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Letter to the Editor

A *de novo CASK* mutation in pontocerebellar hypoplasia type 3 with early myoclonic epilepsy and tetralogy of Fallot

To The Editor:

In a male patient with pontocerebellar hypoplasia (PCH) type 3, previously described in this journal [1], we identified a novel hemizygous frameshift mutation in *CASK* (c.227_228del [p.Glu76Valfs*6]), which occurred *de novo*. *CASK* at Xp11.4 encodes a calcium/calmodulin-dependent serine protein kinase belonging to the membrane-associated guanylate kinase protein family. Heterozygous loss-of-function mutations in *CASK* cause X-linked intellectual disability (ID), microcephaly, and pontocerebellar hypoplasia in female patients [2]. In male patients, hemizygous hypomorphic *CASK* mutations have been found in ID and FG syndrome; cases had developmental delay, a characteristic face, and complete or partial agenesis of the corpus callosum [3,4]. On the other hand, hemizygous loss-of-function mutations in *CASK* have been reported to cause more severe phenotypes: one case with profound developmental delay, ID, and PCH (c.278 + 1G>A) and two cases showing cerebellar hypoplasia and Ohtahara syndrome (OS) (c.1A>G or deletion of exon 2) [5,6].

The present case showed a suppression-burst pattern during the infantile period on electroencephalography and frequent myoclonus from the first day of birth, which were compatible with early myoclonic encephalopathy (EME). OS, which is characterized by early-onset seizures, epileptic spasms, and a suppression-burst pattern on electroencephalography, shares some clinical features with EME. Recently, mutations in *STXBP1* have been identified in patients with OS and three patients with EME [7]. Our findings further demonstrate that loss-of-function *CASK* mutations in males cause early-onset epileptic encephalopathy and PCH, and support the idea that OS and EME may share the same genetic basis.

The patient is also complicated with tetralogy of Fallot, which has never been described in association with *CASK* mutation. Further studies are required to investigate the causal relationship between loss-of-function mutations in *CASK* and congenital heart disease in males.

In summary, a novel frameshift *CASK* mutation was identified in a patient with PCH, EME, and tetralogy of Fallot.

Conflict of interest

None of the authors has any conflict of interest to disclose. The study sponsors played no role in the study design; in the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the report for publication.

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FULL-LENGTH ORIGINAL RESEARCH

Clinical spectrum of early onset epileptic encephalopathies caused by *KCNQ2* mutation

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Summary

Purpose: ***KCNQ2* mutations have been found in patients with benign familial neonatal seizures, myokymia, or early onset epileptic encephalopathy (EOEE). In this study, we aimed to delineate the clinical spectrum of EOEE associated with *KCNQ2* mutation.**

Methods: **A total of 239 patients with EOEE, including 51 cases with Ohtahara syndrome and 104 cases with West syndrome, were analyzed by high-resolution melting (HRM) analysis or whole-exome sequencing. Detailed clinical information including electroencephalography (EEG) and brain magnetic resonance imaging (MRI) were collected from patients with *KCNQ2* mutation.**

Key Findings: **A total of nine de novo and one inherited mutations were identified (two mutations occurred recurrently). The initial seizures, which were mainly tonic seizures, occurred in the early neonatal period in all 12 patients. A suppression-burst pattern on EEG was found in most. Only three patients showed hypsarrhythmia on EEG; eight patients became seizure free when treated with carbamazepine, zonisamide, phenytoin, topiramate, or valproic acid. Although the seizures were relatively well controlled, moderate-to-profound intellectual disability was found in all except one patient who died at 3 months.**

Significance: **De novo *KCNQ2* mutations are involved in EOEE, most of which cases were diagnosed as Ohtahara syndrome. These cases showed distinct features with early neonatal onset, tonic seizures, a suppression-burst EEG pattern, infrequent evolution to West syndrome, and good response to sodium channel blockers, but poor developmental prognosis. Genetic testing for *KCNQ2* should be considered for patients with EOEE.**

KEY WORDS: ***KCNQ2*, Ohtahara syndrome, Early onset epileptic encephalopathy, Mosaic, Ion channel.**

Early onset epileptic encephalopathies (EOEEs) are characterized by developmental impairment and disastrous seizures starting from early infancy. Several genes have been demonstrated to be involved in the pathogenesis of EOEE: *ARX* in Ohtahara syndrome (OS) and West syndrome (WS), *CDKL5* in WS, *STXBP1* in OS, *SLC25A22* in early myoclonic encephalopathy (EME), and *SCN1A* in Dravet syndrome (Claes et al., 2001; Stromme et al., 2002; Kalscheuer et al., 2003; Molinari et al., 2005; Kato et al., 2007; Saitsu et al., 2008). There are many infants who do not strictly fit the electroclinical parameters of these known syndromes (Holland & Hallinan, 2010). Identification of

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causative mutations associated with EOEE and its particular phenotypes is useful for genetic counseling and potentially for patient management.

KCNQ2 encodes the potassium channel subunit Kv7.2, and its mutations have been shown to cause benign familial neonatal seizures (BFNS) with a favorable prognosis (Biervert et al., 1998; Singh et al., 1998). In addition, rare sporadic and familial cases have been reported with neonatal-onset seizures and poor outcomes associated with *KCNQ2* mutations (Borgatti et al., 2004; Steinlein et al., 2007). Recently, de novo *KCNQ2* mutations have been found in a substantial proportion of patients with neonatal epileptic encephalopathy (Weckhuysen et al., 2012), confirming the association of *KCNQ2* mutations with intractable seizures with poor outcomes. Some cases showed a suppression-burst pattern on EEG, tonic seizures, and profound intellectual disability, resembling OS. We also found three de novo missense mutations in *KCNQ2* in 3 of 12 patients with OS by whole-exome sequencing (WES) (Saitsu et al., 2012a). Therefore, it is likely that de novo *KCNQ2* mutations are one of the common causes of EOEE, including OS.

In this study, to delineate the clinical spectrum of EOEE caused by *KCNQ2* mutations, we screened for *KCNQ2* mutations in 239 patients with EOEE.

Patients and Methods

Patients

A total of 239 patients with EOEEs (51 patients with OS, 104 with WS, 4 with EME, and 80 with unclassified epileptic encephalopathy with an age of onset <1 year) were analyzed for *KCNQ2* mutations by high-resolution melting (HRM) analysis or WES (30 of 50 patients with OS) (Saitsu et al., 2012a,b). The diagnosis was made based on clinical features and characteristic patterns on EEG. Mutations of *STXBP1* or *ARX* had been excluded in all or male patients in advance, respectively. *KCNQ3* mutations were excluded only in 30 patients with OS by WES. We obtained detailed clinical information on all the 12 patients with a *KCNQ2* mutation, an EEG from 10 patients, and brain MRI or computed tomography (CT) images from 9 of them.

Mutation analysis

Genomic DNA was obtained from peripheral blood leukocytes by standard methods, amplified by GenomiPhi version 2 (GE Healthcare, Little Chalfont, United Kingdom), and used for mutation screening. Exons 1–17, covering the entire *KCNQ2* coding region (transcript variant 1, NM_172107.2), were examined by HRM analysis or direct sequencing. Samples showing an aberrant melting curve pattern by HRM analysis were sequenced. Polymerase chain reaction (PCR) primers and conditions are available upon request. All novel mutations were verified in the original genomic DNA sample. For the families showing de novo mutations, parentage was confirmed by microsatellite analysis, as previously described (Saitsu et al., 2008). Appropriate biologic parentage was confirmed if three or more informative markers were compatible and the other markers showed no discrepancies.

Whole-exome sequencing

DNAs were captured using the SureSelect^{XT} Human All Exon v4 Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and sequenced with four samples per lane on an Illumina HiSeq2000 (Illumina, San Diego, CA, U.S.A.) with 101-bp paired-end reads. Image analysis and base calling were performed by sequence control software real-time analysis and CASAVA software v1.8 (Illumina). Reads were aligned to GRCh37 with Novoalign (Novocraft Technologies, Selangor, Malaysia); duplicate reads were marked using Picard (http://picard.sourceforge.net/index.shtml) and excluded from downstream analysis. Local realignments around small insertions or deletions and base quality score recalibration were performed using the Genome Analysis Toolkit (DePristo et al., 2011). Single-nucleotide variants and small insertions or deletions were identified using the Genome Analysis Toolkit and were annotated using ANNOVAR (http://www.openbioinformatics.org/annovar/) (Wang et al., 2010).

