

Basic research

New substitutions of mitochondrial DNA in Iranian autistic children

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Abstract

Introduction: Autism spectrum disorders (ASDs) are neurodevelopmentally complex diseases with causative de-novo and inherited genetic factors. They contain a range of cognitive and behavioral conditions such as Asperger's syndrome, pervasive developmental disorder and autism. Our study subjects were children with autistic behaviors (15–60 CARS Score).

Material and methods: The DNA extraction process was done using a GeNet Bio DNA extraction kit, and the region of interest was amplified using independent PCR runs. After purification of PCR products, both strands were sequenced by the Big Dye Termination system. The automated sequencing on an ABI 3700 was directly determined with a capillary sequencer machine. Both primers' sequencing results were analyzed using a bioinformatics tool, Sequencher Software 5.

Results: In the population we studied, the variant G9055A (located at ATP6) was reported to be pathogenic (CAAD > 20 and PolyPhen shows it to be probably damaging). In this variant amino acid alanine converts to threonine. A to T substitutions induce accumulation of amyloid fibril in the brain because threonine prefers to form a β sheet as a necessary stage in the amyloidogenic process.

Conclusions: In our study of patients with autism, we found one case having an interesting association with amyloidosis. It is hoped that by finding such markers, the children will be treated with more certainty.

Key words: mtDNA, *ATPase 6/8*, amyloidogenic processes.

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmentally complex diseases with causative de-novo and inherited genetic factors. They include a range of cognitive and behavioral conditions such as Asperger's syndrome, pervasive developmental disorder and autism. The etiology of autism is unclear but it is known that it arises in early childhood. The disease symptoms include impaired reciprocal social interactions, isolated interests, epilepsy, motor defects, gastrointestinal abnormalities, aggression, hyperactivity and sleep and mood disorders [1–4]. Among these, ste-

reotypical behavior, impaired verbal or nonverbal communication and delayed social interaction are the main symptoms [5].

mtDNA-related disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), mood disorder and schizophrenia are specially challenging because of the two different genes (nuclear DNA and mtDNA) involved in the pathogenesis [6]. These types of disorders can occur due to tRNA point mutations or deletions of mtDNA. The cells have thousands of mtDNA molecules. Mutations in the mtDNA result in heteroplasmy (mixture of wild-type and mutant mtDNA). By dividing heteroplasmic cells, these two types of mtDNA randomly spread between cells. Finally, diseases that occur due to defects in mtDNA are inherited from the mother to the children [1, 7].

The prevalence of autism in the USA is estimated to be 1 in 110 individuals. In Iran, the prevalence of autism in 5-year-old children with mitochondrial diseases is 6.26 in 10,000. This is less than one percent of all autistic patients. Also studies of mtDNA show that humans originated in Africa [8, 9]. In mammals, the mitochondrion is the only organelle with its own genome. In the mitochondria, the electron transport chain (ETC) is coded by mtDNA and nDNA. The mtDNA is 16.5 kb and there are 10s-1000s of copies of it in each cell. It has 37 genes that code for 13 subunits of complexes I, III, IV, and V. The remaining subunits are encoded by the nuclear genome [7, 10].

The electron transport chain (ETC) has five complexes among which *ATPase 6/8* is a part of the complex V ATP synthase. In addition to these genes, there are 2 rRNA and 22 tRNA genes which are necessary for the synthesis of polypeptides. As a result, producing mitochondrial energy would definitely be affected by any mutation in the coding of mtDNA. Genetic variation in the mtDNA is a probable cause of autism. The mtDNA is abundant in the brain. It produces energy and is essential for the brain's function [4, 7, 10].

There are few biological markers for diagnosis of autism. That's why a clinical diagnosis is solely based on behavioral observation. Up to now, treating patients with autism is done by using risperidone and aripiprazole, the only drugs that are approved by the FDA. Both of these drugs are atypical antipsychotics which are used to treat irritability, hyperactivity and aggression [3].

ATPase 6/8 genes and also the relationship between autism and *tRNA^{Leu}* and *tRNA^{Lys}* have been investigated in this study [5]. Because of the importance of this disease and increasing prevalence of it, the main objective of this study is to identify variants of *ATPase 6/8* that play a role in producing energy and variants which occur in tRNA as potential risk factors.

Material and methods

Subjects and samples

We selected all children with autistic behaviors (15–60 CARS Score) without attention to presence or absence of similar behaviors in their relatives and assessed their blood (with complete consent) to detect mtDNA gene variants. We analyzed patients with a mean age of 3–5 years who in total comprised 6 girls and 25 boys.

