**Association of Transcription Factor Gene LMX1B with Autism**

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**Abstract**

Multiple lines of evidence suggest a serotonergic dysfunction in autism. The role of LMX1B in the development and maintenance of serotonergic neurons is well known. In order to examine the role, if any, of LMX1B with autism pathophysiology, a trio-based SNP association study using 252 family samples from the AGRE was performed. Using pairwise tagging method, 24 SNPs were selected from the HapMap data, based on their location and minor allele frequency. Two SNPs (rs10732392 and rs12336217) showed moderate association with autism with p values 0.018 and 0.022 respectively in transmission disequilibrium test. The haplotype AGCGTG also showed significant association (p = 0.008). Further, LMX1B mRNA expressions were studied in the postmortem brain tissues of autism subjects and healthy controls samples. LMX1B transcripts was found to be significantly lower in the anterior cingulate gyrus region of autism patients compared with controls (p = 0.049). Our study suggests a possible role of LMX1B in the pathophysiology of autism. Based on previous reports, it is likely to be mediated through a serotonergic mechanism. This is the first report on the association of LMX1B with autism, though it should be viewed with some caution considering the modest associations we report.

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**Introduction**

Autism and other developmental disabilities, clinically referred to as autism spectrum disorders (ASDs), are characterized by impairments in communication skills and social interaction, and the presence of repetitive stereotyped behaviors and interests. It is typically diagnosed by the age of three and has a prevalence rate of 60-70 per 10,000 children in broader diagnostic criteria as per the most recent estimates [1]. ASDs are considered to be among the most heritable of all psychiatric disorders. A recent largest population based twin study comprised of 10,895 twin pairs, reported 80% heritability for ASDs [2], confirming the previously reported heritability estimates [3,4]. Linkage, candidate gene and whole genome association studies have suggested several genes and chromosomal regions associated with the disorder. However, none of these known causes individually account for more than 1–2% of the cases, and specific genetic mechanisms underlying the heritability of the disorder still remain largely cryptic. It was found that many different genetic changes in unrelated genes can cause indistinguishable ASD features; this genetic heterogeneity necessitates the need to look for more potential candidate genes associated with the disorder.

The LIM homeodomain transcription factor 1b (LMX1B) was initially characterized as a key regulator of the normal dorsoventral patterning in the developing limbs [5]. Several mutations reported in this gene have been found to lead to the pleiotropic phenotype, the nail patella syndrome [6–8]. Later, the role of Lmx1b in the development and maintenance of serotonergic (5HTergic) neurons in the central nervous system (CNS) was reported, and thereafter, underlying mechanisms were studied in detail. Lmx1b knock-out mice were found to be lacking the entire central 5HTergic neurons [9,10]. Further, it was shown that overexpression of Lmx1b enhances differentiation of mouse embryonic stem cells into 5HT neurons [11]. In addition to its role in the development of central 5HTergic neurons, Lmx1b is also required for the normal biosynthesis of 5HT in adult brain, and possibly for the regulation of normal functions of 5HTergic neurons [12].

A role of 5HTergic system in the pathophysiology of autism was proposed based on following observations, a) hyperserotonemia in the whole blood cells and platelets of 25–50% of patients with autism [13,14], b) depletion of tryptophan, the 5HT precursor, in ASD patients increased some stereotype behaviors associated with the disorder [15], c) treatment with selective serotonin reuptake inhibitors has shown to be effective in ameliorating the repetitive and/or...
compulsive behaviors in some autistic individuals [16] and d) recent neuroimaging studies have shown low levels of brain 5HT synthesis in autistic children [17] and reduction in serotonin transporter (SLC6A4) binding in different brain regions of both children and adults with the disorder [18,19]. Compliant with these reports, several genetic association studies involving genes in the 5HT metabolic pathway were also attempted. While several SLC6A4 polymorphisms were shown to be associated with the disorder in some studies [20,21], others failed to replicate the findings [22].

