Original article

HLA-DR4 gene expression in a sample of Egyptian autistic children and their mothers: is it a risk factor?

Background: *Autism spectrum disorder (ASD) is a biologically based neurodevelopmental disorder without a known cause. However, some autistic children experience immune malfunction and numerous studies noted that probands with autism and their mothers had higher HLA-DR4 frequencies than their fathers did. Probably, the mother's HLA-DR4 activity contributes to the phenotype of autism in her fetus. The purpose of this work was to investigate any potential link between the HLA-DR4 gene and autism.* ***Methods:*** *We conducted a controlled cross-sectional study on 100 subjects enrolled from the Children's Hospital of Ain Shams University. They comprised four groups: 25 autistic children, 25 mothers of the autistic children, 25 healthy children as a control group, and 25 mothers of the healthy children. All children underwent a detailed history taking, general and neurological examination, and IQ assessment using the Stanford Binet scale. Molecular HLA-DR typing was assessed in all subjects. The diagnosis of ASD was established using the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) criteria. Severity was assessed using the childhood autism rating scale (CARS).* ***Results:*** *45.5% of the autistic patients demonstrated the HLA DR4 allele compared to 36% of the control children. Up to 9.1% of cases of ASD had a double DR4 allele compared to 4% of the controls but with no significant statistical difference in-between. Also, 48 % of the autistic children's' mothers had the HLA DR4 allele compared to 24% of the control mothers but the difference did not reach a significant difference. On the other hand, the HLA DR3 haplotype was present in only 6.8 % of the patients' alleles compared to 24% of controls ($p<0.05$).* ***Conclusion:*** *The link of some HLA alleles to autism indicates the possible contributing role of these alleles to autoimmunity in some autistic children. Wider scale studies are needed to validate our findings.*

Key words: Autism, HLA-DR4, gene, children, autoimmunity

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INTRODUCTION

Known to be a neurodevelopmental illness, autism spectrum disorder (ASD) is prevalent, highly heritable, diverse, and has cognitive traits at its core. Although there is no known cause of ASD, various genetic and non-genetic risk factors have been linked to the condition.[1] It develops and is typically diagnosed before 36 months of age and persists throughout the life span. The diagnosis of ASD is based on typical symptoms, including repetitive behaviors and impaired social communication and engagement, as there are currently no diagnostic biomarkers available. Many pathomechanisms have been postulated to explain these behaviors, including changes in brain structure and function as well as synapse abnormalities.[2] A clinical diagnosis is based on behavior, using the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) as the gold standard.[3]

Studies have demonstrated that the etiology of ASD is influenced by both hereditary and environmental variables. The common and unusual copy number or single nucleotide variants in genes encoding proteins involved in brain development, which are crucial for the creation and function of neurons and synapses, have been discovered using an array of comparative genomic hybridization and whole exome/genome sequencing investigations. 25–35% of ASD patients have an established genetic cause.[4]

The human major histocompatibility complex HLA is located on the short arm of chromosome 6. It is known to be the most polymorphic genetic system in humans. The biological role of the HLA class I and class II molecules is to present processed peptide antigens.[5] The human MHC is divided into

three regions: The class I region contains the classical HLA-A, HLA-B, and HLA-C genes that encode the heavy chains of class I molecules. The class II region consists of a series of subregions, each containing A and B genes encoding α and β chains, respectively[6]. The most important function of human leukocyte antigen (HLA) molecule is in the induction, regulation of immune responses and the selection of the T cell repertoire.[7]

Some autistic children experience an immune malfunction, which may be a key etiological factor. Neuroinflammation, differences in the total number and frequency of immune cells and their subsets, adaptive and innate immune dysfunction, altered levels of immunoglobulin, and the presence of autoantibodies, which have been found in a significant number of cases with ASD, are just a few of the immune-ASD puzzle pieces that may be mechanistically contributing to the pathogenesis of these disorders.[8] Human leukocyte antigen (HLA) genes and their byproducts are thought to play a role in the mechanism. HLA genes are a component of the major histocompatibility complex, which also contains genes essential for the immunological response. Possibly during pregnancy, the mother's HLA-DR4 activity contributes to the phenotype of autism in her fetus. Numerous studies noted that probands with autism and their mothers had higher HLA-DR4 frequencies than their fathers did.[9]

