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Ｇ．知的財産権の出願・登録状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

厚生労働科学研究費補助金（難治性疾患等克服研究事業）
ATR-X症候群の臨床研究および基礎研究のための基盤整備に関する研究

分担研究報告書

3D-FISH法を用いた*ATRX*遺伝子とαグロビン遺伝子の空間配置の解析による ATR-X症候群の病態解明に関する研究

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研究要旨
研究要旨 ATR-X症候群患者由来の培養細胞を用いて、*ATRX*遺伝子領域（Xq21.1）とαグロビン遺伝子領域（16p13.3）の空間配置の特性を3D-FISH法により検討した結果、16pとXq染色体テリトリーの高頻度な隣接（chromosome kissing）が観察され、健常者のものと異なることが見出された。BACクローンを用いた3D-FISH法により遺伝子キッシングが見られるかどうか、空間配置相互作用の観点から引き続き検討を進める。

A．研究目的

ATR-X症候群の責任遺伝子はXq21.1に局在するATRX遺伝子であり、ATRXタンパク質のエピジェネティクス制御の破綻によってαサラセミア、精神遅滞などを特徴とした多彩な症状を呈すると考えられている。本分担研究では、ATRX遺伝子が存在するX染色体長腕特異的ペインティングプローブ及びαサラセミアを引き起こす原因となるαグロビン遺伝子領域が存在する16番染色体短腕特異的ペインティングプローブ、および関連する遺伝子領域を含んだBAC DNAクローンを調整し、3D-FISH法により関連する染色体テリトリー（CT）と遺伝子領域の空間配置の特性を調べることを目指した。

B．研究方法

ATR-X症候群患者由来の培養繊維芽細胞及び健常人由来の培養繊維芽細胞を用いて、3次元構造を維持した細胞核の固定を行い、16pとXqペインティングプローブおよびBAC DNAプローブをハイブリダイズされ、共焦点レーザースキャン顕微鏡により、画像スキャンを行い、両CTと遺伝子領域の空間配置解析を行った。

（倫理面への配慮）

ATR-X症候群患者由来および対照としての健常人由来の培養繊維芽細胞の使用に際して、すでに個人情報の連結不可能匿名化がなされ、研究倫理上、品質管理上、ともに十分配慮されている。

C．研究結果

3D-FISH法により、16p CT、Xq CTおよびATRXとHBA遺伝子領域の空間配置の解析を行った。その結果、ATR-X症候群患者由来の細胞核では、16pとXq CTの高頻度な隣接（chromosome kissing）が観察された（約57%）。しかしながらATRXとHBAの両遺伝子間での遺伝子キッシングはこれまでのところ観察されなかった（14細胞核より）。CTの空間配置が関連する遺伝子発現に何らかの影響を及ぼしている可能性が考えられ、遺伝子キッシングについての観察を引き続き進める。

D．考察

ATRX遺伝子はクロマチンリモデリング因子であるATRXタンパク質をコードしている。そのエピジェネティクス制御の破綻が、核内CTの空間配置の異常に結びつき、αグロビン遺伝子の不適切な遺伝子発現の原因の一つとなっていると考えられる。今後も各種BAC DNA領域とペインティングプローブを組み合わせた3D-FISH法により、ATRX遺伝子領域の空間的な相互作用領域の詳細を明らかにしていく。

E．結論

ATR-X症候群の患者由来の細胞核では、16p CTとXq CTのkissing現象が高頻度に観察されたが、遺伝子間でのkissing現象は見出されておらず、検討を続ける。

G．研究発表

1. 論文発表　なし
2. 学会発表　なし

H．知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録　なし
3. その他　　特になし

Ⅲ．研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

Neuroradiologic Features in X-linked α-Thalassemia/Mental Retardation Syndrome

T. Wada, H. Ban, M. Matsufuji, N. Okamoto, K. Enomoto, K. Kurosawa, and N. Aida

ABSTRACT

BACKGROUND AND PURPOSE: X-linked α-thalassemia/mental retardation syndrome (Mendelian Inheritance in Man, 301040) is one of the X-linked intellectual disability syndromes caused by mutations of the *ATRX* gene and characterized by male predominance, central hypotonic facies, severe cognitive dysfunction, hemoglobin H disease (α-thalassemia), genital and skeletal abnormalities, and autistic and peculiar behavior. More than 200 patients in the world, including >70 Japanese patients, have been diagnosed with ATR-X syndrome.

