Schizencephaly is a brain malformation disorder characterized by one or more full-thickness clefts through the cerebral cortex. While initial reports suggested that EMX2 mutations are a common cause of schizencephaly, more recent evidence suggests that EMX2 mutations are not a common cause of this malformation. To determine the frequency of EMX2 mutations in patients with schizencephaly, we sequenced EMX2 in a cohort of 84 affected probands. No pathologic mutations were identified in this cohort, suggesting that EMX2 mutations are an uncommon cause of schizencephaly.

Key words: schizencephaly; EMX2; congenital brain malformation

acid substitutions and other intronic mutations were also reported. However, no EMX2 mutations are reported in subsequent studies of patients with schizencephaly, and two groups were unable to identify EMX2 mutations in cohorts of 15 or 17 individuals [Barkovich et al., 2001; Granata et al., 2005], respectively. Thus, the extent to which EMX2 mutations contribute to schizencephaly remains uncertain [Barkovich et al., 2005; Granata et al., 2005; Guerrini and Filippi, 2005]. To estimate the contribution of EMX2 mutations to schizencephaly and to provide accurate recurrence risks, we sought to determine the prevalence of EMX2 mutations in a large cohort of affected individuals.

MATERIALS AND METHODS

Subjects
A schizencephaly case series was assembled from two independent studies conducted at Beth Israel Deaconess Medical Center and Boston Children's Hospital (Boston, MA) and the California Birth Defects Monitoring Program (CBDMP, Berkeley, CA). For the Boston study, the protocol was reviewed and approved by the Institutional Review Board at BIDMC and Boston Children's Hospital (n = 31). All affected individuals and brain imaging studies were reviewed and interpreted by a clinical neurologist and/or neuroradiologist. Patients with schizencephaly from the California study (n = 53) were described previously [Curry et al., 2005]. Infants diagnosed with schizencephaly were born during 1985–2001 and ascertained by active surveillance conducted by the staff of the CBDMP. Schizencephaly diagnoses were confirmed by a review of medical records and interpretations of brain imaging studies by a clinical geneticist. Each patient was computer matched to a corresponding dried newborn blood spot archived by the Genetic Disease Branch, California Health Department. Genotyping for this study was approved by the State of California Health and Welfare Agency Committee for the Protection of Human Subjects.

Genomic DNA and EMX2 Sequencing
Genomic DNA was extracted from peripheral whole blood lymphocytes according to the manufacturer's protocols (Qiagen, Valencia, CA). For the CBDMP subjects, genomic DNA was extracted from dried newborn blood spots and subject to genomewide linear amplification (Qiagen). Sequencing primers were designed to flank the exons and adjacent intron boundaries of EMX2 (Primer 3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ www.cgi). EMX2 sequencing coverage was performed as described [Brunelli et al., 1996; Tietjen et al., 2005]. EMX2 exons and adjacent intron boundaries were PCR amplified from genomic DNA, and PCR products were purified using the Ampure kit (Agencourt, Beverly, MA), and sequenced bidirectionally by fluorescent dye-terminator chemistry (Seqwright, Houston, TX). For the CBDMP subjects with heterozygosity at Ala35, unamplified genomic DNA was also sequenced and confirmed the presence of the polymorphism. Primer sequences for the three EMX2 exons are: E01F, ACAAACAGGTCCCCAATTCTCGTCC; E01R, CTTGGGAGGCTGGACCTTAGATCG; E02F, GTGAGCCCTTTGGGAGGAC; E02R, GCACCTACAGCCCCTTTCTG; E03F, GGAGGCTGGACCTTATGGACT; E03R, GTGAACGTGTATGCCTTGGTTTG.

Electronic Database Resources
Previously reported SNPs in the EMX2 gene and EMX2 SNP frequencies in control populations were obtained from dbSNP build 126 (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov).

RESULTS
We assembled a large schizencephaly case series from patients enrolled in a research study at Boston Children’s Hospital and Beth Israel Deaconess Medical Center and ascertained by the California Birth Defects Monitoring Program [Croen et al., 1991] (Fig. 1; see Methods and Materials). Although detailed case histories were not available for ~25% of patients, we attempted to exclude as many patients as possible where schizencephaly could have resulted from environmental causes such as maternal care (e.g., reported substance abuse or attempted pregnancy termination), non-developmental vascular insults (e.g., in utero loss of a monozygotic sibling), or possible viral infection (e.g., calcifications reported along schizencephalic clefts) [Barkovich and Kjos, 1992; Dominguez et al., 1992; Iannetti et al., 1998; Sener, 1998; Roccella and Testa, 2005]. In total, 84 individuals with schizencephaly and a variety of cleft patterns were ascertained (Table I). Familial schizencephaly was very rare in the case series; however, two affected half-siblings were included (data not shown).

