Clinical and Experimental Nephrology, Original Article

Onset mechanism of a female patient with Dent disease 2

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The number of words in the manuscript: 3505 words

Abstract:

Background: Approximately 15% of patients with Dent disease have pathogenic variants in the *OCRL* gene on Xq25-26, a condition that is referred to as Dent disease 2 (Dent-2). Dent-2 patients sometimes show mild extrarenal features of Lowe syndrome, such as mild mental retardation, suggesting that Dent-2 represents a mild form of Lowe syndrome. To date, 8 female patients with Lowe syndrome have been reported, but no female Dent-2 patients have been reported.

Methods: In this study, we performed genetic testing of the first female Dent-2 patient to detect the presence of an *OCRL* variant. Aberrant splicing was demonstrated by in vivo, in vitro and in silico assays, and skewed X-chromosome inactivation (XCI) in our patient and asymptomatic mothers of 3 Lowe patients with the heterozygous *OCRL* variant was evaluated by HUMARA assays using genomic DNA and RNA expression analysis.

Results: Our patient had an *OCRL* heterozygous intronic variant of c.1603-3G>C in intron 15 that led to a 169-bp insertion in exon 16, yielding the truncating mutation r.1602_1603ins(169) (p.Val535Glyfs*6) in exon 16. HUMARA assays of leukocytes obtained from this patient demonstrated incompletely skewed XCI (not extremely skewed). On the other hand, the asymptomatic mothers of 3 Lowe patients demonstrated random XCI. These results may lead to our patient's Dent-2 phenotype.

Conclusions: This is the first report of a female patient clinically and genetically diagnosed with Dent-2 caused by an *OCRL* heterozygous splicing site variant and skewed XCI. Skewed XCI may be one of the factors associated with phenotypic diversity in female patients with Lowe syndrome and Dent-2.

Key words: Lowe syndrome, Dent disease, X-chromosome inactivation, OCRL, genetic counseling

Introduction:

The classic form of oculocerebrorenal syndrome of Lowe (Lowe syndrome; OMIM#309000) is an X-linked multisystemic disorder characterized by the triad of congenital cataracts, severe intellectual impairment, and renal proximal tubular dysfunction that usually leads to end-stage renal disease (ESRD) [1, 2]. Lowe syndrome is caused by mutations in the *OCRL* gene, which is located on Xq25-26, and encodes OCRL-1, an inositol polyphosphate 5-phosphatase [3, 4]. The prevalence of Lowe syndrome is very low, estimated as 1 in 500,000 to 1,000,000 individuals [5].

Dent disease is an X-linked isolated renal proximal tubulopathy[6]. Approximately 15% of patients with Dent disease have pathogenic variants in the *OCRL* gene, a condition that is referred to as Dent disease-2 (Dent-2; OMIM#300555) [7, 8]. Although Dent disease is characterized by isolated tubulopathy, some Dent-2 patients show mild extrarenal features of Lowe syndrome such as mild mental retardation, suggesting that Dent-2 represents a mild form of Lowe syndrome [4, 9, 8]. To date, 8 female patients with Lowe syndrome have been reported [10-16] (Table 1), but no female patients with Dent-2 have been reported to date.

In this study, we performed genetic analyses to detect the presence of an *OCRL* variant in-an attempt to determine the onset mechanism of Dent-2 in the first female Dent-2 patient. At present, the molecular mechanism underlying the difference in the clinical severity of Lowe syndrome and Dent-2 is unknown. The results of this study suggest a possible molecular mechanism for the difference between Lowe syndrome and Dent-2.

Materials and methods:

Isolation of genomic DNA and total RNA from leukocytes

Peripheral blood leukocytes were isolated from the patient and from her parents, and genomic DNA was extracted from the leukocytes using the QuickGene Mini 80 system (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the protocol provided with the kit. The RiboPure Blood Kit (Invitrogen, Carlsbad, CA, USA) and an RNA stabilization agent (RNAlater; Invitrogen) were used to extract total RNA from the leukocytes. The obtained genomic DNA was subsequently used for targeted sequencing, Sanger sequencing, and HUMARA assays.

