Association between schizophrenia with ocular misalignment and polyalanine length variation in PMX2B

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The increased incidence of minor physical anomalies (MPAs) in schizophrenia is the fundamental basis for the neurodevelopmental hypothesis of schizophrenia etiology. Ocular misalignment, or strabismus, falls into the category of MPAs, but this phenotype has not been assessed in schizophrenia. This study reveals that a subtype of strabismus, constant exotropia, displays marked association with schizophrenia ($P = 0.00000000906$). To assess the genetic mechanisms, we examined the transcription factor genes ARIX (recently identified as a causative gene for syndromic strabismus) and its parologue, PMX2B. We identified frequent deletion/insertion polymorphisms in the 20-alanine homopolymer stretch of PMX2B, with a modest association between these functional polymorphisms and constant exotropia in schizophrenia ($P = 0.029$). The polymorphisms were also associated with overall schizophrenia ($P = 0.012$) and more specifically with schizophrenia manifesting strabismus ($P = 0.004$). These results suggest a possible interaction between PMX2B and other schizophrenia-precipitating factors, increasing the risk of the combined phenotypes. This study also highlights the unique nature of the polyalanine length variations found in PMX2B. In contrast with other transcription factor genes, the variations in PMX2B show a high prevalence, with deletions being more common than insertions. Additionally, the polymorphisms are of ancient origin and stably transmitted, with mild phenotypic effects. In summary, our study lends further support to the disruption of neurodevelopment in the etiology of schizophrenia, by demonstrating the association of a specific MPA, in this case, constant exotropia with schizophrenia, along with molecular variations in a possible causative gene.

INTRODUCTION

The view that neurodevelopmental abnormalities are involved in, at least partially, the etiologies of schizophrenia has become prevalent, as can be seen from a range of epidemiological, clinical and neurobiological evidence (1). One facet of such supporting evidence is the observation of a significantly higher prevalence of minor physical anomalies (MPAs) in schizophrenic patients than in healthy controls (reviewed in 2). MPAs involve slight dysmorphic features representing subtle alterations in the development of various ectoderm-derived bodily structures in the mouth, eye, ear, global head, hand and foot areas. MPAs are believed to develop during the first and/or early second trimesters of gestation (3). As the bodily structures involved in
the expression of MPAs typically share an embryonic origin with that of the brain (4), an organ of ectodermal origin, MPAs represent potentially valuable indices of disturbances in early neuronal development. MPAs may result from both genetic factors and environmental influences, such as complications during pregnancy (2). The findings that MPAs are particularly common among schizophrenic patients with a family history of the disorder (5), and that siblings display significantly more MPAs than normal subjects (6) represent evidence of a genetic effect.

MPAs in schizophrenia have been scored using the Waldrop scale (7), including modified versions (8) and additional or different items (9). The Waldrop scale was originally devised for use in children with Down’s syndrome, and is a standardized tool for assessing 18 features of the head, eyes, ears, mouth, hands and feet. However, the study of MPAs in schizophrenia is still in the exploratory phase, and the instruments used to measure MPAs have been criticized for their inherent limitations, including content. For eye assessments, the items that have been addressed thus far include telecanthus, epicanthus (7), heterochromia and ptosis (6). Abnormalities in eye position, such as the presence of strabismus, have not been addressed in previous studies. Isolated non-syndromic strabismus affects 1–5% of the general population (10). Most forms of strabismus are multifactorial in origin, with possible inherited components. Strabismus can result from errors in developmental co-ordination of cranial nerves innervating the extraocular muscles, namely the oculomotor (nIII), trochlear (nIV) and abducens nerves (nVI). These cranial nerves differentiate from neural crest cells in the embryonic stage (11). Once formed in the developmental stage, strabismus persists into adult life and is readily detected on simple visual examination, as with other MPAs. We therefore set out to compare the prevalence of strabismus in schizophrenia and mentally normal cohorts in this study, to determine whether this developmentally minor anomaly is associated with schizophrenia.

