

ELECTRONIC LETTER

No association between *HOXA1* and *HOXB1* genes and autism spectrum disorders (ASD)

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Autism (MIM 209850) is an early onset neurodevelopmental disorder with a prevalence rate of at least 5 in 10 000 people¹⁻³ and belongs to a group of heterogeneous diseases known as autism spectrum disorders (ASD), including autism, Asperger syndrome, and the prototypical pervasive development disorder-not otherwise specified (PDD-NOS).⁴ Affected subjects have impairment in reciprocal communication and social interaction which are accompanied by repetitive and stereotyped behaviours and interests.

Twin and family studies have shown a strong underlying genetic predisposition for autism⁵⁻⁸ with linkage to regions 2q, 7q, 16p,⁹⁻¹² and 15q.¹³⁻¹⁵ The greatest linkage is to 7q31.⁹ 16 17 However, to date no single gene has been identified as being responsible for autism and it is suggested that as many as 15 loci are likely to contribute to its aetiology.^{18 19}

Because autism is a neurodevelopmental disorder, genes involved in early formation of the brain and nervous system are good candidates to study for this complex disorder. HOX genes encode a class of transcription factors known as homeobox genes which are involved in regulating neural migration during embryogenesis.^{20 21} Furthermore, analysis of mice mutants have shown that HOX genes, specifically *HOXA1* and *HOXB1*, function together in the development of the hindbrain.^{22 23} *HOXA1* and *HOXB1* are located on chromosomes 7p^{24 25} and 17q²⁵ in humans, respectively.

Recently, Ingram *et al*²⁶ investigated the frequency of *HOXA1* and *HOXB1* variants in 57 white autistic probands from singleton or multiplex families and 119 unrelated adults as controls. They studied a variant of the *HOXA1* gene (A218G) which changes the codon for one histidine in a series of histidine repeats to an arginine (H73R). They also studied a common variant in the *HOXB1* gene which includes a 9 base insertion after base 88 (referred to as the INS allele), which codes for histidine-serine-alanine. They concluded that these HOX genes, especially *HOXA1*, might play a role in the susceptibility to autism. Furthermore, they suggested an interaction between the two HOX genes, gender, and susceptibility to autism.

For their multiplex families, Ingram *et al*²⁶ reported a significant deviation from Hardy-Weinberg equilibrium for the *HOXA1* genotype ratios in their ASD group. Furthermore, a significant deviation from Mendelian expectation in gene transmission was detected among the affected offspring. By applying transmission disequilibrium test analysis of *HOXA1* genotypes in 110 multiplex autism families, Li *et al*²⁷ found no deviation from Hardy-Weinberg equilibrium in either the parents or offspring in their study.

Herein, we investigate the suggested association between autism and the two HOX genes in 35 subjects with ASD and 35 unrelated controls. Furthermore, we examine the presence or absence of associations between the observed ratio for the *HOXA1* and *HOXB1* genotypes and gender, phenotypic features, and ASD type.

Key points

- *HOX* genes are neurodevelopmental genes involved in early brain formation. Allelic variations have been reported in the human *HOXA1* and *HOXB1* genes including a single base change (A218G) leading to an amino acid substitution (H73R) and a nine base insertion (INS) at base 88, respectively.
- Recent evidence from a study of 57 autistic probands suggested a role for the two *HOX* genes in the susceptibility to autism.
- To investigate further whether allelic variations of the *HOXA1* and *HOXB1* genes are overrepresented in autistic subjects, we screened 35 mostly sporadic probands with autism spectrum disorders (ASD) and 35 unrelated subjects without a history of mental retardation or autism as controls.
- The results of our current study did not support an association between *HOXA1* and *HOXB1* gene variants and ASD.

