THE CORRELATION FISH – KARYOTYPING IN PRENATAL DIAGNOSIS

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Keywords: Fluorescence in situ hybridization (FISH), Aneuploidy, Amniocentesis.
Abstract: Antenatal detection the chromosome abnormalities in high risk pregnancies and correlation between karyotype analysis and FISH (Fluorescent In Situ Hybridization). Amniotic fluid karyotyping and FISH have been offered to pregnant women with genetic risk, using the standard method and GTG banding techniques. Were found 22 abnormal karyotypes: 13 cases with numerical abnormalities (13 homogenuous aneuploidies: trisomies – 3 cases of 47,XX+21, 3 cases of 47,XY+21; 2 cases of 47,XY+18, 1 case of 47,XXY, 2 cases of 47,XXX and monosomies – 1 case of 45,X0; 1 triploidy - 69 XXX), 1 structural abnormality, one case of 46, XY, der(14;21)(q10;q10) +21) and 8 normal variants (3 cases of 46, XX inv(9)(p11;q13); 1 case with 46,XY inv (3)(p11;q11.2); 2 cases with 46,XX inv(3)(p11; q11.2), 1 case 46,XY inv(3)(p11; q11.2), and 1 case of 46,XY inv (3)(p11;q11.2) inv(9)(p11;q13)). This report confirms the importance of karyotyping and FISH in prenatal diagnosis, FISH being much more important for prenatal diagnosis due to the short time of results.

INTRODUCTION

Aneuploidy is a common event in pregnancy with a wide spectrum of medical consequences ranging from the lethal to the benign. The most common chromosomal abnormalities in newborns are trisomies 21, 18, 13, monosomy X and other sex chromosome aneuploidies (Divane et al 1994).

Trisomy 21 (Down Syndrome). Trisomy 21 was the first chromosomal abnormality described in humans (Lejeune et al.,1959). The phenotype was delineated by John Langdon Down in 1866 and is referred to today as Down syndrome (Down, 1866). It is the most common single known cause of mental retardation. The frequency in the general population is approximately 1 in 700.

Trisomy 18 (Edwards Syndrome). Trisomy 18 is the second most frequent autosomal chromosome abnormality and occurs in about 1 in 5000 births. It is named after John H. Edwards, who first described the syndrome in 1960 (Edwards et al., 1960). The syndrome has a very low rate of survival. It is impossible to predict the exact prognosis of an Edwards Syndrome child during pregnancy or the neonatal period (Chen, 2008). The median life span is five to fifteen days (Rodeck et al. 1999; Zoler et al., 2003).

Trisomy 13 (Patau Syndrome) was first described by Patau et al. in 1960 (Patau et al.,1960). The incidence is estimated to be 1 in 12,000 births. It is seen slightly more in females than in males.

Trisomy X. Triple X syndrome occurs in around 1 in 1000 girls (Nielsen and Wohlert,1991) . It was first described by Jacobs et al. (1959). Unfortunately, the term originally used for this cytogenetic abnormality was “superfemale,” which gives a misconception of the syndrome and is no longer in use.

Trisomy XXY. Klinefelter’s Syndrome is the most common sex chromosome disorder (James et al.,2005) The condition exists in roughly 1 out of every 1000 males. One in every 500 males have an extra x chromosome but do not have the syndrome.

Monosomy (Turner syndrome) Most monosomies are embryologically lethal, the only exception known in humans is monosomy X (45,X). The 45,X genotype, complete or mosaic, is found in 1 per 2,500 female births with a downward trend in frequency with increasing maternal age.

The aneuploidies presented above can account for up to 95% of live born chromosomal abnormalities (Whiteman and Klinger 1991). Diagnosis of chromosomal abnormalities in fetus is one of the most important challenges in modern perinatology.

MATERIALS AND METHODS

A total of 594 amniotic fluid samples from 2008 to 2009 were subjected to interphase FISH as well as karyotyping. Amniocentesis is an ultrasound-guided invasive prenatal diagnosis procedure usually performed after 14 weeks gestational age for determination of fetal karyotype, molecular, and biochemical abnormalities.