MATERIALS AND METHODS: We reviewed the brain MRI and/or CT findings of 27 Japanese patients with ATR-X with *ATRX* mutations retrospectively.

RESULTS: The findings were categorized into 5 types: 1) nonspecific brain atrophy (17/27); 2) white matter abnormalities, especially around the trigones (11/27); 3) widespread and scattered white matter abnormalities (1/27); 4) delayed myelination (4/27); and 5) severe and rapidly progressive cortical brain atrophy (1/27).

CONCLUSIONS: This is the first report on a comprehensive study of brain MRI/CT findings of ATR-X syndrome. Our findings suggest that the ATRX protein seems to be involved in normal myelination. The classification will require revisions in the near future, but it will be helpful in establishing the relationship between *ATRX* mutation and brain development and understanding the ATRX protein function in the brain.

ABBREVIATIONS: ADD domain = ATRX-DNMT3-DNMT3L; ATR-X (*ATRX*) = X-linked α-thalassemia/mental retardation

X-linked α-thalassemia/mental retardation syndrome (Mendelian Inheritance in Man, 301040) is one of the X-linked intellectual disability syndromes and is due to mutations of the *ATRX* gene on Xq13.3, encoding a SWI/SNF-like chromatin remodeling protein.[1] The ATRX protein has 2 functionally important domains: the zinc-finger motif (ADD) and the highly conserved chromatin-remodeling domain, where the *ATRX* mutations cluster.[2] Although the pathophysiologic mechanism of ATR-X syndrome is not yet completely known, ATR-X syndrome is one of the chromatin diseases (which include Rett, Coffin-Lowry, and Rubinstein-Taybi syndromes), and a disturbance of the epigenetic mechanism is suggested.[3]

More than 200 patients in the world, >70 of whom are Japanese, have been diagnosed with ATR-X syndrome.[2,4] ATR-X syndrome seems to be a rare disease; however, we estimate that the prevalence of ATR-X syndrome is 1/30,000–40,000 neonate boys, which is much higher than previously reported estimations (K. Kurosawa, unpublished data 2012). Therefore, more patients remain to be diagnosed because 7–9 patients with ATR-X should be diagnosed every year based on the fact we have 1 million neonates born every year in Japan.

In 2010, we established the ATR-X Syndrome Network Japan (http://kcmc.jp/ATR-X/index.html) for patients and their families, and we have surveyed patients with ATR-X syndrome in Japan. The ultimate purpose of our clinical research is to establish diagnostic criteria, which would facilitate further clinical study of individuals with molecularly proved ATR-X, assist in the evaluation of those who appear to exhibit the clinical features of ATR-X with no ATRX mutations, and establish the management of ATR-X syndrome.

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Paper previously presented in the poster session at: American Society of Human Genetics hosting the 12th International Congress of Human Genetics, October 11–15, 2011; Montreal, Quebec, Canada.

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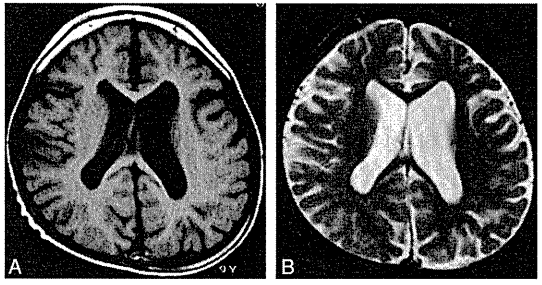

FIG 1. Type 1. Nonspecific brain atrophy. T1WI (*A*) and T2WI (*B*) of a 9-year-old patient (case 4) with an *ATRX* mutation of the ADD domain in exon 6 (c.390_391 ins A; E131fs). Nonprogressive diffuse cortical brain atrophy and ventricular enlargement due to loss of white matter volume are shown.