The genes responsible for strabismus have long remained quite unknown. However, Nakano et al. (12) recently reported homozygous mutations in ARIX (PHOX2A) in congenital fibrosis of the extraocular muscles type 2 (CFEOM2), which accompanies strabismus. The ARIX paralogue, PMX2B (PHOX2B) (12), is 100% identical to ARIX within the homeo-domain and 71% identical over the whole gene. The two proteins show an overlapping pattern of expression, including co-expression in the nIII and nIV cranial nerve nuclei (13) that control eye alignment. Both genes are also known to be involved in the development of catecholaminergic neurons (13). We therefore screened for polymorphisms in these two candidate genes and evaluated the contribution of detected variants to risk of strabismus and schizophrenia.

RESULTS

Strabismus in schizophrenia and controls

Much of the difficulty in studying strabismus lies in the use of varying definitions and measures of strabismus (14). For the purpose of the present study, strabismus was defined as ocular misalignment in which both eyes are not directed to the object of regard (for details see Materials and Methods). This includes both misalignment in the primary position (straight ahead gaze) and eccentric gaze. All subjects defined as displaying strabismus in this study manifested concomitant strabismus without any systemic abnormalities: angle of misalignment was approximately the same for all directions of gaze (i.e., none of the subjects demonstrated incomitant/paralytic strabismus). Concomitant strabismus is the most common form of ocular motility defect, and has been the target of numerous epidemiological studies (14). The observed concomitant strabismus was divided into subtypes according to the direction of squint and based on whether the condition was constant or intermittent, as different mechanisms have been suggested for different forms of strabismus. The complication of strabismus as a whole displayed a highly significant association with schizophrenic cohorts compared to control subjects ($P = 0.0000161$; Table 1).

When subtypes of strabismus were inspected, this marked association was attributable to the over-representation of constant exotropia in schizophrenia ($P = 0.00000000906$; Table 1, Fig. 1). The odds ratio for constant exotropia in schizophrenia was 20.6 (95% confidence interval, 5.03–56.2). Age of onset of schizophrenia in cohorts with (mean ± SD, 24.4 ± 6.5 years) and without (mean ± SD, 26.1 ± 9.5 years) strabismus did not differ significantly ($P = 0.625$ using the Mann–Whitney test).

Analyses of candidate genes

Strabismus is widely acknowledged as displaying genetic components, although the etiology is multifactorial (14). Confirming this notion, disruption of ARIX protein, a homeo-domain transcription factor, has recently been reported to cause strabismus as one symptom in CFEOM2 (12). We therefore first examined ARIX in the 24 schizophrenic patients manifesting constant exotropia (Table 1). However, no polymorphisms were detected.

As ARIX has a close parologue, PMX2B, this gene was the next to be screened in the same samples. Human PMX2B (paired mesoderm homeobox 2b, also known as NBPhox) encodes a transcription factor with a paired-like homeodomain (15). The chromosomal assignment of the gene was first reported to be 5p12–p13 (15), but later amended to 4p12–13 by GenBank (accession no. AB015671). We further refined the location to 4p13, by fluorescence in situ hybridization using the BAC clone RP11-227F1910, which spans the PMX2B and by radiation hybrid mapping using the Stanford G3 panel (http://shgc-www.stanford.edu/RH/index.html) (linked to SHGC4-435, LOD 13.6). The PMX2B protein contains two polyalanine regions, comprising nine (Ala9) and 20 alanines (Ala20), both located downstream of a homeobox domain, and none of which are present in ARIX (Fig. 2). Interestingly, mutation screening detected variations in length of the Ala20 tract, with variant alleles derived from an in-frame deletion of alanine residues and an insertion. Examination of all schizophrenia ($n = 346$) and control samples ($n = 542$) revealed three different mutated alleles: $-15$ bp (−5 Ala), $-21$ bp (−7 Ala) and $+6$ bp (−2 Ala) (mutated alleles: 5.9% in schizophrenia, 4.8% in controls) (Figs 3 and 4). Initial analysis using ordinal denaturing acrylamide gels detected bands migrating to a −3 base position (Fig. 5). Use of c’dGTP in the PCR reaction mixtures to
breakdown hydrogen bonds in the highly GC template revealed that the 3-base shift was induced by a c.762A-to-C substitution (Ala254Ala) (Figs 2 and 5). We also observed that the alleles having both 15 bp deletion and c.762C migrated at a 18 base position. All the alleles with 21 bp deletion and 6 bp insertion displayed c.762A.

