ELECTRONIC LETTER

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

Z Talebizadeh, D C Bittel, J H Miles, N Takahashi, C H Wang, N Kibiryeva, M G Butler

J Med Genet 2002;39:e70(http://www.jmedgenet.com/cgi/content/full/39/11/e70)

Autism (MIM 209850) is an early onset neurodevelopmental disorder with a prevalence rate of at least 5 in 10 000 people 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). 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 autism with linkage to regions 2q, 7q, 16p, and 15q. The greatest linkage is to 7q31. 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.

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. Furthermore, analysis of mice mutants have shown that HOX genes, specifically HOXA1 and HOXB1, function together in the development of the hindbrain, and HOXA1 and HOXB1 are located on chromosomes 7p and 17q in humans, respectively.

Recently, Ingram et al. 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. 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. 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.

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 criteria for pervasive development disorder-not otherwise specified (PDD-NOS). 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) 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

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

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

<table>
<thead>
<tr>
<th>Subject No</th>
<th>HOXA1</th>
<th>HOXB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>A/A (+/+)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>A/G (+/+)</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>A/A (+/+)</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>A/A (+/+)</td>
<td>(+/INS)</td>
</tr>
<tr>
<td>67</td>
<td>A/A (+/+)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>73</td>
<td>A/A (+/+)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>74</td>
<td>A/ (+/+)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>77</td>
<td>A/A (+/INS)</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>A/A (+/+)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>81</td>
<td>A/G (INS/INS)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>92</td>
<td>A/A (+/INS)</td>
<td></td>
</tr>
<tr>
<td>93</td>
<td>A/G (+/+)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>A/A (+/+)</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>A/A (+/+)</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>A/A (+/+)</td>
<td></td>
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<tr>
<td>117</td>
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<tr>
<td>125</td>
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<td>(+/+)</td>
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<tr>
<td>163</td>
<td>A/A (INS/INS)</td>
<td>(+/+)</td>
</tr>
<tr>
<td>172</td>
<td>G/G (+/+)</td>
<td></td>
</tr>
<tr>
<td>178</td>
<td>A/A (+/INS)</td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>A/A (+/INS)</td>
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</tr>
<tr>
<td>194</td>
<td>A/ (+/+)</td>
<td>(+/)</td>
</tr>
<tr>
<td>199</td>
<td>A/A (+/INS)</td>
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<tr>
<td>211</td>
<td>A/G (+/+)</td>
<td></td>
</tr>
<tr>
<td>214</td>
<td>A/ (+/+)</td>
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<td></td>
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<tr>
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<td>(+/)</td>
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<td>276</td>
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<td>(+/+)</td>
</tr>
<tr>
<td>281</td>
<td>A/A (+/+)</td>
<td></td>
</tr>
</tbody>
</table>

Standard PCR was performed using intronic primers to amplify the first exons of the HOXA1 and HOXB1 genes according to published protocols.² 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 10.1 was used to perform the statistical analysis. All p values were taken to be significant at <0.05.

DNA sequencing

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

Table 3  DNA sequencing analysis results for the HOXA1 and HOXB1 genes for subjects with autism spectrum disorders (ASD)

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<td>(+/)</td>
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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)²⁵ 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.²⁵ 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
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. 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 duplications (ACAGCGCCC) at position 88 referred to as the INS allele. The variant frequency of 0.24 and 0.21 was found in 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. 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. 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 3 Genotype (count and percentage) and allele frequency of the HOXA1 and HOXB1 allelic variants in autism spectrum disorders (ASD) and controls

<table>
<thead>
<tr>
<th>Genotypes HOXA1</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>0.83</td>
</tr>
<tr>
<td>A/G</td>
<td>0.77</td>
</tr>
<tr>
<td>G/G</td>
<td>0.80</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>95% confidence intervals (CI)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXA1 (A/G allele)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASD (current study) &amp; control (current study)</td>
<td>-0.10% to 0.16%</td>
<td>Not significant</td>
</tr>
<tr>
<td>HOXB1 (+/INS allele)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASD (current study) &amp; control (Ingram et al.)</td>
<td>-0.28% to 0.29%</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

*If the zero is included in a CI, frequency of the variant is not significantly different from the two compared groups.
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 binding 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 **HOXB1** 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**. 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. 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. and Li et al. 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. reported an increased risk for the A/G genotype of **HOXA1** among their probands, while Li et al. did not find any deviation from the expected genotype ratios.

Ingram et al. reported a deviation from Hardy-Weinberg equilibrium for the **HOXA1** gene in their ASD group. By contrast, Li et al. detected no deviation from Hardy-Weinberg equilibrium in either the parents or offspring. Li et al. studied only multiplex autism families while the subjects screened by Ingram et al. 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., 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. It is noted that the majority of the subjects studied by Ingram et al. 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. 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.
ACKNOWLEDGEMENTS
The authors wish to thank Dr Stephen Simon for statistical advice.

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REFERENCES