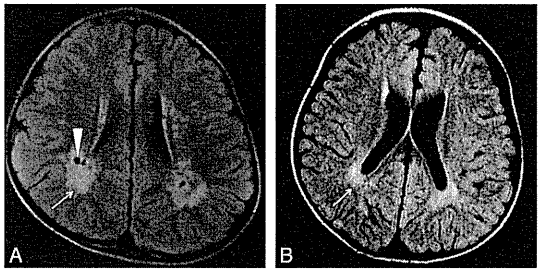

FIG 2. Type 2. White matter abnormalities, especially around the trigones. FLAIR image of a 4-year-old patient (case 22) (*A*) with an *ATRX* mutation of the chromatin-remodeling domain in exon 19 (p.V1624M), and a 35-month-old patient (case 21) (*B*) with an *ATRX* mutation of the chromatin-remodeling domain in exon 19 (p.A1622V). Increased signal intensity on T1WI/FLAIR in the periventricular region, especially around the peritrigonal area (*arrow*), and enlargement of perivascular space (*arrowhead*) are seen.

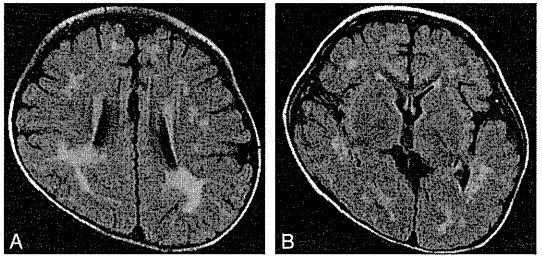

FIG 3. Type 3. Widespread and scattered white matter abnormalities. FLAIR image of a 12-month-old patient (case 1) with an *ATRX* mutation of a nucleotide substitution in 5′-UTR. Note high signal intensity on FLAIR/T2WI in the white matter, especially in the peritrigonal area and deep white matter, not in a diffuse but in a widespread and scattered pattern.

Because few comprehensive studies of brain MRI/CT of ATR-X syndrome are available, we propose here the classification of brain MRI/CT findings in the case of ATR-X syndrome.

MATERIALS AND METHODS

We reviewed the brain MRI/CT findings of 27 Japanese patients with ATR-X who exhibited *ATRX* mutations retrospectively. The images came from our medical center and from referrals sent to our center for consultation and spanned 1994–2012. They were evaluated by a pediatric neuroradiologist, a pediatric radiologist, or pediatric neurologists. The mutations of patients 5–18 reside in the ADD domain; those of patients 20–24, in the chromatin-remodeling domain. Other mutations are outside these 2 domains, and all of these mutations cause loss-of-function mutations (On-line Table). Their ages at the time of the evaluated MRI/CT ranged from 4 months to 54 years of age. MR imaging of the brain was performed at 1.5T or 3T at multiple medical centers. Axial T1WI, T2WI, and FLAIR or CT scans were evaluated. DWI was examined in a limited number of patients. Patients with severe prematurity and clinical signs of neonatal hypoxic-ischemic injury that would result in white matter and/or cortex abnormalities on MRI or CT scans were excluded from this study.

RESULTS

The MRI/CT findings and *ATRX* mutations of 27 patients with ATR-X are summarized in the On-line Table. The brain CT scans/MRI of all 27 patients whose scans were available showed some abnormal findings: Nonspecific brain atrophy (type 1, Fig 1) was shown in 17 of 27 patients (63%); a high intensity of white matter especially around the trigones, or the terminal zone, with/without multiple small spheric foci on FLAIR/T2WI (type 2, Fig 2) was seen in 11 patients (41%). Type 2 with hypoplasia of the cerebellar vermis was shown in 1 patient (case 22), and type 2 with hypoplasia of the corpus callosum and ventricular enlargement was seen in 1 patient (case 25). Compared with type 1, type 2 seemed more frequent in patients with mutations in the chromatin-remodeling domain. Four had hypoplasia of the corpus callosum. Furthermore, widespread and scattered white matter abnormalities on FLAIR (type 3, Fig 3) were seen in 1 patient (case 1). Delayed myelination (type 4, Fig 4) was shown in 4 (15%), and severe and rapidly progressive cortical brain atrophy with ventricular enlargement (type 5, Fig 5) was seen in 1 patient (case 27). No alterations of cerebral diffusion

could be observed on DWI in any patients with types 3, 4, and 5.

