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Research article

Association of cystathionine beta synthase gene polymorphism with cognitive disorders in autistic children

Mohammed M. El Shafae[1], Jehan H. Sabry[2], Eman G. Behiry[3], Sara A. Elshahat[4] , Maha S. Zaki[5], Nora N. Esmaiel[6]

[1,2,3,4]*Clinical and Chemical Pathology Department, Benha Faculty of Medecine, Benha University.*
[5]*Clinical Genetics at Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre, Cairo, Egypt.*
[6]*Researcher of human molecular Genetic, molecular genetics and Enzymology Department, Human Genetics and Genome Research Division, National Research Centre.*

Key words: Autism; CBS; Homocystiene; G573A (rs73906420), CBS C699T (rs234706).

***Corresponding Author: Nora N. Esmaiel,** Researcher of human molecular Genetic, molecular genetics and Enzymology Department, Human Genetics and Genome Research Division, National Research Centre.

Abstract

Folate, methionine and trans-sulfuration pathways and enzymes` are playing an important role in the pathophysiology of autism. Cystathionine beta synthetase (CBS) is a key enzyme of these pathways that associated with a lot of diseases such as brain atrophy and worsening neurological impairment in various central nervous system (CNS) disorders. CBS gene polymorphisms have been reported as a risk factor for neurodevelopment disorders and psychiatric disease. **Aim:** Hence the present study was designed to investigate the relationship between CBS gene polymorphisms from one side, and autism and the autistic behavior from another side. **Methods:** we sequenced the DNA fragment between exon 8 and exon 10 in CBS gene by using the polymerase chain reaction followed by direct sequencing methods in 40 autistic and 40 control children. **Results:** We found two polymorphisms CBS C699T (rs234706) and G573A (rs73906420). The frequency distribution of mutant and compound genotypes allele (T/T and C/T+T/T) of CBS C699T (rs234706) were (27.5%) and (52.5%) in the autism patients, respectively with a significantly higher association in autistic children; compared to controls (p=0.003 and 0.043). Also C/T showed significantly least frequency associated with sleep disorders and GIT disorders (p=0.016 and 0.001). No significant association was found between CBS genotypes and severity of the autism disorders. G573A (rs73906420) polymorphism was observed only in two autistic patients. **Conclusion:** This study demonstrates a role for CBS (C699T) polymorphism in sleep and GIT disorders and provides further support to the idea that CBS (C699T) gene polymorphism increased risk for autism spectrum disorders (ASD).

Introduction

Autism is a complex neuro-developmental disorder causing a disturbance in social communication interactions and marked by restricted, repetitive behaviors, interests, or activities [1]. Autism Spectrum Disorders (ASD) appears before the age of two of childhood [2]. The latest autism prevalence studies with a male to female ratio were 1 in 110 male children of 4:1 female [3]. The Middle East ranged from 1.4 per 10000 in Oman [4], to 29 per 10,000 in the United Arab Emirates [5]. The etiology of ASD may be genetic, environmental, autoimmune or oxidative stress. Autism has conclusively described as a highly heritable neuropsychiatry disorder in Family and twin studies [6].
There is a critical connection between incidence of ASD and irregularities in folate-dependent one-carbon metabolism and trans-sulfuration, where increase plasma homocyctien level; reduced levels of plasma sulfate and subsequent reduced sulfation capacity are among the most consistent findings in autism research [7]. Whereas, methylation capacity and increased oxidative stress are reduced in people with ASD compared to age-matched controls [8].

Cystathionine β-synthase (CBS) deficiency and/or dysfunction causes homocystinuria, it is an autosomal recessive disorder of sulfur amino acid metabolism. Cystathionine β-synthase protein is a pyridoxal 5′ phosphate dependent enzyme and catalyzes the condensation of homocysteine with serine to form cystathionine [9]. The Biochemical studies revealed that homocysteine and methionine plasma concentrations were elevated, homocysteine in urine was increased and decreased levels of cystathionine and cysteine in body fluids, because of CBS enzyme dysfunction [10]. Patients with homo-cystinuria often display different symptoms, including, disorder of central nervous system (mental retardation, convulsions and psychiatric disturbances) and other manifestations [9]. It has been reported that mutations in the CBS gene caused homocystinuria due to the disruption of enzyme activity which consequently results in increased levels of homocysteine [11].
The human CBS gene is located on chromosome 21q22.3, consists of 30 kb of genomic DNA, and a total of 23 exons [12], only exon 1–14 and 16 encode the CBS protein. In the CBS gene, more than 140 different mutations have been

identified and reported as disease-causing. The most prevalent of these mutations are located in exon 3, 8 and 10 [14].