TA cloning

For measurement of the ratio of wild-type and mutant alleles of patient 272's mother, PCR products using maternal DNA as a template was subcloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.). 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′-TAAAACGACGGCCAGTGAAT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACCATGA-3′) primers were used for amplification, and M13 forward primer was used for sequencing.

Standard protocol approvals, registrations, and patient consents

The experimental protocols were approved by the institutional review boards for ethical issues of Yokohama City University School of Medicine and Yamagata University Faculty of Medicine. Informed consent was obtained from the families of all patients.

Results

Identification of *KCNQ2* mutations

The 10 mutations found in 12 patients are summarized in Table S1. A total of 10 mutations in 12 patients were identified (two mutations occurred recurrently). All the mutations are missense changes. In 11 patients the mutation

arose de novo, and in one patient the mutation (c.854C>A) was transmitted from the mother, who had epilepsy and possessed the mutation as a somatic mosaic (Fig. S1). All the mutations were absent from our in-house 212 control exomes. One mutation (p.Arg333Trp) was identical to the de novo mutation found in a patient with neonatal tonic seizures and poor developmental outcome (Schmitt et al., 2005). Five mutations (p.Ala265Val, p.Gly290Ser, p.Ala294Val, p.Arg553Trp, p.Arg553Leu) caused alteration of amino acid residues at which different missense changes (p.Ala265Pro, p.Gly290Asp, p.Ala294Gly, and p.Arg553Glu) have been reported in patients with neonatal seizures (Steinlein et al., 2007; Weckhuysen et al., 2012). The mutations were distributed from the transmembrane S4 domain to the C-terminal cytoplasmic domain of the encoded protein (UniProtKB O43526).

Clinical features of patients with *KCNQ2* mutation

KCNQ2 mutations were assigned to two patients with unclassified EOEEs and 10 patients with OS, including one patient showing the transition from OS to WS, in whom tonic seizures started 2–3 days after birth but the first medical examination was at 3 months. Detailed clinical information was obtained from all 12 patients (Table S1). The mothers of two patients had a history of epilepsy. Nine patients showed initial symptoms such as seizures or poor feeding within a few days, and also demonstrated initial epileptic attacks within a week. Tonic seizures were initially seen in 11 patients on an hourly or daily basis. Initial EEG studies showed a pattern of suppression-burst in 10 patients with a diagnosis of OS, 4 of whom had an asymmetric pattern and 4 of whom had a brief period of electrodecremental response (Fig. 1). Epileptic spasms arose in four patients, and hypsarrhythmia on EEG was noted in three patients. The most frequently used antiepileptic drug was phenobarbital. Eight patients became seizure-free with a treatment of a particular antiepileptic drug (patients 205, 14, 272, 232, and 168) or combinations of them (patients 297 and 17), or intramuscular injections of adrenocorticotropic hormone (patient 304). The most recent EEG displayed focal or multifocal paroxysmal discharges in eight patients, whereas diffuse paroxysms were seen in only two patients and hypsarrhythmia in one patient. One patient died at 3 months after palliative care. All patients showed intellectual disability from moderate to profound developmental delay. Two patients were able to walk without support, and seven patients were bedridden. Normal brain images were confirmed in three of nine patients for whom MRI findings were studied; the other six patients showed abnormal hyperintensities in the globus pallidus, particularly on T_1-weighted images in the neonatal period and on T_2-weighted images in early infancy. Mild atrophy of the frontal lobe and a thin corpus callosum were seen in three patients at later stages (Fig. S2).

Discussion

We identified 10 *KCNQ2* mutations in 12 patients with EOEEs (10 cases with OS, two cases with unclassified EOEEs), suggesting that *KCNQ2* mutations are one of major causes for OS in contrast to *KCNQ3* mutations, which are causative for BFNS as well, but not for EOEEs in our cohort and the previous report (Weckhuysen et al., 2012). Consistent with the results of previous reports, all *KCNQ2* mutations found in EOEE patients in this study were missense changes (Saitsu et al., 2012a; Weckhuysen et al., 2012). In contrast, frameshift or nonsense mutations in *KCNQ2* are more frequently found among patients with BFNS (Singh et al., 2003; Steinlein et al., 2007); the electrophysiologic properties of mutant cells suggest that haploinsufficiency of the ion channel is the main mechanism of BFNS (Rogawski, 2000; Volkers et al., 2009). There are few experimental data on the function of mutant KCNQ2 in EOEE patients, but a missense mutation p.Ser247Trp within the fifth transmembrane region, found in a mother with BFNS and her son with "early epileptic encephalopathy due to undetermined etiology" has been studied (Dedek et al., 2003). This mutation results in a dominant negative effect on the current amplitude of homomeric wild-type and mutant *KCNQ2* constructs. There may be common pathomechanisms between BFNS and EOEE, although further study is needed.

Six of 10 mutations occurred at amino acid residues in which the same or different missense changes have been previously reported: three amino acids (Arg333, Ala265, and Gly290) are mutated in patients with neonatal tonic seizures and poor developmental outcome (Schmitt et al., 2005; Weckhuysen et al., 2012), and two (Ala294, Arg553) are mutated in patients with BFNS. However, the mutations in patients with EOEEs did not cluster in any particular domain of the encoded protein, making it difficult to identify genotype–phenotype correlations. In mice, a combination of a subclinical mutation of *Kcnq2* and a mild mutation of *Scn2a* exacerbates the phenotype (Kearney et al., 2006). It is possible that genetic modifiers contribute to the severity of phenotypes caused by de novo *KCNQ2* mutations as well as a dominant negative effect.

The initial clinical symptoms of the patients with EOEEs and *KCNQ2* mutation in this study are generally similar to the findings for BFNS. No significant prenatal or perinatal history except seizures can be found. The onset of seizures is within a week of birth, and tonic seizure is the most frequently observed initial type. In our cohorts, apparent autonomic symptoms, such as facial flushing, pale face, apnea, or poor feeding were noticed prior to evident seizures, as is seen in patients with BFNS (Ronen et al., 1993). Although some patients with BFNS experience tens of seizures per day, they seem to be even more frequent in patients with EOEEs and *KCNQ2* mutation.

