Short Report

Paternal mosaicism of an \textit{STXBP1} mutation in OS


Ohtahara syndrome (OS) is one of the most severe and earliest forms of epilepsy. We have recently identified that the \textit{de novo} mutations of \textit{STXBP1} are important causes for OS. Here we report a paternal somatic mosaicism of an \textit{STXBP1} mutation. The affected daughter had onset of spasms at 1 month of age, and interictal electroencephalogram showed suppression-burst pattern, leading to the diagnosis of OS. She had a heterozygous c.902+5G>A mutation of \textit{STXBP1}, which affects donor splicing of exon 10, resulting in 138-bp insertion of intron 10 sequences in the transcript. The mutant transcript had a premature stop codon, and was degraded by nonsense-mediated mRNA decay in lymphoblastoid cells derived from the patient. High-resolution melting analysis of clinically unaffected parental DNAs suggested that the father was somatic mosaic for the mutation, which was also suggested by sequencing. Cloning of PCR products amplified with the paternal DNA samples extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutation, respectively. This is a first report of somatic mosaicism of an \textit{STXBP1} mutation, which has implications in genetic counseling of OS.

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

Ohtahara syndrome (OS), also known as early infantile epileptic encephalopathy with suppression-burst, is one of the most severe and earliest forms of epilepsy (1). It is characterized by early onset of seizures, typically frequent epileptic spasms, seizure intractability, characteristic suppression-burst patterns on electroencephalogram (EEG), and poor outcome with severe psychomotor retardation (2, 3). Brain malformations such as cerebral dysgenesis or hemimegalencephaly are often associated with OS, but cryptogenic or idiopathic OS is found in a subset of OS patients, in whom genetic aberrations might be involved (4). Mutations in \textit{ARX} gene have been found in several male patients with OS (5–8). We have recently found \textit{de novo} mutations in \textit{STXBP1} (encoding syntaxin binding protein 1, also known as MUNC18-1) in individuals with cryptogenic OS (9). A microdeletion involving \textit{STXBP1} and various kinds of point mutations including missense, frameshift, nonsense, and splicing mutations have been found in about one-third of Japanese cases with cryptogenic OS (10). We have showed that both missense mutations and
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A splicing mutation result in haploinsufficiency of \textit{STXBP1}: degradation of \textit{STXBP1} proteins with missense mutations and nonsense-mediated mRNA decay (NMD) associated with an aberrantly spliced mRNAs (10).

Here we describe a family with an affected daughter with an \textit{STXBP1} mutation and healthy parents. Parental analysis indicates that the father is somatic mosaic for the mutation. Detailed molecular analysis is presented.

\textbf{Materials and methods}

\textbf{Patient and her parents}

The 1-year-old girl is a product of unrelated healthy parents. There is no history of epilepsy in her parents. She was born at term without asphyxia after an uneventful pregnancy. Her physical and neurological findings were normal until vomiting, which was supposed to be a pre-symptomatic event of seizures, was observed at 25 days of age, and her seizures started at 37 days of age, consisting of brief tonic spasms, occasionally in cluster, followed by vomiting and subtle seizures, such as head extension, upward eye gazing, and vocalization, with increased muscle tone of her extremities for a few seconds. According to suppression-burst pattern on EEG (Fig. 1a,b), she was diagnosed as OS. Brain magnetic resonance imaging (MRI) showed normal brain structure (Fig. 1c–f). Seizures were refractory to antiepileptic drugs, such as high-dose phenobarbital, phenytoin, zonisamide, pyridoxal phosphate, valproic acid, ketogenic diet, and potassium bromide. Injection of adrenocorticotropic hormone (ACTH) was partially effective. She was hypertonic and could not control her head or smile. At 6 months of age, a mild rigospastic quadriplegia was noted. Developmental milestones were profoundly delayed.

