
GENETIC AND ENVIRONMENTAL INFLUENCES ON AUTISM

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ABSTRACT

Polymorphisms of genes encoding enzymes involved in float metabolism have long been hypothesized to be maternal risk factors for autism. In addition to genetic background, the exposure to mercury whether organic or inorganic can give rise to the symptoms of autism spectrum disorders (ASD) such as impairments in social interaction, communication difficulties and repetitive behaviors and interests.

Our objective was to investigate the association between metallothionein (MT) gene polymorphism and levels of mercury in the hair of Egyptian autistic children. Thirty-one autistic children diagnosed by DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition criteria) were subjected to hair mercury measurement using Atomic Absorption Spectrometry (AAS) and were compared to polymorphism of genes MT1A and MT1E matched healthy children.

The present study revealed that there was a highly significant increase in the mean hair mercury level in autistic patients than controls (0.79 ± 0.56) ppm vs. (0.13 ± 0.08) $P < 0.001$ associated with polymorphism in MT1A (AG genotype). There was high significant difference in MT1E (GG genotype) between males and females ($P < 0.001$) while in control were non significant. The results of this study highly support the increase in the mean hair mercury level in autistic children compared to controls.

Key words: Autism, Mercury, Genetics

INTRODUCTION

The autism spectrum describes a range of conditions classified as neuro development disorders in the fifth revision of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5). These disorders are characterized by social deficits and communication difficulties, stereotyped or repetitive behaviors and interests, sensory issues, and in some cases cognitive delays. (American Psychiatric Publishing, 2013).

While genetic factors are clearly important, as indicated by high concordance rates among twins and siblings, they alone cannot account for an epidemic that developed in the relatively short period of 10-20 years (Hebert and Russo, 2006). Thus environmental factors are very likely to account for the major portion of the increased prevalence of autism. Exposure to xenobiotics is an inevitable feature of contemporary life driven by an ever increasing number of threatening chemicals found in air, water and food supplies and other materials we come in contact with during our daily routine (Death *et al.*, 2008). Mercury is a well-known neurotoxin. There are three kinds of mercury exposure: elemental mercury, as well as poisoning inorganic and organic mercury which are the most toxic forms (Chany *et al.*, 2007). More over it is possible that individual vulnerability to toxic environmental exposure varies according to additive and interactive effects of multiple genetics, and poly morphisms that negatively affect detoxification capacity (Lichtens *et al.*, 2010). Because the risk of developmental toxicity with low dose environmental chemicals is likely to be influenced by individual genetic

determination of susceptibility (Gurney and Isere, 2013), it was of interest to evaluate autistic children for the frequency of polymorphic variants that affect the tissue distribution and toxicity of mercury (Bernard *et al.*, 2002; Blanchard *et al.*, 2006 and Windham *et al.*, 2006).

AIM OF WORK

To investigate the association between metallothionein gene polymorphism and levels of mercury in the hair of Egyptian autistic children.

SUBJECTS AND METHODS

Subjects: This is a case-control study conducted on 31 autistic children who were diagnosed by DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders Ver.4) following up at Child and Adolescent Psychiatry Clinic at Children Hospital, Faculty of Medicine, Ain Shams University. They were 22 males and 9 females, their ages ranged from 2 to 11 years and their mean age (\pm SD) was (4.73 \pm 2.008). The control group included 30 healthy children, 22 males and 8 females, their mean age (\pm SD) was (5.05 \pm 2.9). They were subjected to the following:

- 1- Full detailed history (Age ,Sex ,.....etc)
- 2- Psychiatric evaluation
 - a) IQ assessment (Intelligence Quotient) using Stanford Benet Intelligence Scale (2006).

Range of IQ:

- | | |
|-------|-----------------------------|
| 20-30 | severe mental retardation |
| 31-49 | moderate mental retardation |
| 50-70 | mild mental retardation |

71-89 below average

90-109 normal IQ

110-125 above average

b) Assessment of severity of autistic symptoms using Childhood Autism Rating Scale (CARS) (Schopler *et al.*, 1994).

3- Recording of the history of environmental exposure to mercury.

