

ORIGINAL ARTICLE

Genetic analysis of contiguous X-chromosome deletion syndrome encompassing the *BTK* and *TIMM8A* genes

Takashi Arai¹, Meina Zhao², Hirokazu Kanegane², Menno C van Zelm³, Takeshi Futatani^{2,4}, Masafumi Yamada⁵, Tadashi Ariga⁵, Hans D Ochs⁴, Toshio Miyawaki² and Tsutomu Oh-ishi⁶

Patients with X-linked agammaglobulinemia (XLA) can present with sensorineural deafness. This can result from a gross deletion that not only involved the Bruton's tyrosine kinase (*BTK*) gene, but also *TIMM8A*, mutations in which underlie the Mohr-Tranebjærg syndrome (MTS). We analyzed the genomic break points observed in three XLA–MTS patients and compared these with deletions break points from XLA patients. Patient 1 had a 63-kb deletion with break points in intron 15 of *BTK* and 4 kb upstream of *TAF7L*. Patients 2 and 3 had 149.7 and 196 kb deletions comprising *BTK*, *TIMM8A*, *TAF7L* and *DRP2*. The break points in patients 1 and 3 were located in *Alu* and endogenous retrovirus (*ERV*) repeats, whereas the break points in patient 2 did not show involvement of transposable elements. Comparison of gross deletion sizes and involvement of transposable elements in XLA and XLA–MTS patients from the literature showed preferential involvement of *Alu* elements in smaller deletions (< 10 kb). These results show further insights into the molecular mechanisms underlying gross deletions in patients with primary immunodeficiency.

Journal of Human Genetics (2011) 56, 577–582; doi:10.1038/jhg.2011.61; published online 14 July 2011

Keywords: *Alu*; *BTK*; Mohr-Tranebjærg syndrome; *TIMM8A*; X-linked agammaglobulinemia

INTRODUCTION

X-linked agammaglobulinemia (XLA) is an inherited primary immunodeficiency characterized by early onset of recurrent bacterial infections, profound hypogammaglobulinemia and markedly reduced circulating B cells.¹ The gene responsible for XLA was identified in 1993,^{2,3} and named Bruton's tyrosine kinase (*BTK*). The *BTK* gene is mapped to the Xq21.3–Xq22 region, encompasses 37.5 kb of genomic DNA and contains 19 exons (the initiation codon is in exon 2). Only 770 bp centromerically of the *BTK* gene is the gene *TIMM8A* (formerly *DDP1*) located; it consists of two exons and produces a 97 amino acid polypeptide.⁴ Mutations in *TIMM8A* cause the rare X-linked neurodegenerative Mohr-Tranebjærg syndrome (MTS), which is clinically characterized by a progressive neurological deficits, including early onset of sensorineural deafness.^{5–7}

A large number of *BTK* mutations, scattered over the entire gene, have been reported and deposited in an international mutation database (<http://bioinf.uta.fi/BTKbase/>). The most commonly found mutations are missense (34%), followed by nonsense mutations (20%). Mutations affecting splice sites were reported in 18% and small insertions and deletions in another 18% of XLA families. Disruption of the *BTK* gene by gross deletions occurs in about 3.5% of XLA families.¹ As *BTK* and *TIMM8A* are positioned in close genomic proximity, gross gene deletions can result in disruption of

both genes causing a contiguous deletion syndrome of XLA and MTS, which has been observed previously in nine families.^{4,8–10}

In this study, we describe three patients with gross deletions including the *BTK* and *TIMM8A* genes. We characterized the deletions and identified the genomic break points by combining comparative genomic hybridization (CGH) array, DNA fluorescence *in situ* hybridization (FISH) and long accurate (LA)-PCR analyses. The results of these investigations provide new insight into the genetic mechanisms causing the XLA–MTS deletion syndrome and allow accurate genetic analysis of patients and carriers.

