Molecular cytogenetics in prenatal diagnosis

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Moleculaire cytogenetica in de prenatale diagnostiek

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit van Rotterdam
op gezag van de rector magnificus
Prof. dr P.W.C. Akkermans M.A.
en volgens het besluit van het college voor promoties

De openbare verdediging zal plaatsvinden op
woensdag 4 maart 1998 om 11.45 uur

Door

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geboren te Hoogstraten

Promotie -commissie

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The studies described in this thesis were performed at the Department of Clinical Genetics of the University Hospital Dijkzigt and the Erasmus University Rotterdam. This project was financially supported by the Rotterdam Foundation of Clinical Genetics.

Printed by Copynomie, Rotterdam

Contents

Chapter I

Introduction

1. Traditional prenatal cytogenetic diagnosis

Prenatal diagnosis (PD) started in the fifties when various groups indicated the possibility of prenatal sex determination in amniotic fluid (AF) cells (Serr et al., 1955; Fuchs and Riis, 1956; Dewhurst, 1956). After the first succesful attempts at AF cell cultivation and karyotyping by Steele and Breg (1966) and Thiede et al. (1966), the first small series of prenatal chromosome analyses were presented (Jacobson and Barter, 1967), and the first chromosome aberrations in cultured AF cells were detected (Valenti et al., 1969). AF cells could also be used for prenatal detection of inborn errors of metabolism (Nadler, 1968). Our centre made an important international contribution towards experience with a large number of biochemical assays, and the development of ultramicrochemical techniques permitting a rapid PD (Galjaard, 1972; Niermeijer, 1975; Galjaard, 1976a, 1979, 1980; Galjaard et al., 1977; Kleijer, 1990). Another important contribution to PD was the finding by Brock and Sutcliffe (1972), that the alpha-fetoprotein level in AF is increased when the fetus has an open neural tube defect.

Amniocentesis is generally performed in the second trimester of pregnancy. The major disadvantage is a midtrimester termination of the pregnancy in case of a fetal chromosome abnormality. This may cause serious psychological stress and burden for the parents (Thomassen-Brepols, 1985). Advances in the technology associated with amniocentesis using high resolution real time ultrasound and improved laboratory methods have made it technically possible to perform amniocentesis prior to 15 weeks of gestation, but the safety and accuracy of this "early amniocentesis" technique has yet to be established (reviewed by Wilson, 1995).

The first attempts at first trimester PD were made by Kullander and Sandahl (1973) and Hahneman (1974) who used long-term cultures of chorionic villi (CV) from first trimester abortions for fetal karyotyping. In 1975, Yamamoto et al. described the use of spontaneous mitoses in CV for chromosome analysis after induced abortions. That same year, a Chinese group (Anshan Iron and Steel Company, 1975) reported fetal sex prediction by sex chromatin analysis in direct preparations of CV during early pregnancy. The origin of spontaneous mitoses in CV was investigated by Watanabe et al. (1978) who found them to be derived from the Langhans cells of the cytotrophoblast and not to be present in syncytiotrophoblast and mesodermal core of CV. In 1983, Simoni et al. described an efficient method to obtain metaphases from chorionic villi cytotrophoblast tissue within a few hours after sampling, making a rapid prenatal cytogenetic diagnosis possible in the first trimester of pregnancy. This led to the first PD of trisomy 21 at 11 weeks of gestation within five hours after sampling (Brambati and Simoni, 1983). In the Netherlands, CVS was first employed in Rotterdam in 1983 (Jahoda et al., 1984; Galjaard, 1985; Sachs et al., 1985).

Nowadays, *second-trimester* amniocentesis and *first-trimester* chorionic villus sampling are two widely used invasive techniques for PD of chromosome aberrations. Invasive implies that specimens are obtained from the fetus or from associated fetal structures or products by needle puncture or biopsy technique, in contrast with non-invasive techniques, such as ultrasound investigation.

1.1 Amniocentesis

Second-trimester amniocentesis is defined as an amniocentesis peformed at 15^{+0}-19^{+6} weeks of gestation with a cytogenetic result available after 10-20 days (Wilson, 1995). This gestational age for the procedure was established in the seventies, when various reports noted that AF could be obtained at this time of pregnancy with an acceptable degree of safety and accuracy (National Institute of Child Health and Human Development, 1976). AF is removed from the intrauterine gestational sac by needle aspiration under continuous ultrasound guidance. About 20 ml of AF is aspirated, which represents 12,5 % of the total volume at 16 weeks (Finegan, 1984). During pregnancy the total number of AF cells per ml increases, although the percentage of viable cells declines, being the highest at 14-16 weeks of gestation (24 %) and it decreases to 9-10 % at 32-36 weeks of gestation (Galjaard, 1976b).

A number of studies have been performed on the different cell types present in uncultured (Huisjes, 1978; Papp and Bell, 1979; Tydén et al., 1981) as well as cultured AF (reviewed by Gosden, 1983; Chang and Jones, 1988). Tydén et al. (1981) studied the origin of cells in mid-gestational AF by using scanning electron microscopy, both of the surface ultrastructural morphology of the AF cells and the fetal surfaces exposed to the AF. They identified four cell populations derived from periderm, umbilical cord, oral and nasal mucosa, and from the vagina, respectively. Non-shedding epithelia, which do not contribute a significant number of cells to the AF, are observed in the respiratory tract, the urinary bladder, and the amniotic membrane.

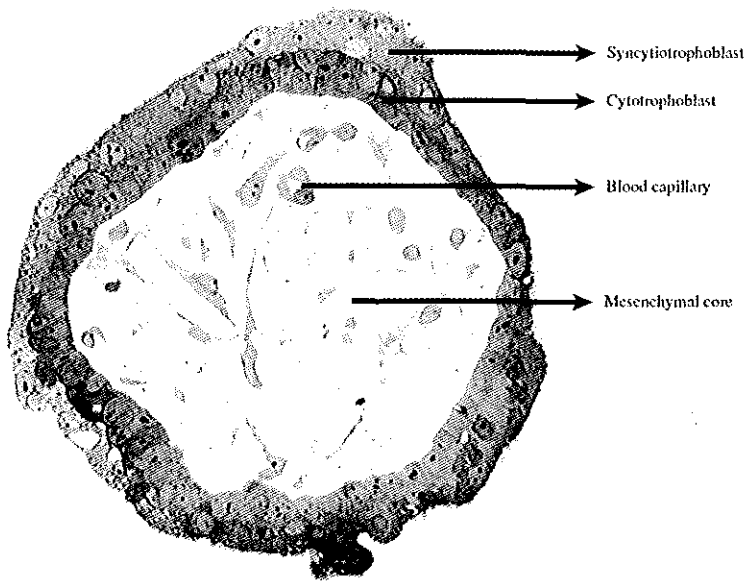
The cells present in the AF have to be cultured, before chromosome slides can be made. The preparation of these cells for karyotyping can be carried out according to two main principles: the " in situ" preparation technique, allowing karyotyping of individual cell colonies, and the "flask method" requiring trypsinization of the cells before harvesting, which obviously disrupts colony integrity (Rooney and Czelpulkowski, 1992). According to our protocol, the cell suspension of 20 ml of AF is seeded over five culture dishes, and the cultures are harvested using the in situ method . Trypsin-Giemsa staining is routinely used for banding of the chromosomes (Seabright, 1971), and we investigate 16 cell colonies per patient.

1.2 Chorionic villus sampling (CVS)

In our PD centre, CV are mainly sampled transabdominally at 11-13 weeks of gestation. There are two reasons for discarding sampling at an earlier gestational age. Firstly, older women (≥36 years), who represent the major indication group for PD, have a higher spontaneous abortion rate in early pregnancy before 12 weeks of gestation which affected the post-procedure fetal loss rate (Jahoda et al., 1987, 1990; Cohen-Overbeek et al., 1990). Secondly, there might be an association between early CVS during the critical stage of development of various organ systems and some vascular disruptive syndromes (Firth et al., 1991, 1994; Jahoda et al., 1993; Los et al., 1996).
CV for prenatal investigations are biopsied from the chorion frondosum. They consist of three

major components: (1) an outer layer of hormonally active syncytiotrophoblast, (2) a middle layer of cytotrophoblast from which the syncytiotrophoblast is derived, and (3) an inner mesodermal core containing blood capillaries (Figure 1.1). A mean of 10-15 mg villi is usually obtained and is necessary for a succesful chromosome preparation. Additional biochemical or DNA-investigations require an extra 20-30 mg of CV.

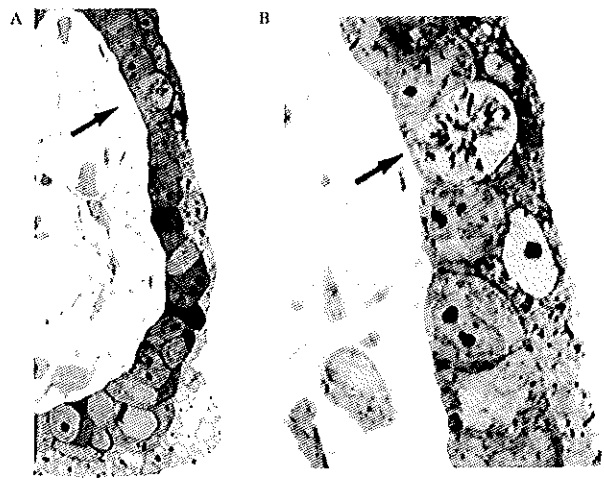
Figure 1.1 Cross section through a chorionic villus, showing its major components.