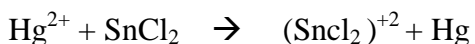
Methods:

1-Measurment of mercury levels in the hair of the children:

A) Hair specimen collection: Hair samples were collected from cases and control by single cutting from the occipital region. The samples were cut to lengths of about 1.5-2 cm using clean stainless steel scissors. Approximately 100 strands of hair (50mg) were used for hair analysis assay. Adhesive paper was placed over the end of the hair strands closest to the scalp. The paper was marked with an arrow indicating the end of hair closest to the scalp. The samples were placed in a sealed plastic bag (McDowell *et al.*, 2004)

B) Hair Hg Analysis: This was done in our study using Atomic Absorption Spectrophotometry (AAS)/hydride system which are one of the most sensitive analytical techniques used for trace element determination.

The determination depends on the formation of atomic mercury at room temperature after reacting with strong reducing agent as stannous chloride SnCl₂ or sodium borohydride is used to liberate Hg as follows:



C) Mercury Reduction: All mercury must be in ionic form, most is present in organic-mercury complexes.

Mercury solutions are unstable, 0.01% $\text{K}_2\text{Cr}_2\text{O}_7$ in 0.8 M HNO_3 is used to prevent mercury loss.

D) Sample Preparation: The samples were cleaned by acetone three times then washed by ultra pure water and dried in an oven at 70° C overnight. The hair samples were then digested for 15 min by adding 4 ml of HNO_3 and 2 ml of H_2O_2 to 0.1 g hair. The digested samples were introduced to Hydride system AAS for mercury analysis (Chakraborty, *et al.*, 1996)

E) Genotyping: Genomic DNA purification used for PCR amplification was extracted from the whole blood sample using a DNA extraction minikit # k0781 thermoscientific.com/onebio made in (Eu) Lithuania kits.

MT1A and MT1E analysis were performed using PCR primers from Jilin University (as a Research Center for Genomic Medicine)

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The Primers for amplification of MT1A gene and control are

F-5' ACTAAGTGTCCCTCTGGGGCTG-3'

R-5' AATGGGTCACGGTT6TATGG-3'

and the other primers for amplification of MT1E gene and control are

F-5'-TCCTCTGGGTCTGGGTTCT-3'

R-5'-GCAATGGCTCAGTGTTGTAT-3'

PCR Amplification was performed in 25 μl reaction mixture with each primer set for which the conditions included on initial denaturation at 94 ° C for 5 min followed by 35-40 cycles of 94 °c for 45 Sec, 55°C for 1 min And 72° C for 1 min and A final elongation at 72°C for 10 min. 15 μl of PCR products was completely digested with 10 units of restriction enzymes, enzyme A fast

digest Pst1 #fd0614 and enzyme E fast digest Nde1#fd0583 (thermoscientific), according to Lina Y. *et al.*, 2008.

Statistical Analysis: Results were analyzed using the Statistical Package of Social Science (SPSS) Computer Software program version 21.0 (Chicago IL, USA), Data were presented in the form of frequencies and percentages. Differences among groups were tested using Pearson's chi-square test. P value of 0.05 indicates significant results, while P value of < 0.001 indicates highly significant results.

RESULTS

There was a highly significant increase in the level of mean hair mercury in patients (0.79 ± 0.56 Ppm) $P < 0.001$ compared to control group (0.127 ± 0.08 ppm) (Table 1), While there was no significant difference regarding IQ and also CARS score (Table 2).

Table (1) shows also the high significant difference between male patients and control ($P < 0.001$) and between female patients and control ($P < 0.001$).

In MT1A, AG genotype was higher in patients than control ($P < 0.001$) (Table 3). The results in Table (5) shows that GG genotype in MT1E is higher in patients than control group. Results also show that allele T ($P < 0.05$) and allele G ($p < 0.001$) were also higher in patients than control group (Table 5). There was no significant association between MT1A and MT1E genotyping with mercury levels (Tables 4 & 6)

Regarding SNP selection and RFLP analysis, we detected 2 SNPs using PCR – RFLP analysis based on the SNP map of MT1A and MT1E genes.

Table (1): Comparison between mercury levels in autistic patients and controls

Mercury level Mean ± SD	Control (ppm)	Patients (ppm)	p-value
Total	0.127 ± 0.08	0.79 ± 0.56	P < 0.001**
Male	0.13 ± 0.08	0.9 ± 0.48	P < 0.001**
Female	0.11 ± 0.06	0.53 ± 0.63	P < 0.001**

** High significant difference

Table (2): Statistical comparison between different groups of cases according to their IQ and CARS regarding to mercury levels

Mercury Levels	IQ. Grades			P
Mean ± SD (ppm)	1*	2*	3*	N.S
	0.836±0.5914	0.708±0.433	0.882±0.422	
Mean ± S.D (ppm)	CARS			N.S
	A*	B*	C*	
	0.967±0.639	0.728±0.445	0.854±0.544	

