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Author Contributions

Conceived and designed the experiments: EK SM N. Matsumoto. Performed the experiments: EK SM. Analyzed the data: EK SM MN YT N. Miyake HS. Contributed reagents/materials/analysis tools: NO. Wrote the paper: EK SM N. Matsumoto.

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FULL-LENGTH ORIGINAL RESEARCH

Targeted capture and sequencing for detection of mutations causing early onset epileptic encephalopathy

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Summary

Purpose: Early onset epileptic encephalopathies (EOEEs) are heterogeneous epileptic disorders caused by various abnormalities in causative genes including point mutations and copy number variations (CNVs). In this study, we performed targeted capture and sequencing of a subset of genes to detect point mutations and CNVs simultaneously.

Methods: We designed complementary RNA oligonucleotide probes against the coding exons of 35 known and potential candidate genes. We tested 68 unrelated patients, including 15 patients with previously detected mutations as positive controls. In addition to mutation detection by the Genome Analysis Toolkit, CNVs were detected by the relative depth of coverage ratio. All detected events were confirmed by Sanger sequencing or genomic microarray analysis.

Key Findings: We detected all positive control mutations. In addition, in 53 patients with EOEEs, we detected 12 pathogenic mutations, including 9 point mutations (2 nonsense, 3 splice-site, and 4 missense mutations), 2 frameshift mutations, and one 3.7-Mb microdeletion. Ten of the 12 mutations occurred de novo; the other two had been previously reported as pathogenic. The entire process of targeted capture, sequencing, and analysis required 1 week for the testing of up to 24 patients.

Significance: Targeted capture and sequencing enables the identification of mutations of all classes causing EOEEs, highlighting its usefulness for rapid and comprehensive genetic testing.

KEY WORDS: Target capture, Sequencing, Mutation, Copy number variation, Genetic testing.

Early onset epileptic encephalopathies (EOEEs), occurring before 1 year of age, are characterized by impairment of cognitive, sensory, and motor development by recurrent clinical seizures or prominent interictal epileptiform discharges (Berg et al., 2010). Ohtahara syndrome (OS), West syndrome (WS), early myoclonic encephalopathy (EME), migrating partial seizures in infancy (MPSI), and Dravet syndrome (DS) are the best known epileptic encephalopathies recognized by the International League Against Epilepsy (ILAE; Berg et al., 2010). However, many infants with similar features do not strictly fit the parameters of these syndromes.

To date, 11 genes have been shown to be associated with EOEEs (Mastrangelo & Leuzzi, 2012). The identification of

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causative mutations associated with EOEEs and their related phenotypes is useful for genetic counseling, and possibly for management of the patients; however, it is time-consuming and arduous to screen all known disease-causing genes one by one using Sanger sequencing or high-resolution melting curve analysis (Wittwer, 2009). In addition, copy number variations (CNVs) involving causative genes can also cause EOEEs (Saitsu et al., 2008; Mei et al., 2010; Saitsu et al., 2011, 2012b). Array comparative genomic hybridization (CGH) and multiplex ligation-dependent probe amplification (MLPA) are well established for the detection of CNVs; however, it is often difficult for array CGH to detect small CNVs such as a single-exon deletion and for MLPA to screen multiple genes at a time (Schouten et al., 2002; Dibbens et al., 2011; Mefford et al., 2011; Stuppia et al., 2012). Therefore, an integrated method that detects both point mutations and CNVs for multiple genes would be useful for comprehensive genetic testing in EOEEs.

Recent progress in massively parallel DNA sequencing in combination with target capturing has facilitated rapid mutation detection (Ng et al., 2009). It has been reported that CNVs involving disease-causing genes in patients with breast or ovarian cancer can be detected by target capture sequencing using the relative depth of coverage ratio (Walsh et al., 2010, 2011; Nord et al., 2011). Targeted capture and sequencing of patients with epileptic disorders has successfully identified potential disease-causing mutations in 16 of 33 patients (Lemke et al., 2012), revealing its efficacy for detecting mutations. However, the detection of both point mutations and CNVs has not been reported in patients with epilepsy.