Taking together, these results provide compelling, though inconsistent evidence for the role of 5HTergic system in the pathophysiologic mechanism of ASDs. In view of the importance of LMX1B in the development of 5-HTergic neurons, it would be interesting to study its role in autism. Here we performed a trio-based study to examine the association of LMX1B with autism. We also assessed any alterations in the expression LMX1B in the postmortem brain samples of autism patients as compared to healthy controls.

Results

Single SNP TDT

Mendelian inheritance inconsistencies were not observed for any of the SNPs. For each SNP, >99% of the genotypes were scored; none of the SNPs showed deviation from HWE.

| Marker | db SNP ID | Genomic Location | Variation* | Location | Minor allele | T (%)  | p-value $^d$ |
|--------|-----------|------------------|------------|----------|--------------|--------|--------------|
| SNP 1  | rs10732392| 129396037        | G:A        | Intron 2 | 0.078        | 48.92  | 0.018        |
| SNP 2  | rs10760444| 129396434        | A:G        | Intron 2 | 0.449        | 48.23  | 0.214        |
| SNP 3  | rs10448285| 129397014        | C:T        | Intron 2 | 0.376        | 50.64  | 0.601        |
| SNP 4  | rs12336217| 129399870        | A:G        | Intron 2 | 0.075        | 48.98  | 0.022        |
| SNP 5  | rs7858338 | 129406644        | T:C        | Intron 2 | 0.26         | 51.61  | 0.085        |
| SNP 6  | rs11793373| 129407543        | G:A        | Intron 2 | 0.252        | 50.6   | 0.513        |
| SNP 7  | rs10819190| 129408513        | G:A        | Intron 2 | 0.414        | 49.56  | 0.739        |
| SNP 8  | rs6478750 | 129409198        | T:C        | Intron 2 | 0.408        | 49.91  | 0.948        |
| SNP 9  | rs12555734| 129411242        | C:A        | Intron 2 | 0.24         | 51.25  | 0.16         |
| SNP 10 | rs13285227| 129413298        | C:T        | Intron 2 | 0.348        | 49.11  | 0.439        |
| SNP 11 | rs946103  | 129413490        | G:A        | Intron 2 | 0.472        | 49.05  | 0.526        |
| SNP 12 | rs12555176| 129414303        | G:T        | Intron 2 | 0.074        | 50.11  | 0.809        |
| SNP 13 | rs7854658 | 129414938        | G:A        | Intron 2 | 0.21         | 50.57  | 0.486        |
| SNP 14 | rs10987386| 129416317        | C:T        | Intron 2 | 0.191        | 49.5   | 0.519        |
| SNP 15 | rs12551234| 129417809        | G:C        | Intron 2 | 0.407        | 49.92  | 0.949        |
| SNP 16 | rs7853174 | 129419990        | G:A        | Intron 2 | 0.394        | 49.04  | 0.452        |
| SNP 17 | rs10819194| 129422023        | G:A        | Intron 2 | 0.422        | 51.78  | 0.189        |
| SNP 18 | rs4322101 | 129428677        | A:G        | Intron 2 | 0.416        | 51.19  | 0.37         |
| SNP 19 | rs7030919 | 129438872        | A:G        | Intron 2 | 0.115        | 49.49  | 0.37         |
| SNP 20 | rs3737048 | 129458092        | G:T        | Intron 6 | 0.107        | 50.39  | 0.474        |
| SNP 21 | rs10987413| 129459438        | G:A        | 3’       | 0.333        | 50.65  | 0.56         |
| SNP 22 | rs10760450| 129459628        | C:T        | 3’       | 0.21         | 50.58  | 0.475        |
| SNP 23 | rs10733682| 129460614        | G:A        | 3’       | 0.486        | 51.27  | 0.41         |
| SNP 24 | rs4083644 | 129461714        | C:T        | 3’       | 0.28         | 49.93  | 0.943        |

T: Transmitted.

