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FULL-LENGTH ORIGINAL RESEARCH

STXBP1 mutations in early infantile epileptic encephalopathy with suppression-burst pattern

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Summary

Purpose: De novo *STXBP1* mutations have been found in individuals with early infantile epileptic encephalopathy with suppression-burst pattern (EIEE). Our aim was to delineate the clinical spectrum of subjects with *STXBP1* mutations, and to examine their biologic aspects.

Methods: *STXBP1* was analyzed in 29 and 54 cases of cryptogenic EIEE and West syndrome, respectively, as a second cohort. RNA splicing was analyzed in lymphoblastoid cells from a subject harboring a c.663 + 5G>A mutation. Expression of STXBP1 protein with missense mutations was examined in neuroblastoma2A cells.

Results: A total of seven novel *STXBP1* mutations were found in nine EIEE cases, but not in West syndrome. The mutations include two frameshift mutations, three nonsense mutations, a splicing mutation, and a recurrent missense mutation in three unrelated cases. Including our previous data, 10 of 14 individuals (71%) with *STXBP1* aberrations had the onset of spasms after 1 month, suggesting relatively later onset of epileptic spasms. Nonsense-mediated mRNA decay associated with abnormal splicing was demonstrated. Transient expression revealed that STXBP1 proteins with missense mutations resulted in degradation in neuroblastoma2A cells.

Discussion: Collectively, *STXBP1* aberrations can account for about one-third individuals with EIEE (14 of 43). These genetic and biologic data clearly showed that haploinsufficiency of *STXBP1* is the important cause for cryptogenic EIEE.

KEY WORDS: *STXBP1*, EIEE, West syndrome, Haploinsufficiency.

Early infantile epileptic encephalopathy with suppression-burst (EIEE), also known as Ohtahara syndrome (Ohtahara et al., 1976), is characterized by early onset of tonic seizures, seizure intractability, characteristic suppression-burst patterns on electroencephalography (EEG), and poor outcome with severe psychomotor retardation (Djukic et al., 2006; Ohtahara & Yamatogi, 2006). We recently found de novo mutations in *STXBP1* (encoding syntaxin binding protein 1, also known as MUNC18-1) in individuals with EIEE (Saitsu et al., 2008). The subjects with *STXBP1* aberrations, including four missense mutations and a 2-Mb microdeletion encompassing *STXBP1*, showed the characteristic feature of EIEE. A mutant protein with a missense change (p.C180Y) showed structural instability with significant thermolability and impaired binding to syntaxin-1A compared with the wild-type (Saitsu et al., 2008). These findings suggest that haploinsufficiency of *STXBP1* causes EIEE.

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West syndrome is one of the most common infantile epileptic syndromes and is characterized by epileptic spasms, arrest of psychomotor development, and hypsarrhythmia on EEG (Kato, 2006). Brain malformations and metabolic disorders were found as underlying causes of symptomatic West syndrome, but many cryptogenic cases remain etiologically unexplained (Kato, 2006). Two causative genes, *ARX* (aristaless related homeobox) and *CDKL5* (cyclin-dependent kinase-like 5), are mutated only in a subset of familial and sporadic cases of West syndrome (Stromme et al., 2002; Kalscheuer et al., 2003; Kato et al., 2003; Weaving et al., 2004; Guerrini et al., 2007; Bahi-Buisson et al., 2008). EIEE and West syndrome are considered as a continuum of epileptic encephalopathies because the majority (75%) of EIEE transit to West syndrome (Yamatogi & Ohtahara, 2002; Ohtahara & Yamatogi, 2006). Specific mutations of *ARX* have been also found in EIEE (Kato et al., 2007), further suggesting a common pathologic mechanism among two syndromes. *STXBP1* mutations would be possibly involved in West syndrome. However, it remains to be determined.

To delineate the clinical spectrum of *STXBP1* mutations, a second cohort consisting of EIEE and West syndrome cases was investigated. Novel *STXBP1* mutations have been found only in EIEE cases. We also characterized biologic aspects of *STXBP1* mutations by using lymphoblastoid cells derived from a patient, and by transient expression of mutant STXBP1 proteins in neuroblastoma2A cells.

Subjects and Methods

Subjects

A total of 29 and 54 Japanese individuals with EIEE and West syndrome, respectively, were newly recruited as a second cohort. Brain malformations were not found in all cases. The diagnosis was made on the basis of clinical features and characteristic patterns on EEG. Experimental protocols were approved by Institutional Review Boards for Ethical Issues at Yokohama City University School of Medicine and Yamagata University Faculty of Medicine. Informed consent was obtained from all individuals and/or their families in agreement with the requirements of Japanese regulations. Clinical aspects of subjects with *STXBP1* mutations are summarized in Table 1.

