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ORIGINAL ARTICLE

CHROMOSOME Y ISODICENTRICS IN TWO CASES WITH AMBIGUOUS GENITALIA AND FEATURES OF TURNER SYNDROME

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ABSTRACT

Karyotype investigations using classical cytogenetics, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) techniques were used for the characterization of Y chromosome structural anomalies found in two patients with ambiguous genitalia and features of Turner syndrome. Both exhibited mosaic karyotypes of peripheral blood lymphocytes. The karyotype was 45,X[90]/ 46,X,idic(Y)(p11.3).ish idic(Y)(wcpY+, DXYS130++,SRY++,DYZ3++,DYZ1++, DYS224++)[10] in one case, and the karyotype was 45,X[65]/46,X, idic(Y) (q11).ish idic(Y)(SRY++, RP11-140H23-)[35] in the other case. Derivative Y chromosomes were different in shape and size and positive for the SRY gene, a common underlying element of ambiguous genitalia phenotypes. These results add new information concerning the role of Y chromosome structural abnormalities in sex determination pathway perturbation which are poorly understood, and highlight the importance of the sex chromosomes integrity for a normal sex phenotype development.

Key words: Ambiguous genitalia; Fluorescence *in situ* hybridization (FISH) techniques; Isodicentric [*idic*(Y)] chromosome; Polymerase chain reaction (PCR) markers ; Turner syndrome features

INTRODUCTION

Normal gender phenotype is determined by normal number and structure of sex chromosomes: XX for females and XY for males. Abnormalities in the number or the structure of sex chromosomes are sometimes associated with a wide spectrum of phenotypes, ranging from almost male through mixed gonadal dysgenesis to female with Turner syndrome [1]. The most common structural abnormalities of the human Y chromosome are dicentrics [1]. Patients with ambiguous genitalia and patients with Turner syndrome, beside a 45,X cell line, frequently have an additional one that contains structural abnormalities of the Y chromosome [2-4]. While the phenotypic gender can be influenced by the 45,X cell line, 4.0-6.2% of female Turner syndrome patients exhibit Y chromosome mosaicism [5-7,9-11]. Phenotypic gender strongly depends on the percentage and distribution of the Y chromosome in the gonads, but in the other tissues mosaicism degree is variable [4,7,11]. However, studies on gonadal tis-

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sue are rarely available for analysis and alternative, more easily accessible tissue is usually studied. On the other hand, structure of the rearranged Y chromosomes plays an indirect role in the phenotypic gender: since in the *idic*Yp the very proximal breakpoint in the *q* arm makes the chromosome more unstable; this results in a higher percentage of 45,X cell line, leading to a female phenotype [8,12]. The *idic*Y chromosome instability may arise from loss of the region containing repetitive sequences at the euchromatin/heterochromatin boundary of their Y chromosome long arm that has a very important stabilizing role [13]. If the breakpoint position is more proximal in the long arm, then the *idic*Yp chromosome instability is greater, the percentage of 45,X cells is higher and gender phenotype is more likely to be female [14]. We here report on cytogenetic and molecular studies of two isodicentric Y [*idic*(Y)] chromosomes identified in two patients with ambiguous genitalia and features of Turner syndrome who have mosaic karyotypes.

MATERIALS AND METHODS

Patients. Case 1 was referred for cytogenetic investigation at 11 years of age, when she started developing as a boy, instead of a girl as her parents had expected. At birth, she was of normal weight and length, but had an obvious genital ambiguity, so that she underwent a clitoroplasty when she was 3 years old.

Physical examination revealed short stature for her age, abnormal body proportions with short extremities and well developed muscles. Mild somatic features of Turner syndrome (short stature, webbed neck, cubitus valgus) were noticed. The external genitalia consisted of a small phallus with hypertrophy of labia majora, two perineal openings without palpable gonads on labioscrotal structures or in the inguinal region. The internal genitalia consisted of an intra-abdominal gonad on the right side, a fibrous structure on the left side, and a small uterus. She was referred for surgical removal of the right gonad. Macroscopic and microscopic examination of this gonad revealed a dysgenetic testis.

