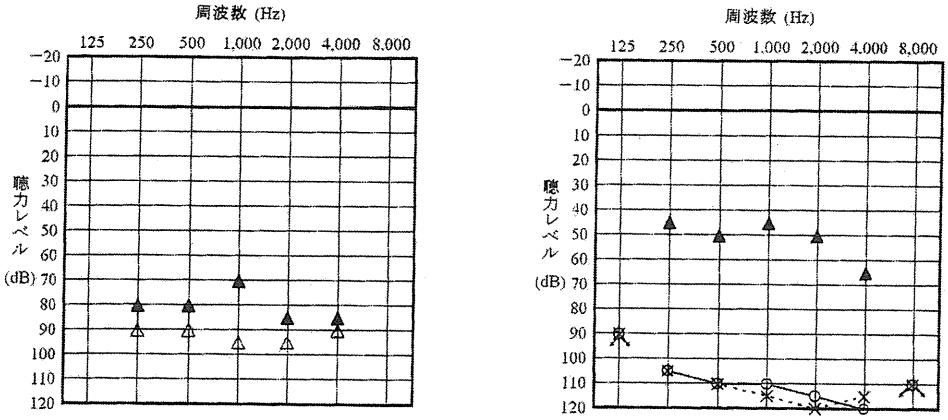
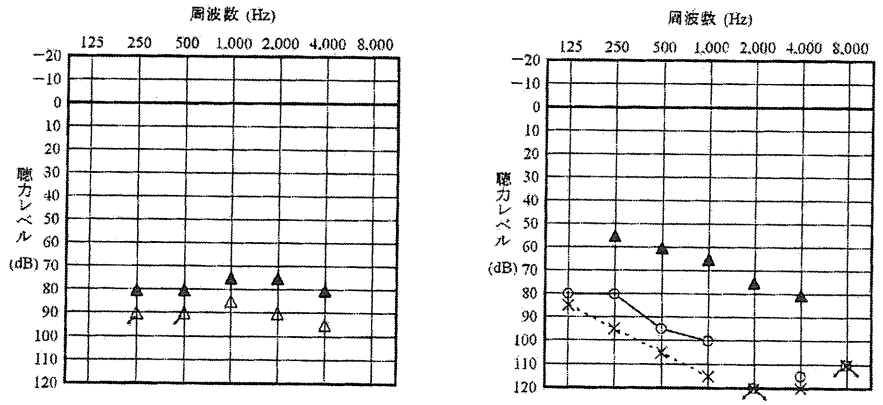


(a) 3歳7カ月COR結果　　(b) 6歳4カ月純音検査結果

図2　症例1の聴力検査結果

(a) 3歳7カ月時COR検査結果：音への反応はっきりせず、閾値も不安定。
(b) 6歳4カ月時純音聴力検査結果：閾値安定するが、音声言語の聞き取りは極めて不良。



(a) 2歳8カ月COR結果　　(b) 5歳9カ月遊戯聴力検査

図3　症例2の聴力検査結果

(a) 2歳8カ月時のCOR検査結果：初診時以降COR検査を反復したが、反応が一定せずに経過。
(b) 5歳9カ月時の遊戯聴力検査結果。検査中、音への反応は確認できるが、日常生活での音声の聞き取りは困難。

伝子解析にて1アレルにミスセンス変異が確認された。現在補聴器を装用して聾学校通学中であるが、音声によるコミュニケーションは困難である。

症例3：初診時0歳3カ月男児

生育歴、既往歴、家族歴：特記すべきことなし

現病歴：自動ABRによるNHSで右側要精査のため精査目的に当院を受診。

初診時検査所見：ABR検査では両側105dBnHLでV波確認できず、両側難聴の診断となる。DPOAEは右一部正常、左は正常で、ASSRでは右90dB、左110dB、CORは40－50dBと、各検査結果に乖離を認めた。図4は1歳7カ月時の聴力検査結果である。

経　過：補聴器装用、療育を開始し、徐々に音声の模倣が出現した。現在2歳6カ月であり、同程度の聴力の他の難聴児と比較すると言語発達はゆっくりで構音は不明瞭な部分も多いが、日常生活は音声によるコミュニケーションが可能となってきている。OTOF遺伝子解析では1アレルにミスセンス変異が確認された。

考　察

ANの中にはいくつかの疾患が含まれており、2008年にNHS 2008 ConferenceからANSDという疾患概念に基づいて、本疾患の乳幼児に対するidentificationとmanagementについてのガイドラインが提唱された[3]。

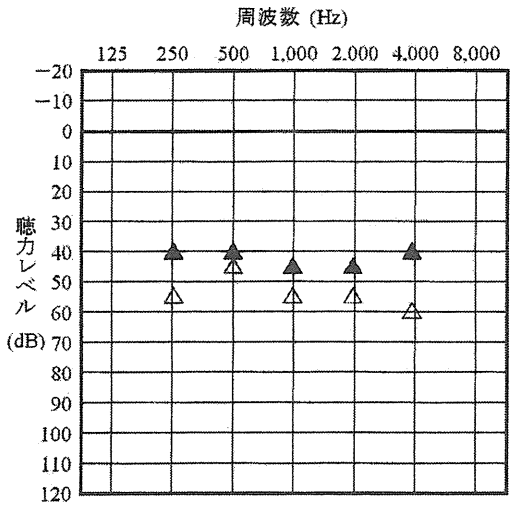


図4　症例3の1歳7カ月時のCOR検査結果

その中では、蝸牛外有毛細胞機能は正常で、聴神経の機能が異常であるものをANSDと呼び、OAEまたは蝸電図が正常あるいはほぼ正常で、ABRが異常のものと定義している。しかし、各症例の経過は様々であり、補聴器や人工内耳が有効な例も報告されている[6]が、語音明瞭度が極端に悪い例も多く、補聴器装用による通常の補聴、療育では効果が十分でないことが多いとされている。OTOF遺伝子変異による難聴症例は、人工内耳の効果があると報告されており[4),5)]、ANが疑われた症例では治療方法の選択のためにも遺伝子検査は有用であると考えられる。

OTOF遺伝子はotoferlin蛋白をコードする遺伝子で、非症候性難聴の劣性遺伝子であり、ANの原因遺伝子の1つである。Otoferlinは蝸牛、前庭、脳に分布し、内有毛細胞のシナプス小胞の膜融合と放出に関与していると考えられている[7]。OTOF遺伝子変異による難聴は、臨床的には先天性非症候性の高度難聴で外有毛細胞が正常であるためOAEは正常でありANと診断される。OTOF遺伝子変異が確認された症例では、その病態から蝸牛神経には異常がなく、補聴器の効果が得られなかった場合でも人工内耳の効果が期待できると考えられる。しかし、今回の症例3や泰地らの報告でもOTOF遺伝子変異による難聴例でも補聴器の有効症例が報告されており[6]、すべてのOTOF遺伝子変異難聴が人工内耳の適応ではないだろう。

Rodriguez-BallesterosらはOTOF遺伝子解析を行い、289例の非症候性で原因不明の言語習得前難聴児中15例(5.2%)に、また多施設研究では非症候性で明らかな原因のない難聴821例中31例(3.8%)にOTOF遺伝子変異を確認している[8]。人種による差はあるが、OTOF遺伝子変異による難聴は、遺伝性難聴の中では、決して頻度の少ない疾患ではないと考えられている。

症例1は2アレルにOTOF遺伝子変異が同定されたが、症例2、3は1アレルのみのOTOF遺伝子変異であった。1アレルのみの変異ではOTOF遺伝子変異が難聴の原因とは断定はできないが、今回の3症例は、ABRの結果とDPOAEの結果に乖離があり、通常の内耳性難聴児の検査所見、臨床経過とは異なる点が多く臨床的にはANと診断できると考えている。2症例の難聴の説明としては、1）今回の遺伝子解析方法では検出できないプロモーター領域の変異やスプライシングに影響を与えるイントロンの変異、あるいは大規模な欠失/重複がある可能性、2）OTOF遺伝子産物であるOtoferin蛋白と共同して作用する他の蛋白質の遺伝子に変異がある可能性、3）症例で見つかったOTOF遺伝子変異の他に難聴の原因があり、今回見つかったOTOF遺伝子変異は偶然保因者であった可能性等が考えられる。しかし、症例2、3ともに臨床所見からはANと診断されるため、何らかのOTOF遺伝子変異が関与が強く疑われ、3）の可能性は低いと考えられた。

ABRが異常でOAEが正常であるANは、外有毛細胞機能は正常である難聴であり、内有毛細胞の異常、内有毛細胞と樹状突起のシナプスの異常、らせん神経節の異常、第Ⅷ脳神経の異常、あるいはこれらの組み合わせ等が考えられる。OTOF遺伝子変異による難聴の場合は、内有毛細胞のシナプス障害であるが、Rodriguez-BallesterosらはOTOF遺伝子変異が確認された難聴41例中TEOAE検査を施行した34例中6例はTEOAEが異常であったと報告している[8]。OAEの消失に関しては、補聴器装用による外有毛細胞の障害などの環境要因や遺伝的要因が考えられているが、その機序は不明である。今回の症例でも、症例1では初診時に正常であったDPOAEが徐々に異常となっており、症例2も新生児期あるいは乳児期に検査していれば両側正常だった可能性があり、検査時にDPOAEが異常であり通常の内耳性難聴のパターンを示す症例の中にも当初はANのパターンを示していた症例が隠れている可能性が示唆された。

難聴児のうちANの比率は乳幼児では10－15.4%、聾学校在籍中の小児では1.6－4%と報告されている[9),10)]。乳幼児では高ビリルビン血症等一過性のANが含まれているために頻度が高くなっている可能性と、経過中にOAEが消失するためにANと診断されない児が多くな

