ORIGINAL ARTICLE

Breakpoint determination of X;autosome balanced translocations in four patients with premature ovarian failure

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Premature ovarian failure (POF) is a disorder characterized by amenorrhea and elevated serum gonadotropins before 40 years of age. As X chromosomal abnormalities are often recognized in POF patients, defects of X-linked gene may contribute to POF. Four cases of POF with t(X;autosome) were genetically analyzed. All the translocation breakpoints were determined at the nucleotide level. Interestingly, *COL4A6* at Xq22.3 encoding collagen type IV alpha 6 was disrupted by the translocation in one case, but in the remaining three cases, breakpoints did not involve any X-linked genes. According to the breakpoint sequences, two translocations had microhomology of a few nucleotides and the other two showed insertion of 3–8 nucleotides with unknown origin, suggesting that non-homologous end-joining is related to the formation of all the translocations.
Journal of Human Genetics (2011) **56**, 156–160; doi:10.1038/jhg.2010.155; published online 9 December 2010

INTRODUCTION

Premature ovarian failure (POF) is a disorder characterized by amenorrhea and elevated serum gonadotropins before 40 years of age. The risk of this disorder or natural menopause before 40 years is approximately 1% of women.[1] Heterogeneous etiology should be involved in POF, such as environmental, autoimmune and genetic factors. X chromosomal abnormalities (partial monosomies and X;autosome-balanced translocations) are often observed in POF patients. These rearrangements cluster at Xq13–q26 called the critical region (for POF).[2,3] The critical region is separated into two: critical region 1 at Xq13–q21 and critical region 2 at Xq23–q26.[2,4] It was suggested that several X-linked loci expressing on both X chromosomes, which were required in a higher dosage for normal ovarian function, were involved in POF.[5] Furthermore, genetic factors for POF may be more complex as X;autosome translocations often disrupt no genes; therefore, other factors, such as position effects on autosomal genes, are proposed.[6] We had an opportunity to analyze four cases of POF each having t(X;autosome). Precise determination of translocation breakpoints in these patients may reveal direct evidence of POF-related genes and mechanisms of the formation of chromosomal translocations. Breakpoint sequences will be presented and discussed in relation to genes and formation process.

MATERIALS AND METHODS

Patients and genomic DNA preparation

A total of four POF patients with t(X;autosome) were recruited to this study. Case 1 had secondary amenorrhea and the other three (cases 2, 3 and 4) presented with primary amenorrhea. Cases 1, 3 and 4 are Japanese and case 2 is Caucasian. Case 2 was reported previously.[7] Chromosome analysis revealed 46,X,t(X;4)(q21.3;p15.2) in case 1, 46,X,t(X;2)(q22;p13) in case 2, 46,X,t(X;4)(q22.1;q12) in case 3 and 46,X,t(X;14)(q24;q32.1) in case 4. All translocations occurred *de novo*. In addition, 11 other POF patients were collected to check candidate gene abnormality. After informed consent was obtained, genomic DNA was prepared from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan). Institutional review board approved the research protocol.

Fluorescence *in situ* hybridization

Metaphase chromosomes were prepared from peripheral blood lymphocytes of POF cases. Bacterial artificial chromosome DNA was labeled with fluorescein isothiocyanate- or Cy3-11-dUTP by Vysis Nick Translation kit (Vysis, Downers

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Received 17 September 2010; revised 10 November 2010; accepted 12 November 2010; published online 9 December 2010

Grove, IL, USA), and denatured at 70 °C for 10 min. Probe–hybridization mixtures (15 μl) were applied to chromosomes, incubated at 37 °C for 16–72 h, and then washed and mounted in antifade solution (Vector, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole. Photographs were taken on an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Southern blot and inverse PCR
Genomic DNA was digested with restriction enzymes: EcoRI and HindIII for case 1 and her parents, SacI and EcoRI for case 2 and a normal female control, BamHI and EcoRI for case 3 and her parents and NdeI and BglII for case 4 and her mother. Probes were made by PCR and labeled using DIG synthesis kit (Roche Applied Science, Basel, Switzerland). Hybridization, wash and detection were performed according to the manufacturer's protocol. Inverse PCR was performed using self-ligated DNA after digestion with EcoRI (cases 1 and 3), SacI (case 2) and BglII (case 4). All the breakpoints were determined by sequencing inverse PCR products. Information of primers used is available on request.