Case 2 was 5 years old when she was admitted for karyotype investigation, because her parents requested a second opinion concerning the previous finding of a mosaic karyotype with Y chromosome in her peripheral blood lymphocytes. Ambiguous genitalia were obvious at birth (peniform clitoris, vaginal vestibule with two perineal openings, labioscrotal structure with palpable gonad on the right side which seemed to be a testis on ultrasonographic examination). Vaginography revealed the presence of a vagina and pelvic ultrasonography showed a normal uterus. At 2 years of age a laparotomy was performed, and the right gonad was removed. On the left side, the presence of a tube and of a gonad with macroscopic appearance of an ovary led to the conservation of that gonad. Macroscopic and microscopic examination of the removed gonad revealed a dysgenetic testis. At 5 years of age, the child showed mild growth delay.

Classical Cytogenetics. Written and informed consent of the patient's parents was obtained. Chromosomal studies were performed from peripheral blood samples, for both subjects and the parents of Case 1. Cell cycle synchronization, GTG- and CBG-banding techniques were applied as described [15]. Karyotype description followed the International System for Human Cytogenetic Nomenclature reccomendations [16]. Microscopic analysis was performed on a NIKON E800 microscope and CCD camera (NIKON DS-2MBWc; Nikon Corporation, Tokyo, Japan), using LUCIA karyotyping software (Laboratory Imaging, Praque, Czech Republic).

Fluorescence *In Situ* Hybridization (FISH). In this study, FISH was carried out with commercial probes according to the manufacturer's instructions. These included: Y-specific painting probe (XCP-Y; MetaSystems GmbH, Altlussheim, Germany); centromeric probe for Y (CEN DYZ3); Yq12(DYZ1) (Vysis Inc., Downers Grove, IL, USA); subtelomeric probes for short arm X and Y locus DXYS130; subtelomeric probes for long arm of X and Y, locus DXYS224 (Q-Biogene Inc., Irvine, CA, USA); locus specific Yp11.3 SRY (Vysis Inc.); DAPI counterstaining. Bacterial artificial chromosomes (BAC) clones RP11-400O10 and RP11-140H23 were used in Case 2.

Selection and Isolation of BAC DNA For Fluorescence *In Situ* Hybridization Analysis. Two BAC clones from RPCI-11 human libraries were selected from NCBI (http://www.ncbi.nlm.nih.gov) databases. The RP11-400O10 clone is localized on Yp11.3 [position 2,709,521-2,838,553 (http://www.ncbi.nlm.nih.gov/entrez/

viewer.fogi?list_uids=10801470] and covers the entire SRY gene. The RP11-140H23 clone is localized on Yq11.2 (position 23,691,183-23,794,414). The BAC clones were supplied by the Wellcome Trust Sanger Institute (Hinxton, Cambridgeshire, UK). The FISH probes were directly labeled with Spectrum Green-dUTP (RP11-140H23) and Rhodamine (RP11-400O10) using commercially available kits (Vysis Inc.; and Roche Diagnostics GmbH, Mannheim, Germany). The FISH analysis was performed on an epifluorescence microscope (NIKON E800; Nikon) equipped with a CCD camera (NIKON DS-2MBWc; Nikon) and dedicated software (NIS BR Elements - LUCIA; Laboratory Imaging). A minimum of five metaphases containing the structurally abnormal Y chromosome were examined with each probe.

Polymerase Chain Reaction (PCR). Molecular studies were performed on genomic DNA that was extracted by a salting out method from fresh peripheral blood collected on EDTA [17]. Two DNA markers were amplified by PCR: ***i)*** a short DNA sequence from the first intron of Amelogenin gene which is localized on X chromosome (Xp22.31-p22.1 region) is 106 bp long, and Y chromosome (Yp11.2 band) is 112 bp long. ***ii)*** The *DYS392 STR* marker which is localized on the long arm of the Y chromosome (Yq11.2, position 21,043,146-21,043,399) and has the $[TAT]_n$ repeat. The primer pair used in this study (STRBase) amplified a 93-125 bp DNA sequence, depending on the existent allele [18].

The amplified DNA sequence, two PCR products should be obtained in the case of male individuals. The PCR mixture consisted of 1X PCR buffer (10X PCR Buffer: 200 mM Tris-HCl, pH 8,4, 500 mM KCl), 1,5 mM $MgCl_2$, 200 μM dNTPs, 200 μM of each primer, 1 unit Taq-DNA polymerase, 1 μg/μL bovine serum albumin (BSA) and sterile water up to 20 μL.