There are two ways of processing CV for cytogenetic studies: the direct and long-term preparation technique:

-the *direct preparation technique*, first described by Simoni et al. (1983), utilizes the spontaneously dividing Langhans cells in the cytotrophoblast of CV. Figure 1.2 shows a cross section through a CV showing the presence of spontaneous mitoses in the cytotrophoblast. Since the introduction of this technique, some modifications were introduced by several investigators (Gibas et al., 1987; Terzoli et al., 1987; Simoni et al., 1990). We use the semi-direct preparation technique described by Gibas et al. (1987).

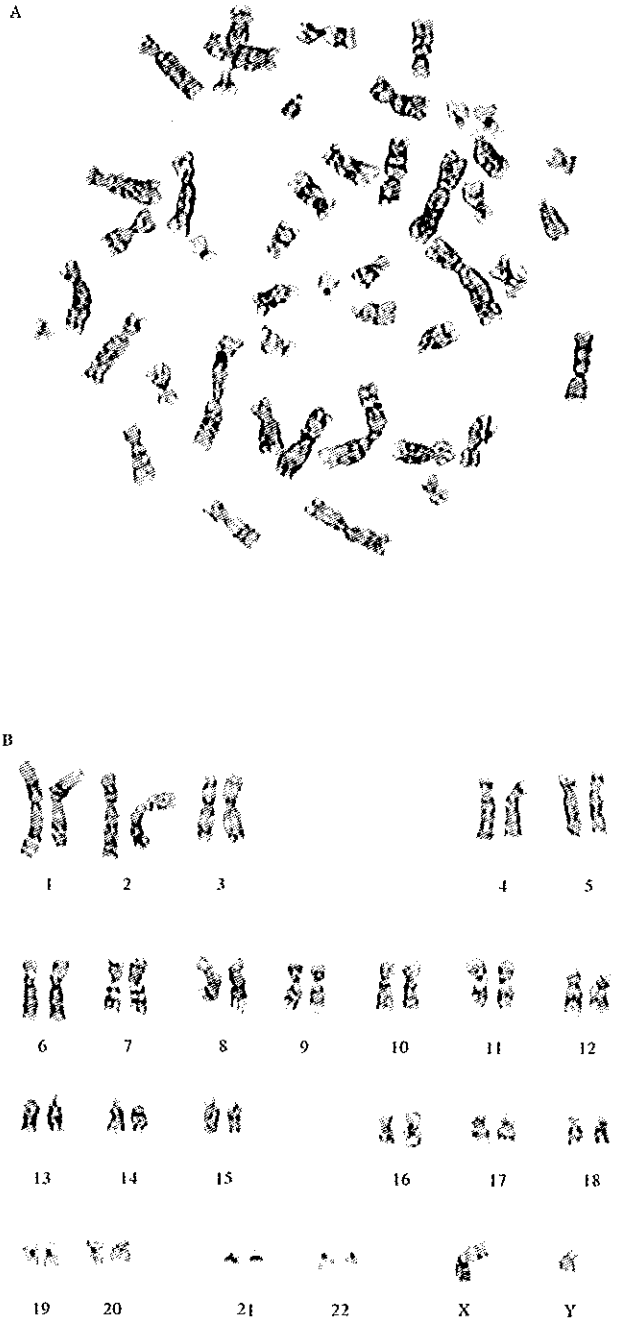
Figure 1.2 Cross section through a chorionic villus, showing a spontaneous mitosis in the cytotrophoblast. A) magnification of 250 ×, and B) of 750 ×.



-the *long-term preparation method* consists of a trypsin (0,05%)-EDTA (0,02%) and a collagenase treatment of the villi before setting up cultures, as described by Smidt-Jensen et al. (1989).

Whereas the metaphases in the direct chromosome preparations are derived from the cytotrophoblast, those in long-term cultures are assumed to be predominantly of mesenchymal origin. Since cytotrophoblast and mesenchymal core of CV have a different embryogenic origin (Crane and Cheung, 1988; Bianchi et al., 1993), karyotyping both cell types represents the investigation of two different compartments. Therefore, most centres for PD prefer to use the direct and long-term preparation method simultaneously to improve the accuracy of PD on CV, which will be discussed extensively in section 1.4.2. This approach, however, requires an amount of CV of at least 20 mg. As the weight of the CV samples in our centre did generally not exceed 10-15 mg during the study period, we could only perform one of both methods. As culturing of CV is very laboureous and time-consuming, delaying the reporting time of a cytogenetic result to the parents, and therefore obviate the main advantage of CVS for prenatal diagnosis, and because of the risk of maternal cell contamination, we prefered the use of the semi-direct method only, eventually followed by amniocentesis for further investigation of uncertain cytogenetic results. Since January 1997 we use both preparation methods for samples of at least 20 mg. Chromosome analysis is routinely performed using trypsin-Giemsa staining (Seabright, 1971). For most indications, we analyse eight cells and count another eight. A metaphase of average quality and its karyotype are shown in figure 1.3.

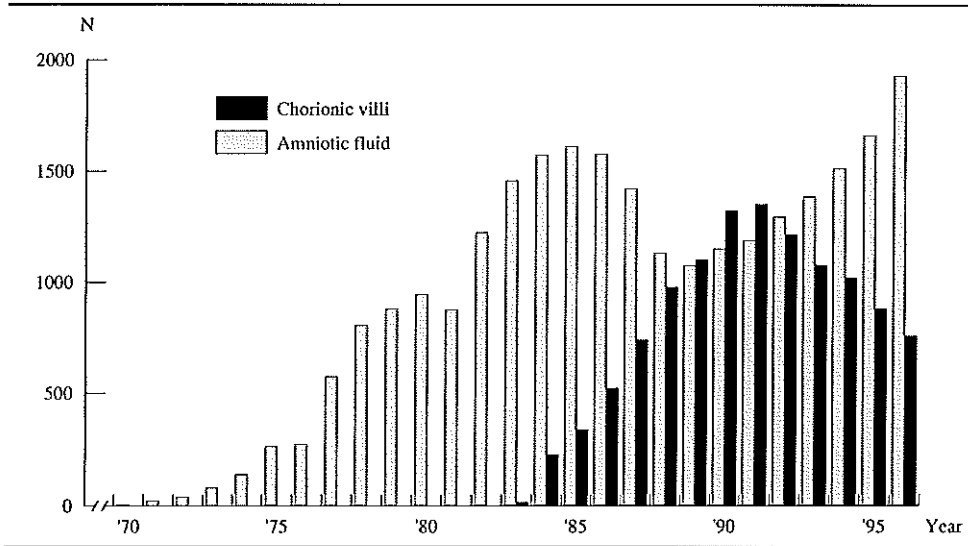
Figure 1.3 Metaphase spread (A) and karyotype (B) of average quality in chorionic villi semi-direct preparation.



1.3 Number of AF and CV samples received in the period 1970-1996

Between 1970 and the end of 1996, 25.073 AF and 11.140 CV samples were recieved in our laboratory for prenatal chromosome analysis (Niermeijer et al., 1976; Galjaard et al., 1982; Sachs et al., 1982; Jahoda et al., 1984, 1985; Galjaard 1985; Sachs et al., 1985, 1990). The evolution of the numbers of AF and CV samples during that time period is shown in figure 1.4. After the introduction of CVS in 1983, the number of AF samples decreased with a concomitant increase in the number of CVS. However, since 1992, a decrease in the number of CV samples occured, together with an increase of AF samples, due to the yet unsolved problem of inducing fetal vascular disruptive syndromes by CVS, as mentioned above, and because of the limited representativity of an abnormal karyotype in CV for the actual fetal karyotype, which will be discussed in section 1.4.2.

Figure 1.4 Number of amniotic fluid and chorionic villi samples received during the time period 1970-1996.



1.4 Limitations of traditional cytogenetic analysis of AF and CV cells

Traditional cytogenetic analysis of AF and CV cells has a few limitations:

1) chromosome abnormalities with *indistinct banding patterns*, such as marker chromosomes or de-novo structural rearrangements, are often difficult to interpret. The additional use of different staining techniques is important for their identification (Sachs et al., 1987), but can

not always give a definite diagnosis.

2) the main limitation of amniocentesis is the *time-consuming cell culturing* which is required for generating sufficient high-quality chromosome spreads, so that a result is only achieved two to three weeks after sampling. This long waiting-time for a result may be a burden on the future parents, especially if the pregnancy is at high genetic risk. In the past, several attempts have been made to use uncultured AF cells for fetal sex determination in pregnancies at risk for X-linked diseases, by demonstration of X and Y chromatin in interphase nuclei (Pearson et al., 1970). However, this method turned out to be unreliable (Gosden, 1983).

3) *chromosomal mosaicism*, defined as the presence of at least two karyotypically different cell lines within an individual (Gosden et al., 1995), may cause interpretation problems, as it may represent true fetal mosaicism or pseudomosaicism (culture artefacts without clinical significance). Discrimination between both phenomena is therefore critical and requires the analysis of a large number of cells, which is often difficult due to limited time and sample size.

4) the detection of a (non)-mosaic chromosome aberration in CV sometimes poses an interpretation dilemma, as the chromosome aberration may be confined to placental tissue (*confined placental mosaicism (CPM)*) and not be present in the fetus itself. If CPM is suspected, follow-up investigations are necessary, including ultrasound examination of the fetus as well as karyotyping AF cells or fetal lymphocytes, which severely delay the reporting time.

5) although most chromosome aberrations that are detected prenatally are numerical abnormalities (trisomy 13, 18, 21, triploidy, sex-chromosomal aneuploidies), structural chromosome rearrangements account for a small but significant proportion of the abnormal karyotypes, and their detection is limited by the *resolution of the light microscope*. Today, chromosome methodology in classical cytogenetics has reached a stage of resolution that allows the detection of chromosome rearrangements involving about 6×10^6 base pairs (Ferguson-Smith, 1988). Smaller changes, such as microdeletions, are difficult or impossible to identify with classical cytogenetic techniques.