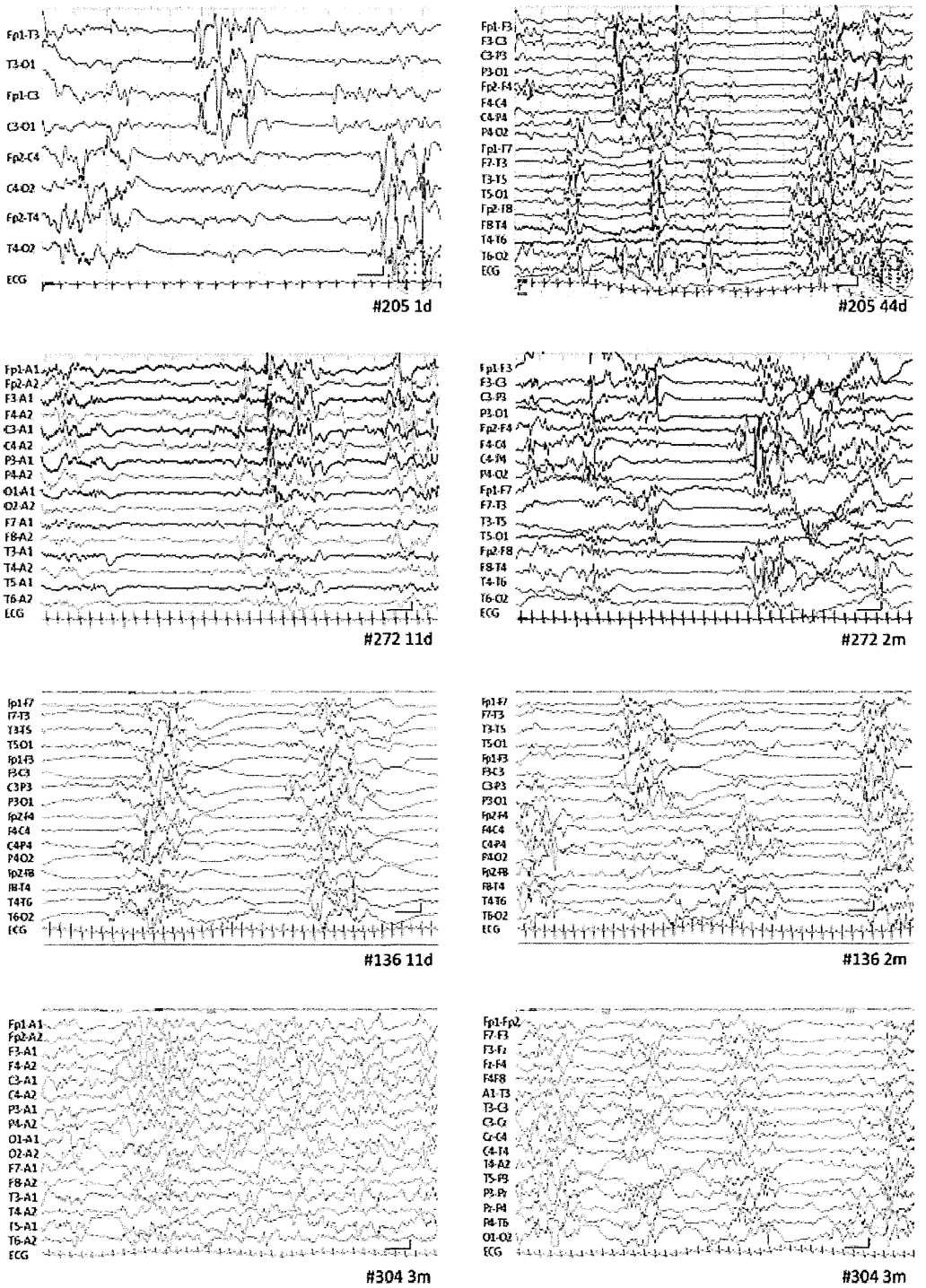

Figure 1.
EEG of patients with a *KCNQ2* mutation. EEG of patient 205 shows an asymmetric pattern of suppression-burst at both 1 day (d) and 44 days of age. EEGs of patients 272 and 136 show patterns of symmetric and asymmetric suppression-burst at 11 days and 2 months (m) of age, respectively. EEG of patient 304 shows hypsarrhythmia for the most part and also electrodecremental discharge that is identical to suppression-burst to some extent. The calibration bar in the lower right-hand corner indicates 1 s (horizontal line) and 100 μV (vertical line).

The most critical finding for discrimination between EOEE and BFNS caused by *KCNQ2* mutation is the suppression-burst pattern on EEG that we observed in 18 (78%) of 23 patients (Saitsu et al., 2012a; Weckhuysen et al., 2012). Another case with *KCNQ2* mutation (p.Ser247Trp), reported by Dedek et al. (2003), also showed a suppression-burst pattern and severe developmental delay, leading to a diagnosis of OS because of the combination of tonic seizures and suppression-burst on EEG. The asymmetrical pattern or brief span of suppression found in our cohort might be characteristic EEG features in patients with EOEE caused by *KCNQ2* mutation.

In contrast to the typical clinical course of OS caused by other etiologies, such as brain malformation or mutations of *ARX* or *STXBP1* (Kato et al., 2007; Saitsu et al., 2008), other features can be observed in patients with OS caused by *KCNQ2* mutation. First, an evolution to WS characterized by epileptic spasms and hypsarrhythmia on EEG is infrequent. Second, medical control of seizures is relatively good. Third, effective antiepileptic drugs for EOEE with *KCNQ2* mutation are unique (Ozawa et al., 2002). Of interest, the primary action of most of the antiepileptic drugs that were useful in our patients—carbamazepine, phenytoin, sodium valproate, and topiramate—is the blockade of voltage-gated sodium channels. Retigabine, which selectively opens the Kv7 potassium channel, might be more effective (Maljevic et al., 2011), possibly improving the neurologic prognosis of EOEE with *KCNQ2* mutation.

Faint but unusual signal changes in the globus pallidus were seen on brain MRI in half of the patients. The abnormal signals disappeared in accordance with developmental age, as reported previously (Weckhuysen et al., 2012), but no associations were found between the hyperintensities and seizure prognosis or involuntary movement. Although the pathologic mechanism of the lesion is uncertain, it might be useful clinically to discriminate the EOEE patients with *KCNQ2* mutation from others.

In summary, our data clearly demonstrate that de novo *KCNQ2* mutations are involved in EOEEs, most of which cases are diagnosed as OS. The seizures responded to some anticonvulsants; however, the neurologic prognosis was very poor. More effective treatments are needed.

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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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Supporting Information

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

Figure S1. Maternal somatic mosaicism of the c.854C>A in the subject 272.

Figure S2. Brain MRI of subjects with a *KCNQ2* mutation.

Table S1. Summary of the clinical features of subjects with a *KCNQ2* mutation.

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SHORT COMMUNICATION

Exome sequencing identifies a novel *INPPL1* mutation in opsismodysplasia

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Opsismodysplasia is an autosomal recessive skeletal disorder characterized by facial dysmorphism, micromelia, platyspondyly and retarded bone maturation. Recently, mutations in the gene encoding inositol polyphosphate phosphatase-like 1 (INPPL1) are found in several families with opsismodysplasia by a homozygosity mapping, followed by whole genome sequencing. We performed an exome sequencing in two unrelated Japanese families with opsismodysplasia and identified a novel *INPPL1* mutation, c.1960_1962delGAG, in one family. The mutation is predicted to result in an in-frame deletion (p.E654del) within the central catalytic 5-phosphate domain. Our results further support that *INPPL1* is the disease gene for opsismodysplasia and that opsismodysplasia has genetic heterogeneity.
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Keywords: exome sequencing; INPPL1; opsismodysplasia

INTRODUCTION

Opsismodysplasia (OMIM 258480) is a rare skeletal dysplasia identifiable at birth. Its clinical features are rhizomelic micromelia and facial dysmorphism, including prominent brow, large fontanels, depressed nasal bridge and small anteverted nose with long philtrum, as well as short feet and hands with sausage-like fingers.[1] Its main radiological features include retarded bone maturation, marked shortness of the bones of hands and feet with concave metaphyses and thin, lamellar vertebral bodies. Some patients show severe phosphate wasting. Autosomal recessive inheritance is the most likely mode of inheritance; to date, at least three consanguineous families with opsismodysplasia are reported.[2–4]

Recently, Below *et al.*[5] performed a homozygosity mapping coupled with whole genome sequencing in a consanguineous family with opsismodysplasia, and identified *INPPL1* (inositol polyphosphate phosphatase-like 1) as a causative gene for opsismodysplasia. They first identified a homozygous missense mutation, p.Pro659Leu, in the consanguineous family, and then found *INPPL1* mutations in additional five unrelated families with opsismodysplasia. We performed a whole exome sequencing for two patients from two unrelated families and identified a homozygous in-frame deletion of *INPPL1* in one family.

SUBJECTS AND METHODS

Subjects and DNA samples

Two families with clinical diagnosis of opsismodysplaisa were included in the study. Family 1 consisted of parents and affected sibs (Figure 1a), and Family 2 consisted of parents and a patient. Genomic DNA was extracted by standard procedures from peripheral blood of the patients and their family members after informed consent. The study was approved by the ethical committee of RIKEN, Yokohama City University, and participating institutions.

Exome sequencing

Six individuals in the two families were analyzed by the whole exome sequence as described previously.[6] Briefly, 3 μg of genomic DNA was sheared by Covaris 2S system (Covaris, Woburn, MA, USA) and partitioned using SureSelect Human All Exon V4 (Agilent technology, Santa Clara, CA, USA) according to the manufacturer's instructions. The exon-enriched DNA libraries were sequenced using HiSeq2000 (Illumina, San Diego, CA, USA) with a 101-bp paired-end reads and a 7-bp index reads. Four samples (2.5 pM each, with different index) were run in one lane. HiSeq Control Software/Real-Time Analysis and CASAVA1.8.2 (Illumina) were used for image analysis and base calling. The mapping was performed to human genome hg19 using Novoalign (http://www.novocraft.com/main/page.php?s=novoalign). The aligned reads were processed by Picard to remove the polymerase chain reaction (PCR) duplicate (http://picard.sourceforge.net). The variants were called

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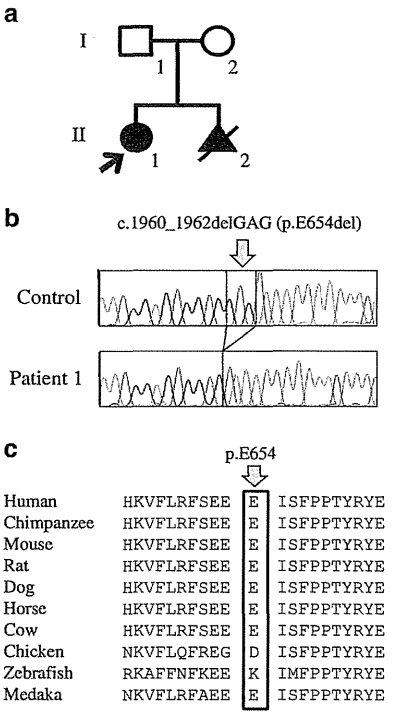

Figure 1 *INPPL1* mutation in a Japanese family with opsismodysplasia. (**a**) Pedigree, (**b**) an in-frame deletion c.1960_1962delGAG (p.E654del) within exon 17 and (**c**) conservation of p.E654 in INPPL1 among different species.

by Genome Analysis Toolkit 1.6-5 (GATK; http://www.broadinstitute.org/gsa/wiki/index.php/Main_Page) with the best practice variant detection with the GAKT v.3 (http://www.broadinstitute.org/gsa/wiki/index.php/Best_Practice_Variant_Detection_with_the_GATK_v3) and annotated by ANNOVAR (23 February 2012) (http://www.openbioinformatics.org/annovar/). Through this flow, common variants registered in dbSNP135 (minor allele frequeny $\geqslant$0.01) (http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=316787363&g=snp135Common&hgTracksConfigPage=configure) were removed.