MATERIALS AND METHODS

Patients

Patient 1 is a 15-year-old Japanese boy without a family history of immunodeficiency or neurological deficits. He was diagnosed with XLA at 7 years of age when he developed recurrent bacterial infections. Deafness was noticed 1 year earlier and gradually worsened.

Patient 2 is a 10-year-old Japanese boy, who has a history of recurrent otitis media and sinusitis since 12 months of age. He had been diagnosed with deafness and autism at 18 months of age. Agammaglobulinemia and lack of circulating B cells were recognized at 8 years of age, and was diagnosed with XLA.

Both patients are doing well on immunoglobulin replacement therapy. They have no dystonia, but their hearing losses are severe and progressive.

¹Division of Clinical Research, Saitama Children's Medical Center, Saitama, Japan; ²Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; ³Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; ⁴Center for Immunity and Immunotherapies, Seattle Children's Research Institute, University of Washington, Seattle, WA, USA; ⁵Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan and ⁶Department of Infectious Diseases, Immunology and Allergy, Saitama Children's Medical Center, Saitama, Japan

Correspondence: Dr H Kanegane, Department of Pediatrics, Graduate School of Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.

E-mail: kanegane@med.u-toyama.ac.jp

Received 3 April 2011; revised 10 May 2011; accepted 11 May 2011; published online 14 July 2011

Patient 3 corresponds to patient 6 who was described in a previous report.⁹ He was diagnosed with XLA at 8 months of age, and was found to have sensorineural hearing loss at 3 years of age. Genetic analysis resulted in the identification of a gross deletion involved the entire coding regions of the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes.

Gene analysis of *BTK*

Informed consent for genetic analysis was obtained from the patients and their parents under a protocol approved by the Institutional Review Board of University of Toyama. *BTK* mutation analysis was performed by direct sequencing of complementary DNA and all 19 exons and exon–intron boundaries using genomic DNA as described previously.^{11,12}

Fluorescence *in situ* hybridization

Peripheral blood mononuclear cells from patients and controls were stimulated in culture with phytohemagglutinin for 72 h, followed by treatment with a 0.075 M KCl solution and fixation with Carnoy's solution (3:1 methanol and acetic acid) for metaphase preparation. A *BTK*–*TIMM8A*-specific genomic probe (5231 bp long) was prepared using the LA-PCR Kit (Takara, Kyoto, Japan) with primer pairs reflecting exon 19 of *BTK* and exon 2 of *TIMM8A* (5'-AGCATTCTGGCATGAATGTTCCCTGAAC-3' and 5'-ATCTCTCCGGGTGCAGATAATAACTG C-3', respectively). In addition, probes were designed to detect the *TAF7L* and *DRP2* genes, which are located centromerically of *TIMM8A*. The *TAF7L*- and *DRP2*-specific genomic probes (5662 and 5975 bp) were prepared similarly by LA-PCR Kit (Takara) with primers derived from exons 2 and 5 of *TAF7L* (5'-GCTTAGGTAGCCACCAACGTGTTGTTGA-3' and 5'-GACGTCCCTGTTTCAACAAGGAATTAGGA-3') and from exons 7 and 12 of *DRP2* (5'-CGTGACTGTATTAAGGGCTCAACCATG-3' and 5'-GCAGTACTTCCTGTACAAAGCAGTTGC-3'), respectively. For FISH analysis, denatured metaphase spreads were hybridized with probes labeled with digoxigenin-11-deoxyuridine triphosphate using a nick translation kit (Roche Diagnostics KK, Tokyo, Japan). The Spectrum Green-labeled X-chromosome probe (DXZ1, Abbott Japan, Tokyo, Japan) was used as a control. The *BTK*–*TIMM8A*-, *TAF7L*- and *DRP2*-specific probes were detected with anti-digoxigenin rhodamine (Roche Diagnostics KK) providing a red signal, whereas DXZ1 was detected by a green signal. Metaphases were counterstained with 4', 6-diamino-2-phenylindole dihydrochloride, and the images of the

hybridization captured by fluorescence microscopy (Carl Zeiss Co., Tokyo, Japan). At least 20 metaphases were observed in each setting.