The time of hospitalization of patients who underwent amniocentesis was at least one day. After the procedure all women were observed for the eventual occurrence of amniotic fluid leakage, fetal loss (is estimated to be one in every 100 to 200 procedures above the background loss rate (Hunter etal., 1987; Tabor et al,1986), bleeding,

abdominal pain and symptoms of infection . The risk of infection introduced at the time of the amniocentesis is estimated to be one to two in 3000 procedures (D'Alton 1994; Romero et al., 1995).

Investigation for chromosomal anomalies was routinely performed by cytogenetic analysis and FISH. The traditional "gold standard" for prenatal diagnosis of chromosome abnormalities is metaphase analysis by G- banding.

FISH uses a fluorescently labeled probe targeted to a unique sequence of DNA where it selectively binds (Klinger et.al 1992). For prenatal samples, FISH is done on uncultured, interphase cells. For purposes of RAD (rapid aneuploidy detection), the probes used are specific for chromosomes 13, 18, 21, X, and Y. Samples are visualized using a microscope; the number of fluorescent signals per cell indicates the number of copies of the targeted chromosome.

The FISH protocol that we used:

For each amniotic fluid sample, usually 5–7 ml of clear amniotic fluid sample was centrifuged for 10 min at 1200 rpm. The pellet was resuspended in 5 ml of trypsin-EDTA, gently vortexed, and incubated at 37°C for 30 min. After centrifugation at 1200 rpm for 10 min was performed. The pellet was resuspended by slowly adding 5-7 ml of prewarmed (37°C) hypotonic solution (0,625%sodium citrate). The tube was then placed in a 37°C incubator for 30 min followed by centrifugation at 1200 rpm for 10 min. The supernatant was removed and the pellet was resuspended by adding 5 ml of fixative (3 : 1 methanol and acetic acid) and gentle mixing. The suspension was kept in a refrigerator (2–8°C) for at least 30 min. Another centrifugation at 1200 rpm for 10 min. Pellet was resuspended in 2 ml of fresh fixative.

Slide preparation for FISH analysis: 1–2 drops of the above cell suspension was dropped on clean, dry slides placed on a slide warmer at 40–42°C and allowed to dry for 30 minutes.

Two hybridisation areas were made for each sample, one area for chromosomes X, Y and 18 and another for chromosomes 13 and 21.

Denaturation and hybridization of the specimen DNA and the probe was performed according to the manufacturer's instruction. Slides were observed under a fluorescence microscope using appropriate filters (green for chromosomes 13 and X, red for chromosomes y and 21, blue for chromosome 18). For result interpretation a minimum count for 100 nucleus was scored and results were interpreted following manufacturer's instructions.

RESULTS AND DISCUSSIONS

The present study for prenatal detection of chromosomal abnormalities in high risk pregnancies was performed using two approaches – FISH and conventional cytogenetics and was conducted among 594 women who underwent amniocentesis between august 2008 and june 2009 at Life Memorial Hospital Medlife, Bucharest. All cases were white Caucasians.

Of the 594 samples, 34,7 % had a maternal age between 31 and 35 years, which was the most common age group, followed by age 36-40 (183, 30,7%), 26-30 (143, 24.1%), 21-25 (35, 5,9%) older than 41-45 (24 , 4.0%) and younger than 20 (3, 0,5%).graphic 1 The aneuploidies were most frequently detected in age 39-40 (5 cases- trisomy 21, and 1 case - trisomy XXY,).

