Journal of Assisted Reproduction and Genetics (2019) 36:769–775
https://doi.org/10.1007/s10815-019-01405-0

GENETICS

Unbalanced X;9 translocation in an infertile male with de novo duplication Xp22.31p22.33

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Received: 23 October 2018 / Accepted: 9 January 2019 / Published online: 24 January 2019

Abstract

Purpose Male carriers of an X-autosome translocation are generally infertile, regardless of the position of the breakpoint on the X chromosome while the pathogenicity of Xp22.3 subtelomeric duplications is under debate. To shed light into this controversy, we present a rare case, of an azoospermic male with no other significant clinical findings, in whom classical cytogenetics revealed additional unbalanced chromosomal material, at the telomere of the long arm of one homolog of chromosome 9.

Methods In peripheral blood specimens of the index case and his parents, we performed GBanding, Inverted-DAPI Banding, AgNOR staining, Telomere specific Fluorescence in Situ Hybridization (FISH), Molecular karyotyping by Multi-color FISH, whole genome SNP microarrays, sub-telomeric MLPA, and transcription analysis of the expression of KAL1 gene by RT-PCR.

Results Multi-color FISH revealed an unbalanced translocation involving the short arm of chromosome X. SNP microarray analysis combined to classical cytogenetics and MLPA demonstrated a de novo 8.796 Mb duplication of Xp22.31-p22.33. Compared to three control specimens, the patient presented significantly elevated expression levels of KAL1 mRNA in peripheral blood, suggesting transcriptional functionality of the duplicated segment.

Conclusions The duplicated segment contains the pseudo-autosomal region PAR1 and more than 30 genes including SHOX, ARSE, STS, KAL1, and FAM9A and is not listed as polymorphic. Our data advocate that duplications of the Xp22.3 region may not be associated with a clinical consequence.

Keywords X-autosome translocation · Unbalanced translocation · Azoospermia · Duplication Xp22.3 · *KAL1*

Introduction

Balanced or unbalanced gonosome-autosome translocations consist a peculiar category of inherited, or de novo, structural chromosome aberrations that exert gender-specific effects. The implication of X-inactivation renders each X-autosome translocation a unique condition, the outcome of which depends upon the sex of the carrier and the rearrangement breakpoints [1]. Although most balanced translocations handicap fertility, only a few disrupt genic territories or may trigger chromosome position effects that may be associated with Mendelian disease [2, 3]. However, if the translocated segment contains the X-inactivation center (XIC), illegitimate spreading of X-inactivation may occur, rendering adjacent autosomal genes inactive [1]. Favorable skewing of X-inactivation, through silencing of the expression of the intact X-chromosome, rescues the individual from conditions analogous to contiguous deletion syndromes [4–6]. Hence, most female carriers of a balanced X-autosome translocation who bear an additional intact chromosome X are phenotypically normal [4, 7]. On the other hand, male carriers of a balanced X-autosome translocation may suffer from a broad spectrum of severe phenotypic abnormalities, or they may appear phenotypically normal; however, they are almost invariably infertile, due to severe defects in spermatogenesis [8, 9].

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In contrast to balanced translocations, most unbalanced genome rearrangements that are detectable by conventional cytogenetics are associated with duplication/deletion syndromes and serious phenotypic abnormalities [10, 11]. Duplications in the Xp22.31 region are frequent findings during routine genetic testing, and in several cases, they were inherited from phenotypically normal parents [12–14]. However, it is not yet clarified if they consist pathogenic or benign variants. For example, duplications at the sub-telomeric Xp have been considered as causative or risk factors for myoskeletal, neurocognitive, or behavioral phenotypes [12–16]. To explain these discrepancies, Liu et al. (2011) proposed a genomic dosage model that implies the combination of two or more genetic alterations in an individual who presents a clinical phenotype, which is otherwise not as severe or not as penetrant [12]. To shed light into this controversy, we report herein an apparently phenotypically normal male with azoospermia, due to a de novo unbalanced translocation t(X;9) leading to a microscopically visible duplication of Xp22.31-p22.33.

