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Sexual Ambiguity Diagnosis: Cytogenetics and Fluorescence *In Situ* Hybridization (FISH)

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Abstract:

The sexual differentiation depends on a succession of events that can be the seat of dysfunctions leading to sexual ambiguity. Sexual development disorders might be caused by genetic (chromosomal) or hormonal anomalies; hence, an accurate diagnosis is required. The purpose of this work is to show the impact of classical method and a molecular technic on sexual ambiguities diagnosis and their eventual complementarity in order to unveil the source of the anomalies. In order to fulfil this aim, we have studied three cases of patients with sexual ambiguities addressed in cytogenetic laboratory of Centre Pierre et Marie Curie, Algeria. Concerning the methodology used to diagnose the ambiguities, we started by the cytogenetic for highlighting any chromosomal aberrations. To refine the results, we used a molecular method restricted to the in situ hybridization by fluorescence (FISH). The results obtained indicate that the karyotype allows the analysis of number and macro structural anomalies that affect the chromosomes, but with a limited resolving power that requires using more precise technics, such as FISH, to highlight chromosomal micro reshuffling in the etiology of human sexual ambiguity. The findings indicate that the combination of classical cytogenetics and molecular diagnostics allows highlighting new genotype as the origin of sexual ambiguities at genic order with different complexity levels such as mosaicism.

Keywords: Sexual ambiguity, Pseudo hermaphroditism, Cytogenetic, Karyotype, FISH.

1. Introduction

The sexual differentiation and genital organs development of both internal and external ones, in addition to the differentiation of secondary sexual character, represent a series of complex events that lead to implement a functional reproductive apparel of a fertile individual (Muczynski, 2011). Sexual differentiations anomalies correspond to congenital chromosomal atypia, gonadic or anatomic sexual development. Due to their several causes, the problem of sexual differentiation anomaly diagnosis will arise at birth with each new born having aspects of external genital organs that are not complying with the norm. These aspects range from posterior penile hypospadias to clitoral hypertrophy. Among these two extremities genital organs are frankly ambiguous (Alaoui Belghiti, 2011).

Human cytogenetic is a recent discipline dating from 1956 with an exact determination of human chromosomes number (Tijo and Levan, 1956). In 1970, the introduction of chromosomes banding technics has improved the resolution and the sensitivity of classic cytogenetic analysis, allowing both number and structure of chromosomes studies (Ferguson-Smith, 1976). Finally, in situ hybridization fluorescence appearance in 1986 and its rapid development provide today a whole range of tools permitting an accurate study and scrutiny of chromosomes and their structure.

Fortunately, these technics made the identification of many autosomal and gonosomal pathologies possible, like sexual ambiguities or sex determinism anomalies (SDA).

It is highly important to show the role of cytogenetic and FISH studies in the sex determinism pathology. This article will present three cases of sexual ambiguities collection from the cytogenetic service in "Centre Pierre et Marie Curie (CPMC) "

2. Patients and Methods

After a clinical consultation for an anamnesis and conducting the therapy, taking pictures of patients then a blood sample were carried out.

A conic tube is used where the phytohemagglutinin is added in the middle of cell culture RPMI (Roswell Park Memorial Institute medium); it will serve both the cytogenetic and the FISH methods. The protocol of the shared part of both technics is demonstrated in figure 1.

Once the culture is conducted, we collect the blocked cells in the metaphase stage to move to both technics, the conventional cytogenetic then to the FISH if necessary.

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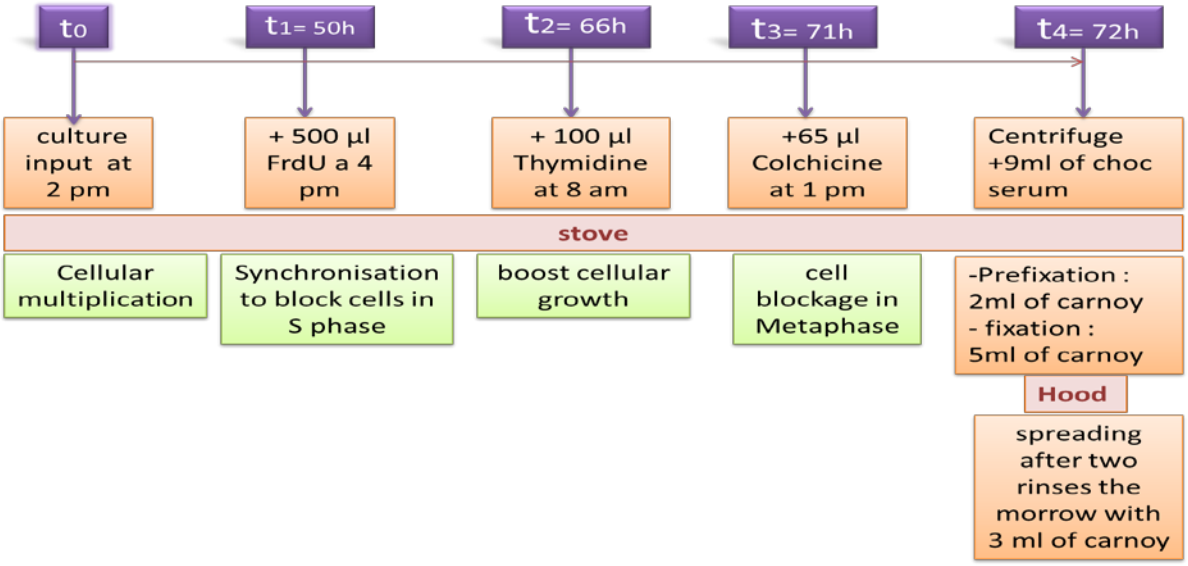