Deletion/insertion polymorphisms of PMX2B were in Hardy–Weinberg equilibrium in all sample groups, and displayed a modest association with constant exotropia in the schizophrenic group [nominal $P=0.029$ by $2 \times 4$ Fisher’s exact test; $P$-value after Bonferroni correction is not significant, when multiple tests for three-way comparisons (Table 2) plus three-way comparisons (Table 3) are considered] and in the combined samples (schizophrenia + controls) (nominal $P=0.017$), but not in controls ($P=1.000$) (Table 2). In schizophrenia, the deleted allele ($–15$ bp) was over-represented in subjects with constant exotropia. The polymorphisms were weakly associated with overall schizophrenia (nominal $P=0.012$), and more specifically with the subset of schizophrenia that carried constant exotropia (nominal $P=0.004$, corrected $P=0.024$) (Table 3). Power analysis showed that the present sample size had powers of 0.757 and 0.912 ($a<0.05$) in an additive model with a genotype relative risk of 1.5 and allele frequencies of 0.1 and 0.2, respectively.

The polyalanines affect the protein function

To understand the functional role of alanine repeats in PMX2B, we generated constructs with total deletion or varying length of the Ala20 sequence and total deletion of the Ala9 sequence (Fig. 6A). Activities as transcription factors under the dopamine β-hydroxylase gene (DBH) promoter were then examined. Deletion of Ala20 or Ala9 sequences reduced luciferase activity to approximately half the normal PMX2B value (Fig. 6B). Deletion of Ala20 produced a larger reduction than deletion of Ala9. Increasing [+5 alanine residues (16)] or decreasing (–1, –5, –6, –7, or –13 alanine residues: we found one schizophrenic patient with a –13 alanine deletion who was unavailable for ophthalmologic examination) the Ala20 stretch also reduced promoter activity, with the greatest change seen in the +5 alanine insertion (16) (Fig. 6C).

During the preparation of our manuscript, Amiel et al. (16) reported an association between CCHS and variants with +5 to +9 alanine expansions within the Ala20 tract of PMX2B. In some of their patients, expansions resulted from de novo mutations, prompting Amiel et al. to suggest unequal crossing-over during meiosis as a mechanism for the mutations (16). In the separate panel of family samples, we analyzed allele transmission, but found no evidence of de novo mutations (transmitted alleles included 259 wild-type and 15 deleted variants). If the polymorphisms detected in this study were attributable to unequal crossing-over, the area surrounding the Ala20 stretch should represent a recombination hot spot, giving rise to an LD gap in this region. Mutation screening and a database search identified nine SNPs in the genomic region surrounding the Ala20 stretch (Fig. 1). LD analysis between these SNPs excluded the possibility of an LD gap (Table 4). Furthermore, analysis of the evolutionary history of haplotypes...
defined by these SNPs and alanine length variations suggested a relatively ancient origin for polymorphisms of the Ala20 stretch (Fig. 7). The amino acid sequence of human PMX2B differs from that seen in mouse (17) by one residue located outside either polyalanine stretch. Orthologous genes in other species have not been reported. We examined the Ala20 homopolymer in mouse \textit{Pmx2b} from each of the parental lines C57BL/6 and C3H/He, in addition to F1 intercrosses \((n = 120\) each), and found no polymorphisms. These results imply that the Ala20 stretch (and the genetic variations in humans) is stably transmitted in both species. However, this stability of transmission differs from the duplications seen in the rare CCHS (16).