Mutation screening

Genomic DNA was obtained from peripheral blood leukocytes according to standard protocols. Mutation screening of 1st to 20th exons covering the coding region of *STXBP1* was performed by high-resolution melt analysis (HRM). Realtime polymerase chain reaction (PCR) and HRM were serially performed in a 12-μl mixture on RotorGene-6200 HRM (Corbett Life Science, Brisbane, Qld, Australia). For the 2nd to 20th exons, PCR mixture contained 1× ExTaq buffer, 0.2 mM each dNTP, 0.25 μM each primer, 1.5 μM SYTO9 (Invitrogen, Carlsbad, CA, U.S.A.), and 0.375 U Ex TaqHS polymerase (Takara Bio, Ohtsu, Japan). For the first exon, the PCR mixture contains 1× PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, 1.5 μM SYTO9, and 0.3 U KOD FX polymerase (Toyobo, Osaka, Japan). PCR primers and conditions are shown in Table S1. PCR samples showing an aberrant melting curve pattern were sequenced as previously described (Saitsu et al., 2008). For all the families showing de novo mutations, parentage was confirmed by microsatellite analysis as described previously (Saitsu et al., 2008).

RNA analysis

Lymphoblastoid cells (LCL) derived from a subject harboring a c.663 + 5G>A mutation was grown in Roswell Park Memorial Institute 1,640 medium supplemented with 10% fetal bovine serum (FBS), 1× Antibiotic-Antimycotic (Invitrogen), and 8 μg/ml tylosin (Sigma, Tokyo, Japan) at 37°C in a 5% CO_2 incubator. After incubation with dimethyl sulfoxide (DMSO) (as vehicle control) or 30 μM cycloheximide (Sigma) for 4 h, total RNA was extracted using TRizol (Invitrogen). One μg total RNA was subjected to reverse transcription using PrimeScript 1st strand synthesis kit with random hexamers (Takara). Minus reverse transcriptase (RT) control with no reverse transcriptase was included in each experiment. PCR was performed in a 15-μl mixture, containing 1 μl cDNA, 1× PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, and 0.3 U KOD FX polymerase (Toyobo). Primer sequences are 5′-CTTTGTGCCACCCTGAAGGAGTACC-3′ in ex7-F and 5′-CAGTGCTATCCACAGGTCGTCGTC-3′ in ex10-R. The control cDNA isolated from a normal LCL sample was used as a reference. PCR products electrophoresed in 2% agarose gel were stained with ethidium bromide, and were analyzed by quantitative densitometry on FluorChem 8,900 (Alpha Innotech, San Leandro, CA, U.S.A.). Experiments were repeated three times. Inhibition of nonsense-mediated mRNA decay (NMD) was estimated according to the density ratios of upper normal and lower aberrant bands with/without 30 μM cyclophosphamide treatment in the culture of the patient's LCL. Statistical analyses were done using the unpaired Student's *t*-test (two-tailed). Each PCR band was sequenced using purified DNA by QIAEXII Gel extraction kit (Qiagen, Tokyo, Japan).

Expression vectors

A fragment containing internal ribosomal entry signal (IRES) and nuclear-localized Flag-DsRed was inserted into pEGFP-C1 vector (Clontech, Mountain View, CA, U.S.A.). Human *STXBP1* cDNA was fused to this vector, achieving dual expression of both N terminal EGFP-tagged STXBP1 and nuclear-localized Flag-DsRed. A wild-type *STXBP1* cDNA, four mutants (c.251T>A, p.V84D; c.539G>A, p.C180Y; c.1328T>G, p.M443R; c.1631G>A, p.G544D) and two normal variants (c.250G>A, p.V84I; c.1292A>T,

