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FULL-LENGTH ORIGINAL RESEARCH

Rare exonic deletions of the *RBFOX1* gene increase risk of idiopathic generalized epilepsy

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Summary

Purpose: Structural variations disrupting the gene encoding the neuron-specific splicing regulator RBFOX1 have been reported in three patients exhibiting epilepsy in comorbidity with other neuropsychiatric disorders. Consistently, the *Rbfox1* knockout mouse model showed an increased susceptibility of seizures. The present candidate gene study tested whether exon-disrupting deletions of *RBFOX1* increase the risk of idiopathic generalized epilepsies (IGEs), representing the largest group of genetically determined epilepsies.

Methods: Screening of microdeletions (size: >40 kb, coverage >20 markers) affecting the genomic sequence of the *RBFOX1* gene was carried out by high-resolution single-nucleotide polymorphism (SNP) arrays in 1,408 European patients with idiopathic generalized epilepsy (IGE) and 2,256 population controls. Validation of *RBFOX1* deletions and familial segregation analysis were performed by quantitative polymerase chain reaction (qPCR).

Key Findings: We detected five exon-disrupting *RBFOX1* deletions in the IGE patients, whereas none was observed in the controls ($p = 0.008$, Fisher's exact test). The size of the exonic deletions ranged from 68 to 896 kb and affected the untranslated 5′-terminal *RBFOX1* exons. Segregation analysis in four families indicated that the deletions were inherited, display incomplete penetrance, and heterogeneous cosegregation patterns with IGE.

Significance: Rare deletions affecting the untranslated 5′-terminal *RBFOX1* exons increase risk of common IGE syndromes. Variable expressivity, incomplete penetrance, and heterogeneous cosegregation patterns suggest that *RBFOX1* deletions act as susceptibility factor in a genetically complex etiology, where heterogeneous combinations of genetic factors determine the disease phenotype.

KEY WORDS: Idiopathic generalized epilepsy, Microdeletion, *RBFOX1*, Genetics.

The idiopathic generalized epilepsies (IGEs) affect up to 0.3% of the general population and account for 30% of all epilepsies (Jallon et al., 2001). Genetic factors play a predominant role in the etiology of common IGE syndromes. Heritability estimates are >80% and recurrence risk for first-degree relatives varies between 4% and 9% depending on the IGE subtype (Helbig et al., 2008). The genetic architecture is likely to display a biologic continuum, in which a small fraction follows monogenic inheritance, whereas the majority of IGE patients presumably display an oligogenic/polygenic predisposition. Molecular genetic studies have identified causative gene mutations in mainly rare monogenic forms of genetic epilepsies. Most of the

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currently known genes for human genetic epilepsies encode voltage-gated or ligand-gated ion channels (Reid et al., 2009; Pandolfo, 2011). Despite extensive research, the majority of genetic variants predisposing to common IGE syndromes remain elusive.

Large-scale analysis of structural genomic variations using high-resolution whole-genome oligonucleotide arrays suggests that copy number variations (CNVs) play a substantial role in about 3% of patients with idiopathic epilepsies (de Kovel et al., 2010; Heinzen et al., 2010; Mefford et al., 2011). Recurrent microdeletions on 15q11.2, 15q13.3, and 16p13.11 increase risk of IGE and a wide range of neurodevelopmental disorders (Helbig et al., 2009; de Kovel et al., 2010; Heinzen et al., 2010; for review see Hochstenbach et al., 2011). The genes deleted by these microdeletions are thought to play a key role in the regulation of neuronal excitation and cortical synchronization.