DNA extraction and PCR amplification

The DNA extraction process was done by a GeNet Bio DNA extraction kit, and the regions of interest were amplified using independent PCR runs. PCR amplification was carried out in a final volume of 25 µl containing 200-300 ng of total DNA and 12.5 µl Cinqen PCR Master Kit Cat. No. PR8251C (CinaGen, Tehran, Iran) and 10 pmol of each primer (Table I). After initial denaturation for 5 min at 95°C, 38 cycles of amplification were performed as follows: 55 s at 95°C, 50 s at 55°C at 60°C and 55 s at 72°C followed by 72°C for 10 min. PCR products were checked for yield and purity on agarose gels (Figure 1).

DNA sequencing

Sequence analysis of PCR products was done after purification of PCR products (PCR product purification kit, Roche). Both strands were sequenced by the Big Dye Termination system in a directly determined automated sequencing on an ABI 3700 capillary sequencer machine using both primers (Macrogen, Seoul, Korea). Sequencing results were analyzed using bioinformatics' tools, Sequencher Software 5.

Results

In this study, 31 samples were examined of which 14 unique variants were detected in *ATPase6/8*

Table I. Primer sequences of study areas

mtDNA genes	Primer sequence F 3'→5'	Primer sequence R 3'→5'	Size [bp]	T _m [°C]
<i>tRNA Lys, ATPase 6 and 8</i>	CTACGGTCAATGCTCTGAAA	TACTATATGATAGGCATGTGA	1078	55
<i>tRNA leu</i>	CTCAACTTAGTATTATACCC	GATGGTGAGAGCTAAGGTCG	363	60

and tRNA. All these variants were homoplasmic. The most frequent variant (58.97%) is related to ATP6 with amino acid change T→A. Overall, ATP6 has the most amino acid replacements, following ATP8 and tRNA^{lys} (2.63%). Some of these variants were reported previously in certain diseases, such as prostate tumor, colonic crypts and thyroid tumor with ATP6 mutation. No variants were found related to tRNA^{leu}. The only pathogenic variant was G9055A (Table II, Figure 2).

Discussion

Fourteen unique amino acid replacements are seen in ATPase 6/8. Four of them encompass non-syn replacements which are seen in ATPase 6. One of them known as G9055A (CAAD > 20 and PolyPhen indicates probably damaging) was shown to be pathogenic according to PolyPhen and CADD scores (Figure 1). ATPase 8 includes synonym replacements. Variants that were found in ATPase 6 have been reported in some cancers previously. G9055A variant is known as a Parkinson disease protective factor [11]. Five percent of children with defects in mitochondria are experiencing autism [12]. As a result, abnormalities in mitochondrial components' function can be used as a biomarker in diagnosis of autism. Studies have shown that the activity of complex V (ATPase 6/8) in brain is higher than in other tissues [13]. This means that variants in the energy-generating pathway may make mitochondria stop producing ATP in the brain [14]. Subsequently, deficiencies in the ener-

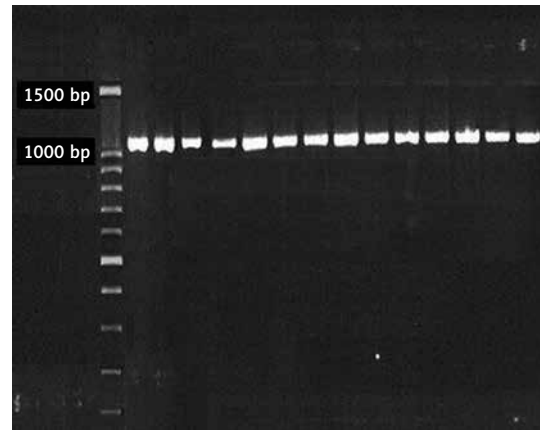


Figure 1. Gene product of tRNA leu/lys and ATPase 6 and 8. All products are 1078 bp

gy-generating pathway as brain neuron fuel might lead to some of the cognitive impairments associated with autism [15]. Polymorphism A8860G appeared as many as 23 times in 31 cases. This polymorphism in the study of Houshmand *et al.* [16] appeared in 36/40 of the autism patients.