Mutation analysis
Genomic DNA was obtained from peripheral blood leukocytes by standard methods and used for mutational screening. Protein coding exons of COL4A6 (exons 1–45), insulin-like growth factor binding protein 7 (IGFBP7) (exons 1–5) and C14orf159 (exons 4–16) were screened by high-resolution melt analysis using LightCycler 480 system II (Roche Applied Science, Tokyo, Japan), except for exon 1 of IGFBP7, which were analyzed by direct sequencing. PCR mixture contained 20 ng genomic DNA, 1× ExTaq buffer, 0.2 mM each dNTPs, 0.3 μM each primer, 0.25 μl SYTO9 (Invitrogen, Carlsbad, CA, USA) and 0.25 U ExTaq HS (Takara, Ohtsu, Japan). PCR was initially denatured at 94 °C for 2 min and cycled 45 times for 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C, and then finalized at 72 °C for 1 min. High-resolution melt analysis was then performed. For exon 1 of IGFBP7, PCR mixture contained 20 ng genomic DNA, 1× GC buffer II, 0.4 mM each dNTPs, 1 μM each primers, 2% dimethyl-sulfoxide and 0.04 U LaTaq HS (Takara), and then PCR was initially denatured at 94 °C for 2 min and cycled 35 times at 94 °C for 20 s, at 60 °C for 20 s, at 72 °C for 1 min, and finalized at 72 °C for 2 min. If a sample showed

an aberrant melting curve pattern, the PCR product was purified using ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced by a standard method using BigDye terminator ver.3 (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Sequences were compared with reference sequences using SeqScape version 2.7 (Applied Biosystems).

X-inactivation assay
Human androgen receptor assay and FRAXA locus methylation assay were performed as described previously,[8,9] with a slight modification. In brief, genomic DNA of patients, their parents and a female control was digested with two methylation-sensitive enzymes, HpaII and HhaI. Subsequently, PCR was performed using digested and undigested DNA with human androgen receptor assay primers (FAM-labeled ARf: 5′-TCCAGAATCTGTTCCAGAG CGTGC-3′; ARr: 5′-CTCTACGATGGGCTTGGGGAGAAC-3′)[10] and FRAXA primers (FAM-labeled FRM1f: 5′-AGCCCCGCACTTCCACCACCAGCTCCT CCA-3′; FMR1r: 5′-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′), electrophoresed on ABI PRISM 3100 Genetic analyzer and analyzed with GeneMapper™ Software version 3.5 (Applied Biosystems).