In this study, we performed targeted capture and sequencing of a subset of 35 genes to detect mutations and CNVs simultaneously in 68 patients with EOEEs. By analyzing the relative depth of coverage ratio, we were able to detect microdeletions, in which the numbers of deleted exons varied from a single exon to all exons of two genes. In combination with rapid sequencing using a benchtop next-generation sequencer, our method provides a fast, comprehensive, and cost-effective method for genetic testing of patients with EOEE.

Methods

Patients

We examined 68 patients (36 male and 32 female) with EOEEs (20 patients with OS, 20 with WS, 3 with EME, 4 with MPSI, 2 with DS, and 19 with unclassified epileptic encephalopathy). Diagnoses were based on clinical features and characteristic patterns on electroencephalography. In 15 of 68 patients (10 male and five female), disease-causing mutations or CNVs had been previously identified in our laboratory, so these mutations were used as positive controls (Table 1) (Saitsu et al., 2008, 2010a,b, 2011, 2012b,c; Nonoda et al., 2013). Genomic DNA was isolated from blood leukocytes according to standard methods. Experimental protocols were approved by the Yokohama City University School of Medicine Institutional Review Board for Ethical Issues. Written informed consent for genetic testing was obtained from the guardians of all tested individuals prior to analysis.

Target capture sequencing and variant detection

A custom-made SureSelect oligonucleotide probe library (Agilent Technologies, Santa Clara, CA, U.S.A.) was designed to capture the coding exons of 35 genes; 5 of them were potential candidates for EOEEs based on unpublished data (for a list of the 30 of 35 genes, see Table 2). We designed 120-bp capture probes with 3× centered probe-tiling, and avoiding 20-bp overlap to repeat region using the Agilent e-Array Web-based design tool. To cover regions

Table 1. Known mutations and copy number variants used as positive controls

	Case	Sex	Chr	Genes	Reported mutations or copy number variants (positive controls)	Type	Deletion size (kb)	Refs
SNVs	27	F	9	*STXBP1*	c.1328T>G (p.Met443Arg)	Missense		Saitsu et al. (2008)
	69	M	X	*CASK*	c.1A>G	Missense		Saitsu et al. (2012b)
	241	M	X	*CDKL5*	c.145G>A (p.Glu49Lys)	Missense		–
Indels	95	M	9	*STXBP1*	c.388_389del (p.Leu130AspfsX11)	Deletion		Saitsu et al. (2010a)
	313	M	X	*CASK*	c.227_228del (p.Glu76ValfsX6)	Deletion		–
	26	F	9	*SPTAN1*	c.6619_6621del (p.Glu2207del)	Deletion		Saitsu et al. (2010b)
	220	M	9	*STXBP1*	c.1381_1390del (p.Lys461GlyfsX82)	Deletion		–
	16	M	9	*SPTAN1*	c.6923_6928dup (p.Arg2308_Met2309dup)	Duplication		Saitsu et al. (2010b)
	309	M	9	*SPTAN1*	c.6908_6916dup (p.Asp2303_Leu2305dup)	Duplication		Nonoda et al. (2013)
CNVs	12	F	9	*STXBP1, SPTAN1*	Del(9)(q33.33–q34.11)	Microdeletion	2150	Saitsu et al. (2008)
	22	M	9	*STXBP1*	*STXBP1* Ex4 deletion	Microdeletion	4.6	Saitsu et al. (2011)
	83	M	X	*CASK*	*CASK* Ex2 deletion	Microdeletion	111	Saitsu et al. (2012b)
	102	F	X	*MECP2*	Del(X)(q28)	Microdeletion		–
	204	M	9	*STXBP1, SPTAN1*	Del(9)(q33.33–q34.11)	Microdeletion	2850	Saitsu et al. (2011)
	214	F	X	*CDKL5*	Del(X)(q22.13)	Microdeletion	137	Saitsu et al. (2011)

SNVs, single nucleotide variants; Indels, insertion/deletions; CNVs, copy number variations.