っているために、頻度に差が生じていると考えられる。

今回の症例は難聴診断当初、正確な聴力閾値の把握に苦慮した。ABR、ASSR、DPOAEに乖離を認めただけではなく、聴性行動上も通常の内耳性難聴児とは異なる経過であった。音への反応が極めて不明瞭であり、CORの条件付けが困難であった。症例1、2では遊戯聴力検査が可能になるまでの期間は、聴力検査時には集中できず、補聴器を装用しても日常生活上も音への反応は極めて不良であった。現在まで3症例には明らかな発達障害は確認されず、これは他の内耳性難聴児と異なるANの特徴であると推測された。ANでは、ASSRが実際の聴力に近い値を示すことも多いとの報告もある[6]が今回の症例では一致しておらず、正確な聴力像は他覚的聴力検査所見と、日常生活の聴性行動の観察から総合的な評価が必要であると考えられた。

まとめ

臨床所見よりANが疑われた難聴児4症例でOTOT遺伝子解析を行い、3症例で変異を認めた。1症例は2アレルに、2症例は1アレルのみの変異が確認された。3症例とも、各検査所見に乖離を認めていたが、臨床経過はやや異なっていた。

NHSの普及により早期にANと診断される難聴児が増加すると考えられたが、臨床経過は様々であること、遺伝子解析が診断の一助となる可能性があること、診断時期によりANと診断されなかった難聴児の中にもANと診断される児と同様の臨床経過を取る児が含まれている可能性あることが示唆された。

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Prenatal molecular diagnosis of a severe type of L1 syndrome (X-linked hydrocephalus)

Clinical article

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Object. The aim of this study was to evaluate the feasibility of prenatal *L1CAM* gene testing for X-linked hydrocephalus (XLH).

Methods. In a nationwide study conducted in Japan between 1999 and 2009, the authors identified 51 different *L1CAM* gene mutations in 56 families with XLH. Of these 56 families, 9 obligate carriers requested prenatal gene mutation analysis for the fetal *L1CAM* gene in 14 pregnancies.

Results. In 2004, new clinical guidelines for genetic testing were established by 10 Japanese genetic medicine–related societies. These guidelines stated that the genetic testing of carriers should be done only with their consent and with genetic counseling. Therefore, because females are carriers, since 2004, *L1CAM* gene analysis has not been performed for female fetuses. The authors report on 7 fetal genetic analyses that were performed at the request of families carrying *L1CAM* mutations, involving 3 female (prior to 2004) and 4 male fetuses. Of the 7 fetuses, 3 (1 male and 2 female) carried *L1CAM* mutations. Of these 3, 1 pregnancy (the male fetus) was terminated; in the other cases, the pregnancies continued, and 3 female and 3 male babies without the XLH phenotype were born.

Conclusions. Prenatal *L1CAM* gene testing combined with genetic counseling was beneficial for families carrying *L1CAM* mutations. *(DOI: 10.3171/2011.7.PEDS10531)*

Key Words • **X-linked hydrocephalus** • ***L1CAM* gene** • **prenatal diagnosis** • **amniotic fluid aspiration** • **chorionic villous sampling**

Congenital hydrocephalus is one of the most common and serious developmental malformations treated in pediatric neurosurgery. Currently, more than 60% of congenital hydrocephalus cases are diagnosed prenatally as fetal ventriculomegaly, because of advances in prenatal imaging techniques. In fetal hydrocephalus cases, the prognosis of isolated ventriculomegaly varies widely, because it is indicative of multiple diseases. Therefore, effective tools for accurately predicting the functional outcome are required.

On the other hand, recent advances in molecular biology have made great contributions toward establishing a molecular diagnosis for some hereditary diseases. In congenital hydrocephalus, the genetic understanding of

Abbreviations used in this paper: AC = amniocentesis; CVS = chorionic villous sampling; PCR = polymerase chain reaction; XLH = X-linked hydrocephalus.

XLH has advanced over the past 20 years, since the first family with XLH due to a mutation in *L1CAM*, which is the gene encoding the neural cell adhesion molecule L1, was reported in 1992.[17] A member of the Ig superfamily of cell adhesion molecules, L1CAM is expressed predominantly in developing neurons.[5] Mutations in the gene for L1CAM are now known to be responsible for many cases of XLH: mental retardation, adducted thumbs, shuffling gait, and aphasia (also known as MASA) syndrome; certain forms of X-linked spastic paraplegia; and X-linked agenesis of the corpus callosum. Therefore, these syndromes have been reclassified as L1 syndrome.[2,4,7–11,17]

The *L1CAM* gene is located on the X chromosome in humans and is composed of 28 exons. The open reading frame has 3825 bp that encode a protein of 1257 amino acids.[5] According to an updated (January 19, 2011) database of *L1CAM* gene mutations [on a website maintained by Yvonne Vos[20] from the Department of Genetics, University Medical Center Groningen, The Netherlands], more than 200 mutations have been found in unrelated families carrying the L1 syndrome. In Japan, we conducted a nationwide investigation of *L1CAM* gene mutations and identified 51 types of *L1CAM* mutations in 56 unrelated families. Some of these mutations have already been reported, and we established neuroradiological criteria for a severe form of L1 syndrome.[8]

With its well-known characteristics, including ventricular enlargement, adducted thumbs, and severe developmental retardation,[22] XLH is relatively easily diagnosed. However, the prenatal differential diagnosis of XLH from other kinds of disease showing isolated ventriculomegaly that have a good outcome is not easy. Therefore, in this study, we evaluated the feasibility of prenatal *L1CAM* gene testing as a clinical tool for XLH.

Methods

Patients and Protocol for Prenatal Gene Testing

We conducted a nationwide *L1CAM* gene analysis and identified *L1CAM* mutations in 60 patients and 41 carriers in 56 families.[8,9] Genetic counselors informed women carrying an *L1CAM* gene mutation of the guidelines for prenatal genetic analysis, and after fully explaining the procedure, obtained informed written consent from carriers who were pregnant. In 2004, new clinical guidelines for genetic testing were established by 10 Japanese genetic medicine–related societies (Guidelines 2004[3]). These guidelines recommend that carriers be tested voluntarily with their informed consent; thus, carrier testing in childhood is prohibited. Therefore, since the release of Guidelines 2004,[3] only male fetuses have been tested (Fig. 1).

This study was approved by the Institutional Review Board of Osaka National Hospital and was performed in accordance with the principles of the Declaration of Helsinki, as well as the ethics guidelines for human genome/gene analysis research devised by the Ministry of Education, Culture, Science, and Technology; the Ministry of Health, Labor, and Welfare; and the Ministry of Economy, Trade, and Industry of Japan.

Procedures for DNA Sampling and Karyotype Testing

Fetal tissues or cells were isolated by CVS at 10–12 weeks of gestation or by AC at 15–16 weeks of gestation. Fetal cells were cultured in vitro, and their karyotypes were examined by standard techniques. The DNA was then extracted from the cultured fetal cells (Fig. 1).

Genetic Analysis

Each mutation site of the *L1CAM* gene was amplified by PCR analysis; full details have been described previously.[8,9] Briefly, 100 ng of genomic DNA was used for PCR amplification. Each PCR product was separated by electrophoresis on a 2% agarose gel and purified using the Power Prep Express Gel Extraction System (Marligen Biosciences, Inc.). The purified products were examined by direct sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), followed by analysis on a DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The DNA sequencing was performed in both directions for each template.

Results

In the cases reported here, 9 obligate carriers request-

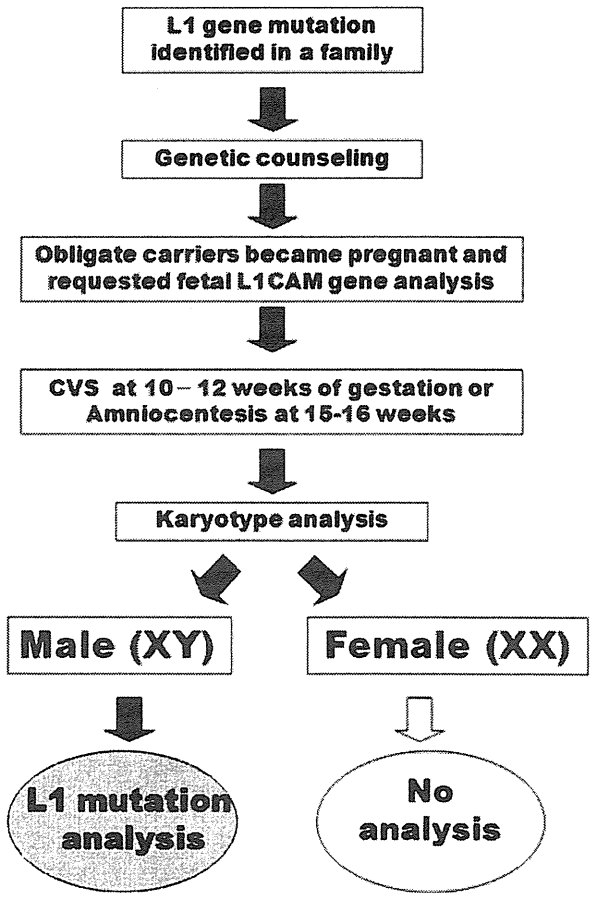

FIG. 1. Flow chart showing the protocol for prenatal *L1CAM* mutation analysis used at Osaka National Hospital.

ed prenatal testing 14 times in total (Table 1). The fetuses were tested for sex, *L1CAM* mutations, or both, using CVS or AC. Of the 14 fetuses, 4 were found to be male and 10 were female. Prenatal genetic testing was performed on 7 of the fetuses—3 females (prior to 2004) and 4 males. Of the 4 male fetuses tested, only 1 (HC53) had an *L1CAM* gene mutation (c.1146C > A, p.Tyr382X). The mother terminated the pregnancy. Of the 3 female fetuses tested, 1 did not carry the mutation, and 2 (HC16, HC13) were heterozygous carriers (c.665delA, c.694+5G > G/A). Prenatal genetic testing was not performed on the other 7 female fetuses, as directed by Guidelines 2004.[3] In 13 of the 14 cases, the mothers continued their pregnancies and delivered healthy babies without an XLH phenotype. The diagnosis was made with perfect accuracy. In 12 of the 14 cases, the DNA was obtained by CVS between 10 and 12 weeks of gestation, and in 2 cases, it was obtained by AC between 15 and 16 weeks of gestation. No maternal or fetal complications occurred during either the CVS or AC procedures. The results of the genetic analysis were available within 3 weeks after CVS or AC.

