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CLINICAL CORNER: CASE REPORT

## Disruption of *HDX* gene in premature ovarian failure

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We present a case of a 19-year-old phenotypically normal girl with premature ovarian failure. Cytogenetic analysis using G banding and fluorescence *in situ* hybridization (FISH) from cultured peripheral blood lymphocytes of the patient and the family revealed a *de novo* X;15 translocation and the imbalance to be 46,X,t(X;15)(Xpter → Xq21::15q11 → 15qter;15pter → 15q11::Xq21 → Xqter).

ish (CEPX+, wcp15+, ISNRPN+, PML+, D15S10+, wcp15-, SNRRN-, PML-)[20]. The X chromosome inactivation (XCI) assay revealed a completely skewed XCI pattern in which selective pressure favors an active maternal allele. The Affymetrix 2.7 M cytogenetics whole-Genome array confirmed the chromosomal imbalance and identified disruption of the *HDX* gene at Xq21, the translocation breakpoint.

**Keywords** *HDX* gene, premature ovarian failure, X chromosome inactivation, X;autosome translocation

**Abbreviations** FISH: fluorescence *in situ* hybridization; XCI: X chromosome inactivation; POF: premature ovarian failure; POI: premature ovarian insufficiency; USG: ultrasonography; FSH: follicle-stimulating hormone; LH: luteinizing hormone; TSH: thyroid-stimulating hormone; *HDX*: highly divergent homeobox; AR: androgen-receptor; CrR: corrected ratio.

### Introduction

Premature ovarian failure (POF; POF1: MIM 311360, POF2: MIM 300511; POF3: MIM 608996) [Beck-Peccoz and Perzani 2006], premature ovarian insufficiency (POI), are characterized by absences of menstruation (primary amenorrhea) or premature depletion of ovarian follicles with elevated gonadotrophin and low estrogen before the age of 40 years (secondary amenorrhea) [Cordts et al. 2011]. Iatrogenic intervention, autoimmune diseases, infections, X chromosome defects, and monogenic defects are possible causes of POF [Beck-Peccoz and Perzani 2006]. There are different types of X chromosome aberrations. These

include monosomy X, trisomy X, partial deletions, and X; autosome translocations. X;autosome translocations are rare chromosomal abnormalities with an incidence rate of 1 in 30,000 in live births [Speroff and Fritz 2005].

Premature ovarian failure is clearly a heterogeneous disorder associated with mutations in *FMR1* [POF1, (OMIM: 311360)] on Xq27.3, *DIAPH2* [POF2A, OMIM: 300511] on Xq21.33 and *POF1B* [POF2B, (OMIM: 300604)] on Xq21.1, *FOXL2* [POF3, (608996)] on 3q22.1, *BMP15* [POF4, (OMIM:300510)] on Xp11.22, *NOBOX* [POF5, (OMIM:611548)] on 7q35, *NIGLA* [POF6, (OMIM:612310)] on 2p13.3, and *NR5A1* [POF7, (OMIM:612964)] on 9q33.3. In this paper, we report a phenotypically normal girl with an X;15 translocation that interrupts the highly divergent homeobox (*HDX*) (RefSeq accession number: NG\_017158.1) gene on Xq21.1.

### Case Report

A 17-year-old girl with secondary amenorrhea was admitted to the Department of Medical Biology, Section of Medical Genetics at Ondokuz Mayıs University. She was the fourth child of healthy non-consanguineous parents. Her body height was 158 cm, and her weight was 51 kg. She menstruated at age of 12 and then at 17 years of age with continued hormone therapy. Ultrasonography (USG) revealed small ovaries with arrested folliculogenesis. Laboratory investigations demonstrated that her hormonal levels were as follows: follicle-stimulating hormone (FSH), 75.6 mU/mL; luteinizing hormone (LH), 100.5 mIU/mL; prolactin, 16.94 ng/mL, and estradiol, 83 pg/mL. FSH and LH were higher than normal. The measurement of thyroid-stimulating hormone (TSH) levels was normal.