Targeted genomic DNA sequencing

Comprehensive analyses were conducted to examine the genes associated with Dent disease, Lowe syndrome and other diseases affecting renal tubule function by NGS using the MiSeq platform (Illumina, San Diego, CA). The sample library for NGS analysis was prepared using the HaloPlex target enrichment system (Agilent Technologies, Santa Clara, CA, USA) according to the protocol provided with the kit. Approximately 225 ng of genome DNA was extracted, subjected to restriction reaction for fragmentation, and hybridized with NGS probes at 54 ℃ for 16 h. The patient's genomic DNA was subsequently amplified by polymerase chain reaction and sequenced using the MiSeq platform. The results were analyzed using SureCall 3.0 (Agilent Technologies).

Sanger sequencing and transcript analysis

The results of the mutational analysis of *OCRL* obtained by targeted sequencing were confirmed using Sanger sequencing. *OCRL* mutation sites in the patient and in her parents were amplified by PCR, and the PCR-amplified products were purified and subjected to direct sequencing using a dye terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) and an automatic DNA sequencer (ABI Prism 3130; Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Some PCR products were subjected to subcloning after purification. For variant descriptions, NG_008638.1 (NM_000276.4) was used as a reference sequence. Total RNA was reverse-transcribed to cDNA using Ecodry Premix (Double Primed; Clontech Laboratories Inc., Mountain View, CA, USA), and cDNA was amplified by RT-PCR using a specific pair of *OCRL* primers to detect abnormal splicing. After 40 cycles of amplification, the PCR products were separated on a 1.5% agarose gel, and the purified products were sequenced using a dye terminator cycle sequencing kit and an automatic DNA sequencer as described above. Semi-quantification of RNA was conducted using capillary electrophoresis (2100 Bioanalyzer; Agilent Technologies).

HUMARA method

The HUMARA assay was performed as previously described [17]. First, 200 ng of genomic DNA from blood leukocytes and urine sediment was digested by a methylation-sensitive enzyme (*Hpa2*) and the same volume of undigested DNA was amplified using a FAM-labeled forward primer and reverse primer specific to regions on either side of the polymorphic CAG repeats. The PCR product was mixed with an internal size standard (GeneScan 500 LIZ dye Size Standard; Perkin Elmer, Applied Biosystems) in deionized formamide and

subjected to automatic DNA sequencing (ABI Prism 3100; Perkin Elmer, Applied Biosystems). Data quantification and visualization were performed using GeneScan software. X-chromosome inactivation (XCI) was evaluated by calculating a corrected ratio that compensated for amplification bias of the shorter allele as previously reported [18]. The corrected ratio was obtained by dividing the ratio of the peak height of the shorter allele to that of the longer allele in the double-digested sample by the ratio of the peak height of the shorter allele to that of the longer allele in the single-digested sample. Results showing a corrected ratio of 80:20 to 90:10 were determined as having a skewed XCI pattern.

In vitro splicing analysis

To create hybrid minigene constructs, we used the H492 vector based on the pcDNA 3.0 mammalian expression vector (Invitrogen, Oregon, USA), using our recently reported method [19, 20]. Genomic DNA from both the patient and control peripheral leukocytes was amplified using primers for *OCRL* IVS16-330 to IVS17+218. The hybrid minigenes were checked by sequencing and transfected into HEK293T and HeLa cells using Lipofectamine®2000 (Thermo Fisher Scientific, MA, USA). After 24 hours, total RNA was extracted from cells using the RNeasy® Plus Mini Kit (QIAGEN GmbH, Hilden, Germany), and PCR was performed. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and subjected to direct sequencing.

Results:

Patient characteristics

The patient is the first daughter of unrelated parents. At 7 years of age, she showed mild proteinuria without hematuria or glycosuria in the school urinary screening system, and she was referred to a regional hospital. At that time, her height was 108.6 cm (-2.1 SD), and her body weight was 19.5 kg (-0.8 SD). Her blood pressure was within the normal range. The patient's hearing was normal and the ophthalmological findings showed no abnormalities; cataracts and glaucoma were absent. The patient had mild mental retardation (IQ; 65-70). Biochemical analysis showed normal levels of total protein (7.5 g/dl) and a normal glomerular filtration rate (123 ml/min/1.73 m^2) without electrolyte abnormalities, metabolic acidosis or hypouricemia. However, mildly elevated levels of creatine kinase (282 IU/l) and lactate dehydrogenase (346 U/l), proteinuria (trace by dip stick, 2.0 g/g creatinine), low-molecular weight proteinuria (urinary β2 microglobulin level; 98300 μg/l), hypercalciuria (urinary calcium to creatinine ratio; 0.5) and aminoaciduria were evident. Renal echography and CT showed no nephrocalcinosis or nephrolithiasis. She had normal female external genitalia and a female karyotype (46,XX). Family screening showed normal urinary findings in her parents and in her two young brothers. The patient is now 8 years old. To date, no abnormal ophthalmological findings have been observed during follow-up.