Illustrative Cases

Cases 10 and 11

The first-born boy (III-1) in this family (HC27; Fig. 2) showed severe hydrocephalus with adducted thumbs. The mother's family history was unremarkable. An *L1CAM* gene analysis of the patient (III-1), his mother (II-2), her sister (II-3), and their maternal cousin (II-4) was performed. The DNA examination revealed that the patient's *L1CAM* gene had a G1829-1 to C transition located 1 bp downstream from the 5′ end of intron 14. This transition was at a splice site, which would cause aberrant splicing in the sixth Ig-like domain of *L1CAM*. The mother (II-2) was heterozygous for the same mutation. The mother's sister and their cousin (II-3 and II-4, respectively) did not carry the mutation. The mother (II-2) was pregnant and requested prenatal genetic testing as part of genetic counseling. At 11 weeks of gestation, CVS and karyotype testing showed that the fetus was female. In accordance with Guidelines 2004,[3] genetic analysis was not performed. The mother continued her pregnancy and delivered a healthy girl (III-2). The mother (II-2) became pregnant again and requested prenatal genetic testing. At 11 weeks of gestation, DNA was obtained by CVS, and karyotype testing showed that the fetus was male. The DNA analysis determined that the fetus did not have an *L1CAM* gene mutation. The mother continued her pregnancy and delivered a healthy boy (III-3).

Case 12

In this family (HC45; Fig. 3), the mother (I-2) was in her third pregnancy. The mother's family history was unremarkable. Her first pregnancy had aborted spontaneously at 8 weeks of gestation (II-1). The second baby, a female (II-2), died in utero due to a twisted umbilical cord at 35 weeks. The third baby was male (II-3), and ventricular dilation was detected at 20 weeks of gestation. An ultrasound echogram revealed bilaterally adducted thumbs. The diagnosis in this fetus was severe hydrocephalus with bilateral adducted thumbs, without family history. The mother chose to terminate her pregnancy. An *L1CAM*

TABLE 1: Case summaries for families in which fetuses underwent prenatal *L1CAM* mutation analysis*

Case No.	Family ID; Origin	DNA Test	Family *L1CAM* Mutation	Sex of Fetus	*L1CAM* Mutation on Gene Testing	Outcome
1†	HC17; Osaka City General Hospital	10-wk CVS	c.1963A>G	F	not detected	37 wks, 2820 g; healthy
2		9-wk CVS	p.Lys655Glu,	F	not done	healthy
3		11-wk CVS	c.924C>T p.Gly308Gly	F	not done	healthy
4†	HC22; Tokyo University	12-wk CVS	c.2872+1G>A	M	not detected	40 wks, 3326 g; healthy
5		12-wk CVS		F	not done	40 wks, 3165 g
6†	HC16; Nagaoka Red Cross Hospital	12-wk CVS	c.665delA p.Lys222fs	F	c.665delA, heterozygous	40 wks, 3215 g; healthy
7†	HC13; Nagoya City University	11-wk CVS	c.694+5G>A	F	c.694+5G>G/A, heterozygous	healthy
8	HC28; Hokkaido University	16-wk AC	c.1373T>A p.Val458Asp	F	not done	healthy
9	HC36; Nagoya City University	10-wk CVS	c.2278C>T p.Arg760X	F	not done	healthy
10 (III-2)	HC27; Nagoya City University	11-wk CVS	c.1829-1G>C	F	not done	healthy
11 (III-3)		11-wk CVS		M	not detected	37 wks, 3260 g; healthy
12	HC45; CRIFM	16-wk AC	c.817-819del p.Thr273del	M	not detected	39 wks, 2652 g; healthy
13 (II-2)	HC53; Hirosaki University	12-wk CVS	c.1146C>A	M	c.1146C>A p.Tyr382X	termination of pregnancy
14 (II-3)		12-wk CVS	p.Tyr382X	F	not done	healthy

* CRIFM = Clinical Research Institute of Fetal Medicine; ID = identifier.
† Treated before the 2004 guidelines for genetic testing were in effect.

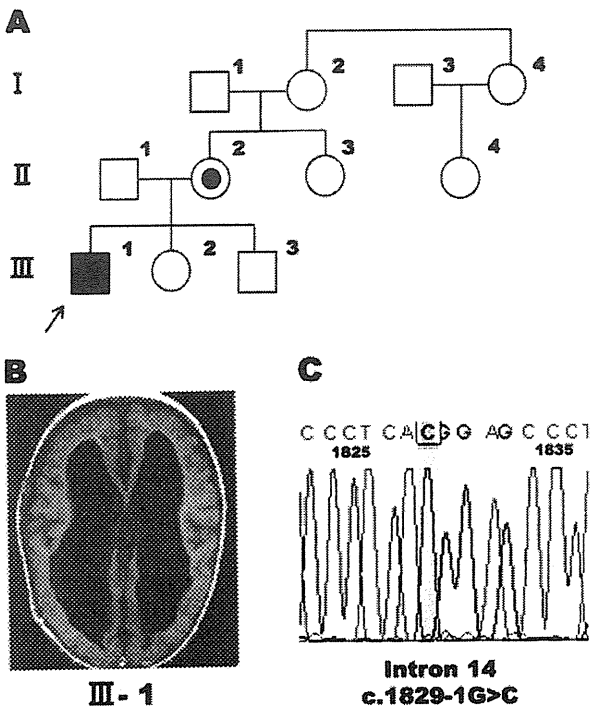

FIG. 2. **A:** Pedigree of family HC27. **B:** Postnatal CT scan of the patient (III-1) showing bilateral severe ventricular dilation. **C:** Result of *L1CAM* gene analysis showing the position of the G to C transition in intron 14. This *L1CAM* mutation was reported previously by Kanemura et al. in 2005.

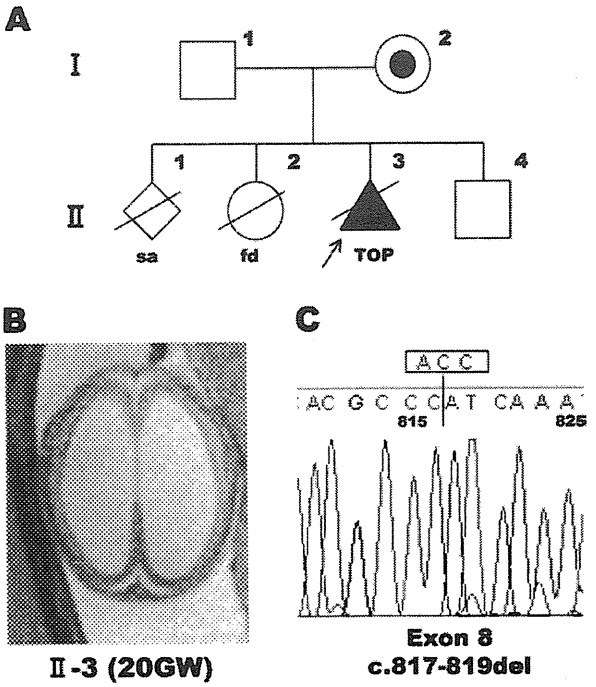

FIG. 3. **A:** Pedigree of family HC45. **B:** Fetal MR imaging of fetus (II-3) at 20 weeks of gestation showing bilateral severe ventricular dilation. **C:** Result of *L1CAM* gene analysis showing the deletion of ACC, which is a novel *L1CAM* mutation. fd = fetal death due to twisting of the umbilical cord; GW = gestational weeks; sa = spontaneous abortion; TOP = termination of pregnancy.

gene analysis of the fetal tissue (II-3) and a sample from the mother (I-2) was performed. We found a deletion of 3 nucleotides, ACC (817–819), in exon 8 of the fetal *L1CAM* gene, which would have deleted threonine 273, in the Ig3 domain of *L1CAM*. The mother was heterozygous for the same mutation. The mother became pregnant again and requested prenatal genetic analysis as part of genetic counseling. Fetal DNA was obtained by AC at 16 weeks of gestation. The fetus was male (II-4) and did not have a mutation of the *L1CAM* gene. The mother continued her pregnancy and delivered a healthy boy. The third baby (II-3) was not included in this series, because XLH was diagnosed in the fetus based on morphological findings on fetal ultrasonography images and not through prenatal genetic testing.

Cases 13 and 14

In this family (HC53; Fig. 4), severe ventricular dilation was detected in a male fetus (II-1) at 24 weeks of gestation. The mother's family history was unremarkable. The boy was delivered early, weighing 3620 g at 37 weeks of gestation. His thumbs were bilaterally adducted. Because XLH was suggested, an *L1CAM* gene analysis of the patient (II-1) and the mother (I-2) was performed. The DNA sequencing revealed that the boy's *L1CAM* gene had a C1146 to A transition in exon 10. This transition would change tyrosine 382 to a stop codon and terminate the L1CAM protein at the fourth fibronectin domain. The mother (I-2) was heterozygous for the same mutation. She became pregnant again and requested genetic analysis of the fetus (II-2) as part of genetic counseling. Fetal DNA was obtained by CVS at 12 weeks of gestation, and the male fetus was determined to have an *L1CAM* gene mutation. Ultimately, the parents chose to terminate the pregnancy. The ventriculomegaly and adducted thumbs were observed postmortem. Subsequently, the mother (I-2) became pregnant and again requested prenatal genetic analysis. The CVS procedure was performed at 12 weeks of gestation, and karyotype testing showed that the fetus was female (II-3). In accordance with Guidelines 2004,[3] genetic analysis was not performed. The mother continued her pregnancy and delivered a healthy girl.