### Results and Discussion

G banding cytogenetic analysis from cultured peripheral blood lymphocytes of the proband revealed a karyotype:

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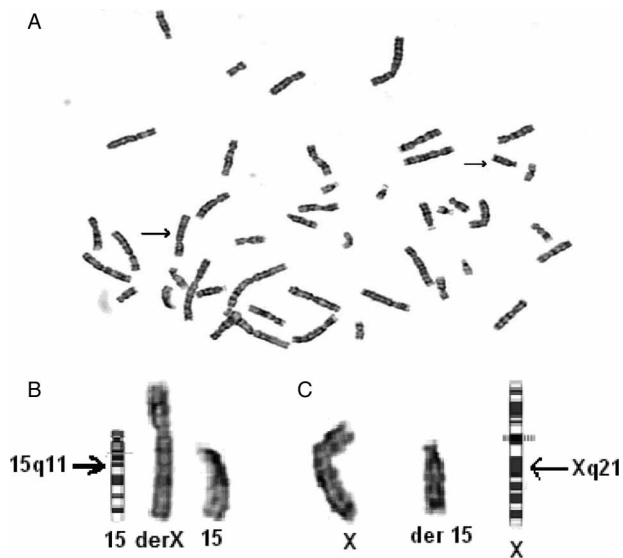


Figure 1. Cytogenetic analysis using G-banding assay from cultured peripheral blood lymphocytes. A) Metaphase image of the patient; upper arrow: der(15) chromosome and lower arrow: der(X) chromosome; B) Chromosome 15 ideogram, der(X)(Xpter → Xq21::15q11 → 15qter) chromosome, chromosome 15, and C) Chromosome 15, der (15)(15pter → 15q11::Xq21 → Xqter) chromosome, and X chromosome ideogram.

46,X,t(X;15)(q21;q11) (Fig. 1 A,B,C). The karyotypes of her mother, father, brother, and sisters were normal. FISH analysis has verified the *de novo* chromosomal imbalance to be 46,X,t(X;15)(Xpter → Xq21::15q11 → 15qter;15pter → 15q11::Xq21 → Xqter)

ish(CEPX+,wep15+, ISNRPN+, PML+, D15S10+, wcp15-, SNRRN-, PML-)[20] (Fig. 2).

Following conventional G-banding and FISH analysis, genome wide cytogenetic array analysis (Affymetrix Cytogenetics 2.7M Whole-Genome array) was used to determine the precise location and size of genomic imbalance. Cytogenetic array results showed the disruption of *HDX* gene at the translocation breakpoint region on the X chromosome

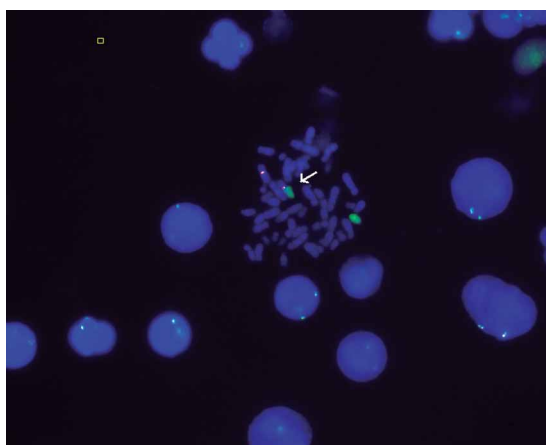


Figure 2. FISH analysis of the patient showing t(X;15). FISH analysis was performed on metaphase spreads of the patient. Red signals show chromosome X centromere and green signals show chromosome 15. Normal X chromosome (red) and chromosome 15 (green) are evident. The arrow indicates derivative (X) chromosome.

(Fig. 3). XCI assay in the family revealed a completely skewed XCI pattern in the patient and random XCI patterns in the sisters as expected (Fig. 4). She was homozygous for the Familial Mediterranean fever E148Q mutation.