**DISCUSSION**

This is the first study to report the frequency of strabismus, an ocular misalignment, in schizophrenia. Constant exotropia, a subtype of strabismus, was found to be extremely prevalent in schizophrenia compared to normal controls. In addition, the rate of overall strabismus (13\%) or constant exotropia (6.9\%) in our schizophrenic cases was higher than that of ptosis (2\%) (6), another MPA of the eye. We therefore propose that strabismus (and the genetic variations in humans) is stably transmitted in both species. However, this stability of transmission differs from the duplications seen in the rare CCHS (16).

of our cases displayed this type of ‘fluctuating’ strabismus. All current schizophrenic patients in this study were maintained on the appropriate medication. Neuroleptics are known to sometimes cause acute dystonic reactions, and involvement of the extraocular muscles may result in oculogyric crisis, wherein the eyes are elevated and ‘locked’ in this position (20). However, this symptom is easily differentiated from strabismus in the clinical situation.

The reason for accumulation of this specific subtype of strabismus (constant exotropia) in schizophrenia is unknown. Comitant strabismus is likely to display an etiologically heterogenous, complex and multifactorial phenotype, possibly with genetically distinct backgrounds according to subtype (reviewed in 14). For instance, Schlossman and Priestley (21) reported the presence of a family history in 50\% of esotropes and 37\% of exotropes. Direction of squint in each family was concordant with that of the affected proband. Waardenburg (22) described families in which exotropia was transmitted through generations, implying dominant transmission. Maumenee and Alston (23) described inheritance of congenital esotropia. Our results suggest an overlap of genetic etiology and developmental trigger for constant exotropia and schizophrenia.

Prevalences of the various forms of comitant strabismus vary widely among populations. Gover and Yankey (24) found prevalences of 2.5 and 0.6\% for strabismus among Caucasians and African Americans, respectively; most of the latter were exotropic. Nordlow (25) examined a Caucasian population, finding prevalences of 2.59\% constant esotropes, 0.93\%...
intermittent esotropes, 0.13% constant exotropes, 0.3% intermittent exotropes and 0.05% hypertropes, for a total of 4% strabismics. Laatikainen and Erkkila (26) reported 2.9% esotropes and 1.7% exotropes in a study of 411 Finnish schoolchildren. Ing and Pang (27) reported the frequency in Asian populations, as 33% esotropia and 67% exotropia. Concordant with their Asian data, occurrence of exotropia (3.5%) is about 2-fold higher than that of esotropia in our Japanese controls (1.5%). Differences between races in the frequencies and types of strabismus may again be attributable to genetic factors, providing further evidence of a genetic contribution. We could not examine the heritability of strabismus in schizophrenia (and controls) in the present case–control study, because of a lack of information on the phenotypic status of parents and siblings, but this would be an important issue to be solved in future studies.

Human PMX2B and its mouse ortholog are expressed in neural crest cell derivatives and play a primary role in the generation and survival of adrenergic neurons and a subpopulation of brainstem motor neurons (13). PMX2B protein is expressed in the nIII and nIV cranial nerve nuclei (13) that control eye alignment. This expression pattern may explain the contribution of functional polymorphisms to the risk of strabismus in schizophrenia. However, PMX2B mutations alone may not be sufficient to induce strabismus, it may require interaction with an additional causative gene(s) and/or environmental factors relevant to schizophrenia, since the mutations were not associated with strabismus in normal subjects. PMX2B regulates the expression of tyrosine hydroxylase and DBH, which are required for the biosynthesis of dopamine and noradrenaline, respectively, in catecholaminergic cells (13). Perturbations in the expression of these enzymes have been linked to the pathophysiology of schizophrenia (28). In line with the pivotal roles of PMX2B in catecholaminergic neurons, variation of the Ala20 length in PMX2B sequence exerted a genetic effect, albeit modest, on the development of overall schizophrenia. A stronger association of PMX2B variation with the subset of schizophrenics who manifested constant exotropia suggests that

![Figure 3. Sequences of Ala20 deletions/insertion mutants. Two different sequences were detected for each of the 6 bp insertion and 21 bp deletion mutants.](https://academic.oup.com/hmg/article/13/5/551/575458)
The study identified one schizophrenic with a 13 alanine deletion, but the patient was unavailable for ophthalmologic examination. The suggestion is that the current Ala20 stretch might have and additional alanine residues (Fig. 1), whereas Ala9 is not.