Distribution of Maternal Age

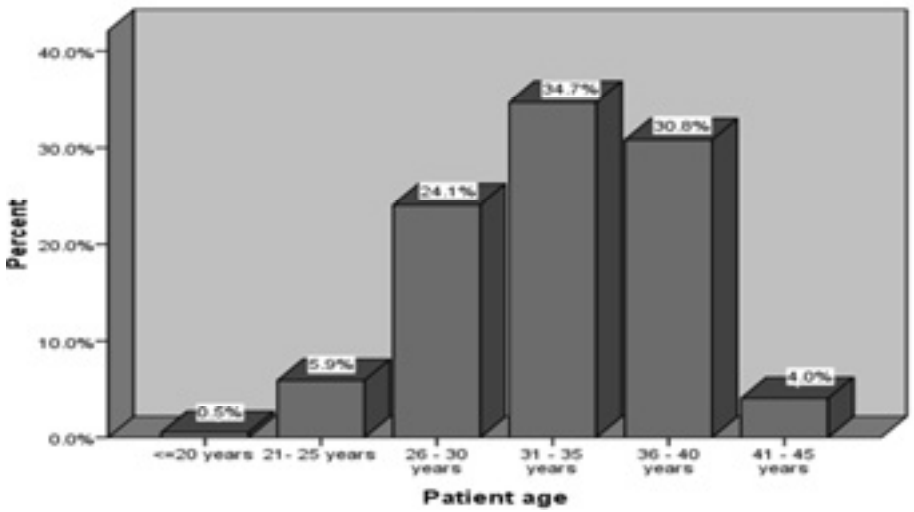

Fig.1. Age distribution of the population screened

Gestational age at the time of the procedure varied from 13 to 24 (amniocentesis was done at 18 gestational weeks in 23.4% of cases, 17 in 22.2%, 19 in 15.7%, 16 in 11.6%, 20 in 9.4%, 21 in 6,6%, 22 in 3.0%, 23 in 3.0%, 24 in 2.0%, 15 in 1,9%,14 in 0.3% and less than 13 in 0.8% (graphic 2).

The aneuploidies were most frequently detected in gestational weeks 17 (6 cases, 46.1%).

Distribution of Gestational Age

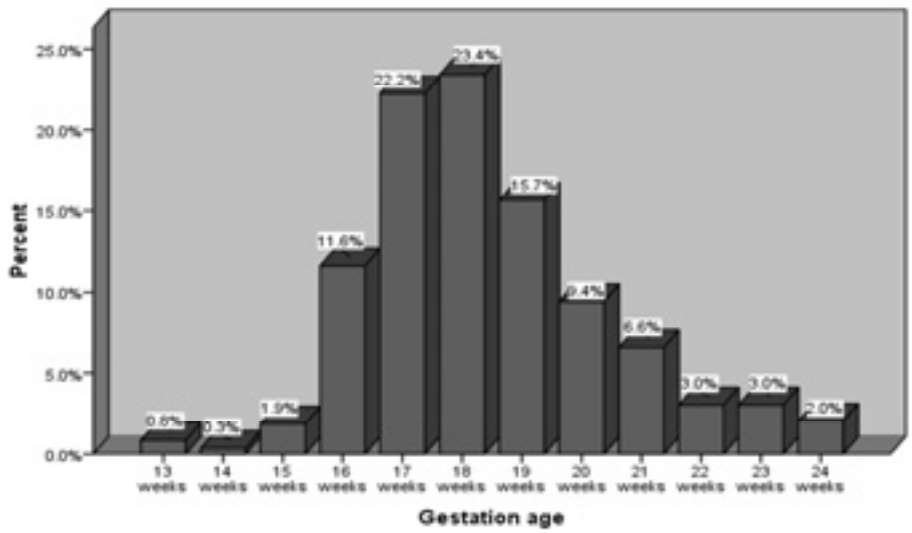

Fig.2. Gestational age distribution of the population screened

The most frequent indications for amniocentesis were:

a) *abnormal results of double or triple test* (69.70% of patients had high risk, 12.79% low risk, 17.51% have not performed any of the two screening tests). Biochemical screening of specific markers in maternal serum is very important for risk stratification as it is increasingly employed to detect additional pregnancies in the low-risk population that need fetal karyotype evaluation. Low levels of alpha-fetoprotein (AFP) and unconjugated estriol and high levels of HCG in maternal serum are associated with increased risk of a chromosomally abnormal pregnancy (Evans 2006) (fig.3).

b) *advanced maternal age* (42.93 % had increased age risk and 57.07% had low age risk). The association of maternal age over 35 years with an increased risk of chromosomally abnormal conceptions is well documented. Maternal age influences the chances of conceiving a baby with Down syndrome. At maternal age 20 to 24, the probability is one in 1562; at age 35 to 39 the probability is one in 214, and above age 45 the probability is one in 19 (Huether, 1998). Although the probability increases with maternal age, 80% of children with Down syndrome are born to women under the age of 35 (Sheth et al.,2007), (fig.4).