In the present study, we reported a patient with POF who has a *de novo* balanced t(X;15) translocation determined by G banding and FISH. Characterization of the transcription breakpoint using genome wide cytogenetics arrays revealed that the translocation interrupts the *HDX* (Refseq accession number: NG\_017158.1) gene on Xq21. *HDX* is a known protein coding gene with 3 isoforms that encodes a highly divergent homeobox protein with unknown function and is not associated with any disease.

Cytogenetic studies have suggested that the long arm of the X chromosome is mainly involved in defects of ovulation [Bione et al. 1998]. To the best of our knowledge five genes were disrupted as a result of interruption by an X;autosome translocation near our critical region have been observed. These are *XPNPEP2* (MIM \*300145) in Xq25, *POF1B* (MIM \*300603) in Xq21.2, *DACH2* (MIM \*300608) in Xq21.3 [Bione et al. 2004], *CHM* (MIM \*300390) in Xq21.2 [Cremers et al. 1990], and *DIAPH2* (MIM \*300108) in Xq22 [Bione et al. 1998]. However, most of the breakpoints mapped in the Xq region described in POF patients are intergenic [Mumm et al. 2001; Prueitt et al. 2002]. Only the *DIAPH2* gene was disrupted by translocation in a family with POF affecting oogenesis [Bione et al. 1998]. In many cases candidate genes were neither expressed in the ovarian follicle nor in oocyte. It has been suggested that expression of these genes may be altered by a position effect of the balanced translocation [Rizzolio et al. 2007]. In our case, the translocation breakpoint falls within the *HDX* which is the only gene in Xq21 that is expressed in the ovary [EMBL-EBI atlas].

Recent studies have improved knowledge in regard to the X;autosome translocations and POF. Bertini et al. [2010] described an infertile woman with primary amenorrhea due to X;autosome translocation involving Xq22 region. The array CGH results excluded deletions and duplications. Bertini et al. [2010] identified the *NFX5* gene which is located on the Xq22.1 region and shows functional homology *fragile X mental retardation 1* (*FMR1*) gene as a POF candidate [Bertini et al. 2010]. Giacomozzi et al. [2010] reported an adolescent girl with a *de novo* unbalanced translocation t(X;15)(q24;q26.3) with partial Xq24 duplication who presented with secondary amenorrhea and absence of pubic and axillary hair. It has been suggested that a distal X breakpoint involving *POF1* locus caused POF without virilization during adolescence [Giacomozzi et al. 2010].

The disruption of a gene at the translocation breakpoint would be the most probable explanation of the phenotype in female balanced X;autosome translocation patients with a completely skewed XCI pattern. However, other mechanisms may be suggested on the cellular, chromosomal, and transcriptional level. First, defective pairing between the X chromosome and the translocated segment of chromosome 15 within the pachytene oocyte may lead to the elimination