Immune abnormalities occur in a substantial number of individuals with ASD. Identifying subgroups with immune system dysregulation and linking specific cellular immunophenotypes to different symptoms would be key to defining a group of patients with immune abnormalities as a major etiology underlying behavioral symptoms. These determinations would provide the opportunity to investigate causative treatments for a defined patient group that may specifically benefit from such an approach.[10]

Regarding the connections between the immune system and the nervous system, immunological factors are thought to exert a role in the pathogenesis of ASD.[11] These neuroimmune interactions begin early during embryogenesis and persist throughout an individual's lifetime, with successful neurodevelopment contingent upon a normal balanced immune response. Autism has been linked with autoimmunity and an association with immune-based genes including human leukocyte antigen (HLA)-DRB1 and complement C4 alleles described. There is potential that such aberrant immune activity during vulnerable and critical periods of neurodevelopment could participate in the generation of neurological dysfunction characteristic of ASD.[10] The aim of this work was to study the possible association of the HLA-DR4 gene and autism in a group of autistic children and their mothers. This may offer explanation to the autoimmune phenomena observed in some autistic children

METHODS

Study population

We designed a controlled cross-sectional study that was conducted on 100 participants attending the Children's Hospital of Ain Shams University. The studied sample comprised four groups of 25 subjects.

Group I: Autistic children.

Group II: Mothers of the autistic children.

Group III: Clinically healthy age and sex-matched children as controls.

Group IV: Mothers of the control group.

Inclusion criteria

Autistic children in the age range of 2-18 years diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition (DSM-V) criteria and Childhood Autism Rating Scale.

Exclusion criteria

Presence of any other psychiatric illness, History of seizure disorders or past history of head injury.

Study measurements

1. Clinical evaluation

All groups included in this study were subjected to detailed history taking, thorough clinical and neurological examination, and IQ assessment using the Stanford Binet scale and Laboratory investigations including CBC and Molecular HLA-DR typing. Children diagnosed with ASD, using the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) criteria. Severity was assessed using the childhood autism rating scale (CARS).

2. Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition (DSM- V)[3]

This manual specifies the required number of symptoms needed in the three core diagnostic areas which include –socialization, communication and repetitive/restricted behaviors and interests.

3. Childhood Autism Rating Scale[12]

The CARS consists of a 15-item behavioral rating scale and utilizes professionals' and parents, or caregivers, observations about their child. Its purpose is to identify children with autism and to distinguish them from other developmentally delayed children. It is especially effective in

discriminating between children with autism and those who are trainable and mentally retarded.

4. Stanford-Binet intelligence scale[13]

This scale is used for the assessment of mental age and for calculating the intelligence quotient (IQ). This test is used to measure the child's cognitive abilities. It is suitable for children aged from 2 to 16 years. The test has two items, the verbal and the performance and the test item is chosen according to the child's abilities. IQ was calculated by dividing the mental age by the chronological age multiplied by 100. A subnormal intellectual function is diagnosed when IQ is below 70.

5. Molecular HLA-DR typing

It was determined by polymerase chain reaction (PCR), using sequence-specific oligonucleotide probes (SSOP) methodology, using the Inno-LiPA kits (Innogenetics N.V), in accordance with the manufacturer's instructions[14]

Ethical Considerations

Informed written consent was obtained from the parents before enrollment and after an explanation of the aim of the study. The study protocol gained the approval of the local Ethics Committee of the Pediatric Department, Ain Shams University - Cairo, Egypt.

Statistical methods

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA) and MedCalc© version 13 (MedCalc© Software bvba, Ostend, Belgium). Numerical variables were presented as mean and SD and between-group differences were compared using the unpaired Student t-test. Categorical variables were presented as numbers and percentages and intergroup differences were compared using the Pearson chi-square test or Fisher's exact test, when appropriate. For ordinal variables, the chi-square test for trend was used. Unadjusted odds ratios were estimated to examine the relation between HLA type and autism. Multivariable logistic regression analysis was done to estimate the odds ratio as adjusted for potential risk factors or confounders. The overall model fit was examined using the -2 log-likelihood test, the Hosmer and Lemeshow test, and the correct classification rate.