\textbf{DNA samples}

Peripheral blood leukocytes from the patient and her parents as well as other tissues from the father were used for this study. Genomic DNA from whole blood, saliva, buccal cells, and nails were isolated using a Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan), an Oragene DNA kit (DNA Genotek, Ottawa, Canada), an ISOHAIR kit (Nippon Gene, Toyama, Japan), and a Gentra Puregene Buccal Cell Kit (Gentra, Minneapolis, MN), respectively. 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 the patient’s parents in agreement with the requirements of Japanese regulations.

\textbf{Mutation analysis and TA cloning}

Mutation screening of \textit{STXBP1} by high-resolution melting (HRM) analysis using RotorGene-6200 HRM (Corbett Life Science, Brisbane, Australia) was performed as previously described (10). Parentage was confirmed by microsatellite analysis (9). For measurement of the ratio of wild-type and mutant alleles, PCR products using paternal DNA as a template were subcloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Cloned fragments were amplified with PCR mixture containing 1× ExTaq buffer, 0.2 mM each dNTP, 0.5 μM each primer, and 0.375 U Ex TaqHS polymerase (Takara Bio, Ohtsu, Japan). M13 forward (5′-TAAAACGACGCCAGTGAT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACCAT-3′) primers were used for amplification, and an ex10-F (5′-AGCTGAGAGGGTTCGAT-3′) primer was used for sequencing.

\textbf{RNA analysis}

RNA analysis using lymphoblastoid cells (LCL) was performed essentially as previously described (10). Briefly, after incubation with dimethyl sulfoxide (as vehicle control) or 30 μM cycloheximide (Sigma, Tokyo, Japan) for 4 h, total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Tokyo, Japan). Two micrograms total RNA was subjected to reverse transcription, and 1 μl cDNA was used for PCR. Primer sequences are ex9-F (5′-CCCCGTGTCCCTGATTGACTTT-3′) and ex12-R (5′-CTGAGGCATCTTCTCAGATCCTTG-3′). Inhibition of NMD was estimated according to the density ratios of lower normal and upper aberrant bands with/without 30-μM cycloheximide treatments in the culture of the patient’s LCL. Two separately extracted RNA samples were used for duplicated experiments, respectively. Data were averaged and the standard deviation was calculated. Statistical analyses were performed using the unpaired Student’s \textit{t}-test (two-tailed). DNA of each PCR band purified by QIAEXII Gel extraction kit (Qiagen, Tokyo, Japan) was sequenced.

\textbf{Results}

Through the screening for \textit{STXBP1} mutations in individuals with cryptogenic OS, we found a patient harboring heterozygous c.902+5G>A mutation. To examine whether the mutation
could affect donor splicing of exon 10, reverse transcriptase (RT)–PCR designed to amplify exons 9–12 was performed using total RNA extracted from LCL derived from the patient (Fig. 2a). A single band (286 bp), corresponding to the wild-type STXBP1 allele, was amplified using a cDNA template from a control LCL (Fig. 2b). By contrast, a longer band was detected from the patient’s cDNA (Fig. 2b). The longer mutant transcript had a 138-bp insertion of intron 10 sequences (Fig. 2c), producing a premature stop codon at amino acid position 302; therefore, the mutant mRNAs are probably to be degraded by NMD (11, 12). The intensity ratio of the mutant compared to the normal band was increased up to 36.3% after treatment with 30 μM cycloheximide, which inhibits NMD, compared to 13.8% in the untreated condition (Fig. 2d). Thus the mutant transcript suffered from degradation by NMD, which would result in haploinsufficiency of STXBP1.

To examine whether the c.902+5G>A mutation occurred de novo, the parental DNA extracted from whole blood were analyzed by HRM. Compared with the mother’s sample, the patient’s sample showed clearly shifted melting curve, indicating that the heterozygous c.902+5G>A mutation could be surely detected (Fig. 3a). Interestingly, the father’s sample showed a slightly shifted melting curve, suggesting that the father may harbor the mutation in mosaic state, which was suggested by sequencing (Fig. 3a,b). Similar melting curves and electropherograms were obtained in DNA extracted from saliva, buccal cells, and nails (Fig. 3a,b). We further investigated the mosaicism by counting wild-type G and mutant A alleles after TA cloning of the PCR product. DNA extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles (i.e. 10.6%, 17.4%, 23.8%, and 33.8% of cells) harbored the mutation, respectively (Fig. 3c).