Table 1. Summary of clinical features of subjects with *STXBP1* mutations

Subject (age)	Sex	Mutation	Dx	Age at onset	Initial symptoms	Initial EEG	Age at onset of spasms	Age at onset of SB pattern	Age at transit to WS	Transition from spasms to other seizures	Transition to other EEG findings	Response to therapy	Development	Neurologic examination	Magnetic resonance imaging
1,751 (3 y)	M	c.1217G>A p.R406H de novo	EIEE	0 d	Bilateral convulsion	SB	3 w	1 m	5 m	No	Multifocal spike and slow wave complex	Intractable, hourly	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 1 m, Mild brain atrophy at 3 y
1,989 (15 m)	M	c.1217G>A p.R406H *de novo*	EIEE	43 d	GTCS with upward eye gazing	SB	2 m	1 m	2 m	~48 d myoclonic seizure	No	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 11 m
2,123 (20 m)	M	c.1217G>A p.R406H *de novo*	EIEE	15 d	Partial seizures (right hemiconvulsion)	Focal spike at P3	2 m	2 m	No	Tonic seizure to myoclonic seizure	Unknown	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 1 m, Mild frontal brain atrophy at 20 m
1,792 (6 y)	F	c.157G>T p.E53X *de novo*	EIEE	2 d	Spasms	SB	2 d	1 w	6 m	Versive seizure after hypoxia at 2 y	Multifocal irregular spikes	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 0 m, Mild ventricular dilatation at 14 m
1,694 (17 m)	M	c.388_389delCT p.L130DfsX11 *de novo*	EIEE	2 m	Secondary generalized seizures initiated from the right face	SB with fluctuated baseline	2 m	2 m	3 m	CPS	Multifocal spike and slow wave complex with desynchronization	Seizure-free after ACTH or VPA with KBr	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 3 m
1,951 (6 m)	F	c.663 + 5G>A *de novo*	EIEE	5 d	Blinking to tonic seizures	SB with fluctuated baseline	1 m	1 m	3 m	Tonic seizure	Left temporal spike and slow wave complex	Seizure free with VB6 for spasms and ACTH for WS	Eye pursuit and smiling from 4 m, Head control and rolling over from 6 m	Moderate MR, Quadriplegia	Normal at 0 m, subdural effusion at 2 m
1,655 (6 m)	M	c.703C>T p.R235X *de novo*	EIEE	1 m	Spasms in cluster	SB	1 m	1 m	No	No	Occipital spikes	Seizure free from 6 m after high-dose PB	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Delayed myelination and brain atrophy
2,103 (11 m)	F	c.747dupT p.Q250SfsX6 *de novo*	EIEE	3 d	Clonic convulsion	Focal spike at C3, Cz, Fz	31 d	1 m	10 m	Partial seizure (abnormal eye movement) and myoclonic seizures	Multifocal spikes	Intractable, hourly	No head control, Smiling from 5 m	Profound MR, Severe spastic quadriplegia	Normal at 1 m, left mild brain atrophy at 3 m
1,979 (10 y)	M	c.961A>T p.K321X *de novo*	EIEE	2 w	Partial seizures	SB	3 w	1 m	3 m	Partial seizure	Multifocal spikes with asymmetric background activity	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 0 m, Brain atrophy and subdural hematoma at 7 m after ACTH

Dx, diagnosis; GTCS, generalized tonic–clonic seizures; SB, suppression-burst; WS, West syndrome; CPS, complex partial seizure; ACTH, adrenocorticotropic hormone; VPA, valproic acid; KBr, potassium bromide; VB6, vitamin B_6; PB, phenobarbital; MR, mental retardation; d, day(s); w, week; m, month(s); y, year(s); 0 w, 0–6 days; 0 m, 0–3 week.

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p.Q431L) were generated as described previously (Saitsu et al., 2008). c.250G>A was registered as single nucleotide polymorphism (SNP) (rs34830702). c.1292A>T was observed in one of 250 normal controls (allele frequency: 1/500), but not in EIEE or West syndrome.

Cell culture, transfection, and immunoblotting

Mouse neuroblastoma 2A (N2A) cells were grown as described previously (Saitsu et al., 2008). For transient expression experiments, N2A cells on glass cover slips (in 24-well plates for microscopic detection) and 3.5-cm culture dish (for immunoblotting) were transfected with 200 and 800 ng of plasmid DNA using FuGene6 reagent (Roche diagnostics, Tokyo, Japan), respectively. After 3 h, culture medium was changed to low serum medium (5% FBS) with 20 μM all transretinoic acid (Sigma) in order to induce neural differentiation, and cells were subsequently cultured for 2 days. For microscopic detection, N2A cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS for 15 min. Cover slips were mounted using Vectashield with DAPI (Vector Laboratories, Youngstown, OH, U.S.A.) and images were visualized with an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Tokyo, Japan), and captured using Axio Vision 4.5 software (Carl Zeiss). The exposure time for enhanced green fluorescent protein (EGFP) and DsRed capture was fixed in a series of experiments to enable direct comparison between different experimental samples. For immunoblotting, N2A cells were washed twice in ice-cold PBS, and lysed in sodium dodecyl sulfate (SDS) sample buffer. Samples were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the polyvinylidene difluoride (PVDF) membrane, and analyzed with anti-Munc18 (for STXBP1 detection, 1:5,000 dilution) (BD Transduction Laboratories, Tokyo, Japan) or anti-Flag M2 (1:2,000 dilution) (Sigma) antibody. Secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Blots were detected using the Supersignal West dura (Pierce, Yokohama, Japan). Chemiluminescence was evaluated by quantitative densitometry using a FluorChem 8,900 (Alpha Innotech). Experiments were repeated three times.

Results

A total of seven novel heterozygous mutations found in six males and three females are presented together with four reported missense mutations in Fig. 1 (Saitsu et al., 2008). The recurrent p.R406H mutation occurred at evolutionary conserved amino acid (Fig. 1). All the mutations are novel and occurred de novo. Parentage was confirmed using several microsatellite markers (data not shown). All the mutations were found only in EIEE cases, but not in West syndrome.