Array CGH analysis

Array CGH analysis was performed using the Agilent kit (Agilent Technologies, Palo Alto, CA, USA) as described.¹³ A total of 1 µg DNA from patients and male controls were double-digested with *RsaI* and *AluI* for 4 h at 37 °C. After column purification, each digested sample was labeled by random priming for 2 h using Cy3-deoxyuridine triphosphate for the patient DNA and Cy5-deoxyuridine triphosphate for the control DNA. Labeled products were purified by Microcon YM-30 filter units (Millipore, Billerica, MA, USA). After probe determination and pre-annealing with Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), hybridization was performed at 65 °C with rotation for 40 h. The hybridized array was analyzed with the Agilent scanner and the Feature Extraction software (v9.5.3.1; Agilent). A graphical overview was obtained using the CGH analytics software (v3.5.14; Agilent). The UCSC Genome Browser was used to retrieve the reference genome sequence (<http://www.genome.ucsc.edu>).

LA-PCR and sequencing

LA-PCR reaction was performed with the appropriate forward and reverse primers that were used to map the gross deletion boundaries as described previously.¹⁴ PCR products were excised from the gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and sequenced on ABI Prism 3130XL sequence detection system (Applied Biosystems, Foster City, CA, USA).

RESULTS

BTK mutation analysis

PCR analysis of genomic DNA resulted in the amplification of *BTK* exons 1–15, but not of exons 16–19 in patient 1, and amplifications of exons 1–5, but not of exons 6–19 in patient 2.

FISH analysis

To confirm a large deletion in the *BTK* gene and to investigate a possible deletion of the *TIMM8A* gene, we performed FISH analysis with a *BTK*–*TIMM8A*-specific probe. Cells from both patients 1 and 2 lacked signals of this probe (Figures 1a and b), indicating a contiguous

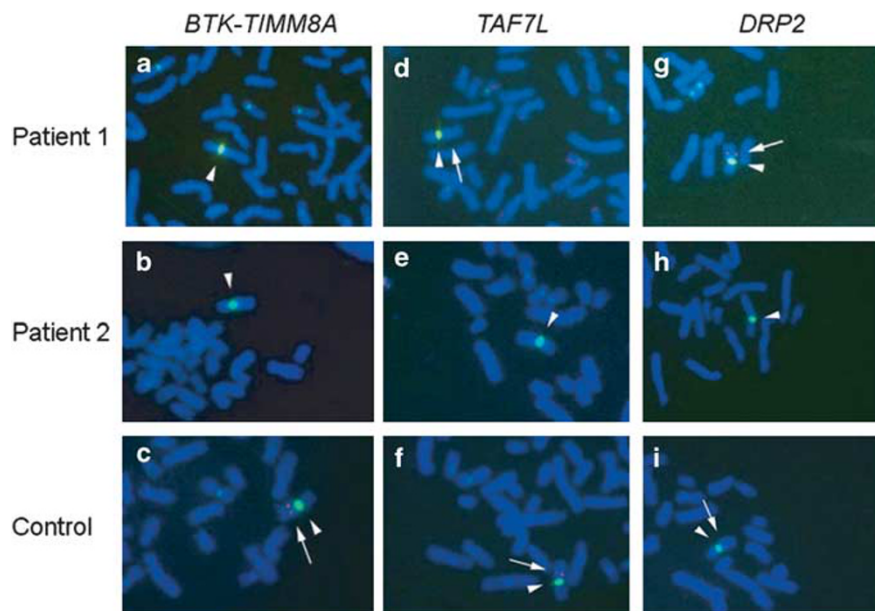


Figure 1 Identification of multigene deletions by fluorescence *in situ* hybridization analysis with Bruton's tyrosine kinase (*BTK*)–*TIMM8A*- (a–c), *TAF7L*- (d–f) and *DRP2*-specific probes (g–i) and the X-chromosome-specific DXZ1 probe in patient 1 (a, d and g), patient 2 (b, e and h) and healthy male control (c, f and i). The DXZ1 probe is shown in green (arrow heads), whereas the *BTK*–*TIMM8A*, *TAF7L* and *DRP2* probes are shown in red (arrows).