The case

A 43-year-old Caucasian male of Greek origin was referred for evaluation of male infertility. His 35-year-old wife had been trying to conceive for 2 years. She had a complete obstetric evaluation that revealed regular ovulatory cycles and normal reproductive anatomy. The couple had unprotected vaginal intercourse at least twice weekly. The patient's past medical history is not remarkable. He has no family history of hypogonadism, cleft palate, or infertility; he has a sister with one healthy child. He is a farmer and has graduated from high school. He has been smoking a packet of cigarettes daily for at least 10 years, and he now occasionally drinks a cocktail. He does not smoke marijuana or use other drugs of abuse. He has never fathered a child. His physical examination was normal (including a normal male voice and normal torso-limb proportions), with a height of 178 cm and a body mass index of 21 kg/m^2, a normal genitourinary examination with normally descended testes that are 12 cc bilaterally (measured by Prader orchidometer), and easily palpable *vasa deferentia*. His laboratory tests (performed on early morning blood samples) included a total testosterone that was 218 ng/dL (normal, 249–836), calculated free testosterone of 87 pmol/L (normal, 220–640), SHBG of 25.1 pmol/L (normal, 13–90), oestradiol < 20 pg/mL (normal, < 50), LH of 2 mIU/mL (normal, 2–14), and FSH of 6 mIU/mL (normal, 1–12). Serum prolactin and iron studies were normal. Repeat blood testing yielded analogous results. Seminal fluid analysis by computer-assisted semen analysis revealed no sperm, but manual inspection under high-power microscopy revealed several immature spermatozoa. Seminal fluid volume was 2.5 cc, with normal pH (≥ 7.2) and fructose levels. Repeat seminal fluid analysis yielded analogous results. Pituitary imaging revealed no hypothalamic or pituitary abnormalities. No evidence of anosmia or hyperosmia was noted.

Cytogenetics and gene expression analysis

Peripheral blood chromosomes were prepared using conventional cytogenetic methods and banded with GTG or inverted DAPI. Routine analysis with a 500–750 band resolution revealed an apparently unbalanced male 46, XY karyotype, bearing at least two additional dark and light staining chromosomal bands of unknown origin at the telomere of the long arm of one homolog 9: add(9)(qter) (Fig. 1a, b). Parental karyotypes were normal. The possible origin of the unknown extra material from satellite regions of acrocentric chromosomes was excluded, by negative Argyrophilic Nucleolar Organizer Region (AgNOR) staining [17] (data not shown). Application of multi-color/FISH combined with inverted DAPI banding revealed that the extra material at the tip of 9q has derived solely from the X chromosome (Fig. 1c). Based on the banding pattern, the karyotype was interpreted according to ISCN 2016 [18], as 46,XY,der(9)t(X;9)(p22.31;q34.3)(wcpX+) or der(9)t(X;9)(q27.2;q34.3)(wcpX+). Telomere-specific PNA FISH ruled out telomere duplication, or the presence of interstitial telomeric repeats (Fig. 1e). No other apparent structural abnormalities were detected. To identify the precise location and the extent of the duplicated segment of the X-chromosome and to rule out additional cryptic genomic imbalances, we applied whole genome SNP (single-nucleotide polymorphism) arrays. This analysis revealed a de novo 8.796 Mb duplication at the Xp22.3region: arr [hg19]Xp22.31p22.33(168551-8964197)x2. No other copy number alterations or variants of uncertain significance were identified (Fig. 2a–c).