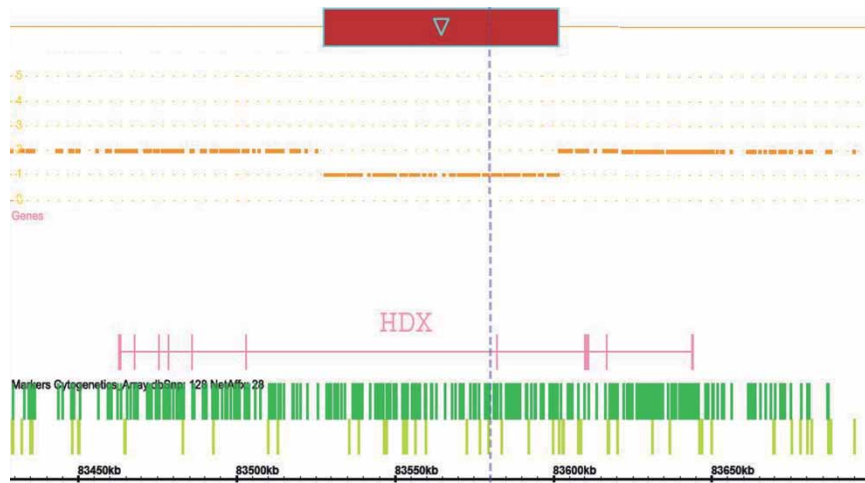


Figure 3. Representation of the translocation breakpoint on X chromosome using Affymetrix CytoGenetics Whole-Genome 2.7M Array. Analysis was performed by Affymetrix GeneChip. CytoGenetics Whole-Genome 2.7M Array using default settings for image analysis, filtration, normalization, and annotation. Visualization of the graphical data was carried out using Chromosome Analysis Suite Software v1.0 software. At the translocated region on X chromosome highly divergent homeobox (*HDX*) gene is disrupted (represented by arrow).

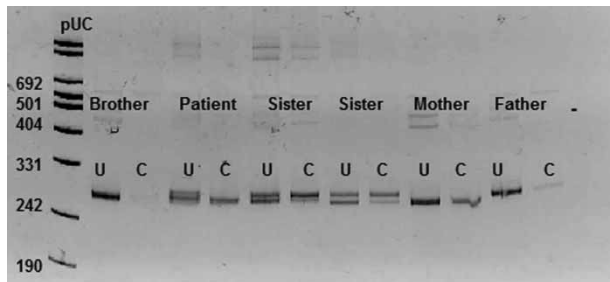


Figure 4. X-chromosome inactivation pattern of the family. Line 1, marker; line 2 and 3; brother of the patient; line 4 and 5, the patient; line 6,7,8,9, sisters of the patient; line 10 and 11 mother of the patient; line 12 and 13, father of the patient, and line 13 and 14, negative control. A single allele in the *Hpa*II digested sample indicating non-random X-chromosome inactivation was observed in the patient (U: uncleaved and C: cleaved).

of germ cells from the pool [Schlessinger et al. 2002]. Second, translocation of regulatory domains to another position on the genome might cause alternations in transcriptional regulation due to 'position effect' [Persani et al. 2009]. Lastly, in female balanced X;autosome translocations carriers, the normal X chromosome is usually inactivated allowing full expression of genes on the translocated segments [Schmidt and Du Sart 1992; Yang et al. 2011]. Female balanced X; autosome translocation carriers are generally phenotypically normal because the normal X chromosome is completely inactivated in these patients as a result of phenotypic plasticity to prevent deleterious monosomy. Similarly, as reported here a balanced X;15 translocation has a completely skewed XCI pattern in which the selective pressure for XCI should be against the translocated autosomal material in order to prevent deleterious monosomy.

In this study, we directly demonstrated that X;15 translocation interrupts the Xq21 *HDX* gene in a phenotypically normal female with POF. The findings and our observations

suggested that *HDX* could be critically important in the development of ovarian follicles or the regulation of ovarian gene expression and alterations of this gene may lead to premature ovarian failure. Further characterization and analysis of the genomic region encompassing *HDX*, in POF patients, as well as its effects on RNA and protein levels in patients should provide information for ovarian failure.

## Materials and Methods

Prior to the study, the patient and her family was informed and gave signed informed consent to participate in this study. All protocols involving the use of human tissue specimens were approved by the Institutional Review Board.

### Cytogenetic analysis

Chromosomal analysis was performed from peripheral blood samples using standard procedures. G banding analysis was performed on metaphases obtained from PHA stimulated lymphocytes from the proband and all of her family members according to standard procedures.

### FISH analysis

FISH was performed on lymphocyte metaphase spreads of the patient using the CEP X SpectrumOrange/Y SpectrumGreen Direct Labeled Fluorescent DNA Probe Kit (Abbott Molecular 07J20-050, Des Plaines, IL) and Whole Chromosome Painting probe 15 (Cytocell, Aquarius®LPP15 G9X, UK).