gene deletion syndrome of XLA and MTS. To study the extent of the deletion further, we performed additional FISH experiments using *TAF7L*- and *DRP2*-specific probes. Lymphocytes from patient 1 showed normal signals for both probes (Figures 1d and g), but cells from patient 2 failed to give signals for either *TAF7L* or for *DRP2* (Figures 1e and h). We conclude that the genomic deletion in patient 1 is restricted to the *BTK* and *TIMM8A* genes, whereas the deletion in patient 2 is considerably larger involving *TAF7L* and *DRP2*.

Array CGH analysis

To study the extent of the deletions in patients 1 and 2, we performed array CGH analysis of genomic DNA. In patient 1, we found an interstitial loss in copy number in the Xq22 region, involving the *BTK* and *TIMM8A* genes, spanning a minimum of 63 kb (Figure 2). In patient 2, we observed a deletion of at least 138 kb including the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes (Figure 2). These results confirmed the FISH analysis of patient 1 having a deletion of *BTK* and *TIMM8A*, and of patient 2 having a deletion involving the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes, similar to what was found for patient 3.⁹

Analysis of the break point junctions

On the basis of FISH and array CGH results, PCR primers were designed to span the putative break points. Sequence analysis of the PCR products from patient 1 revealed that recombination had occurred between a site in intron 15 of *BTK* and a site 4.4 kb upstream of *TAL7L* (Figure 3). It is an unequal crossover, which shares a 22-bp stretch of 100% homology and resulted in the deletion of 63 kb. To determine whether the break points were located in transposable elements, we analyzed the ± 1000 bp genomic sequences flanking the break point regions against reference collection repeats.¹⁵ The results revealed that both the distal and proximal deletion break points of patient 1 are located within short interspersed element of the *Alu* subclass (Figure 4).

In patient 2, the deletion extended from a site 4.4 kb upstream of *DRP2* to a site in intron 5 of *BTK*, encompassing 149.7 kb (Figure 3). Break point junction analysis revealed 3 bp microhomology between the two break point regions. Neither of the break points was located in DNA sequences derived from transposable elements (Figure 4).

The gross deletion in patient 3 encompassed 196 kb, and the junction showed microhomology between the 5' and 3' break points (Figure 3). The 5' break point was located upstream of the *DRP2* gene

in a repeat derived from an endogenous retrovirus 2 (ERV2), whereas the 3' break point in intron 1 of *BTK* was not located in a transposable element, but close to an *Alu* element (Figure 4).

Thus, two of the three break point regions of the XLA–MTS patients we studied showed involvement of transposable elements. Although six out of eight break points of *BTK* deletions were found to be located in an *Alu* element in previous studies,^{14,16,17} we observed *Alu* element involvement only in patient 1.

Transposable element involvement in XLA and XLA–MTS

It was previously shown that *BTK* gross deletion break points were frequently located in *Alu* elements. To study whether gross deletions resulting in XLA or XLA–MTS are derived from similar deletion mechanisms, we analyzed all reported gross deletions in XLA and XLA–MTS patients (Figure 5).^{9,14,16,17} Including our patients, we collected data from five XLA and six XLA–MTS deletions. The deletion size clearly differed: XLA–MTS patients, as expected, had much larger disease-causing deletions. As *BTK* and *TIMM8A* are in close genomic proximity, a deletion <10 kb can already disrupt both genes.