DISCUSSION

This is the first report on a comprehensive study of brain CT/MRI findings of patients with ATR-X syndrome, though some case reports or personal accounts mentioned some findings of brain MRI/CT, including mild cerebral atrophy, partial or complete agenesis of the corpus callosum, and hypoplasia of the white matter.[5,6] Our results suggest that brain MRI/CT can present a broader spectrum of abnormalities in both white and gray matter than has been expected in ATR-X syndrome. Some of our patients with ATR-X were referred to our medical center because their MRI/CT scans showed white matter abnormalities, or myelination; and for some, leukodystrophy was suspected as their diagnosis. Therefore, we categorized the MRI/CT findings of 27 patients with ATR-X into 5 types from the point of view of white matter lesions, or myelination.

More patients showed nonspecific brain atrophy (type 1) on brain MRI/CT. This result agrees with the finding that, in a review of 168 patients with ATR-X syndrome, 77% presented with microcephaly.[7] This brain atrophy in ATR-X syndrome is not progressive, and it seems to be due to the reduced production of neurons or glia in the perinatal period, not to some destructive process. This characterization was supported by a study by Bérubé et al[8] by using a conditional targeting approach with mice, showing that *ATRX* is a critical mediator of cell survival during early neuronal differentiation and that increased neuronal loss may contribute to severe mental retardation.[8]

Types 2 and 3 suggest an abnormality of the white matter, or myelination. Type 2 includes white matter abnormalities, especially around the trigones, with increased signal intensity showing on T2WI/FLAIR in the periventricular region. Persistently high signal intensity in this peritrigonal area, or the terminal zones, is seen throughout the first decade of life, and it is sometimes very difficult to differentiate from white matter injury resulting from prematurity, which results in periventricular leukomalacia. Moreover, a layer of myelinated white matter is present between the trigones of the ventricle and the terminal zones in healthy patients.[9] The findings shown in our patients with ATR-X differ from slow myelination in these areas or terminal zones, being normal variants in their distribution and signal intensity. Because these patients did not present clinically with apparent neonatal asphyxia and their findings do not show other signs suggesting periventricular leukomalacia, these white matter abnormalities may be relatively frequent findings in CT/MRI of ATR-X syndrome.

Especially type 3, with its widespread and scattered white matter abnormalities, strongly suggests that normal expression of the ATRX protein is involved in normal myelination because the patient's mutation is a nucleotide substitution in 5′-UTR and ATRX expression should be reduced, though the ATRX protein structure is normal. Actually, red cells of case 1 had more hemoglobin H inclusions (5%) than those of other patients, and he had hypospadias. Most interesting, his development was much better than that of typical patients with ATR-X, and he can walk and speak a few words. This suggests that the quantity of the ATRX protein can affect α-globin expression, genital development, and myelination, but the quality of the ATRX protein is more important for intellectual ability. Another possibility is that the site of the mutation may be more important for *ATRX* expression in the oligodendroglia, which is involved in myelination. The delayed myelination in type 4 also supports the idea that *ATRX* is important for normal myelination.

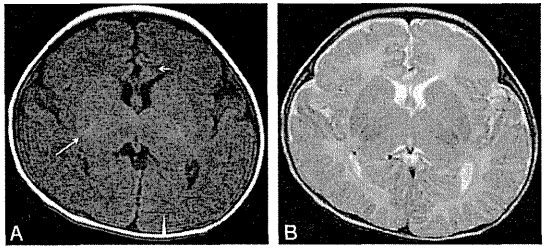

FIG 4. Type 4. Delayed myelination. T1WI (*A*) and T2WI (*B*) of a 4-month-old patient (case 26) with an *ATRX* mutation in exon 35 (c.7156C>T, p.Arg2386Stop). Myelination appears only at the posterior limb of the internal capsule (*long arrow*) on T1WI. At 4 months of age in a healthy infant, high intensity should extend from the junction of the anterior limb of the internal capsule at the callosal genu (*short arrow*) all the way back to the visual cortex (*arrowhead*) along the internal capsule and optic radiations.