Detection of pathogenic intronic variants of *OCRL*

Comprehensive genetic analysis of *CLCN5* and *OCRL* using genomic DNA isolated from peripheral leukocytes obtained from the patient and her parents demonstrated that the patient had an *OCRL* heterozygous intronic variant c.1603-3C>G in intron 15 that has not been reported previously, whereas neither of her parents

possessed this variant (Figure 1a). The variant (c.1603-3C>G) has not been registered in any of several variant databases including 1000 Genomes, gnomAD, dbSNP, HGVD and ClinVar. In intron 17 of the *OCRL* gene, the mother had a benign variant (c.1879+38_1879+39insTCC, dBSNP:rs34007381), while the father did not have this benign variant (Supplementary Figure 1a). Genetic analysis of our patient, including subcloning of PCR products to separate the paternal and maternal alleles, demonstrated that this benign variant was not found in the allele with the de novo mutation in intron 15 (Supplementary Figure 1b). Based on these findings, the de novo mutation in our patient is located on the X chromosome she received from her father. The patient and her parents had no mutation in *CLCN5*. These genetic and clinical features were compatible with Dent-2; however, the pathogenicity of this intronic variant (c.1603-3C>G) and the onset mechanisms of Dent-2 in female patients were not clear.

In the in vivo analysis, electrophoresis of RNA transcripts from peripheral blood leukocytes demonstrated that the control (Ct) exhibited a single band, while the patient's (PT) transcript exhibited a double band: one of the bands, which might be derived from the normal allele, was the same size as the Ct band, and the other, which might be derived from the mutant allele, was 169 bp larger (Figure 1b and 1c). Furthermore, this larger band was expressed more strongly than the Ct band in the patient. Semiquantitative analysis revealed a ratio of normal to abnormal mRNA expression of 26:74, suggesting a mildly skewed XCI pattern (Figure 1b). In silico analysis (Shapiro's score and human splicing finder) revealed that the splice site variant (c.1603-3C>G) disrupted the original acceptor site and resulted in the generation of an aberrant acceptor site that led to a 169-bp insertion; this was revealed to be a truncating mutation, r.1602_1603ins(169) (p.Val535Glyfs*6), in exon 16

(Figure 1d). In vitro splicing experiments (minigene assay) (Figure 2a) revealed that the patient's (PT) sequence exhibited a complete 169-bp insertion compared to the wild type (Figure 2b).

X-chromosome inactivation analysis

In both our patient and her parents, the HUMARA assay was performed using genomic DNA derived from blood leukocytes and urinary sediment (Figure 2c). This assay demonstrated that our patient had a skewed XCI pattern, with a paternal to maternal allele activation ratio 85:15 in leukocytes and 81:19 in urine sediment. Skewed XCI was also observed in her mother's leukocytes. On the other hand, the asymptomatic mothers of 3 Lowe patients with heterozygous *OCRL* variant demonstrated random XCI (Supplementary Figure 2).

Discussion:

Our patient is the first female to be clinically and genetically diagnosed with Dent-2. She has short stature and mild developmental retardation without congenital cataract; the clinical features of her disease correspond to a relatively more severe phenotype for Dent-2 but a much milder phenotype for Lowe syndrome [5, 9].

Comprehensive genetic analysis of the *OCRL* gene in our patient demonstrated the presence of a heterozygous intronic variant of c.1603-3G>C in intron 15 that led to a 169-bp insertion in exon 16, which produced the truncating mutation r.1602_1603ins(169) (p.Val535Glyfs*6) in exon 16. Studies of genotype-phenotype diversity in Lowe syndrome and Dent-2 patients have shown that nearly all truncating mutations of

the *OCRL* gene associated with Lowe syndrome are located in exons 8-24, which encode the inositol polyphosphate 5-phosphatase, ASH and RhoGAP-like domains, whereas the majority of truncating mutations that cause Dent-2 are located in exons 1-7, which encompass the PH domain [5, 21]. Therefore, our patient might have manifested Lowe syndrome if an “extremely” skewed XCI or X/autosome translocation had occurred, as previously reported in a female patient with Lowe syndrome (Table 1) [10, 16, 14]; instead, our patient manifested the Dent-2 phenotype.