Sub-telomeric multiplex ligation-dependent probe amplification (MLPA) assays, including the SHOX gene, confirmed the array results in the patient (Fig. 2d) and ruled out microdeletion/microduplication in the mother. Hence, the karyotype of the patient was finally amended according to ISCN 2016 as de novo 46,XY,add(9)(q34.3).ish der(9)t(X;9)(p22.31;q34.3)(wcpX+).arr[hg19] Xp22.31p22.33(168551-8964197)x2. Based on the heterozygous composition of SNP variants of the duplicated Xp22.3 segment (Fig. 2e), we presume that the rearranged chromosome 9 derived from an exchange of genomic material between the telomere of the long arm of a maternal chromosome 9 and the short arm of one of the X chromosomes. The non-rearranged maternal X chromosome, together with the rearranged chromosome 9, was transmitted to the index case.

The duplicated region contains six OMIM disease genes, namely, ARSE, CSF2RA, KAL1, NLGN4X, SHOX, and

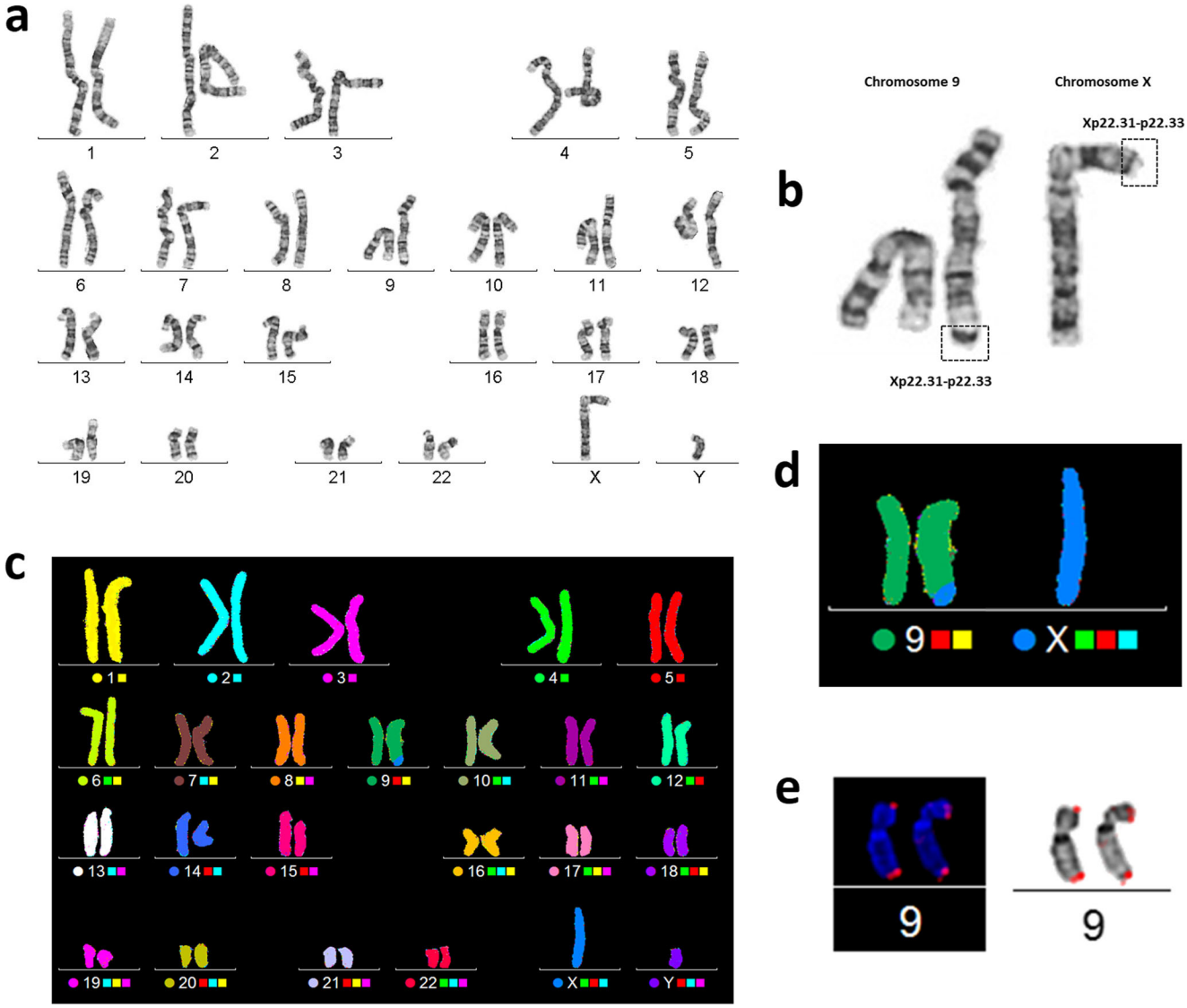

Fig. 1 Representative GTG banded karyotype of the azoospermic patient (**a**) and the unbalanced t(X;9) enlarged (boxes indicate translocation breakpoints) (**b**). Pseudo-colored M-FISH karyotype of the index case and enlarged image of the translocation partners (**c**, **d**). Telomere-specific FISH rules out telomeric duplication (**e**)