6) *maternal cell contamination* (MCC) of the sample may lead to incorrect sex prediction and potentially to a false negative diagnosis. It is regularly found in CV long-term cultures (frequency of 1 % to 3 %) (Ledbetter et al., 1992a; Smidt-Jensen et al., 1993; ACC working Party on Chorionic Villi in Prenatal Diagnosis, 1994), although it is extremely rare in AF cell cultures (frequency of 0 % to 0,3 %) (Benn et al., 1983; Hsu and Perlis, 1984; Bui et al., 1984; Worton and Stern, 1984) and CV semi-direct preparations (frequency of 0 % to 0,4 %) (Simoni et al., 1986; Ledbetter et al., 1992a; ACC Working Party on Chorionic Villi in Prenatal Diagnosis, 1994).

Limitations 3 and 4 will be further discussed in detail in sections 1.4.1 and 1.4.2, respectively.

1.4.1 Chromosomal mosaicism in AF cell cultures

The accurate diagnosis of chromosomal mosaicism in AF cell cultures represents a problem in PD. True fetal mosaicism may go undetected if an insufficient number of AF cells is analysed. A false positive diagnosis is also possible, since a chromosome abnormality can arise in-vitro and may not reflect the actual fetal chromosome constitution; this situation is designated pseudomosaicism. True chromosomal mosaicism is generally defined as the presence of an identical chromosome abnormality in at least two independently cultured dishes, whereas pseudomosaicism involves multiple cell colonies with the same chromosome aberration restricted to one culture dish (pseudomosaicism type C), one colony with a chromosome abnormality with the other colonies of the sample being normal (pseudomosaicism type B), or a partial abnormal colony (pseudomosaicism type A)(Boué et al., 1979). Four large collaborative studies (Hsu and Perlis, 1984; Worton and Stern, 1984; Bui et al., 1984; Hsu et al., 1996) provide probably the best data available on mosaicism and pseudomosaicism in AF cell cultures. The combined frequencies of chromosome mosaicism and pseudomosaicism ranged from 3,4 % to 8,5 % in these four large surveys.

Due to the relatively high incidence of pseudomosaicism and the fear of missing a case of true chromosome mosaicism, most laboratories use a two-stage approach in the work-up of AF cell culturing and karyotyping for the differentiation of true mosaicism from pseudomosaicism (Hsu and Perlis, 1984; Cheung et al., 1990). In most cytogenetic laboratories the routine karyotyping involves the examination of 10-16 cell colonies from multiple in situ culture dishes. If all cells show a normal karyotype, a normal result is reported. If one or more cell colonies in one culture dish show an abnormality, additional colonies from other dishes are studied. This two-stage approach has been considered more efficient and more cost-effective in work-up for chromosome mosaicism than routinely analysing a large number of cells in every AF sample (Cheung et al., 1990). The number of additional cell colonies that need to be evaluated to exclude true chromosome mosaicism at a given confidence level can be derived from tables proposed by Hook (1977), or those developped by Cheung et al. (1990), Featherstone et al. (1994), and Sikkema-Raddatz et al. (1997).

Hsu et al. (1992) proposed three different levels of work-up for the exclusion of potential mosaicism, depending on the chromosome abnormality involved, based on available karyotype/phenotype correlation data: extensive, moderate, and no additional work-up (table 1.1). Extensive and moderate additional work-up mean the analysis of 24 and 12 cell colonies, respectively, from multiple in situ culture dishes, not including the colonies from the culture dish in which the abnormal colony/colonies were found. According to the tables of Hook (1977), this allows the detection of 12 % and 24 % mosaicism at a 95% confidence level, respectively (Hsu et al., 1992).

The two-stage approach offers a high degree of confidence in excluding mosaicism. Benn et al. (1984) made a crude estimate of the extent to which true mosaicism might be interpreted as pseudomosaicism, or entirely missed, based on data from the US survey (Hsu and Perlis, 1984). It was concluded that at the most 4,5 % of cases of true mosaicism may be completely missed and up to 7 % could be misdiagnosed as pseudomosaicism. Examples of both

situations are described in the literature (Wolstenholme et al., 1988; Cheung et al., 1988; Terzoli et al., 1990; Vockley et al., 1991; Schneider et al., 1994; Hanna et al., 1995; Wolstenholme, 1996).

Table 1.1. Proposed guidelines for work-up of possible pseudomosaicism/mosaicism, using the in situ method (Hsu et al., 1992)

A.	**Indications for extensive work-up**
1.	Autosomal trisomy involving chromosomes 8, 9, 12, 13, 14, 15, 18, 20, 21, or 22 (Sco, Mco)
2.	Unbalanced structural rearrangement (MCo)
3.	Marker chromosome (MCo)
B.	**Indications for moderate work-up**
4.	Autosomal trisomy involving chromosomes 1, 2, 3, 4, 5, 6, 7, 10, 11, 16, 17, or 19 (Sco, Mco)
5.	Unbalanced structural rearrangement (SCo)
6.	Marker chromosome (SCo)
7.	Extra sex chromosome (SCo, MCo)
8.	45, X (SCo, MCo)
9.	Balanced structural rearrangement (MCo)
10.	Monosomy (other than 45,X) (SCo, MCo)
C	**No additional work-up**
11.	Balanced structural rearrangement (SCo)
12.	Break at centromere with loss of one arm (SCo)
13.	All single cell abnormalities

Note.- Sco: single colony/single dish; MCo: multiple colonies/single dish

1.4.2. Confined placental mosaicism

Since fetus and placenta originate from the same zygote, their chromosomal complement is expected to be the same. However, in 1-2 % of viable pregnancies studied by CVS, the cytogenetic constitution of fetus and placenta is different. *Firstly*, a (non-) mosaic chromosome abnormality may be confined to the placenta and be not present in the fetus. This phenomenon is called confined placental mosaicism (CPM). It was first described by Kalousek and Dill (1983) in term placentas of infants born with unexplained intrauterine growth retardation (IUGR) and it was soon recognised in first trimester CV after the introduction of CVS for prenatal cytogenetic studies (Simoni et al., 1985; Mikkelsen et al., 1985; Vejerslev and Mikkelsen, 1989; Leschot et al., 1989; Sachs et al., 1990; MRC Working Party on the Evaluation of Chorionic Villus Sampling, 1991; Ledbetter et al., 1992a; Teshima et al., 1992; Breed, 1992; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Wang et al., 1994; Wolstenholme et al., 1994; Pittalis et al., 1994; Leschot et al., 1996; Hahnemann and Vejerslev, 1997). Three different types of CPM, according to the compartments of the CV involved, can occur: **type I** (confinement of chromosome abnormality to cytotrophoblast ((semi)-direct preparations) of CV), **type II** (confinement of abnormality to mesenchymal core (long-term cultures) of CV), and **type III**

(both cytotrophoblast and mesenchymal core abnormal) (Kalousek, 1990; Kalousek et al., 1992). *Secondly*, the converse pattern, normal CV results and a (non-) mosaic abnormal karyotype in the fetus, has also been observed, although it is extremely rare and it seems to be mainly restricted to the (semi-) direct preparation method (Martin et al., 1986; Simoni et al., 1987; Leschot et al., 1988; Miny et al., 1988; Ledbetter et al., 1992; Pittalis et al., 1994; Hahnemann and Vejerslev, 1997). To the best of our knowledge, only two cases of false negative results of both methods ((semi-) direct and long-term preparation method) were described by Pindar et al. (1992) and Pittalis et al. (1994).