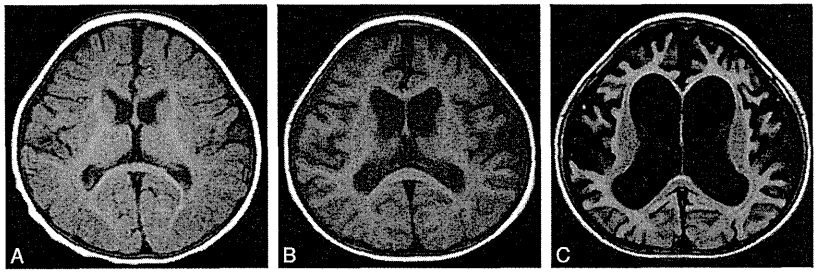

FIG 5. Type 5. Progressive brain atrophy. Sequential change of brain MR imaging of patient 27 with an *ATRX* mutation in int 35 (c.7200 + 4A>G, p.L2401fs) at 6 months (T1WI) (*A*), 14 months (T1WI) (*B*), and 34 months (FLAIR) (*C*).

High signal on T2WI/FLAIR and no alteration on DWI in the white matter of these patients meaning that there is no cytotoxic edema but increased water content in the white matter region, and these findings may suggest the prematurity of the blood-brain barrier in ATR-X syndrome, though our data have limitations in that not all MRI/CT scans include diffusion imaging or contrast enhancement.

Most interesting, 2 patients with ATR-X with partial duplications of the *ATRX* gene, resulting a severe reduction of *ATRX* messenger RNA and absence of the ATRX protein, presented with normal brain MR imaging findings and agenesis of the corpus callosum, respectively.[10] The former patient was evaluated before his death at 4.5 months of age, and it was difficult to detect white matter abnormalities. For the latter patient, although the age when the MR imaging was evaluated was 6 months, we cannot specify his age. He could have been too young for evaluation of myelination on MR imaging, and agenesis of the corpus callosum is the ultimate result of white matter abnormality. That finding does not contradict our theory that *ATRX* is important for normal myelination. However, more data should be forthcoming to clarify the relation between the ATRX protein and white matter or myelination.

Progressive brain atrophy in type 5 is an exceptional finding of CT/MRI in ATR-X syndrome. The patient with type 5 presented with a severe developmental delay and intractable epilepsy following West syndrome, which is rare in ATR-X syndrome. He had no episodes of hypoxic encephalopathy. Although it is possible that he may have had some other pathologic conditions, ATR-X syndrome should be considered in the differential diagnosis of patients with progressive brain atrophy on brain MRI/CT. His mutation was in int 35, or the last intron, resulting in skipping exon 35, which consists of 126 base pairs, and the introduction of a 43-amino-acid-deleted ATRX protein. This truncated protein may be related to his severe intellectual disability or epileptic condition.

A gray matter abnormality seems more frequent in those patients with *ATRX* mutation in the ADD domain, or exons 8, 9, and 10, and a white matter abnormality in the chromatin-remodeling domain, or exons 18 to 31. These results would indicate that mutations in the ADD domain produce more severe and permanent psychomotor deficiencies than those in chromatin-remodeling domains, though the clear phenotype-genotype correlation remains to be established.[11] However, conclusion may be too hasty because we have studied fewer cases with *ATRX* mutations in the chromatin-remodeling domains.

A number of intellectual disorders have been identified whose gene products regulate chromatin and chromosome architecture, and ATR-X syndrome is a disease of chromatin remodeling, as well as Rett syndrome (*MeCP2*) and Cornelia de Lange syndrome (*SMC1A, SMC3,* and *NIPBL*).[3,12] These 3 diseases share clinical manifestations, including severe intellectual disabilities, and ATRX, MeCP2, and cohesion proteins, respectively, indicating that these syndromes directly or indirectly interact with each other. Although these syndromes share a common final pathway to their pathogenesis, there are no common characteristic findings on brain MRI/CT among these diseases, except for brain hypoplasia or atrophy. These abnormal signals in the white matter on MR imaging seem to be relatively specific findings in ATR-X syndrome.

We propose that these findings should be included as associated (<50%) or supporting features of the diagnostic criteria for ATR-X syndrome. We will evaluate the brain MR imaging/CT of additional patients to validate these findings and update the consensus for the neuroradiologic features as a diagnostic criteria of ATR-X syndrome in the near future.

CONCLUSIONS

This is the first report on a comprehensive study of brain MRI/CT findings of ATR-X syndrome. We consider ATR-X syndrome a differential diagnosis in patients with intellectual disabilities whose brain MRI shows abnormal signals in the white matter. The ATRX protein seems to be involved in normal myelination. The classification will require revisions in the near future but will be helpful to establish the relationship between the *ATRX* gene mutation and brain development and to understand the ATRX protein function in the brain.