*Common allele is listed first.

$^a$Based on the parental genotypes of 252 trios.

$^b$% of common allele is listed, $^c$ Computed on the basis of likelihood ratio test; significant p-values (<0.05) are indicated in bold italics.

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The results of TDT analysis are shown in Table 1. rs10732392 (p = 0.018; OR = 1.764; 95% CI for OR 1.095–2.042) and rs12336217 (p = 0.022; OR = 1.748; 95% CI for OR 1.076–2.841) showed significant associations with autism. However, these associations did not withstand the multiple testing correction. Overtransmission was observed for the minor allele A (62.82%) of rs10732392 and for minor allele G (62.67%) of rs12336217.

LD analysis

LD analysis based on D’ values identified six distinct haploblocks across LMX1B gene. The first block consists of SNPs 01 to 06, the second block SNPs 08 and 09, the third block 10 and 11, fourth block 12 to 16, fifth block 18 and 19 and the sixth block included SNPs 20 to 22 (Figure 1).

Haplotype TDT

The results of haplotype TDT is given in Table 2. Based on the LD structure of LMX1B, associations of haplotypes in the six haploblocks were analysed. The haplotype AGCGTG of the first block showed significant association with autism (p = 0.008).

LMX1B expression in the postmortem brains

No significant difference in age, sex and postmortem intervals was observed between autism and control groups in all the brain samples.
regions (ACG, MC and THL). There was a significant difference in LMX1B expression between the autism and control group in the ACG (p = 0.049) (Figure 2). Expression was significantly lower in autism groups with a fold change of \((2^{-\Delta\Delta CT}) 0.43\). No LMX1B expression could be detected in the other two brain regions (MC and TH).

**Discussion**

In this study, we examined the association of the transcription factor gene LMX1B with autism in Caucasian population. In the trio-based study, we found nominal associations for two SNPs (rs10732392 and rs12336217) and a haplotype with autism. To the best of our knowledge, this is the first study which reported an association between LMX1B and autism; a previous study reported the association between LMX1B and schizophrenia [23], which is also a neurodevelopmental disorder. Both the SNPs which are found to be associated with the disorder are located in the introns (intron 2) and may lack any direct functional importance. We also found that the LMX1B mRNA expression in general, is rather low in adult brain; detected only in ACG. However, LMX1B mRNAs were found to be significantly lower in the ACG of autistic brains than the similar regions of control brain tissues.

Multiple lines of evidence suggested a serotoninergic dysfunction in many patients with autism, although the results are still inconclusive. Involvement of several transcription factors are reported in the 5HTergic differentiation. In mammalian CNS, a sequential activation of transcription factors in the hindbrain, starting with the regulation of the expression of Nkx2.2 by the Shh signaling pathway, has been proposed [9]. It was observed that 5HT neurons are absent in the mice lacking Nkx2.2 [24]. It occupies the highest hierarchical position in the genetic cascade that involved in the development of 5HT neurons. Another transcription factor Pet1, expressed in the post mitotic 5HT neurons was reported to be the terminal differentiation factor, which acts in the final step of the transcriptional cascade that establishes the final identity of 5HT neurons. Mice lacking Pet1 had 70–80% fewer 5-HT neurons than normal mice. The Lmx1b ablation does not affect the expression Nkx2.2 and Shh [9,25] putting these factors upstream of Lmx1b. However, during development, Lmx1b precedes pet1, and Lmx1b knock-out mice showed loss of Pet1 expression [10]. In vivo, Pet1 expression was increased in neurons overexpressing Lmx1b [11]. Thus, Lmx1b has been proposed as an essential link between Nkx2.2 and Pet1 in the genetic cascade that controls the early specification and terminal differentiation of 5HTergic neurons in the hindbrain. Lmx1b expression was shown to be the rate limiting step in this cascade of events for specifying the 5HT phenotype [11]. Further, Lmx1b, together with Pet1, is also involved in the serotonin metabolism as it controls a set of molecules essential for the serotonin synthesis (TPH2), vesicular transport (VMAT2) and reuptake after synaptic release (SLC6A4) in the developing as well as adult brain [10,12].