Clinical features of *STXBP1* aberrations

Detailed clinical information of individuals with *STXBP1* mutations is summarized in Table 1. All nine individuals were born at term without asphyxia except for the subject 1,751 who had the short umbilical cord. Their body weight, height, and head circumference were normal at birth other than the subject 1,792 with mild microcephaly [30.5 cm, −2.0 standard deviation (SD)]. Epileptic spasms were preceded by other seizure types including partial seizures in seven subjects (other than 1,792 and 1,655), and initial EEG in two subjects showed focal epileptic discharges (2,123 and 2,103). Only one subject demonstrated suppression-burst pattern on EEG (Fig. 2A) in the neonatal period. Transition to West syndrome was observed in seven subjects with EIEE. Although seizures were intractable in six subjects, three subjects responded to medication, such as adrenocorticotropic hormone (ACTH) injection, vitamin B_6, high-dose phenobarbital, and valproic acid. All subjects demonstrated severe psychomotor developmental delay, and only two subjects had the social smile. Most subjects presented with normal brain at the first magnetic resonance imaging (MRI), and then showed mild brain atrophy, which might be influenced by ACTH injection, after 1 year (Fig. 2B).

Abnormal splicing and nonsense-mediated mRNA decay

To observe mutational effects of c.663 + 5G>A (intron 8), reverse transcriptase PCR was performed using total RNA extracted from LCL derived from the subject 1,951. PCR primers were designed to amplify exons 7 to 10 (Fig. 3A). Only one band (338 bp), corresponding to the wild-type *STXBP1* allele, was amplified using a cDNA template from a control LCL (Fig. 3B). In contrast, a smaller band was detected from the subject's cDNA (Fig. 3B). Direct sequencing of both fragments revealed that exon 8 was skipped in the abnormal band (Fig. 3B), resulting in the insertion of nine new amino acids followed by a premature stop codon at position 203. As intensity of the smaller band was significantly weak (Fig. 3B), NMD may be involved (Maquat et al., 1981; Shyu et al., 2008). Intensity ratio of mutant versus normal band was 29% in untreated condition. The ratio was raised up to 67% after 30-μM cycloheximide treatment preventing NMD (Fig. 3C), suggesting that the early truncated mutant mRNA underwent degradation by NMD.

Degradation of mutant STXBP1 proteins

We previously demonstrated that intense fluorescence signals in clusters likely representing protein aggregation were observed in approximately 20% of N2A cells transiently expressing mutant EGFP-STXBP1 (p.V84D, p.C180Y, p.M443R, and p.G544D), suggesting structural instability of STXBP1 proteins with missense mutations (Saitsu et al., 2008). The other 80% of cells showed diffuse