SUBJECTS AND METHODS

Subjects

The study sample consisted of 35 unrelated patients with an autistic disorder (27 males and eight females; 31 white, three black, and one other) referred to the Autism Center at the University of Missouri-Columbia Hospitals and Clinics between 1994 and 2000. The average age was 9.5 years with an age range of 1.7 to 37.3 years. All patients met DSM-IV (Diagnostic and Statistical Manual of Mental Disorders - IV)²⁸ and CARS (Childhood Autism Rating Scale)²⁹ criteria for the diagnosis of an autism spectrum disorder including autism (25), Asperger syndrome (3), and PDD-NOS (7). Patients were ascertained from the entire state for diagnosis, medical management, and recommendations for behavioural and school placement. There was no ascertainment bias towards more or less phenotypically abnormal, mentally retarded, or multiplex subjects, and no exclusion of any person who met autism diagnostic criteria specified by the DSM-IV and the CARS criteria. Independent diagnostic evaluations were conducted by the clinic director and author (JHM), a child psychiatrist, and a neuropsychologist. The DSM-IV criteria were graded on the basis of behavioural questions and observations prepared for use in this study. CARS testing was carried out independently by the neuropsychology team. The results were compared and in any case where there was a disparity, the subject was discussed jointly to reach a conclusion. A subset of patients were evaluated by the Autism Diagnostic Interview-Revised (ADI-R)³⁰ and in all cases the ADI-R confirmed the previous diagnosis.

Clinical evaluation

The Autism Center diagnostic evaluation used a standard data set for the collection of historical data including prenatal,

Table 1 Demographic and clinical characteristics of our subjects with autism spectrum disorders (ASD): number and frequency

Gender (n=35)	
Male	27 (77%)
Female	8 (23%)
Race (n=35)	
White	32 (90%)
Black	3 (8%)
Other	1 (2%)
Diagnosis (n=35)	
Autism	25 (72%)
PDD-NOS	7 (20%)
Asperger	3 (8%)
IQ (n=25)	
Average	=66
<70	16 (64%)
≥70	9 (36%)
MRI finding (n=20)	
Abnormal	4 (20%)
Normal	16 (80%)
EEG (n=16)	
Abnormal	5 (31%)
Normal	11 (69%)
History of seizures (n=34)	
Yes	8 (24%)
No	26 (76%)
Language regression (n=33)	
Yes	13 (39%)
No	20 (61%)

teratogen exposure, perinatal, developmental, language, behaviour, and health history, including allergies and traumas, medication, dietary, metabolic, neurological, and family history. All pertinent records including school, therapy, and IEP reports as well as psychological, developmental, and medical testing were reviewed. A detailed history of the onset of autistic symptoms was obtained including the age of onset of each symptom and whether delays and/or losses occurred in language, gross motor and fine motor activities, or social interactions.

Laboratory tests included G banded chromosomes, DNA for fragile X, urine metabolic screen, organic acids, urine and serum amino acids, short chain fatty acids, thyroid profile, comprehensive metabolic profile, haematological profile, and differential and lead level. Brain MRI was performed in 20 subjects and an EEG in 16 of the subjects. Neurological and physical examinations were performed including standard morphological measurements of the head, face, hands, feet, and body proportions, and dermatoglyphic analysis.^{31–33} The skin was also examined with a Woods lamp. All study data were entered into a fully searchable relational database. Demographic and clinical characteristics of these autism subjects are summarised in table 1.

In addition, we studied the DNA from 35 unrelated subjects (27 male and 8 female; 32 white and three black) without a history of mental retardation or autism. While the ASD and control groups contained the same race and gender ratio, they did not match for age. The control group consisted of older subjects with 33 of 35 subjects (94%) being over 18 years of age to ensure that they were sufficiently mature to show features of autism spectrum disorders, if present. These subjects included parents of children with Prader-Willi syndrome and subjects with a history of giant cell tumour of bone or deafness. The frequency of the *HOXA1* and *HOXB1* variants was determined by direct DNA sequencing in each group (ASD and control) and statistically analysed.

DNA sequencing

DNA sequencing analysis was performed on genomic DNA isolated from peripheral blood of ASD and control subjects.