Priority scheme

On the basis of the hypothesis that opsismodysplasia is inherited in an autosomal recessive manner, variants were filtered by following conditions using the script created by BITS (Tokyo, Japan). For the homozygous mutation model: (1) variant allele frequency (variant alleles/total alleles) in probands $\geqslant$0.8, (2) variant allele frequency in parents $\leqslant$0.8, (3) excluding synonymous changes and (4) excluding the variants observed in our in-house database (n = 429). For the compound heterozygous mutation model: (1) mutation allele frequency in probands: 0.2–0.8, (2) variant allele frequency in parents $\leqslant$0.8, (3) excluding synonymous changes, (4) excluding the variants observed in our in-house database (n = 429) and (5) selecting genes with compound heterozygous change. After combining variants selected by both models, genes commonly found in the two families were searched.

Sanger sequencing

We performed Sanger sequencing to confirm the deletion identified in the proband of Family 1 by the exome sequencing. We amplified exon 17 by PCR using primer sequences, 5′-AAGCACAAGGTCTTCCTTCGATTCA-3′ and 5′-CCATACCCTTGACCCAAATTCTTGAT-3′. We directly sequenced the PCR product using an Applied Biosystems 3730xl DNA analyzer (Life Technologies, Forster City, CA, USA). For the patient in Family 2, we screened 28 exons of *INPPL1* and exon–intron boundaries by direct sequencing of PCR products from genome DNA. The primer sequences are available on request.

Evaluation of polymorphism

We used the invader assay coupled with PCR[7] to exclude the possibility of polymorphism in 188 Japanese general populations. The deletion was evaluated by databases, PROVEAN v.1.1 (http://provean.jcvi.org/genome_submit.php), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and 1000 genomes (http://www.1000genomes.org/). We used Evola website to investigate the conservation of p.E654 of INPPL1 (http://www.h-invitational.jp/hinv/ahg-db/index.jsp).

RESULTS

Exome sequencing

By the whole exome sequencing, 3.8–5.1 Gb sequences uniquely mapped to all human RefSeq coding region were obtained. For all subjects, at least 95.9% of all coding regions were covered in five reads depth and more (Supplementary Table 1). No candidate genes that had mutations in the two families were identified.

Because *INPPL1* mutations have recently been identified in opsismodysplasia,[5] we checked *INPPL1* mutations in the exome sequence data. Five or more reads covered 100% of its coding regions (Supplementary Table 1). A homozygous deletion, c.1960_1962 (p.E654del), was found in the proband of Family 1 (Figure 1a). However, this deletion had been excluded as a candidate mutation because no *INPPL1* variant likely to be a mutation was detected in Family 2.

Confirmation and evaluation of c.1960_1962delGAG

We confirmed the deletion by direct sequence of PCR product from genomic DNA in the proband of Family 1 (Figure 1b). Next, we performed the invader assay coupled with PCR in the family. The parents were compound heterozygous for the deletion and the affected sibs were homozygous for it. The deletion was not found in 188 Japanese controls and in the public databases. The E654 is conserved between different species (Figure 1c). It is within the central catalytic 5-phosphate domain, but located at the position far from active site (25 amino acids) and within a loop region, which is thought to have structural flexibility in general. Inositol polyphosphate 5-phosphatase domain (ipp5c) of yeast synaptojanin in complex with inositol (1,4)-bisphosphate and calcium ion (PDB ID 1i9z) is the most analogous structure to the human INPPL1 catalytic domain among the currently available structures; however, its sequence identity with the human INPPL1 catalytic domain is low (26%). These make the structural assessment of the mutation equivocal. The PROVEAN database showed that p.E654del had a deleterious function against the gene product (score: −12.1).

Mutation screening of *INPPL1* in Family 2

We screened the *INPPL1* mutation in the patient of Family 2 by direct sequencing of the entire coding exons and their flanking regions. A total of nine SNPs were found, but no mutation was found in the patient.

Clinical information of the patients with the *INPPL1* mutation

The proband of Family 1 (II-1 in Figure 1a) was a 9-year-old girl born to non-consanguineous healthy parents. Family history was unremarkable. She was referred to one of us because fetal echogram revealed short extremities. She was born at 40 weeks' of gestation. Her birth weight was 2119 g (<3 percentile), length 38.0 cm (<3 percentile) and head circumference 35.1 cm (<3 percentile). She had a wide fontanelle, widely patent sutures, frontal bossing, flat nasal

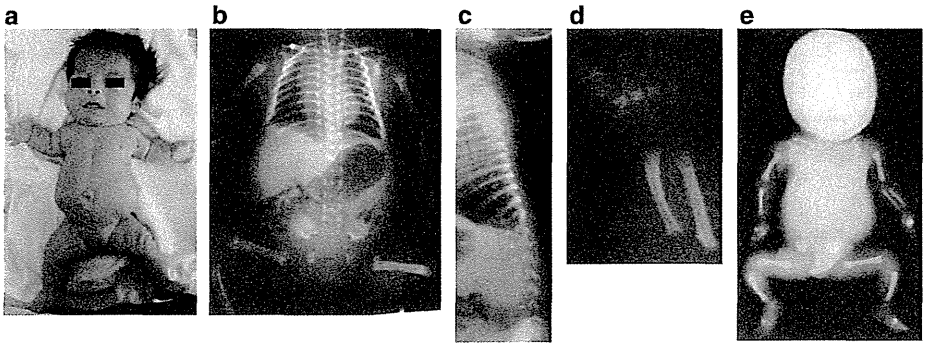

Figure 2 Phenotype of patients in Family 1. (**a**) Appearance of the proband in Family 1. Rhizomelic micromelia, frontal bossing, flat nasal bridge, low set ears, anteverted nostrils, micrognathia, narrow thorax and distended abdomen were noted. Radiographs of the proband (II-1) at birth (**b–d**) and the aborted fetus (II-2) (**e**). Characteristics of opsismodysplasia including retarded bone maturation, shortness of the bones of hands and feet, concave metaphyses and thin, lamellar vertebral bodies were noted.

bridge, low set ears, anteverted nostrils, micrognathia, narrow thorax and distended abdomen, and her extremities were remarkably short (Figure 2a). Her respiratory activity was weak and inspiratory wheezing was noted. Tracheal intubation became necessary 4 h after birth. Radiological investigations of her skeleton showed characteristics of opsisimodysplasia (Figures 2b–d). She was repeatedly admitted because of respiratory insufficiency due to infections. At 2 years of age, tracheotomy was performed to care for respiratory problems. She was noticed to show low serum phosphate levels at around 1 year and since then had been treated on phosphate supplements and/or alfacalcidol (1α-OH-D_3). At age 9 years, her height was 65 cm (<−6 s.d.) and weight 9 kg (−4 s.d). Her intellectual development was normal and was attending an elementary school.

In the second pregnancy, similar conditions were found by a fetal echogram. Artificial abortion was carried out. The post-mortem radiograph showed skeletal findings similar to the proband (Figure 2e).