RESULTS
Breakpoint sequences
Using fluorescence in situ hybridization analysis of metaphase chromosomes, we could identify Bacterial artificial chromosome clones spanning translocation breakpoints in each patient: RP11-636H11 at Xq22.3 (case 1), RP11-815E21 at Xq22.3 (case 2), RP11-589G9 at 4q12 (case 3) and RP11-904N19 at Xq24 (case 4). Southern blot analysis could identify aberrant bands in all the patients (Figure 1) and subsequent inverse PCR successfully cloned all breakpoints in the four cases. Breakpoint sequences are shown in Figure 2. Junction sequencing of der(X) and der(4) in case 1 revealed a 4192-bp deletion of chromosome X (UCSC genome browser coordinates March 2006 version: chr. X: 107 322 866–107 327 057 bp) and 7082-bp deletion (chr. 4: 11 846 359–11 853 387 bp) of chromosome 4, respectively. In addition, five unknown nucleotides were recognized at the der(X)
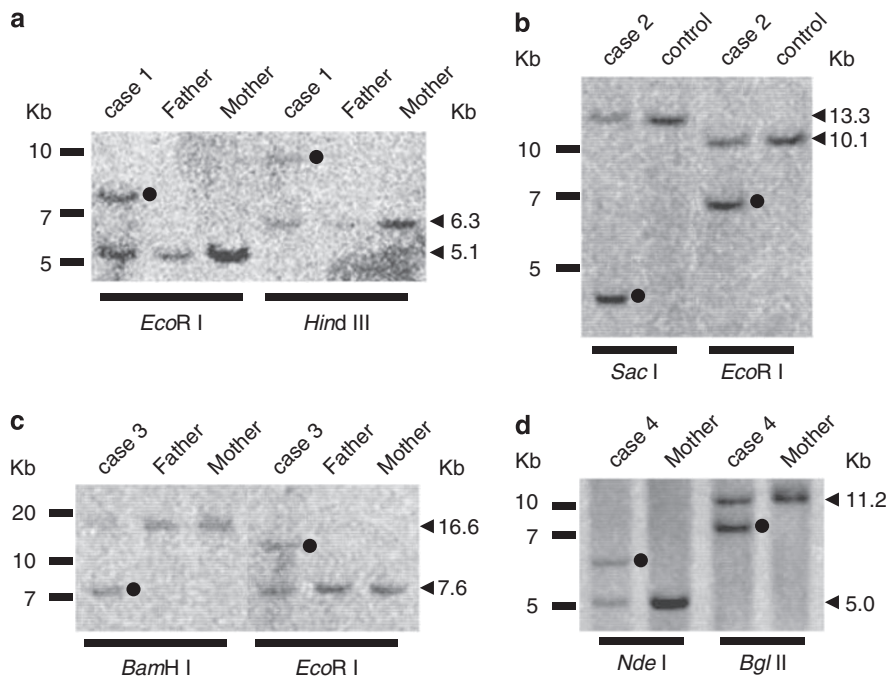
Figure 1 Southern blot analysis of four cases. (a) case 1, (b) case 2, (c) case 3 and (d) case 4. Aberrant bands are indicated with dots.
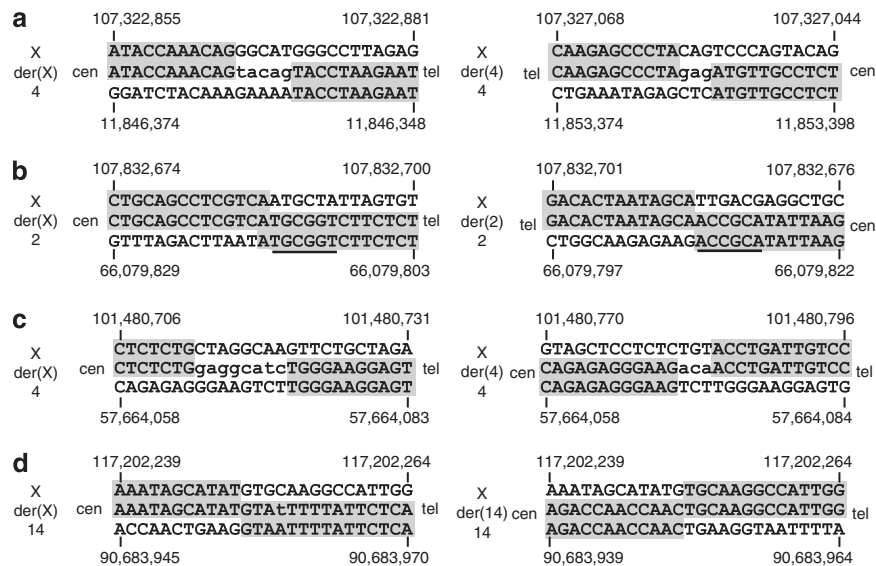
Figure 2 Breakpoint sequences of t(X;autosome) in four cases. (**a**) case 1, (**b**) case 2, (**c**) case 3 and (**d**) case 4. Top, middle and bottom sequences indicate those of normal X, derivative and normal autosomal chromosomes. Upper and lower cases indicate nucleotides of known and unknown origin, respectively. Matched sequences are with gray shadow. Underline indicates duplicated sequence. Numbers are based on the nucleotide position of the UCSC genome browser coordinates March 2006 version.

