Background
Premature ovarian failure (POF) is an ovarian defect characterized by the premature depletion of ovarian follicles before the age of 40 years, and its aetiology is still unknown in most cases. Coulon et al. examined 1858 patients with premature ovarian failure, and the age-specific incidence was based on the results: under 40 years of age the incidence was 1:100, and under 30 years of age the incidence was 1:1000 [1]. In addition to a reduction in the duration of fertility, there are other important health issues that may be associated with POF such as an increased risk of overall mortality, cardiovascular diseases, osteoporosis and autoimmune disorders such as diabetes or problems with the thyroid or adrenals [2]. In particular, an association between POF and abnormalities of the X chromosome has been reported several times, and another cause, which can be associated with POF, is the balanced X/autosomal translocations despite their generally neutral clinical effect [3]. In fact, the breakpoints of these aberrations are distributed over the whole X
The causes including rearrangements or large deletion of chromosome Xq13.3-Xq21.1 (POF2) [8]. The above-carried out due to suspected premature ovarian failure/primary ovarian insufficiency (POF/POI). Her menses ceased at the age of 25. The first menses (menarche) started at the age of 12. There is no pregnancy or assisted reproductive procedure appearing in her history. Other illnesses, genetic disorders, mental retardation have never occurred in the family. The menopause of her mother occurred at the age of 53. The patient's body type is average, BMI: 19.4 (average: 18.5 to 24.99). During the examination the disease met the criteria for premature ovarian failure/primary ovarian insufficiency (POF/POI): secondary amenorrhea, ovarian failure before the age of 40, levels of FSH > 40 IU/l in two different measurements and low estrogen levels. The patient has never had surgery significantly affecting both ovaries; ovarian toxic medications have never been used (cytostatic treatment). The patient's mother has normal karyotype and her father was already deceased. For this case study we presented the details of the molecular and cytogenetic analyses at the index patient which were performed by G-banding; FISH, Southern-blotting, Repeat Primed PCR and array-CGH technique.

Methods

Chromosome and fluorescence in situ hybridization analysis
Chromosome analysis was performed on stimulated peripheral blood cultures on metaphase cells with trypsin and Wright Giemsa stain. Fluorescence in Situ Hybridization (FISH) analysis was carried out on methanol/acetic acid-fixed suspensions. Slide preparation for FISH was made according to standard techniques. X, Y centromere specific probes as well as X Painting probe (Cytocell, United Kingdom) were used for evaluation of sex chromosomes and their possible hidden structural abnormalities. Spectrum Green CEP X and Spectrum Red SRY gene specific probe (Abbott, Germany) were used to detect chromosome X copy number and to control the presence of the SRY gene on the chromosome, respectively. Karyotypes and FISH results were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2013).

Southern blot
Genomic DNA from the patient was isolated from peripheral lymphocytes by the simple salting-out procedure. DNA was subjected to restriction enzyme digestion with EcoRI and the methylation sensitive enzyme EagI followed by Southern blot analysis and hybridization using the DNA probe SbB12.3 [12]. In unaffected females, two bands are visible: a 2.8 Kb fragment corresponding to the unmethylated X and a 5.2 kb allele representing the methylated X chromosome.

Repeat primed PCR
The exact number of CGG repeats was determined by Repeat Primed PCR technology (Amplidex, Asuragene).

Case presentation
Genetic testing of the 27 year old female patient was carried out due to suspected premature ovarian failure/primary ovarian insufficiency (POF/POI). Her menses ceased at the age of 25. The first menses (menarche) started at the age of 12. There is no pregnancy or assisted reproductive procedure appearing in her history. Other illnesses, genetic disorders, mental retardation have never occurred in the family. The menopause of her mother occurred at the age of 53. The patient's body type is average, BMI: 19.4 (average: 18.5 to 24.99). During the examination the disease met the criteria for premature ovarian failure/primary ovarian insufficiency (POF/POI): secondary amenorrhea, ovarian failure before the age of 40, levels of FSH > 40 IU/l in two different measurements and low estrogen levels. The patient has never had surgery significantly affecting both ovaries; ovarian toxic medications have never been used (cytostatic treatment). The patient's mother has normal karyotype and her father was already deceased. For this case study we presented the details of the molecular and cytogenetic analyses at the index patient which were performed by G-banding; FISH, Southern-blotting, Repeat Primed PCR and array-CGH technique.

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The genomic DNA sample was diluted (20 ng/μl), and then for PCR was used 2 μl. The PCR reaction was carried out in three primers: the FMR1 gene-specific primers (forward and reverse, FAM-labeled) and a CGG repeat specific primer. The PCR products were separated by size, using capillary electrophoresis. The statistical analyzes were performed with FMR1AnalysisMacro_version 2.1.1 software and GeneMapper software.