Based on XCI analysis using genomic DNA and mRNA, our patient had a mildly skewed XCI pattern (not extremely XCI). The pattern of XCI can affect the clinical severity of X-linked disorders in females [17]. In mammals, X-linked gene products can be dosage-compensated in females by inactivation of one of the two X chromosomes in developing female embryos [10]. XCI is usually random; therefore, heterozygous females with X-linked recessive disorders usually show mild to no clinical phenotype compared to male patients. However, some such females show a complete phenotype; this can be caused by several mechanisms, including cytogenetic abnormalities (e.g., a reciprocal translocation involving the X-chromosome, 45,X karyotype, uniparental disomy and extremely skewed XCI) [22]. Our patient showed a skewed, but not extremely skewed, XCI that induced incomplete inactivation of the mutant allele. On the other hand, the asymptomatic mothers of 3 Lowe patients with heterozygous *OCRL* variant demonstrated random XCI (Supplementary Figure 2). It was suggested that this difference in genetic background is the reason our patient manifested Dent-2. The results also indicate that skewed XCI may be one of the factors associated with phenotypic diversity in female patients with

X-linked disorders such as Lowe syndrome and Dent-2, suggesting that *OCRL* gene mutations may cause two different diseases.

Conclusions:

This study is the first report of a female patient clinically and genetically diagnosed with Dent-2 caused by an *OCRL* heterozygous splicing site variant and skewed XCI. Skewed XCI may be one of the factors associated with phenotypic diversity in female patients with Lowe syndrome and Dent-2.

Compliance with Ethical Standards:

Conflict of interest: The authors declare that they have no conflicts of interest to report.

Research involving human participants or animals:

Our patient is a patient at Hokkaido University Hospital and this analysis was performed at Kobe University Graduate School of Medicine. All procedures performed in this study were reviewed and approved by the Institutional Review Board of Hokkaido University Hospital (IRB approval number 019-0412) and Kobe University Graduate School of Medicine (IRB approval number 019-301).

Informed assent and consent: Informed assent was obtained from the patient and informed consent was obtained from her parents.

Acknowledgments:

This study was supported by Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (subject ID: 19K17297 to Nana Sakakibara, 17H04189 to Kazumoto Iijima and 19K08726 to Kandai Nozu). We thank Ichiro Kobayashi and Yukiyo

Ohshima (Department of Pediatrics, KKR Sapporo Medical Center, Sapporo, Japan) for providing us with medical information on the patient and her family. We also thank Tomohiko Yamamura and Tomoko Horinouchi (Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan) for conducting the genetic examination. We thank American Journal Experts for editing a draft of this manuscript.

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FIGURE LEGENDS

Figure 1. Genetic analysis of the *OCRL* gene.

a. Results of Sanger sequencing of the *OCRL* gene. Genetic analysis of *OCRL* using genomic DNA isolated from peripheral leukocytes demonstrated that the patient had a novel *OCRL* heterozygous splicing site variant (NG_008638.1 (NM_000276.4): c.1603-3C>G) in intron 15, whereas neither of her parents had a mutation in *OCRL*.

b. Transcript analysis of the patient's peripheral blood leukocytes. The panel shows the electrophoresis results in which the control (Ct) exhibited a single band, while the patient's transcript exhibited a double band: one transcript was the same size as Ct, and the other was 169 bp larger. This larger band was expressed more strongly than the Ct band in our patient, suggesting a skewed XCI pattern.

c. Schematic of the splicing pattern found in our patient. A 169-bp insertion was found between exon 15 and exon 16.

d. In in silico analysis (Shapiro's score and human splicing finder), the splice site variant (c.1603-3C>G) disrupted the original acceptor site and resulted in the generation of an aberrant acceptor site that led to a 169-bp insertion and revealed the truncating mutation r.1602_1603ins(169) (p.Val535Glyfs*6).

