Comprehensive *EMX2* Genotyping of a Large Schizencephaly Case Series

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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. © 2007 Wiley-Liss, Inc.

Key words: schizencephaly; *EMX2*; congenital brain malformation

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INTRODUCTION

Schizencephaly is a congenital brain malformation whose hallmark is the presence of one or more clefts spanning the pial and ependymal surfaces of one or both cerebral hemispheres [Barkovich et al., 2005; Granata et al., 2005; Guerrini and Filippi, 2005]. Schizencephalic clefts were first described by Yakovlev and Wadsworth [1946a,b] as fused in a pial-ependymal seam (closed lip) or separated with the resulting space filled with cerebrospinal fluid (open lip). Schizencephaly is associated with several clinical features of varying severity including developmental delay, mental retardation, epilepsy, and motor deficits [Barkovich and Kjos, 1992; Packard et al., 1997; Barkovich, 2000; Denis et al., 2000; Curry et al., 2005]. This brain malformation is rare among the general population with a recently estimated prevalence of 1.54 per 100,000 individuals [Curry et al., 2005].

Schizencephaly can arise from a number of environmental factors including maternal trauma, substance abuse, viral infection, in utero vascular accidents in monozygotic twins, and other vascular disruptions [Yakovlev and Wadsworth, 1946a,b; Barkovich and Kjos, 1992; Dominguez et al., 1992; Iannetti et al., 1998; Sener, 1998; Rocella and Testa, 2003; Curry et al., 2005]. However, reports of familial schizencephaly, including some reports describing similar clinical and radiological features among affected individuals, raise the possibility of one or more genetic causes [Hosley et al., 1992; Hilburger et al., 1993; Haverkamp et al., 1995; Tietjen et al., 2005]. Several reports implicate the *EMX2* transcription factor as a causative gene for schizencephaly [Brunelli et al., 1996; Faiella et al., 1997; Granata et al., 1997; Cecchi, 2002]. A total of 18 patients were sequenced for *EMX2* in these studies, and 13 (72%) were found to have various heterozygous mutations including deleterious frameshift, splicing, or deletion mutations. Heterozygous mutations of synonymous and non-synonymous amino

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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 medial records and interpretations of brain imaging studies by a clinical geneticist. Each patient was computer matched to a corresponding dried newborn screening 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 genome-wide 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 heterozygousity at Ala_{35}, unamplified genomic DNA was also sequenced and confirmed the presence of the polymorphism. Primer sequences for the three *EMX2* exons are: E01F, ACAAACGAGTCCCCAATTCTCGTCC; E01R, CTTGGAAGCGATGACCCAGATATCG; E02F, GTGAGCCCTTGGGAGGAC; E02R, GCACTTACAGCCCCTTTCTG; E03F, GGAGGCTGGACCTTAGGACT; E03R, GTGAACGTGTATGCGGTTTG.

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, 2003]. 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*

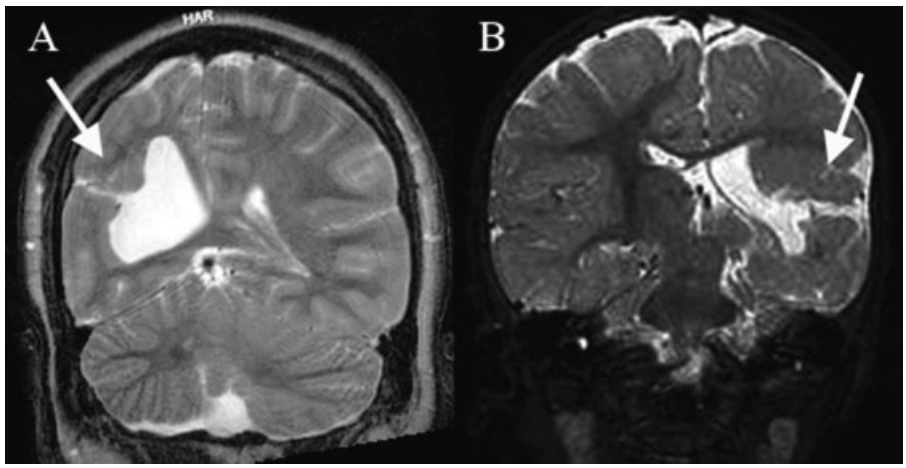

FIG. 1. A large case series of patients with schizencephaly. Representative MRIs of case series individuals with (**A**) closed-lip and (**B**) open-lip schizencephalic clefts (arrows).

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 (Ala_{182} and Arg_{156}, respectively) nor splicing properties of *EMX2*. We detected a variant allele for one patient who is heterozygous at Ala_{182}, consistent with the frequency of allelic variation for this SNP among normal individuals (Table II). Population data is unavailable for the Arg_{156} 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 Ala_{35}. 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 Ala_{35} 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 Ala_{35} 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

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

SNP	Variant	Control dataset (%)	Schizencephaly case series (%)
rs424112 (Ala_{182})	C	98.4	98.8
	T	1.6	1.2
rs2240776	A	49.0	55.6
	T	51.0	44.4

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 Ala_{35}. 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 Ala_{35} 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

Barkovich AJ. 2000. Congenital malformations of the brain and skull. In: Barkovich AJ, editor. Pediatric Neuroimaging. Philadelphia: Lippencott, Williams & Wilkins. Vol. 3e. p 289–292.

