The use of fluorescence in situ hybridization in the diagnosis of hidden mosaicism: apropos of three cases of sex chromosome anomalies

SUMMARY

FISH has been used as a complement to classical cytogenetics in the detection of mosaicism in sex chromosome anomalies. The aim of this study is to describe three cases in which the final diagnosis could only be achieved by FISH. Case 1 was an 8-year-old 46,XY girl with normal female genitalia referred to our service because of short stature. FISH analysis of lymphocytes with probes for the X and Y centromeres identified a 45,X/46,X,idic(Y) constitution, and established the diagnosis of Turner syndrome. Case 2 was a 21-month-old 46,XY boy with genital ambiguity (penile hypospadias, right testis, and left streak gonad). FISH analysis of lymphocytes and buccal smear identified a 45,X/46,XY karyotype, leading to diagnosis of mixed gonadal dysgenesis. Case 3 was a 47,XYY 19-year-old boy with delayed neuromotor development, learning disabilities, psychological problems, tall stature, small testes, elevated gonadotropins, and azoospermia. FISH analysis of lymphocytes and buccal smear identified a 47,XYY/48,XXYY constitution.

SUMÁRIO

FISH tem sido usado como um complemento para a citogenética clássica na detecção de mosaicismo em anormalias de cromossomos sexuais. O objetivo deste trabalho é descrever três casos nos quais o diagnóstico final só foi obtido por meio de FISH. O caso 1 é uma menina de 8 anos, 46,XY, com genitália feminina normal, encaminhada ao nosso setor devido a baixa estatura. A análise de linfócitos por FISH com sondas centroméricas de X e Y identificou a constituição 45,X/46,X,idic(Y) e estabeleceu o diagnóstico de síndrome de Turner. O caso 2 é um menino de 21 meses, 46,XY, com ambiguidade genital (hipospadia peniana, testículo à direita e gônada disgenética à esquerda). FISH de linfócitos e mucosa oral identificou o cariótipo 45,X/46,XY, levando ao diagnóstico de disgenesia gonadal mista. O caso 3 é um rapaz de 19 anos, 47,XYY, com atraso de desenvolvimento neuromotor, dificuldade de aprendizado, problemas psicológicos, altura pequena, gonadotrofinas elevadas e azoospermia. FISH de linfócitos e mucosa oral identificou a constituição 47,XYY/48,XXYY. Os casos 1 e 2 ilustram o fenotípico variabilidade do mosaico 45,X/46,XY e a importância da detecção da linhagem 45,X na avaliação e na condução dos casos. No caso 3, a função gonadal anormal pode ser explicada pela linhagem 48,XXYY. O uso de FISH na prática clínica é particularmente relevante quando classificativa cognitiva análise mostra normal ou incerto resultados em pacientes com features of sex chromosome aneuploidy.

Andréa Trevas Maciel-Guerra 1,2, Juliana De Paulo 1, Ana Paula Santos 1, Guilherme Guaragna-Filho 3, Juliana Gabriela Ribeiro Andrade 1,2, Adriana Aparecida Siviero-Miachon 4, Ângela Maria Spinola-Castro 4, Gil Guerra-Júnior 2,3

1 Departamento de Genética Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (FCM-Unicamp), Campinas, SP, Brazil
2 Grupo Interdisciplinar de Estudos da Determinação e Diferenciação do Sexo (GIEDDS), FCM-Unicamp, Campinas, SP, Brazil
3 Departamento de Pediatria, Unidade de Endocrinologia Pediátrica, FCM-Unicamp, Campinas, SP, Brazil
4 Departamento de Pediatria, Unidade de Endocrinologia Pediátrica, Escola Paulista de Medicina, Universidade Federal de São Paulo (Unifesp-EPM), São Paulo, SP, Brazil

Correspondence to: Andréa Trevas Maciel-Guerra
Departamento de Genética Médica, FCM-Unicamp
13083-887 – Campinas, SP, Brazil
atmg@uol.com.br

Received on July/31/2012
Accepted on Oct/4/2012

Arq Bras Endocrinol Metab. 2012;56(8):545-51

Arq Bras Endocrinol Metab. 2012;56(8):545-51
INTRODUCTION

Sex chromosome anomalies are the most frequent chromosome abnormalities in newborns, with an overall incidence of 1:448 (1). They can be either structural or numerical, and the latter comprise monosomy (45,X or Turner syndrome) and polysomies (47,XXY or Klinefelter syndrome; 47,XXX; 47,XYY and rare cases of 48,XXXX; 48,XXXY; 48,XXYY; 49,XXXX; and 49,XXXXY chromosome constitutions) (2). While individuals with a 47,XXX or 47,XYY karyotype may have few or no physical or developmental problems, in monosomy and other polysomies, the clinical picture usually include growth disorders, primary hypogonadism and (or) multiple congenital anomalies; mental deficiency and behavioral problems are also frequent features in polysomies with more than three sex chromosomes (2-7) (Table 1).