ACG region plays important role in the pathophysiology of autism as shown by previous reports [26,27]. Our positron emission tomography studies had shown that a reduction in SLC6A4 binding in the cingulate cortices is associated with an impairment of social cognition in autistic subjects [19]. The present finding of reduced LMX1B expression in the ACG of autism.
Table 2. Haplotype associations of SNPs belonging to the six LD blocks of LMX1B, in 252 trios.

| Block | Haplotype* | Frequency | T(%) | Individual p-value | Permutation p-value† | Block p-value‡ |
|-------|------------|-----------|------|--------------------|---------------------|---------------|
| Block 1 (SNPs 01–06) | GGTATG | 0.355 | 51.67 | 0.6291 | 1 | |
| | GACATA | 0.25 | 48.81 | 0.7487 | 1 | |
| | GACACG | 0.244 | 45.71 | 0.2568 | 0.994 | |
| | AGCGTG | 0.073 | 66.13 | **0.0079** | 0.114 | |
| | GACATG | 0.052 | 51.42 | 0.8461 | 1 | |
| | GGTACG | 0.014 | 30.77 | 0.1658 | 0.97 | 0.096 | |
| Block 2 (SNPs 08–09) | CC | 0.406 | 50.23 | 0.9432 | 1 | |
| | TC | 0.353 | 54.03 | 0.2242 | 0.987 | |
| | TA | 0.239 | 44.23 | 0.1255 | 0.892 | 0.258 | |
| Block 3 (SNPs 10–11) | CG | 0.525 | 48.4 | 0.6123 | 1 | |
| | TA | 0.345 | 52.71 | 0.4094 | 1 | |
| | CA | 0.126 | 48.79 | 0.8046 | 1 | 0.731 | |
| Block 4 (SNPs 12–16) | GGCGA | 0.379 | 53.41 | 0.3114 | 0.998 | |
| | GGCGG | 0.209 | 45.31 | 0.2362 | 0.991 | |
| | GACCG | 0.201 | 48.99 | 0.8072 | 1 | |
| | GGTCG | 0.119 | 55.41 | 0.2624 | 0.994 | |
| | TGTGC | 0.071 | 48.81 | 0.8455 | 1 | 0.595 | |
| Block 5 (SNPs 18–19) | AA | 0.58 | 52.42 | 0.4476 | 1 | |
| | GA | 0.304 | 47.25 | 0.1587 | 0.966 | |
| | GG | 0.112 | 53.61 | 0.4772 | 1 | 0.354 | |
| Block 6 (SNPs 20–22) | GGC | 0.35 | 55.39 | 0.111 | 0.868 | |
| | GAC | 0.332 | 48.19 | 0.59 | 1 | |
| | GGT | 0.21 | 47.63 | 0.5365 | 1 | |
| | TGC | 0.107 | 46.45 | 0.4947 | 1 | 0.512 | |

‡: Transmitted / (Transmitted + Untransmitted).
†: 10,000 permutations.
*All possible combinations of haplotypes with frequency >0.01. †Significant p-values (<0.05) are indicated in bold italics.
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Figure 2. LMX1B expression in the brain. LMX1B expression in the anterior cingulate gyrus region of the brain of autism patients compared to that of control samples.
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autism group, therefore, could have some deleterious effects on the serotonergic system, given the role of LMX1B in the differentiation of 5HT neurons in developing brain, and in the maintenance of 5HT system in adult brain.

In conclusion, we report a possible association of the transcription factor LMX1B with autism pathogenesis. However, our results should be interpreted with some caution, given the limitations in sample size of postmortem brain samples and the modest associations we found in genetic and gene expression studies.