For all the above, we aimed to study the effect of CBS polymorphisms on the autistic behavior and if it is considered as a risk factor of autism.

Experimental

Subjects

The case controlled study was carried out during the period from December 2015 to December 2016.

Eighty children with their age ranging from 3-6 years, referred from the National Research Centre (NRC) and Pediatrics Department at Benha University Hospital were included; the control group included 40 apparently healthy children matched for age and sex and 40 autistic children with cognitive disorders. All the parents of children recruited for the study gave their informed written consent after being given an explanation of purposes, nature, and potential risks of the study.

Autistic children were diagnosed using Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV-R) [14], and Childhood Autism Rating Scale (CARS) [15]. All cases were subjected to detailed history taking including three generation pedigrees construction, with detailed peri-natal history.

DNA extraction

Blood samples were collected under complete aseptic condition in vacationers containing Disodium ethylene diamine tartaric acid (Na2EDTA) as anticoagulant. We isolated DNA according to the procedures of the DNA isolation kit iNtRON G-spin Total DNA extraction kit (50 preps); catalogue number 17045, Korea (https://www.intronbio.com/eg/).

Genotyping by Sanger sequencing method

Primers Designing

We designed the primers used in this study to cover exon 8, 9, 10 and exon-intron boundaries of CBS gene sequence using NCBI Primer-BLAST tool *(http://www.ncbi.nlm.nih.gov/ tools/primer-blast).* The Forward primer was 5': AATTTTGGAATCCCACAGAAC CCTC and Reverse was 3': AGGAGAGGGCAAGAGAT GTGTA.

Mutation Analysis

Mutation analysis of our fragment in CBS gene was performed by conventional PCR followed by DNA sequencing. The amplification was performed in a reaction mixture of 50 µl containing approximately 2 µl genomic DNA, 25 µl PCR Master mix (2x) containing Taq DNA polymerase, dNTPs,10Mm buffer containing 2mM MgCl2 (iNtRON-Korea), 10 pico mole from each primer and 22 µl Distilled Water. The amplified products were 710 pb had electrophoresis on 2% agarose gel containing ethidium bromide and visualized by ultraviolet (UV) light trans-illumination. The PCR products were purified by wash steps then, DNA is eluted in a low salt buffer or elution buffer. DNA adsorbs specifically to silica membrane of a MEGA quick spin column, then sequenced directly by ABI3730XL sequencer in LGC genomic GmbH, 12459 Berlin/ Germany *(*www.igcgroup.com*).*

Statistical analysis

The statistical analysis of data was done using excel program (Microsoft Office 2013) and IBM SPSS (statistical package for social science) program (SPSS, Inc, Chicago, IL) version 20.

Qualitative data were presented as frequency and percentage. Chi square and Fisher's exact tests were used to compare groups. Quantitative data were presented by mean, SD, median and range. Comparisons between two groups were done using t-test or Man Whitney (for non-parametric), while comparison between more than two groups were done using ANOVA or Kruskal Wallis tests (for non-parametric). Deviations from Hardy–Weinberg equilibrium expectations were determined using the chi-squared test. Odds ratio and 95% confidence interval were calculated. Ordinal and logistic regression analyses were done for prediction of risk factors. N.B: p is significant if <0.05 at confidence interval 95%.

Results and Discussion

Results

In this study we use a designed primer that cover a fragment from exon 8 -exon 10 and their introns boundaries to study any genetic variant could be found in this region by using direct sequencing technique. We found two polymorphisms CBS C699T (rs234706) shown in (Figure 1), and G573A (rs73906420) shown in (Figure 2).

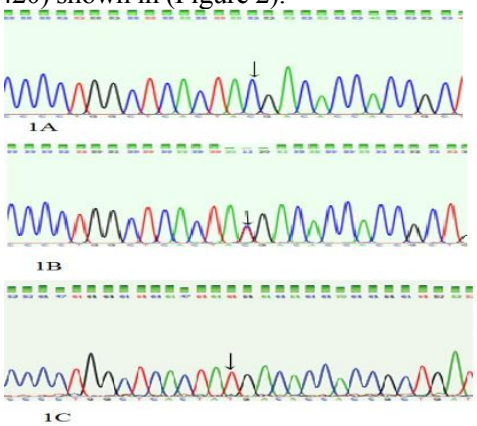

Figure 1. Sequence chromatograms of CBS C699T polymorphism genotypes. 1a shows the wild type C/C. 1b

show the heterozygous allele C/T. 1c show the homomutant allele T/T.