Table 2 DNA sequencing results for the *HOXA1* and *HOXB1* genes for subjects with autism spectrum disorders (ASD)

Subject No	<i>HOXA1</i>	<i>HOXB1</i>
7	A/A	(+/+)
38	A/G	(+/+)
58	A/A	(+/+)
66	A/A	(+/INS)
67	A/A	(+/+)
73	A/A	(+/+)
74	A/A	(+/+)
77	A/A	(+/INS)
80	A/A	(+/+)
91	A/G	(INS/INS)
92	A/A	(+/INS)
93	A/G	(+/INS)
100	A/A	(+/+)
101	A/A	(+/+)
103	A/A	(+/+)
117	A/A	(+/INS)
125	A/A	(+/+)
165	A/A	(INS/INS)
172	G/G	(+/+)
178	A/A	(+/INS)
189	A/A	(+/INS)
194	A/G	(+/+)
199	A/A	(+/INS)
211	A/G	(+/+)
214	A/G	(+/+)
217	A/G	(INS/INS)
235	A/A	(+/INS)
237	A/A	(+/+)
263	A/G	(+/+)
265	A/G	(+/+)
267	A/G	(+/+)
271	A/A	(+/+)
275	A/A	(+/+)
276	A/A	(INS/INS)
281	A/A	(+/+)

Standard PCR was performed using intronic primers to amplify the first exons of the *HOXA1* and *HOXB1* genes according to published protocols.²⁶ For direct DNA sequencing, PCR products were purified by Microcon ultrafiltration (Amicon, Beverly, MA). DNA sequencing was performed on the PCR products using an ABI 373 Sequencer and following manufacturer's recommendations.

Statistics

The allele frequency for both *HOXA1* and *HOXB1* genes was compared between the ASD and control groups. Confidence intervals were used to determine if a difference existed between the two proportions. A 2×3 table and an extension of Fisher's exact test were used to examine for associations. The SPSS statistical software version 10.1³⁴ was used to perform the statistical analysis. All p values were taken to be significant at <0.05 .

Protein profile

To investigate the impact of the observed amino acid substitution on secondary protein structure for *HOXA1*, a protein profile analysis program (MacVector software version 7.0)³⁵ was used to predict protein surface probability. The input for this program is the segment of amino acids while the output is a calculated value between 0 (that is, the amino acid is definitely buried in the interior of the protein) to 1 (that is, the amino acid is definitely exposed at the surface of the protein).

A 40 residue segment of the *HOXA1* protein including the 73 residue in its middle was analysed with MacVector.³⁵ The protein surface probability was predicted separately for the A and G allele. This analysis was used to determine whether the observed amino acid substitution (H73R) has a significant

Table 3 Genotype (count and percentage) and allele frequency of the *HOXA1* and *HOXB1* allelic variants in autism spectrum disorders (ASD) and controls

	Genotypes <i>HOXA1</i>			Allele frequency	
	A/A	A/G	G/G	A	G
Current study					
ASD (n=35)	24 (69%)	10 (29%)	1 (2%)	0.83	0.17
Control (n=35)	21 (60%)	12 (34%)	2 (6%)	0.77	0.23
Ingram <i>et al</i> ²⁶					
ASD (n=57)	35 (61%)	21 (37%)	1 (2%)	0.80	0.20
Control (n=119)	93 (78%)	26 (22%)	0 (0%)	0.89	0.11

	<i>HOXB1</i>			Allele frequency	
	+/+	+/INS	INS/INS	+	INS
Current study					
ASD (n=35)	22 (63%)	9 (26%)	4 (11%)	0.76	0.24
Control (n=35)	21 (60%)	13 (37%)	1 (3%)	0.79	0.21
Ingram <i>et al</i> ²⁶					
ASD (n=57)	30 (53%)	25 (44%)	2 (4%)	0.75	0.25
Control (n=119)	72 (60%)	42 (35%)	5 (4%)	0.78	0.22

Table 4 Comparison of the HOX genes allelic variants in autism spectrum disorders (ASD) and various controls

Groups	95% confidence intervals (CI)	Significance*
<i>HOXA1</i> (A/G allele)		
ASD (current study) & control (current study)	-29% to 16%	Not significant
ASD (current study) & control (Ingram <i>et al</i> ²⁶)	-10% to 25%	Not significant
ASD (current study) & ASD (Ingram <i>et al</i> ²⁶)	-28% to 12%	Not significant
<i>HOXB1</i> (+/INS allele)		
ASD (current study) & control (current study)	-34% to 13%	Not significant
ASD (current study) & control (Ingram <i>et al</i> ²⁶)	-26% to 10%	Not significant
ASD (current study) & ASD (Ingram <i>et al</i> ²⁶)	-37% to 4%	Not significant

*If the zero is included in a CI, frequency of the variant is not significantly different from the two compared groups.

impact on accessibility of the segment of the *HOXA1* protein encompassing this amino acid change.