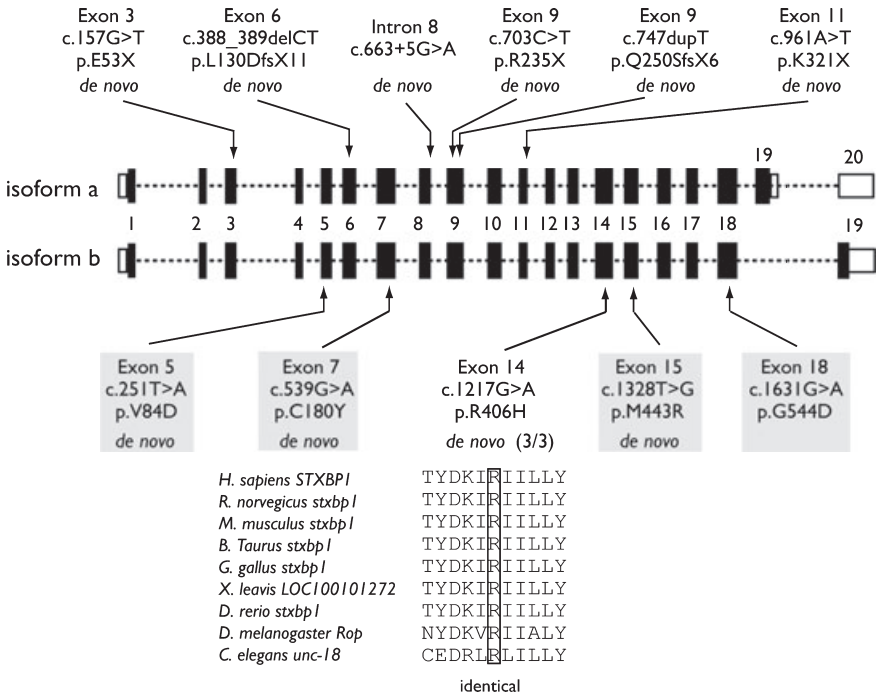

Figure 1.
Summary of *STXBP1* mutations found in Japanese individuals with EIEE. Schematic representation of *STXBP1* consisting of at most 20 exons (untranslated region and coding region are open and filled rectangles, respectively). There are two isoforms, a (GenBank accession number, NM_003165) with exon 19, and b (NM_001032221) without exon 19 of isoform a. The location of mutations is indicated by arrows. A total of 11 different mutations were presented: missense mutations below the gene scheme and the other types of mutations above the gene. Four mutations previously reported are in shadow (Saitsu et al., 2008), and seven without shadow are newly found in this study. All the seven novel mutations occurred de novo. The recurrent p.R406H missense mutation found in three unrelated subjects occurred at evolutionary conserved amino acids. CLUSTALW website (http://align.genome.jp/) was used to align homologs of different species.

cytosolic protein distribution similar to that expressing the wild-type, but the signal intensity was much weaker, implying possible protein degradation. To observe mutant protein degradation, a dual-expression vector of EGFP-STXBP1 and nuclear Flag-DsRed (EGFP-STXBP1-IRES-nuclear Flag-DsRed) was generated. Two days after transfection, the wild-type EGFP-STXBP1 was expressed in cytosol, but not in nucleus or plasma membrane as described previously (Saitsu et al., 2008). Notably, in mutant EGFP-STXBP1 transfected cells (p.V84D, p.C180Y, p.M443R, and p.G544D), EGFP signals were almost absent, whereas nuclear DsRed was expressed at comparable levels to that of wild-type (Fig. 4A). Two normal variants, p.V84I and p.Q431L, were expressed in a manner similar to that of the wild-type, with less intensity for a p.Q431L variant (Fig. 4A). Decreased level of mutant STXBP1 expression was confirmed by immunoblotting using Munc18 antibody (Fig. 4B, top). Transfection efficacy or amount of protein loading was similar in all cases based on the level of Flag-DsRed (Fig. 4B, bottom).

Discussion

We could successfully find a total of seven novel mutations: two frameshift mutations (2-bp deletion and 1-bp insertion), three nonsense mutations, a splicing mutation, and a recurrent missense mutation. Transcripts associated with frameshift, nonsense, and splicing mutations are likely to be degraded by NMD. In the case harboring c.663 + 5G>A, aberrant splicing associated with NMD was demonstrated in LCL derived from the subject. Moreover, mutated STXBP1 proteins underwent degradation in N2A cells. Variable expression of mutated STXBP1 proteins has been also reported in HeLa cells (Ciufo et al., 2005), suggesting that degradation mechanism of mutated STXBP1 proteins may be common in mammalian cells. Considering these genetic and biologic data presented here as well as a complete deletion of *STXBP1* in one EIEE case (Saitsu et al., 2008), haploinsufficiency of *STXBP1* consistently results in EIEE.