DISCUSSION

Below *et al.*[5] examined *INPPL1* in a total of 12 unrelated families with opsismodysplasia and found its mutations in seven families. The list of mutation includes missense, nonsense and splicing mutations; all are predicted to be loss of function mutations. In one family, we also found a deletion mutation in *INPPL1* that is predicted to be a loss of function mutation, but in another family, we could not detect an *INPPL1* mutation. These results further support the results of the previous study that *INPPL1* is the disease gene for opsismodysplasia and that opsismodysplasia has genetic heterogeneity.[5] In retrospect, the patient of Family 2 showed significant platyspondyly, yet some of the radiographic features for opsismodysplasia that include hypoplasia of the base of the skull on lateral views and lateral spikes of the acetabular roof were absent. Further, the fragmented epiphyses and coning of the distal femora are not characteristically seen in opsismodysplasia. This case is also different from the other cases with an opsismodysplasia phenotype that do not have *INPPL1* mutations (Prof. Debora Krakow, personal communication). Further collection of *INPPL1* mutation-proven cases would help in defining the phenotype of opsismodysplasia. While we were preparing the manuscript, another study reporting the identification of *INPPL1* as the cause of opsismodysplasia was published.[8] It reports identification of the *INPPL1* mutation in all 10 families examined.

INPPL1 (also known as SHIP2) is a member of the inositol 5′-phosphatase family that hydrolyzes phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) and generates phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2).[9] *INPPL1* encodes a 142-kDa protein with a variety of protein interaction domains, including an N-terminal SH2 domain, a central catalytic 5-phosphatase domain, a C-terminal proline-rich domain, an NPXY site and a sterile a motif domain in the C-terminal region.[10] At least 12 proteins of binding partners for INPLL1, such as Shc, APS, filamin and EphA2, have been identified.[10] The genes for these binding partners are good candidates for the disease gene for the opsismodysplasia-like phenotype.

Biological roles of INPPL1 remain unclear. *INPPL1* expression is particularly high in heart, skeletal muscle and placenta.[11,12] Its proposed roles are cell adhesion and spreading, actin cytoskeletal remodeling and receptor internalization. INPPL1 negatively regulates insulin signaling through its catalytic PtdIns(3,4,5) P_3 5-phosphatase activity.[9] The *INPPL1*$^{-/-}$ mice show a shortened snout and grow more slowly than wild-type littermates.[13] After 6 weeks of age, they showed a substantial reduced body length and body weight; however, radiographic analysis showed no gross skeletal deficit. Further studies are necessary to clarify the role of *INPPL1* in skeletal development and homeostasis.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

A De Novo Deletion at 16q24.3 Involving *ANKRD11* in a Japanese Patient With KBG Syndrome

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KBG syndrome is a rare autosomal dominant congenital syndrome comprising developmental delay with various neurological involvements, macrodontia of the upper central incisors, characteristic facial dysmorphism, and skeletal anomalies. *ANKRD11* was recently identified as the gene responsible for this syndrome. To date, there have been only five KBG syndrome families described, each carrying a single base substitution or a 1- to 14-bp deletion of this gene. Here, we present a patient with clinically confirmed KBG syndrome carrying a de novo 690-kb deletion at 16q24.3 involving part of *ANKRD11*. He had characteristic facial appearance, macrodontia of the upper central incisors, hand anomalies, delayed bone age and intellectual impairment without autistic features. Interestingly, the deleted region overlaps with the critical region for 16q24.3 microdeletion syndrome. We discuss the clinical entities of KBG syndrome and 16q24.3 microdeletion syndrome from a clinical and genetic point of view. © 2013 Wiley Periodicals, Inc.

Key words: *ANKRD11*; KBG syndrome; 16q24.3 microdeletion syndrome; de novo deletion

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INTRODUCTION

KBG syndrome (OMIM 148050) is a multiple congenital anomaly/intellectual disability syndrome characterized by macrodontia of the upper central incisors, distinctive facial dysmorphism, skeletal anomalies, and developmental delay [Herrmann et al., 1975]. Fifty-nine patients have been reported since it was first described in 1975 [Sirmaci et al., 2011]. Clinically KBG syndrome has been recognized as an under-diagnosed cause of intellectual disability, short stature, or dental/skeletal abnormalities because its clinical features are mild and not specific [Sirmaci et al., 2011]. Thus, diagnostic criteria for KBG syndrome have been proposed. At least four out of eight major criteria (macrodontia, characteristic facial appearance, hand anomalies, neurological development, delayed bone age, costovertebral anomalies, short stature, and the presence of a first-degree relative with this syndrome) should be fulfilled for diagnosis [Skjei et al., 2007]. Recently, ankyrin repeat domain 11 (*ANKRD11*) was reported to be the causative gene for KBG syndrome, with five heterozygous nucleotide substitutions or a 1- to 14-bp deletion identified among the patients [Sirmaci et al., 2011]. Here, we present a patient with KBG syndrome carrying a de novo 690-kb heterozygous deletion at 16q24.3 involving a part of *ANKRD11*. Interestingly, this deletion overlaps with the critical region for 16q24.3 microdeletion syndrome [Marshall et al., 2008; Willemsen et al., 2010; Isrie et al., 2012; Youngs et al., 2011]. We compare the clinical and genetic aspects of our patient with those of previously reported 16q24.3 microdeletion syndrome patients.

MATERIALS AND METHODS

After obtaining written informed consent, genomic DNA was extracted from blood leukocytes using QuickGene 610-L (FUJIFILM, Tokyo, Japan). Copy number analysis was performed

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using an Affymetrix Cytogenetics Whole-Genome 2.7M Array (Affymetrix, Santa Clara, CA). Quantitative real-time PCR using a Quantifast SYBR Green PCR kit (Qiagen, Germantown, MD) on a Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia) was performed to confirm the copy number change. The relative ratios of genomic DNA copy number between the patient and his parents were calculated using Rotor-Gene 6000 Series software (Corbett Life Science) with the standard curve method. Duplicate experiments were performed twice. Experimental protocols were approved by the Committee for Ethical Issues at Yokohama City University School of Medicine.

CLINICAL REPORT

The patient was the firstborn of a nonconsanguineous 38-year-old father and 34-year-old mother. He was born by cesarean because of a breech position at 37 weeks and 5 days of gestation. The family history was unremarkable. His birth weight was 2,356 g (−1.1 SD), birth length 46 cm (−0.9 SD), and occipitofrontal circumference (OFC) 33.6 cm (+0.5 SD). Transient tachypnea and generalized hypotonia were noted as a neonate. His developmental milestones were delayed. He sat unassisted at 18 months and started to walk at 31 months. At the age of 3, he was referred to Osaka Medical Center and Research Institute for Maternal and Child Health because of developmental delay and dysmorphic features.

Upon physical examination at age 4 years, his weight was 13.3 kg (−1.4 SD), height 100.6 cm (−0.3 SD), and OFC 51.5 cm (+0.6 SD). Facial dysmorphism was noted, including hypertelorism, epicanthal fold, ptosis, anteverted nostrils, long philtrum, and wide eyebrows with synophrys. At a glance, his face looked asymmetrical. His upper and lower central incisors were apparently wider than normal. Oligodontia and fusion of the incisors were observed. Both lower central incisors had central clefts (Fig. 1a). Brachydactyly of the fifth fingers was present. On neurological examination, his standing position was unstable suggesting mild truncal ataxia, while intentional tremor or dysmetria were not observed in his extremities. His eye movement was normal without any nystagmus. The deep tendon reflexes were positive within normal range. He showed no epileptic seizures. He had moderate intellectual impairment. Developmental Quotient at age 4 years was 40. His speech delay was so remarkable that he could speak almost no significant words at this age. However, he had good communication skills and used sign language for social interaction. He could play cooperatively with other kids such as playing house, point things to others, and make eye contact with others. He did not have tendency toward inflexibility or insistency. He was not hyperactive. Thus, he was considered to have no autistic features according to the DSM–IV TR criteria. Routine laboratory findings were normal. Short tubular bones of the hand were observed with a delayed bone age by X-ray examination. Brain magnetic resonance imaging showed hypoplasia of the cerebellar vermis, especially in the lower segment with a large communication between the fourth ventricle and the cysterna magna. Decreased white matter in both sides of cerebral hemispheres was observed without any heterotopic grey matter (Fig. 1b). Electroencephalogram revealed asymptomatic multifocal epileptic discharges. He fulfilled five of the eight diagnostic criteria (macrodontia of the upper central incisors, characteristic facial appearance, hand anomalies, neurological involvement, and delayed bone age) and was clinically diagnosed with KBG syndrome.