Structural variations disrupting the gene encoding the neuronal splicing regulator *RBFOX1* (also assigned as *A2BP1, HRNBP1*, or *FOX1*) have been reported in three patients exhibiting epilepsy in comorbidity with autism, intellectual disability, or pontocerebellar hypoplasia (Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011). The *RBFOX1* gene is located in the chromosomal region 16p13.3 to which a linkage locus for photoparoxysmal response in families of IGE subjects has been mapped (Pinto et al., 2005). The *RBFOX1* gene plays a key role in the regulation of neuronal excitation and influences susceptibility of epilepsy (Gehman et al., 2011; Voineagu et al., 2011). The *RBFOX1* protein regulates splicing of many neuronal transcripts by binding the sequence (U)GCAUG in introns flanking alternative exons (Jin et al., 2003; Auweter et al., 2006; Voineagu et al., 2011; Fogel et al., 2012). A number of *RBFOX1* target transcripts (e.g., *SNAP25*, *SCN8A*, *GRIN1*, *GABRG2*, *DCX*, *GAD2*, *KCNQ2*, *SLC12A5*, *SV2B*, *SYN1*) have been implicated to play a role in epileptogenesis (Barnby et al., 2005; Corradini et al., 2009; Papale et al., 2009; Pandolfo, 2011; Fogel et al., 2012; Veeramah et al., 2012) and show differentially spliced RNA transcripts in *Rbfox1* knockout mice (Gehman et al., 2011) Notably, brain-specific homozygous and heterozygous *Rbfox1* knockouts in mice do not alter brain morphology but display spontaneous seizures and a dramatic epileptogenic response to kainic acid resulting in status epilepticus (Gehman et al., 2011). Consistent with the splicing alterations in mice, a RNA interference–mediated 50% knockdown of *RBFOX1* transcripts in human neurons changes the alternative splicing pattern and expression of primarily neuronal genes involved in synapse formation and function (Voineagu et al., 2011; Fogel et al., 2012).

The present candidate gene association study tested whether exon-disrupting deletions of *RBFOX1* increase risk of common IGE syndromes. We found a significant excess of exon-disrupting deletions of the *RBFOX1* gene in IGE patients compared to population controls. Familial cosegregation analysis implicates that exon-disrupting *RBFOX1* deletions represent susceptibility factors that increase risk of IGE but are not sufficient for the expression of IGE in most of the families.

Subjects and Methods

Study participants

The study protocol was approved by the local institutional review boards of the participating centers, and all study participants gave informed consent. The patients with common IGE syndromes were recruited as a concerted effort of epilepsy genetics programs integrated in the European EPICURE Project (http://www.epicureproject.eu; EPICURE Consortium et al., 2012). Phenotyping and diagnostic classification of IGE syndromes were carried out according to standardized protocols (available at: http://portal.ccg.uni-koeln.de/ccg/research/epilepsy-genetics/sampling-procedure). Patients with IGE exhibit unprovoked generalized seizures but are typically otherwise normal and have no anatomic brain abnormalities (Commission on Classification & Terminology of the International League Against Epilepsy, 1989; Nordli, 2005). Accordingly, the ascertainment scheme applied in this multicenter study did not include IGE patients with severe intellectual disability (no basic education, permanently requiring professional support in their daily life). All subjects of the case–control association cohorts were typed by the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, U.S.A.). To ensure highly confident CNV calls, we excluded all individuals (351 of 4,015) carrying a genome-wide excess of more than 50 microdeletions (size >40 kb, coverage >20 markers) prior to the assessment of *RBFOX1* microdeletions (Elia et al., 2012).

The case–control sample included in this candidate gene CNV study comprised 1,408 unrelated patients with IGE of self-identified Northwestern European ancestry (869 females/539 males; childhood absence epilepsy [CAE] n = 413, juvenile absence epilepsy [JAE] n = 207, unspecified idiopathic absence epilepsy [IAE] n = 7, juvenile myoclonic epilepsy [JME] n = 557, epilepsies with generalized tonic–clonic seizures alone [EGTCS] n = 224) and 2,256 German population controls (1,077 females/1,179 males). Array data of 2,256 German control subjects were obtained from the PopGen biobank (University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany) and the KORA (Cooperative Health Research in the Region of Augsburg) research platform representing epidemiologically recruited cohorts from the Northern (Schleswig-Holstein, PopGen) and Southern (Augsburg, KORA) regions of Germany. The control subjects have not been screened for epilepsy or other neurodevelopmental disorders. EIGENSTRAT principal component analysis (Price et al., 2006) was applied to remove ancestry outliers and to match the European ancestry of the case–control cohorts (EPICURE Consortium et al., 2012).