Discussion

In 2005, guidelines for the diagnosis and management of fetal hydrocephalus were published in Japan.[15] This was the first set of guidelines related to the prenatal diagnosis of congenital disease to be published in Japan, and it is widely accepted by obstetricians, pediatrics, neurosurgeons, and, especially, patients' families. According to the data in a nationwide survey for congenital hydrocephalus conducted by the Research Committee for Intractable Hydrocephalus in Japan, 55% of congenital hydrocephalus cases have been detected prenatally as fetal hydrocephalus. However, fetal hydrocephaly varies widely in long-term outcome, largely because a wide variety of conditions is responsible for this phenotype. Although other malformations may be exclud-

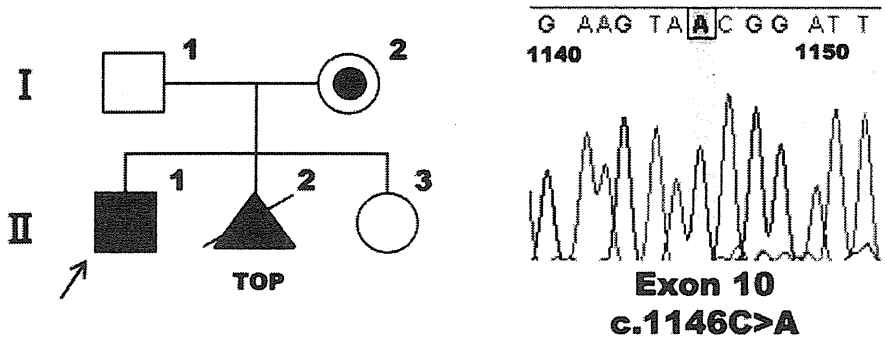

Fig. 4. Case summary of HC53. **Left:** Pedigree of family HC53. **Right:** Result of *L1CAM* gene analysis showing the C to A transition, which is a novel *L1CAM* mutation.

ed by fetal ultrasonography or fetal MR imaging, an initial finding of isolated ventriculomegaly may be XLH, corpus callosum agenesis, lissencephaly, chromosomal anomaly, hydrocephalus due to viral infection, or fetal intracranial hemorrhage. For prenatal counseling, a precise diagnosis obtained by fetal sonography, fetal MR imaging, and toxoplasmosis, rubella, cytomegalovirus, herpes simplex, and HIV (also called TORCH) screening is very important. Prenatal *L1CAM* gene analysis can play an important role in obtaining an exact diagnosis when there is a finding of isolated ventriculomegaly.

To date, more than 200 mutations of the *L1CAM* gene have been identified in families with L1 syndrome. In a nationwide analysis of the *L1CAM* gene in Japan, we identified *L1CAM* gene mutations in 56 families with XLH, including 63 male patients and 43 female carriers. There were no cases of males who carried an *L1CAM* mutation but showed no symptoms of XLH. We previously reported on 26 of these families.[8] In that paper, we evaluated the clinical prognosis of the individuals with an *L1CAM* gene mutation. In all the known cases of XLH with an *L1CAM* mutation, patients showed severe retardation, both physical and mental, without any intrafamilial variability.[8,9,22] In contrast, mutations in the *SHH* gene, which is responsible for some types of holoprosencephaly, cause a wide variety of phenotypes, from no symptoms to severe holoprosencephaly.[14] Renier et al.[16] reported that XLH always shows the poorest outcome of any prenatal noncommunicative hydrocephalus.

Precise serial observation by fetal sonography can detect ventriculomegaly at approximately 19 weeks of gestation, but prenatal *L1CAM* gene detection enables us to determine whether a fetus has XLH as early as 13 weeks of gestation. This 6-week difference not only saves the carrier mother from tremendous added anxiety, but also from severe physical stress in the case of pregnancy termination. In Japan, the termination of pregnancy is legally allowed before 21 weeks of gestation. If we can diagnose XLH only by using morphological findings, this time limit becomes critical. In male fetuses of a mother carrying an *L1CAM* mutation, 50% could have severe hydrocephalus, making prenatal molecular genetic diagnosis with genetic counseling beneficial for a family with XLH.

The prenatal molecular genetic diagnosis of L1 syndrome was first reported by Jouet and Kenwrick[6] in 1995. Since then, several case reports have been published.[1,12,13,18,21] This is the first report in which prenatal *L1CAM* gene analysis was systematically performed.

As previously reported, most *L1CAM* mutations are private, meaning they occur within a single family. We identified 51 types of mutations in 56 unrelated families; in other words, there were 51 different types of mutations at different sites of the *L1CAM* gene that were unique to each family. Within the 56 families in which we identified *L1CAM* gene mutations, only 4 types were found in multiple families: 3 types were each found in 2 unrelated families, and 1 type was found in 3 unrelated families. Only half of the 56 families had a family history of hydrocephalus; the others were sporadic cases. Novel mutations were found in 38 (68%) of 56 families. These observations indicate that *L1CAM* mutations have no hot point.

Most Western researchers have used single-strand conformation polymorphisms as their primary screening method.[11,19] We initially used this as a screening method, but it failed to identify obvious *L1CAM* gene mutations in 6 families. Therefore, we switched to the direct sequencing method—DNA sequencing is now our standard technique for detecting genetic mutations, and it can examine all the L1CAM exons rapidly and reproducibly. The present results suggest that direct sequencing should be used to avoid false negatives and improve the accuracy of *L1CAM* genetic analysis. This is especially important in prenatal diagnosis, which requires perfect accuracy and prompt results if the parents are to be able to decide whether to continue the pregnancy.

The *L1CAM* gene is composed of 28 exons and a 3825-bp open reading frame. Therefore, direct sequencing of the entire gene requires at least 4 weeks. In the case of families in which an *L1CAM* gene mutation has been previously detected and analyzed, it only takes 1 week to analyze the few exons known to be involved. Therefore, prenatal gene analysis is limited to carriers already diagnosed with an *L1CAM* mutation. Performing *L1CAM* gene analysis in all cases of fetal hydrocephalus detected by ultrasonic echogram is not possible at present, but it will become more feasible as the cost and speed of analysis improve.

Conclusions

Prenatal *L1CAM* genetic testing with genetic coun-

seling has been useful for families carrying *L1CAM* mutations.

Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Yamasaki. Acquisition of data: Nonaka, Suzumori, Nakamura, Fujita, Namba, Kamei, Yamada, Pooh, Tanemura, Sudo, Nagasaka. Analysis and interpretation of data: Yamasaki, Nonaka, Yoshioka, Shofuda, Kanemura. Drafting the article: Yamasaki. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Yamasaki. Administrative/technical/material support: Yoshioka, Shofuda, Kanemura. Study supervision: Yamasaki, Kanemura.

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RESEARCH ARTICLE

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A Loss-of-Function Mutation in the *SLC9A6* Gene Causes X-Linked Mental Retardation Resembling Angelman Syndrome

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SLC9A6 mutations have been reported in families in whom X-linked mental retardation (XMR) mimics Angelman syndrome (AS). However, the relative importance of *SLC9A6* mutations in patients with an AS-like phenotype or XMR has not been fully investigated. Here, the involvement of *SLC9A6* mutations in 22 males initially suspected to have AS but found on genetic testing not to have AS (AS-like cohort), and 104 male patients with XMR (XMR cohort), was investigated. A novel *SLC9A6* mutation (c.441delG, p.S147fs) was identified in one patient in the AS-like cohort, but no mutation was identified in XMR cohort, suggesting mutations in *SLC9A6* are not a major cause of the AS-like phenotype or XMR. The patient with the *SLC9A6* mutation showed the typical AS phenotype, further demonstrating the similarity between patients with AS and those with *SLC9A6* mutations. To clarify the effect of the *SLC9A6* mutation, we performed RT-PCR and Western blot analysis on lymphoblastoid cells from the patient. Expression of the mutated transcript was significantly reduced, but was restored by cycloheximide treatment, indicating the presence of nonsense mediated mRNA decay. Western blot analysis demonstrated absence of the normal NHE6 protein encoded for by *SLC9A6*. Taken together, these findings indicate a loss-of-function mutation in *SLC9A6* caused the phenotype in our patient.

Key words: *SLC9A6*; sodium/hydrogen exchanger 6; Angelman syndrome; X-linked mental retardation; nonsense mediated mRNA decay

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INTRODUCTION

SLC9A6 mutations were first reported by Gilfillan et al. [2008] in families exhibiting an X-linked mental retardation (XMR) syndrome mimicking Angelman syndrome (AS). Angelman syndrome is characterized by severe developmental delay with absent or minimal speech, ataxia, easily provoked laughter, epilepsy, and microcephaly. The syndrome is caused by loss-of-function of the *UBE3A* gene which is subject to genomic imprinting. Patients with *SLC9A6* mutations resemble patients with AS, but also demonstrate distinctive clinical features including cerebellar atrophy, slow progression of symptoms, increased glutamate/glutamic acid peak on magnetic resonance spectroscopy (MRS), and lack of characteristic abnormalities seen AS patients examined using electroencephalography (EEG). Following the first report in 2008, in 2010 Schroer et al. reported two other families with AS due to *SLC9A6* mutations, and confirmed the findings of Gilfillan et al.