Materials and Methods

Subjects

DNA samples from trio families recruited to the Autism Genetic Resource Exchange [28] were used for the single nucleotide polymorphism (SNP) association study. We selected 252 trio families with male offspring scored for autism. Only Caucasians (white) were selected and non-idiopathic autism cases were excluded.

Brain samples

Frozen postmortem brain tissues from autistic patients and controls were provided by the Autism Tissue Program (ATP; Princeton, NJ; http://www.autismtissueprogram.org/) and Harvard Brain Tissue Research Center (HBTRC; Belmont, MA; http://www.brainbank.mclean.org/). Tissues were obtained from three brain regions important in cognitive and behavior processing namely a) anterior cingulate gyrus (ACG- 8 autism and 13 controls), b) motor cortex (MC- 7 autism and 8 controls), and c) thalamus (THL-8 autism and 9 controls). The demographic features of the samples are described in Table 3.

Selection of SNPs

LMX1B, located in 9q33.3 (129,376,748 – 129,463,311), is 86.56kb in size and consists of eight exons. The genomic structure is based on the UCSC (http://www.genome.ucsc.edu) assembly of the human genome. SNPs for the association studies were selected using the information from international HapMap project (http://www.hapmap.org) and National Centre for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP). On the basis of their genomic locations and minor allele frequencies (MAF >0.1), 24 SNPs were selected (Figure 3; Table 1), using the pair-wise tagging option of Haploview.v4.1 (http://www.broad.mit.edu/mpg/haploview).

Genotyping

Assay-on-demand/Assay-by-design SNP genotyping products (ABI, Foster City, CA, USA) were used to score SNPs, based on the TaqMan assay method [29]. Genotypes were determined in ABI PRISM 7900HT Sequence Detection System (SDS) (Applied Biosystems), and analyzed using SDS v2.0 (ABI).

Statistical Analysis

PedCheck v1.1 (http://www.watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inheritance inconsistencies in

Table 3. Postmortem brain tissue information.

| Sample ID* | Diagnosis | Age (years) | Gender | PMI (hours) | Race       | Cause of death         | Brain regions b |
|------------|-----------|-------------|--------|-------------|------------|------------------------|-----------------|
| UMB 818    | Control   | 27          | M      | 10          | Caucasian  | Multiple injuries      | ACG             |
| UMB 1065   | Control   | 15          | M      | 12          | Caucasian  | Multiple injuries      | ACG, THL        |
| UMB 1297   | Control   | 15          | M      | 16          | African American | Multiple injuries | ACG, MC, THL   |
| UMB 1407   | Control   | 9           | F      | 20          | African American | Asthma           | ACG, MC, THL   |
| UMB 1541   | Control   | 20          | F      | 19          | Caucasian  | Head injuries          | ACG, MC, THL    |
| UMB 1649   | Control   | 20          | M      | 22          | Hispanic   | Multiple injuries      | ACG, MC, THL   |
| UMB 1708   | Control   | 8           | F      | 20          | African American | Asphyxia, multiple injuries | ACG, MC, THL |
| UMB 1790   | Control   | 13          | M      | 18          | Caucasian  | Multiple injuries      | ACG             |
| UMB 1793   | Control   | 11          | M      | 19          | African American | Drowning       | ACG, MC, THL |
| UMB 1860   | Control   | 8           | M      | 5           | Caucasian  | Cardiac Arrhythmia     | ACG             |
| UMB 4543   | Control   | 28          | M      | 13          | Caucasian  | Multiple injuries      | ACG, MC, THL |
| UMB 4638   | Control   | 15          | F      | 5           | Caucasian  | Chest injuries         | ACG             |
| UMB 4722   | Control   | 14          | M      | 16          | Caucasian  | Multiple injuries      | ACG, MC, THL |
| UMB 797    | Autism    | 9           | M      | 13          | Caucasian  | Drowning               | ACG, THL        |
| UMB 1638   | Autism    | 20          | F      | 50          | Caucasian  | Seizure                | ACG, MC, THL |
| UMB 4231   | Autism    | 8           | M      | 12          | African American | Drowning      | ACG, MC, THL   |
| UMB 4721   | Autism    | 8           | M      | 16          | African American | Drowning       | ACG, MC, THL |
| UMB 4899   | Autism    | 14          | M      | 9           | Caucasian  | Drowning               | ACG, MC, THL |
| B 5000     | Autism    | 27          | M      | 8.3         | NA         | NA                     | ACG, MC, THL   |
| B 6294     | Autism    | 16          | M      | NA          | NA         | NA                     | ACG, MC, THL   |
| B 6640     | Autism    | 29          | F      | 17.83       | NA         | NA                     | ACG, MC, THL   |