junction as well as three unknown nucleotides at der(4) (Figure 2a). Sequences of der(X) and der(2) in case 2 indicated six nucleotides (chr. 2: 66 079 810–66 079 816 bp) were duplicated (Figure 2b). In case 3, 71 nucleotides (chr. X: 101 480 713–101 480 782 bp) were deleted, and unknown eight nucleotides were inserted in der(X), and unknown three nucleotides were also recognized in der(4) (Figure 2c). In case 4, a nucleotide in chromosome X (chr. X: 117 202 250 bp) and five nucleotides in chromosome 14 (chr. 14: 90 683 951–90 683 955 bp) were missing (Figure 2d). The locations of X-chromosome breakpoints are shown in Figure 3. Translocation breakpoints disrupted *COL4A6* at Xq22.3 in case 1 (Figures 3a and b, Table 1), *IGFBP7* at 4q12 in case 3 (Table 1) and *C14orf159* at 14q32.12 in case 4 (Table 1). Other breakpoints did not involve any functional genes. Adjacent genes to breakpoints (less than 100 kb away) are *COL4A5* (5 kb away at Xq22.3) and *IRS4* (30 kb away at Xq22.3) in case 2, *NXF2* (12 or 21 kb away at Xq22.1) in case 3 and *KLHL13* (67 kb away at Xq24) in case 4 (Table 1). *COL4A6*, encoding collagen type IVα6, was the only disrupted X-linked gene in our POF patients.

X-inactivation assay
Human androgen receptor assay in cases 2 and 3 and FRAXA assay in cases 1 and 4 clearly indicated skewed X inactivation in all cases (100% in case 1, 94% in case 2, 98% in case 3 and 100% in case 4) and random patterns in their mothers available for this study (20–80%). Eleven other POF patients also showed random inactivation patterns (30–70%).

Mutation search
Considering accumulation of X-chromosome structural abnormalities in POF, X-chromosomal genes disrupted by rearrangements would be the primary target of this study. As *COL4A6* was completely disrupted in case 1 (Figure 3b), we started analyzing *COL4A6* as a candidate in 11 other POF patients. We found one heterozygous missense change, c.1460G > T (p.Gly487Val) (Figure 3c). This mutation was not recognized in 247 ethnically matched female controls (494 alleles).

Web-based SIFT (http://sift.jcvi.org/) and PolyPhen (http://genetics.bwh.harvard.edu/pph/) did not indicate harmful effects of the amino-acid change on protein function: 0.26 by SIFT (predictable functional damage is < 0.05) and 'benign' by PolyPhen, but the amino acid was evolutionarily conserved (Figure 3d). The Gly487 was located between the (Gly–X–Y)*n* repeats. Parental origin of the change could not be confirmed as parental samples were unavailable. As *IGFB7* at 4q12 and *C14orf159* at 14q32.12 were also disrupted, both genes were analyzed in the 11 POF patients, but no mutation was found.

DISCUSSION
In this study, we could successfully determine the translocation breakpoints at nucleotide level in all the four cases analyzed. *COL4A6* at Xq22.3 in case 1, *IGFBP7* at 4q12 in case 3 and *C14orf159* at 14q32.12 in case 4 were disrupted. No genes were disrupted in case 2. Importantly, *COL4A6* was the only X-linked gene that was our primary target as a causative gene for POF. One missense change with benign nature outside the functional repeats was found in another POF patient who showed random X inactivation (35%).