Figure 2. Minigene assay transcript analysis and X-chromosome inactivation analysis.

a. Schematic of the hybrid minigene. The H492 vector contains two cassette exons, A and B, with multiple cloning sites, including NheI and BamHI restriction sites. The H492 vector also contains a cytomegalovirus

enhancer-promotor and a bovine growth hormone gene (BGH) polyadenylation site. An insertion fragment consisting of either the wild-type exon 16 or exon 16 with the intronic mutation (c.1603-3C>G) plus the flanking intron sequences of *OCRL* was inserted into the multicloning site within an intervening intron between two exons (exon A and B) of the minigene construct (H492) built in the pcDNA 3.0 mammalian expression vector (Invitrogen) as previously reported [23]. The arrows show the positions of the primers used in the RT-PCR assay.

b. Electrophoresis and schematic for the analysis of transcripts obtained from the minigene constructs. The left panel shows the electrophoresis result. Transcripts of the minigene containing the patient sequence from both HEK293 and Hera cells were 169-bp larger than those of the minigene containing the control sequence. The right panel shows the 169-bp insertion between exon 15 and exon 16 in the patient sample.

c. XCI analysis by HUMARA assay in peripheral leukocytes and urine sediment. This assay revealed that our patient had a skewed XCI pattern, with a ratio of 85:15 in leukocytes and a ratio of 81:19 in urine sediment. Our patient's mother also had a skewed XCI.

SUPPLEMENTARY FIGURE LEGENDS

Figure 1. The de novo mutation in our patient is located on the paternal X chromosome

a. In intron 17 of the *OCRL* gene, the mother had a benign variant (c.1879+38_1879+39insTCC, dBSNP:rs34007381), while the father did not have this benign variant.

b. Genetic analysis of our patient demonstrated that this benign variant was not found in the allele with the de novo mutation in intron 15 that was separated by subcloning of the purified PCR products.

Figure 2. Relationship between the degree of skewedness of X-chromosome inactivation and clinical severity in female cases with heterozygous *OCRL* mutations

a. Clinical and genetic characteristics of 3 Lowe patients whose mothers have heterozygous *OCRL* mutations.

b. XCI analysis by HUMARA assay in peripheral leukocytes in the mothers of 3 Lowe patients with the heterozygous OCRL variant. All of them demonstrated no skewed XCI.

c. Summary of XCI in female cases with heterozygous *OCRL* mutation.

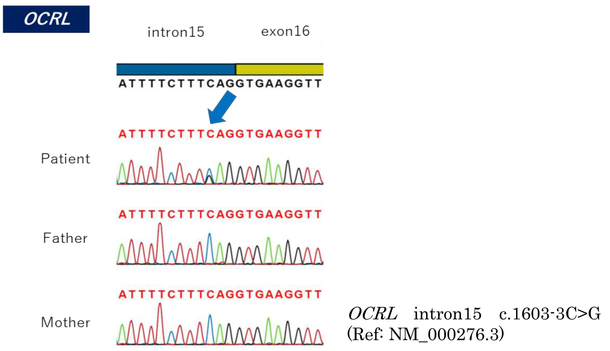
Table 1. Previously reported female patients with Lowe syndrome

Authors	Family history of Lowe syndrome	Ocular abnormalities	Intellectual impairment	Renal proximal tubular dysfunction	Karyotype	*OCRL* mutation	X-chromosome inactivation
Scholten et al [10]	+	+	ND	+	ND	ND	ND
	+	+	ND	+	ND	ND	ND
Svorc et al [11]	-	+	+	+	46,XX	ND	ND
Sagel et al [12]	-	+	+	+	46,XX	ND	ND
Cyvin et al [14]	-	+	+	+	46,XX	ND	ND
Hodgson et al [13]	-	+	+	+	46,XX,t(14;17)(q24;q23), t(X;3)(q25;q27)	ND	X/autosome translocation
Mueller et al [15]	-	+	+	+	46,XX,t(X;20)(q26.1;q11.2)	ND	X/autosome translocation
Cau et al [9]	-	+	+	+	46,XX	IVS14+2T>G	extremely skewed XCI

