FISH TECHNIQUE USEFULNESS FOR THE EVALUATION OF TURNER SYNDROME IN PATIENTS WITH SHORT STATURE

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Summary
Turner syndrome is very heterogeneous, short stature often being the only clinical sign. In this pathology, classical karyotype is the gold standard in genetic investigation for the positive diagnosis, and it is recommended to be performed in every girl with unexplained short stature. We investigated by standard and molecular cytogenetics a girl who presented for short stature not explained by common medical reasons. Standard karyotype showed homogeneous monosomy, 45,X, FISH technique revealing a mosaicism undetected before, 45,X/46,XX, the level of mosaicism for the second cell line being of 22%. This difference could be explained by an augmented number of cells analyzed by FISH, thus improving the cytogenetic diagnosis. FISH technique is useful in medical practice, complementary to the classical techniques due to a greater resolution and for the detection of the mosaicism.

Keywords: Turner syndrome, short stature, X chromosome, FISH technique, karyotype

Introduction
Turner syndrome is observed in 1:2500 female newborn, the characteristic phenotype being characterized by dysmorph, short stature, hypogonadism and internal malformations (Bondy, 2006). Turner syndrome is very heterogeneous, the clinical spectrum ranging from a complete form to an almost asymptomatic picture, short stature being present in the majority of cases. The phenotype of the patients with Turner syndrome is explained by the aneuploidy himself or by the haploinsufficiency of the genes which escape from the mechanism of X inactivation (Cabrol, 2007; Ranke and Saenger, 2001; Zinn and Ross, 1998). The study of structural abnormalities permitted us to determine the role of certain genes which intervene to explain some phenotype features of Turner syndrome, thus: the loss of the distal extremity on Xp leads to short stature and to typical skeleton abnormalities, due to the haploinsufficiency of the SHOX gene, situated on the pseudoautosomal region of X chromosome (Xp11-22) and of Y chromosome (Yp11) (Cabrol, 2007; Kosho et al., 1999; Pienkowski, 2004; Ogata et al., 2001); the ovarian function is related to the genes situated on the long arm of X, Xq26 (POF1) and Xq13-21 (POF2) (Cabrol, 2007); the congenital lymphedema is associated with Xp11.4 region, cardiac malformations and aortic coarctation being presumed to be caused by the haploinsufficiency of the lymphogenic gene (Cabrol, 2007).

The change of the vision concerning Turner syndrome has been remarked in the last years due to the knowledge of the great variability of its clinical expression. On the other hand, this entity should not be considered a devalorized handicap, due to the therapeutic progress. Actually, in Turner syndrome there is the possibility to treat the growth retard by growth hormones, there is also a better approach to pharmacological feminization and the possibility of maternity by egg cell donation from wealthy women and in vitro fertilization. Therefore, we may say that this pathology is not what it was a while ago and it benefitted from a therapeutic revolution. By a precocious diagnosis and therapy, the quality of life of these patients should be improved. Beyond all the aspects, a correct and precocious diagnosis also permits
to diagnose and treat the associated pathologies. A better informing is therefore necessary to know this progress.

As a consequence, in the developed countries it has been observed in the last 10-20 years, a progressive growth of the number of cases yearly diagnosed, by more careful investigation of the short stature and by better prenatal and postnatal genetic diagnosis, using a complex cytogenetic techniques (Fluorescent in situ Hybridization - FISH)(Pienkowski, 2004; Ogata et al., 2001; Stochholm et al., 2006). The tendency of lower age at the diagnosis has also been observed, the majority of cases being diagnosed in antenatal and postnatal period until the age of 10-12 years (in Belgium the average age at diagnosis was in 1991 of 11.2 years, and in 2005 of 6.6 years, the average height at diagnosis being -2.9 DS in 1991, and -2.4DS in 2005) (Massa et al., 2005; Carel et al., 2004).

However, in Romania, there are many undiagnosed cases of Turner syndrome, partly because of an inadequate clinical evaluation and on the other hand due to the lack of advanced cytogenetic techniques. They are diagnosed tardily, often at the age of puberty (14-16 years) or later, thus delaying the institution of a treatment which, otherwise, could ameliorate significantly the disease (Grigorescu-Sido, 2000).

There is still no consensus on warning clinical sign that should lead to an indication of karyotype. However, for an early diagnosis it is generally recommended to perform a karyotype in every girl with unexplained short stature, because it can often be the only sign in Turner syndrome (Savendahl and Davenport, 2000). In literature it is described that, among the girls with short stature, up to 19% have Turner syndrome (Lam et al., 2002; Gicquel et al., 1998; Moreno-Garcia et al., 2005).