Mosaicism in sex chromosome anomalies can be present in a variety of combinations. This is particularly true in TS, in which about 5% of the cases may have normal or structurally abnormal Y chromosome in the karyotype, e.g., 45,X/46,XY; 45,X/47,XYY; and 45,X/46,idic(Y) (8,9). Mosaicism with a 45,X cell line and a second cell line containing a normal or abnormal Y chromosome leads to a wide spectrum of phenotypes, including streak gonads and female genitalia, dysgenetic testes and sex ambiguity, and normal virilized male with infertility. In addition, these patients may have somatic anomalies characteristic of TS (10). Other mosaics of sex chromosome anomalies, including monosomic, normal and (or) polisomic cell lines, may lead to intermediate phenotypes (2).

Fluorescence in situ hybridization (FISH) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It has been incorporated to clinical practice as a valuable tool for the identification and definition of chromosome abnormalities, and can be applied to metaphase chromosomes preparations and (or) interphase cell nuclei (11).

Within the group of sex chromosome anomalies, FISH analysis has been a complement to classical cytogenetics for the identification of marker chromosomes (12), and for a more specific and sensitive detection of mosaicism (13,14). Moreover, the study of mucosa cells has also been proposed as a quick and reliable test for the diagnosis of hidden mosaicism (15). This is particularly relevant when classical cytogenetic analysis brings normal or uncertain results in patients with

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Incidence</th>
<th>Mental retardation</th>
<th>Behavioral disorders</th>
<th>Status</th>
<th>Gonadal function</th>
<th>Congenital anomalies/ Additional medical problems</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>45,X</td>
<td>1:2,130 F</td>
<td>-</td>
<td>-</td>
<td>Short</td>
<td>Hypergonadotropic hypogonadism</td>
<td>Dysmorphic picture, CV and renal anomalies, autoimmune disorders</td>
<td>1,2</td>
</tr>
<tr>
<td>47,XXY</td>
<td>1:576 M</td>
<td>Greater frequency when compared with normal men</td>
<td>Greater frequency when compared with normal men</td>
<td>Tall</td>
<td>Hypergonadotropic hypogonadism</td>
<td>Minor physical findings, varicose veins, DVT, diabetes mellitus, autoimmune disorders</td>
<td>1,2,6</td>
</tr>
<tr>
<td>47,XXY</td>
<td>1:851 M</td>
<td>Greater frequency when compared with normal men</td>
<td>Greater frequency when compared with normal men</td>
<td>Tall</td>
<td>Usually normal</td>
<td>Minor physical findings</td>
<td>1,2,6</td>
</tr>
<tr>
<td>47,XXX</td>
<td>1:897 F</td>
<td>Greater frequency when compared with normal women</td>
<td>Greater frequency when compared with normal women</td>
<td>Tall</td>
<td>Usually normal</td>
<td>Unknown frequency of premature ovarian failure</td>
<td>1,2,6</td>
</tr>
<tr>
<td>?</td>
<td>?</td>
<td>+</td>
<td>Variable</td>
<td>Short</td>
<td>Hypergonadotropic hypogonadism</td>
<td>Dysmorphic picture, CV anomalies</td>
<td>6,16</td>
</tr>
<tr>
<td>48,XXXX</td>
<td>1:100,000-1:150,000 M</td>
<td>+</td>
<td>+</td>
<td>Long</td>
<td>Hypergonadotropic hypogonadism</td>
<td>Dysmorphic picture, CV and renal anomalies, type II diabetes, seizures, DVT</td>
<td>3,6,7</td>
</tr>
<tr>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>Short</td>
<td>Hypergonadotropic hypogonadism</td>
<td>Dysmorphic picture, CV and renal anomalies, type II diabetes, seizures, DVT</td>
<td>4,6,7</td>
</tr>
</tbody>
</table>

+ = present; - = absent; CV = cardiovascular; DVT = deep vein thrombosis; F = females; M = males.
features of sex chromosome aneuploidy. The aim of this study is to describe three cases of sex chromosome anomalies in which the final diagnosis could only be achieved by FISH with specific probes for X and Y chromosomes.