RESULTS

The results of the present study showed that there was a higher frequency of autism among boys (80%) than girls (20%). The autistic children were aged between 3 and 8 years and there was no statistically significant difference between the autistic patients and controls in terms of age, parental consanguinity, birth weight, time of delivery, age of weaning, prenatal, natal, and postnatal history, and immunization. Family history of similar cases was negative except in 2 (8.0%) autistic patients. The mean duration of breastfeeding in the autistic cases was significantly lower than that of the control group (Table 1).

Most of the patients' CARS scores were in the mild to moderate range (88%) while three patients had scores in the moderate to severe range representing (12%) of cases (fig. 1). Our results showed that 48% of our patients with mild to severe mental retardation, 40% with below average mentality and 12% with normal mentality (fig.2). The autistic patients were comparable to controls in terms of deodorant use, use of Anti-D or dental amalgam and consumption of fish (Table 2).

Frequencies of different HLA DR alleles in the studied autistic and control groups were demonstrated in (table 3). Frequencies of different HLA DR alleles in the studied autistics' mothers and controls were demonstrated in (table 4). HLA DR 3 frequency in the autistic patients' alleles is significantly less than those of controls (Table 5). There were non-statistically significant differences between the autistic patients and controls and their mothers as regards the frequency of the HLA DR4 allele (Table 6). A statistically significant higher frequency of HLA DR13 among autistic patients compared to controls (Table 7). The multivariable logistic regression model for risk factors of autism was done for the included variables, only male gender (OR, 5.53; 95% CI, 1.02 to 30.0; p-value, 0.047), a child having A-DR13 genotype (OR, 5.08; 95% CI, 1.03 to 25.08; p-value, 0.046), were independent risk factors for autism (table 8).

Table 1. Comparison between autistic cases and controls in terms of the studied clinical variables

Variables		**Autism (n=25)**	**Control (n=25)**	**p-value**	**Significance**
Gender	Male	(20)80%	(13) 52%	0.037*	**S**
	Female	(5) 20%	(12) 48%		
Age (yrs)		4.5 (1.5)	3.7 (1.4)	0.079†	NS
Age of onset	18-36 months	21(84%)	--	--	
	> 36 months	4(16%)			
Parental consanguinity		2 (8.0%)	3 (12.0%)	1.0‡	NS
Relevant prenatal history		2 (8.0%)	0 (0.0%)	0.490*	NS
Time of delivery (Term/Preterm)		23/2	23/2	1.0*	NS
Birth weight (kg)		3.0 (0.2)	3.0 (0.4)	0.414†	NS
Breastfeeding (Months)		13.5±7.2	17.1±4.9	0.001†	**HS**
Age of weaning (Months)		6.1±3.1	5.7±0.9	0.292†	NS

*Pearson chi-square test; †Unpaired t-test. ‡Fisher's exact test; data are presented as number (%), ratio, or mean (SD).