RESULTS

G-banded chromosomal analysis at the 550-band level of the patient's peripheral blood lymphocytes indicated a normal karyotype (46,XY; data not shown). High-resolution microarray analysis revealed a 690-kb heterozygous deletion at 16q24.3 (46,XY.arr 16q24.3 (88641808–89332049)x1 dn; UCSC Human Genome build 37; Fig. 1c). Although none of probes directly indicated deletion of *ANKRD11*, its 3′ portion was suspected to be deleted. Thus, we performed quantitative real-time PCR and confirmed de novo deletion of *ANKRD11* exon 13 (Fig. 1d).

DISCUSSION

Here, we describe the first KBG patient carrying a de novo microdeletion involving *ANKRD11*. Based on its nuclear localization in neurons, ANKRD11 has been proposed to function in neuronal plasticity by interacting with nuclear receptor complexes and modifying transcriptional activation [Sirmaci et al., 2011]. Interestingly, there have been several reports of patients with intellectual impairment and autism spectrum disorder with a variety of

FIG. 1. a: Facial and dental appearance of the patient. The left-hand picture shows hypertelorism, epicanthal fold, anteverted nostrils, long philtrum, and wide eyebrows with synophrys. The right-hand picture shows the upper and lower central incisors being apparently wider than normal. The lower central incisors have central clefts. b: Brain magnetic resonance imaging of the patient at 3 years old. The T1-weighted sagittal image (left) revealed hypoplasia of the lower segment of the cerebellar vermis with a large communication between the fourth ventricle, and the cysterna magna. The T1-weighted axial image (right) showed decreased white matter in both sides of cerebral hemispheres with the enlarged lateral ventricles. c: Analysis of the 16q24.2–q24.3 deletion. High-resolution microarray analysis revealed a 690-kb deletion at 16q24.2–q24.3. In the upper part, the *x, y* axis indicate the physical position of the probe, the probe signal intensity (log_2 ratio) on chromosome 16, respectively. The middle part displays a close-up of the deleted region, showing the chromosomal band, and the physical position of each probe. The thick dark gray horizontal bar indicates the deleted region detected by microarray analysis, the light gray bars indicate the suspected deleted region for which no probes were available. The lower part shows the deletions previously described in patients with 16q24.3 microdeletion syndrome or KBG-like syndrome, along with the RefSeq genes. Youngs et al. did not report the precise physical position of the deletion in their patient (shown as a light gray dotted bar). The shortest region of overlap among these deletions contains only *ANKRD11* and overlaps with the suspected deleted region in our patient. d: Quantitative real-time PCR analysis of exons 13 and 4 of *ANKRD11*. The *y* axis shows the relative ratio of the copy number. De novo deletion of exon 13 was confirmed in the patient. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmga]

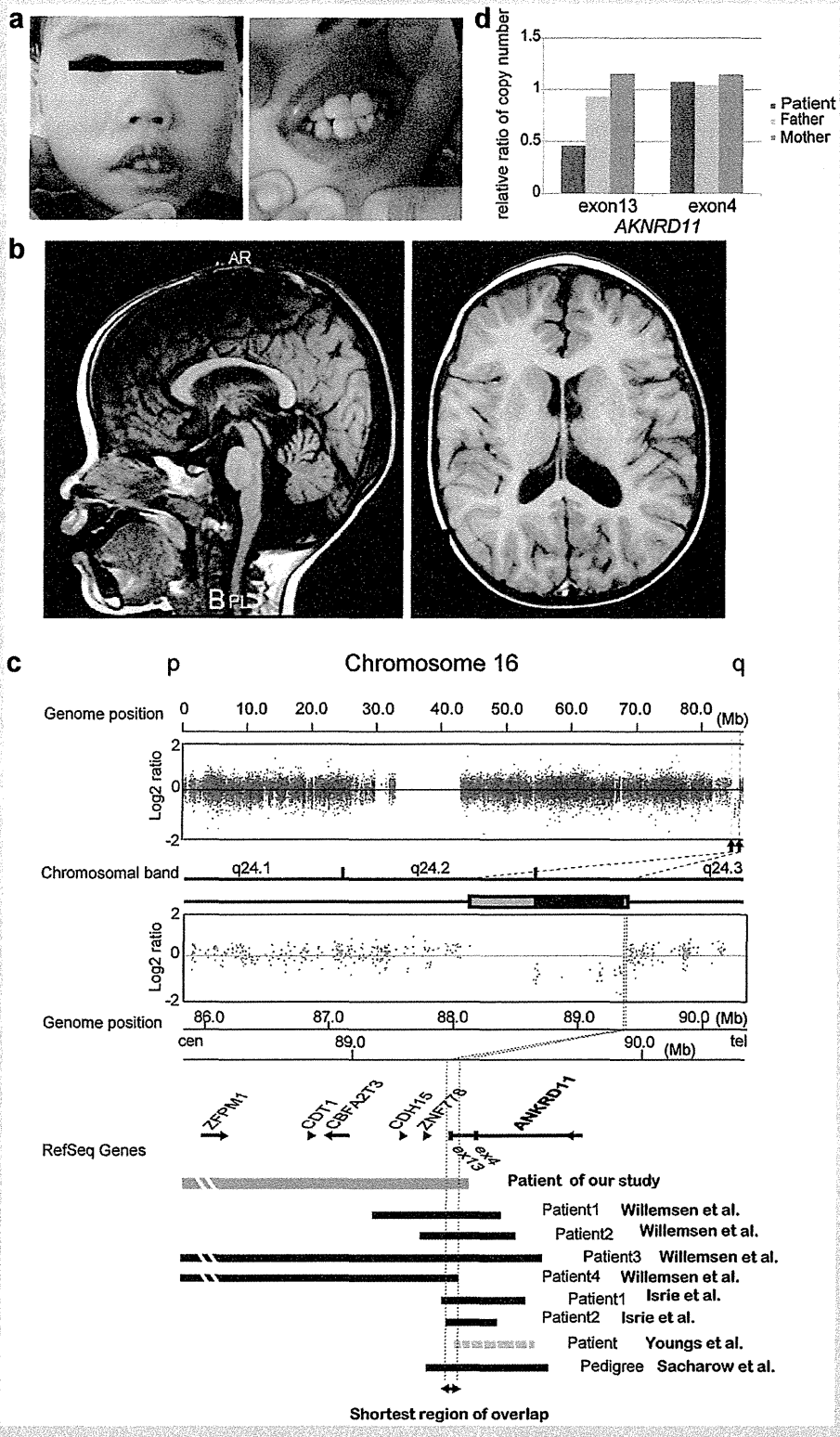
a
d
relative ratio of copy number
1.5
1
0.5
0
exon13
exon4
AKNRD11
Patient
Father
Mother
b
AR
BPL
c
p
Chromosome 16
q
Genome position
0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(Mb)
Log2 ratio
2
0
-2
Chromosomal band
q24.1
q24.2
q24.3
Log2 ratio
Genome position
86.0
87.0
88.0
89.0
90.0 (Mb)
cen
89.0
90.0 (Mb)
tel
ZFPM1
CDT1
CBFA2T3
CDH15
ZNF778
ANKRD11
RefSeq Genes
ex13
ex4
Patient of our study
Patient1 Willemsen et al.
Patient2 Willemsen et al.
Patient3 Willemsen et al.
Patient4 Willemsen et al.
Patient1 Isrie et al.
Patient2 Isrie et al.
Patient Youngs et al.
Pedigree Sacharow et al.
Shortest region of overlap

FIG. 1.

dysmorphic features carrying a de novo microdeletion of 16q24.3 [Marshall et al., 2008; Willemsen et al., 2010; Isrie et al., 2012; Youngs et al., 2011]. The region of overlap among these patients has been narrowed down to 28.3 kb (nucleotides 89335228–89363602; UCSC Human Genome build 37), in which the only protein-coding gene is *ANKRD11* (Fig. 1c). The 16q24.3 microdeletion has been proposed to be a novel locus for autosomal dominant intellectual impairment and autism spectrum disorder. Although dental information is scarce among the patients with 16q24.3 microdeletion syndrome, the other clinical features, such as facial dysmorphism or skeletal abnormalities, are similar to those of KBG syndrome (Table I). Recently, familial patients with a KBG-like syndrome carrying an inherited 16q24.3 deletion containing *ANKRD11* were reported [Sacharow et al., 2012]. They had sufficient clinical findings to fulfill the diagnostic criteria for KBG syndrome. The deletion in our KBG syndrome patient overlaps with this critical region at 16q24.3 (Fig. 1c).