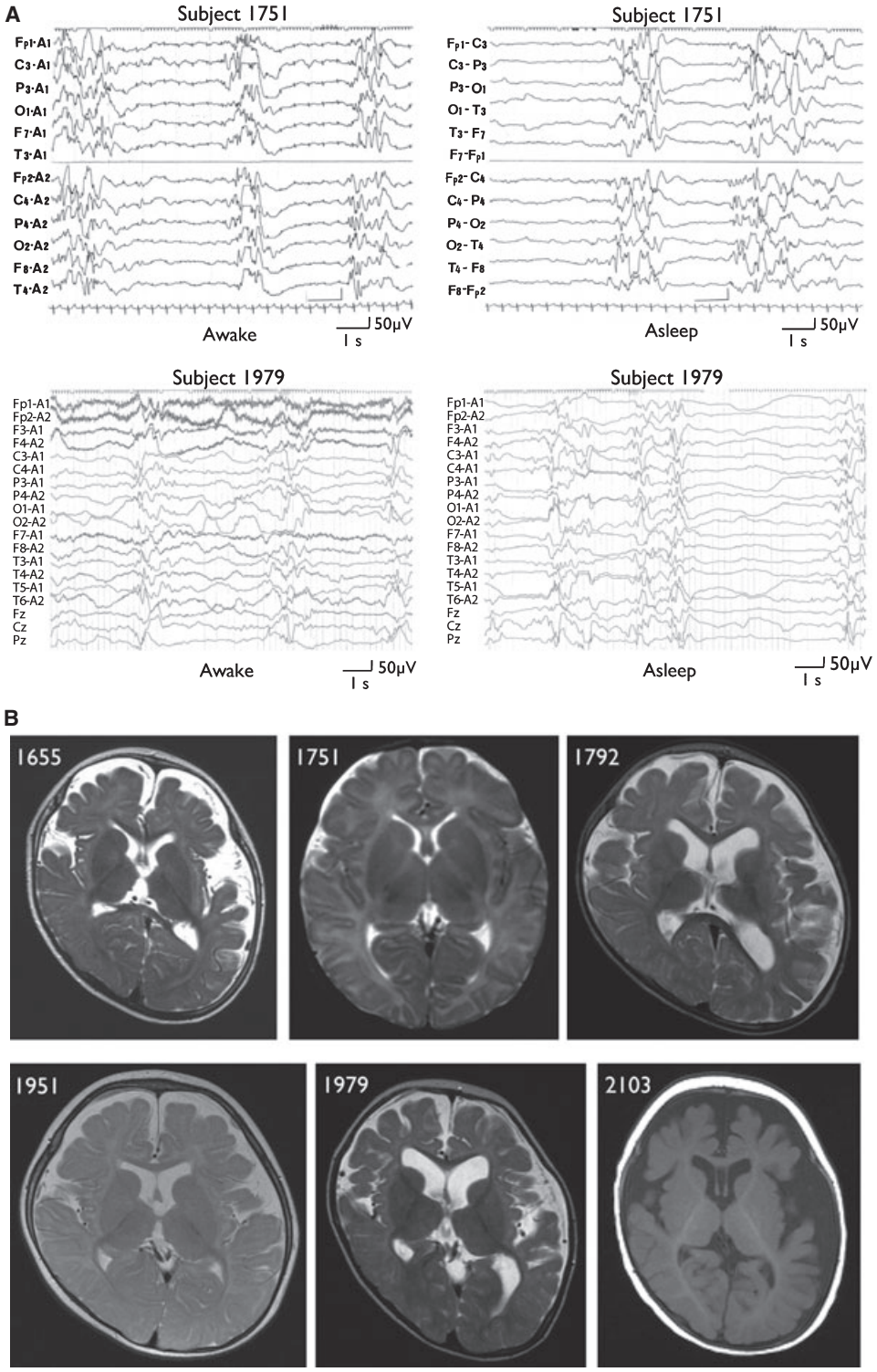

Figure 2.
EEG and brain MRI of subjects with *STXBP1* mutations. (**A**) EEG of subjects 1,751 and 1,979 at 1 month of age. Suppression-burst pattern characterized by diffuse bursts of irregular spikes and slow waves lasting 1 to 3 s with low-amplitude background lasting 2–5 s are seen during both sleep and wake. (**B**) T_2-weighted brain MRI scan of subjects 1,655 at 9 months of age, 1,751 at 1 month, 1,792 at 15 months, 1,951 at 1 month, and 1,979 at 15 months, and T_1-weighted MRI scan of subject 2,103 at 5 months. Mild dilation of lateral ventricles is seen in patients with 1,792, 1,951, 1,979, and 2,103, but none shows brain malformation.