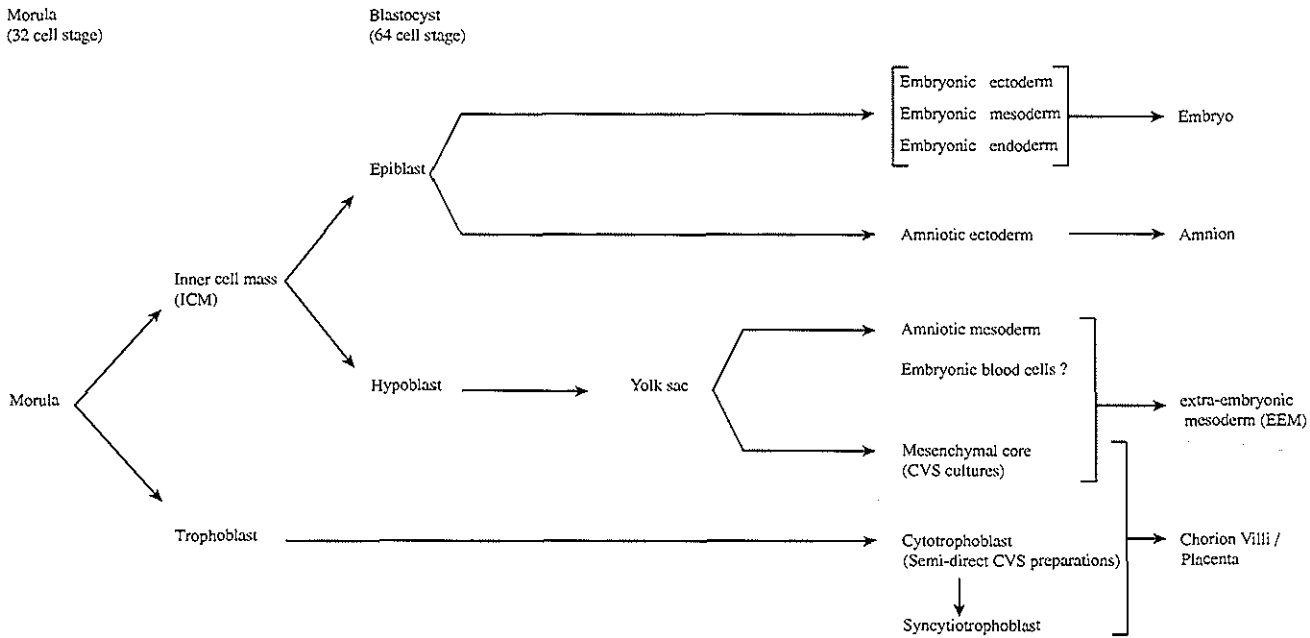
Early embryonic development

During early embryogenesis, a complex sequence of events will lead to the formation of distinct embryonic and extra-embryonic components (figure 1.5). The inner cell mass (ICM) and cytotrophoblast can first be distinguished in the late morula (32 cells). Blastocyst formation is apparent at the 64-cell stage. The blastocyst consists of an outer layer of trophoblast cells, which will give rise to the cytotrophoblast of the placenta (studied in semi-direct CV preparations), and an ICM, now represented by approximately 16 cells, of which three to four cells will develop into the embryo, whereas the remaining cells will form the extraembryonic mesoderm (studied in long-term CV cultures) (Markert and Petters, 1978; Crane and Cheung, 1988; Bianchi et al., 1993). Chromosomal mosaicism is caused by abnormal cell division arising in early embryonic development. While early division errors may cause generalized mosaicism, later errors, affecting specific cell lineages, will lead to a mosaic conceptus with mosaicism confined to either the placenta or the embryo/fetus. So time and place of a postzygotic mitotic error in a chromosomal normal or abnormal situation will determine the pattern of mosaicism. Pittalis et al. (1994) proposed a detailed classification of all theoretical combinations of karyotypes in the various placental and fetal compartments (cytotrophoblast, mesenchymal core, and fetus/embryo), and they were grouped in 11 categories. All but one were found in a consecutive series of 4860 CVS diagnoses, demonstrating a considerable cytogenetic variability along the trophoblast-embryo axis.

Origin of trisomy CPM

CPM involving a trisomy, representing about 50 % of all CPM cases, may result from a somatic duplication of a whole chromosome in placental progenitor cells originating from a diploid zygote (mitotic CPM), or from a trisomic conceptus with loss of the extra chromosome in embryonic, but not in placental progenitor cells (meiotic CPM); this phenomenon is called trisomic zygote rescue (Kalousek and Vekemans, 1996). A mitotic origin of CPM likely results in low levels of mosaicism (Wolstenholme, 1996; Robinson et al., 1997), whereas meiotic CPM was shown to be significantly correlated with high levels of abnormal cells in both placental lineages (Robinson et al., 1997). Meiotic CPM can theoretically be associated with the phenomenon of fetal uniparental disomy (UPD), i.e., both chromosomes of a chromosome pair derived from one parent only (Engel, 1980). The chance is 66,7 % that one of the chromosomes from the parent contributing two copies will disappear, and 33,3 % that the one from the parent contributing one copy will be lost; in this last situation both chromosomes left are derived from one parent only (Engel and Delozier-Blanchet, 1991) (figure 1.6).

Figure 1.5 Early embryonic development, according to Crane and Cheung (1988), and Bianchi et al. (1993).



Depending on the meiotic division in which the non-disjunctional error occurred, and on the extent of crossing-over between the homologues of the chromosome pair, prior to that meiosis, UPD can be heterodisomic, isodisomic, or combined hetero/isodisomic (Engel, 1980; Engel and Delozier-Blanchet, 1991).

Fetal confirmation rate of CVS mosaicism

If chromosomal mosaicism is detected in first trimester CV, the overall fetal confirmation rate is about 10 % (Pittalis et al., 1994; Phillips et al., 1996) [compared with 60-70 % when found in AF cell cultures (Hsu et al., 1992)]. It has been demonstrated that the risk of fetal mosaicism is related to two factors: the cell type in which the abnormality is seen and the type of chromosome abnormality. *Firstly*, it has repeatedly been shown that cultures of CV mesenchymal core are more likely to reflect the fetal chromosomal constitution than the direct cytotrophoblast preparations, which is explained by the embryonic models proposed by Crane and Cheung (1988) and Bianchi et al. (1993). Pittalis et al. (1994) found that the predictive value for an abnormal fetal karyotype rised from 12,1 % for mosaic anomalies observed in direct preparations, to 27,3 % for those observed in the culture method, to 66,7 % for mosaic karyotypes detected in both methods. Therefore, many investigators advocate the use of both direct cytotrophoblast preparations and mesenchymal core cultures to improve accuracy of fetal chromosome studies in CV preparations (Sachs et al., 1990; Ledbetter et al., 1992a; Teshima et al., 1992; Pittalis et al., 1994). *Secondly*, the incidence of generalized mosaicism also varied according to the type of chromosome abnormality. A mosaic polyploidy on CVS was confirmed in about 4 % of cases. When a marker chromosome was involved, it was confirmed in the fetus in 27 % of cases. Mosaicism involving the common trisomies (13, 18, 21) were found in fetal tissues in 19 % of cases in contrast to uncommon trisomies (3, 5, 7, 8, 9, 16, 20, 22), which account for about 40 % of placental mosaicism and which were confirmed in the fetus in only 3 % of cases (Phillips et al., 1996).

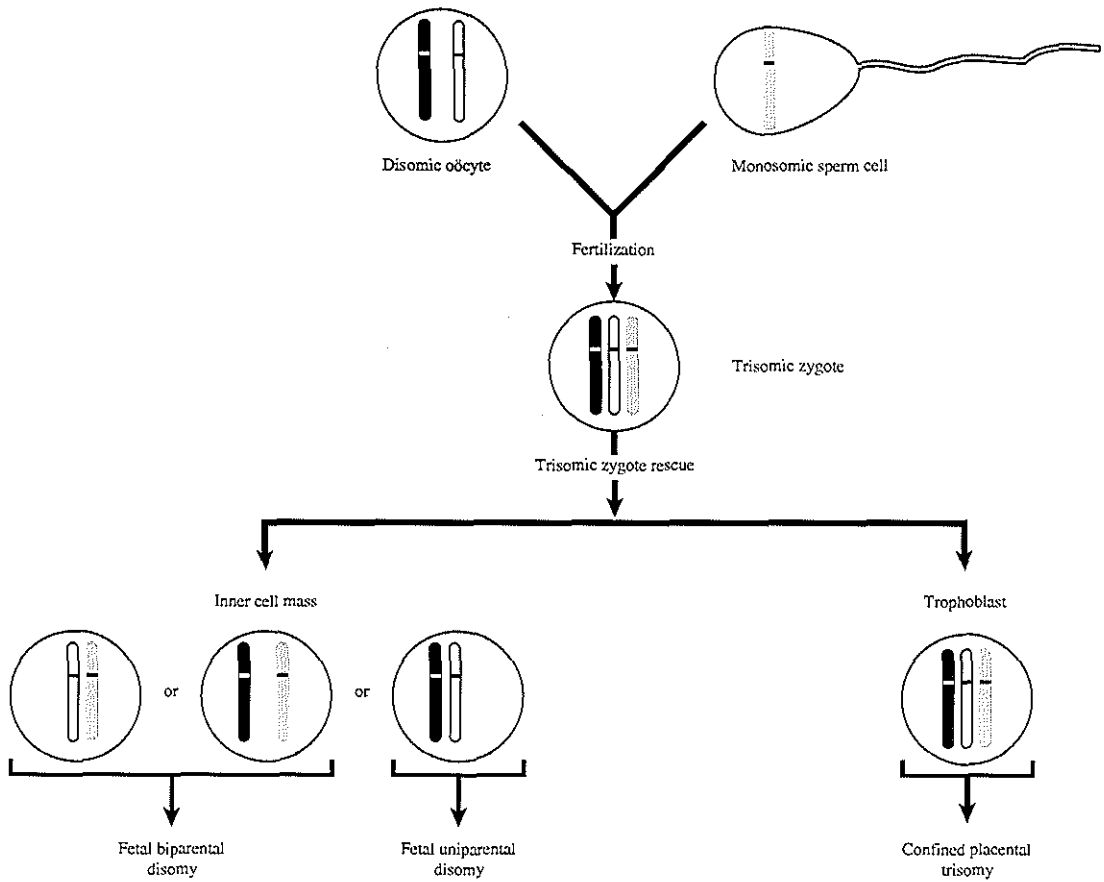
Pregnancy outcome in cases of CPM

If chromosomal mosaicism is detected in CV, follow-up studies are necessary to verify the fetal karyotype. However, even if the chromosome abnormality is found to be absent in AF cells, a normal pregnancy outcome can not be ensured. Although the majority of pregnancies with CPM proceed uneventfully (Leschot et al., 1996), a number of cases were found to be associated with IUGR (Kalousek et al., 1991; Kalousek, 1993; Wolstenholme et al., 1994), fetal loss (Goldberg et al., 1990; Breed et al., 1991; Wapner et al., 1992), or poor perinatal outcome (Johnson et al., 1990; Brandenburg et al., 1996). This may be explained by three mechanisms:

(1) placental function may be disturbed by the presence of cytogenetically abnormal cells (Kalousek, 1993; Wolstenholme, 1994), as also indicated by the reported association between an abnormal profile of maternal serum markers and CPM, especially CPM 16 (Vaughan et al., 1994; Zimmerman et al., 1995; Tantravahi et al., 1996)

(2) UPD may be present in the disomic cell line in case of trisomy CPM, and depending on the chromosome involved, it may be harmful to the fetus. UPD may affect human

Figure 1.6 Schematic presentation of trisomic zygote rescue, resulting in confined placental trisomy and fetal uniparental disomy in one third of the cases



development if imprinted regions, known to exist on some chromosomes, are involved (Ledbetter and Engel, 1995). It may also lead to recessive disorders in case of (partial) isodisomy, or autosomal dominant diseases in case of heterodisomy, if the parent contributing both homologues of a chromosome pair is affected by such a disorder.