Interestingly, all deletions <10 kb involved *Alu* elements, whereas only two out of six deletions >10 kb had break points located in *Alu* elements (Figure 5). These results imply that *Alu* elements are more frequently associated with smaller deletions, whereas other elements, such as long interspersed elements and ERV elements, seem to be more frequently associated with larger deletions. As XLA–MTS deletions are on average larger than XLA deletions, they show less frequently involvement of *Alu* elements.

DISCUSSION

The contiguous gene deletion syndrome involving *BTK* and *TIMM8A* has previously been described in 12 patients from nine unrelated families with deletion sizes ranging from 20–196 kb.^{8–10} Of these, only three break point junctions have been cloned.^{14,16} In this study, we describe three patients with 63, 150 and 196 kb large deletions at the Xq22 region, which included *BTK* and *TIMM8A*.

Short interspersed elements of the *Alu* subclass are the most frequently occurring interspersed repeat elements in the human genome: the 280-bp sequence occurs approximately every 4 kb in the human genome.¹⁸ Mispairing between such repeats has been shown to be frequent causes of deletions and duplications. *Alu/Alu*-mediated genomic rearrangements are classical homologous recombination that result in a loss or gain in the number of nucleotide bases,

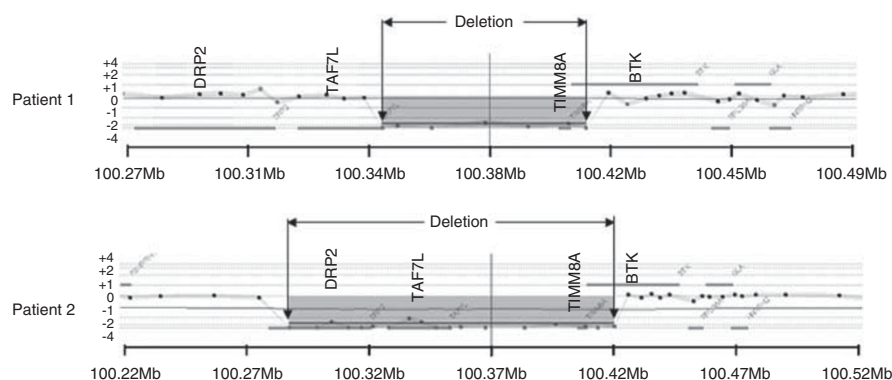


Figure 2 Array comparative genomic hybridization analysis of the X-chromosomes of patient 1 (upper panel) and patient 2 (lower panel). Grey belts indicate regions of continuously reduced copy number around Xq22. Upper panel discloses a deletion of at least 65.8 kb including the Bruton's tyrosine kinase (*BTK*) and *TIMM8A* genes in patient 1, and lower panel discloses a deletion of at least 138 kb including the *BTK*, *TIMM8A*, *TAF7L* and *DRP2* genes in patient 2. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