**Discussion**

To date, 13 point mutations and one deletion of STXBP1 have been reported in individuals with OS (9, 10). Thirteen out of fourteen deletion/mutations were confirmed as de novo
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Fig. 2. The c.902+5G>A mutation causing abnormal splicing associated with nonsense-mediated mRNA decay (NMD). (a) Schematic representation of the genomic structure from exons 9 to 12 of \textit{STXBP1}. Exons, introns and primers are shown by gray boxes, dashed lines and arrows, respectively. The mutation in intron 10 was colored in red. Sequences of exon and intron are presented in upper and lower cases, respectively. (b) Reverse transcriptase (RT) – PCR analysis of the patient with c.902+5G>A and a normal control. Two PCR products were detected from the patient’s cDNA: lower was the wild-type (WT) transcript and upper 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. (c) Sequence of mutant amplicons clearly showed a 138-bp insertion of intron 10 sequences and a premature stop codon (asterisk) in the mutant transcript. (d) Quantitative analysis of the NMD inhibition by CHX based on the data shown in (b). *p = 0.00186 by unpaired Student’s \textit{t}-test (two-tailed). Averages of duplicated experiments using two distinctive RNA samples, respectively, are shown with error bars (standard deviation).

Fig. 3. Paternal somatic mosaicism of the c.902+5G>A mutation. (a) Melting curves of PCR products. Compared with the mother’s sample (black), the patient’s sample (gray) showed largely shifted melting curve. The father’s sample from blood (red), saliva (green), and buccal cells (blue) showed slightly, but distinctly shifted melting curves. (b) Electropherograms of the c.902+5G>A mutation (arrow) showed mosaicism of the mutation in the father. (c) Allele frequencies counted by TA cloning of PCR products and sequencing. DNA extracted from blood, saliva, buccal cells, and nails of the father showed that 5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutant A allele. The numbers of colonies corresponding to each allele are indicated within bars.

Thus, somatic and germline mosaicism of \textit{STXBP1} mutations should be carefully taken into account especially for genetic counseling of familial OS cases.

We have successfully identified the paternal somatic mosaicism of an \textit{STXBP1} mutation by HRM. DNA from blood indicated that the mosaic ratio is as low as about 5%; therefore, HRM could be very sensitive in detecting low-ratio mosaicism. HRM is a rapid and simple approach to detect heteroduplexes (13). It only requires the addition of a saturating dye before PCR. By HRM analysis of the PCR products, the sensitivity of successful detection of heterozygotes is nearly 100% (13). It should be noted that the sensitivity of HRM to

events (paternal DNA was unavailable for one remaining mutation). Many OSs are sporadic, probably because of their poor outcome with severe psychomotor retardation; however, some X-linked familial cases have been reported with ARX mutations (6, 8). Here we have showed a paternal somatic mosaicism of an \textit{STXBP1} mutation. Although DNA from the semen of the father could not be analyzed in this study, the identical c.902+5G>A mutation found in both the father and the affected daughter indicated that the father should possess the mutation in germ cells as a mosaic state, suggesting recurrence risks.
detect somatic changes or heteroplasmy is much better than that of DNA sequencing (14, 15): HRM could detect the level of somatic mosaicism down to 5–10% (15). However, the ability to detect low percentage heterodeuplex of PCR products may vary among mutations. Although the heterozygous c.902+5G>A mutation showed largely shifted melting curve, we experienced some heterozygous mutations only showing slightly shifted melting curve, in which we may not be able to detect the mosaicism. Therefore, optimization of HRM analysis for each mutation would be recommended especially to examine parental samples.

In conclusion, we firstly described the paternal somatic mosaicism of an STXBP1 mutation. The percentage of mosaicism was quite low (5–17%), and no minor problems like dexterity, intelligence (cognition), behavior or psychological state were recognized in the father. The information described here was quite useful for future genetic counseling of this family.

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