c) *fetal malformations* found during ultrasound examination (43.77% of patients had no ultrasound reports for pregnancy in their discussion with genetic counselor, so ultrasound evolution of those fetuses is unknown, 9,43.% had ultrasound malformations and 46,80% have no malformations. Ultrasonography now has a considerable role in prenatal diagnosis. Certain major ultrasonographic defects are fairly specific: for example, holoprosencephaly predicts the likelihood of trisomy 13, fetal hydrops/cystic hygroma predicts monosomy X or trisomy 21, and an endocardial cushion defect or duodenal atresia predicts trisomy 21. The minor marker of increased nuchal translucency (actually, this separation of the skin from the underlying tissue can extend from as far as the occiput down to the lower back) is less specific. Cardiac malformations generally have a frequent association with fetal aneuploidy, as do certain renal defects (McKinlay and Sutherland ,2004) reviewed 1800 cases in which an anomaly (an actual malformation, or a minor marker of aneuploidy) had been detected at ultrasonography, and assembled a table of risks of aneuploidy according to the findings (fig.5).

Indications for amniocentesis:

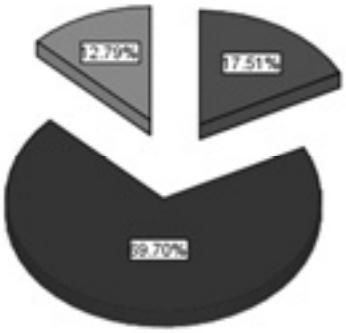

Fig. 3: Abnormal results of double or triple test

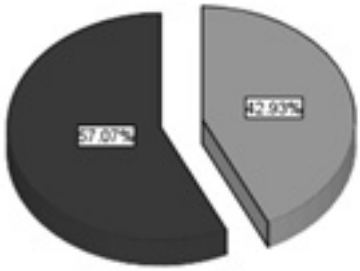

Fig. 4: Advanced maternal age

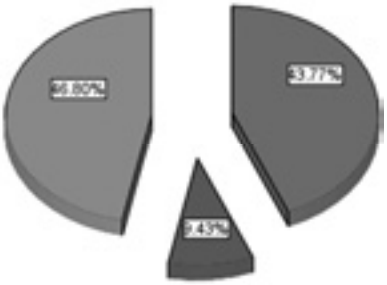

Fig. 5: Ultrasound markers

From these indications, 2 aneuploidies (trisomy 21, 1 case; trisomy 18, 1 case were first indicated by abnormal results in maternal serum screening test, 3 (trisomy 18, 1 case; triploidy 1 case; monosomy 1 case) by abnormal ultrasound findings and 5 (trisomy 21, 5 cases) by abnormal results in maternal serum screening combined with advanced maternal age.

Of the total 594 patients, 510 opted for fetal karyotyping, 515 opted for FISH and 440 opted for both analyses. Karyotyping results were categorized into normal karyotypes (96.08 % of patients) and abnormal karyotypes (3.92% of patients). Abnormal karyotypes were divided into common aneuplodies of chromosomes (21, 18, X and Y) and other chromosomal abnormalities (inversions and translocations). One of the frequent occurrences in chromosome rearrangements is pericentric inversion of the chromosome 9 inv(9)(p11q13), which some scientists consider as a variant of normal karyotype. Although it seems not to correlate with abnormal phenotypes there have been many controversial reports indicating that it may lead to abnormal clinical conditions such as infertility and recurrent pregnancy loss (Mansouri et al., and Amiel et al.,2001). The incidence of pericentric inversion of the chromosome 9, inv(9)(p11q13),is said to be about 1% to 1.65% in the general population (Teo et al.,1995) Table 1 shows the details of the 20 cases with chromosomal abnormalities.