RESULTS

The variants of the two HOX genes were identified in both the ASD and control DNA samples (tables 2 and 3). The frequency of each gene variant was compared between the ASD and control groups. For *HOXA1*, the A218G allele frequency was 0.17 in the ASD group and 0.23 in the control group. To investigate the impact of this A to G change and the resulting amino acid substitution, we examined surface probability of the *HOXA1* peptide. A 40 residue segment in the middle of the protein which includes the H73R substitution was analysed with the MacVector Sequence Analysis Software.³⁵ An increase of 0.06 was detected for the protein surface probability which represented a change from 0.56 to 0.62 in the three residues upstream and downstream of this position.

Screening of the *HOXB1* gene showed the 9 bp tandem duplication (ACAGCGCCC) at position 88 referred to as the INS allele. The variant frequency of 0.24 and 0.21 was found in our ASD and control groups, respectively.

No significant differences were detected for the observed genotype and allele frequencies of the *HOXA1* and *HOXB1* variants between the ASD and control groups (table 4). In addition, there was no association between the two HOX gene variants and ASD ($p=0.38$, Fisher's exact test). Furthermore, no deviation from Hardy-Weinberg equilibrium was observed for either gene in the ASD and control groups while comparing the observed and expected genotypes.

We also tested for possible associations between the HOX gene variants and gender, phenotypic features (IQ, MRI findings, EEG, history of seizures, and language regression), and ASD type. No significant differences were detected based on the available clinical data (table 5).

Owing to our limited sample size, we were not able to detect small changes between our groups. However, it shows a reasonable level of precision. According to a post hoc power calculation, the current sample size would yield us 82% power for detecting a doubling of a frequency between our ASD and control groups.

DISCUSSION

To investigate a possible association between the *HOXA1* and *HOXB1* gene variations and autism, we screened the two HOX genes in 35 autistic probands and 35 control subjects. The same allelic variations of *HOXA1* and *HOXB1* reported by Ingram *et al*²⁶ were identified in our two study groups (ASD and unrelated controls).

The frequencies of different alleles and genotypes in our autism population were compared with the control group. The control group consisted of the same gender ratio and approximately the same ethnic background as our ASD group.

The result of our study does not support the presence of an association between the *HOXA1* or *HOXB1* genes and autism, unlike the study reported by Ingram *et al*.²⁶ Furthermore, statistical analysis also showed lack of any interaction between the observed allelic variants in the two HOX genes and autism.

The A218G variant of *HOXA1* changes the histidine at position 73 to an arginine (H73R) in a series of 10 histidine

Table 5 Comparison of the *HOXA1* and *HOXB1* allelic variants based on the clinical characteristics of the subjects with autism spectrum disorders (ASD)*

	<i>HOXA1</i>			<i>HOXB1</i>		
	A/A	A/G	G/G	+/+	+/INS	INS/INS
Gender (n=35)						
Male	19	8	0	17	6	4
Female	5	2	1	5	3	0
IQ (n=25)						
<70	13	2	1	11	4	1
≥70	3	6	0	5	3	1
MRI finding (n=20)						
Abnormal†	3	0	1	2	1	1
Normal	10	6	0	8	5	3
EEG (n=16)						
Abnormal	4	1	0	2	1	2
Normal	5	5	1	7	2	2
Seizures (n=34)						
Yes	6	1	1	3	4	1
No	17	9	0	18	5	3
Language regression (n=33)						
Yes	7	5	1	11	2	0
No	15	5	0	10	6	4

*No statistical differences were identified between the allelic variants and the clinical data using an extension of Fisher's exact test.