SUBJECTS AND METHODS

Clinical reports

Case 1
This girl was referred to the University Hospital of Universidade Federal de São Paulo (Unifesp) at the age of 8 years because of short stature. On physical examination, height was 2.25 standard deviations below the mean, and there were some Turner syndrome stigmata: low posterior hairline, low-set ears, epicanthic folds, high-arched palate, micrognathia, webbed neck, scoliosis, genu varum, and hyperconvex nails. External genitalia were typically female. There were no cardiovascular or renal anomalies; thyroid function and the growth hormone – insulin-like growth factor axis were both normal. Ultrasound examination revealed hypoplastic uterus, and gonads located in the pelvic region.

Her karyotype was 46,XY (50 cells), and sequencing of the SRY gene did not show any mutation. She underwent bilateral gonadectomy, and histopathology revealed streak gonads. Hormone replacement therapy with female hormones began when she was 12 years old.

When the girl was 18 years old, her short stature and TS stigmata, together with the absence of SRY mutations, which could enable the diagnosis of complete XY gonadal dysgenesis, led to FISH analysis of lymphocytes using the alpha-satellite probes for the X (DXZ1) and Y (DYZ3) centromeres, which identified mosaicism with a 45,X cell line (Figures 1A and 1B, Table 2). In addition, there were two red signals from the Y centromere probe both in metaphase chromosomes and interphase nuclei, indicating an isodicentric Y chromosome. Thus, the karyotype of the girl was defined as 45,X/46,XY,idic(Y), and the diagnosis of Turner syndrome was established.

Case 2
This 21-month-old boy was referred to the University Hospital of Universidade Estadual de Campinas (Unicamp) because of genital ambiguity. He was born at term from healthy and nonconsanguineous parents after an uneventful pregnancy, with birth weight of 2,850 g and length 47 cm. He had normal neuromotor development and history of painful urination. He had a normal 6-year-old sister, and family history was unremarkable.

On physical examination, weight (10.1 kg) was slightly above the 3rd percentile, and height (77 cm) below the 3rd percentile. He had low-set and posteriorly rotated ears, a 3-cm phallus with chordee, penile hypospadias. The right gonad was palpable in the scrotum, and the left one was not.

Laboratory investigation revealed a 46,XY karyotype (50 cells) and prepubertal levels of FSH (1.05 mIU/mL), LH (< 0.1 mIU/L), and testosterone (< 0.02 ng/mL). After a human chorionic gonadotropin (hCG) stimulation test (three intramuscular injections of 2000 IU on successive days) testosterone rose to 1.9 ng/mL, which is consistent with the expected pattern for the male sex.

A few months later, he underwent hypospadias repair and surgical exploration. The left gonad was located near the internal inguinal ring; biopsies of this gonad and its adnexa revealed prepubertal testis, segments of epididimus and uterine tube, and fibromuscular tissue compatible with Müllerian remnants. Further biopsies of the left gonad showed only fibrous tissue devoid of germ cells, while biopsies of the right gonad showed prepubertal testis. The left gonad was then removed, and histopathology revealed a streak gonad.

The child was diagnosed as having XY partial gonadal dysgenesis and was followed up thereafter in the pediatric endocrinology service. Growth deficiency became more pronounced during follow-up, but there were no other associated conditions, and ultrasound examination did not reveal any renal anomalies. At the age of 10.9 years, height was 127.5cm (z = -2.3; mean target height: 175 cm), and weight 29.5 kg (z = -1.1).

FISH analysis using centromere probes for the X and Y chromosomes was done both in lymphocytes and buccal smear cells, and identified mosaicism with a 45,X cell line (Figure 1C, Table 2). Thus, the patient’s karyotype was found to be 45,X/46,XY and his diagnosis was changed to mixed gonadal dysgenesis (MGD).

Case 3
A 19-year-old boy was referred to the University Hospital of Unicamp due to tall stature, small testes,
and elevated gonadotropins. He was born at term after an uneventful pregnancy with a birth weight of 2,950 g and length of 49 cm. He had delayed neuromotor development, learning disabilities, and difficulties in relationship with his peers. There was also a history of right inguinal hernia repair when he was 11 years old. He was the younger of two sons of a consanguineous couple, and there were no similar cases in the family.

On physical examination, height was 194 cm (z = 2.43; mean target height: 176 cm), and weight 76 kg (z = 0.47). There was no dysmorphic picture, no gynecomastia, and external genitalia were typically male, with a 11.5-cm phallus (slightly above -2.0 standard deviations), and both testes palpable in the scrotum with reduced volume (right: 12 cm³; left: 10 cm³). Pubic hair was on Tanner stage P5.