Array CGH analysis
Array CGH analysis was performed according to the manufacturer's protocol on genomic DNA ISCA plus design array of Nimblegen Roche containing 1.4 M probes per sub array. This microarray provides a mean average resolution of approximately 15–20 Kb on chromosomes to detect chromosomal imbalances throughout the whole genome. The CGH protocol involves independent labeling of the patient (test DNA) and the reference genomic DNA (Human Genomic DNA, Promega Madison, WI U.S.A.) with Cy3 and Cy5 dyes using a NimbleGen Dual-Color DNA Labelling Kit (Roche NimbleGen Inc.). Cohybridization of these DNAs to a NimbleGen CGH arrays were performed for 72 hrs at 42°C. Following hybridisation, the array was washed and dried at room temperature using the wash buffer kit (Roche NimbleGen Inc.). Array CGH was scanned on NimbleGen MS 200 microarray scanner and data was extracted and analysed using NimbleScan software and SignalMap and Deva 1.1 software (Roche NimbleGen Inc.). DNA CNVs were mentioned as gain or loss as a linear ration, and the length of the variation was given in megabase (Mb).

Results
For the POF/POI affected patient, the G-banded analysis based on 30 metaphases revealed two cell lines, the largest has a structural (Xq deletion) and the smallest a numerical (X monosomy) chromosome abnormality. The mosaic karyotype was the following: 46,XX,del(X)(q21q28)[25]/45,X[5] (Figure 1). The patient's mother has normal karyotype and as the father was already deceased. This deletion in a male would be incompatible with life and so we can stated that the deletion is "de novo". The FISH examination with the X centromere/SRY specific probe-based on 200 interphase cells-detected two X chromosomes in 90% of cells and X monosomy in 10% of cells and no SRY signals respectively (Figure 2a).
whole painting chromosome X FISH probe did not disclose X chromosome balanced translocation and identified a normal and a smaller size X chromosome in 88% and one normal size X chromosome in 12% of cells (Figure 2b).

For southern blot in this case we detected one FMR1 allele of X chromosome which was the 2.8 Kb size and unmethylated, and the 5.2 Kb methylated allele was not detected (Figure 3). For southern blot analysis for the index patient we can detect only the active X chromosome so this is why we had to make the Repeat-primed PCR in order to identify the CGG number and the exact allele number. Repeat-primed PCR analysis revealed a peak, which corresponds to a 23-CGG, and we can detect only one FMR1 gene allele. The method is also suitable for detection of AGG sequences interrupting CGG repeats. The AGG repeats stabilize the CGG repeats containing sequences. The more the number of AGG interruptions, the less likely it is to grow in the next generation of the number of CGG repeats. At the index patient we determined only one AGG interruption (Figure 4). Regarding the result of the cytogenetics analysis we identified a large deletion on the X chromosome (measure: 67.355 Mb) and in order to identify the exact breakpoints, we made the array CGH technique and we defined an X chromosome loss that is located at ChrX:87842016–155255380 (ChrX q21.31-q28) based on the Human genome GRCh37/hg19 assembly (Figure 5).

Discussion
A 27 year old woman with premature ovarian failure including ceased menses at the age of 25 and elevated FSH? 40 mIU/ml and low estrogen levels. She carried a 67.355 Mb deletion on one of the X chromosomes and the exact breakpoints boundaries were identified by an oligonucleotide aCGH analysis. In this region, in total 795 genes were located and up until now, ten genes (POF1B; BHLHB9; DACH2; DIAPH2, FMRI1; FMR2; XPNPEP2; PGRMC1, CENP1, BCORL1) have been identified as the ones associated with POF (Table 1). The frequencies of these genes in POF are different, the...
highest value is 3-15% at the FMR1 gene premutation cases [13,14] and followed PGRMC1 variants with 1–5% prevalence [15] and for the other six genes the frequencies are still unknown in POF/POI disease. The other genetic aspect which can effect the POF/POI phenotype is the chromosomal anomalies. These aberrations can be liable for the POF/POI phenotype in 8.8-33% of women [16] and 10-15% of the cases are X chromosome abnormalities, such as numerical and structural aberrations (deletions, inversions and X/autosome translocations) [17,18]. Theoretically, fertility impairment in patients with chromosomal abnormalities can be explained in various ways. First of all, chromosomal anomalies might disrupt a gene that is important for gonadal function [19] and structural rearrangements involving the X chromosome may disrupt the normal pairing at meiotic arrest [3]. Focus on the candidate genes that may be implicated in the POF phenotype, the first gene that we must exam, is the POF1B gene. The function of this gene is still unknown, although it binds to actin and has some homology to myosin heavy chain, so it has a remarkable role in the chromosome pairing procedure. This gene product is expressed in the ovaries during early embryonic development [20] and the mutation of the POF1B gene affects the actin binding action that may be obligate at meiotic chromosome pairing and apoptosis [21]. Deletion of the other candidate gene the BCORL1 may lead to insufficient repression of apoptosis resulting in atresia of ovarian

![Figure 4 Picture of repeat primed PCR analysis.](image)

Repeat-primed PCR analysis revealed a peak, which corresponds to a 23-CGG with only one AGG interruption.