The *SLC9A6* gene is located on Xq26.3, and encodes the ubiquitously expressed Na^+/H^+ exchanger protein member 6, NHE6. The NHE protein family consists of nine members and includes

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NHE1-5 which is found in the plasma membrane, and NHE6-9 which is found in the membranes of intracellular organelles such as mitochondria and endosomes. NHE6 is predominantly present in the early recycling endosome membranes, and is believed to have a role in regulating luminal pH and monovalent cation concentration in intracellular organelles [Brett et al., 2002; Nakamura et al., 2005]. Moreover, Roxrud et al. demonstrated that NHE6 in combination with NHE9 participated in regulation of endosomal pH in HeLa cells by means of the procedure of co-depletion of NHE6 and NHE9 [Roxrud et al. 2009], indicating the significant role of NHE6 in fine-tuning of endosomal pH in human cells. In the brain, exocytosis from recycling endosomes is essential for the growth of dendritic spines which grow during long-term potentiation (LTP). In the absence of recycling endosomal transport, spines are rapidly lost, and LTP stimuli fail to elicit spine growth [Park et al., 2006]. Thus, NHE6 has an important role in the growth of dendritic spines, and also in the development of normal brain wiring. Thus far, five *SLC9A6* mutations have been reported in six AS families; two nonsense mutations, one inframe deletion, one frameshift deletion, and one splicing mutation [Gilfillan et al., 2008; Schroer et al., 2010]. The precise pathogenesis by which these mutations produce disease remains to be clarified.

The aim of this study was to clarify the incidence and importance of *SLC9A6* mutations in AS-like patients and patients with XMR, and to shed light on the molecular pathogenesis of disease due to *SLC9A6* mutations.

MATERIALS AND METHODS

Enrolled Patients

We examined 22 affected Japanese males clinically suspected of having AS but who lacked the genetic abnormalities reported in AS (AS-like cohort). These patients had AS excluded by having negative results for the *SNURF-SNRPN* DNA methylation test (which identifies a deletion, uniparental disomy, or imprinting defect) and *UBE3A* mutation screening (performed as described previously) [Saitoh et al., 2005]. We also examined DNA samples from 104 Japanese patients suspected of having XMR (XMR cohort). The XMR samples were collected as a part of a project for the Japanese Mental Retardation Consortium [Takano et al., 2008]. This study was approved by the Institutional Review Board of Hokkaido University Graduate School of Medicine, and written informed consent was obtained from the parents of the enrolled patients.

Mutation Analysis of the *SLC9A6* Gene

We amplified each exon, including exon–intron boundaries, of the *SLC9A6* gene using polymerase chain reaction (PCR), and all amplicons were directly sequenced on an ABI 3130 DNA analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator V.1.1 Cycle Sequencing Kit (Applied Biosystems). *SLC9A6* encodes two alternatively spliced transcripts produced from alternative splicing donor sites in exon 2 which give rise to a long form designated as variant 1, and a short form called variant 2. Variant 1 and variant 2 code for NHE6.1 (isoform a) and NHE6.0 (isoform b), respectively (Fig. 1). The primers were designed to amplify each transcript variant. The primers sequence used for amplification and sequencing are available on request. Genomic DNA (10 ng) extracted from peripheral blood was amplified in a total PCR volume of 20 µl containing 1× buffer, 0.4 µM of each primer (forward/reverse), 0.18 mM dNTPs, 0.5 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems). The PCRs for all exons except exon one were performed at 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then one cycle at 72°C for 7 min. The high CpG content of exon 1 required it to be amplified in a total reaction volume of 20 µl containing 1× buffer, 0.4 µM of each primer, 0.2 mM dNTPs, 0.4 U Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland), and 3% DMSO. The thermocycling conditions for exon 1 were 98°C for 3 min followed by 35 cycles of 98°C for 10 sec, 65°C for 30 sec and 72°C for 30 sec and then one cycle of 72°C for 5 min. The PCR products were purified with Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) prior to sequencing. All mutations are referred to in relation to reference sequence NM_001042537.

Cell Culture and Cycloheximide Treatment

Epstein–Barr virus (EBV)-transformed lymphoblastoid cells lines were established from peripheral blood cells using standard methods. To prevent potential degradation of transcripts containing premature translation termination codons (PTCs) by nonsense mediated mRNA decay (NMD), lymphoblastoid cells from the patient with the *SLC9A6* mutation and normal controls were treated with 100 µg/ml cycloheximide (CHX) (Sigma, St. Louis, MO). This compound interferes with NMD through inhibition of protein synthesis [Aznarez et al., 2007]. CHX or a 0.1% DMSO control vehicle was used 4 hr prior to RNA extraction from the cell lines [Carter et al., 1995].

RT-PCR

Total RNA from cultured lymphoblastoid cells from the patient and four normal controls, was extracted using the RNAqueous® Kit (Applied Biosystems). Reverse transcription was performed using 100 ng of total RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a total reaction volume of 20 µl containing 1× Random primers, 4 mM dNTP mix, 2.5 U of Multiscribe™ Reverse Transcriptase, and 1 µl of RNase Inhibitor. The reactions were incubated at 25°C for 10 min, then at 37°C for 120 min and then followed by 85°C for 5 min to inactivate the reverse transcriptase. Complementary DNA was then amplified using a primer set designed to amplify exon 2–5; forward 5′-GTCTTTTGGTGGGCCTTGT-3′, reverse 5′-GTCCCGTTACC-TTCATCAG-3′. PCR products for NHE6.1 (transcript variant 1) and NHE6.0 (transcript variant 2) were 399 and 303 bp, respectively.

Real-Time Quantification of *SLC9A6* mRNA

To measure *SLC9A6* transcript variant 1 and variant 2, both of which are alternative splicing products, primers and TaqMan® MGB probes were designed with Primer® Express Software (Applied Biosystems; Fig. 1). The Primer and MGB probe sequence

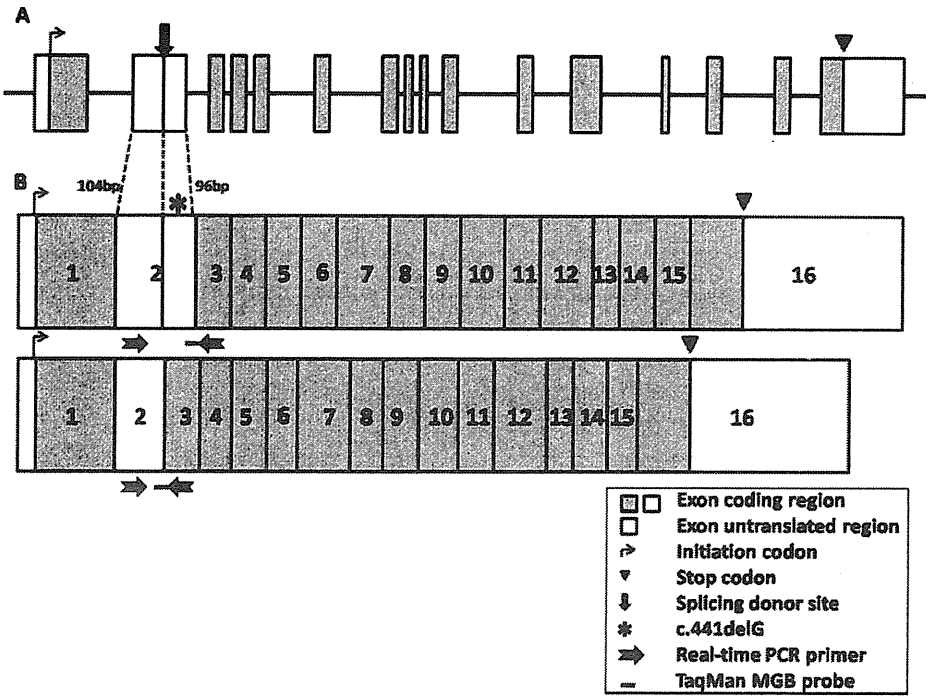

FIG. 1. A: Genomic structure of the *SLC9A6* gene. B: Two alternatively spliced transcripts of the *SLC9A6* gene. Above: *SLC9A6* transcript variant 1 (encodes NHE6.1 or isoform a). Below: *SLC9A6* transcript variant 2 (encodes NHE6.0 or isoform b). The location of the *SLC9A6* mutation in our patient is shown with *. Primers and probes used in real-time quantitative PCR are shown (horizontal arrows).

for variant 1 were forward primer 5′-TGAGTATATGCTGAAAGGAGAGATTAGTTC-3′, reverse primer 5′-GATAGGAGGAAGTAATATGTTGAAAAATACTTC-3′, TaqMan MGB probe 5′-CTTAGAAAGGTTACTTTTGATCC-3′; and for variant 2 forward primer 5′-CTGTGAAGTGCAGTCAAGTCCAA-3′, reverse primer 5′-GATAGGAGGAAGTAATATGTTGAAAAATACTT-3′, TaqMan MGB probe 5′-CTACCTTACTGGTTACTTTTGA-3′. Human *GAPDH* MGB probe and primers purchased from Applied Biosystems were used as the internal control. Patient cDNA was transcribed from 10 ng of total RNA in a total volume of 25 µl containing 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each primer (sense/antisense) and 0.25 µM of probe. Thermocycling was 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Real-time quantitative PCR was performed using the ABI PRISM 7700 (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used for relative quantification.

Western Blot Analysis

HeLa cells and cultured lymphoblastoid cells from the patient, mother and normal controls were washed with phosphate buffered saline and suspended in lysis buffer (phosphate buffered saline containing 1% Triton-X, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, and 1 µg/ml leupeptin). HeLa cells expressing the NHE6.1 were used as a control. The cells were disrupted by sonication and centrifuged at 20,000*g* for 10 min at 4°C. The supernatants were then resolved by SDS-polyacrylamide electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). NHE6 was detected with rabbit polyclonal anti-NHE6 antibody [Ohgaki et al., 2008], anti-rabbit IgG antibody conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA) and chemiluminescence reagent (ECL Western Blotting Detection System; GE Healthcare, Waukesha, WI).

RESULTS

Identification of a *SLC9A6* Mutation

We identified only one male patient with a frameshift mutation (c.441delG, p.S147fs) in exon 2, out of 22 male patients in the AS-like cohort (Fig. 2). This frameshift mutation causes a PTC. His healthy mother was heterozygous for the mutation.