STS. To investigate if the duplicated genome segment retained transcriptional functionality, we extracted RNA from whole peripheral blood and performed real-time PCR to quantify the m-RNA levels of *KAL1* gene [20]. Our index case showed 4 times more elevated expression of *KAL1*mRNA, as compared to three karyotypically normal fertile male of the same ethnicity and age, suggesting elevated transcription and functionality of the duplicated gene (data not shown).

Discussion

This is a rare case report of an azoospermic male with unbalanced X-autosome translocation leading to a microscopically visible duplication of the terminal region of the short arm of chromosome X. The duplicated segment of 8.796 Mb contains the pseudo-autosomal region PAR1 and encompasses more than 30 transcribed genes. Deletions, or loss-of-function mutations of genes located at Xp22.31p22.33, have been associated with serious hereditary diseases such as X-linked ichthyosis (*STS*), chondrodysplasia punctata (*ARSE*), and the Kalman syndrome (*KAL1*), as well as with skeletal dysplasias, or short stature (*SHOX*) [19–22]. Contiguous gene syndromes due to deletion of the *STS*, *ARSE*, *SHOX*, and *KAL* genes have been reported [23–25].

Despite the severe effects of haploinsufficiency of genes located at Xp22.3, there is increasing evidence that duplications in this region may represent benign variants with no significant pathognomonic nature [12, 16]. Indeed,