In another study, Beke et al. (2013) performed a genome-wide analysis for X chromosome abnormalities in a patient with POF. The NimbleGen ISCA plus CGX design profile for X chromosome is shown in Figure 5. The ideogram (a) delineates genomic regions with the cytogenetic bands on the X chromosome. The CGX workflow (b) showed a 67.355 Mb loss which presented one copy. The affected region contains 1818 genes and we visualized some of them. The blue and yellow dots depict the normalized ration on every probe on the X chromosome. The ideogram listed genes and positions which are affected this patient. This affected region contains 1818 genes and we visualized some of them. The blue and yellow dots depict the normalized ration on every probe on the X chromosome. The ideogram listed genes and positions which are affected this patient. This affected region contains 1818 genes and we visualized some of them. We signed with bold font those genes, which can x play a roll to induce the POF/POI phenotype.
Table 1 POF associated genes at the critical region on chromosome X which are affected at patient

| Gene acronym | Gene name | Chromosome localisation | OMIM | Phenotype | Protein |
|--------------|-----------|-------------------------|------|-----------|---------|
| POF1B        | Premature ovarian failure1B | ChrXq21.1-q21.2 | 300603 | Premature ovarian failure 2B | This gene is expressed at trace levels in mouse prenatal ovary and is barely detectable or absent from adult ovary, in human and in the mouse respectively. |
| BHLHB9      | Basic helix-loop-helix domain-containing class B 9 | ChrX q21.1 | 300608 | Premature ovarian failure | Other members of this gene family encode proteins which function as transcription factors, either enhancing or inhibiting transcription depending on the activity of the DNA binding proteins. |
| DACH2       | Drosophila dashund | ChrX q21.2 | 300608 | Premature ovarian failure | This gene is one of two genes which encode a protein similar to the Drosophila protein dashund; a transcription factor involved in cell fate determination in the eye, limb and genital disc of the fly. |
| DIAPH2      | Homologue drosophila | ChrXq21.33 | 300108 | Premature ovarian failure | The product of this gene belongs to the diaphanous subfamily of the formin homology family of proteins. This gene may play a role in the development and normal function of the ovaries. |
| CENPI       | Centromeric protein 1 | ChrXq22.1 | 300435 | Involved in the gonadal tissue response to FSh and assembly of the kinetochore | It has a critical role in chromosome segregation and with deletions candidate for human X-lined disorders of gonadal development and gametogenesis. |
| PGRMC1      | Progesteronerceptor membrane component-1 | ChrXq24 | 300435 | Premature ovarian failure | This gene encodes a putative membrane-associated progesterone steroid receptor. The protein is expressed predominantly in the liver and kidney. |
| BCORL 1     | BCL6 Corepressor-like 1 | ChrXq25-q26.1 | 300686 | BCORL1 interacted with class II histon deacetylases suggesting that they are involved in its function as a corepressor | Deletion of BCORL 1 gene may potentially lead to insufficient repressor of apoptosis resulting in atresia of ovarian follicles. |
| XPNPEP2     | Propyl aminopeptidase | ChrXq26.1 | 300145 | Angioedema induced by ACE inhibitors, susceptibility to | XPNPEP2, the X-linked gene that encodes membranous aminopeptidase P (APP), has been reported to associate with APP activity. The membrane aminopeptidase P (XPNPEP2) is largely limited in distribution to endothelia and brush border epithelia. |
| FMR1        | Fragile X mental retardatin 1 | ChrXq27.3 | 309550 | Fragile X syndrome Fragile X tremor/ataxia syndrome Premature ovarian failure 1 | The protein encoded by this gene binds RNA and is associated with polysomes. The encoded protein may be involved in mRNA trafficking from the nucleus to the cytoplasm. |
| FMR2/ AFF2  | Fragile X mental retardation 2 | ChrXq28 | 300806 | Mental retardation, X-linked, FRAXE type | This gene encodes a putative transcriptional activator that is a member of the AF4/FMR2 gene family. |
For the chromosome X there is another candidate gene (CENP1) that has been shown to have a critical role in chromosome segregation and the deletion of this gene may cause cell death. It has also been suggested that these structural abnormalities may exert an epigenetic effect influencing the expression of X-linked or autosomal ovary-expressed genes [22]. As the molecular study revealed, the BCORL1 gene has a repressor activity through an association with histone deacetylase, suggesting that they are involved in its function as a corepressor. Disposing the histone associated epigenetic system; the BCORL1 gene may play a role in the epigenetic modification but as scientific literature suggests, genes interruption is not the major cause of pathological phenotype. In particular the breakpoint in the X-autosome translocation in the POF2 interval falls outside gene coding regions, and it has been suggested that the observed effect on expression of ovary and oocyte autosomal and X-linked genes flanking the translocation breakpoints may arise as a consequence of long range effects on promoter activity [22]. Second, some of these genes can influence hormone levels and tissue response to hormones that may impact on efficient oocyte development and maturation. For example, in addition to a role in centromere formation, the CENP1 gene is involved in gonadal tissue response to FSH [23].