No mutation in the *SLC9A6* gene was identified in the XMR cohort. However, two common polymorphisms (rs2291639, rs2307131), and one putative novel polymorphism in intron 12 (c.1692 +10 A>G) were detected.

Clinical Features of the Patient With the *SLC9A6* Mutation

The affected male patient at birth suffered from mild neonatal asphyxia, however he had no other perinatal problems. His parents

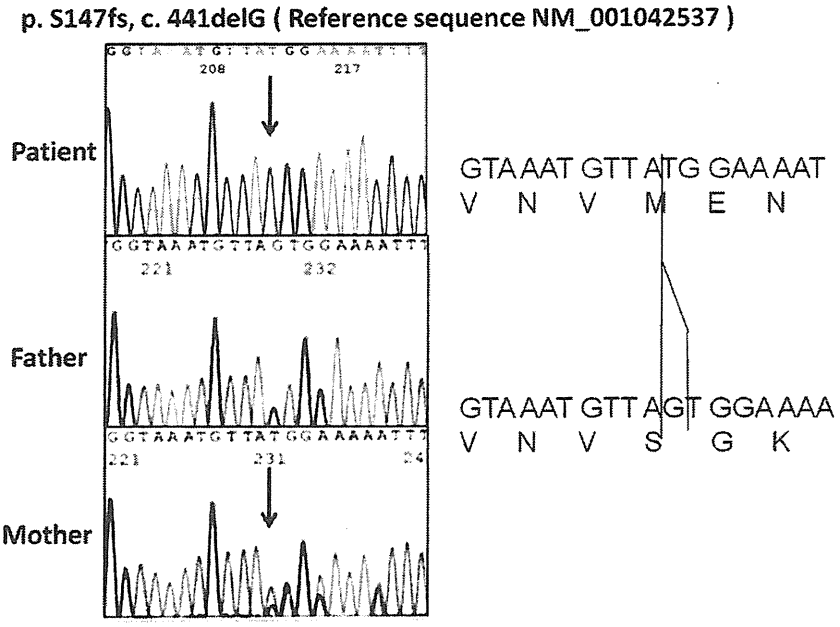

FIG. 2. Chromatographs showing the *SLC9A6* mutation in our patient, and the equivalent genomic region in both his parents. The mutation c.441delG is located in exon 2 and is only present in transcript variant 1. His mother was heterozygous for this mutation, while his father did not have the mutation. This mutant transcript leads to premature protein truncation. The mutation is described relative to reference sequence NM_001043537. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-485X]

were non-consanguineous and he did not have any family history of neurological diseases. Although formal clinical assessment was not conducted to the mother, she is healthy and does not have intellectual disability. His clinical features are summarized in Table I. He showed typical findings of AS; severe developmental delay with absence of verbal language, generalized hypotonia, easily provoked laughter, epilepsy, ataxia, strabismus, and microcephaly. His occipitofrontal head circumference at birth was 33.8 cm (+0.4 SD), but his head growth has decelerated into 51.5 cm (−3.0 SD) at 18 years of age. He acquired head control at three months of age, sat and crawled at 6 months of age, and walked unassisted at 18 months of age. His first epileptic attack occurred at 4 years of age. After this first attack, he lost his ability to walk until he was 5 years old. His epileptic attacks consisted of multiple types of seizures, and they were difficult to control with ACTH or several anti-epileptic drugs. TRH treatment improved his awakening and activity levels, and he transiently acquired the ability to walk. However, subsequently his ability to walk was lost, probably due to exacerbation of ataxia. His deep tendon reflex was not increased and no other features of spasticity or peripheral neuropathy were identified. His EEG findings included a background frequency of 5–6 Hz theta waves and spontaneous appearance of 3 Hz diffuse high voltage slow waves. TRH did not change the frequency of his seizures or his EEG findings. He showed no cerebellar atrophy on magnetic resonance imaging (MRI) at 5 years of age. MRS was not performed. He had a normal G-banding karyotype.

Downregulation of the *SLC9A6* Variant 1 in the Patient With the Mutation

The identified mutation c.441delG is located in exon 2 and is only present in variant 1 (Fig. 1). Therefore, the mutation only affects NHE6.1, leaving NHE6.0 intact. Reverse transcriptase PCR demonstrated that *SLC9A6* variant 1 mRNA expression decreased in our patient (Fig. 3A) compared to that in four normal controls. On the other hand, variant 2 expression was increased in the patient compared to the controls. To further investigate mutant *SLC9A6* gene expression, real-time quantitative PCR (qPCR) was performed using cDNA from the patient and normal controls. Quantitative PCR confirmed that *SLC9A6* variant 1 was significantly downregulated in the patient, while it was not downregulated in normal controls (Fig. 4A). Furthermore, the *SLC9A6* variant 2 mRNA in the patient was significantly increased compared to normal controls (Fig. 4B).

Nonsense Mediated Decay Was Involved in the Downregulation of Mutant *SLC9A6* in the Patient

To investigate the possible involvement of NMD in the downregulation of mutant *SLC9A6* in the patient's lymphoblastoid cells, we treated the cells with CHX. After CHX treatment, the expression level of *SLC9A6* variant 1 increased compared to normal control samples on RT-PCR (Fig. 3B). It was also proved that the expression level of variant 1 was significantly increased by performing qPCR, while the expression level in normal control samples

TABLE I. Clinical Findings in Affected Males Previously Reported and Our Patient

Family number: report affected males number (examined number)	1: Gilfillan et al. [2008] 3 (3)	2: Gilfillan et al. [2008] 2 (1)	3: Gilfillan et al. [2008] 3 (3)	4: Gilfillan et al. [2008], Christianson et al. [1999] 16 (4)	5: Schroer et al. [2010] 6 (6)	6: Schroer et al. [2010] 1 (1)	Our patient
Development and behavior							
Profound delay	+	+	+	+	+	+	+
Verbal language absent	+	+	+	+	+	+	+
Easily provoked laughter	+	+	+	+	3/6	−	+
CNS findings							
Epilepsy	+	+	+	+	+	+	+
Ataxia	+	+	+	+	NR	NR	+
Hyperkinetic movements	2/3	−	+	−	2/6	NR	−
Strabismus	+	+	+	+	5/6	+	+
Physical findings							
Microcephaly	+	+	+	3/4	5/6	+	+
Open mouth + drooling	2/3	+	+	NR	4/6	+	+
Swallowing difficulty	2/3	+	1/3	1/4	NR	+	−
Flexed arms	+	NR	1/3	+	3/6	−	−
Electroencephalography							
Epileptiform activity	+	+	+	+	+	+	+
Background activity	10–11 Hz	1.5–3 Hz	4–7 Hz	3–6 Hz to 11–14 Hz	NR	α rhythm	5–6 Hz
Brain MRI/autopsy							
Cerebellar atrophy	1/3	NR	NR	2/4	2/6	+	−
Mutation	p.E287_S288del c.936_941delAAAGTG	p.R500X c.1574C → T	p.V176_201del c.679 +1 delGTAA	p.H203fs c.684_685delAT	p.R500X c.1574C → T	p.Q437X c.1391C → T	p.S147fs c.441delG

+, present with all the patients; −, not present; NR, not recorded.

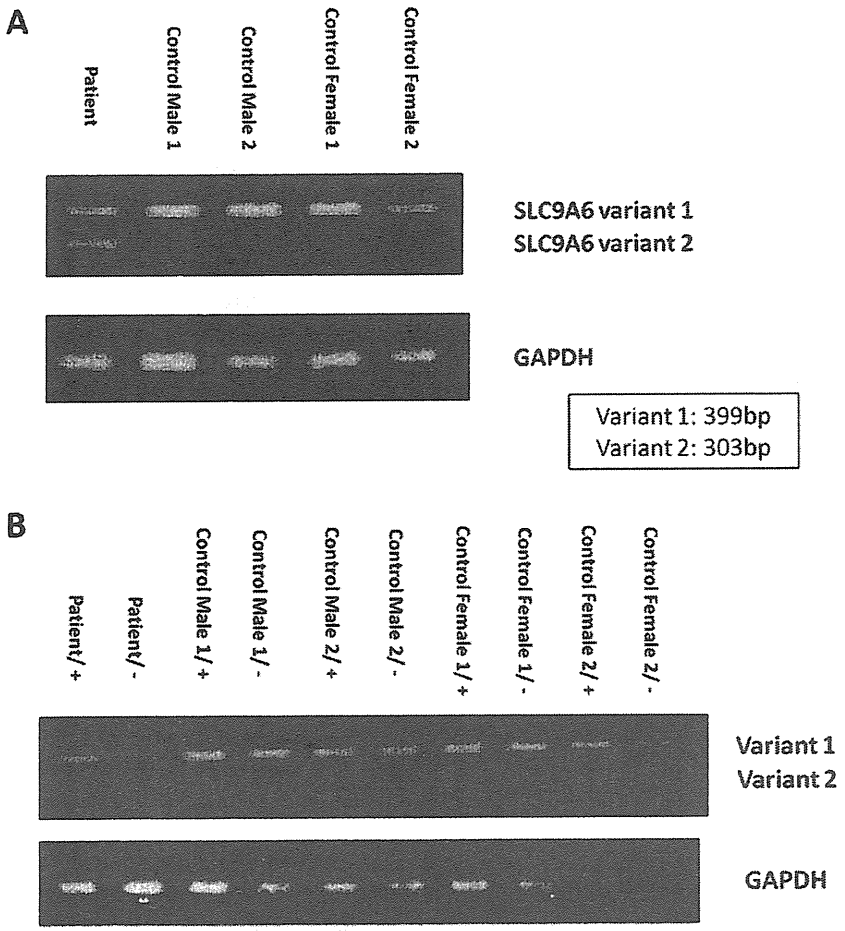

FIG. 3. RT-PCR amplification of the *SLC9A6* gene. A: *SLC9A6* variant 1 mRNA expression was decreased in the patient compared to that in four normal controls. On the other hand, variant 2 expression was increased in the patient compared to that in the controls. B: CHX treatment increases the mutant *SLC9A6* variant 1 mRNA expression, leading to similar expression levels in the patient and four normal controls samples. (+) After CHX treatment, (−) no CHX treatment.

was unchanged (Fig. 4A). The expression level of *SLC9A6* variant 2 increased in all samples after CHX treatment, however the increase was significant only in control samples (Fig. 4B).