Abbreviations: ND, not determined; XCI, X-chromosome inactivation

Figure 1

a



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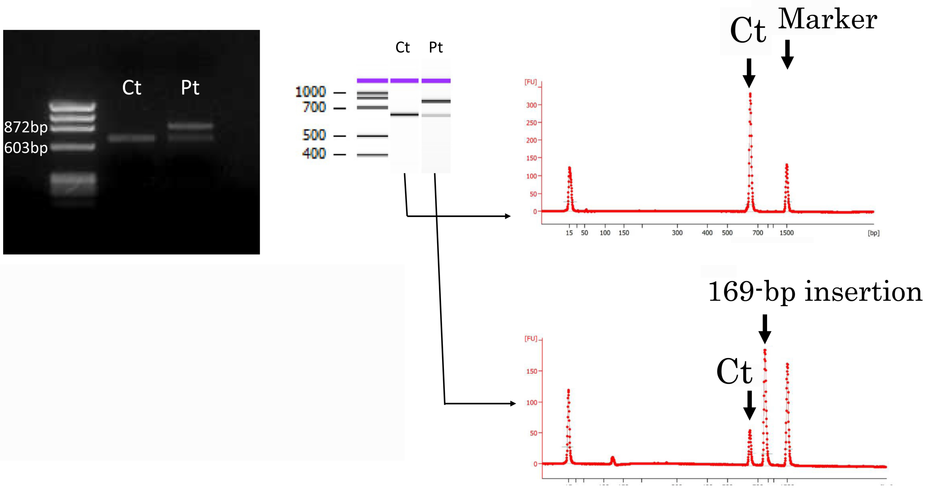
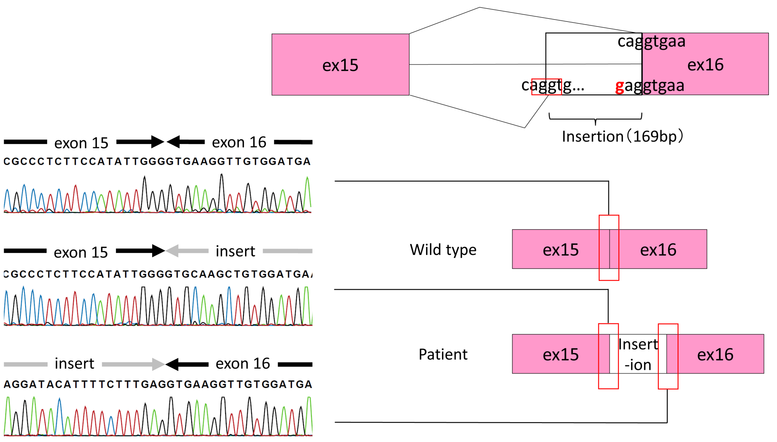
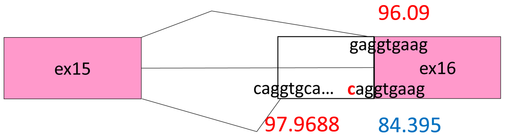