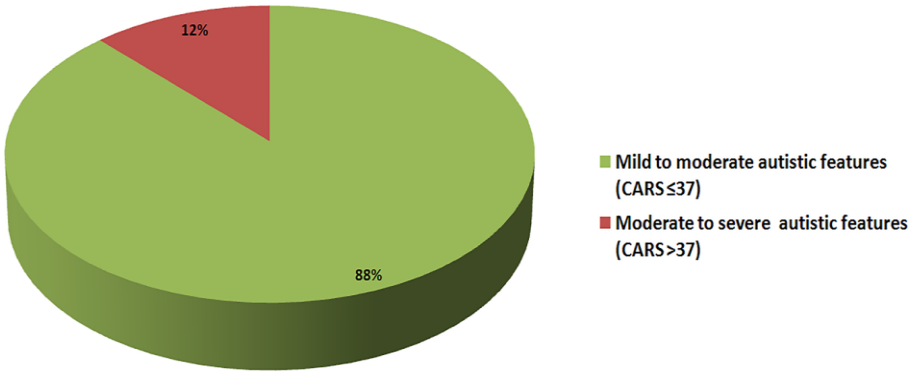

Figure 1. CARS score in the studied autistic patients

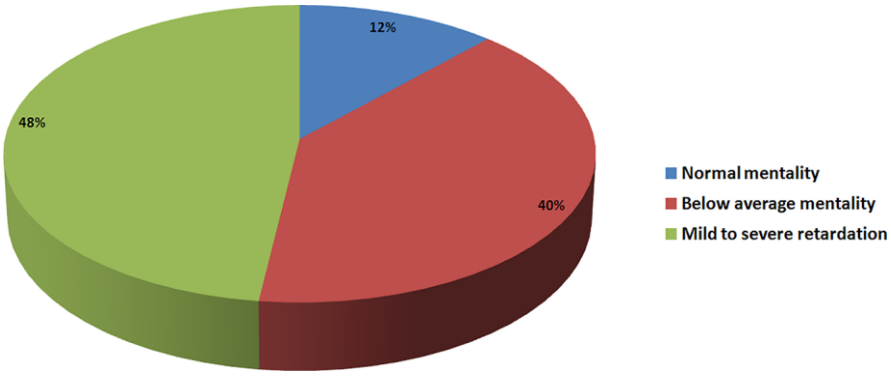

Figure 2. CARS score in the studied autistic patients

Table 2. Comparison between autistic children and controls concerning risk factors among their mothers

Variables	Autism group (n=25)	Control group (n=25)	p-value
Deodorant use	12 (48.0%)	11 (44.0%)	0.777*
Cooking with aluminum utensils	16 (64.0%)	8 (32.0%)	0.024* Significant
Use of amalgam	3 (12.0%)	1 (4.0%)	0.609†
Consumption of fish	7 (28.0%)	8 (32.0%)	0.758*
Anti-D immunization	0 (0.0%)	0 (0.0%)	NA

*Pearson chi-square test; †Unpaired t-test. ‡Fisher's exact test; data are presented as number (%), ratio, or mean (SD).

Table 3. Frequencies of different HLA DR alleles in the studied autistic and control groups

Haplotype	Patients (44 alleles)		Controls (5o alleles)	
	No. of patients' allele	%	No. of controls' allele	%
HLA DR1	1	2.273	2	4
HLA DR 3	3	6.818	13	26
HLA DR 4	12	27.27	10	20
HLA DR7	1	2.273	2	4
HLA DR 8	2	4.545	0	0
HLA DR 9	1	2.273	0	0
HLA DR10	1	2.273	4	8
HLA DR 11	5	11.36	9	18
HLA DR 13	13	29.55	6	12
HLA DR 14	4	9.091	2	4
HLA DR 15	1	2.273	2	4
Total	44	100	50	100

Table 4. Frequencies of different HLA DR alleles in the studied autistics' mothers and controls

Haplotype	Autistics' mothers (50 alleles)		Controls (50 alleles)	
	No. of allele	%	No. of allele	%
HLA DR1	1	2	1	2
HLA DR 3	5	10	10	20
HLA DR 4	13	26	7	14
HLA DR7	2	4	5	10
HLA DR 8	5	10	2	4
HLA DR10	3	6	6	12
HLA DR 11	9	12	8	16
HLA DR 13	12	24	8	16
HLA DR 14	3	6	2	4
HLA DR 15	0	0	1	2
Total	50	100	50	100

Table 5. Variation of HLA-DR3 frequency between autistic and control children and their mothers

Variables		**Patient Alleles (44)**	**Control Alleles (50)**	**p-value ¶**
Child's HLA-DR3 frequency	Heterozygous for HLA-DR3	1	7	0.015 Significant
	Homozygous for HLA-DR3	1	3	
Total		3 (6.8%)	13 (26%)	
Mothers' HLA-DR3 frequency	Heterozygous for HLA-DR3	5	10	0.262
	Homozygous for HLA-DR3	0	0	
Total		5 (10%)	10 (20%)	