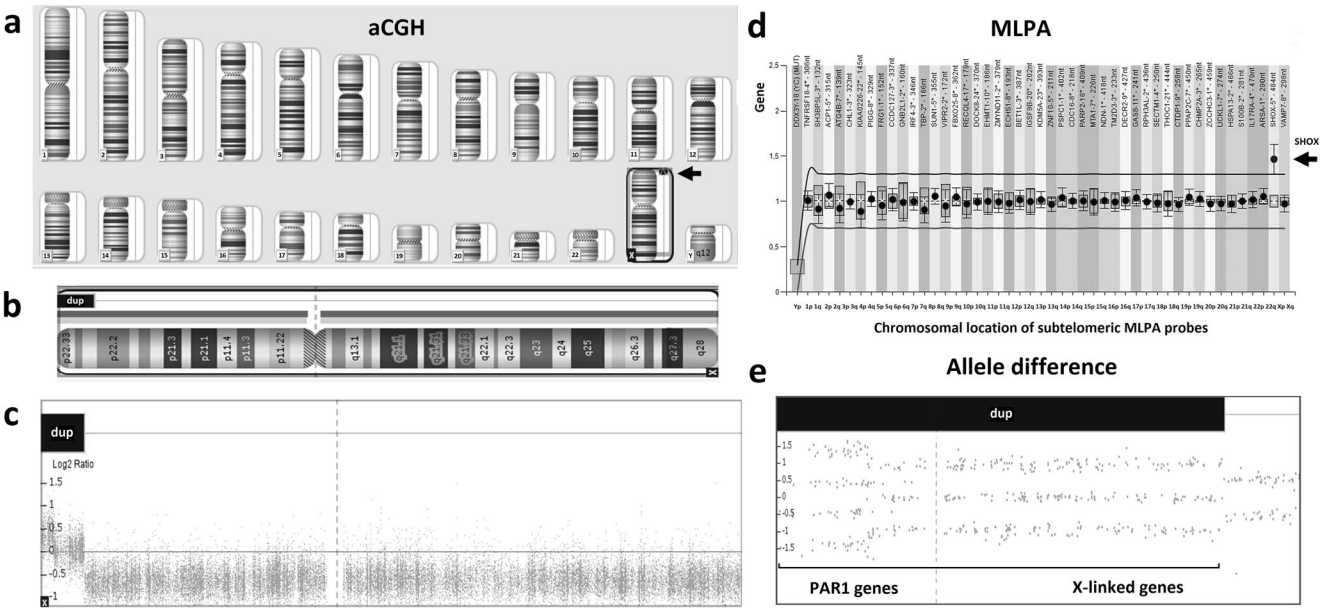

Fig. 2 SNP microarray results of the index case identify duplication of Xp as the sole significant copy number alteration (**a**). The duplicated region contains the PAR1 and a small segment of the X-specific genic territories (**b**, **c**). Confirmation of the duplication of SHOX by a customized MLPA kit assay that also excludes cryptic sub-telomeric imbalances involving most human chromosome sub-termini (**d**). Increased allele heterozygosity into the duplicated region supports origin from a parental X-chromosome different from the one transmitted in the index case (**e**)

duplications of the steroid sulfatase (*STS*) gene, located in PAR1, have been reported in both males and females but without clinical significance [25, 26]. Likewise, duplications of *SHOX* have been described in apparently normal individuals with short, normal, or tall stature and no skeletal anomalies or neurodevelopmental delay [27–29]. However, there are several reports suggesting an association of microduplications at the *STS* and *SHOX* loci with skeletal dysplasias, or autism spectrum disorders [14, 30–32]. Esplin et al. (2013) presented nine individuals from five families (five males and four females) with maternally inherited Xp22.31 duplications involving the STS gene [14]. The size of the Xp22.31 duplications ranged from 294 to 1.6 Mb and was associated with developmental delay, talipes anomalies, seizures, and/or feeding difficulties [14]. There are no reports on the pathogenicity of copy number gains of the *ARSE* region. Little is known about over-dosage effects of the *KAL1* gene located at the boundaries of PAR1 and the X-linked territories [19, 20]. Deletions and loss-of-function mutations of *KAL1* are associated with Kallmann syndrome, a disorder characterized by hypogonadotropic hypogonadism and anosmia/hyposmia [33]. A hemizygous tandem duplication of 110,967 bp on Xp22.31, encompassing the promoter region and the first two exons of *KAL1*, increased dramatically *KAL1* expression levels in peripheral blood and was associated with hyperosmia, ectrodactyly, genital anomalies, facial dysmorphism, and mild intellectual disability [20].

Translocated genome segments can activate chromosome position effects that may disturb gene expression either by altering the number or location of regulatory elements or by changing the architectural landscape of a gene or a genic cluster [34–36]. In the present case, the Xp22.33p22.31 translocated segment does not include the X-inactivation center (XIC). However, its apparent juxtaposition to the highly heterochromatic sub-telomere of 9q might have activated telomere position effects capable to rescue the hypermorphic expression of the duplicated genes and to explain the lack of significant clinical symptomatology, other than azoospermia [36–38]. We investigated this possibility, by examining the expression levels of the *KAL1* gene [20]. Our patient presented significantly elevated expression of *KAL1* mRNA, suggesting transcriptional functionality of the translocated region. Apparently, in our index case, the hypermorphic expression of the *KAL1* gene and probably of most of the functional genes included in the duplicated region Xp22.31p22.33 does not exert important clinical significance. As far as infertility is concerned, it is possible that the azoospermia phenotype stems from the well-established meiotic deficiencies of X-autosome translocations and is most probably independent of the Xp22.31p22.33 duplication [39]. Our observations suggest that in males, large Xpter duplications spanning the complete PAR1 region up to distal X-linked territories including the *KAL1* and *FAM9A* genes may not be associated with a clinical consequence.