Decreased Expression of the NHE6 Protein From Mutant *SLC9A6*

Western blotting was performed to investigate expression of the NHE6 protein in the homogenate of lymphoblastoid cell lines from the patient and his mother. As a result, protein expression of NHE6.1 was not detected in the patient (Fig. 5A,B). The same NHE6.1 was detected in HeLa cells and cells from the patient's mother as well as in the controls. NHE6.0, which was expected to be 10–20 kDa smaller than NHE6.1on SDS–PAGE [Ohgaki et al., 2008], was not detected in any sample (Fig. 5B).

DISCUSSION

In this study we investigated 22 male AS-like patients and 104 male patients with XMR, and identified only one AS-like patient with a *SLC9A6* frameshift mutation. This result further confirms *SLC9A6* is not a major cause of AS-like cases, as reported by Fichou et al. [2009]. Although the number of patients with XMR in this study was small, *SLC9A6* is likely to account for only small proportion of XMR cases.

Patients with *SLC9A6* mutations reported by Gilfillan et al., exhibit cardinal features similar to those of AS including severe developmental delay, mental retardation with absent or minimal use of words, easily provoked laughter, ataxia, epilepsy, hyperkinetic movement, nystagmus, and microcephaly.

Gilfillan et al. also identified possible features of difference between these patients and AS patients, including slow progression of symptoms, thin body, cerebellar atrophy, increased glutamate/

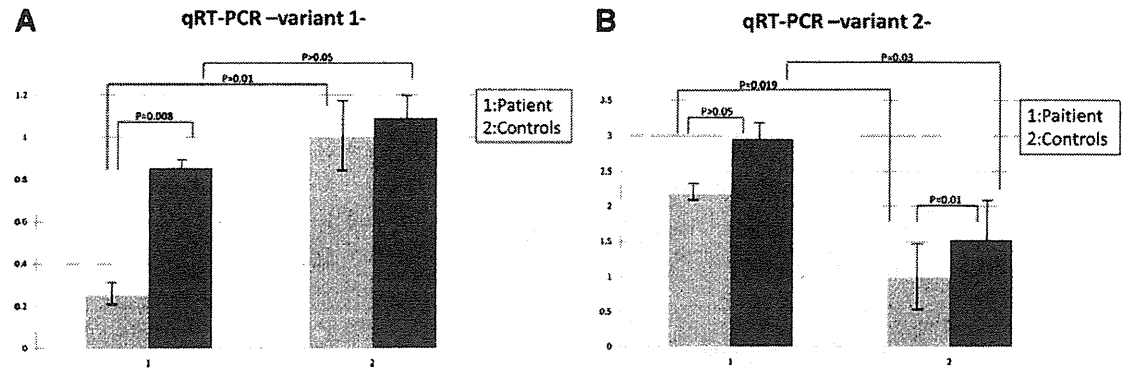

FIG. 4. Real-time quantitative PCR in samples from cell lines from the patient and four normal controls containing two males and two females. The light gray bars indicate the expression levels of *SLC9A6* before CHX treatment, while deep gray bars after CHX treatment. We performed statistical analysis using paired and unpaired Student's t-test. Error bars show standard deviation. A: The *SLC9A6* variant 1 was significantly downregulated in samples from the patient while it was not downregulated in samples from four normal controls. After CHX treatment, expression level of the *SLC9A6* variant 1 mRNA in the patient's sample was significantly increased. B: The *SLC9A6* variant 2 in the patient's sample was significantly increased compared to normal controls. Expression level of *SLC9A6* variant 2 increased in all samples after CHX treatment, but a significant increase was only seen in samples from controls.

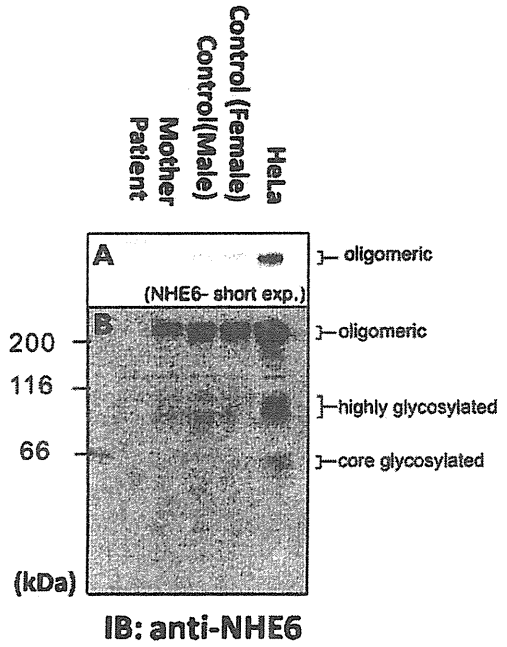

FIG. 5. Protein expression of NHE6 in cultured lymphoblastoid cells and HeLa cells. In the patient, no protein expression of NHE6 isoforms was detected with Western blotting using anti-NHE6 antibody. A: A cropped image taken using a short exposure time demonstrating the oligomeric form of NHE6. Protein size in kDa is shown by numbers on the left of the image. B: A chemiluminescence image of Western blotting taken with a longer exposure time.

glutamic acid peak on MRS, and rapid frequency of 10–14 Hz waves on EEG (Table I). Our patient lost his ability to walk although he did not demonstrate spasticity, demonstrating a slowly progressive clinical course consistent with findings in Gilfillan's report. Indeed, slow progression may be a distinctive clinical feature for patients with *SLC9A6* mutations. One of the families which Gilfillan et al. investigated was previously reported by Christianson et al. [1999], and designated as Christianson syndrome. Schroer et al. reported patients with Christianson syndrome, and they showed that the patients demonstrated an AS-like phenotype. However, while the clinical features of our patient were consistent with those of most patients previously reported by Gilfillan, there were differences including the EEG findings and lack of cerebellar atrophy. Despite this, our patient did meet the diagnostic criteria for AS [Williams et al., 2006]. Therefore, this study further demonstrated that a patient with a *SLC9A6* mutation may resemble patients with AS. Further, this striking similarity between patients with AS and those with *SLC9A6* mutations suggests a possible relationship between the gene function of *UBE3A* and *SLC9A6* in the developing brain.

Our patient's mutation created a frameshift resulting in 7 missense amino acids followed by a stop codon. This mutation was present only in *SLC9A6* transcript variant 1. *SLC9A6* mRNA has two transcript variants caused by alternative splicing in exon 2 (Fig. 1), but the role of each variant has not been clarified. The mutation detected in our patient only affects variant 1 sequence, but the phenotype of the patient was as severe as those in previously reported patients. Therefore, our finding suggests that the NHE6.1 plays an important role in brain function.

Nonsense mediated decay is involved in regulating the expression of alternatively spliced forms containing PTCs [Lareau et al., 2007; Ni et al., 2007]. Since the identified mutation was predicted to result in a PTC, we speculated that NMD could be involved in disease pathogenesis. The result of qRT-PCR showed a significant

decrease in *SLC9A6* variant 1 mRNA expression in the patient sample. This reduction was restored by CHX treatment, while *SLC9A6* variant 1 expression was unaltered by CHX treatment in normal control samples. Expression of *SLC9A6* variant 2 in the patient on the other hand, was significantly increased compared to that in control samples, however it was not influenced by CHX treatment. Therefore, the c.441delG mutation in the patient seems to have modified the alternative splicing pattern, leading to an increase in variant 2 expression. Alternatively, low variant 1 could trigger a regulatory feed back on transcription causing the apparent increase in variant 2 expression. A mutation causing premature protein truncation could alter the splicing pattern and lead to exon skipping, use of alternative splice sites, and intron retention [Hentze and Kulozik, 1999; Mendell and Dietz, 2001]. Our results indicated that the c.441delG mutation caused a PTC altered the splicing pattern, and activated NMD machinery then downregulated *SLC9A6* variant 1 expression.

As protein NHE6.1 was not detected, this indicates an absence of intact NHE6.1. NHE6.0 was also not detected. These findings conclusively indicated that the identified mutation should cause total loss-of-function. Recently, Garbern et al. identified cases with an in-frame deletion of three amino acids, who showed milder dysmorphic features and higher gross motor abilities than those in cases previously reported [Garbern et al., 2010]. Their in-frame deletion should not cause total loss-of-function but create a mildly dysfunctional protein. Therefore, severe phenotypes including severe developmental delay and progressive neurological deterioration may be caused by truncated mutations and less severe phenotypes may be caused by missense or in-frame mutations, and such mild phenotypes are likely missed in patients with mild developmental delay.

Given that the *SLC9A6* variant 2 was upregulated, we speculated that upregulated variant 2 might partially compensate for the absence of NHE6.1. However, we could not establish the upregulation of the NHE6.0 protein, rather it was not detected in the patient's lymphoblastoid cells. NHE6.0 may be unstable compared to NHE6.1. Alternately, NHE6.0 translation may be inhibited. Further investigation is required to definitively answer this question.

NHE6 is found in the membranes of early recycling endosomes and transiently in plasma membranes. Its distribution is regulated by RACK1 [Ohgaki et al., 2008]. Recycling endosomal trafficking is essential for the growth of dendritic spines during LTP in the brain [Park et al., 2006]. The function of the protein product of *UBE3A*, E3 ubiquitin ligase, is also associated with dendritic spine morphology. Mice with a maternal null mutation in *Ube3a* are also reported to have defects in LTP, and manifest motor and behavioral abnormalities [Jiang et al., 1998]. In a recent study, *Ube3a* deficient mice demonstrated dendritic spine dysmorphology [Dindot et al., 2008]. Thus, *UBE3A* and *SLC9A6* could interact in a common pathway involved in dendritic spine development, with a mutation in either leading to an AS-like phenotype.