RBFOX1 microdeletion screening

For all DNA samples of the case–control cohorts, we assessed the signal intensities of 1.8 million probe sets on the Affymetrix Genome-Wide Human SNP Array 6.0. CNV analysis of all Affymetrix SNP 6.0 arrays was performed at the Cologne Center for Genomics, using the algorithm implemented in the Affymetrix Genotyping Console version 4.1.1. To achieve high accuracy in CNV calling across Affymetrix SNP 6.0 arrays processed at different laboratories, microdeletion screening was restricted to deletions covered by at least 20 probes and spanning >40 kb in size (Pinto et al., 2011).

Array-based screening of *RBFOX1* deletions captured all deletions affecting the genomic sequence of the *RBFOX1* gene (chr16:6,069,131-7,763,339, human genome build 37/hg19). The *RBFOX1* gene is located in the chromosomal region 16p13.3 and consists of 21 exons (NM_018723.3), which form six mRNA transcripts encoding five known protein isoforms (Fig. 1; *RBFOX1* exon annotation adapted from Fogel et al., 2012). Notably the 5′-terminal exons 1, 2, 3, 1B and part of exon 4 are untranslated (Fig. 1). All potential *RBFOX1* microdeletions were manually inspected for the regional SNP heterozygosity state and log2 ratios of the signal intensities to exclude technical artifacts. Subsequently, the copy number state of all *RBFOX1* microdeletions identified by the array-based CNV analysis was examined by real-time quantitative PCR (qPCR), using seven TaqMan CNV assays covering the 5′-terminal *RBFOX1* exons 1-4 (Life Technologies, Carlsbad, CA, U.S.A.; Fig. S1).

Statistical analysis

Case–control association analysis was carried out using a two-sided Fisher's exact test.

Results

Detection of *RBFOX1* deletions in patients with IGE and controls

Microdeletions (size >40 kb, coverage >20 markers) affecting the genomic sequence of the *RBFOX1* gene were found in 8 (0.6%) of 1,408 individuals with IGE, whereas two deletions were observed in 2,256 controls ($p = 0.017$, Fisher's exact test; odds ratio [OR] 6.4, 95% confidence interval [CI] 1.2–62.35; Figs 1 and S1). The size of the deletions ranged from 41 to 896 kb. All 10 *RBFOX1* deletions were located in the 5′-terminal *RBFOX1* region encompassing the untranslated exons 1–4. Remarkably, the two *RBFOX1* deletions observed in the controls were both located in intronic sequences and were smaller (41 and 56 kb) than the deletions observed in the IGE patients (68–896 kb; Fig. S1). Specifically exon-disrupting *RBFOX1* deletions were present in 5 (0.35%) of 1,408 individuals with IGE (Table 1) and none was detected in 2,256 controls ($p = 0.008$, Fisher's exact test; Figs 1 and S2). The hemizygous copy number state of all *RBFOX1* deletions detected by the array-based CNV scan could be confirmed in the IGE patients by TaqMan qPCR assays. DNA samples of the control subjects were not available for qPCR validation.

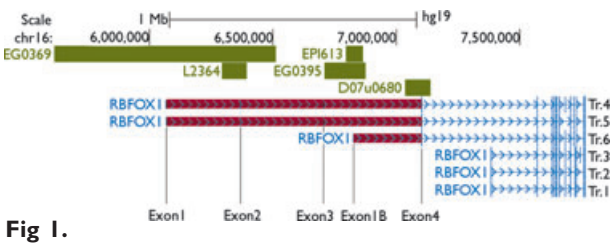

Fig 1.
Exon-disrupting *RBFOX1* deletions. Overview of exon-disrupting *RBFOX1* deletions, genomic localization, and overview of the transcript variants (hg19). The largest transcript variants 4–5 (NM_018723.3, NM_001142333.1) cover almost the entire 1.7 Mb of the gene and encode isoforms 4 and 5. The third largest transcript variant 6 also encodes isoform 4 (NM_001142334.1), whereas transcript variants 1, 2, and 3 cover only approximately 380 kb of the 3′-region of the gene. Green bars represent microdeletion size and location for each individual IGE patient. The red bars indicate the untranslated 5′-terminal *RBFOX1* exons. Tr., Transcript variant ID. The exon annotation refers to the genomic organization of *RBFOX1* as shown in Fogel et al. (2012).
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Table 1. *RBFOX1* exon-disrupting deletions in IGE index patients