Data are presented as numbers (%); ¶ Fisher's exact test

Table 6. Variation of HLA-DR4 frequency between autistic and control children and their mothers

Variables		**Patient Alleles (44)**	**Control Alleles (50)**	**p-value**
Child's HLA-DR4 frequency	Heterozygous for HLA-DR4	8	8	0.228* NS
	Homozygous for HLA-DR4	2	1	
Total		12 (27%)	10 (20%)	
Mothers' HLA-DR4 frequency	Heterozygous for HLA-DR4	11	5	0.145* NS
	Homozygous for HLA-DR4	1	1	
Total		13 (26%)	7 (14%)	

Data are presented as numbers (%); * Pearson chi-square test; NS: Non-significant

Table 7. Variation of HLA-DR13 frequency between autistic and control children and their mothers

Variables		**Patient Alleles (44)**	**Control Alleles (50)**	**p-value**
Child's HLA-DR13 frequency	Heterozygous for HLA-DR13	9	6	0.042 Significant
	Homozygous for HLA-DR13	2	0	
Total		13(29.5%)	6 (12%)	
Mothers HLA-DR13 frequency	Heterozygous for HLA-DR13	8	8	0.454
	Homozygous for HLA-DR13	2	0	
Total		12 (24%)	8 (16%)	

Data are presented as numbers (%); ¶ Fisher's exact test.

Table 8. Multivariable logistic regression model for risk factors of autism

Variable	**B**	**SE**	**OR**	**95% CI**	**P-value***
Male gender	1.71	0.86	5.53	1.02 to 30.00	0.047 **S**
Cooking with aluminum utensils	1.18	0.71	3.25	0.80 to 13.15	0.099 NS
A child having HLA-DR4 genotype	-0.67	0.87	0.51	0.09 to 2.81	0.440 NS
A child having HLA-DR13 genotype	1.62	0.82	5.08	1.03 to 25.08	0.046 **S**

B: regression coefficient; SE, standard error; OR: odds ratio; 95% CI, 95% confidence interval; *Adjusted for other covariates using multivariable logistic regression.

DISCUSSION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with biological roots. It is defined by deficits in two key areas; the lack of social interaction and communication and the restrictions on repetitious behaviors, interests, and activities.[3]

In the present study, there was a higher frequency of autism among boys (80%) than girls (20%) with a male/female ratio of 4.5:1. Emphasizing our results, research focusing on the prevalence and characteristics of ASD in the USA reported that ASD was 4.2 times as prevalent among boys as among girls.[15] Also, Fernell et al. reported a male-to-female ratio of 5.5:1 in Sweden.[16] Very few studies reported female predominance among the studied autistic cases.[17]

The autistic patients in our series were comparable to the controls in terms of age, parental consanguinity, birth weight, time of delivery, age of weaning, prenatal, natal, postnatal history, and immunization. The family history did not reveal similar cases except in 2 (8.0%) autistic patients. Postnatal hypoxia and fathers who were older than average at the time of conception were significant risk factors associated with the development of ASD.[18]

The mean values of the breastfeeding duration of our autistic cases were significantly lower than that of the control group. In agreement with our findings, Aloufi et al. noted that the percentage of autistic children who were never breastfed was higher than that of non-autistic children, and the majority of mothers of autistic children breastfed their infants for less than six months.[17]

We observed a significantly higher frequency of delayed mental, motor and speech development among autistic patients as compared to the control group. According to published data, the specific presenting symptoms of autism are delayed speech, loss of eye contact, in attention to the mother and preferring to play alone.[19] Motor deficits are common and prevalent in neurodevelopmental disorders, particularly autism spectrum disorder (ASD). Furthermore, difficulties in social communication are likely a result of motor deficits, which may be the first indicator of altered development in ASD.[20]