Table 2. Sequence performance for 30 target genes

Gene	Cytoband	No. of coding exons	Mean read depth	%bases above 5× depth (%)	%bases above 10× depth (%)
ARHGEF9	Xq11.1–q11.2	10	206	100	100
ARX	Xp21.3	5	44	59.4–94.4	38.7–90.6
CASK	Xp11.4	27	201	95.9–100	95.9–100
CDKL5	Xp22.13	20	238	100	100
COL4A1	13q34	52	287	98.3–100	98.3–100
COL4A2	13q34	47	190	100	99.1–100
FOXG1	14q12	1	231	86.5–100	81.1–96.4
GABRG2	5q34	11	300	92.3	92.3
GRIN2A	16p13.2	13	310	100	100
KCNQ2	20q13.33	17	135	100	97.7–100
MAGI2	7q21.11	22	255	96–98.3	94.5–97.5
MAPK10	4q21.3	12	304	100	100
MECP2	Xq28	3	217	96.2	96.2
MEF2C	5q14.3	10	270	100	100
NTNG1	1p13.3	9	298	100	100
PCDH19	Xq22.1	6	212	100	100
PLCB1	20p12.3	32	293	100	100
PNKP	19q13.33	17	208	100	98.5–100
PNPO	17q21.32	7	210	100	100
SCN1A	2q24.3	26	345	100	100
SCN2A	2q24.3	26	323	100	100
SLC25A22	11p15.5	9	121	100	100
SLC2A1	1p34.2	10	209	100	98.8–100
SNPH	20p13	4	179	100	100
SPTAN1	9q34.11	56	277	100	100
SRGAP2	1q32.1	20	320	96.6	96.6
ST3GAL5	2p11.2	8	302	93.6–100	93.6–99.9
STXBP1	9q34.11	20	306	100	100
SYN1	Xp11.23	13	131	93.4–100	81–100
SYP	Xp11.23	6	146	100	99.1–100

where we could not design probes with the above settings, some probes from the SureSelect Human All Exon 50-Mb kit (Agilent Technologies) were added to the probe libraries. A total of 2,738 probes, covering 156 kb, were prepared. Exon capture, enrichment, and indexing were performed according to the manufacturer's instructions. Twenty-four captured libraries were mixed and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, U.S.A.) with 150-bp paired-end reads. Image analysis and base calling were performed using the Illumina Real Time Analysis Pipeline version 1.13 and CASAVA software v.1.8 (Illumina) with default parameters. Sequence reads were aligned to the reference human genome (GRCh37: Genome Reference Consortium human build 37) with Novoalign (Novocraft Technologies, Selangor, Malaysia). After conversion of the SAM file to a BAM file with SAMtools (Li et al., 2009), duplicate reads were marked using Picard (http://picard.sourceforge.net/) and excluded from downstream analysis. Local realignment around insertion/deletions (indels) and base quality score recalibration were performed using the Genome Analysis Toolkit (DePristo et al., 2011). Single-nucleotide variants (SNVs) and indels were identified using the Genome Analysis Toolkit UnifiedGenotyper and filtered according to the Broad Institute's best-practice guidelines v.3 except for HaplotypeScore filtering. We excluded variants found in 147 exomes from healthy individuals previously sequenced in our laboratory. Variants were annotated using ANNOVAR (Wang et al., 2010). Candidate disease-causing mutations were confirmed by Sanger sequencing on a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The Human Gene Mutation Database professional 2012.3 (BIOBASE GmbH, Wolfenbuettel, Germany) was used to check whether the variants had been previously reported.