c



d

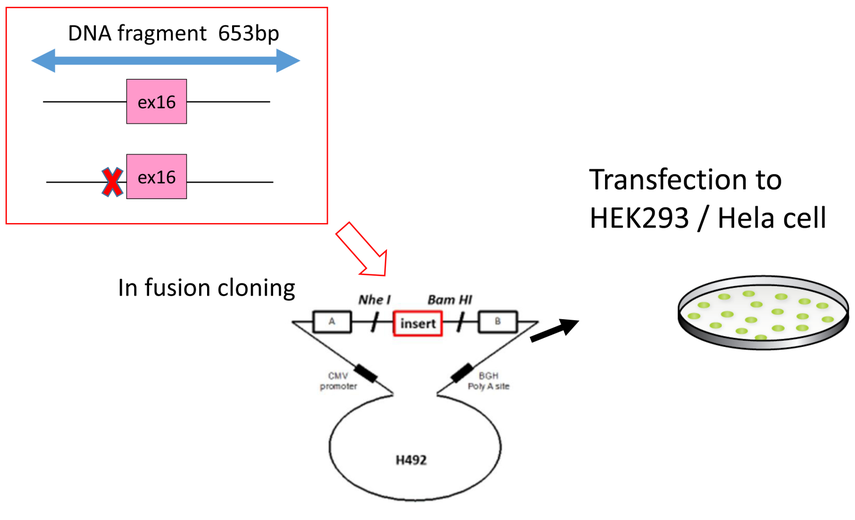
Shapiro's score



Human splicing finder

Figure 2

a



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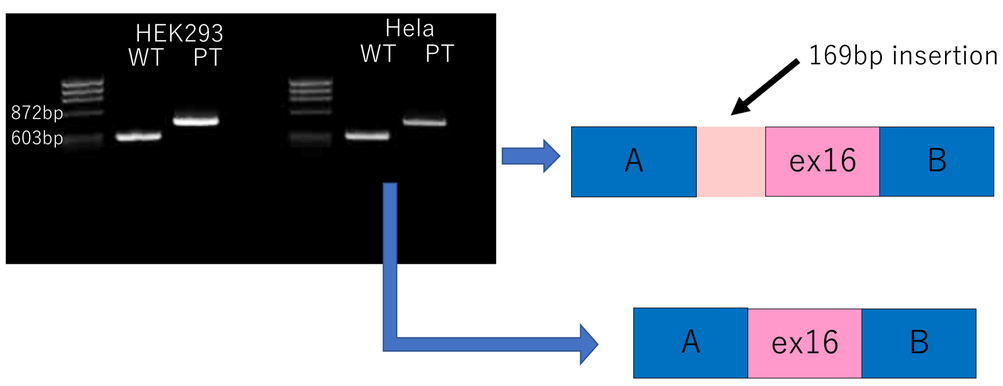
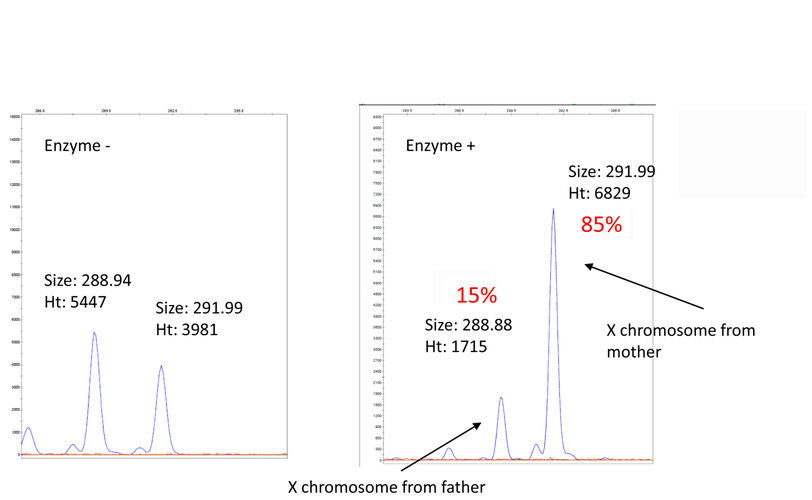
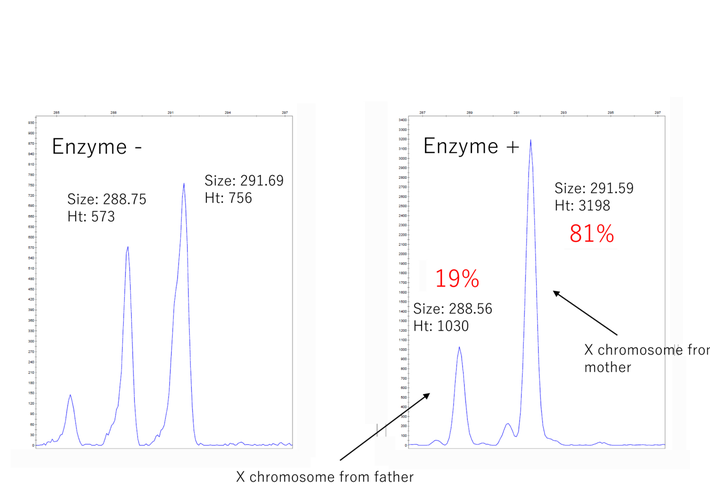


c

Patient

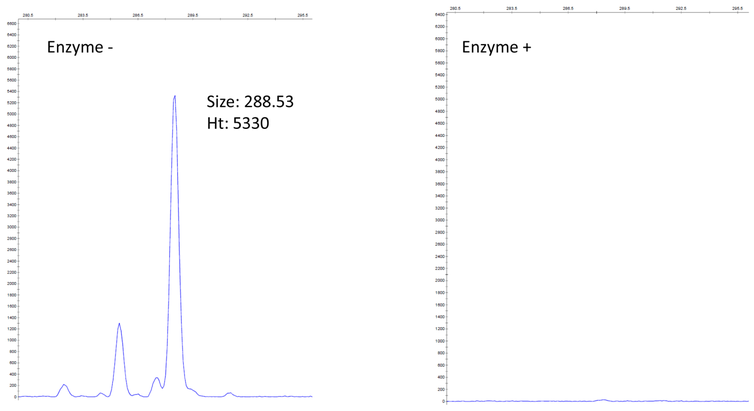


Peripheral leukocyte



Urine sediment

Father



Mother