### Cytogenetics array analysis

Molecular cytogenetic array analysis was performed in DNA sample obtained from the patient, according to the manufacturer's protocol for Affymetrix GeneChip® CytoGenetics 2.7M Whole-Genome Array (Affymetrix Inc. Santa Clara, CA, USA) with average genome coverage of approximately one



marker per 735 bp. The array consisted of approximately 2.7 million markers, including 2,361,876 non-polymorphic markers and 400,103 single nucleotide polymorphic markers for unbiased coverage of the genome. The microarray image data was extracted using the Command Console Software 3.0.1 (AGCC) (Affymetrix Inc.). Data were analyzed using Chromosome Analysis Suite Software v1.0 (ChAS) (Affymetrix Inc.) with a threshold SNP QC  $\geq$  1.1 MAPD  $\leq$  0.27. Duplication or loss of chromosome number at given loci was considered significant only if the duplication or loss of the chromosome exceeded 100 kb. The genomic locations were retrieved from the National Center for Biotechnology Information (NCBI) build 28 (hg 18). The gene located at the translocated X chromosome region is *HDX* gene and on chromosome 15 includes: *LOC727832*, *LOC727924*, *CXADRP2*, *LOC283767*, *POTEB* [MIM 608912], *GOLGA8D*, *LOC646214*, *LOC650137*, *BCL8* [MIM 601889], *OR4M2*, *OR4N4*, *GOLGA8C*.

### X-chromosome inactivation analysis

DNA was extracted from 200  $\mu$ l venous blood sample by NucleoSpin Blood kit as in the protocol supplied by the manufacturer (Macherey-Nagel, Düren, GERMANY) and evaluated for quality and concentration using ND-1000 spectrophotometer (Nanodrop, Wilmington, USA) and agarose gel electrophoresis.

Genotyping of the highly polymorphic CAG repeat in the androgen-receptor (AR) was performed to assess the XCI pattern [Allen et al. 1992]. The DNA was divided into two identical aliquots, one of which was incubated overnight at 37°C with methylation-sensitive restriction enzyme *HpaII* (MBI Fermentas, Vilnius, Lithuania) for the digestion of unmethylated (or active) alleles. A second restriction enzyme, *RsaI* (MBI Fermentas), which recognizes a four base pair sequence not present in the amplified region of the AR locus was also included in the reaction to facilitate the *HpaII* digestion process. The other aliquot of the DNA was digested with *RsaI* alone as a control. Male DNA with cytogenetically verified 46,XY karyotype was used as a control for incomplete digestion. After restriction enzyme digest, residual DNA was amplified by using the primers 5'-GTC CAA GAC CTA CCG AGG AG-3' and 5'-CCA GGA CCA GGT AGC CTG TG-3'. PCR products were separated on 8% denaturing 29:1 acrylamide/bisacrylamide gel for 3 h at 15W. Densitometric analysis of the alleles was performed at least twice for each sample using the appropriate software (MultiAnalyst version 1.1; Bio-Rad, Hercules, California). Since the number of cycles could be critically important for the outcome of densitometric analyses, samples were subjected to 30 cycles of amplification during PCR. Products were visualized by ethidium bromide staining, and densitometric analysis of the alleles was performed at least twice for the MultiAnalyst version 1.1 software. A corrected ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the non-predigested sample for normalization of the ratios that were obtained from the densitometric analyses. The use of CrR compensates for preferential amplification of the shorter

allele when the number of PCR cycles increases. A skewed population is defined as a cell population with greater than 80% expression of one of the AR alleles. This corresponds to CrR values of  $< 0.33$  or  $> 3$ .

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**Declaration of interest:** The authors report no conflict of interest.

**Author contributions:** Cytogenetic analysis and editing of manuscript: GO; Preparation of the manuscript and cytogenetic analysis: SG; XCA and preparation of related parts in manuscript: OEO; FISH analysis: AT; XCA and editing of manuscript: TO; Clinical evaluation of the patient: IK.

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