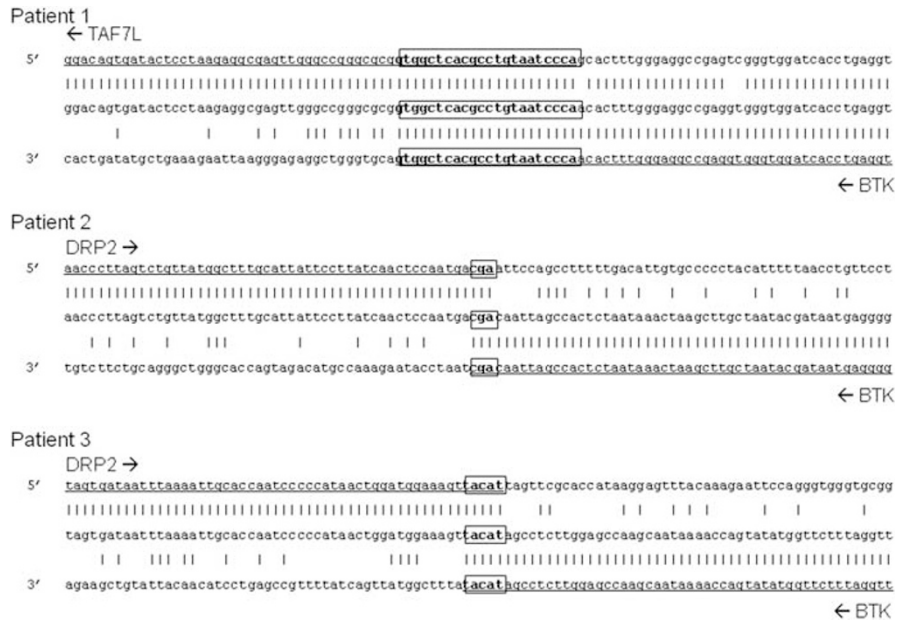


Figure 3 Sequences of the gross deletion break point junctions of three patients compared with control sequences. The upper, middle and lower panels indicate patient 1, patient 2 and patient 3. Microhomology regions at the junctions are boxed. BTK, Bruton's tyrosine kinase.

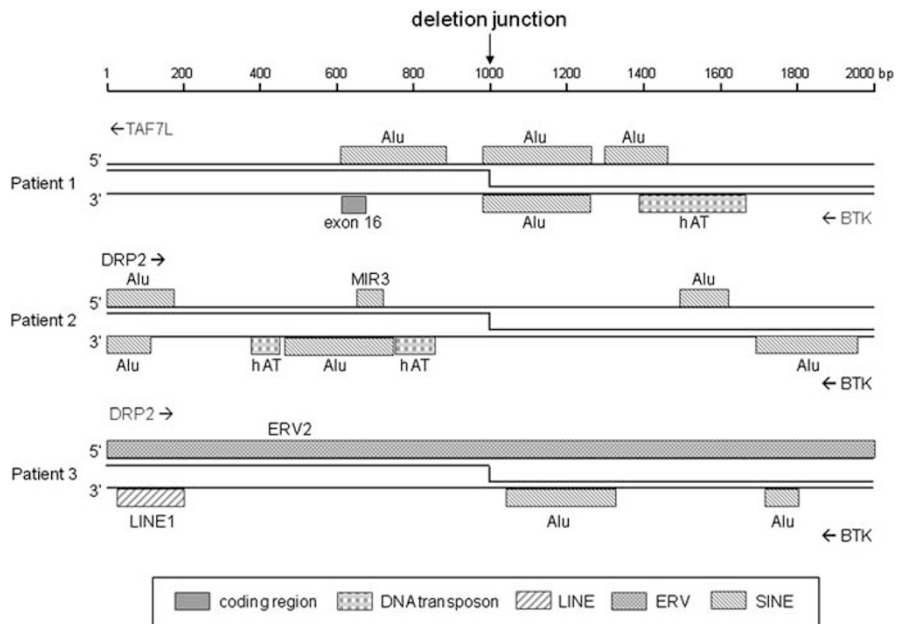


Figure 4 A \pm 1000 bp flanking the gross deletion break points of patient 1 (upper panel), patient 2 (middle panel) and patient 3 (lower panel). Besides coding elements, four types of transposable elements are indicated: DNA transposons, endogenous retrovirus (ERV), long interspersed element (LINE) and short interspersed element (SINE). BTK, Bruton's tyrosine kinase.

causing approximately 0.3% of human genetic diseases.^{19,20} Recently, the break point junctions of eight XLA patients with a *BTK* gross deletion were analyzed for the presence of transposon-derived repetitive elements. Most of the break points were located in *Alu* elements.¹⁴ These observations have suggested a general role for *Alu* sequences in promoting recombination in the *BTK* gene. Besides deletions, *Alu*-mediated recombination may also promote genomic duplications in *BTK*, depending on how the break points are joined.¹⁶ The 5' and 3' break points in patient 1 were located in *Alu* elements,

similar to most XLA patients with gross deletions affecting the *BTK* gene.^{14,16,17} The break points of patients 2 and 3, however, were not located in *Alu* elements, and, intriguingly, the homology regions were very small repeat fragments of only 3 or 5 bp nucleotides, implying that the deletion was not due to an unequal homologous recombination.