NON-SIGNIFICANT P > 0.05

1* Mild To Sever	= 20-70	Cases = 17	54.84%
2* Below Average Mentality	= 71-89	Cases = 12	38.71%
3* Normal Mentality	= 90-109	Cases = 2	6.45%
A* MILD	= 21-27	Cases = 3	9.384%
B* MODERATE	= 28-33	Cases = 17	56.25%
C* SEVERE	≥34	Cases = 11	34.38%

Table (3): Comparison between MT1A genotypes, alleles among autistic patients and controls

Genotyping, alleles	Patients			control			P< statistical
	Male	Female	Total alleles	Male	Female	Total alleles	
Alleles							
-A	22	10	62	0	0	60	0.001
-G	22	8		40	20		0.001
Genotypes							
1 GG (118bp) (homozygous)	0			30			NS
2 AG (165bp) (heterozygous)	31			0			(0.001)
3 AA (283bp) (homozygous)	0			0			N.S.
Total	31			30			

Table (4): Comparison between mercury levels in different MT1A genotypes, alleles among autistic patients and controls

Genotyping, alleles	Patients			Control			Mercury level Mean \pm S.D (ppm)	P-value
	Male	Female	Total alleles	Male	Female	Total alleles		
Alleles	22	10	62	0	0	60	0.181 \pm 0.55	NS p>0.05
	22	8		40	20		0.77 \pm 0.57	
Genotypes								
1 GG (homozygous)	0			30			0.79 \pm 0.56	NS
2 AG (heterozygous)	31			0			0.79 \pm 0.56	N.S.
3 AA (homozygous)	0			0			0	0
Total	31			30				

Table (5): Comparison between MT1E genotypes, alleles among autistic patients and controls

Genotyping, alleles	Patients			Control			P< statistical
	Male	Female	Total alleles	Male	Female	Total alleles	
Alleles							
-T	4	2	62	18	10	60	P< 0.001
-G	40	16		22	10		P< 0.001
Genotypes	male	Female		male	female		
1 TT (63bp) (homozygous)	2	1		0	0		NS
2 GT (216bp) (heterozygous)	0	0		18	10		NS
3 GG (279bp) (homozygous)	20	8		2	0		P < 0.05
Total	31			30			

Table (6): Comparison between mercury levels in different MT1E genotypes, alleles among autistic patients and controls

Genotyping, alleles	Patients			Control			Mercury level Mean \pm S.D (ppm)	P-value
	Male	Female	Total alleles	Male	Female	Total alleles		
Alleles								
-T	4	2	62	18	10	60	0.181 \pm 0.55	N.S.
-G	40	16		22	10		0.8 \pm 0.58	N.S.
Genotypes	Male	Female		Male	Female			
1 TT (homozygous)	2	1		0	0		0.83 \pm 0.56	N.S.
2 GT (heterozygous)	2	0		18	10		0	N.S.
3 GG (homozygous)	20	8		2	0		0.8 \pm 0.58	N.S.
Total	33			30				

Using PCR based restriction fragment length polymorphism method in two of the MT genes (MT1A and MT1E genes) and single nucleotide polymorphisms (SNPs) of those genes. Thirty one autistic children were tested for the frequency distributions of the G allele. Particularly two restriction endonuclease enzymes (Pst1 and NDE1) which cut in SNPs-were used in this study. Different genotypes in both patients showed a high significant polymorphism.

Pst1 end nuclease using primer rs 8052394 cleaves the PCR product at the recognition site (rs) 5`CTGCA/G3`at fragment lengths 118/165/283 at GG/GA/AA respectively. NDE1 endonuclease using primer rs 708274

cleaves the PCR product at the recognition site (rs) 5`CA/TATG3` at fragment lengths 63/216/279 at TT/TG/GG respectively.

DISCUSSION

The present study was conducted on 31 autistic children (90% of autistic children were diagnosed before the age of 3 years while the remaining 10% after the age of 3 years). Autistic group age range from 2-11 years with mean + SD average age 4.73 ± 2.008 years. These finding are agreement with (Gray and Tongue, 2001) who found that parents become concerned about autistic behavior at age 12-30 months.

While (Mandell *et al.*, 2002) found that there is often wide variation in the age at which children present for diagnosis to a specialized developmental centre. One cause for these delays may be due to the lack of access to care, whether for diagnosis or to obtain necessary therapy in certain socioeconomic groups.