Our study revealed that 48% of patients had mild to severe mental retardation (IQ= 20-70), 40% had below average mentality (IQ= 71-89) and only 12% had normal mentality (IQ= 90-109). A relevant study reported comparable results; only 5 autistic patients (8.3%) had a normal mentality, 33 autistic patients (55%) had mild to severe retardation and 22 patients (35.7%) had below average mentality[19]. Alternatively, another publication implied that over half of individuals with ASD have average or higher IQs, and that boys with ASD are more likely than girls to have IQs that are average or above. ASD patients with higher IQs often face the possibility of going undiagnosed.[21]

According to the CARS score, 88% of our sample had mild to moderate autism while 12% were moderate to severe; an observation that is different than what was published by Elbaz et al. where 57% of cases had a severe degree of autism, 28% had a moderate degree, and 15% had a mild degree.[22]

HLA DR3 frequency in the autistic patients' alleles was significantly less than those of controls while there was no significant difference between the autistic patients and controls and their mothers concerning the frequency of the HLA DR4 allele. A significantly higher frequency of HLA DR13 was observed among autistic patients compared to controls.

The human leukocyte antigen (HLA), a complex genetic system that encodes proteins that primarily regulate immune/inflammatory processes, can be implicated in neurodevelopment and neuroplasticity, particularly through microglia regulation and synaptic pruning. Consequently, this highly polymorphic gene region has recently emerged as a major player in the etiology of several major psychiatric disorders including autism spectrum disorder.[23]

As infections and autoimmune diseases are linked to the autism spectrum disorders (ASD), numerous studies have suggested a potential role of the HLA system in ASD.[24] The transmission disequilibrium test (TDT) indicated that the ASD probands inherited the DR4 allele more frequently than expected ($p = 0.026$) from the fathers. The TDT also revealed that fewer DR13 alleles than expected were inherited from the mother by ASD probands ($p = 0.006$), suggesting that DR4 and DR13 are linked to ASD.[25] Similarly, HLA typing indicated that mothers and their sons had a significantly higher frequency of DR4 than normal control subjects. These findings are consistent with a hypothesis that prenatal maternal-fetal immune interaction can affect fetal brain development. Such immune interactions may involve HLA and related genes in both genetic and epigenetic mechanisms during pregnancy.[26] However, recent HLA haplotype-based analysis have identified both risk

and protective HLA haplotypes regarding autism[24] For instance, the celiac disease-associated HLA-DRB*11 ~ DQB1*07 haplotype is associated with autism risk, especially in patients with high scores for social and non-verbal functioning.[27] Also, a protective status is conferred by the HLA 8.1 AH, and it is expected to be associated with decreased complement C4 and synaptic pruning especially as cortical thickness abnormalities were amply documented in autism.[28] Finally, in a subset of patients with regressive autism, a subgroup known to have particularly pronounced immune dysfunctions, protection was afforded by a class II sub-haplotype, namely HLA-DPA1*01~ DPB1*04.[29]

Nevertheless, other studies could not find a significant association between any HLA haplotypes and ASD. Although HLA haplotypes might exert a pathogenic role in the neurodevelopmental process in ASD patients, particularly after microbial infection, any HLA haplotypes as markers of genetic susceptibility to ASD in the Iranian population could not be suggested.[30] A recent very large Genome-Wide Association Study (GWAS) failed to detect MHC-linked signals in ASD.[31] These results may be due to the heterogeneity of the disease and/or the paucity of post-GWAS HLA imputation research in ASD. Thus, exploring the risk factors that may have pathogenic association with ASD showed that children with the HLA-DR13 genotype had significant association with autism by using multivariable logistic regression analysis adjusted for potential risk factors or confounders.

CONCLUSIONS

HLA-DR13 in children may have a significant tendency for association with autism while HLA-DR3 seems to have a protective relation to autism. We did not observe a significant association between HLA-DR4 and ASD both in autistic children and their mothers. The potential link of some HLA alleles to autism and their possible contributing role to autoimmunity in a category of autistic children should be further traced. The results of our pilot study are limited by technical difficulties and the relatively small sample size. Consequently, further wider scale investigations are needed to validate our conclusions and to provide closer insight into the observed associations.

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