*Autism Tissue Program (ATP) identifier. 

bBrain regions for which, each sample was available. 

M: Male; F: Female, PMI: Postmortem interval, ACG: Anterior cingulate gyrus; MC: Motor cortex; THL: Thalamus; NA: Not available.

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the trio genotype data. SNPs were tested for Hardy–Weinberg Equilibrium (HWE) using Haploview. SNP associations were examined by transmission disequilibrium test (TDT), using the TDTPHASE option of UNPHASED v2.403 (http://portal.libio.org); expectation maximization (EM) algorithm was used to resolve uncertain haplotypes, to infer missing genotypes and to provide maximum-likelihood estimation of frequencies.

A linkage disequilibrium (LD) plot was constructed using the D’ values. Pair-wise LD values between SNPs were estimated using Haploview. Subsequently, associations of haplotypes (frequency >0.01) belonging to the various haploblocks of LMX1B were also examined using Haploview.

Extraction of RNA from brain tissues
The brain tissues were homogenized by ultrasonication and total RNA was extracted using TRIZol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer’s protocol. The RNA samples were further purified using RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer’s instructions. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 nm and 280 nm) of RNA were estimated with a NanoDrop ND-1000 Spectrophotometer (Scrum, Tokyo, Japan).

Quantitative real-time reverse transcriptase PCR (qRT-PCR)
ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) was used to synthesize first-strand cDNA from the total RNA according to the manufacturer’s protocol.

RT-PCR primers for LMX1B (NM_001174146.1) (F-cctttgagcagctaaaacgct, R-gggactgaatttcccagcaa) and endogenous reference GAPDH (NM_002046.3) (F-atcacaagcctgctgac, R-tggacctgtgacgctatg) were designed using primer express v2.0 (Applied Biosystems). SYBR Green qRT-PCR assays were performed using Quantitect SYBR Green PCR kit (Qiagen).

Association of LMX1B with Autism
Conceived and designed the experiments: IT KN AA SS NM. Performed the experiments: IT AA SS. Analyzed the data: IT AA AA KY Y. Iwata KS HM KI TS TY. Wrote the paper: IT KN AA NM. Contributed reagents/materials/analysis tools: TT MT Y. Iwata KS HM KI TS TY. Performed the experiments: IT AA SS. Contributed reagents/materials/analysis tools: TT MT Y. Iwata KS HM KI TS TY. Contributed reagents/materials/analysis tools: IT KN AA NM. We thank Dr. Jane Pickett, Director of Brain Resources and Data, Autism Tissue Program, for facilitating brain tissue collection. Human tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. Tissue samples were also provided by the Harvard Brain Tissue Resource Center, which is supported in part by PHS grant number R 24 MH 068855. We thank Ms. Tae Takahashi for technical assistance.

Author Contributions
Conceived and designed the experiments: IT KN AA SS NM. Performed the experiments: IT AA SS. Analyzed the data: IT AA AA KY Y. Iwata KS HM KI TS TY. Wrote the paper: IT KN AA NM.

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