In summary, MPAs have been found to display increased prevalence in a range of neurodevelopmental disorders other than schizophrenia, including learning disabilities, congenital speech, hearing impairments, attention deficit hyperactivity disorder (35) and autism (36). Investigation of the prevalence of strabismus in those neuropsychiatric disorders, and assessment of the role of PMX2B variations in strabismus conveyed by these illnesses and in the development of the illnesses themselves may therefore prove worthwhile.

**MATERIALS AND METHODS**

**Subjects**

For ophthalmologic examinations, 346 schizophrenia (mean age 42.8 ± 8.3 years) and 542 mentally healthy controls (mean age 42.5 ± 11.0 years) were recruited. All subjects were collected from a single geographic area in central Japan. Diagnosis of schizophrenia was achieved by direct interview, based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (37), with consensus from at least three experienced psychiatrists. All available medical records were taken into consideration. All patients underwent computer tomography examination of the brain to exclude organic abnormalities. None of the current subjects displayed mental retardation or congenital central hypoventilation syndrome (CCHS) (16). Control subjects were recruited from among volunteers documented as free of psychoses. A total of 124 families were recruited separately to test for transmission stability of PMX2B variants. These comprised 80 families with one offspring plus parents, 18 with two offspring plus parents and five with three offspring plus parents. Biological parentage in all families was confirmed by examining 444 highly polymorphic microsatellite markers from Japanese cohorts (mean heterozygosy = 0.73) (38). The present study was approved by the Ethics Committees of RIKEN and Hamamatsu Medical University, and all participants provided written informed consent.

**Ophthalmologic examination**

Clinical assessments were made by trained medical doctors (Tomoko Toyota, Yoshio Minabe and Kiyoshi Yoshitugu) in a blind study, and confirmed by an independent and experienced ophthalmologist (Hajime Fujikura). The Hirschberg test and two kinds of cover tests (a cover–uncover test and an alternating cover test) were conducted to detect and classify strabismus and to exclude heterophoria. None of the strabismus sufferers displayed incomitant strabismus, accommodation esotropia, or systemic abnormalities including developmental and metabolic defects, brain damage or mental retardation.

**Mutation screening of ARIX and PMX2B**

ARIX comprises three exons with the initiation codon in exon 1 and a stop codon in exon 3 (39). We screened the coding region, flanking introns and promoter sequences (1210 bp upstream from the reported 5' end of exon 1) (39), using PCR amplification and sequencing of genomic DNA from 24 schizophrenia samples who showed constant exotropia (Table 1).

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**Figure 4.** Fragment analysis of polymorphic Ala20 length genotypes. Four different Ala20 variants were detected: three in-frame deletions (−15, −21 and −39 bp) and one in-frame insertion (+6 bp) within the homopolymeric stretch.
For detecting mutations in PMX2B, an initial screen was performed on the coding region, flanking introns and promoter sequences (130 bp upstream from the reported 5' end of exon 1) (15), using the same samples as in ARIX. To identify single nucleotide polymorphisms (SNPs) for use in linkage disequilibrium analysis, we analyzed a region up to 2204 bp upstream from the 5' start of the gene, introns 1 and 2 and 1913 bp downstream from the 3' end, by examining 30 additional

Figure 5. Effect of c.762A>C polymorphism on aberrant mobility shift in a denaturing gel. Ala20 templates with a c.762C migrate at a -3 base position in the absence of 7-deaza-2'-deoxyguanosine triphosphate (c'dGTP). This aberrant -3 base allele peak was removed on the addition of c'dGTP to the PCR reaction mixture (A). Without c’dGTP, 17% of total non-deletion/insertion alleles displayed the -3 base migration. Diagram of sequences (anti-sense strands) shows the c.762A>C polymorphism (B). Secondary structure of the DNA fragment spanning the Ala20 portion was predicted using GENETYX-MAC version 11 software (GENETYX Corporation, Tokyo, Japan) (C). Minimum free energy generated by the secondary structure was -78.3 kcal/mol for the fragment with c.762A and -83.5 kcal/mol for the fragment with c.762C. The fragment with c.762C was deemed to retain at least partial secondary structure even under denaturing conditions. This remnant secondary structure is apparently destroyed when the fragment is PCR-amplified in a reaction mixture containing c’dGTP.
samples. Information on primer sequences used in this study is available on request.