The purpose of this paper was to highlight the role of FISH technique in cytogenetic diagnosis of Turner syndrome. Case. An 8-yr-old girl presented with short stature. Auxological parameters at birth showed a weight of 2900g (-0.6DS) and a length of 47 cm (-1.5DS) at 38 weeks of gestational age. Statural growth rate was always poor. It isn’t mentioned a family history of short stature, midparental height being of 171 cm (+1.3DS). Clinical exam showed: height of 112 cm (-2.5 DS), weight of 24 kg (+3.2 DS), bilateral short 4th metacarpal. Routine laboratory investigations performed for the short stature reveals no changes. Thus, genetic tests were indicated to characterize sex chromosomes.

Material and methods

Cytogenetic techniques were done on the lymphocytes of the peripheral blood. 10 cells were examined, and for 4, karyotype was performed. In FISH techniques, specific probes for X and Y centromeres were used (Vysis®, Abbott, Abbott Park). The threshold for the diagnosis of a mosaic was 2%. 200 interphase cells and 50 metaphases were examined by FISH.

The protocol for standard karyotype included: culture peripheral blood lymphocytes (culture environment with phytohemaglutinun), use of colchicines to block the cells in metaphase, hypotonic shock with a solution of KCl, fixation with acetic acid:methanol (1:3), spreading on the slide.

FISH technique consisted in: pretreatment of the slide by plunging in successive baths of PBS 1X (5') – PFA (10') – PBS 1X (5'), then treatment with HCl 0.01 N and pepsin at 37°C (8'); suspension in successive baths of PBS 1X (2') – PFA (10') – PBS 1X (2'); dehydration in 70% (2'), 85% (2'), 100% (2') alcohol bath; application of the XY centromeric probe – 75°C (5'); maintaining the slide overnight in a hybridizing room at 37°C; slides lavage in a solution of SSC (3M de NaCl + 0.3 M sodium citrate) 0.4X, than at 70°C (2'); slides suspension in SSC 2X solution; DAPI application and examination in microscopy.

Results and discussion

Standard cytogenetic evaluation revealed a 45,X, homogeneous monosomy. FISH technique evidenced the karyotype 45,X/46,XX, the second normal cell line being
observed in 22% cells. Regarding Hook’s tables, we observe that by the analysis of 10 cells, as it has been done in standard cytogenetic analysis, we can detect a level of mosaicism superior to 25% with 95% confidence and a inferior level of mosaicism cannot be excluded (Hook, 1977). 22% level of mosaicism was identified only by FISH and not by standard cytogenetics, because it was based on the analysis of a greater number of cells, 200 metaphases and 50 interphases, which allowed the detection of a mosaicism level superior to 2% (Hook, 1977).

Diagnostic guidelines in Turner syndrome recommend to perform a cytogenetic analysis in every girl with short stature, after the exclusion of common causes (Bondy, 2006; Savendah and Davenport, 2000). Chromosomal analysis in this case is recommended to be done on 10 cells and in 4 cells a karyotype should be done. In the laboratories which have FISH techniques available, it is indicated to do a supplementary analysis of a least 100 interphase cells and 10 metaphases for the mosaicism detection, complementary to standard cytogenetic techniques.

FISH technique is based on hybridization by complementarities of a single stranded DNA-probes, fluorescently marked, with a specific region of a chromosome. It can mark chromosomes in metaphase, interphase nuclei, elongated chromosomes, even chromatin fiber. Metaphase cells most often used in FISH are cultured lymphocytes, also the cells obtained from bone marrow and cultured fibroblasts. Interphase cells which are most often used in FISH technique are the amniocytes, lymphocytes, buccal swab cells or histopathological fragments. The analysis of the results is done using a microscope, analyzing fluorescent signals number per cell. To exclude 10% mosaicism a minimum of 100 cells should be examined.

An advantage of FISH technique would be that it can be applied in interphase cells, thus ameliorating the technical steps in interpreting the results. This is particularly important in prenatal diagnosis, its rapidity indicating that as a technique of first choice. Actually, the karyotype is the golden standard in aneuploidies diagnosis, having the maximum of sensibility and specificity of detecting them. One advantage of performing a complete karyotype is that it allows the global study of the chromosomes, revealing numeric or structural abnormalities, larger than 5Mb.