(3) the abnormal cell line is in fact not confined to the placenta, but also present in the fetus. Several cases of placental mosaicism with normal results in AF cells, but with confirmation of the chromosome abnormality in fetal tissues, have been described (Miny et al., 1991; Ledbetter et al., 1992a; Phillips et al., 1996).

Some factors are likely to be of significance in terms of predicting the effects of CPM:

1) Robinson et al. (1997) found a significant correlation between a meiotic origin of placental trisomy and adverse pregnancy outcome. Because a meiotic origin was found to correlate with high levels of trisomy in both placental lineages and UPD, it is difficult to distinguish whether an abnormal outcome is due to the UPD in fetal and/or placental tissues itself or to the presence of excessive trisomic cells in the placenta.

2) the involvement of some specific chromosomes may also be of significance in predicting fetal outcome. Leschot et al. (1996) advocated a careful clinical follow-up in cases of CPM involving the chromosomes 13, 16, and 22. Trisomy 16 is one of the most prevalent chromosome aberration involved in CPM, and is associated with maternal UPD 16 in one third of such cases (Kalousek et al., 1993). CPM 16, with or without fetal UPD, is associated with fetal loss later in pregnancy or IUGR, but can be compatible with a viable pregnancy (Wolstenholme, 1995). Trisomy 7 occurs at similar frequencies as trisomy 16 at CVS. However, Kalousek et al. (1996) found that most cases of CPM 7 seem to be the result of somatic duplication within the placental lineage, and only to be associated with IUGR in case of meiotic CPM 7 with UPD 7 in the fetus. Recently, Shaffer et al. (1996) reported on nine cases of CPM for trisomy 2, two of which were associated with severe IUGR. A biparental origin of the fetal chromosomes 2 was established in all cases. CPM 2, like CPM 16, seems to be associated with IUGR only if high levels of trisomic cells are present in the placenta, regardless of the parental origin of the remaining chromosome pair in the fetus. Further cytogenetic and molecular evaluation of cases with CPM for each chromosome will finally result in an improved prediction of pregnancy outcome in cases of prenatally diagnosed CPM.

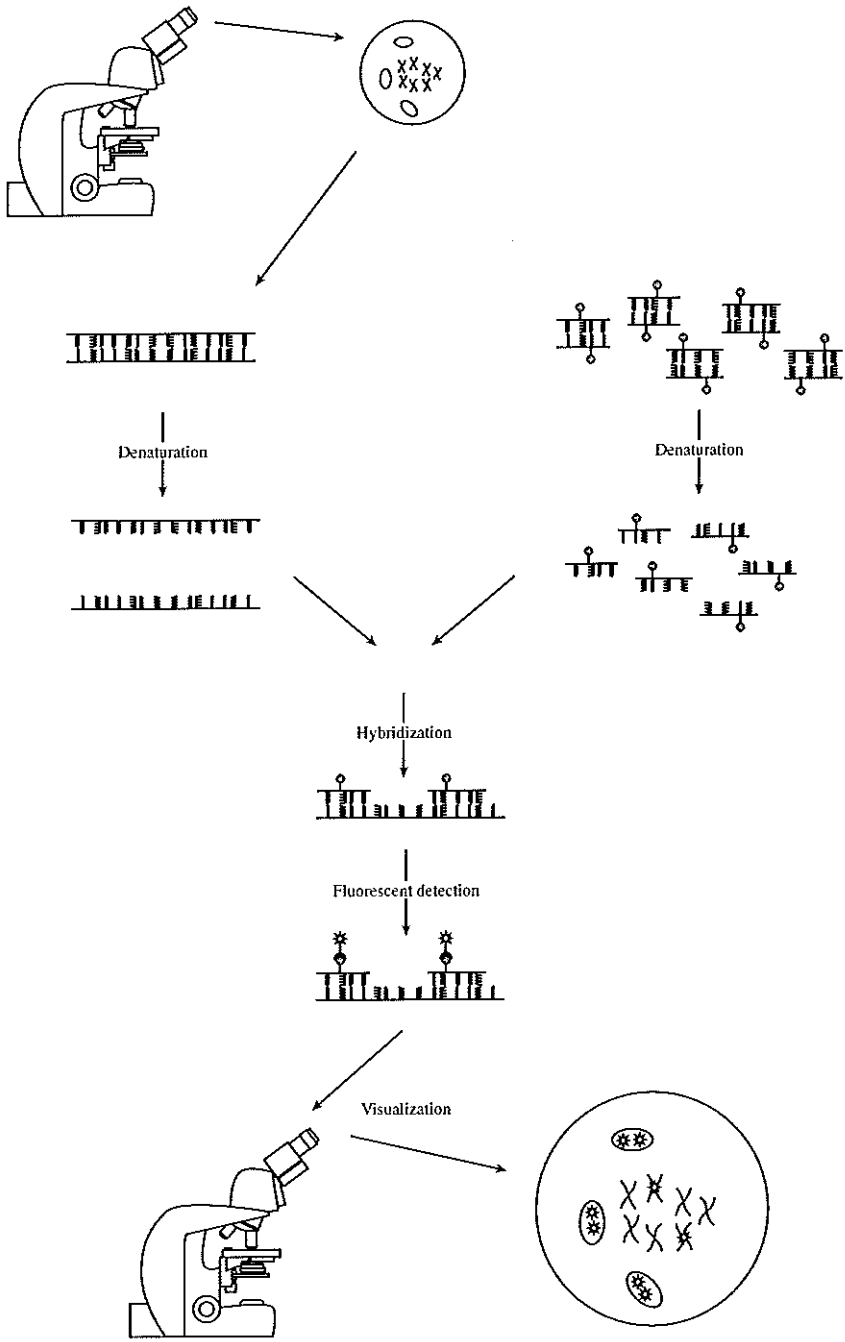
2. Molecular cytogenetics

Most of the limitations of traditional prenatal cytogenetic analysis, as described in section 1.4, can be overcome with a molecular cytogenetic technique known as in situ hybridization (ISH). It involves the detection of a specific nucleic acid sequence, either RNA or DNA, in a chromosome preparation. Early work with this technique, which was developed by Gall & Pardue (1969) and John et al. (1969), made use of DNA probes labelled with a radioactive isotope (radiolabelled in situ hybridization, RISH). In the 1980s, the ISH technique became accessible to routine cytogenetic laboratories by replacement of the radioative labels by a

fluorescent label (**fluorescent in situ hybridization, FISH**) (Cremer et al., 1986; Hopman et al., 1986). The principle of FISH is depicted in figure 1.7. Briefly, target-DNA (in nuclei and/or chromosomes) and labelled chromosome specific probe-DNA are denatured (the DNA becomes single-stranded). Probe DNA is applied to the chromosome slide, to allow hybridization of probe-DNA to target-DNA. After removing any remnants of probe-DNA that is unbound or bound with poor homology, the target-DNA-probe-DNA-complex is subsequently detected.

FISH allows the detection of chromosomal rearrangements at the submicroscopical level, such as microdeletions (Desmaze et al., 1993; Lowery et al., 1995), as well as identification of chromosomes or chromosomal segments of unknown origin (Sachs et al., 1992; Van Hemel et al., 1992; Callen et al., 1992). However, the major advantage of this technique is its application to interphase nuclei, which is called interphase cytogenetics (Hopman et al., 1988; Cremer et al., 1988), and it was first introduced in the field of cancer research, since most tumors have low mitotic indexes. Fluorescent detection of chromosome specific probes enabled the recognition of the centromeres on the chromosomes as clearly localized and brightly fluorescent spots in metaphase spreads and in nuclei. This provided the means of rapidly enumerating the copy number of chromosomes in interphase nuclei. Since interphase FISH can be applied to nuclei of cultured as well as uncultured cells, it soon became clear that this technique had the potential of improving speed of prenatal diagnosis in AF cells, allowing a rapid cytogenetic evaluation of the fetus (Klinger et al., 1992; Zheng et al., 1992; Ried et al., 1992; Ward et al., 1993; Divane et al., 1994; Philip et al., 1994).
As pointed out in previous sections (1.4.1 and 1.4.2), the detection of chromosomal mosaicism in AF cell cultures as well as in CV may pose an interpretation dilemma. Differentiation between pseudomosaicism in AF cultures or local mitotic division errors in CV, and mosaicism requires the evaluation of a large number of cells in the sample, which is often difficult or impossible due to time constraints and a limited amount of metaphases that is suitable for analysis. An elegant and efficient way for differentiation between both situations is the use of FISH (Schwartz et al., 1993; van den Berg et al., 1997). It can rapidly provide information on the presence of a specific chromosome aberration in a large number of cells, since it allows the analysis of metaphases of lesser quality, not suitable for chromosome analysis, as well as interphase cells, non-accessible for karyotyping.
Discrimination between CPM and generalized mosaicism may require follow-up investigations, such as cytogenetic analysis of AF and /or fetal blood cells. These follow-up studies prolonge severely the definite reporting time. Since FISH allows analysis of uncultured cells, it makes a rapid differentiation between confined and generalized mosaicism possible. If the chromosome aberration is found to be confined to the placenta, and if it involves a trisomy, study of the parental origin of both homologues of the involved chromosome pair in AF cells is essential, since CPM may be associated with fetal UPD (Kalousek and Barrett, 1994). Since FISH does not permit differentiation between the paternal and maternal inherited chromosome, other molecular techniques have to be used. Polymerase chain reaction (PCR) amplification of polymorphic microsatellite repeat markers permits the investigation on the parental origin of both homologues of a chromosome. Moreover, it may also allow the identification of the parental origin of an unbalanced chromosome aberration as well as of the extent of a deleted chromosome segment, if relevant FISH probes are not available.