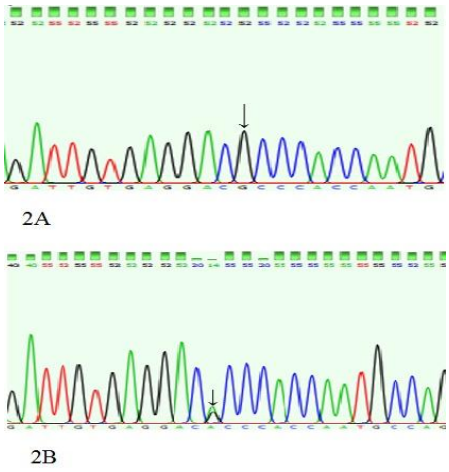

Figure 2. The sequence chromatograms of CBS G573A polymorphism. 2a show the heterozygous allele G/A and 2b show the wild type allele G/G.

CBS C699T (rs234706)

The distribution frequency of the CBS C699T genotypes were, the wild type C/C found in (70 %) of the control cases where it was found in (47.5%) of the ASD cases. The mutant allele T/T didn't found in the control cases, while it presented in (27.5%) of the ASD cases. The combined genotypes hetero and mutant allele C/T+T/T presented in (52.5%) of the ASD cases but in (30%) of the control. The mutant allele T/T and C/T+T/T combined genotypes had a significant frequency ($p_=$ 0.003 and 0.043). Also T allele showed significantly higher frequency ($p_=$0.001) in ASD cases. Data regarding genotype distributions are shown in (Table 1 & 2).

When we studied the distribution of the different types of CBS genotypes with the clinical data we found that, in GIT disorders we have a significantly higher distribution of hetero C/T genotype ($p_=$0.001) as well as combined C/T+T/T genotypes ($p_=$0.004). C/T showed significantly least frequency associated with sleep disorders ($p_=$0.016), and most frequent with NICU admission ($p_=$0.049). Head circumference showed significantly lower level associated with mutant genotype (TT or CT+TT) ($p_=$0.28 and 0.011). The severity of the autistitic behavior shows no significant association to CBS genotypes. (Table 2).

Table 1. Chi-square test was done for the distribution of the CBS (C699T) polymorphism between ASD cases and control cases

	Control N=40		Cases N=40		Crude	
	N	%	N	%	*p*	OR
CC	28	70	19	47.5		R
CT	12	30	10	25	0.580	1.2
TT	0	0	11	27.5	0.003	5.08
CT+TT	12	30	21	52.5	0.043	2.579
C	68	85	48	60	0.001	3.436
T	12	15	32	40		

OR: dds ratio

P significant distribution of each genotype between the studied groups by chi-square test. Statistical significance was at $p \leq 0.05$

Table 2. Statistical analysis of the CBS C699T polymorphism distribution according to clinical factors

	CC N=19		CT N=10		TT N=11		CT+TT N=21		*P1*	*P2*
Age (years)	M=4.417	S.D= 1.2003	M=4.420	S.D =1.2621	M=4.282	S.D =1.3593	M=4.348	S.D =1.283	0.955	0.861
Males	17	89.5	10	100	9	81.8	19	90.5	0.452	1
Females	2	10.5	0	0	2	18.2	2	9.5		
Consanguinity	5	26.3	2	20	7	63.6	9	42.9	0.075	0.333
Family history of psychological disease	8	42.1	2	20	2	18.2	4	19.0	0.363	0.112
History of NICU admission	6	31.6	4	40	0	0	4	19.0	0.049	0.473
Head circumference	49	48-51	49	47-50	48	45-50	48	45-50	0.028	0.011
Abnormal EEG	7	36.8	4	40	5	45.5	9	42.9	0.917	0.698
Abnormal MRI	0	0	0	0	2	18.2	2	9.5	0.128	0.488
GIT disorders	5	26.3	10	100	5	45.5	15	71.4	0.001	0.004
Hyperactivity	13	68.4	8	80	7	63.6	15	71.4	0.824	0.836
sleep disorders	17	89.5	4	40	9	81.8	13	61.9	0.016	0.069
CARS (severity)	35	33-48	33	29-44	43	31-50	38	29-50	0.067	0.929

P1: comparison between 3 genotypes, Statistical significance was at $p \leq 0.05$.