Figure 1. Illustration of the common part of karyotype and FISH with the different steps of lymphocytes culture**.**

2.1. Conventional cytogenetic (karyotype).

In this work, the R karyotype banding treatment is discussed. It is the thermal denaturation moderated at 87°C lasting 20 to 25 minutes in an ionic environment Earl PH 6.5 (Earl's balanced salt solution).

Chromosomes banding is observed with a fluorescence microscope (Zeiss MOTORISE) with a low magnification (10 x) in order to spot mitosis, then (100 x) by using immersion oil. The capture is done by image acquisition software (Meta System IKAROS) that allows the chromosomes classification (Figure 2).

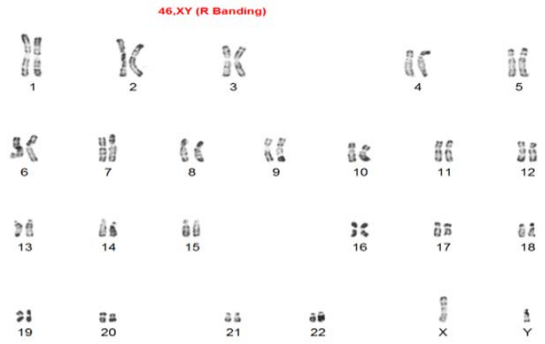


Figure 2. Normal karyotype in R bands (the Picture of cytogenetic laboratory, CPMC)

2.2. The FISH method

The in situ hybridization by fluorescence (FISH) technic consists of hybridizing a fluorescent molecular probe with a chromosomal target on a glass slide (Pinkel et al, 1988). We put 10 µl of probe on the sample. The sample and probe denaturation is made in the ThermoBrite to 75°C during 2 min the capture is done by an image acquisition softeware (Meta System ISIS).

3. Results

3.1. Patient A

3.1.1. Clinical

Patient A is from a 3rd degree consanguineous marriage. 10 years old (1m 36, 35kg) is a targeted case of sexual ambiguity therapy. The patient represents a male phenotype with ambiguous genital organs: Micro penis, absence of the left testicle, but the right testicle position is normal. Hormonal analysis report reveals that the rate of testosterones is low. Family antecedents: notion of ambiguity in a paternal female cousin

3.1.2. Cytogenetic and FISH results

The patient's standard Karyotype shows a presence of xx gonosomes (Figure 3). In order to determine precisely the cause of sexual ambiguity, we have realised a FISH to look for the major gene of testicular determinism carried by the short arm of y chromosome called sexual determining region of y chromosome (SRY). We have used a SRY YP 11.2 (Cytocell) probe coloured in red, a witness probe of the Y chromosome: DYZ1 (cyto cell) coloured in green and a witness probe DXZ1 (Cytocell) of X chromosome coloured in blue.

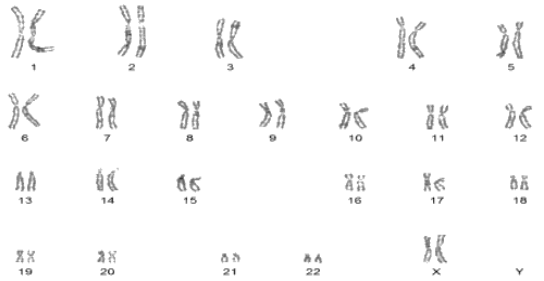


Figure 3. R bands Karyotype (46XX) of patient A.

The FISH result indicates that there is a presence of a blue signal corresponding to x chromosome and a red signal corresponding to SRY gene , the chromosomal formula of this patient is then (46, xx ish YP 11.2 (SRY)+) (Figure 4).