For the index patient as we mentioned before using the Southern blot assay, we detected a normal and active X chromosome and the absence of the methylated FMR1 allele, according to Lyon hypotheses, should be inactivated in 50% of cells [24]. As scientific studies described, the haploinsufficiency of the genes, which are located in the missing region on one of the chromosome X, could be a promising explanation for the POF disease background, especially when it involves Xq28. A haploinsufficient gene is described as needing both alleles to be functional in order to express the wild type. The lack of expression of those missing genes that normally escape X inactivation may threaten ovarian function [25]. Based on the scientific studies we can conclude that with this POF, the affected patient may be configured by abnormal X chromosome pairing and the epigenetic modification [26]. The epigenetic effects would be supportive if the patient had a POF affected relative carrying the same deletion. In this case we confirmed a “de novo” X-chromosome deletion, so we cannot make any phenotype comparison. We conclude that cytogenetic analysis might be the first step in the investigation of POF/POI, as it might make some subsequent analysis steps unnecessary (i.e. Southern blot and PCR).

**Conclusion**

Here we present a patient affected with POF disease, where molecular and cytogenetic analyses revealed that she was a carrier of a large deletion spanning from Xq21.31-q28. Sample DNA of the POF phenotype affected women were collected so as to identify the exact molecular genetics background. As the scientific studies suggested, the most frequent gene mutation that can cause the POF phenotype, is the CGG triplet repeat number increasing which denoted that these females are the permutation status carrier. For the index patient we confirmed a large deletion on one of the X chromosomes and we couldn’t detect premutation status at the FMR1 gene. In order to identify the exact breakpoints of the X chromosome deletion, we made the array CGH analyses. As we verified the deletion on the database, we realised that this deletion region consisted of 795 genes, and 10 of them were considered as POF associated genes. Comparing the phenotype and the molecular genetic results, we concluded that for this patient, we established the POF1 disease and offering the prenatal diagnostic at possible future pregnancies is crucial.

**Consent**

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

**Abbreviations**

BCORL1: BCL 6 corepressor-like-1; BHLHB9: Basic helix-loop-helix domain-containing class B; CENP1: Centromeric protein 1; CGG: Citosine-guanine-guanine trinucleotide; CGH: Comparative genomic hybridisation; CNV: Copy number variation; DIAPH2: Drosophila diaphanous homolog 2; DIAPIH: Drosophila diaphanous homolog 2; FISH: Fluorescence in situ hybridisation; FMR1: Fragile mental retardation 1 gene; FMR2: Fragile X mental retardation 2; FMRP: Fragile mental retardation protein; FRAXA: Fragile X syndrome; FSH: Follicle stimulating hormone; FXTAS: Fragile X-associated tremor ataxia syndrome; PGRMC1: Progesterone receptor membrane component-1; POF: Premature ovarian failure; POF1B: Premature ovarian failure 1B; XPNEP2: Propyl aminopeptidase gene.

**Competing interest**

The authors declare that they have no competing interests.

**Authors’ contributions**

AB cared for the patient. AB and HP contributed to data collection and the first draft of the manuscript. HP and AB performed Repeat Primed PCR, IH and GF performed FISH examination, JC and AM performed cytogenetic examination, JJR, VK read and approved the final manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

The authors wish to thank the patient with POF disease for providing her DNA sample and personal data. Written informed consent was obtained from the patient. The authors are grateful to Éva Gönczi, Margit Czimbalmos, Eszter Kis, Tóth Zsuzsa, Linda Gyurcső-Deák, Krisztina Császár, Ágota Szepesi for their valuable technical help.

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