P 2: comparison between CT, TT versus CC, Statistical significance was at p ≤ 0.05. M: mean, S.D=standard deviation

Table 3. Clinical data of CBS G573A polymorphism

Case no	CBS C699T genotype	CBS G573A	sex	Consanguinity	CARS	Degree of autism	EEG	MRI	GIT disorders	Sleep disorders	HC
Case 1	C/C	G/A	M	-ve	48	Severe	Abnormal	Abnormal	+ve	-ve	47
Case2	C/T	G/A	M	-ve	33	Mild	Abnormal	Not available	+ve	-ve	47

G573A (rs73906420)

This polymorphism found only in two autistatic cases with heterozygous allele G/A. The clinical data of the two cases are shown in (Table 3). Where one has a normal allele of the C699T polymorphism but it is a severe autism case while the other case which has the two polymorphisms C699T and G573A was a mild autism degree. The two cases have abnormal EEG, GIT disorders and sleep disorders.

Discussion

Both genetic and epigenetic factors play an important role in the rate and severity of classic autism and autism spectrum disorders (ASDs) [16]. For neuronal function, folic acid, vitamin B12 and B6 are essential and have been linked to increased risk of neuro-developmental disorders and psychiatric disease. Cystathionine β Synthase (CBS) enzyme and gene are involved in B vitamin absorption, metabolism and function. Any disturbances in the enzyme or polymorphisms of genes have been linked to increased incidence of psychiatric and cognitive disorders [17, 18].

There isn't much studing done on CBS polymorphisms and autism despite of its key role in the folate and Hcy metabolism and their effect on neuropsychiatric disorders. In the present study, we investigate the effect of CBS polymorphisms on autism patients and their autistic behavior. Also we aimed to know if it acts as a risk factor of autism or not.

Our results showed that there is a significant distribution of the homozygous and heterozygous mutant allele comparing with control cases.

In a study performed on schizophrenia patients, Korovaitseva [19] reported that, CBS gene mutations were associated with decreased CBS activity leading to elevated plasma homocysteine. Also in another study done by Golimbet [20, 21], they found that polymorphism in CBS gene was associated with increased risk of schizophrenia, changes in attention and auditory evoked potentials. Our results showed that, the CBS C699T polymorphism is associated with the cognitive disorder in ASD children where, mutant and heterozygous alleles have a significant distribution than wild allele.

The results of the present study revealed that C699T polymorphism showed significant association with high score of (CARS) and clinical data from studied ASD participants compared to C/C genotypes. Also, TT, CT+TT genotypes and T allele of C699T polymorphism showed significantly higher frequency in autism cases. Autism cases showed significantly higher frequency of GIT disorders, sleep disorders, and most frequent with admission in neonatal Intensive Care Unit (NICU). In studies performed by [22,23], they found that CBS polymorphisms have been reported in major depression, so C699T polymorphism may be a risk factor for autism and increases their autistic behaviors. Also, American Psychiatric *As*sociation, and Main [2,24] reported that, CBS gene is associated with vascular function reduction, systemic oxidative stress, brain atrophy, and worsening neurological impairment in various central nervous system (CNS) disorders, i.e. autism, epilepsy, Parkinson's disease, Alzheimer's disease, and dementia. More than 140 different disease-causing mutations have been identified in the CBS gene, [13].

The G573A polymorphism was found also in the fragment under consideration. The C699T was found in (52.5%) of the cases while G573A was found only in two cases. Tilley [25] sequenced the DNA from 96 patients with myelomeningocele (MM) to identify novel potential disease causing variants across the 17 exons of the CBS gene. They were the first to describe allele frequencies for the known SNP G573A (rs73906420) that hadn't been reported. They found this SNP in 2 of 96 Caucasian American M patients and 1 from 93 Mexican American MM.

Due to the low presentation of G573A (rs73906420) polymorphism in the studied patients, it didn't give us the possibility to study its correlation with ASD incidence and behavior. But we could take in our consideration that the two patients had abnormal EEG and GIT disorders. One of these patients had the two heterozygous polymorphism G573A (rs73906420) and C699T polymorphism (CT+GA).

Conclusion

In conclusion, our data suggest that C699T polymorphism may contribute to an elevated risk for autism, and may increase the autistic behavior. G573A (rs73906420) polymorphism was found in our study group, but it needed to be studied in a large scale to elucidate its effect on autism. More studies about gene-gene and gene-gene-environment interactions are recommended which could be more powerful than SNP-by-SNP approaches. Haplotype-based association studies are recommended for gene-gene and gene environment interactions which may give an answer

about the complex relationship between folate pathway and the risk of autism.

Declarations

All the parents of children recruited for the study gave their informed written consent after being given an explanation of purposes, nature, and potential risks of the study. The authors declare that there is no conflict of interests' regarding the publication of this paper. The authors certify that no funding has been received for the conduct of this study and/or preparation of this manuscript. The author certify that all persons who have made substantial contributions to the work reported in the manuscript (e.g. data collection, data analysis, or writing or editing assistance)

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