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SHORT COMMUNICATION

A case report of two brothers with ATR-X syndrome due to low maternal frequency of somatic mosaicism for an intragenic deletion in the *ATRX*

Hiroko Shimbo[1], Shinsuke Ninomiya[2], Kenji Kurosawa[3] and Takahito Wada[1,4]

In clinical practice, it is important to diagnose the carrier state of female patients with X-linked diseases for genetic counseling to calculate the recurrent risk of offspring. Because some X-linked diseases show high rates of gonadal mosaicism, this diagnosis is sometimes difficult, when there are few offspring in a family and no mutation is detected in the maternal genomic DNA. Here, we report two male siblings with ATR-X syndrome carrying an intragenic deletion of 78.6 kb involving exons 2–5 out of the 35 exons in the *ATRX*, as revealed by PCR amplification of these exons. The mother was expected to be an obligate carrier, but we could not confirm her as a mutation carrier by quantitative PCR (qPCR) for the exons. However, we identified the breakpoint of *ATRX*, and qPCR with breakpoint-specific primers revealed gonosomal mosaicism, with a relative frequency of the mutation of <1% in genomic DNA of her peripheral blood. For these obligate carriers of X-linked disease, we should aggressively investigate the maternal genomic status, not only because her genetic condition is important for estimating the recurrent risk of her offspring but also because a diagnosis of her gonosomal mosaicism can render negligible the possibility that her female siblings are carriers. We should reconfirm that a female who has a risk of being a carrier has a gonosomal or somatic mutation, even if she is an obligate carrier or apparently harbors a mutation.
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INTRODUCTION

X-linked α-thalassemia mental retardation syndrome (ATR-X syndrome; MIM# 603040) is characterized by severe intellectual disability, dysmorphic facies, hypotonia, genital and skeletal abnormalities and downregulation of the α-globin genes (α-thalassemia).[1] More than 80 patients have been molecularly diagnosed in Japan and more than 200 patients worldwide.[2] Most of their mutations are clustered in two functionally important regions: the ADD (ATRX-DNMT2-DNMT3) and helicase domains, resulting in loss of function.[2]

Generally, in sporadic cases of severe X-linked disease with zero-fitness, the probability that the mother is a mutation carrier is 2/3 and the probability that the patient has a *de novo* mutation is 1/3.[3] Thus, it is important to diagnose the mother's mutation carrier state to calculate the probability of the recurrence risk in the offspring. On the other hand, even if no mutations are detected in genomic DNA extracted from their peripheral blood, we should consider the possibility of germline, somatic or gonosomal mosaicism. Germline and gonosomal mosaicism have been recently reported in several other X-linked diseases,[4] and two cases of mosaicism, including gonosomal and germline, have been documented among 20 families of sporadic cases with ATR-X syndrome.[5]

Here, we report the case of two brothers affected with ATR-X syndrome, having an intragenic deletion involving exons 2–5 of the 35 exons in the *ATRX*, and their mother exhibiting gonosomal mosaicism, with the mutant allele at <1% incidence in the peripheral blood. This finding renders negligible the probability that his mother's female siblings are carriers of the mutation.

CASE REPORT

Case 1 (Figure 1; III-2) is a 9-year-old boy. He was suspected of having ATR-X syndrome at the age of 2 years on the basis of severe motor and psychiatric developmental delay, characteristic hypotonic facies, spaced teeth and presence of HbH inclusions in his brilliant cresyl blue (BCB)-stained peripheral blood. PCR of all exons and exon–intron boundaries was performed with specific primers, and PCR amplifications were not observed for exons 2–5 out of all 35 exons. His genomic DNA showed a deletion of exons 2–5 in the *ATRX*, confirming his diagnosis molecular genetically. His mother's mutation carrier state could not be diagnosed. He had received surgery for undescended testes. He has a 1-week vomiting episode annually. He had gastroesophageal reflux during his infancy but has grown out of it. He was diagnosed as having epilepsy and takes valproic acid. He has recently begun walking with assistance.