Copy number analysis using target capture sequence data

Copy number changes were analyzed based on the relative depth of coverage ratios (Nord et al., 2011). Raw coverage on the target regions was calculated by SAMtools using BAM files, in which duplicate reads were excluded. Raw coverage was normalized and corrected for GC content and bait capture bias. Next, the ratios were calculated by comparing the sample-corrected coverage to the median-corrected coverage for the other 23 samples. A sliding window (20 bp) was used to identify CNVs for which the majority of bases had a ratio ≤ 0.6 (loss) or ≥ 1.4 (gain). We visually inspected the ratio

data and judged whether the call was true or likely to be a false positive. A flow chart of our variant detection and copy number analysis scheme is illustrated in Fig. S1.

Genomic microarray analysis and cloning of deletion breakpoints

The microdeletion involving *SCN1A* and *SCN2A* was confirmed using a CytoScan HD Array (Affymetrix, Santa Clara, CA, U.S.A.) according to the manufacturer's protocol. Copy number alterations were analyzed using the Chromosome Analysis Suite (ChAS; Affymetrix) with NA32 (hg19) annotations. The junction fragment spanning the deletion was amplified by long polymerase chain reaction (PCR) using several primer sets based on putative breakpoints according to the microarray data. Long PCR was performed in a 20-μl volume, containing 30 ng genomic DNA, 1× buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, and 0.3 U KOD FX polymerase (Toyobo, Osaka, Japan). The deletion junction fragments were obtained using the following primers: #409-F (5′-TCCACAGTTTACAAACATCTTTTCATGG-3′) and #409-R (5′-AGAAATTGGCTTGGTCAGTACCAGCA-3′) (1.6-kb amplicon). PCR products were electrophoresed on agarose gels stained with ethidium bromide, purified with ExoSAP (USB Technologies, Cleveland, OH, U.S.A.), and sequenced with BIGDYE TERMINATOR CHEMISTRY v.3 according to the manufacturer's protocol (Applied Biosystems).

Results

Target capture sequencing yielded an average of 26 Mb per sample (range 17–41 Mb per sample) on the target regions, resulting in an average read depth of 255 (range across all samples: 173–437). The coverage of the protein-coding sequences of the 30 target genes is shown in Table 2. Overall, 98.6% of targeted coding sequence bases were covered by 10 reads or more; however, some genes such as *ARX* and *FOXG1* were less well covered because of embedded repeat sequences (Fig. S2). To validate the performance of target capture sequencing for detecting mutations and CNVs, we analyzed 15 samples in which disease-causing mutations or microdeletions had been identified previously in our laboratory (Saitsu et al., 2008, 2010a,b, 2011, 2012b; Nonoda et al., 2013). All nine control point mutations and six control microdeletions were detected (Table 1; Fig. 1). These data indicate that our target capture sequencing method was able to detect both point mutations and microdeletions, including deletion of a single exon.

Examination of 53 previously unresolved EOEE patients by targeted capture and sequencing revealed mutations in 12 patients (Table 3). Every patient harbored a different