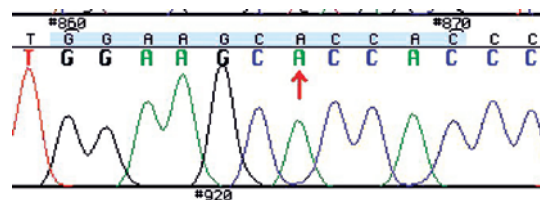


Figure 2. G9055A substitution in ATP6

Table II. Unique mutations in tRNA and ATPase6/8

Nucleotide position	Locus	Amino acid change	Frequency (%)	MutationTaster	CADD Score	PolyPhen	Reported in other disease
A8860G	MT-ATP6	T→A	23 (74)	Polymorphism	6.12	Benign	
G8697A	MT-ATP6	M→M	2 (6.44)	Disease_causing	8.57	-	Thyroid tumor
G8994A	MT-ATP6	L→L	2 (6.44)	Disease_causing	9.35	-	
T8614C	MT-ATP6	L→L	1 (3.22)	Polymorphism	1.96	-	
G8557A	MT-ATP6	A→P	3 (9.7)	Polymorphism	8.62	Benign	Colonic crypts
G8392A	MT-ATP8	W→W	1 (3.22)	Disease_causing	11.6	-	
T8473C	MT-ATP8	P→P	1 (3.22)	Polymorphism	4.64	-	
C8434T	MT-ATP8	I→I	1 (3.22)	Disease_causing	12.92	-	
T8419C	MT-ATP8	L→L	1 (3.22)	Disease_causing	3.55	-	
T8937C	MT-ATP6	L→L	1 (3.22)	Disease_causing	4.58	-	
A8653G	MT-ATP6	P→V	1 (3.22)	Polymorphism	0.01	Benign	Prostate tumor
T8877C	MT-ATP6	F→F	1 (3.22)	Disease_causing	5.13	-	
G9055A	MT-ATP6	A→T	1 (3.22)	Polymorphism	22.6	Probably damaging	PD protective factor*
A8331G	tRNA ^{lys}	A→G	1 (2.56)	Polymorphism	9.67	-	

*Parkinson disease protective factor.

But it is necessary to take a closer look at the G9055A variant. In this variant, the amino acid alanine converts into threonine at position 9055 of ATP6 that shows pathogenesis. A to T substitutions induce accumulation of amyloid fibril because threonine prefers to form a β sheet, as a necessary stage in amyloidogenic processes [17].

Previously the secretion of amyloid- β precursor protein α form (sAPPA) in patients with mild-to-moderate autism was reported [18]. N-truncated amyloid- β causes the production of reactive oxygen species and lipid peroxidation which leads to mitochondrial injury, contributing to abnormal neuronal function [19]. Detecting specific mitochondrial variants which cause excessive expression of β -amyloid in autistic children is a great step in autism diagnosis and treatment. Previous studies showed that threonine amino acid plays an important role in amyloidosis [20]. There are at least 25 proteins forming the amyloid structure [21] which creates beta sheets. Making these sheets is an important step in amyloidosis. Theoretically valine is an amino acid that tends to create beta sheets [22]. In this study, a variant that led to the replacement of alanine with threonine was found. The main focus of our discussion is with regard to this variation.

Children with mild autism express β -amyloid at two or more times the levels of children without autism and this level exceeds four times in children with severe autism. This causes β -amyloid accumulation in the brain and leads to neurotoxicity and impaired transmission of nerve impulses [23, 24]. Also, some studies have reported an association of autism with cancer [25]. In one study, it was reported that people with autism are more susceptible to prostate cancer in comparison with controls [26]. This suggests that individuals with autism have cancer risk due to the commonality of some genes in the cancer pathway [27]. Also dysfunction in ETC can increase the level of reactive oxygen species (ROS) and as a risk factor for cancer, promotes tumor progression [14, 28].

Another study reported G9055A as one of the most significant variants in the risk of breast cancer progression, pancreatic cancer and tubular and villous adenomas with the frequency of 10%, 57% and 100% respectively. This variant is located in a protected area which has a destructive effect on the protein structure [29]. Overall G9055A variant has been reported in a few studies but merely as a simple report, without detailed assessment of its association with autism [1]. But, regarding previous related studies we find a correlation between this variant and amyloidosis and a correlation between amyloidosis and autism.

In conclusion, we conclude that defects in mitochondria affect multiple organs such as muscu-

lar and gastrointestinal systems and the central nervous. These organs are affected in children with autism [30]. We can take steps for better recognition by mitochondrial genetic markers in these patients. In our study among the variations in autistic patients one case has an interesting association between G9055A and amyloidosis. We hope that through finding more biomarkers, autism diagnosis and treatment can be performed with more certainty.

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Conflict of interest

The authors declare no conflict of interest.

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