Genotyping of PMX2B polymorphisms

The genomic region encoding the 20 alanine (Ala20) tract was amplified using fluorescently labeled forward primer (5’-AACC CGGCAAGGGCGGCGACGA, 3’ end at nt c.726) and reverse primer (5’-GAA GGACCCCAAGCGGAAT, 3’ end at nt c.854), rTaq polymerase (Takara, Tokyo, Japan) and MasterAmp K buffer (Epicentre Technologies, Madison, WI). The mouse Ala20 tract of PMX2B gene was examined in 120 C57BL/6 mice, 120 C3H/He mice and 120 of the F1 progeny, using PCR-based techniques (40). The gene promoter for dopamine β-hydroxylase (DBH) (41) was amplified using a primer set designed from the genomic sequence (accession no. AC001227), then cloned into the pGL3-basic reporter vector (Promega, Madison, WI). Constructs lacking the entire Ala20 and Ala9 regions were generated using PCR-based techniques (40).

Transfection and luciferase assay

HepG2 cells were purchased from the Riken Cell Bank (Tsukuba, Japan). The plasmid mixture was prepared by combining 1.3 μg of construct DNA (pIRES-neo2-PMX2B: pGL3-basic-DBH promoter = 400 μg: 900 μg), 100 μg of pRL-TK as an internal control and 2.5 μl of LipofectAMINE2000 in 100 μl of OPTI-MEM (Invitrogen). Transfections were performed using LipofectAMINE2000 (Invitrogen) according to the instructions of the manufacturer. Transcriptional assay was performed using the PicaGene Dual SeaPansy kit in accordance with the manufacturer’s instructions (Toyo Ink, Tokyo, Japan). Luciferase activity was measured using a luminometer Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany).

Statistical analyses

Phenotype–genotype association tests were assessed using the $\chi^2$ test, or Fisher’s exact test where appropriate. Linkage disequilibrium (LD) statistics were calculated using COCAPHASE (42) (http://www.hgmp.mrc.ac.uk/~fhudbrid/software/), and estimation

Table 2. Strabismus and the Ala20 polymorphism of PMX2B gene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strabismus</th>
<th>n</th>
<th>Genotype counts</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0/0</td>
<td>0/−15</td>
</tr>
<tr>
<td>Control</td>
<td>Constant exotropia</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>No strabismus</td>
<td>515</td>
<td>472</td>
<td>33</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>Constant exotropia</td>
<td>24</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No strabismus</td>
<td>300</td>
<td>266</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>Constant exotropia</td>
<td>41</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No strabismus</td>
<td>815</td>
<td>738</td>
<td>65</td>
</tr>
</tbody>
</table>

$^a$0 indicates the normal allele, and − or + denotes the variant alleles with the indicated number of deletions (−) or insertions (+) of Ala20-coding nucleotides. $^b$P-value for association was calculated by Fisher’s exact test.

Table 3. Schizophrenia and the Ala20 polymorphism of PMX2B gene

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Strabismus</th>
<th>n</th>
<th>Genotype counts (%)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0/0</td>
<td>0/−15</td>
</tr>
<tr>
<td>Total schizophrenia</td>
<td>No strabismus</td>
<td>324</td>
<td>283 (87)</td>
<td>38 (12)</td>
</tr>
<tr>
<td>Total schizophrenia</td>
<td>With strabismus</td>
<td>300</td>
<td>266 (88)</td>
<td>32 (11)</td>
</tr>
<tr>
<td>Total schizophrenia</td>
<td>Without strabismus</td>
<td>24</td>
<td>17 (71)</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Controls $^a$</td>
<td>No strabismus</td>
<td>515</td>
<td>472 (92)</td>
<td>33 (6.4)</td>
</tr>
</tbody>
</table>

$^a$0 indicates the normal allele, and − or + denotes the variant alleles with the indicated number of deletions (−) or insertions (+) of the Ala20-coding nucleotides. $^b$P-value for association was calculated by Fisher’s exact test. $^c$Subjects who had strabismus were excluded.
of haplotype frequencies and assessment of Hardy–Weinberg equilibrium were performed using Arlequin software (http://lgb.unige.ch/arlequin/). Genotype data from 100 males and 100 females were used for LD and haplotype analyses.