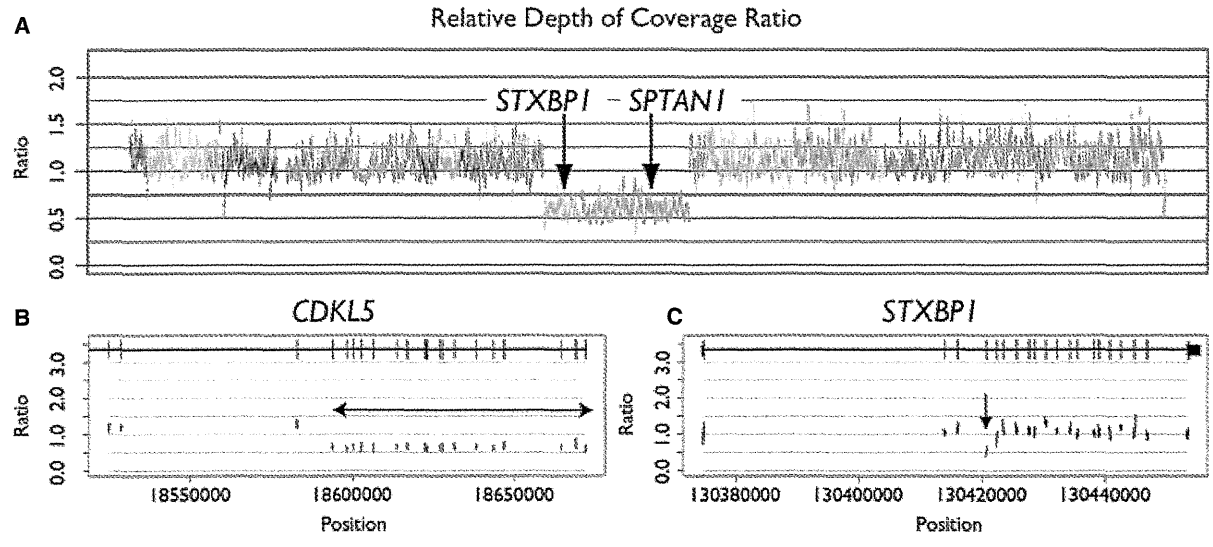

Figure 1.
Detection of three known microdeletions by target capture sequencing. (**A**) Relative depth of coverage ratio for patient 12. Coverage ratios for each target gene are indicated by different colors. A microdeletion including *STXBP1* and *SPTAN1* is clearly observed. (**B**, **C**) Relative depth of coverage ratio for patient 214 in the *CDKL5* region and patient 22 in the *STXBP1* region, respectively. Black vertical lines indicate exons and horizontal lines indicate introns (top). Red vertical lines show bait regions that were judged to be "deleted." A number of exons of *CDKL5* were deleted in patient 214 (bidirectional arrow in **B**), and a single exon of *STXBP1* was deleted in patient 22 (arrow in **C**).

Table 3. Mutations in 53 patients with EOEEs detected by targeted capture and sequencing

	Case	Sex	Diagnosis	Chr	Gene	Mutation	Type	Deletion size (kb)	Inheritance	References
SNVs	329	M	OS/EME	9	*STXBP1*	c.247-2A>G	Splice site		De novo	–
	402	M	OS	9	*STXBP1*	c.902+1G>A	Splice site		De novo	Milh et al. (2011)
	423	F	OS	9	*STXBP1*	c.246+1G>A	Splice site		De novo	–
	403	F	MAE or DS	2	*SCN1A*	c.580G>A (p.Asp194Asn)	Missense		Not found in the mother	Mancardi et al. (2006)
	415	F	EOEE	2	*SCN1A*	c.3714A>C (p.Glu1238Asp)	Missense		Not determined	Harkin et al. (2007)
	416	M	EOEE	X	*CDKL5*	c.533G>A (p.Arg178Gln)	Missense		De novo	Liang et al. (2011)
	418	F	WS, severe hypotonia	2	*SCN2A*	c.632G>A (p.Gly211Asp) in NM_001040143 (variant 3)	Missense		De novo	–
	244	F	Epilepsy + PCH	X	*CASK*	c.55G>T (p.Gly19X)	Nonsense		De novo	–
	404	F	EOEEs	X	*MECP2*	c.844C>T (p.Arg282X)	Nonsense		De novo	–
Indels	336	F	OS	9	*STXBP1*	c.1056del (p.Asp353ThrfsX3)	Deletion		De novo	–
	397	F	DS	2	*SCN1A*	c.342_344delinsAGGAGTT (p.Phe114LeufsX6)	Deletion–insertion		De novo	–
CNV	409	F	MPSI	2	*SCN2A*, *SCN1A*	Microdeletion	Microdeletion	3,726	De novo	–

OS, Ohtahara syndrome; EME, early myoclonic encephalopathy; MAE, myoclonic astatic epilepsy; DS, Dravet syndrome; WS, West syndrome; PCH, pontocerebellar hypoplasia; MPSI, malignant migrating partial seizures in infancy; SNVs, single nucleotide variants; CNVs, copy number variations; EOEEs, early onset epileptic encephalopathies.