After an initial DNA denaturation step at 94°C for 2 min., 35 cycles were performed, each consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The reaction was terminated with a 7 min. extension at 72°C. The PCR reactions were performed in a UNO II Biometra® Thermocycler (Biometra Biomedizinische Analytik GmbH, Goettingen, Germany). The PCR products were demonstrated by electrophoresis on 8% PAA (polyacrylamide) gel and silver-stained [19]. All PCR assays were carried out in duplicate, and control reactions were also performed with DNA extracted from a normal male, from a normal female, and a negative control (with water).

RESULTS

More than 100 metaphase spreads were analyzed from each subject. In Case 1, GTG-banded karyotyping revealed the presence of two cell lines: a hypodiploid line with monosomy X in 90% of the cells, and a pseudodiploid line with *idic*(Y) in 10% of cells. The karyotype formula was established as a mosaic with 45,X[90]/46,X,idic(Y)(p11.3) [10] (Figure 1a). The karyotypes of both parents were normal.

The staining pattern of the constitutive heterochromatin (HC) revealed by CBG-banding, exhibited a mirror image distribution on the derivative Y chromosome (Figure 1b), suggesting that the error occurred during gametogenesis, before the spermatid stage, or more probably, during the first division after fertilization. We propose that initially, both centromeres were functional, each being attached to the dividing spindle, and in migration to the opposite poles, the *idic*(Y) lagged behind and did not appear in the metaphase plate. In this way, a great majority of the daughter cells lack the *idic*(Y) chromosome.

The FISH analysis was performed in several successive steps to extend the range of molecular characterization of the chromosome Y derivative. Fluorescence *in situ* hybridization with a Y-specific painting probe showed the identity of the entire derivative isochromosome to be Y (Figure 1c). Double color FISH with an SRY DNA probe (Yp11.3) and a centromeric probe for X, DXZ1 (XCEN) showed two signals for the SRY gene located on derivative Y and one for the centromeric region of a normal X chromosome (Figure 1d). Dual color FISH with centromeric probes DYZ3 (YCEN) and DYZ1 (Yq12) exhibited two signals with a mirror image appearance (Figure 1e). The FISH results obtained with subtelomeric probes for the Xp and Yp locus DXYS130 plus Xq and Yq, locus DXYS224, are shown in Figure 1f. Two signals for the Yp subtelomeric probe were detected in the median region of an abnormal Y chromosome. The signal for subtelomeric locus Xq and Yq was detected on the normal location on Xq, but on both ends of the *idic*(Y) chromosome.