Family	Index patient	Deletion size (kb)	Breakpoints at chr16 (Mb)	Diagnosis/age-at-onset/seizure types	Familial comorbidity
1	EG0369	896	5.616–6.512	CAE:3/abs,7/GTCS	Developmental delay, LD, sudden death
2	EPI613	68	6.797–6.865	JAE:1/FS,15/abs,15/GTCS	No neuropsychiatric disorders
3	D07u0680	103	7.035–7.138	JME:16/myo,16/GTCS	ASD, LD, myopia
4	EG0395	165	6.709–6.874	CAE:3/abs, 3/GTCS	No neuropsychiatric disorders
–	L2364	100	6.294–6.394	JME:14/myo,14/GTCS	No neuropsychiatric disorders

Diagnosis: ASD, autism spectrum disorder; CAE, childhood absence epilepsy; EGTCS, epilepsy with generalized tonic–clonic seizures alone; FS, febrile seizure; JME, juvenile myoclonic epilepsy; IGE, idiopathic generalized epilepsy; LD, learning disability; Seizure types: myo, myoclonic seizure; abs, absence seizure.
Survey on *RBFOX1* exon-disrupting deletions in index patients with idiopathic generalized epilepsies.
Values indicate the age-of-onset in years.

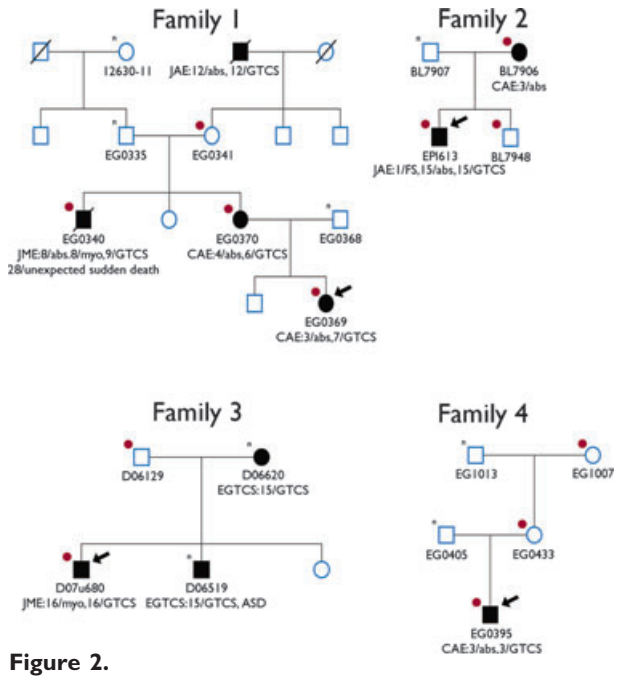

Figure 2.
Familial segregation of the exonic *RBFOX1* deletions. Familial segregation of exon-disrupting *RBFOX1* microdeletions. Deletion carriers of *RBFOX1* are marked with a red dot, "n" indicates an analyzed sample without deletion. Crossed individuals, deceased; black symbols, individuals affected by IGE; CAE, childhood absence epilepsy; JME, juvenile myoclonic epilepsy; EGTCS, epilepsy with generalized tonic–clonic seizures alone; FS, febrile seizures; myo, myoclonic seizures; abs, absence seizures; ASD, autism spectrum disorder.