In case 1, based on the skewing of X inactivation, der(X) should be active and normal X should be inactive. Thus, *COL4A6* is predicted to be functionally null in case 1 as the active allele is disrupted by the translocation. Collagen type IV is an essential component of basement membrane, consisting of six distinct α-chains (α1–α6) encoded by *COL4A1* to *COL4A6*. These six genes are located in three pairs with head-to-head orientation, *COL4A1*/*COL4A2* on chromosome 13, *COL4A3*/*COL4A4* on chromosome 2 and *COL4A5*/*COL4A6* on chromosome X. The chains interact and assemble with specificity to form three distinct patterns: α1α1α2, α3α4α5 and α5α5α6.[11] The α5- and α6-chains are found in the basement membrane of skin, smooth muscle and kidney.[12] Two transcripts of *COL4A6* are known, isoforms A and B (Figure 3b). The protein structure of collagen type IV contains an amino-terminal collagenous domain (also called 7S domain), a triple-helical region (Gly–X–Y) and a carboxyl-terminal
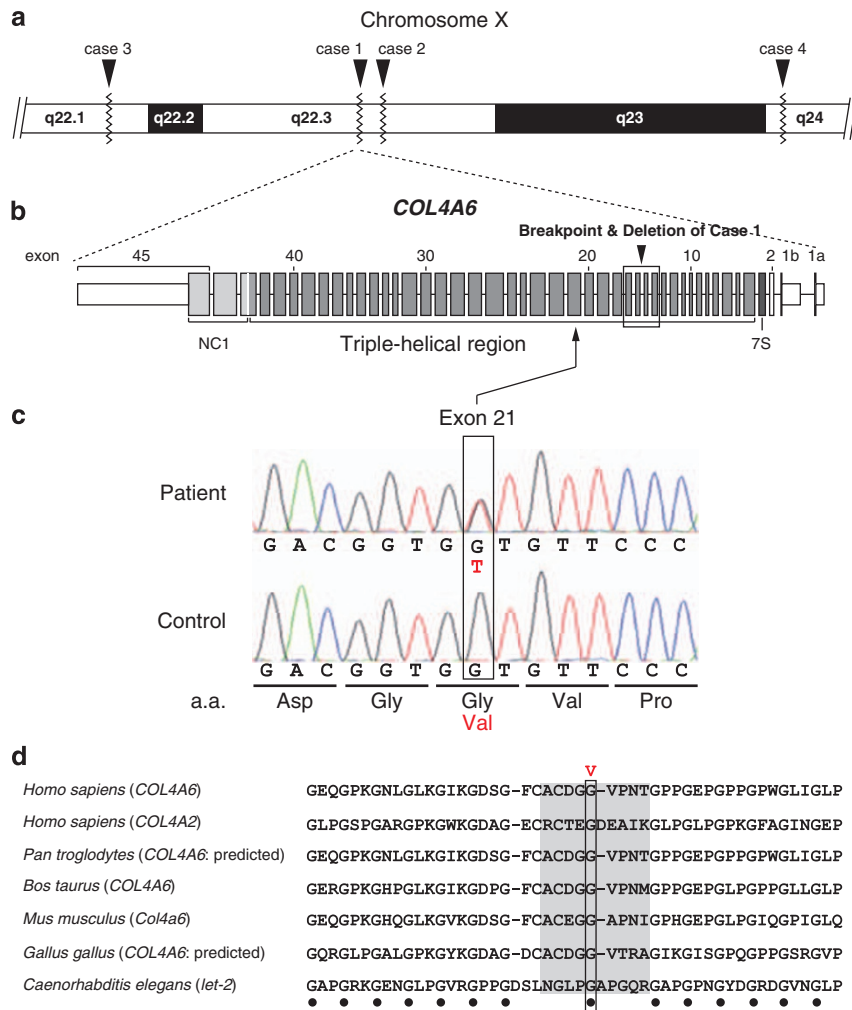
Figure 3 Location of the X-chromosome breakpoints and the *COL4A6* gene. (**a**) Breakpoint locations (zigzag lines) of four cases around Xq22.1–q24. (**b**) Schema of the *COL4A6* gene. Boxes are exons with numbering. White, dark gray, gray and light gray boxes indicate UTRs, 7S domain, triple-helical region and non-collagenous (NC1) domain, respectively. Breakpoint of the translocation with associated deletion is shown above boxes. (**c**) Heterozygous missense mutation, c.1460G>T (p.Gly487Val) at exon 21, is shown in the upper panel and wild-type sequence is shown in the lower panel. a.a.: amino acid. (**d**) Evolutionary conservation of the Gly487. CLUSTALW (http://align.genome.jp/) was used for this analysis. Dots show perfect conservation. Gray box is a space between the Gly–*X*–*Y* repeats.