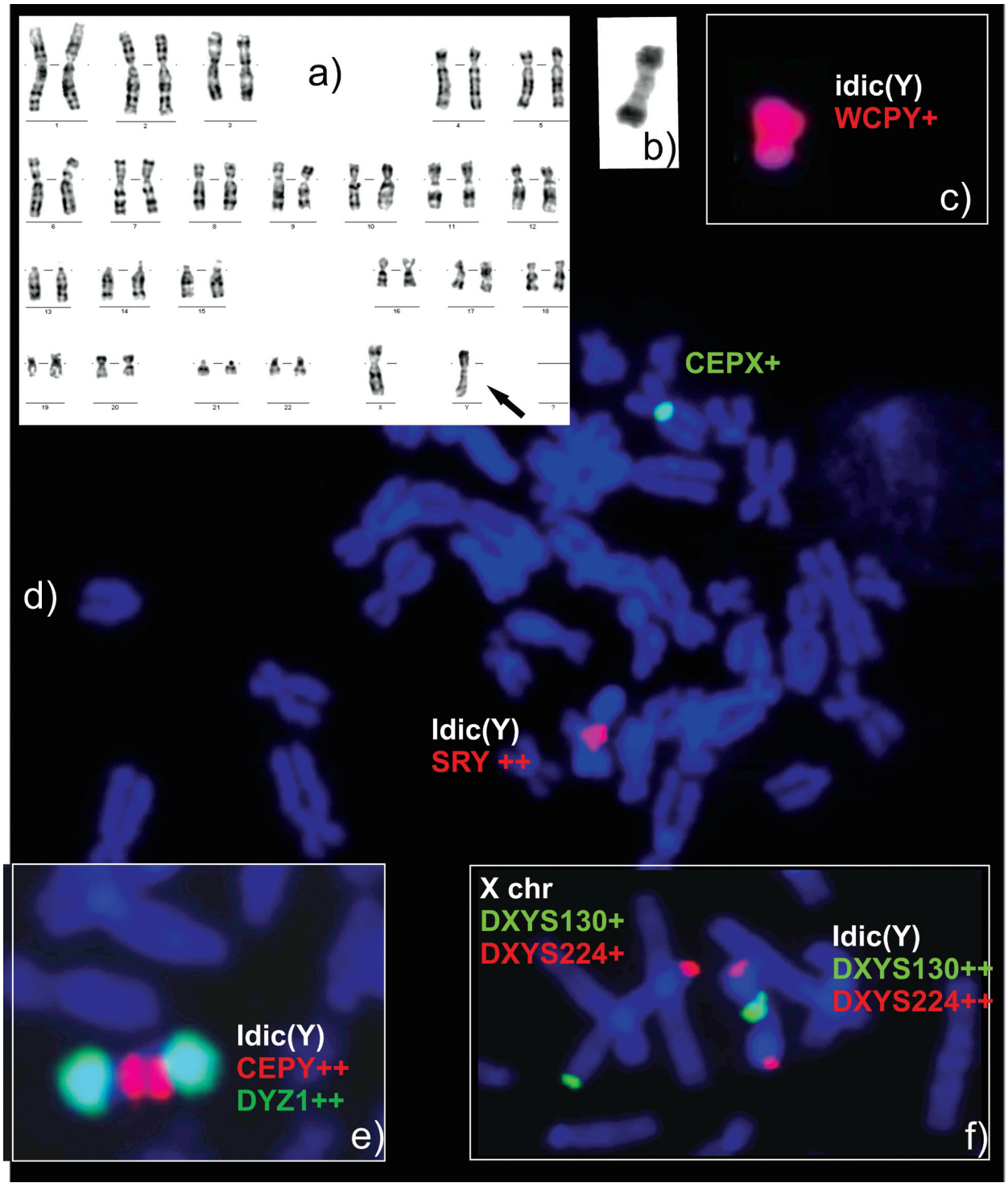

Figure 1. Cytogenetic and FISH results in Case 1. **a)** GTG-banded karyotype with 46 chromosomes and isodicentric Yq pointed by arrows; **b)** CBG banding illustrating *idic*(Yq) with symmetric distribution of constitutive (HC) blocks for centromeres and for the long arms of Y; **c)** Y-specific painting probe (XCP-Y; MetaSystems) for *idic* (Yq); **d)** FISH with red labeled SRY (Yp11.3) and green labeled DXZ1 (XCEN; Vysis Inc.), showing two copies of SRY on *idic*(Y)(q); **e)** FISH with red labeled DYZ3 (YCEN; Vysis Inc.) and green labeled DYZ1 (Yq12), illustrating two copies of each locus; **f)** FISH with subtelomeric probes for X and Y chromosomes, locus DXYS130 (green) and DXYS224 (red); the normal chromosome X shows one signal for each locus; *idic*(Y)(q) shows two copies of each locus.