Figure 1.7 Principal of fluorescent in situ hybridization (FISH).



Chapter II

Application of FISH for prenatal cytogenetic diagnosis

1. Aim of the experimental work

Traditional cytogenetic analysis of chorionic villi (CV) and amniotic fluid (AF) samples has a few limitations which were described in section 1.4 of chapter 1. The experimental work, presented in this chapter, was aimed at the solution of some of these limitations, and represented the introduction of the fluorescent in situ hybridization (FISH) technique in our laboratory as an adjunctive prenatal diagnostic tool.

Firstly, we introduced FISH for the *identification* of structural chromosome aberrations, such as marker chromosomes and de-novo unbalanced chromosome abnormalities, which could not be adequately characterized with classical banding techniques (publications I and II), as well as for further characterization of structural chromosome rearrangements, and for detection of subtle familial chromosome aberrations involving small chromosomal segments or chromosomal parts with similar banding patterns (section 3.1).

Secondly, we introduced FISH for the *rapid detection of chromosome aberrations in uncultured AF cells* if a quick result is necessary (section 3.2). This is the case when
(1) fetal anomalies are detected by ultrasound (US) which are suspicious of chromosomal aneuploidy, and gestational age comes close to 24 weeks (termination of pregnancy legally not allowed after 24 weeks) or in case of impending birth (publications III, and IV).
(2) a chromosome aberration is detected in a CV sample which is suspected to be confined to the placenta, but might be present in the fetus as well (discrimination confined placental mosaicism (CPM)/generalized mosaicism)(publication VII).
(3) low-level mosaicism is detected in an initial AF sample, but the number of cell colonies is too small to confirm true mosaicism. In these cases, FISH is applied to uncultured AF cells of a follow-up AF sample for rapidly verifying the presence of mosaicism.
(4) maternal age is $\geq$ 44 years, if triple test results indicate a risk of Down syndrome of $\geq$ 5 %, or in case of twin pregnancies of advanced gestational age ($\pm$ 16 weeks)

Thirdly, interphase FISH was introduced for studying *chromosomal mosaicism* in cultured AF cells, and semi-direct CV slides, especially for differentiation between mosaicism and pseudo-mosaicism or local mitotic division errors, respectively (publication V; section 3.3);

Fourthly, we introduced FISH for the detection of *submicroscopical deletions*, such as at 17p13.3 (type I lissencephaly, Miller-Dieker syndrome) (Ledbetter et al., 1989), 7q11.23 (Williams syndrome) (Lowery et al., 1995), and 22q11 (CATCH22) (Desmaze et al., 1993). In these cases the couple already has a child with such a deletion or with the associated syndrome, or one of the parents is a carrier, or US investigations are suspicious for such a deletion (publication VI; section 3.4).

After a short description of the FISH procedure, as used in our laboratory (section 2), the possibilities and limitations of FISH as a diagnostic tool will be evaluated for each of the four indications separately, based on our experience during four years (1993-1996) (section3).

2. Materials and methods of the FISH technique

2.1 Slide preparation and pretreatment

The slides that we use for FISH can be the same slides that are used for chromosome analysis. The preparation of these chromosome slides has been previously described (sections 1.1 and 1.2). Slides of uncultured amniotic fluid cells (AF cells) are specifically prepared for FISH analysis, as described in publication III, with a small, but essential modification since 1995: after preparation of the slides, the cells are swollen by a short immersion in 70 % formamide in 2× standard saline citrate (SSC), followed by a 1 min wash in phosphate buffered saline (PBS), which improved hybridization efficiency and signal intensity (J.G. Wauters, University of Antwerp-U.I.A., Antwerpen, personal communication; Fidlerová et al., 1994).

Before hybridization, the metaphase and interphase cells in *unstained preparations* need pretreatment with RNase and a protease (pepsin) to improve accessibility of the target DNA in chromosomes and nuclei for the probe DNA (pubications I, and IV). *Trypsin-Giemsa stained slides* are destained prior to hybridization, according to Klever et al. (1991), and do not need this pretreatment.

2.2 DNA probes and labelling

The probes used in the experimental work are of three general categories: *repeat-sequence probes, whole chromosome probes*, and *locus-specific probes*.

-The *repeat-sequence probes* we use are almost all chromosome specific centromere probes, detecting DNA sequences that are tandemly repeated several hundred to several thousand times in the (peri-)centromeric regions of the chromosomes. Most of these sequences are in the alpha-satellite, or satellite III families.

-The *whole chromosome probes* are chromosome specific composite probes whose individual elements have sequence homology at many sites along one specific chromosome, enabling the fluorescent staining of an entire chromosome. These probes are referred to as "painting" probes or WCPs (whole chromosome paints), since they are used to paint whole chromosomes. These probes contain families of repeat sequences (Alu and Kpn) that are shared by other chromosomes, resulting in aspecific hybridization signals. To achieve specific staining these sequences are prevented from hybridizing by a preannealing step with unlabelled blocking DNA [total human genomic DNA or Cot-1 DNA (Boerhinger Mannheim GmbH, Mannheim, Germany)] before adding the probe-DNA mixture to the target-DNA. This method is referred to as chromosome in situ suppression (CISS) hybridization (Lichter et al., 1988; Pinkel et al., 1988).

-The *locus-specific probes* are typically single copy probes homologeous to specific targets

ranging in size from 15 Kb to > 500 Kb. According to the insert length, different vectors can be used, with cosmids, able to harbour 35 to 45 Kb of foreign DNA, being the most popular for FISH. Preannealing with blocking DNA is necessary to prevent hybridization of repeat sequences in these probes. They produce small signals which are sometimes difficult to detect in uncultured cells. By using cosmid contigs (overlapping cosmids) or cosmid cocktails (non-overlapping cosmids) signal intensity may be improved.

Most of the repeat-sequence and cosmid probes that we use, are kind gifts from their creators. The painting probes that we used until 1994, were the Bluescribe plasmid libraries from Dr. J.W. Gray (Laurence Livermore National Laboratory, Livermore, CA, USA)(Collins et al., 1991). Nowadays we use commercially available painting probes (Cambio Ltd., Cambridge, UK). The probes with their chromosomal localization, and their reference or source are listed in table 2.1.

Table 2.1 Probes used for FISH, their chromosomal localization, and reference or source

Probe	Chromosomal localization	Reference or source
A. Repeat-sequence probes		
pUC1.77	1qhet	Cooke and Hindley (1979)
pα3.5	3 cen	Waye and Willard (1989)
pG-A16	1, 5, 19 cen	Hulsebos et al. (1988)
pα7t1	7 cen	Waye et al. (1987a)
αp8	8 cen	Donlon et al. (1986)
pHuR98	9 qhet	Moyzis et al. (1987)
pH8	10 cen	Devilee et al. (1988)
pLC11A	11 cen	Waye et al. (1987b)
pα12H8	12 cen	Looijenga et al. (1990)
D12Z3	12 cen	Oncor, Gaithersburg, MD, USA
L1.26	13 and 21 cen	Devilee et al. (1986)
c237	14 and 22 cen	C. Meijers, Institute of Paediatric Surgery, Erasmus University, Rotterdam
pTRA-20	15 cen	Choo et al. (1990)
pTRA-25	15 cen	Choo et al. (1990)
D15Z1	15psatIII[1]	Higgins et al. (1985)
pHuR195	16qhet	Moyzis et al. (1987)
p17H8	17cen	Waye and Willard (1986)

Table 2.1 continued

Probe	Chromosomal localization	Reference or source
L1.84	18 cen	Devilee et al. (1986)
p3.4	20 cen	Waye and Willard, (1989)
D20Z1	20 cen	Oncor, Gaithersburg, MD, USA
p190.22	22 cen	Rocchi et al. (1994)
pBamX5	X cen	Willard et al. (1983)
pDP97	Y cen	Wolfe et al. (1985)
RPN1305X	Yq12	Lau (1985)
CEPX/CEPY(satIII)	Xcen/Yq12	Vysis, Downers Grove, IL, USA
r521	NORs[2]	B. Bakker, Dpt. of Clinical Genetics, University Hospital Leiden
B. Painting probes		
WCP[3] 1-22, X, Y	1-22, X, Y	Cambio Ltd., Cambridge, UK
M28	12p	Zhang et al. (1989)
C. Locus-specific probes		
WSCR (Elastin Williams Syndrome chromosomal region)	7q11.23	Oncor, Gaithersburg, MD, USA
tel 7q	7q36-qter	Oncor, Gaithersburg, MD, USA
cFK2,6	13q14-q21	F. Kooy, Medische Genetica Groningen
cW23, and cW9D	16p13.3	European Chromosome 16 Tuberous Sclerosis Consortium (1993)
c179-2,4,9	17p13.3	Ledbetter et al. (1992b)
tel 20p	20p13-pter	Oncor, Gaithersburg, MD, USA
CB21c1	21q11	Van Opstal et al. (1995)
cCMP21.a	21q22.2-q22.3	Zheng et al. (1992)
M51	22q11	Mulder et al. (1995)
cos39	22q11	Aubry et al. (1993)
sc11.1	22q11	Halford et al. (1993)
sc4.1	22q11	Carey et al. (1992)
58B12	22q11.2	Lekanne Deprez et al. (1995
M69	22q13	Mulder et al. (1995)

Table 2.1 continued

Probe	Chromosomal localization	Reference or source
Arcos2,4	Xq11-q12	J. Trapman, Dept. Of Pathology, Erasmus University, Rotterdam
cAL24	Xp21.2	Blonden et al. (1989)
cpq23.1	Xpter and Ypter	L. Blonden, Dpt. Human Genetics
c7B2	Xq28	M. D'Urso, International Institute of Genetics and Biophysics, Naples, Italy
pDP105	Yp/(Yq)[4]	Disteche et al. (1986)
LOR2.6.4	Yq11	Ma et al. (1992)
p49f	Yq11.2	Ngo et al. (1986)

Note.-[1] satIII= satellite III DNA in short arm of chromosome 15;[2] NOR= nucleolus organizer region in p-arm of all acrocentric chromosomes; [3]WCP= whole chromosome paint;[4] Signals on Yp (interval 3) and Yq (interval 6), with the latter mostly not observed with FISH

The ultrasound probe set

We used a specific set of probes for detection of the most common aneuploidies (trisomy 13, 18, 21, triploidy, sex-chromosomal aneuploidies) in uncultured AF cells of pregnancies complicated by fetal anomalies (the so-called *US (ultrasound) probe set*)(table 2.2) (see section 3.2.1).