Table 1 Genes within a 100-kb distance from translocation breakpoints

Case	Chromosome X	Autosome
1	*COL4A6*	None
	[q22.3]	[4p15.33]
2	*COL4A5* (5 kb)	None
	IRS4 (30 kb)	
	[q22.3]	[2p14]
3	*NXF2*[a] (12 or 21 kb)	*IGFBP7*
	[q22.1]	[4q12]
4	*KLHL13* (67 kb)	*C14orf159*
	[q24]	[14q32.12]

Round and square brackets indicate a distance from a breakpoint and chromosomal location, respectively.
Underlined genes are disrupted by breakpoints.
[a]*NXF2* is mapped to two adjacent segmental duplications.

non-collagenous (NC1) domain (Figure 3b).[13] We found a missense change, c.1460G>T (p.Gly487Val), in exon 21 in another POF patient (Figure 3c). Although this change is not found in 247 Japanese controls, its benign nature is suspected based on the web-based programs, the location outside the functional repeats and random X inactivation leading to the production of normal α6-chain. Parental samples were unfortunately unavailable to test the origin of the nucleotide change.

COL4A6 abnormality is known to be related to Alport syndrome with diffuse leiomyomas (AL-DS). *COL4A6* deletions in AL-DS are limited to exons 1, 1′ and 2 always together with *COL4A5* deletion in diverse extent.[14,15] In this paper, we first describe a POF patient (case 1) with disruption of only *COL4A6* not involving *COL4A5*. The inactivated normal X chromosome as well as the der(X) with disrupted *COL4A6* should lead to functionally null status in the patient. Extracellular matrix proteins (including COL4A6) have been shown to alter Leydig cell steroidogenesis *in vivo*, implying that Leydig cell steroidogenic

activity and matrix environment are interdependent.[16] Therefore, *COL4A6* depletion in ovarian extracellular matrix may alter normal steroidogenesis even in the ovary and have been possibly the cause of POF, especially in case 1. So far, there have been at least eight POF genes registered in OMIM: *FMR1* at Xq27.3 (POF1, OMIM no. 311360), *DIAPH2* at Xq22 (POF2A, OMIM no. 300511), *POF1B* at Xq21 (POF2B, OMIM no. 300604), *FOXL2* at 3q23 (POF3, OMIM no. 608996), *BMP15* at Xp11.2 (POF4, OMIM no. 300510), *NOBOX* at 7q35 (POF5, OMIM no. 611548), *FIGLA* at 2p12 (POF6, OMIM no. 612310) and *NR5A1* at 9q33 (POF7, OMIM no. 312964). Furthermore, *XPNPEP2* at Xq25,[17] *DACH2* at Xq21.2[18] and *CHM* (Xq21.2)[19] have also been described as being disrupted by translocations. *COL4A6* may possibly be an additional X-linked gene related to POF.

Two autosomal genes were disrupted: a gene encoding *IGFBP7* at 4q12 and *C14orf159* on 14q32.12. *IGFBP7* (also known as *IGFBP-rP1* or *MAC25*) is a secreted 31-kDa protein, belonging to the IGFBP superfamily. *IGFBP7* is involved in proliferation, senescence and apoptosis. Recently, it is reported that *IGFBP7* loss has a functional role in thyroid carcinogenesis.[20] *C14orf159* is a hypothetical protein with unknown function. Both disrupted genes were relatively expressed in ovary based on the GeneCards database (http://www.genecards.org/). We could not find any sequence aberrations in either gene among other POF patients. Further analysis might be necessary in relation to POF.

According to the precise breakpoint locations in all the cases reported here, *COL4A5* and *IRS4* (case 2), *NXF2* (case 3) and *KLHL13* (case 4) were localized near to breakpoints (within less than a 100-kb distance). All the adjacent genes except for *KLHL13* are shown to be expressed in human ovary in the GeneCards database. Interestingly, it was suggested that IRS4 protein expression was decreased in theca cells of polycystic ovary syndrome[21] and IGFBP7 protein suppressed estrogen production in granulose cells.[22] Reduced expression of these genes owing to the position effects by translocations could affect to normal ovarian function.

On the basis of the breakpoint sequences, two translocations (in cases 2 and 4) had microhomology (defined as the presence of the same short sequence of bases) of a few nucleotides and the other two (in cases 1 and 3) showed insertion of 3–8 nucleotides of unknown origin, suggesting that non-homologous end-joining is related to the formation of all the translocations in our patients.[23]

In conclusion, we could determine four t(X;autosome) breakpoints at the nucleotide level. We found that only one X-linked gene, *COL4A6*, was disrupted, resulting in functionally null status. All the four translocations are formed by non-homologous end-joining.

ACKNOWLEDGEMENTS

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