.

ROC curve analysis was performed to calculate the cutoff value of mtDNA copy number in the two groups. The graph obtained from the ROC curve analysis is shown in Figure 4. Data obtained from the graph provided a p value of 0.005. Moreover, we observed that a cutoff value of 45 predicted the risks of ADHD development with 66% sensitivity and 59% specificity, with $p<0.005$.

Table 1. Distribution of mtDNA Copy Number by Groups, Presentations and Comorbidities

	Mean	mtDNA Copy Number Standard Deviation	p
Groups			0.002^a
Case	57.623	24.827	
Control	44.204	18.926	
ADHD Presentations			0.391 ^b
Combined	58.558	26.368	
Predominantly Inattentive	60.939	16.881	
Predominantly Hyperactive	41.308	11.803	
Comorbidity			0.225 ^a
Absent	59.627	24.698	
ODD	49.427	24.781	

^a Student t-test, ^b One-way ANOVA

Table 2. Multiple Regression Model for mtDNA Copy Number

Variables	Nonstandard Coefficients B	Standard Error	Standard Coefficients Beta	t	p
Constant	54.874	20.344		2.697	0.008
Groups	-15.450	4.360	-0.338	-3.544	0.001
Total Income of Family	-4.649	2.507	-0.174	-1.855	0.066
Physical Illness of Mother	11.854	7.347	0.148	1.613	0.110
Smoking of Mother	-2.459	6.192	-0.038	-0.397	0.692
Smoking of Father	2.025	4.461	0.044	0.454	0.651
Medical Problems at Birth	4.674	4.835	0.095	0.967	0.336

*Regression Model R²=0.14

*In this box plot graph, significant mtDNA copy number difference can be seen between groups

Figure 3. The mtDNA Copy Numbers of Groups

DISCUSSION

We observed that the mean mtDNA copy number of the children in the case group was significantly higher (by 1.3 times) than that of the children in the control group. To the best of our knowledge, this is the first study to examine the relationship between ADHD and mtDNA copy number. Analysis of patients from Childhood Autism Risk from Genes and Environment

Figure 4. ROC Curve of mtDNA Copy Number

(CHARGE) study showed that the mtDNA copy number of children with ASD was 1.4-times higher than that of normally developing children; however, this difference was not statistically significant. Moreover, this study showed that the incidence of mtDNA deletions was 2.6-times higher in children with ASD than in normally developing children (Wong et al. 2016). Another case report of patients from the CHARGE study showed that the mtDNA copy number of children

with autism spectrum disorder (ASD) was 1.5-times higher than that of healthy children (Napoli et al. 2014). In the present study, the mtDNA copy number of children with ADHD was 1.3-times higher than that of normal children, which was similar to that observed for children with ASD. Similar to autism researches, higher mtDNA copy number in ADHD group may suggest mitochondrial dysfunction in the etiopathogenesis of ADHD (Bai et al. 2004).

Although there are a number of factors such as changes in mitochondrial biogenesis, mitochondrial DNA replication machinery, mitophagy that may cause mtDNA copy number change, mtDNA copy number may be an indicator of MD (Clay Montier et al. 2009, Malik & Czajka 2013, Malik et al. 2011). Only a few studies have assessed the role of MD in ADHD development. A recent study by Verma et al. compared the activities of cytoplasmic hybrid neuroblastoma cells (called cybrid cells) generated by placing platelets from patients with ADHD and control subjects with neuroblastoma cells lacking mitochondria (Verma et al. 2016). Results of this study showed that ATPase 6/8 transcription level, mitochondrial complex V activity, cellular and mitochondrial respiration, and mitochondrial membrane potential were lower and oxidative stress was higher in cybrid neuronal cells prepared from the platelets of patients with ADHD than in those prepared from the platelets of control subjects. Thus, the result of this study suggested that mitochondrial pathology is a risk factor of ADHD (Verma et al. 2016), thus supporting the role of MD in ADHD development.

Many studies have investigated changes in the mtDNA copy number of patients with psychiatric disorders. Some studies have reported higher mtDNA copy number in the peripheral lymphocytes and postmortem brain tissues of patients with ASD than in those of control subjects (Chen et al. 2015, Gu et al. 2013). Chen et al. suggested that increased mtDNA copy number was associated with increased replication and decreased degradation of mtDNA because of acute oxidative stress response (Chen et al. 2015). Similarly, Cai et al. reported that the mtDNA copy number of patients with MDD was higher than that of control subjects (Cai et al. 2015). However, they also suggested that the change in the mtDNA copy number affected intercellular communication between hypothalamo-pituitary axis and corticosteroid pathways, leading to MDD development.

Several hypotheses have been proposed to explain the association between MD and psychopathology. The first hypothesis is the direct damage of mtDNA caused by increased oxidative stress, which results in MD. Oxidative stress disrupts protein and chromatin structures by breaking down lipids, proteins, and nucleic acids and by disrupting membrane lipid integrity, thus inducing MD, and apoptosis, and eventually psychopathologies (Avery 2011, Garcia-Giménez et al. 2012, Martínez et al. 2010, Wang, Yang & Yi 2012). The second hypothesis is the effect of oxidative stress on mitochondrial energy production. ADP and H₂O are produced from

ATP during intense energy production in the mitochondria of neurons. Neurons are extremely sensitive to oxidative stress. Studies suggest that neurons of patients with psychiatric disorders are more vulnerable to MD, ROS (e.g., superoxide anions and hydrogen peroxide that cannot be eliminated appropriately), and environmental stimuli (Davies 1995, DiMauro & Schon 2008, Mattson & Liu 2002, Rich 2003).

Although the power value of the sample size in the present study was sufficient to represent all ADHD cases, the sample size was not sufficient to compare among the different ADHD presentation types. A general limitation of mtDNA studies involving peripheral blood samples rather than brain tissue samples also applies to the present study. Moreover, we could not conduct a next-generation sequencing analysis for a more detailed examination and detection mtDNA deletions because of the high cost associated with this genetic test.

CONCLUSIONS

The results of the present study showed that increased mtDNA copy number was significantly correlated with ADHD development, indicating that MD may contribute to the etiopathogenesis of ADHD. However, the high mtDNA copy number detected in patients with ADHD should be validated using different tissue samples. Moreover, follow-up studies should be performed using postmortem brain tissues. In addition, studies involving whole-exome sequencing should be performed to re-evaluate the relationship between MD and ADHD to understand the etiology of this disorder.

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