Laboratory investigation in two different occasions (19 and 19.5 years) revealed high levels of FSH (17 mIU/mL and 16.14 mIU/mL, normal male range, NR, 1.5-12.4), normal to elevated LH (8.30 mIU/mL and 10.65 mIU/mL, NR 1.7-8.6), and normal testosterone (16.93 and 11.00 pg/mL; NR 8.8-27.0). His karyotype was 47,XYY (50 cells), and sperm count revealed azoospermia.

As the picture of hypergonadotropic hypogonadism, microrchidia, and infertility is not typical of the 47,XYY syndrome, FISH analysis using centromere probes for the X (DXZ1) and Y (DYZ3) centromeres from Kreatech Biotechnology in case 1, and from Cytocell® in cases 2 and 3, according to the manufacturers’ instructions.

Our protocol on buccal smear samples was based on Shouman and cols. (17) and was modified as follows: before hybridization, slides were pretreated with 2X SSC for 2 min at 73°C, and with pepsin (Sigma) 0.005% in 10 mM HCl for 40 min at 37°C (Sigma) to remove excess keratin and cytoplasm; washed with phosphate-buffered saline (PBS 1X) and PBS 1X supplemented with 37% formaldehyde; and dehydrated with 70%, 85% and 100% ethanol for 1 minute each. Slides were denatured in 70% formamide/2X SSC for 2 min at 73°C, and dehydrated in washes of 70%, 85% and 100% ethanol for 1 minute each. After hybridization, slides were washed with 2X SSC for 5 min at 37°C, 0.4X SSC/0.15% Tween 20 for 2 min at 73°C, and dehydrated in washes of 70%, 85% and 100% ethanol for 1 minute each. After hybridization, slides were washed with 2X SSC at room temperature before counterstaining with DAPI (Cytocell®).

A hundred to 165 metaphase cells, and 200 to 2,000 interphase cells from peripheral blood lymphocytes and (or) buccal smears were analyzed for each patient.

### Table 2. Details of FISH results for each case

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One X signal (%)</td>
<td>One X and one Y signals (%)</td>
<td>One X and two Y signals (%)</td>
</tr>
<tr>
<td>Blood metaphase</td>
<td>25 (25)</td>
<td>75 (75)</td>
<td>152 (92.1)</td>
</tr>
<tr>
<td>Blood interphase</td>
<td>27 (13.5)</td>
<td>173 (86.5)</td>
<td>62 (6.2)</td>
</tr>
<tr>
<td>Blood metaphase</td>
<td>15 (10.4)</td>
<td>129 (89.6)</td>
<td>841 (84.1)</td>
</tr>
<tr>
<td>Blood interphase</td>
<td>159 (15.9)</td>
<td>841 (84.1)</td>
<td></td>
</tr>
<tr>
<td>Buccal cells</td>
<td>234 (23.4)</td>
<td>766 (76.6)</td>
<td></td>
</tr>
<tr>
<td>Buccal cells</td>
<td>20 (4.3)</td>
<td>131 (28.5)</td>
<td></td>
</tr>
</tbody>
</table>

Molecular cytogenetics and hidden mosaicism

Cytogenetic and FISH analyses

Chromosome analyses were performed on peripheral blood lymphocytes using standard techniques and G-banding. FISH studies were performed on blood samples using the alpha-satellite probes for the X (DXZ1) and Y (DYZ3) centromeres from Kreatech Biotechnology in case 1, and from Cytocell® in cases 2 and 3, according to the manufacturers’ instructions.
DISCUSSION

Chromosomal mosaicism is defined as the presence of two or more karyotypically different cell lines in the same individual, and is the result of post-zygotic nondisjunction, anaphase lag, or structural rearrangement. If it occurs when the embryo has only a few cells, mosaicism is more evenly spread throughout the body and may be detectable in most cell lines and resulting tissues; when it occurs in a cell after extensive differentiation, mosaicism may be confined to only one or few tissues.