Barkovich AJ, Kjos BO. 1992. Schizencephaly: Correlation of clinical findings with MR characteristics. AJNR Am J Neuroradiol 13:85–94.

Barkovich AJ, Kuzniecky RI, Jackson GD, Guerrini R, Dobyns WB. 2001. Classification system for malformations of cortical development: Update 2001. Neurology 57:2168–2178.

Barkovich AJ, Kuzniecky RI, Jackson GD, Guerrini R, Dobyns WB. 2005. A developmental and genetic classification for malformations of cortical development. Neurology 65:1878–1887.

Brunelli S, Faiella A, Capra V, Nigro V, Simeone A, Cama A, Boncinelli E. 1996. Germline mutations in the homeobox gene *EMX2* in patients with severe schizencephaly. Nat Genet 12: 94–96.

Cecchi C. 2002. *Emx2*: A gene responsible for cortical development, regionalization and area specification. Gene 291: 1–9.

Croen LA, Shaw GM, Jensvold NG, Harris JA. 1991. Birth defects monitoring in California: A resource for epidemiological research. Paedriatr Perinat Epidemiol 5:423–427.

Curry CJ, Lammer EJ, Nelson V, Shaw GM. 2005. Schizencephaly: Heterogeneous etiologies in a population of 4 million California births. Am J Med Genet Part A 137A:181–189.

Denis D, Chateil JF, Brun M, Brissaud O, Lacombe D, Fontan D, Flurin V, Pedespan J. 2000. Schizencephaly: Clinical and imaging findings in 30 infantile cases. Brain Dev 22:475–483.

Dominguez R, Aguirre Vila-Coro A, Slopis JM, Bohan TP. 1992. Brain and ocular abnormalities in infants with *in utero* exposure to cocaine and other street drugs. Am J Dis Child 146:278–279.

Faiella A, Brunelli S, Granata T, D'Incerti L, Cardini R, Lenti C, Battaglia G, Boncinelli E. 1997. A number of schizencephaly patients including 2 brothers are heterozygous for germline mutations in the homeobox gene *EMX2*. Eur J Hum Genet 5:186–190.

Granata T, Farina L, Faiella A, Cardini R, D'Incerti L, Boncinelli E, Battaglia G. 1997. Familial schizencephaly associated with *EMX2* mutation. Neurology 48:1403–1406.

Granata T, Freri E, Caccia C, Setola V, Taroni F, Battaglia G. 2005. Schizencephaly: Clinical spectrum, epilepsy, and pathogenesis. J Child Neurol 20:313–318.

Guerrini R, Filippi T. 2005. Neuronal migration disorders, genetics, and epileptogenesis. J Child Neurol 20:287–299.

Haverkamp F, Zerres K, Ostertun B, Emons D, Lentze MJ. 1995. Familial schizencephaly: Further delineation of a rare disorder. J Med Genet 32:242–244.

Hilburger AC, Willis JK, Bouldin E, Henderson-Tilton A. 1993. Familial schizencephaly. Brain Dev 15:234–236.

Hosley MA, Abroms IF, Ragland RL. 1992. Schizencephaly: Case report of familial incidence. Pediatr Neurol 8:148–150.

Iannetti P, Nigro G, Spalice A, Faiella A, Boncinelli E. 1998. Cytomegalovirus infection and schizencephaly: Case reports. Ann Neurol 43:123–127.

Packard AM, Miller VS, Delgado MR. 1997. Schizencephaly: Correlations of clinical and radiologic features. Neurology 48:1427–1434.

Roccella M, Testa D. 2003. Fetal alcohol syndrome in developmental age. Neuropsychiatric aspects. Minerva Pediatr 55:63–69.

Sener RN. 1998. Schizencephaly and congenital cytomegalovirus infection. J Neuroradiol 25:151–152.

Tietjen I, Erdogan F, Currier S, Apse K, Chang BS, Hill RS, Lee CK, Walsh CA. 2005. *EMX2*-independent familial schizencephaly: Clinical and genetic analyses. Am J Med Genet Part A 135A: 166–170.

Yakovlev PL, Wadsworth RC. 1946a. Schizencephalies: A study of the congenital clefts in the cerebral mantle. I. Clefts with fused lips. J Neuropathol Exp Neurol 5:116–130.

Yakovlev PL, Wadsworth RC. 1946b. Schizencephalies: A study of the congenital clefts in the cerebral mantle. II. Clefts with hydrocephalus and lips separated. J Neuropathol Exp Neurol 5:169–206.