Familial segregation and comorbidity analysis of the exonic *RBFOX1* deletions

The segregation of exonic *RBFOX1* deletions identified in the IGE index cases was tracked in four families (Fig. 2). The copy number status of the *RBFOX1* was assessed in 20 available family members using TaqMan qPCR assays (Table S1). In total, 12 family members carried an exon-disrupting *RBFOX1* deletion (6 females, 6 males). All *RBFOX1* deletions identified in the IGE index patients were inherited. Overall, the deletions were transmitted five times maternally and one time paternally. Seven of 12 deletion carriers were affected by IGE, and five carriers were clinically unaffected. Seven of nine IGE patients investigated carried an exonic *RBFOX1* deletion. In families 1 and 2, exonic *RBFOX1* deletions were detected in all investigated family members with IGE. In contrast, the *RBFOX1* deletion identified in the IGE index patient did not cosegregate in family 3. The phenotypic features of the IGE syndromes of the seven affected exonic deletion carriers did not differ from those IGE patients lacking a *RBFOX1* deletion. Notably, six of seven IGE patients with *RBFOX1* exon-disrupting deletions exhibited typical absence seizures. Comorbidity with other neuropsychiatric disorders was observed in families 1 and 3. In family 1, the index patient EG0369 was affected by a classical CAE but also exhibited neurodevelopmental problems with delayed speech and attention and memory problems resulting in a learning disability that required special education. Learning disability also occurred in the IGE-affected siblings of family 3 (D07u680 and D06519). However, only D07u680 carried a *RBFOX1* deletion, whereas the *RBFOX1* deletion was missing in sibling D06519, who was affected by IGE since the age of 15 years but also had pervasive developmental disorder, which is part of the diagnostic group of autism spectrum disorders. Moreover, vision impairment due to a strong myopia was present in the IGE-affected mother and all three siblings in family 3. Comorbidity with neuropsychiatric disorders was not reported in families 2 and 4. Magnetic resonance imaging scans of three IGE patients carrying an exonic *RBFOX1* deletion (family 1, EG0340; family 2, EPI613; family 4, EG0395) did not reveal any structural abnormalities of the brain, other than for bifrontal lesions in patient EG0340 due to a traumatic brain contusion occurring 16 years after the onset of the IGE.

Discussion

The present candidate gene CNV study revealed a significant excess of intronic and exonic deletions affecting the neuron-specific *RBFOX1* gene in patients with IGE compared with population controls. Specifically, we found *RBFOX1* exon-disrupting deletions in 5 (0.35%) of 1,408 IGE patients, whereas none was detected in 2,256 controls. Considering the key role of the splicing regulator *RBFOX1* in the control of neuronal excitation and seizure susceptibility (Gehman et al., 2011), the present findings suggest that rare microdeletions affecting the *RBFOX1* gene increase the risk of common IGE syndromes.

The four exonic *RBFOX1* deletions tested for familial segregation were all inherited. They differed considerably in size, ranging from 68 to 896 kb, and were all located in the 5′-terminal *RBFOX1* region encompassing the untranslated exons 1–4 (Fig. S1). The *RBFOX1* 5′-terminal exons represent highly conserved genomic sequences (Fig. S3) and are predominantly expressed in brain, suggesting that the 5′-terminal *RBFOX1* region contains important regulatory elements (Damianov & Black, 2010). Consistent with our findings, the structural genomic variations of the *RBFOX1* gene reported previously in three single patients with neurodevelopmental disorders and epilepsy also disrupted the 5′-terminal *RBFOX1* exons (Bhalla et al., 2004; Martin et al., 2007; Gallant et al., 2011). Moreover, a female with autism carrying a deletion of *RBFOX1* exon 1 due to a de novo translocation t(15p;16p) displayed a significantly reduced *RBFOX1* mRNA expression in lymphocytes (Martin et al., 2007). Accordingly, a similar reduction in *RBFOX1* mRNA expression can be expected in the

members of IGE-multiplex family 1 carrying the large 896 kb microdeletion that deletes the *RBFOX1* exons 1–2. IGE-multiplex family 1 is of particular interest because of the consistent cosegregation of the IGE trait with the *RBFOX1* deletion (Fig. 2). Notably, none of the previously identified IGE-associated microdeletions at 15q11.2, 15q13.3, and 16p13.11 (Helbig et al., 2009; de Kovel et al., 2010; Heinzen et al., 2010; for review see: Hochstenbach et al., 2011) was found in the IGE index patients carrying a *RBFOX1* deletion.