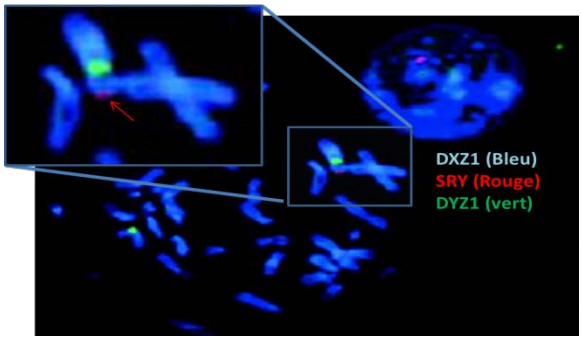


Figure 4. Bicolored FISH, centromere probe of X chromosome and red probe for the SRY gene

3.2. Patient B

3.2.1. Clinical

Patient b is from a 3rd degree consanguineous marriage, 28 years old (1m82, 70Kg). She presents ambiguous external genitalia with a gynecomastia of reduced volume and size with normal nipples. The pelvic abdominal ultrasound imaging indicates the presence of both ectopic testicles of low inguinal region. The hormonal analysis report shows high levels of Follicle stimulating hormone (FSH) and testosterone.

3.2.2. Cytogenetic and FISH results

The R Karyotype bands of the B patient reveal a normal male karyotype where there is no detection of Chromosomal aberration. Even though the results of karyotype confirm the suspected diagnosis, a FISH was used to look for the SRY gene.

The results of this FISH reveal three signals, a blue signal (X) , a green signal (Y) and a red signal (SRY) that confirm the presence of this gene that is localised in YP 11.2. The chromosomal formula of this B patient is then (46xy ish YP11.2 (SRY+).

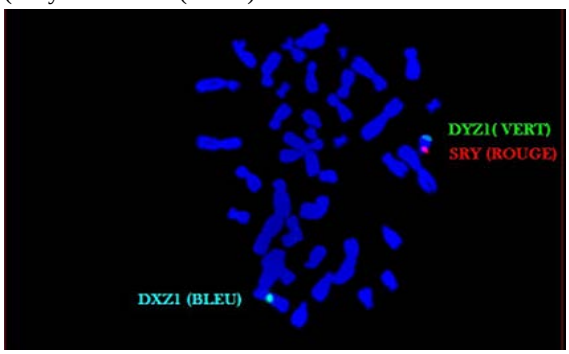


Figure 5. FISH results showing that the presence of red signal corresponds to the presence of the SRY gene.

3.3. Patient C

3.3.1. Clinical

The patient C is from a consanguineous marriage, 4 years old, presenting an advanced staturo-ponderal. He was addressed in cytogenetic laboratory due to sexual ambiguity suspicion linked to a congenital hyperplasia of the adrenal glands.

The genital exam reveals a well-developed penis according to the patient age which measures 8cm accompanied by hypospadias that signifies a hyper-pigmented scrotum of palpable empty testicles. The abdo-pelvic ultrasound imaging reveals the presence of female internal genital organs (left lateralised uterus).

3.3.2. Cytogenetic and FISH results

The standard karyotype of this patient reveals a mosaicism that constitutes three different cell populations : (66%) of mitosis studied presents a normal female karyotype 46 XX, (19%) presents a normal male karyotype 46 XY, and the third population (15%) presents an extra X chromosome so a 47 XXY karyotype (Figure 6). To get more precise results, a FISH was conducted to find the SRY gene. The results of this FISH are normal for the 46XX population, presence of two blue signals corresponding to X chromosomes with no presence of both signals red and green.

Concerning the 46 XY population, presence of a green signal corresponding to Y chromosome, a blue signal corresponding to X chromosome and the absence of the red signal allows deducing that there was no probe hybridization with the SRY gene.

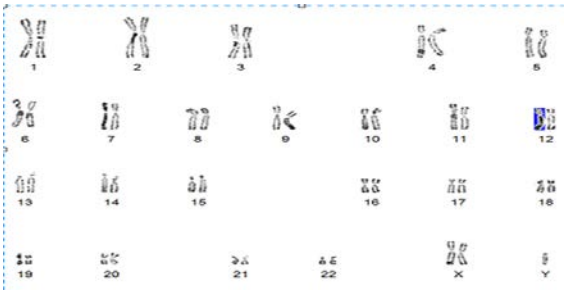
Regarding the 47 XXY population, there is a presence of two blue signals corresponding to X chromosomes and a green signal corresponding to Y chromosome. The absence of the red signal reveals that this SRY gene have been deleted (Figure 7).