mutation. Of these 12 mutations, 9 were single-nucleotide variants (2 nonsense, 3 splice-site, and 4 missense mutations) and two were small indels leading to frameshifts. The other mutation was a microdeletion. All these 11 point mutations were confirmed by Sanger sequencing. Four of the mutations (*STXBP1* c.902+1G>A, *SCN1A* c.580G>A, *SCN1A* c.3714A>C, and *CDKL5* c.533G>A) have been reported in individuals with EOEEs, so are recurrent (Mancardi et al., 2006; Harkin et al., 2007; Azmanov et al., 2010; Liang et al., 2011; Milh et al., 2011). Nine of the 11 mutations occurred de novo. The other two could not be tested because the paternal sample for one patient (*SCN1A* c.580G>A) and parental samples for another patient (*SCN1A* c.3714A>C) were unavailable.

CNV analysis of the 53 patients revealed a microdeletion involving *SCN1A* and *SCN2A* at 2q24.3 in patient 409 (Fig. 2A). To investigate this mutation further, we performed genomic microarray analysis and identified an approximately 3.7-Mb microdeletion (Fig. 2B). The deletion contained 13 RefSeq genes including *SCN2A* and *SCN1A*. Breakpoint-specific PCR analysis of the patient and her parents confirmed that the rearrangement occurred de novo (Fig. 2C). The sequence of the junction fragment confirmed a 3,726,029-bp deletion (chr2: 164,420,771–168,146,801) (Fig. 2D).

Discussion

Several bench-top high-throughput sequencing platforms are now available (Glenn, 2011; Loman et al., 2012; Quail et al., 2012). We selected Illumina MiSeq because it provides reasonable sequence throughput (1.6 Gb per run), a low error rate, a short run time (27 h), and sufficiently long reads (150 bp). We captured genomic DNA fragments of target genes by 3× tiling complementary RNA oligonucleotide probes (Nord et al., 2011) and sequenced 24 samples per MiSeq run, achieving sufficient coverage (a mean read depth of 255) over the target regions. This high coverage enabled us to detect point mutations and CNVs simultaneously, and long reads enabled us to detect small indels (Krawitz et al., 2010). Mapping by Novoalign, we were able to detect indels ranging in size from a 10-bp deletion to a 9-bp duplication.

By evaluating depth of coverage ratios (Nord et al., 2011), we detected six control microdeletions and one novel microdeletion, ranging in size from 4.6 kb to 3.7 Mb. To date, CNVs causing EOEEs have been analyzed by array CGH and MLPA (Mulley & Mefford, 2011). Array CGH can detect genome-wide CNVs, but its standard resolution is relatively low (>10 kb). On the other hand, MLPA can detect CNVs in specific genes, including single exon deletions; however, it is difficult to screen many genes at a time because MLPA is limited to 50 target exons per reaction (Stuppia et al., 2012). In addition, copy number analysis using MLPA can be affected by single nucleotide variants and indels in regions corresponding to the MLPA probes (Stuppia et al., 2012). In contrast, targeted capture and sequencing can analyze all targeted genes to detect mutations and CNVs simultaneously. CNVs as small as a single exon can be identified. Because all the procedures—from