In the current study 70.96% Of Autistic children were male and 29.04% were female with male/female ratio 2:4. This in consistence with (Geier D.A and Geier M.R, 2007) who found that the male/female ratio of patients diagnosed with ASDs is at least 3: 1.

Also (David and Skuse, 2000) found that males were at least four times more likely to develop autism than females and among relatives with a broader autistic phenotype males are predominate.

In respect to IQ level in the present study 54.84 % of autistic children were mild to severe mentally retarded, 38.7% were below average IQ level, and 6.45% had normal IQ level.

While (Hurley and Levites, 2007) speculate that much of the recent advance in ASD has been with persons who are intellectually normal.

The current study showed an significant increased hair mercury levels among autistic group compared to controls ($p < 0.001$) in agreement with (Fido and Al Soad, 2005) who reported that autistic children had a higher levels of mercury than non autistic ($p < 0.001$) children.

In contrary with our results (Holmes *et al.*, 2003) found that mercury levels in the hair of autistic children were significantly lower than non autistic controls group, they suggested that autistic children retain mercury in their body due to impairments in detoxification path ways.

In the current study there was no significant difference in mean levels hair mercury among different autistic and their IQ levels

These findings supported by the study of (Axelrod *et al.*, 2001) who concluded that IQ does not represent all neurodevelopment deficits associated with mercury exposure.

While (Wright *et al.*, 2006) reported that heavy metals such mercury are a particularly high concern low levels are associated with neurological impairments including lower IQ.

The present study showed that higher hair mercury levels are associated with decreased severity of the autism 0.967 ± 0.639 (table 2) NS $p > 0.05$, these findings are in agreement with (Holmes *et al.*, 2003) who found that the lower the severity of autism the higher the levels of hair mercury.

In the present study autistic males had a significant increase in the mean mercury level (0.9 ± 0.48) ($p < 0.001$) than that of mercury level (0.53 ± 0.6) in autistic female. Similar to our study (Yasatake *et al.*, 2003) who reported that mercury level in the hair of males was higher than that of females.

This study also revealed that there was an association between polymorphism MT1A and Mt1E with autism by showing higher incidence when compared with the control group.

However (Yong *et al.*, 2008) reported that MT1A does not have any association with increased susceptibility to autism among their population sample.

In the current study, there is no estimation of MT1E gene frequency alleles and mercury level in autistic children ($p > 0.05$) as a risk factor of autism among Egyptian population.

As regards MT1E polymorphism, our results showed that all the patients carried the GG genotype, compared to controls. (Grarap and Andersen, 2007) study showed that G allele is associated with increased risk of having a child with autism.

This is in contrast to (Copped *et al.*, 2006) who demonstrated the presence of GG genotype among their sample of patients. However, (Chany A *et al.*, 2005) found no significant with MT1E.

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التأثير الجيني والبيئي على حدوث التوحد

[١]

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المستخلص

التعدد الجيني للأنزيمات المشاركة في التمثيل الغذائي لحمض الفوليك تعد من عوامل الخطر لمرض التوحد. هذا بالإضافة إلى أن التعرض العضوي والغير عضوي للزئبق يؤدي إلى تضاعف أعراض طيف التوحد. تهدف هذه الدراسة إلي التحقق في ما إذا كان هناك ارتباط بين تعدد أشكال جين الميتالوثيونين ومستويات الزئبق في الشعر في أطفال التوحد المصريين. أجريت الدراسة على إحدى وثلاثين مريض توحد تم تشخيصهم على أساس DSM IV (الدليل التشخيصي والإحصائي للاضطرابات العقلية).

تم قياس نسبة الزئبق فى الشعر عن طريق استخدام جهاز مطياف الامتصاص الذرى وتم
مقارنتهم كما تم إجراء مقارنة بين نسبة الزئبق فى الشعر والأشكال المتعددة لجين الميتالوثيونين A
والميتالوثيونين E وتم مطابقتهم بأمثالهم من الأطفال الأصحاء.
أوضحت النتائج وجود زيادة كبيرة فى متوسط مستوى الزئبق فى مرضى التوحد مقارنة بالأطفال
الأصحاء و أيضا زيادة كبيرة فى الميتالوثيونين A و E بين المرضى مقارنة بالأطفال الأصحاء.
أسفرت الدراسة عن وجود زيادة عالية فى متوسط نسبة الزئبق فى شعر مرضى التوحد مقارنة
بالأطفال الأصحاء.