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CLINICAL REPORT

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West Syndrome Associated With Mosaic Duplication of *FOXG1* in a Patient With Maternal Uniparental Disomy of Chromosome 14

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FOXG1 on chromosome 14 has recently been suggested as a dosage-sensitive gene. Duplication of this gene could cause severe epilepsy and developmental delay, including infantile spasms. Here, we report on a female patient diagnosed with maternal uniparental disomy of chromosome 14 and West syndrome who carried a small supernumerary marker chromosome. A chromosomal analysis revealed mosaicism of 47,XX, + mar[8]/46,XX[18]. Spectral karyotyping multicolor fluorescence in situ hybridization analysis confirmed that the marker chromosome was derived from chromosome 14. A DNA methylation test at *MEG3* in 14q32.2 and microsatellite analysis using polymorphic markers on chromosome 14 confirmed that the patient had maternal uniparental disomy 14 as well as a mosaic small marker chromosome of paternal origin containing the proximal long arm of chromosome 14. Microarray-based comparative genomic hybridization analysis conclusively defined the region of the gain of genomic copy numbers at 14q11.2-q12, encompassing *FOXG1*. The results of the analyses of our patient provide further evidence that not only duplication but also a small increase in the dosage of *FOXG1* could cause infantile spasms. © 2011 Wiley-Liss, Inc.

Key words: West syndrome; maternal uniparental disomy; chromosome 14; supernumerary marker chromosome; *FOXG1*; mosaic duplication

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INTRODUCTION

Mutations in *FOXG1* on chromosome 14 are associated with the congenital variant of Rett syndrome [Shoichet et al., 2005; Jacob et al., 2009]. Recently, *FOXG1* was described as a dosage-sensitive gene. The duplication of this gene could cause severe epilepsy and developmental delay, including infantile spasms [Yeung et al., 2009; Brunetti-Pierri et al., 2011]. Maternal uniparental disomy 14 (upd(14)mat) is characterized by pre- and postnatal growth retardation, neonatal hypotonia, small hands and feet, feeding difficulty, precocious puberty, and truncal obesity [Kotzot and Utermann, 2005; Mitter et al., 2006]. Upd(14)mat syndrome demonstrates a Prader-Willi-like phenotype during infancy [Mitter et al., 2006; Hosoki et al., 2009] but complications of seizures are rarely observed. Upd(14)mat is reported in carriers of Robertsonian translocations involving chromosome 14 and is also found in patients with normal karyotypes and supernumerary marker chromosomes (SMCs) [Mitter et al., 2006]. The presence of SMCs has often increased chromosome dosage, which results in the increased expression of dosage-sensitive genes.

To add new insight regarding the genetic cause of West syndrome phenotype, we report on a female patient diagnosed with upd(14)mat and West syndrome who carried a small SMC derived from the chromosome 14q11.2 to 14q12 region encompassing *FOXG1*.

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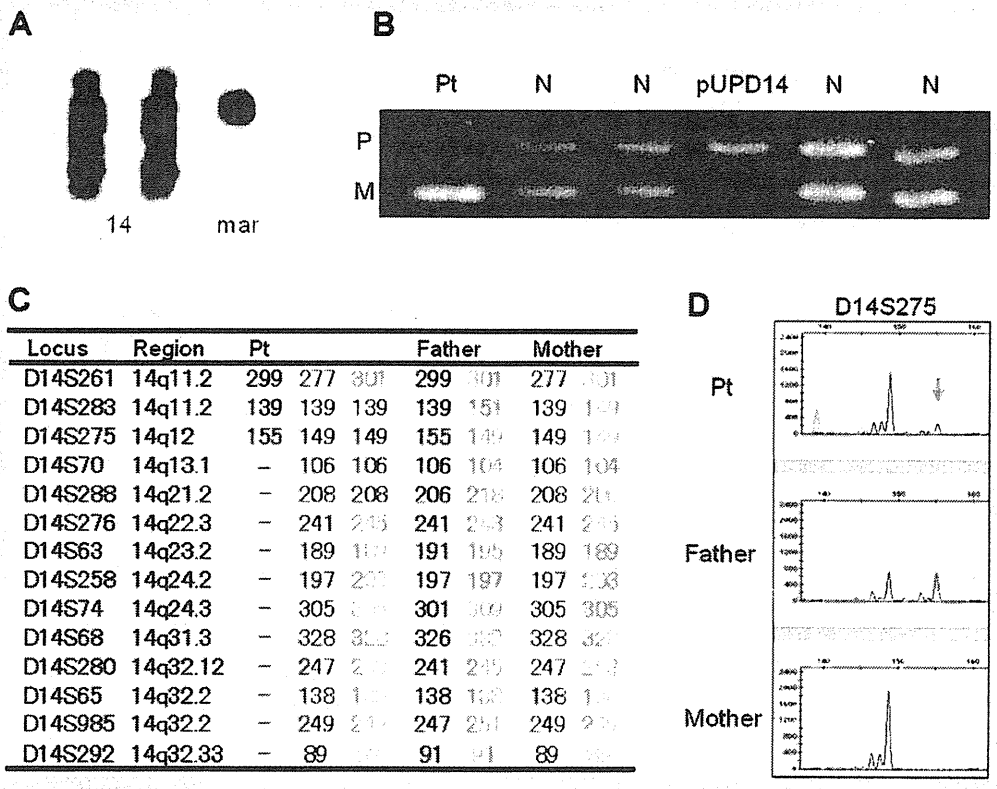
CLINICAL REPORT

The female patient was the first daughter born to healthy, non-consanguineous Japanese parents with an unremarkable family history. Intrauterine growth retardation was noted during pregnancy. The child was delivered at 40 weeks and 5 days of gestation by cesarean because of cervical insufficiency. At birth, birth weight (BW) was 2,140 g (−2.4 SD), birth length (BL) was 48 cm (−0.4 SD), and occipitofrontal head circumference (OFC) was 28 cm (−3.9 SD). After delivery, the infant had episodic vomiting and was admitted to the neonatal intensive care unit. She received nasogastric tube feeding for 11 days due to feeding difficulty. During infancy, she had hypotonia. At 4 months, she developed epileptic seizures with upward eye deviation, and, at 5 months, infantile spasms. Her electroencephalogram (EEG) showed hypsarrhythmia. At 5 months, she was diagnosed as having West syndrome, and referred to our hospital. When she was admitted, her BW was 5.6 kg (−1.9 SD), BH was 62 cm (−1.1 SD), and OFC was 41 cm (+0.2 SD). She had mild dysmorphic features including a frontal bossing, small mouth, and small hands. A hemangioma on the left forehead was noted. A neurological examination revealed mild hypotonia without muscle weakness. A brain MRI and comprehensive metabolic screening were normal. Infantile spasms were not controlled despite an optimal dose of sodium valproate and zonisamide. Treatment with adrenocorticotropic hormone (ACTH) was started at age 6 months and successfully controlled her seizures. Subsequently, clobazam was added to improve her EEG, and she had no relapse of infantile spasms until she was 6 years old. Her EEG at 5 years 11 months was normal. At 3 years 11 months, her BH was 87.5 cm (−3.9 SD). Because of her short stature, growth hormone therapy was started at age four and was effective. The patient had mild psychomotor delay. At age six, she was able to speak a few words. Her intelligent quotient by a modified Binet method was 40 at age 5 years and 8 months.

Cytogenetic and Molecular Genetic Analysis

A chromosomal analysis was performed using the G-banding of cultured lymphocyte and spectral karyotyping (SKY) multicolor fluorescence in situ hybridization (FISH) method. Through the analysis of 26 metaphase cells, we found that the patient had a mosaic chromosome of 47,XX, + mar[8]/46,XX[18] (Fig. 1A). The origin of the SMC was not identified by conventional G banding.



Locus	Region	Pt			Father		Mother	
D14S261	14q11.2	299	277	301	299	301	277	301
D14S283	14q11.2	139	139	139	139	151	139	139
D14S275	14q12	155	149	149	155	149	149	155
D14S70	14q13.1	–	106	106	106	104	106	104
D14S288	14q21.2	–	208	208	206	210	208	210
D14S276	14q22.3	–	241	245	241	243	241	245
D14S63	14q23.2	–	189	189	191	195	189	189
D14S258	14q24.2	–	197	207	197	197	197	203
D14S74	14q24.3	–	305	309	301	309	305	305
D14S68	14q31.3	–	328	332	326	330	328	332
D14S280	14q32.12	–	247	251	241	245	247	251
D14S65	14q32.2	–	138	142	138	142	138	142
D14S985	14q32.2	–	249	251	247	251	249	251
D14S292	14q32.33	–	89	91	91	91	89	91

FIG. 1. Cytogenetic and molecular genetic examinations of chromosome 14. A: G-banding of chromosome 14 and the marker. B: *MEG3* methylation test. The *MEG3* methylation test demonstrated that the patient showed only a maternal unmethylated signal. P, paternal methylated signal; M, maternal unmethylated signal; Pt, patient; N, normal control; pUPD14, paternal uniparental disomy 14. C: Microsatellite analysis using polymorphic markers on chromosome 14. Putative haplotypes are indicated by color. The patient showed a combination of maternal uniparental heterodisomy and isodisomy of the entire chromosome 14, as well as additional paternal inheritance for only the proximal long arm of chromosome 14 (shown in blue). D: Fragment analysis at D14S275. Fragment analysis at D14S275 showed a small peak of paternal inheritance (indicated by arrow) showing the mosaic status of the marker of paternal origin. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833].