We sequenced the EMX2 coding region and adjacent intronic regions that have been reported previously to harbor schizencephaly-causing mutations (see the online Fig. 1A at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html) [Brunelli et al., 1996]. After sequencing all 84 individuals in the case series, no clear deleterious EMX2 mutations were identified including sequence insertions or deletions, frameshift mutations, splicing mutations, non-synonymous point mutations, or other mutations that have been described in EMX2 [Brunelli et al., 1996; Faiella et al., 1997; Granata et al., 1997]. By combining the original study populations with more recent reports of EMX2
sequencing [Barkovich et al., 2001; Granata et al., 2005], the prevalence of schizencephaly mutations is predicted to be 13/50, or 26%. Our data (0/84, or 0%) are statistically incompatible with the combined estimates of these previous EMX2 mutation prevalence rates ($P < 0.0001$).

In fact, very little EMX2 sequence variation was found within the present case series. Only two SNPs in the EMX2 coding sequence are reported (rs424112 and rs8192642, see the online Fig. 1A at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). Neither variant, however, is predicted to change the protein sequence (Ala182 and Arg156, respectively) nor splicing properties of EMX2. We detected a variant allele for one patient who is heterozygous at Ala182, consistent with the frequency of allelic variation for this SNP among normal individuals (Table II). Population data is unavailable for the Arg156 SNP; however, its variability has been reported in both affected individuals and unaffected relatives [Brunelli et al., 1996; Granata et al., 2005]. Only one SNP located in the second intron immediately before Exon 3 was observed with high variability in our cohort (rs2240776, see the online Fig. 1A at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html), and its variability is also consistent with reported allelic frequencies of normal individuals (Table II). Other highly variable SNPs are reported in noncoding and untranscribed regions of EMX2 but are located outside the range of our sequencing efforts.

Of note, we did identify a previously unannotated SNP within the coding sequence of Exon 1 that was heterozygous in two subjects (see the online Fig. 1 at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). The variant is an A → T nucleotide change in the degenerate position of Ala35. This nucleotide substitution is not detected in >350 control chromosomes of European and Middle-Eastern origin and so we cannot rule out the possibility of its specificity to individuals with schizencephaly. However, the Ala35 nucleotide substitution is not predicted to change the EMX2 protein sequence or introduce or abolish a splice site (see the online Fig. 1B at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html and data not shown). Moreover, assuming the Ala35 substitution is causative for schizencephaly, the prevalence of EMX2 mutations in our case study remains statistically inconsistent with the combined estimates of previous EMX2 mutation rates ($P < 0.005$).

**DISCUSSION**

The prevalence of EMX2 mutations in schizencephaly patients has not been established. Early reports suggested that EMX2 mutations may account for

| Table I. Types of Clefts Found in Individuals Within the Schizencephaly Case Series |
|-----------------------------------|-----|-----|-----------------|-----|-----|
| Unilateral cleft                  | Number | Percent | Bilateral cleft | Number | Percent |
| Unilateral closed lip             | 7     | 10.8  | Bilateral closed lip | 3     | 4.6    |
| Unilateral open lip               | 11    | 16.9  | Bilateral open lip | 21    | 32.3   |
| Multiple open and closed lip      | 1     | 1.5   | Bilateral left open lip | 1  | 1.5    |
|                                   |       |       | Bilateral right open lip | 1 | 1.5    |
| Unilateral unknown lip            | 12    | 18.5  | Bilateral unknown lip | 8     | 12.3   |
| Total unilateral cleft            | 31    | 47.7  | Total bilateral cleft | 34    | 52.3   |
as much as ~72% of schizencephaly cases, as evidenced by the presence of heterozygous mutations in 13/18 subjects [Brunelli et al., 1996; Faiella et al., 1997; Granata et al., 1997]. However, more recent reports suggest that the number of schizencephaly cases that can be attributed to *EMX2* mutations is much lower [Barkovich et al., 2001, 2005; Granata et al., 2005].

By performing *EMX2* genotyping on the largest reported schizencephaly case series to date (n = 84), we did not identify any clear pathological *EMX2* mutations. We did identify a novel SNP in two individuals that is located within the degenerate base position of Ala35. However, the significance of this polymorphism for *EMX2* function and schizencephaly is not clear. Moreover, similar, synonymous rare variants within the *EMX2* coding sequence are found in public sequence databases of normal individuals and unaffected relatives [Brunelli et al., 1996; Granata et al., 2005]. It is thus probable that the Ala35 nucleotide substitute is also a rare but benign variant.

Taken together, our data indicate that pathological *EMX2* mutations are not a common cause of schizencephaly. We suggest that future investigations of individuals and families with schizencephaly consider both additional genetic loci and environmental factors in trying to untangle the etiologies of this serious brain malformation.

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### REFERENCES


**TABLE II. EMX2 SNPs in Control Datasets and Schizencephaly Case Series**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Variant</th>
<th>Control dataset (%)</th>
<th>Schizencephaly case series (%)</th>
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<tr>
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