The potential functional alterations of the four smaller deletions involving the *RBFOX1* 5′-terminal exons 2–4 and exons 1B and 4 remain elusive (Figs 1 and S1). In particular, IGE-multiplex family 3 does not show a co-segregation of the IGE-trait with the 163 kb spanning deletion affecting exon 4. Similar heterogeneous cosegregation patterns, incomplete penetrance, and variable phenotypic expressivity have been observed for the recurrent 15q13.3 microdeletion, representing the strongest genetic risk factor for IGE (OR 68; 95% CI 29–181) identified so far (Dibbens et al., 2009; Helbig et al., 2009; de Kovel et al., 2010; Mefford et al., 2011; Mulley et al., 2011). Together these lines of evidence support an oligogenic/polygenic heterogeneity model for the genetic architecture of the majority of common IGE syndromes and other common neurodevelopmental disorders. Accordingly, the effect of each genetic risk factor alone is not sufficient to express IGE phenotypes, but the interactive effects of heterogeneous sets of rare and low frequency susceptibility factors together promote ictogenesis and epileptogenesis (Dibbens et al., 2007). Depending on the heterogeneous composition of genetic risk factors, the phenotypic expression of exonic *RBFOX1* microdeletions is likely to exhibit extensive phenotypic variability as observed for the large recurrent microdeletions at 1q21.1, 15q11.2, 15q13.3, 16p11.2, 16p13.11, and 22q11.2 (Coe et al., 2012). These pathogenic microdeletions seem to affect normal neurodevelopment, resulting in an impaired homeostatic regulation of neuronal networks. In combination with other genetic susceptibility factors, a set of genomic structural variations may contribute to the genetic variability and phenotypic overlap of a wide spectrum of common neuropsychiatric diseases sharing a neurodevelopmental pathogenesis (Coe et al., 2012). With regard to the pivotal role of RBFOX1 in regulating both splicing and transcriptional networks in human neurodevelopmental processes (Fogel et al., 2012), the highly variable spatiotemporal expression of the *RBFOX1* gene in differentiating human neurons (Gehman et al., 2011; Fogel et al., 2012; Lin et al., 2012) and the variable expressivity of the large number of downstream gene transcripts may also contribute to the pleiotropic effects of exon-disrupting *RBFOX1* deletions. In line with the oligogenic/polygenic heterogeneity model, we observed a familial comorbidity with other neurodevelopmental disorders, such as learning disability and autism spectrum disorder, in two families (1 and 3) with *RBFOX1* exon-disrupting deletions (Table 1, Fig. 2). Taking into account that the ascertainment scheme for subjects with IGE applied in this study leads to an exclusion of individuals with severe intellectual disability or predominant neuropsychiatric disorders, comorbidity of generalized seizures with other neurodevelopmental disorders should be more common.

In summary, the present candidate gene CNV study of the neuron-specific splicing regulator gene *RBFOX1* suggests that microdeletions affecting the untranslated 5′-terminal *RBFOX1* exons increase risk of common IGE syndromes. The present findings warrant further studies to replicate an involvement of *RBFOX1* in the genetic predisposition of IGE syndromes and other common neurodevelopmental disorders and to elucidate the pathogenic mechanisms of epileptogenesis resulting from RBFOX1-mediated alterations of the splicing process of neuronal genes.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Appendix 1

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Appendix 2

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1. Overview of all deletions affecting the *RBFOX1* gene including locations of the TaqMan qPCR assays.

Figure S2. Raw signal intensity data of all samples carrying exon-disrupting deletions affecting the *RBFOX1* gene.

Figure S3. UCSC Genome Browser *RBFOX1* regulatory annotation tracks.

Table S1. Epilepsy phenotype of individuals examined by qPCR and overview of all *RBFOX1* deletions validated by TaqMan qPCR assays.