The chromosomal formula of this patient is:

(mos 46,XX [71] ish Yp11.2 (SRY-)/46,XY [20] ish Yp11.2 (SRY-)/ 47,XXY [16] ish Yp11.2 (SRY)).

(A)

(B)



(C)

Figure 6. R karyotype bands reveals three cellular populations, (A) 46, XX, (B) 46, XY et (C) 47, XXY.

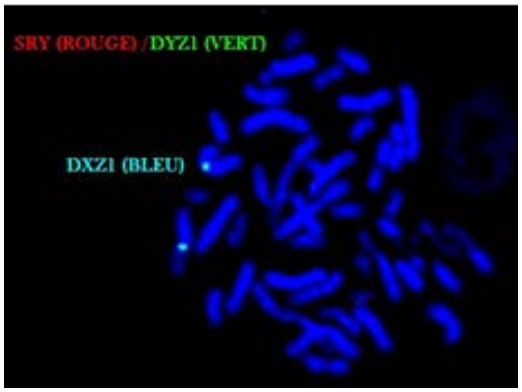


Figure 7. FISH: the red signal absence corresponds to the SRY gene absence.

This patient presenting with a male phenotype having three distinct cellular populations clarifies that this mosaicism has occurred in the post zygotic phase by a mitotic segregation anomaly where the population with an extra X chromosome drift, whereas the patient FISH reveals a normal result for the 46XX population (absence of the SRY gene) and also the SRY gene deletion in both populations 46, XY et 47, XXY.

4. Discussion

The patient A presents a male phenotype with 46XX karyotype; after looking for the SRY gene by the FISH, we highlighted a small segment of the short arm terminal part of Y chromosome on one of the two X chromosomes that seems as a consequence of a terminal exchange between DNA identical sequence of short arms X and Y chromosomes at the level of specific sites called PAR homologous regions (pseudo autosomal region) Xp-Yp (Jack, 2003); explained hereby a homologous genetic combination initiated precisely at the leptotene stage by double–strand breaks during meiosis (meiotic crossing over) that is the process in which the genetic material is exchanged between homologous chromosomes (Jack, 2003; Guichaoua *et al*, 2009). The presence of SRY is sufficient for the testicular determinism. Thus, it can be a male sex with two X chromosomes whenever one of X chromosomes carries the SRY gene, which explains the observed phenotype (Stephen, 2004)**.**

The patient B presents with a male karyotype 46 XY associated with a female phenotype. The SRY gene implies its expression in this patient, which leads to the testicles development. For more precise analyses, the patient was addressed in the molecular biology laboratory.

Although this gonadic intersexual case is rarely encountered, the XY karyotype (positive SRY) is associated with a lateral hermaphroditism with one testicle. This anomaly form can be linked to a female phenotype. The origin of this anomaly can be related to mutation in sex determinism gene interacting with the SRY gene; for example, mutations and translocations in SOX 9 gene are sometimes responsible for sex reversion from male sex to female sex (Kuttenn et al, 2003). Autosomes deletions are also implied, such as 9p24 deletion leading to reversion or sexual ambiguity (Paget, 2001).

In the patient C, the gene deletion implies its non-expression in this patient, which explains part of the phenotype observed (absence of testicles and presence of uterus). Indeed, a conduction of the FISH is recommended on a large number of cellular populations to find out which of the population carries the SRY gene that will make the patient male phenotype explanation possible.

All genital organs' discovery is a traumatising event for both parents at their baby's birth and for the ambiguous people themselves. According to the ill formation type, functional consequences may prevent any sexual activity, harm the couple's life, and trouble fertility (Bazin, 2002).

In this study, the medical care consists of Chromosomal, endocrine and radiologic examinations. Thus, sexual ambiguity diagnosis is a very delicate process that necessitates an interdisciplinary collaboration (Gueniche *et al*, 2008).

The karyotype makes an overall vision of the genome possible, but there are limits due to the existence of cryptic reshuffle that cannot be highlighted by this technic. However, actually we have molecular cytogenetic technics such as the FISH CGH-array and other technics that have a large contribution like gathered information on the DNA-break point that are exactly localised where the indication can be guided to highlight the extra genital anomalies.

In addition to the molecular diagnostics mentioned above, currently DNA sequencing is widely used due to its accuracy and its resolution in structure and mutation of gene determination leading the ambiguity anomalies to occur.

During this work, it has been possible to combine different cytogenetic technics and appraise their the limits and disadvantages in order to use them as a response to a sexual ambiguity suspicion raised by a therapist, being frequent or rare in the population where these molecular processes are highly unavoidable.

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