A comparison of the deletion size and the presence of transposable elements resulting in gross deletions causing XLA and XLA-MTS strongly suggests that *Alu* elements are mainly involved in <10 kb

Patient	Disease	Schematic representation of genomic deletion	Deletion size (kb)	Transposable element	reference
1	ID113	XLA	2.6	Alu	14
2	0850	XLA	2.8	Alu	16
3	P4	XLA	6.1	Alu	17
4	2430	XLA-MTS	7.5	Alu	16
5	ID434	XLA	8.2	Alu	14
6	ID440	XLA	11.5	-	14
7	2433	XLA-MTS	11.9	Alu	16
8	0703	XLA-MTS	38.2	LINE1	16
9	Patient1	XLA-MTS	63	Alu	This study
10	Patient2	XLA-MTS	149.7	-	This study
11	Patient3	XLA-MTS	196	ERV2	9

Figure 5 Schematic representation of all molecularly characterized gross deletions in patients with XLA and XLA-MTS. The patients are listed according to the size of the gross deletion and for each patient, the disorder (XLA or XLA-MTS) is given as well as the involvement of transposable elements. BTK, Bruton's tyrosine kinase; ERV, endogenous retrovirus; LINE, long interspersed element; MTS, Mohr-Tranebjærg syndrome; XLA, X-linked agammaglobulinemia.

deletions. As most deletions underlying XLA-MTS are >10kb, the involvement of *Alu* elements in deletions causing XLA-MTS is less frequent than in those associated with XLA. Thus, it is likely that the majority of the deletions underlying XLA-MTS arise from mechanisms that are different from those underlying XLA.

We used a combined approach of DNA FISH, array CGH and LA-PCR to characterize the break point regions in our patients and clone the break point regions. In doing so, we were able to establish a genetic basis of the disease. This is important for patient care with respect to genetic counseling and carriership analysis in family members. Wide use of DNA FISH and array CGH analysis to establish genetic abnormalities will support higher rates of gross lesions. This will not contribute to patient care, but to general understanding of complexity and frequency of these types of genetic lesions.

In patients 2 and 3, the *TAF7L* and *DRP2* genes were deleted in addition to *BTK* and *TIMM8A*. *TAF7L* encodes an RNA polymerase II TATA-box-binding protein-associated factor II protein, which is expressed only in male spermatogonia and may have a role in premeiotic stages of mammalian spermatogenesis.²¹ The *DRP2* gene encodes the dystrophin-related protein 2, which is a member of the dystrophin family of proteins performing a critical role in the maintenance of membrane-associated complexes at points of intercellular contact in vertebrate cells.²² It is expressed principally in the brain and spinal cord. Disruption of the *DRP2*-dystroglycan complex is followed by hypermyelination and destabilization of the Schwann cell-axon unit in *Prx* (-/-) mice.²³ However, no human pathology has been reported due to any mutation in these genes. Both patients presented with recurrent infections and hearing loss as typical symptoms of contiguous gene deletion syndrome of XLA and MTS, and showed no pathological findings which might be associated with the deletion of *TAF7L* and *DRP2* genes. Therefore, these genes might not have a critical function in humans or other proteins might compensate for their loss.

In conclusion, sensorineural hearing loss in XLA should raise concerns about a possible contiguous gene deletion syndrome. Array CGH analysis combined with FISH analysis can provide a more accurate diagnosis of large deletions. Not only transposable elements and microhomology, but also other mechanisms may contribute to gross rearrangements involving the *BTK* gene.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Ministry of Health, Labour and Welfare of Japan. We thank Chikako Sakai, Hitoshi Moriuchi and Noboru Hosogai for their excellent technical assistance. We are also grateful to the support, cooperation and trust of the patients and their families.

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