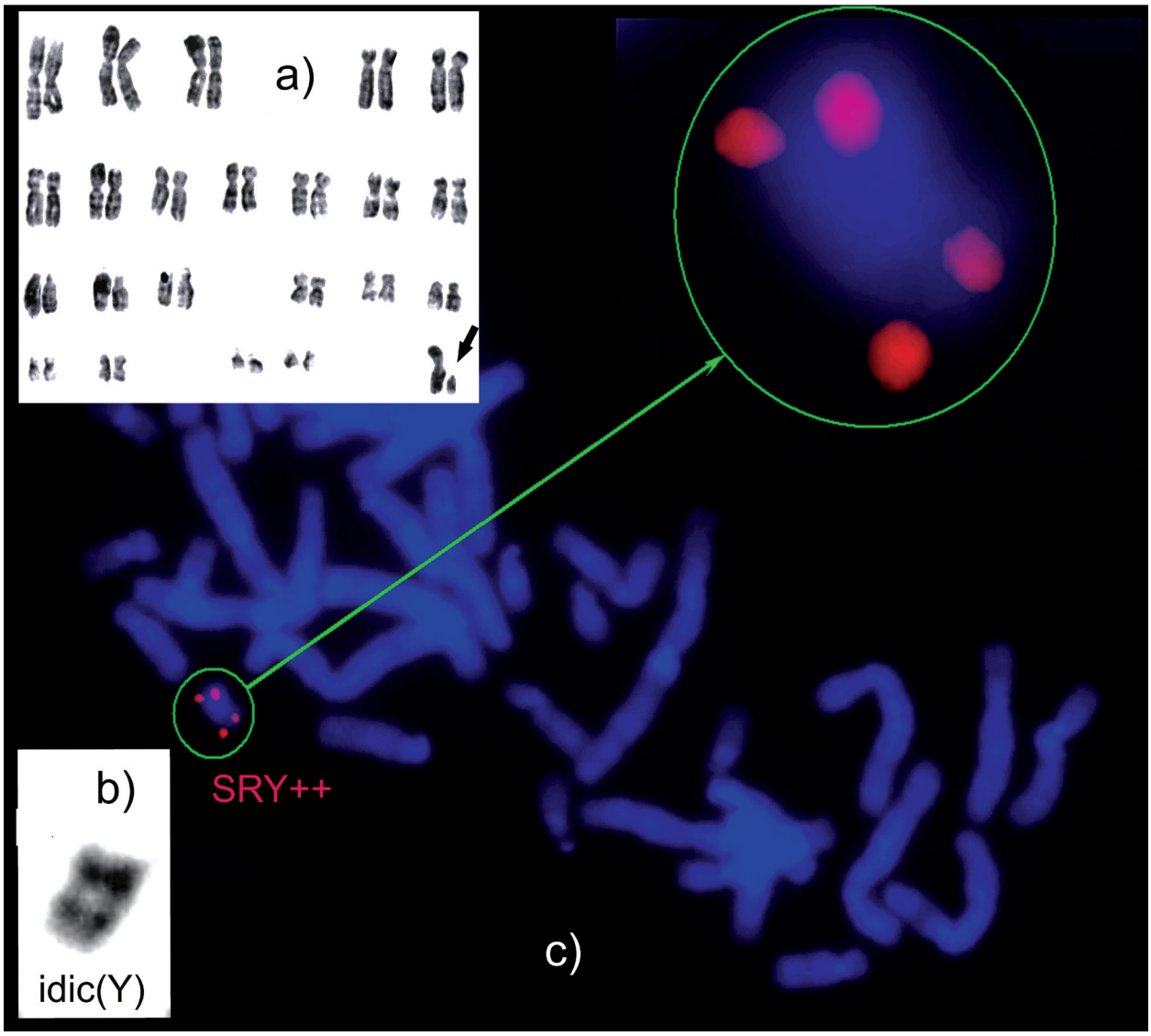

Figure 2. a) Cytogenetic and FISH results in Case 2. **a)** GTG-banded karyotype with 46,X,idic(Y)(q11); **b)** CBG-banding illustrating symmetric distribution of centromeric HC blocks in *idic*(Y)(p); **c)** FISH with SRY probe (red) and RP11-140H23 BAC-FISH probe (green) showing two copies of the SRY locus and absence of the RP11-140H23 locus.

The breakpoint on both chromatids was located subtelomerically on the *p* arm, distal from the SRY gene locus in the Y euchromatic region. The presence of duplicated active SRY genes may explain the male features development. The karyotype in this case is 45,X[90]/46,X,idic(Y) (p11.3). ish idic(Y)(p11.3)(wcpY+,DXYS130++,SRY++, DYZ3++,DYZ1++,DYS224++)[10].

Two PCR products were obtained (106 bp from the X chromosome and 112 bp from the Y chromosome) with the Amelogenin gene probes (Figure 3a). The marker, *DYS392* microsatellite was present in Case 1 (Figure 3b). All methods that we used for characterization of the derivative Y chromosome clearly demonstrated that it is an isodicentric that resulted from an almost entire Y chromosome, and the breakpoint was located distal from subtelomeric locus DXYS130 in Yp.