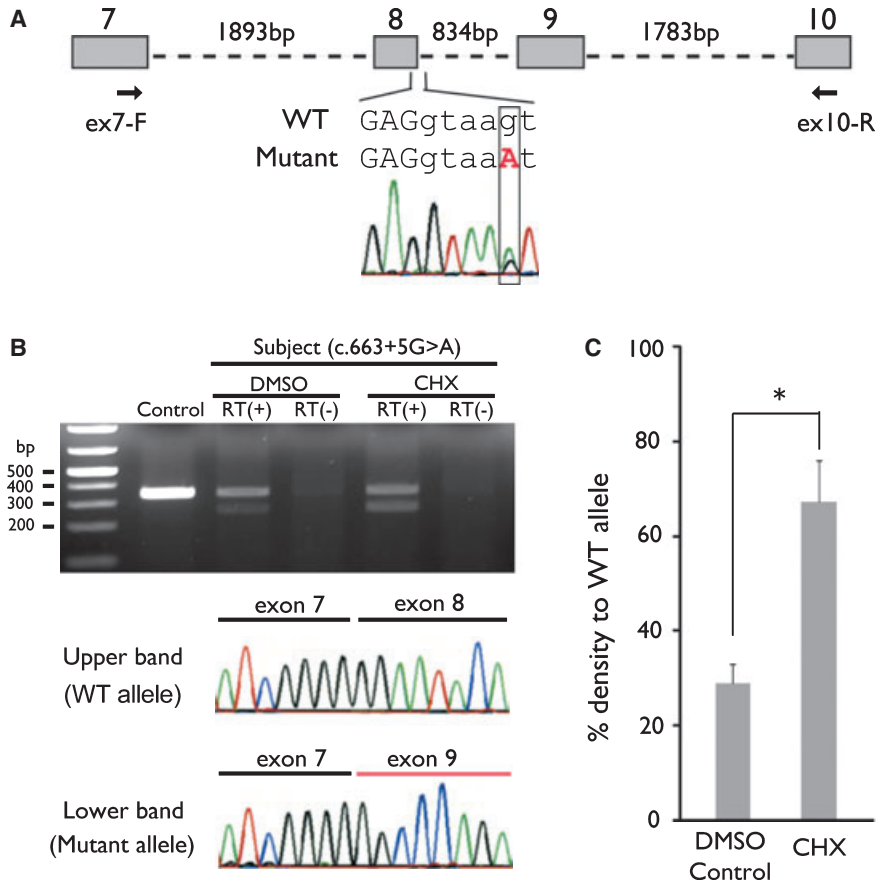

Figure 3.
The c.663 + 5G>A mutation causing abnormal splicing associated with NMD. (**A**) Schematic representation of the genomic structure from exons 7–10 of *STXBP1*. Exons, introns, and primers are shown by boxes, dashed lines, and arrows, respectively. The mutation in intron 8 was colored in red. Sequences of exon and intron are presented in upper and lower cases, respectively. (**B**) RT-PCR analysis of subject 1,951 with c.663 + 5G>A and a normal control. Two PCR products were detected from the subject's cDNA: upper was the wild-type (WT) transcript and lower was the mutant. Only a single WT amplicon was detected in a control. The mutant amplicon was significantly increased by 30 μM cycloheximide (CHX) treatment compared to DMSO treatment as a vehicle control. RT (+): with reverse transcriptase, RT (−): without reverse transcriptase as a negative control. Sequence of WT and mutant amplicons clearly showed exon 8 skipping in the mutant allele. (**C**) Quantitative analysis of the NMD inhibition by CHX based on the data shown in (**B**). *p = 0.0023 by unpaired Student's *t*-test, two tailed. Averages of three repeated experiments are shown with error bars (SD).

The subjects with *STXBP1* mutations in this study showed distinctive features of EIEE, such as early onset seizures, typically frequent epileptic spasms, suppression-burst pattern on EEG, transition to West syndrome after a few to several months, and severe developmental delay, as described in our previous report (Saitsu et al., 2008). Taken together, 14 individuals with EIEE have been found to be associated with *STXBP1* aberrations. A detailed analysis of clinical features revealed that the age at onset of epileptic spasms is rather later in subjects with *STXBP1* aberrations compared to the 16 original subjects reported by Yamatogi and Ohtahara (2002). Only 29% of the subjects (4 of 14) in our series had the onset of spasms within 1 month in contrast to 75% (12 of 16) in the series of Yamatogi and Ohtahara (2002). Although the subjects with *STXBP1* aberrations should be diagnosed as EIEE, the late onset of spasms would suggest that *STXBP1* aberrations could cause an intermediate epileptic encephalopathy between EIEE and West syndrome. The presence of two subjects showing suppression-burst pattern with fluctuated baseline, which could be regarded as intermediate pattern between suppression-burst and hypsarrhythmia, may support this idea.

Another interesting feature is the presence of myoclonic seizures, which are thought to be rather rarely observed in EIEE cases, in three subjects with *STXBP1* mutations (1,989, 2,123, and 2,103). Myoclonic seizures are the main ictal symptom of early myoclonic encephalopathy (EME), which is another epileptic syndrome showing suppression-burst patterns on EEG (Engel, 2006). The prevailing initial seizure type is a main difference between EIEE and EME:

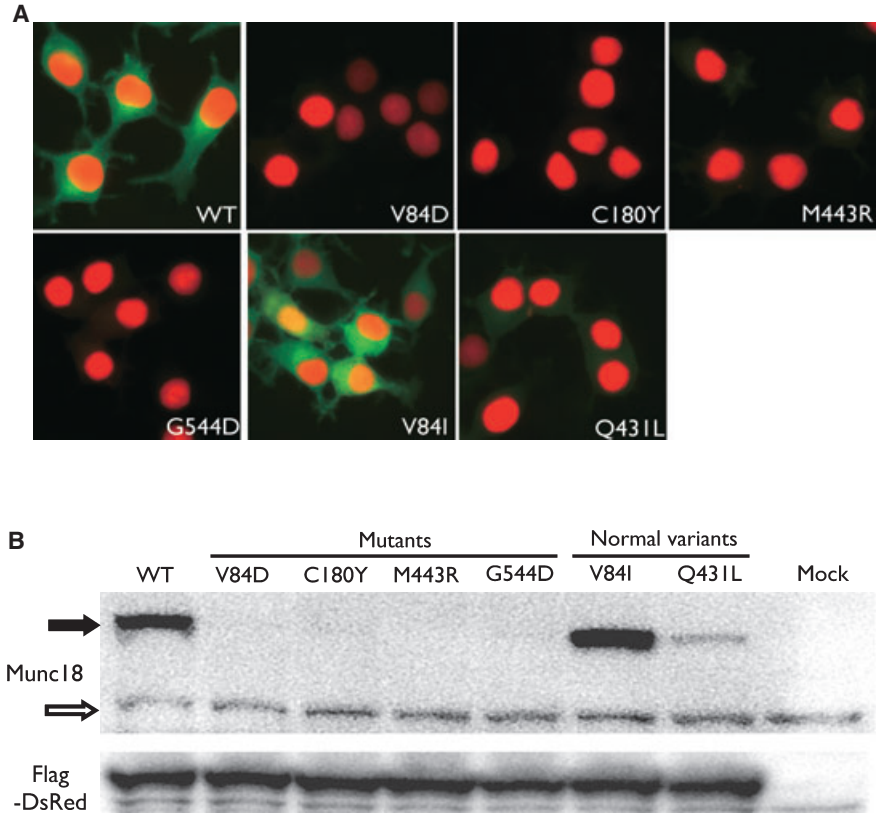

Figure 4.
Degradation of mutant EGFP-STXBP1 proteins in N2A cells. (**A**) The wild-type (WT) EGFP-STXBP1 was expressed in cytosol, but not in nucleus or in plasma membrane. In contrast, cells transfected with mutant EGFP-STXBP1 (p.V84D, p.C180Y, p.M443R, and p.G544D) showed almost absent EGFP signals, whereas nuclear DsRed was expressed at levels comparable to that of WT. The p.V84I variant registered as SNP (rs34830702) was expressed similarly to the WT. The p.Q431L variant found in a normal control was expressed in a similar pattern but weakly compared to the WT. Exposure time for EGFP and DsRed capture was fixed, enabling direct comparison between different samples. (**B**) Immunoblot analysis of mutant STXBP1 proteins by using a monoclonal Munc18 antibody (top). Upper and lower bands represent EGFP-STXBP1 and endogenous STXBP1 proteins, respectively. Expression of four mutant STXBP1 proteins was not detected, whereas WT and two normal variants could be detected. The observed differences in expression were not due to either transfection or loading differences, because the level of Flag-DsRed was similar in all cases (bottom). Mock, no transfection.

tonic seizures in EIEE and myoclonic seizures in EME. However, EIEE and EME have common features, and it is often difficult to distinguish between them. These three subjects can be diagnosed as EME when myoclonic seizures dominate. Therefore, it is possible that *STXBP1* could be also causative for EME. In terms of genotype–phenotype relationship, we found no difference in clinical data between seven subjects with missense mutations and seven subjects with microdeletion, premature termination codon, or splicing mutations. This finding is supported by our experimental data that both missense mutations and a splicing mutation resulted in haploinsufficiency of *STXBP1*: degradation of STXBP1 proteins with missense mutations and NMD associated with an aberrant splicing.

Recently, Hamdan et al. (2009) reported that two de novo *STXBP1* mutations, p.R388X and c.169 + 1G>A, were identified in 2 of 95 individuals with mental retardation and nonsyndromic epilepsy (2%), suggesting that clinical spectrum of *STXBP1* mutations may be broader. However, it is clear that EIEE is the core phenotype of *STXBP1* aberrations in this Japanese cohort as one-third of EIEE cases harbored its mutations. It is also noteworthy that none of West syndrome cases possessed *STXBP1* mutation, suggesting that subjects with initial West syndrome is rarely caused or not caused by *STXBP1* abnormality.

How haploinsufficiency of *STXBP1* leads to infantile epileptic encephalopathy remains to be elucidated. *STXBP1* abnormalities suggest a novel story in which impaired synaptic vesicle release is involved in pathogenesis of epilepsy. Although no seizures have been reported in *Stxbp1* heterozygous knockout mice (Verhage et al., 2000), they showed impaired synaptic function due to reduced size and

replenishment rate of readily releasable vesicles (Toonen et al., 2006), suggesting that heterozygous deletion of *Stxbp1* indeed affected synaptic function in mice. It is possible that the absence of seizures in *Stxbp1* heterozygous knockout mice might be due to the different genetic background. Because *Stxbp1* mutants have been backcrossed for at least six generations to a C57BL/6 background (Toonen et al., 2006), it would be interesting to examine whether seizures would occur in other genetic background. Alternatively, effect of gene dosage alterations of *STXBP1/Stxbp1* may vary between humans and mice: Humans might be more susceptible than mice; therefore, loss of function of one allele could cause seizures only in humans but not in mice. Appropriate mice models by neatly manipulating gene dosage of *Stxbp1* may mimic human phenotype and enable detailed analysis of pathogenesis of infantile epileptic encephalopathy in relation to impaired synaptic function.

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Disclosure

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. None of the authors has any conflict of interest to disclose.

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Supporting Information

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

Table S1. PCR conditions and primer sequences.

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