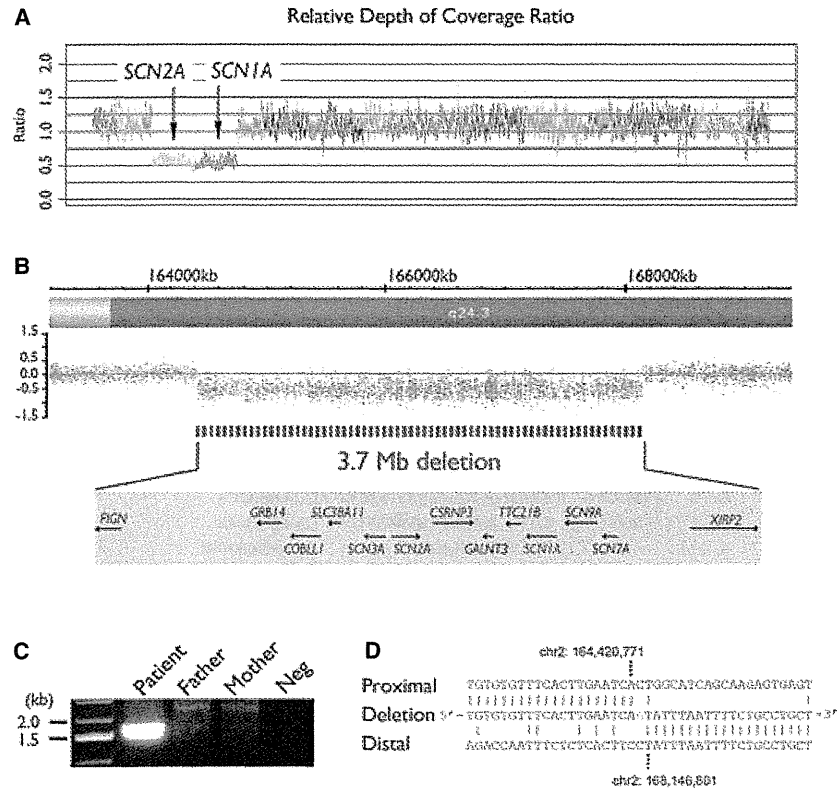

Figure 2.
A 3.7-Mb microdeletion including *SCN2A* and *SCN1A* in patient 409. (**A**) Relative depth of coverage ratio for patient 409 indicates a microdeletion encompassing *SCN2A* and *SCN1A*. Different colors distinguish the target genes. (**B**) The array profile clearly shows a 3.7-Mb microdeletion at 2q24.3 in this patient. Thirteen RefSeq genes, including *SCN2A* and *SCN1A*, lie within the microdeletion (bottom). (**C**) Breakpoint-specific PCR analysis of the patient's family. Primers flanking the deletion were able to amplify a 1,607-bp product from the patient only, indicating that the translocation occurred de novo. (**D**) Deletion junction sequence. The top, middle, and bottom strands show the proximal, deleted, and distal sequences, respectively. A single inserted nucleotide (colored in red) was identified at the breakpoint.

the capture of target genes to the detection of mutations and CNVs—can be done within a week, our workflow provides a fast, sensitive, and comprehensive genetic testing method for patients with epilepsy.

Whole-exome sequencing will reveal novel mutations in unexpected genes in patients with EOEEs. For example, *KCNQ2* mutations, which cause benign familial neonatal seizures (Biervert et al., 1998; Charlier et al., 1998), were identified in patients with OS by whole exome sequencing (Saitsu et al., 2012a). Similarly, screening known and potential candidate genes in patients with EOEEs will reveal novel mutations in unexpected genes, in addition to mutations in well-known genes.

In our target capture analysis, some exons of genes such as *ARX* and *FOXG1* were insufficiently sequenced because repeat sequences hampered the design of capture probes. Repeat sequences also interfere with appropriate mapping of sequence reads, resulting in low coverage. For these exons, Sanger sequencing should be added for complete analysis.

In conclusion, a rapid and efficient system of target capture sequencing can be applied to the comprehensive genetic analysis of EOEEs. Point mutations, small indels, and CNVs are all detected by this method, confirming the potential of this approach for efficient genetic testing.

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Disclosure

None of the authors has any conflicts 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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Supporting Information

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

Figure S1. Flow chart of our variant detection and copy number analysis scheme.

Figure S2. Insufficient coverage of reads in two genes rich in repetitive sequences.

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

MLL2 and *KDM6A* Mutations in Patients With Kabuki Syndrome

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