The chromosome analysis of Case 2 showed a mosaic karyotype with a hypodiploid cell line 45,X and a diploid cell line bearing an abnormal Y chromosome. The mosaic karyotype is: 45,X[65]/46,X idic?(Yp)[35]. Figure 2a shows a karyotype with 46,X,idic(Y)(q11). By C-banding, only centromeric

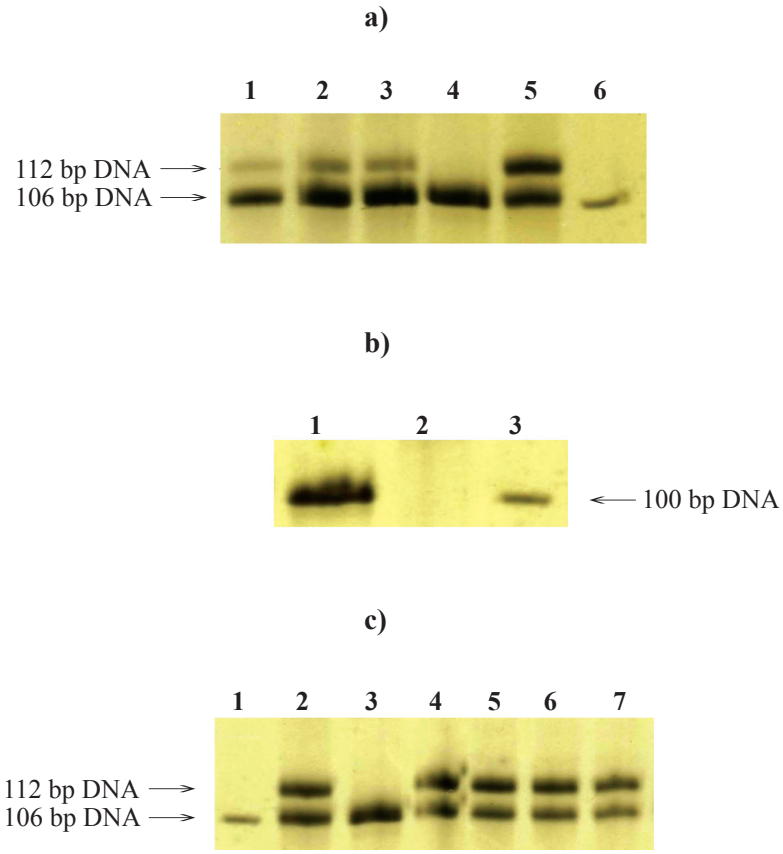

Figure 3. a) The Amelogenin gene on non denaturing 8% polyacrylamide gel for Case 1. Lanes 1-3: Case 1 with two DNA sequences, 106 bp-DNA sequence from the X chromosome and 112 bp-DNA sequence from the Y chromosome (three different PCRs); lane 4: female positive control; lane 5: male, positive control; lane 6: the 100 bp DNA marker. **b)** The DYS392 marker on non denaturing 8% polyacrylamide gel. Lane 1: Case 1-positive; lane 2: Case 2-negative; lane 3: the 100 bp DNA marker. **c)** The Amelogenin gene sequence on non denaturing 8% polyacrylamide gel for Case 2. Lane 1: the 100 bp-DNA marker; lane 2: male, positive control; lane 3: female, positive control; lanes 4-7: Case 2 with two DNA sequences, 106 bp-DNA sequence from the X chromosome and 112 bp DNA sequence from the Y chromosome (four different PCRs).

HC was revealed, but not constitutive HC characteristics for the Yq chromosome (Figure 2b).

Fluorescence *in situ* hybridization with the SRY probe showed two copies of the SRY locus distributed symmetrically on the distal ends of isodicentric (Y)(p) (Figure 3c). No signals for the RP11-140H23 probe (q11.2) were observed, showing that the breakpoint was located proximally to this locus. Isodicentric Y chromosome with fusion in Yq11, may be pseudoisodicentric.