Table 2.2 Ultrasound (US) probe set used for FISH on uncultured amniocytes in cases of ultrasound abnormalities during the years 1993-1996

Probe (chromosome) /Year	pBamX5 (X)	RPN1305X (Y)	CEPX/ CEPY (X/Y)	L1.26 (13/21)	cFK2,6 (13)	L1.84 (18)	CB21c1 (21)	cCMP21.a (21)
1993	*	*		*		*	*	
1994	*	*		*		*	*	
end of 1994	*	*				*	*	*
1995	*	*				*	*	*
end of 1995	*	*			*	*	*	*
1996	*	*			*	*	*	*
july 1996			*		*	*	*	

Note.- see table 2.1 for localization and reference of the probes

The composition of this set changed during the years, as some probes, after a while, turned out to be unreliable for interphase cytogenetics. Two probes were used during the whole 4-year period: CB21c1 and L1.84, for chromosome 21 and chromosome 18 detection, respectively. We investigated extensively the utility of CB21c1 on a series of uncultured AF and blood samples, since a chromosome 21 specific probe was not yet available at the time we started interphase cytogenetics (see appendix publication IV). L1.84 hybridizes to an alpha satellite repeat sequence in the centromeric region of chromosome 18 (Devilee et al., 1986). Centromere probes are often applied for interphase cytogenetics as they produce bright signals. However, the copy number of the repeat sequence that they detect is a highly polymorphic trait and sometimes appears to be too small to produce any signal, which may potentially lead to a false negative result. However, we only had some minor problems with L1.84 (see section 3.2.1).
One of the changes involved substitution of L1.26 (13/ 21 centromere probe), which we initially used for chromosome 13 detection, by a chromosome 13 specific probe (cFK2,6) (a kind gift of Dr. Frank Kooy, Medische Genetica Groningen). The reason for this substitution was the recurrent finding of false positive and false negative FISH results, due to polymorphisms in the centromeric regions of the acrocentric chromosomes (see section 3.2.1; Verma and Luke, 1992; Mizunoe and Young, 1992; Cacheux et al., 1994; Verlinsky et al., 1995). A separate hybridization of X centromere and Y heterochromatine probe was replaced by a simultaneous dual-color hybridization of X and Y probe [CEP (chromosome enumeration probe) X/CEPY (satIII) probe mixture] (VYSIS, Downers Grove, IL, USA). This system allows an accurate identification of maternal cells in the AF sample, as well as of an exceptional 45,X/46,XY mosaic (see section 3.2.1, and 3.2.2). During one and a half year, we used two probes for chromosome 21 detection [CB21c1, hybridizing to 21q11 (see appendix publication IV), and cCMP21.a, mapping to the Down specific region (Zheng et al., 1992)], in order to minimize the risk of a misdiagnosis in case of an exceptional unbalanced reciprocal translocation of chromosome 21 with breakpoints distal to 21q11. However, as we experienced progressive technical problems with cCMP21.a, we precluded this probe from further FISH investigations.

Probes are labelled by nick translation using either biotin-11-dUTP (Gibco BRL, Gaithersburg, MD, USA), biotin-16-dUTP or digoxigenin (DIG)-11-dUTP (Boerhinger Mannheim GmbH, Mannheim, Germany). The commercial probes are purchased already labelled with either biotin or DIG, for indirect detection with fluorescently labelled antibodies, or with a fluorochrome for direct detection.

2.3 Probe hybridization and detection

The commercial probes are all processed according to the manufacturer's instructions. For the non-commercial probes we use the hybridization protocol described in publication I (whole chromosome plasmid libraries, centromere probes), and publication IV (cosmid probes).
Fluorescent detection of the probes depends on probe labeling: detection of biotin labelled probes is described in publications I and IV. DIG labelled probes are detected with one layer of anti-DIG-FITC (1:40 to 1:100)(Boehringer Mannheim GmbH, Mannheim, Germany) in

0,5 % blocking milk in 0,1 M Tris/0,15 M NaCl/0,05 % Tween-20.
Mounting of the slides is described in publication I. The concentration of the fluorescent counterstains propidium iodide (PI) and 4',6' Diamidino-2-Phenylindole (DAPI) varies according to cell type and probe (0,05 µg-0,5 µg/ml PI and 0,015 µg-0,15 µg/ml DAPI)

2.4 Evaluation of the slides

Slides are examined under a Leica Aristoplan epifluorescence equipped microscope and, images are captured with the Genetiscan ProbeMaster system (Perceptive Scientific International Ltd. (PSI), Chester, UK) including a Xybion cooled CCD 24-bit colour camera. According to our own standards, metaphase FISH analysis requires the examination of 10 cells. Interphase FISH analysis involves the counting of at least 50 nuclei of uncultured cells, and 200 nuclei of cultured cells (including CV cells in semi-direct preparations). We use the interphase scoring criteria as proposed by Hopman et al. (1988):

(1) nuclei should not overlap
(2) cells should not be asymmetrically covered by cytoplasm
(3) minor hybridization spots should not be counted
(4) fluorescent spots or patches of fluorescence may only be included when the signals are completely separated from each other
(5) spots in a paired arrangement (split spots), close to each other, are counted as one

For interpretation of interphase FISH results, a statistical approach is necessary. Therefore, signal distribution profiles of the most commonly used probes in our laboratory were generated on a series of CV and uncultured AF samples with normal karyotypes. Statistical analysis of these data was used to determine the 95 % confidence interval of the one-sided upper reference limit (97,5 %) for the proportion of cells with an abnormal number of signals for each of the probes and for each tissue, according to Lomax et al. (1994). This cut-off level is used to discriminate the normal state from the lowest detectable level of monosomy and trisomy mosaicism (table 2.3). For probes that are only occasionally used, a normal control sample, simultaneously processed with the test sample, is used for interpretation of the results.

Table 2.3 Statistical analysis of normal diploid controls: one-sided upper reference limit (97,5 %) and corresponding 95 % confidence interval (CI) for the proportion of uncultured AF and CV cells with an abnormal signal number

Probe	N controls		N signals	One-sided upper reference (97,5 %) limit (95 % CI)	
	AF	CV		AF	CV
pα3.5	12	6	3	3,6 (2,2-5,0)	4,9 (2,7-7,0)
pα7t1	18	11	3	4,6 (3,2-5,9)	4,0 (2,5-5,6)
pHuR98	16	7	3	4,9 (3,5-6,3)	2,2 (1,2-3,3)
pLC11A	6	5	3	0 (0-0)	1,8 (0,9-2,7)
pα12H8	-	5	3	-	2,5 (1,1-4,0)
cFK2,6	67	5	3	5,0 (4,3-5,8)	6,0 (3,6-8,4)
L1.26	50	-	5	18,4 (15,3-21,6)	-
pHuR195	14	16	3	4,9 (3,2-6,5)	6,6 (4,6-8,5)
L1.84	80	12	3	3,6 (3,0-4,0)	2,7 (2,0-3,5)
CB21c1	65	5	3	8,7 (7,5-10)	3,3 (1,1-5,4)
M51	5	8	1	17,9 (9,5-26,3)	10,6 (7,5-13,7)
			3	11,7 (4,8-18,6)	5,7 (3,7-7,8)
pBamX5	♀: 25	10	1	13,6 (10,8-16,4)	9,3 (6,5-12,1)
			3	4,0 (2,8-5,1)	2,9 (1,8-4,1)
	♂: 15	5	2	4,1 (3,0-5,2)	7,6 (3,6-11,6)
CEPX/CEPY(satIII)	♀: 11	5	1	6,3 (3,9-8,6)	17,4 (9,7-25,1)
			3	0 (0-0)	2,6 (1,3-3,9)
	♂: 13	8	1	4,6 (3,0-6,1)	3,3 (0,8-5,8)
			3	0 (0-0)	0 (0-0)

3. Results and discussion

3.1 FISH for identification of structural chromosome aberrations

Appendix publication I

Cytogenetic problems in PD arise when de novo unbalanced structural chromosome aberrations or marker chromosomes, i.e., extra chromosomes of unknown origin, are present. Conventional banding techniques, such as Ag-NOR (nucleolus organizer region) banding, C (centromere) banding, and DA-DAPI (Dystamycine- 4',6'Diamidino-2-Phenylindole) stai-ning are important (Sachs et al., 1987), but can not always give a definite diagnosis. FISH has made further identification of these chromosome abnormalities possible, as shown for a few cases in appendix publication I. These identification studies allowed a more detailed counselling of the future parents, and a better understanding of the fetal pathology.

Between 1993 and the end of 1996, we used the FISH technique for identification of extra chromosomal material in the karyotype (3.1.1). Additionally, FISH showed to be a useful tool for characterization of the breakpoints of a structural chromosome rearrangement, and for confirmation of an uncertain cytogenetic result (because of poor chromosome quality) (3.1.2), and for the detection of subtle familial chromosome aberrations in semi-direct CV preparations (3.1.3).