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He cannot speak any meaningful words but can understand simple words and situations around him. His weight, height and head circumference are 18.3 kg (−1.9 s.d.), 116.3 cm (−3.1 s.d.) and 47.2 cm (−2.5 s.d.), respectively.

Case 2 (Figure 1; III-3) is a 5-year-old boy, a younger brother of Case 1. At birth, he presented with multiple congenital anomalies, including tetralogy of Fallot, heart anomaly, complete tracheal ring, bilateral severe deafness, total blindness due to bilateral persistent hyperplastic primary vitreous, hypospadias and bilateral undescended testis. He had operations in infancy for tetralogy of Fallot and gastroesophageal reflux. He had epilepsy at the age of 4 years. He shows very severe psychomotor developmental delay and central

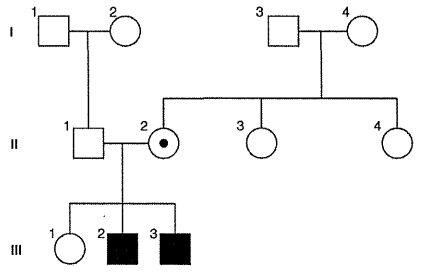

Figure 1 Family tree. III-2 and III-3 are patients. Their mother, II-2, is healthy and a carrier of the mutation in gonosomal mosaicism.

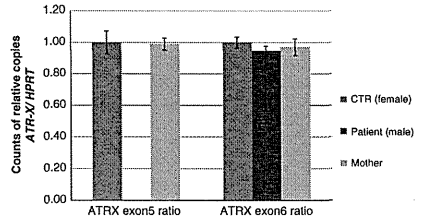

Figure 3 The result of quantitative PCR using primers for exons 5 and 6. It did not detect a copy number difference between mother and a control for exon 5, which was deleted in the patient. *HPRT* (X-linked) is the reference gene. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

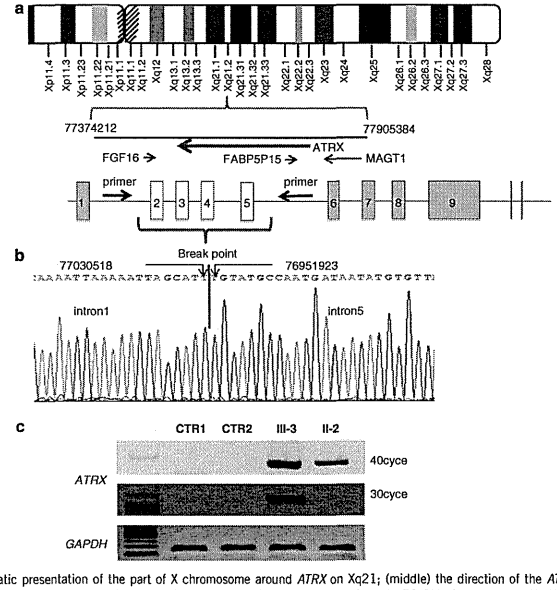

Figure 2 (**a**) (top) Schematic presentation of the part of X chromosome around *ATRX* on Xq21; (middle) the direction of the *ATRX*; and (bottom) a schema of the deleted exons of the *ATRX* detected in the patient. The deletion spans approximately 78.6 kb (chromosome X, NC_000023.10: 77030517-76951924). (**b**) Sequence chromatogram showing breakpoint of genomic DNA from patient III-3. (**c**) PCR using the primers flanking the deletion from intron 1 to intron 5 in the *ATRX* amplifies 1141-bp products in 40 cycles (top) and 30 cycles (middle). *GAPDH* is the reference gene (bottom). CTR1 and CTR2 are normal control samples. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

hypotonic facies. He can roll over but cannot sit unassisted. He cannot speak any meaningful words. His clinical condition is much severer than that of his brother and initially ATR-X syndrome was not suspected for his diagnosis.

He was referred to our medical center at 5 years. Approximately, 60% of his BCB-stained peripheral blood revealed HbH inclusions, and he was diagnosed to have the same mutation in the *ATRX*. His weight, height and head circumference are 10.185 kg (−3.0 s.d.), 95.5 cm (−3.4 s.d.) and 39.7 cm (−6.4 s.d.), respectively.