Materials and methods

Classical cytogenetics

Chromosome preparations from peripheral blood were obtained by conventional methods. For karyotypic analysis, we combined inverted DAPI staining and G-banding. G-banding was performed after treatment with 0.25% trypsin (Gibco) and Giemsa (Carl Roth GmbH, Karlsruhe, Germany) staining. For inverted DAPI banding, slides were counterstained and mounted with 0.6 μg/ml DAPI in Vectashield antifade medium (Vector Laboratories, Burlingame, CA). Cytogenetic analyses were performed using a × 63 magnification lens on a fluorescent Axio-Imager Z1, Zeiss microscope, equipped with a MetaSystems charge-coupled device (CCD) camera, and the MetaSystems Isis/Ikaros software. Karyotypes were recorded in the extended form according to International System for Human Cytogenetic Nomenclature (ISCN) 2016.

Argyrophilic nucleolar organizer region

AgNOR staining was performed according to Howell and Black [17]. Briefly, methanol/acetic acid-fixed cell pellets were resuspended and dropped on wet slides that were left to age overnight. The slides were then incubated in a mixture of 2% gelatin (Sigma-Aldrich) in 1% formic acid (Merk-Millipore) to 50% silver nitrate solution (Sigma-Aldrich) (volumes 1:2) and then washed with double-distilled water. Finally, air-dried slides were mounted with Entellan (Merck-Millipore) and metaphases were photographed in a Zeiss Axiophot2 using the Ikaros Software (Metasystems).

Telomeric peptide nucleic acid fluorescence in situ hybridization

Telomeric PNA-FISH was performed as described in Roumelioti et al. [40].

Multicolor fluorescence in situ hybridization

For M-FISH, we used the 24XCyte Kit from MetaSystems GmbH. Staining was performed according to the manufacturer's instructions. All FISH preparations were mounted and counterstained with Vectashield antifade medium containing 0.6 μg/ml DAPI (Vector Laboratories). Molecular cytogenetic analyses were performed at 630× magnification using the Isis software (MetaSystems) and a microscope (Axio Imager Z1; Carl Zeiss) equipped with a set of six fluorochrome filters and a MetaSystems charge-coupled device camera.

SNP arrays

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). SNP array was performed using Affymetrix CytoScan®750K Array (Affymetrix Inc., CA, USA), according to the manufacturer's protocol. Analysis of array results was performed using Chromosome Analysis Suite (ChAS; version 3.1). All genomic coordinates were based on human reference sequence (NCBI Build 37). Genes and Online Mendelian Inheritance in Man (OMIM) references were from RefSeq and OMIM entries, respectively.

Multiplex ligation-dependent probe amplification

DNA was extracted from patient's and his mother's peripheral blood via conventional methods. The analysis was performed using the P036 and P070 microdeletion/microduplication syndromes probe mix according to the manufacture's protocol and software (MRC HOLLAND).

Quantification of KAL1 expression

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) of the proband and three control samples according to the method of Chomczyński and Sacchi (1987) [41]. In brief, RNA samples were transcribed into cDNA using Superscript III (Invitrogen) according to the manufacturer instructions. A Light Cycler 2.0 (Roche Diagnostic, Germany) was used as real-time PCR for the quantification of target (exons 2–3, KAL1 gene) as reference gene we used porphobilinogen deaminase PBGD. The primers used for KAL1 and for PBGD were KAL1 EX2-3F: TTTGGTGCCAGAATCACAAG, EX2-3R: CTCAGGTCCCCTGATTCCTT PBGD: F: GGAGCCATGTCTGGTAACGGCA R: GGTACCCA CGCGAATCACTCTCA.

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