Table 1: Chromosomal abnormality in cytogenetic study

Chromosomal abnormality	***Disorders***	**Karyotype**	***Frequency***	**No. of cases**
Numerical	Trisomy 21	47,XX+21 47,XY+21	0,58% 0.58%	3 3

	Trisomy 18	47,XY+18	0,20%	1
	Trisomy X	47,XXX	0,39%	2
	Trisomy XXY	47,XXY	0,20%	1
	Triploidy	69,XXX	0,20%	1
Structural	Inversions	46,XX,inv(9)(p11q13) 46,XY,inv(9)(p11q13) 46,XY,inv(3)(p11q11.2) 46,XX,inv(3)(p11q11.2) 46,XY,inv(3)(p11q11.2)(9)(p11q13)	0.58% 0,20% 0,39% 0,20% 0,20%	3 1 2 1 1
	Translocations	46,XY,der(14;21)(q10q10)	0,20%	1
Total	-	-	3.92%	20

Since the 1970s, karyotyping of fetal cells cultured from amniotic fluid has been the gold standard technique for the prenatal diagnosis of chromosomal disorders. Standard cytogenetic analysis performed on fetal cell samples identifies chromosome aneuploidies and rearrangements with approximately 99.5% accuracy (Baruch and Evans, 2002). However, a significant limitation of this technique is that cells have to be cultured, leading to a delayed result (commonly between 14 and 21 days). Waiting for chromosome analysis can be especially stressful for the patients (Evers-Kiebooms et al., 1988; Johnson et al., 1992)

FISH results were categorized into normal FISH (97.87% of patients) and abnormal FISH (2.13% of patients). In 0,97 % it was trisomy 21 (fig.6) , in 0.19 % monosomy 45 X (fig.10), in 0.39% trisomy X,(fig.8) in 0.19% trisomy XXY (fig.9) and 0.39% trisomy 18 (fig.7).

In one case which was identified as trisomy of the chromosome 21, karyotyping identified a derivative chromosome 14 - 46,XY,der(14;21)(q10q10).

FISH analysis identified this abnormality as trisomy because the probe used is specific for the region which was translocated to chromosome 14 and actually this aberration has a phenotypic expression as chromosome 21 trisomy. FISH limitation in detecting structural abnormalities is one major point which makes it a good complementary technique and not the standard analysis.

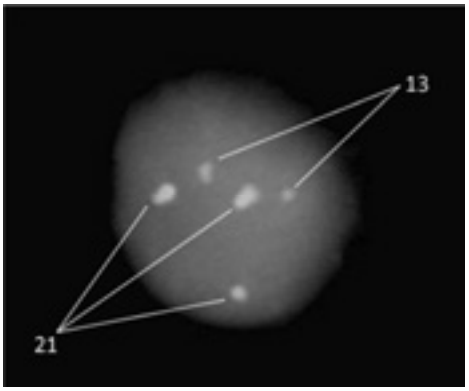

FIG. 6 Interphase nucleus from uncultured amniocytes by FISH shows two green (13 chromosome) and three red (21 chromosome) - trisomy 21

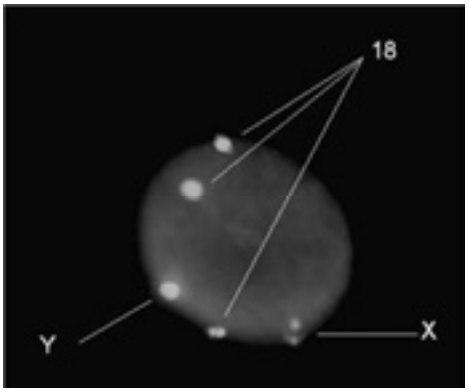

FIG. 7 Interphase nucleus from uncultured amniocytes by FISH shows one green (X chromosome), one red (Y chromosome) and three blue (18 chromosome) – trisomy 18

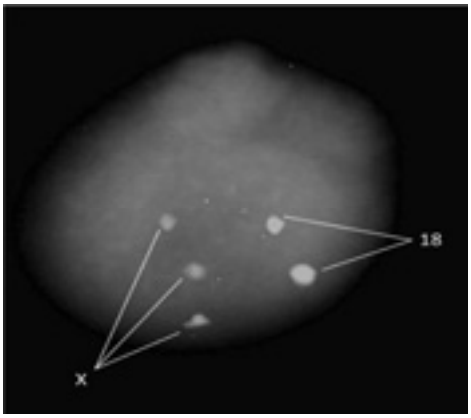

FIG. 8 Interphase nucleus from uncultured amniocytes by FISH shows three green (X chromosome) and two blue (18 chromosome) - triple X