RESULTS

Diagnosis of the patient (III-3) by PCR

We could not amplify exons 2–5 of the 35 exons in *ATRX* in the genomic DNA extracted from the peripheral blood of the patient (III-3), indicating an intragenic deletion involving exons 2–5 in *ATRX* as well as in his elder brother (III-2). His cDNA shows that the 3′ end of exon 1 abuts the 5′ end of exon 6, indicating exon skipping of exons 2–5 resulting in a 350-bp deletion (Supplementary Figure S1). This transcript is expected to be translated into a prematurely truncated protein, p.Ser7Argfs*13.

Identification of the breakpoint

We designed PCR primers to amplify the flanking region of the deleted regions of genomic DNA from the patient (Figure 2a). No amplification of amplicons using the primers was observed in a normal control (Figure 2c).

We sequenced the PCR product and identified the 5′ end at the breakpoint in intron 1 and the 3′ end of intron 5, with primers flanking the deleted region. The deletion spans approximately 78.6 kb (chromosome X, NC_000023.10: 77030517-76951924) (Figure 2b).

Diagnosis of the mother's carrier state

Quantitative PCR (qPCR) using primers for each exon did not detect a copy number difference between exon 5, which was deleted, and exon 6, which was not deleted (Figure 3).

Primers flanking the deletion amplified an 1144-bp product in both the patients and the mother at 40 cycles but not in the mother at 30 cycles (Figure 2c). These findings suggest that the mother carried the same deleted mutation by gonosomal mosaicism, considering that she has two sons with ATR-X.

Estimation of the relative frequency of the mutant allele

Using qPCR with primers flanking the deletion, we estimated at $<1\%$ the relative frequency of the mutant allele in genomic DNA from peripheral blood of the mother. A dilution series of known template concentrations of the patient's genomic DNA was used to establish a standard curve. The standard curve showed a linear form ($R^2 = 0.9996$) (Supplementary Figure S2).

DISCUSSION

We report two affected brothers of ATR-X syndrome with an intragenic deletion of 78.6 kb involving exons 2–5 in the *ATRX*, resulting from their mother's gonosomal mosaicism. It was technically difficult to confirm her mosaicism by qPCR for each exon of the *ATRX*, because the relative frequency of the mutant allele was low, at $<1\%$. After we had identified the breakpoint in the patients' genomic DNA and designed primers straddling the breakpoint, we could easily detect the mutant allele in her genomic DNA.

When we diagnosed the first patient by the usual PCR, we did not know the mother's carrier state. The second patient was diagnosed as having ATR-X syndrome with the same mutation, and their mother was expected to be an obligate mutation carrier. There were two problems in diagnosing her carrier state, which are as follows: first, the mutation was a deletion that was not detected by conventional PCR and sequencing, and second, the relative frequency of the mutant allele in the peripheral blood was too low for the copy number to be determined by qPCR.

In fact, the information about the mother's mosaicism does not aid in genetic counseling to calculate the recurrent risk of subsequent offspring, because she is an obligate mutation carrier. But this information is important for genetic counseling of her two sisters (Figure 1, II-3 and II-4), because her mutation must have occurred *de novo* after fertilization, and the probability that her sisters carry the same mutation is expected to be negligible. Therefore, their genetic counseling was not required.

In clinical practice, somatic, gonadal or gonosomal mosaicism presents several problems, particularly if a patient's mother does not have the mutant allele in her blood; isolated gonadal mosaicism can be suspected, if the same mutant allele is transmitted to a second offspring and he is found to be affected. The relative frequency of the mutant allele in the mosaicism of her peripheral blood is not helpful for estimating the frequency in her ova. In our case, the relative frequency of the mutant allele in the ova could be $<1\%$ as in the peripheral blood or could be much higher, approximately 50%.

It is often difficult to contact the families of maternal female siblings and provide them with clinical information. The present study highlights the importance of thoroughly diagnosing the mother's mutation carrier status, even if only one offspring is affected or she does not exhibit the mutation. These findings allow us to decide whether to inform her female siblings and their families.

In the next-generation sequencing era, we will encounter more patients or carriers with mosaicism of mutant alleles.[6] We should reconsider mosaicism from the standpoint of genetic counseling.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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V．班構成員名簿

「ATR-X症候群の臨床研究および基礎研究のための基盤整備に関する研究」
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