Cases 1 and 2 illustrate the phenotypic variability of the 45,X/46,XY mosaicism, and the differential diagnosis that may arise during investigation. The absence of a 45,X cell line in the classical karyotype indicated, in case 1, the hypothesis of 46,XY complete gonadal dysgenesis (XY CGD), characterized by streak gonads and female internal and external genitalia and, in case 2, 46,XY partial gonadal dysgenesis (XY PGD), characterized by the finding of two dysgenetic testes, or a dysgenetic testis and a streak gonad and genital ambiguity (18). In both disorders, there may be mutations in the SRY gene (19,20) (which are more frequent in XY CGD) or the NR5A1 gene (21,22), but the origin may also be unknown (which is more frequent in XY PGD). Also, in both disorders, streak gonads must be removed, as well as dysgenetic testes that are not located in the scrotal folds, due to the high risk of neoplastic transformation (gonadoblastomas and dysgerminomas) (23).

Growth deficiency was the main clinical feature indicating the possibility of hidden mosaicism in both cases, together with dysmorphic features suggestive of TS in case 1. The use of FISH enabled these hypotheses to be confirmed, also enabling proper management and follow-up of the children. In case 1, final diagnosis was TS, defined cytogenetically by the presence of an X chromosome, and total or partial absence of the second sex chromosome; and defined clinically by a female phenotype with short stature and primary hypogonadism in most cases, highly variable dysmorphic picture, and associated conditions, such as cardiovascular and renal anomalies and hypothyroidism (24).

In case 2, the final diagnosis of MGD was based on partial testicular dysgenesis and genital ambiguity in-
distinguishable from that of XY PGD, but associated with a 45,X/46,XY karyotype, and TS features (25). Thus, management and follow-up must be identical to those of TS; in addition, in both TS and MGD, the use of growth hormone may lead to improvement in final height (24,25). Regarding sex hormone replacement, female hormones are necessary in most cases of TS, and in the cases of MGD reared as females, in whom bilateral gonadectomy must be performed. On the other hand, in patients with MGD with a male sex assignment, maintenance of at least one testis may allow spontaneous male puberty, though secondary failure of Leydig cells may require therapy with testosterone (25).

The use of FISH in case 1 also enabled the detection of a structural anomaly of the Y chromosome (dentricism), which is frequently observed in 45,X/46,XY mosaicism (26), and had not been described in classical karyotyping of the girl. This anomaly predisposes to the loss of the Y chromosome during mitotic divisions, thus generating the 45,X cell line (27).

In case 3, delayed neuromotor development, learning disabilities and psychological problems, though infrequent in 47,XYY syndrome, were compatible with this chromosome constitution. However, low testicular volume, hypergonadotropic hypogonadism, and azoospermia are not usually found in individuals with this karyotype. The detection of the 48,XXYY cell line by FISH explained these findings, which are common in this polysomy (Table 1). In individuals with chromosomal mosaicism the proportion of normal to abnormal cell lines usually varies among tissue types (28), and this was also observed in case 3.

Few cases of 47,XYY/48,XXXY mosaicism are found in literature (29,30); the clinical picture of two of them (29) was very similar to the present case: tall stature, mental retardation, behavior problems, and physical features of Klinefelter syndrome.

The finding of this second cell line in case 3 did not modify the management of this patient, who needs monitoring of testosterone levels and, if necessary, androgen replacement (7), though there may be concerns about worsening of behavior with this therapy (31).

It is worth commenting that the proportion of 45,X cell line in metaphases analyzed by FISH in cases 1 and 2 (25% and 10%, respectively) was greater than expected when one considers that the analysis of 50 cells in the karyotype in both cases enabled the exclusion of mosaicism equal to 6% or greater, with 95% confidence (32). However, FISH analysis was done many years after karyotyping in both cases (10 years in case 1, and 9 years in case 2). As already pointed out, structural anomaly of the Y chromosome in case 1 predisposes to loss of this chromosome during mitotic divisions (27), and this continuous loss may lead to an increasing proportion of the 45,X cell line. Regarding case 2, in vivo studies have shown that the percentage of mosaicism in TS can change dramatically over time (33,34).

Although FISH has been widely used in research, this technique is rarely used in clinical practice to evaluate individuals whose clinical picture is suggestive of a sex chromosome anomalies, since most cases may be solved by classical karyotyping. However, FISH is a useful tool to investigate atypical cases, such as the ones described here. The clinician must keep in mind that mosaicism is a frequent feature of sex chromosome anomalies, particularly TS, and that the use of FISH also in interphase cells from buccal smears may extend the investigation to a different tissue, replacing skin biopsy and fibroblast culture in a reliable, fast, and non-invasive manner (35).

Acknowledgments: we are grateful to the Cytogenetics Laboratory of the Department of Medical Genetics of Universidade Estadual de Campinas (Unicamp). This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) (2011/50189-7).

Disclosure: no potential conflict of interest relevant to this article was reported.

REFERENCES