Although these two syndromes share clinical manifestations, there may be some differences between the two. For example, the detailed manifestation of neurological involvement looks somehow different. In 16q24.3 microdeletion syndromes, intellectual impairment is relatively mild and autistic spectrum disorders are frequently observed [Willemsen et al., 2010; Isrie et al., 2012]. On the other hand, in KBG syndrome, intellectual disability ranges from mild to severe, and common behavioral disturbances are hyperactivity, attention deficit or easy frustration rather than autistic features [Brancati et al., 2006]. At this point, our patient and the familial patients reported by Sacharow et al. [2012] are very significant in that they have 16q24.3 microdeletion, but their neurological symptoms are closer to those of KBG syndrome. Haploinsufficiency of *ANKRD11* has to be confirmed in 16q24.3 microdeletion syndrome to clarify whether these two syndromes are different. Clinical differences might be due to the ambiguous diagnostic criteria for KBG syndrome or to additional deleted gene(s) being associated with other features in 16q24.3 microdeletion syndrome. To resolve this, it is necessary to study further patients with these two syndromes.

The unique finding of our case is hypoplasia of cerebellar vermis, especially in the lower segment. The deleted region in 16q24.3 of our patient did not contain any known causative genes for cerebellar hypoplasia. In the literature review, there are two patients with KBG syndrome that had hypoplastic lower segment of cerebellar vermis. One of them had difficulty in keeping the standing position or walking because of cerebellar ataxia [Zollino et al., 1994]. Thus, we speculate that hypoplasia of the cerebellar vermis, especially in the lower segment, is a rare complication of KBG syndrome. Since there has not been any case of 16q24.3 microdeletion syndrome with this finding, it might be one of the hallmarks for KBG syndrome.

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TABLE I. Comparison of the Clinical and Genetic Features of Patients With KBG Syndrome and 16q24.3 Microdeletion Syndrome

		Sacharow et al.			Isrie et al.		Willemsen et al.				
Clinical information	**Our patient Proband**	**Proband**	**Mother**	**Youngs et al. Proband**	**Patient 1**	**Patient 2**	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**Sirmaci et al. Family 1 (II-1)**
Clinical diagnosis	KBG syndrome	KBG-like syndrome		16q24.3 microdel	16q24.3 microdel		16q24.3 microdel				KBG syndrome
Genetic background	16q24.3 deletion	16q24.3 deletion		16q24.3 deletion	16q24.3 deletion		16q24.3 deletion				*ANKRD11* intragenic mutation
Sex	M	M	F	M	F	M	M	M	M	M	M
1. Macrodontia	+	NA	+	unk	unk	unk	unk	unk	unk	+	+
2. Characteristic facial anomaly	+	+	+	+	+	+	+	+	+	+	+
3. Hand anomalies	+	+	+	−	+	+	−	−	−	+	+
4. Neurological involvement	+	+	+	+	+	+	+	+	+	+	+
Intellectual disability	+	+	−	+	+	+	+	+	+	+	+
ASD	−	−	−	+	−	−	+	+	+	+	−
ADHD/Learning disability	−	+	+	−	+	+	−	−	−	−	−
Seizure	−	−	−	−	−	+	+	−	+	−	+
5. Delayed bone age	+	−	NA	unk	unk	unk	unk	unk	unk	unk	+
6. Costovertebral anomalies	−	−	unk	unk	unk	−	+	unk	unk	unk	+
7. Postnatal short stature	−	−	+	+	+	+	+	+	+	+	+
8. 1st degree relative with KBG	−	+	+	−	−	−	−	−	−	−	+
Number of criteria fulfilled	5	4	6	3	4	4	4	3	3	5	8

ASD, autistic spectrum disorder; ADHD, attention deficit hyperactivity disorder; M, male; F, female; NA, not applicable; unk, unknown; 16q24.3 microdel, 16q24.3 microdeletion syndrome.

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Pathogenic mutations in two families with congenital cataract identified with whole-exome sequencing

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Purpose: Congenital cataract is one of the most frequent causes of visual impairment and childhood blindness. Approximately one quarter to one third of congenital cataract cases may have a genetic cause. However, phenotypic variability and genetic heterogeneity hamper correct genetic diagnosis. In this study, we used whole-exome sequencing (WES) to identify pathogenic mutations in two Korean families with congenital cataract.
Methods: Two affected members from each family were pooled and processed for WES. The detected variants were confirmed with direct sequencing.
Results: WES readily identified a *CRYAA* mutation in family A and a *CRYGC* mutation in family B. The c.61C>T (p.R21W) mutation in *CRYAA* has been previously reported in a family with congenital cataract and microcornea. The novel mutation, c.124delT, in *CRYGC* may lead to a premature stop codon (p.C42Afs*60).
Conclusions: This study clearly shows the efficacy of WES for rapid genetic diagnosis of congenital cataract with an unknown cause. WES will be the first choice for clinical services in the near future, providing useful information for genetic counseling and family planning.

Congenital cataract is one of the most frequent causes of visual impairment and childhood blindness worldwide, with an estimated incidence of 2.49 per 10,000 live births by the age of 1 year in the United Kingdom [1]. Congenital cataract is also the leading cause of treatable blindness in childhood. Good outcomes have been reported in children undergoing surgery before 6 weeks of age in bilateral cases [2]. Early diagnosis in the postnatal unit is important for obtaining good visual function.

Many causes have been considered for congenital cataract: intrauterine infection, exposure to drug or radiation in pregnancy, gene defects, chromosomal disorders, metabolic disease, and trauma [3]. Approximately one quarter to one third of congenital cataract cases may have a genetic cause and often follow a Mendelian inheritance pattern, with autosomal dominant traits more common than autosomal recessive and X-linked traits [4,5]. Inter- and intrafamilial phenotypic variability has been reported in cases of inherited congenital cataract [6,7]. It may occur as an isolated eye anomaly, in association with other ocular anomalies, or as part of a systemic disorder. Congenital cataracts are caused by mutations in various types of genes: lens-related crystallin genes (*CRYAA*, *CRYAB*, *CRYBB1*, *CRYBB2*, *CRYBB3*, *CRYBA1*, *CRYBA4*, *CRYGC*, *CRYGD*, and *CRYGS*), membrane protein genes (*GJA3*, *GJA8*, *MIP*, and *LIM2*), cytoskeleton-related genes (*BFSP1* and *BFSP2*), and transcription factor genes (*FOXE3*, *HSF4*, *MAF*, *PITX3*, and *PAX6*) [8]. Weisschuh et al. reported that mutations in crystallin genes occupied 50% of all mutations in known disease-causing genes [9], suggesting that mutations in the crystallin genes are particularly abundant.

Whole-exome sequencing (WES) targeting all the protein-coding genes is powerful and cost-effective for dissecting the genetic basis of diseases [10]. WES is particularly useful for identifying pathogenic mutations for Mendelian disorders for which conventional approaches are difficult (such as when most cases are sporadic).

In this report, we performed WES on two Korean families with congenital cataract inherited in an autosomal dominant fashion. We identified pathogenic mutations in both families and demonstrated the diagnostic utility of WES in congenital cataract.

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METHODS

Clinical report: The two Korean families with congenital cataract have been described previously (Figure 1A) [11]. Samples from family A with 6 affected (3 females and 3 males) and 3 unaffected members (1 female and 2 males) and family B with 3 affected (1 female and 2 male) and 1 unaffected (female) members were collected at Seoul National University College of Medicine. In family A, the proband (MC41) was diagnosed with congenital cataract and microphthalmia. Other ocular anomalies were noted, including nystagmus, amblyopia, glaucoma, and esotropia. The cousin of the proband (MC42) showed congenital cataract but no microphthalmia. Nystagmus and amblyopia were also noted. In family B, the older sister (MC13, the proband) and the younger brother (MC14) showed congenital cataract. Other ocular anomalies were found, including nystagmus and amblyopia. Systemic abnormalities, intellectual disability, and developmental malformation were unrecognized, and other possible causes such as trauma, intrauterine infection, exposure to drug or radiation, and metabolic disease were unlikely to be involved in both families.

Whole-exome sequencing: Blood was collected from antecubital veins of family members and subsequently treated with a Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen) for preparing genomic DNA of leukocytes. Experimental protocols were approved by the Institutional Review Board for Ethical Issues at Yokohama City University School of Medicine and the Committee for Ethical Issues on the Human Genome and Gene Analysis, Seoul National University. Informed consent was obtained from all individuals.