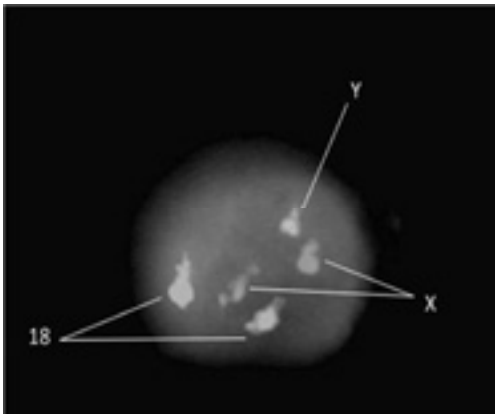

FIG. 9 Interphase nucleus from uncultured amniocytes by FISH shows two green (X chromosome), one red (Y chromosome) and two blue (18 chromosome) – Klinefelter syndrome

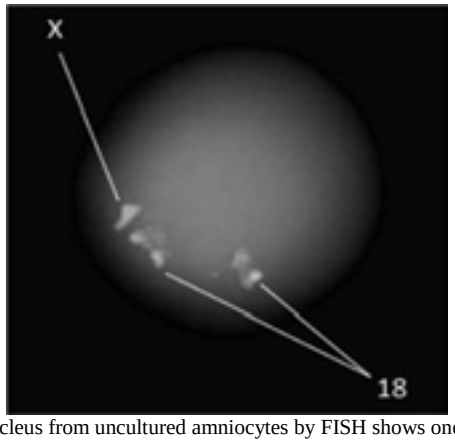

FIG. 10 Interphase nucleus from uncultured amniocytes by FISH shows one green (X chromosome) and two blue (18 chromosome) - Turner syndrome

Compared to cytogenetic studies, the sensitivity of the test FISH to detect aneuploidies in this study was 100%. Chromosomal abnormality, such as 46,XX,inv(9)(p11q13) and 46,XY,inv(3)(p11q11.2) cannot be detected by interphase FISH analysis. **Table 2** shows the details of the 11 cases with FISH abnormalities.

Table 2: Chromosomal abnormalities detectable by FISH

Chromosomal abnormality	***Disorders***	***Frequency***	**No. of cases**
Autosomal abnormalities	Trisomy 21	0.97 %	5
	Trisomy 18	0,39%	2
Sex chromosome abnormalities	Trisomy X	0,39%	2
	Monosomy X0	0,19%	1
	Trisomy XXY	0,19%	1
Total	-	2.13%	11

Several studies have reported successful application of FISH on interphase cells for rapid prenatal diagnosis. Rapid detection of prenatal aneuploidy using interphase FISH on a large scale were successfully initiated (Klinger et al.,1992). Their studies formed the basis of the clinical protocols for the application of FISH to prenatal diagnosis.

Fluorescence *in situ* hybridization (FISH) introduced more than a decade ago, as a potentially powerful tool in clinical cytogenetics (Cremer et al., 1986), can provide a rapid and relatively reliable detection of aneuploidy of these chromosomes (Jalal et al., 1998).

FISH analysis of uncultured amniocytes offers an informative result, in most cases, in 24-48 h. Rapid results may be crucial for important clinical decision-making in some cases and are helpful in decreasing the anxiety level in most patients with an abnormal maternal serum screening and increased risk for trisomy. However, it has been demonstrated that in case of not performing karyotype analyses this will lead to a significant number of false negative results related to other unbalanced abnormalities (Caine et al., 2005).

CONCLUSIONS

All chromosomal aneuploidies and the majority of structural chromosomal abnormalities (deletion, translocation) can be prenatally detected by conventional karyotype.

FISH analysis performed on uncultured amniocytes is important due to the fact that results generally can be obtained in the first 48 hours from amniocentesis.

In this study we have investigated the correlation between karyotype analysis and FISH and concluded that there was a 100% correlation between the results obtained.

Consequently, aneuploidy screening of uncultured amniotic cells with direct FISH is important for prenatal diagnosis due to short time of result delivery which is very important for the anxiety management of the patients.

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Date of manuscript submission
18/08/2010