Standard karyotype has also disadvantages. One would be the time required for cells culture (2-3 days in lymphocytes, 2-3 weeks in amniocytes), and for chromosome analysis. Therefore, it has been tried to obtain such molecular cytogenetic techniques, which can be used in the absence of cell culture and thus allowing faster results. The main disadvantage of these molecular techniques, as FISH, is that they do not allow the detection of other chromosome abnormalities outside the target anomalies.

By conventional cytogenetic techniques, the karyotypes observed in patients with Turner syndrome are in half of the cases 45, X, in approximately 15% - numeric mosaic of X (45, X/46, XX; 45, X/46, XX/47, XXX; 45, X/47, XXX); in 30% - structural abnormalities of X, homogeneous or in mosaic (i(Xq); i(Xp); del(Xp), del(Xq), r(X)) and in 5% anomalies implying Y chromosome, normal or reshuffled, homogenous or in mosaic (Pienkowski, 2004).

By these classical techniques it cannot always be established a correlation karyotype-phenotype, cases being evidenced with typical phenotype of Turner syndrome, but with normal karyotype, in these situations the karyotype with FISH being indicated. It was observed, by using this technique, that the frequencies of the mosaics is greater (some authors suggest that more than 98% of cases are mosaics, and that only the mosaics fetus are viable), the great variability of Turner phenotype, being connected with the proportion of the number of cells 45, X (Ranke and Saenger, 2001; Kosho et al., 1999). The severity of the phenotype depends on the complexity of the mosaic, the patients which
present a 45,X/46,XX karyotype having a better prognosis than those with homogenous 45, X karyotype (Abulhasan et al., 1999). The patients with more attenuated clinical traits, such as the patients who have only short stature, have a greater frequency of mosaics, compared to those diagnosed in the first years of life, with a more severe phenotype, in which a greater frequency of homogeneous X monosomy has been observed (Ogata et al., 2001, Gicquel et al., 1998). The dissociation between phenotype and karyotype is probably caused by the mosaics that are not diagnosed. The exact evaluation of the mosaic could demonstrate a better correlation between karyotype and phenotype.

Thus, the new techniques of cytogenetic using in situ hybridization are indispensable for the detection of some mosaics or structure abnormalities (Abulhasan et al., 1999, Van Dyke and Wiktor, 2006). Even if the karyotype effectuated on the lymphocytes of the peripheral blood is usually adequate, if there is a powerful clinical suspicion of Turner syndrome, in contrast with a normal karyotype on lymphocyte, the second tissue should be analyzed, such as skin tissue (Saenger et al., 2001; Battin et al., 2003).

Also, the cytogenetic exam by FISH may better reveal the abnormalities of structure of sex chromosome. By FISH technique the origin of the marker chromosomes can be established: X or Y. It can also be observed the deletion of the SHOX gene (Rappold et al., 2002).

FISH technique is also useful in Y chromosome detection. The research activities from the last years, indicate the fact that the presence of Y chromosome fragments in the patients with Turner syndrome imply up to 30% risk of developing gonadoblastoma (in situ cancer of germinal cells, associated with dysgerminoma in 50-60% of cases) (Gravholt et al., 2000; Cantoa et al., 2004). The investigation of Y chromosome is necessary to be made in all the patients with Turner syndrome, even more in the presence of virilization signs or when a marker chromosome is detected (Gravholt et al., 2000; McDonough and Tho, 2006).

Among the clinical uses of FISH techniques, it can be mentioned, in the cases of prenatal diagnosis, the detection of 13, 18, 21 or sex chromosomes aneuploidy, or in the postnatal period, in genetic diagnosis of microdeletions syndrome, sexual development anomalies, short stature, mental retardation and also in oncology. The main limitation of FISH technique is that it is an expensive technique, not suitable for automation, as molecular biology techniques are, which could allow costs minimizing.

It can be affirmed that FISH technique presents similar specificity and sensibility with standard karyotype when we refer to targeted diagnosis of different aneuploidies (Grimshaw et al., 2003), and in mosaicism detection it ameliorates the technical steps for the diagnosis.

**Conclusions**

Genetic evaluation is required in any patient with short stature, FISH technique being very useful. These techniques are complementary to the classical cytogenetics, improving the diagnosis of chromosome abnormalities, particularly the mosaicism and structural abnormalities. These techniques are useful for a precise genetic diagnosis, which is important for the evolution and prognosis, in terms of final height, sexual development and fertility of Turner patients, these data being particularly important in the genetic advice, in antenatal and postnatal period.

**References**