Because the DNA samples were limited, 1.5 μg of DNA from each of two affected members in the respective families were combined, and were processed using a SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA) to generate exome libraries. The libraries were sequenced with one lane per sample of the flow-cell on an Illumina GAIIx (Illumina Inc., San Diego, CA) with 107-bp paired-end reads, according to the manufacturer's instructions. Image analysis and base calling were performed with Sequence Control Software with Real-Time Analysis (Illumina) and CASAVA software v1.7 (Illumina). Reads were aligned and mapped to the human reference genome sequence (University of California Santa Cruz [UCSC] Genome Browser hg19, National Center for Biotechnology Information [NCBI] genome sequence website build 37) using MAQ [12] and NextGENe software v2.00 with sequence condensation by consolidation (SoftGenetics, State College, PA). Single nucleotide variants (SNVs) were called using MAQ and NextGENe. Small insertions and deletions were detected using NextGENe. Called SNVs were annotated with SeattleSeq Annotation. Candidate variants were confirmed with Sanger sequencing with a 3130xL or 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA). The Human Gene Mutation Database (HGMD; Biobases, Wolfenbuettel, Germany) was used to check whether the variants had been previously reported. Polymorphism Phenotyping (PolyPhen-2), Sorting Intolerant from Tolerant (SIFT), and MutationTaster were used to evaluate variants in terms of sequence conservation, chemical change, and likelihood of pathogenicity.

RESULTS

With WES, we attained more than 86% target coverage by ten reads or more (Appendix 1). We adopted a prioritization schema to identify the pathogenic mutation in each pooled sample as follows (Table 1). First, we excluded the variants registered in the Single Nucleotide Polymorphism database (dbSNP132) or the 1000 Genomes project. Then, SNVs commonly detected with MAQ and NextGENe were selected as highly confident variants. In family A, we identified 671 non-synonymous or canonical splice site change SNVs along with 100 small insertions or deletions. We surveyed these for mutations in the 26 known congenital cataract genes and 19 anophthalmia or microphthalmia genes (Appendix 2). We found a heterozygous mutation (c.61C>T [p.R21W]) in exon 1 of *CRYAA* (NM_000394.2), which was confirmed with Sanger sequencing (Figure 1B,C; Table 1). The mutation occurred at an evolutionarily conserved amino acid (Figure 1D), and was previously reported in a family with congenital cataract and microcornea [7]. The mutation completely cosegregated with the cataract phenotype in this family (Figure 1A).

In family B, we similarly identified 454 non-synonymous or canonical splice site SNVs, and 135 small insertions or deletions (Table 1). We found a novel heterozygous frameshift mutation, c.124delT (p.C42Afs*60) in *CRYGC* (NM_020989.3), and confirmed the presence of the mutation in MC13 but not in MC13b with Sanger sequencing (Figure 1E,F; Table 1). Although we pooled DNA from MC13b and MC13 based on our initial clinical information (Figure 1A), MC13b was actually unaffected (because of an error in information transfer). After the phenotypic information for this family was corrected, the mutation completely cosegregated with the cataract phenotype, as confirmed with Sanger sequencing (Figure 1A). This 1-bp deletion would be expected to result in the insertion of 60 new amino acids after the mutation site with a premature stop codon at position 102 (p.C42Afs*60). This mutation was not found in the

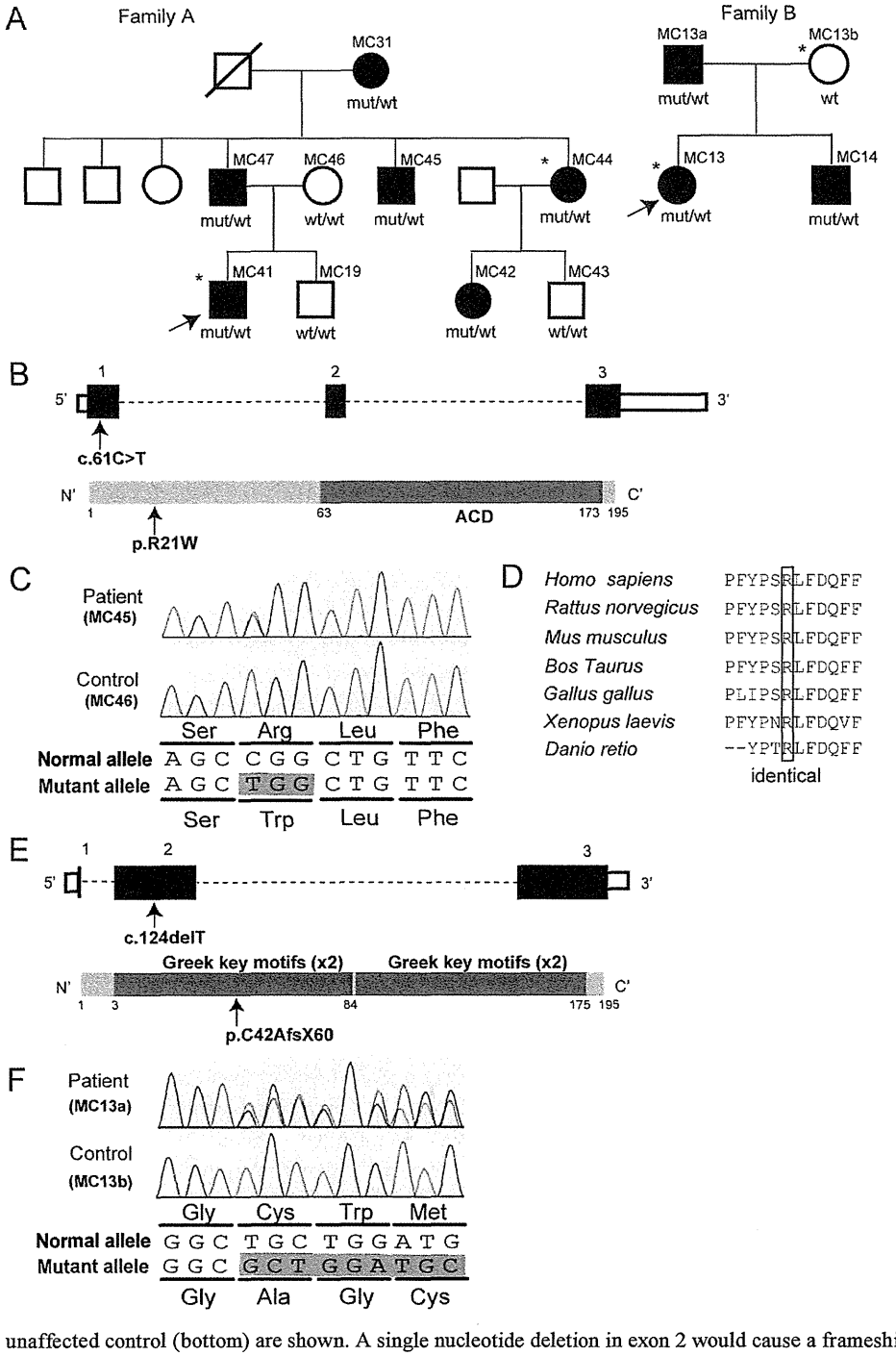

Figure 1. *CRYAA* and *CRYGC* mutations in two Korean families. **A**: Pedigrees of families A and B are indicated. Black and open symbols denote affected and unaffected individuals, respectively. The asterisk shows samples used for whole-exome sequencing. The mutations cosegregate with the phenotype. **B**: Schema of the *CRYAA* gene (top) and the CRYAA protein (bottom) is presented. The untranslated regions and coding region are shown as open and filled rectangles, respectively. The location of the c.61C>T mutation is indicated with an arrow. CRYAA contains an N-terminal region, an α-crystallin domain (ACD, dark gray box), and a C-terminal region. **C**: Electropherograms of the mutation in the affected patient (top) and the unaffected control (bottom) are shown. A single nucleotide change in exon 1 results in an amino acid alteration. **D**: The missense mutation occurred at an evolutionarily conserved amino acid. Homologous sequences were aligned using CLUSTALW. **E**: Schema of the *CRYGC* gene (top) and the CRYGC protein (bottom) is presented. The untranslated regions and the coding region are shown as open and filled rectangles, respectively. The location of the c.124delT mutation is indicated with an arrow. CRYGC contains two domains each composed of two Greek-key motifs (dark gray boxes). **F**: Electropherograms of the CRYGC mutation in the affected patient (top) and in the unaffected control (bottom) are shown. A single nucleotide deletion in exon 2 would cause a frameshift. mut, mutant allele; wt, wild-type allele.

National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) Exome Variant Server that contains data from more than 5,400 exomes, or among our in-house exome data from 135 individuals.