### Table 4. Pairwise linkage disequilibrium estimations between polymorphisms in the PMX2B gene

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>IVS1-385G&gt;A (SNP1)</th>
<th>IVS1-115G&gt;A (SNP2)</th>
<th>IVS2+101A&gt;G (SNP3)</th>
<th>IVS2-404-405delAG (SNP4)</th>
<th>c.1618insT (SNP5)</th>
<th>c.2309G&gt;A (SNP6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>SNP2</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>SNP3</td>
<td>0.984</td>
<td>0.984</td>
<td>0.968</td>
<td>—</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>SNP4</td>
<td>0.984</td>
<td>0.984</td>
<td>0.954</td>
<td>—</td>
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<td>1.000</td>
</tr>
<tr>
<td>SNP5</td>
<td>0.969</td>
<td>0.969</td>
<td>0.846</td>
<td>0.846</td>
<td>0.831</td>
<td>—</td>
</tr>
<tr>
<td>SNP6</td>
<td>0.86</td>
<td>0.858</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values above the diagonal show standardized $D'$ in 200 unrelated subjects, calculated by using the COCAPHASE program. Values below the diagonal show $r^2$ (squared correlation coefficient).

The polymorphisms used in this linkage disequilibrium analysis (SNPs1–6) were those whose minor allele frequencies were more than 3% (also see Fig. 1). The Ala20 stretch is located between SNP4 and SNP5.

### Figure 6. Functional consequences of PMX2B mutant alleles. Preparation of PMX2B deletion/insertion mutants is shown (A). A, alanine. Effects of Ala20 and Ala9 stretch deletions were examined under luciferase assay. Reporter constructs containing the DBH promoter were co-transfected into HepG2 cells with either normal PMX2B or deletion mutants. Luciferase activity of each construct was normalized by the internal control, pRL-TK. Activity of the normal PMX2B was defined as 1. Results shown represent means± SEM for at least three separate transfections, each run in triplicate. pGL3-basic is a promotorless negative control vector. *$P < 0.01$ by Tukey–Kramer multiple comparison test (B). Assay of Ala20 mutants was performed under the same conditions as in B (C). *$P < 0.01$ by Tukey–Kramer test.

### CLUSTALW (program for multiple alignments and tree-making; http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) (43) and TreeView ver.1.6.6 (program for displaying phylogenies; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (44) software
was used to depict the evolutionary history of haplotypes in a phylogram. Power analysis was performed using the Genetic Power Calculator (http://stagen.iop.kcl.ac.uk/gpc/) (45).

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5. SnP1

SNP2

SNP3

SNP4

Ala10

SNP5

SNP6

frequency

haplotype 1 A A G D 1 1 A 0.0784

haplotype 2 A A G D 2 1 A 0.1303

haplotype 3 G G A I 1 D A 0.0282

haplotype 4 G G A I 1 D G 0.0483

haplotype 5 G G A I 2 D 0.0379

haplotype 6 G G A I 3 D G 0.0608

Figure 7. Phylogram of haplotypes in PMX2B. For nomenclature of SNPs 1–6, see Figure 1. For SNP4, ‘D’ indicates deletion and ‘I’ denotes insertion. For Ala10, allele 1 = c.762A, allele 2 = c.762C, allele 3 = 15-bp deletion. Six different haplotypes covered 97.9% of the total number of haplotypes.


45. Purcell, S., Cherny, S.S. and Sham, P.C. (2003) Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics, 19, 149–150.