The karyotype in this case is: 46,X,idic(Y)(q11).ish idic(Y)(SRY++,RP11-140H23-). Two PCR products were obtained (106 bp from the X chromosome and 112 bp from the Y chromosome) for the Amelogenin gene (Figure 2c), showing the presence of the short arm of the Y chromosome sequence p11.2 which is located proximally to the SRY gene locus.

The second PCR marker, DYS392 microsatellite, produced negative results (Figure 3b). We infer that the very small isodicentric, identified in 35% of metaphases, lost a large part of the long arm of Y chromosome, but retained the SRY gene on p11. In this case, the derivative Y is different in size and shape from that of Case 1, but had similar consequences in phenotype features. These results demonstrated that the derivative chromosome was an isodicentric one that resulted from the short arm of

the Y chromosome, with the breakpoint in the long arm and the fusion point proximal to the DYS392 locus in the euchromatic region q11.

DISCUSSION

There is great discrepancy between phenotype-karyotype in individuals having a chromosomal mosaicism involving one single line 45,X, or two or more lines with structural abnormalities of the Y chromosome. In Case 1, 10% of cells contained isodicentric Y chromosome derived from the long arm. In Case 2, the mosaic karyotype showed elevated number of cells (35%) bearing an abnormal Y derived from the short arm of the Y chromosome. Irrespective of the size and shape of the two isodicentrics, both cases were SRY-positive on FISH analysis. All methods used for characterization of the derivative Y chromosome clearly demonstrated that in our cases, both the percentage of cells bearing isochromosomes and breakpoint location in isodicentrics were completely different.

In Case 1, the breakpoint in the derivative Yq chromosome was placed distally from the subtelomeric locus DXYS130 in Yp. We must emphasize that this patient harbors a significant population of 45,X cells (90%), while the cells with 46,X,idic(Y)(p11.3) constitute 10%. Previously reported results showed that the percentage of cells with dicentric Yq chromosome was markedly higher being more stable than the Yp dicentric [14].

The question that arises from our results is: why was an isodicentric composed of almost the entire Y chromosome not able to sustain a normal male phenotype? We speculate that very soon after fertilization, both centromeres of the isodicentric Y chromosome were functional. Loss of this chromosome through failure of correct mitotic segregation and anaphase lag, produced increasing amounts of the 45,X clone. These results suggest a novel mechanism for the formation of the 45,X Turner syndrome, and highlight an important potential risk for generation of phenotypic anomalies frequently associated with sex chromosome mosaicism, including ambiguous genitalia [20].

In Case 2 the 46,X,idic(Y)(q11) cells constituted 35% with a fusion point proximal to the DYS392 locus in the euchromatic region q11. Thus, the position of the q arm breakpoint in the isodicentric Yp chromosome did not seem to influence stability of derivative Y, as had been proposed on the hypothesis that the more proximal the location of the breakpoint on the Yq arm, the less stable the dicentric Yp chromosomes and hence higher numbers of 45,X cells [13,15].

The cell lines containing the dicentric Yp chromosome with the breakpoint proximal on the q arm were more prevalent (35%) than in Case 1, in which fewer cells contained *idic*(Y)(p11.3) (10%) and the breakpoint was situated distally from the subtelomeric locus DXYS130 in Yp. We agree therefore, that "multiple areas of Y specific repeat sequences along the Y chromosome q arm are susceptible to breakage and reunion and in these cases formation of dicentrics" [21].

We have demonstrated in two patients with ambiguous genitalia and features of Turner syndrome different numbers of cells bearing isodicentrics of Y chromosome in peripheral blood lymphocytes. Fluorescence *in situ* hybridization and PCR allowed a precise evaluation of the mosaicism and using molecular markers, characterization of the chromosomal breakpoints in derivative Y chromosomes. Despite their difference in shape and size, the two derivative Y chromosomes retained the SRY gene, which seems to be the common element of ambiguous genitalia phenotype.

Reporting of these rare chromosomal abnormalities is important to increase our knowledge of genotype-phenotype correlations and add valuable information when associated with a comprehensive review of the literature. Our results confirm the necessity for cytogenetic investigation of each patient with an abnormal gender phenotype. The undesirable consequence of chromosome Y structural abnormalities on a carrier's phenotype highlights the importance of sex chromosome integrity for normal gender phenotype development. Shape and size differences of a Y chromosome may distort the gender determination pathway.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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