†Abnormal MRI seen in our subjects included encephalomalacia, atrophy of the optic chiasm, small periventricular white matter, and cerebellar hypoplasia.

repeats. The H73R is a conservative substitution since both amino acids are basic. Our preliminary protein analysis indicates only a subtle change in surface probability of the segment of protein in the presence of the H73R substitution. This stretch of histidine residues may be involved in DNA protein binding²⁶ which would require surface accessibility. Based on our basic computer prediction it is unlikely that the histidine to arginine substitution would have a dramatic impact on accessibility of this site. Our protein profile analysis did not suggest a significant change in surface accessibility; however, a functional analysis of this *HOXA1* variant could be done to investigate whether the observed amino acid change has any impact on the function of the *HOXA1* protein.

We detected one autistic and two control subjects with the G/G genotype for the *HOXA1* gene. This observation suggests that the homozygous G/G did not have a deleterious effect. Interestingly, all three people with the G/G genotype were black. Thus, three out of a total of six black subjects (50%) who were screened in our study had the G/G genotype for *HOXA1*. This suggests that the G allele is more common in the black population than in whites.

The *HOXB1* gene consists of a repeated sequence of nine bases in exon 1. The wild type or more common allele has two copies of this tandem repeat while the INS variant has three copies. Insertion of the nine base segment would produce three amino acids (histidine, serine, and alanine) in the open reading frame (ORF) of the *HOXB1* gene without changing the rest of the ORF.

This specific human *HOXB1* variant was previously reported by Faiella *et al.*²⁶ They also reported on the presence of one copy of the nine base sequence in seven mouse strains while the gibbon and rhesus monkey had three copies. To date, no functional data have been reported on this *HOXB1* variant. However, the presence of different copies of the nine base sequence in different organisms suggests that an extra copy of this repeated sequence is unlikely to have impact on the function of the *HOXB1* gene.

We constructed a 2 × 3 table to examine the presence of an association between (1) gender or clinical characteristics (ASD type, IQ, MRI finding, EEG, history of seizures, and language regression) in our ASD subjects and (2) the observed allelic variants. The data shown in table 5 did not produce significant associations. In addition, when analysing IQ data, we

grouped the ASD subjects into mentally retarded (IQ score <70) and not mentally retarded (IQ score ≥70). A statistical analysis for a 2 × 3 table representing the IQ**HOXA1* association produced a p value of 0.08 using an extension of Fisher's exact test.

In addition, both Ingram *et al.*²⁶ and Li *et al.*²⁷ studied possible mating types in both multiplex and simplex families to examine transmission of different allele variants from the parents to their affected children. Ingram *et al.*²⁶ reported an increased risk for the A/G genotype of *HOXA1* among their probands, while Li *et al.*²⁷ did not find any deviation from the expected genotype ratios.

Ingram *et al.*²⁶ reported a deviation from Hardy-Weinberg equilibrium for the *HOXA1* gene in their ASD group. By contrast, Li *et al.*²⁷ detected no deviation from Hardy-Weinberg equilibrium in either the parents or offspring. Li *et al.*²⁷ studied only multiplex autism families while the subjects screened by Ingram *et al.*²⁶ were mixtures of multiplex and singleton ASD cases. We examined an association of the HOX genes in a group of mainly singleton ASD subjects. In agreement with Li *et al.*²⁷ we did not observe any deviation from the expected genotype ratios in either the ASD or control groups for either *HOXA1* or *HOXB1*.

Our lack of association of *HOXA1* and *HOXB1* gene variants and ASD did not agree with the report by Ingram *et al.*²⁶ It is noted that the majority of the subjects studied by Ingram *et al.*²⁶ were familial (91%) while our autism group consisted of mainly singleton probands (85%). In addition, different methods of genotype screening were used in the two studies. Ingram *et al.*²⁶ used a DNA sequencing ladder and/or restriction enzyme digestion of PCR fragments to determine genotype status. We used automated DNA sequencing of amplified PCR fragments. It is possible that there may be a different detection rate between DNA sequencing and restriction enzyme analysis.

Furthermore, autism is a clinically heterogeneous disorder. Possibly, different diagnostic criteria may result in recruitment of a divergent category of subjects for analysis. Lastly, considering the complex nature of autism, it is more likely that several genes may contribute to autism and it is unlikely to result from a single gene or family of genes (that is, HOX genes). Additional studies are needed to address further the possible association of these neurodevelopmental genes and ASD.

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