3.1.1 Identification of extra chromosomal material of unknown origin (tables 2.4 and 2.5)

Extra chromosomal material of unknown origin may be the result of an unbalanced structural chromosome aberration (translocation, duplication, insertion) or a marker chromosome. The latter pose a problem in prenatal counselling procedures, as they present a heterogeneous group of chromosomes with varying phenotypic consequences, depending on their chromosomal origin: they may cause severe anomalies, but also may have no phenotypic effect on the carrier. Before the introduction of FISH, the chromosomal origin of most markers remained obscure and the risk of phenotypic abnormality has been estimated in relation to size, staining properties, level of mosaicism, and familial occurence (Buckton et al., 1985; Sachs et al., 1987; Warburton, 1991). The risk of abnormal development in familial cases was predicted to be very small (Gardner and Sutherland, 1989; Sachs et al., 1987), whereas the risk in de novo cases has been estimated to be 13 % (Warburton, 1991).

The introduction of FISH has provided the possibility of resolving the heterogeneity of this group of chromosomes (Callen et al., 1990a and b, 1991, 1992; Crolla et al., 1992; Blennow et al., 1993, 1994; Plattner et al., 1993; Leana-Cox et al., 1994; Brøndum-Nielsen and Mikkelsen, 1995). These studies led to the identification of the most commonly observed marker chromosomes in PD [inv dup (15), i(18p), i(12p), and del(22q)] and their associated phenotypes (Robinson et al., 1993; Callen et al., 1990a; Schinzel, 1991; McDermid et al., 1986), facilitating genetic counselling of the prospective parents.

Table 2.4 FISH for identification of marker chromosomes

Case	Probes[1]	FISH result
1	r521, αp8, pHuR98, pH8, pLC11A, pα12H8, pHuR195, p17H8, L1.84, pG-A16, p3.4	ISH der(16)(pHuR195+)
2	US probe set[2] (pBamX5, RPN1305X, L1.26, L1.84, CB21c1), WCP12, M28, D12Z3, pα12H8	ISH i(12)(q10)(WCP12+,M28+,D12Z3-,pα12H8-)[17]/ del(12)(q10)(WCP12+,M28+,D12Z3-,pα12H8-)[6] [3]
3	US probe set (pBamX5, RPN1305X, L1.84, CB21c1), M51, sc4.1, L1.26	ISH der(13 or 21)(L1.26+)
4	r521, L1.26, c237, pTRA-20, P190.22	ISH mar(r521+)

Note.- [1] See table 2.1 for chromosomal localization and references of the probes; [2] US probe set= set of probes used to screen uncultured AF cells in cases of ultrasound (US) abnormalities (see table 2.2); [3] see Los et al. (1995)

Table 2.5 FISH for identification of an extra chromosomal segment

Case	Cytogenetic result	Probes[1]	FISH result
1, 2	21ps+	r521	ISH 21ps+ (r521++)
3, 4, 5, 6	22ps+	r521	ISH 22ps+ (r521++)
7	13ps+	r521	ISH 13ps+ (r521++)
8	15ps+	r521	ISH 15ps+ (r521++)
9	add (7q)	WCP7, 7qter	ISH dup(7)(WCP7+,7qter×1)
10	add (1q)	WCP1	ISH dup(1)(WCP1+)
11	add (8p)	WCP7, WCP8, WCP18	ISH der(8) t(7;8)(p13;p23)(WCP8+,WCP7+)
12	add (16q)	WCP16, WCP11, WCP12	ISH der(16) t(11 or12;16)(WCP16+, mix (WCP11,WCP12)+)
13	add (Xp)	WCPX, WCPY, cpq23.1, cAL24, pBamX5, c7B2	ISH inv(X)(p22.3q26)(WCPX+,cpq23.1st,cAL24mv,pBamX5mv,c7B2st)

Note.- [1] see table 2.1 for chromosomal localization and references of the probes

Figure 2.1 21ps+. A) partial karyotype of cultured AF cells. B) FISH signals on all acrocentric chromosomes, including 21ps+ (arrow), after hybridization with r521, detecting NORs in the p-arm of the acrocentric chromosomes.

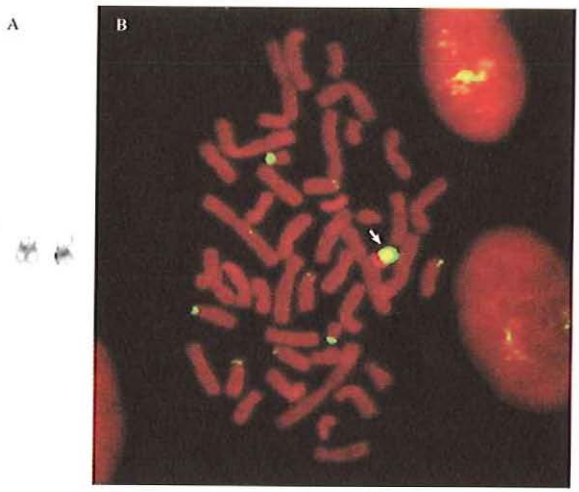
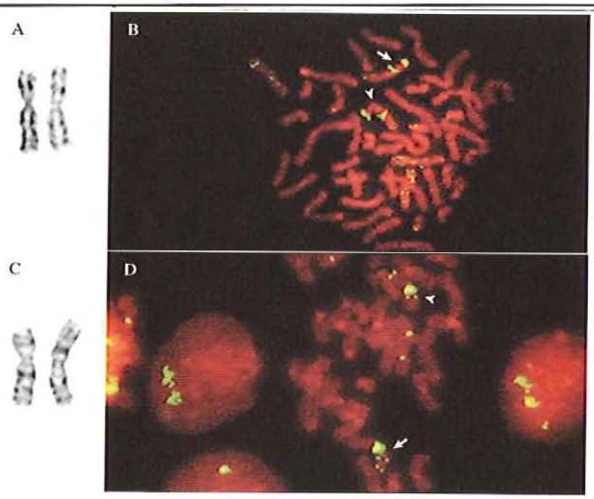


Figure 2.2 inv(X)(p22.3q26). A) partial karyotype of CV cells, and C) of AF cells, showing inv(X) on the right. B) metaphase spread of CV with FISH signals on normal chromosome X (arrowhead) and inv(X) (arrow) after hybridization with WCPY: two aspecific signals are seen on different chromosome arms (Xpter and Xq13) of the normal , and on the same chromosome arm of the abnormal X chromosome, indicative for an inversion. D) metaphase spread of AF cells: FISH signals on normal (arrowhead) and abnormal (arrow) X chromosome after simultaneous hybridization with pBamX5 (X centomere), c7B2 (Xq28), and cAL24 (Xp21.2), showing that c7B2 and cAL24 hybridize to the same chromosome arm of the abnormal X chromosome, confirming an inversion in the X chromosome.



However, identification of marker chromosomes using FISH with chromosome specific probes, as we did, has limitations. Of the ten cases of marker chromosomes that were encountered during four years, FISH could only elucidate the chromosomal origin in four cases (table 2.4). In dayly practice, we were confronted with two problems. Firstly, the number of available chromosome slides and the time necessary to perform FISH studies showed to be the main limitating factors. In most of the larger prenatal studies, marker chromosomes were identified retrospectively, without time constraints and without a limitation of the number of available chromosome preparations (Blennow et al., 1994; Brøndum-Nielsen and Mikkelsen, 1995). Secondly, whenever the marker showed not to be one of the four most common markers, the counselling of the parents still remained uncertain, and based on the classical characteristics such as size, staining properties, mosaicism, and familial occurence, than on the FISH result. For instance, the marker in case one (table 2.4) was found to be positive with the 16 heterochromatin probe, but the presence of any euchromatine could not be excluded. Therefore, the parents decided to terminate the pregnancy on the basis of this uncertainty. The probe that showed to be very useful for counselling procedures was r521, detecting nucleolus organizer regions (NORs) in the short arm of all acrocentric chromosomes. It demonstrated whether the marker was satellited or not, irrespective of the active or inactive state of the satellites in contrast with the Ag-NOR banding technique, with satellited markers having a better prognosis than non-satellited markers (Warburton, 1991).
Similarly, FISH with r521 showed the presence of a normal polymorphism in eight cases showing extra chromosomal material in the short arm of one of the acrocentric chromosomes (table 2.5) (figure 2.1). On the basis of these results, the prospective parents could be reassured. The remaining five cases of table 2.5 all displayed an unbalanced structural chromosome aberration, indicative for a duplication or a translocation. Our identification strategy involved FISH with the relevant whole chromosome paint (WCP), and if a duplication was excluded, WCPs of other chromosomes were applied, depending on the banding pattern of the extra chromosomal part. The advantages of FISH are clearly illustrated in case 15. Chromosome analysis of CV semi-direct preparations revealed an add(Xp) chromosome (figure 2.2A). FISH with WCPX resulted in a fluorescent staining of the whole abnormal X chromosome, initially indicative for a duplication. Further hybridization with WCPY indicated the presence of an inversion in chromosome X; two aspecific WCPY hybridization signals were seen on the normal X chromosome at Xpter and Xq13, as described by the probe supplier (Cambio Ltd., Cambridge, UK), but the abnormal X chromosome showed both aspecific signals on the same chromosome arm (figure 2.2B). FISH with locus specific X probes (cpq23.1, cAL24, pBamX5, and c7B2), applied to cultures of a subsequent AF sample, confirmed an inversion (X)(p22.3q26)) (figure 2.2C and D).

Recently, new FISH techniques have been developed that allow a more efficient and accurate identification of any extra chromosomal material in the karyotype. Firstly, the *micro-FISH* technique, which involves the microdissection of one or a few markers, followed by an amplification of the dissected fragments by a degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), and hybridization of the PCR product to normal metaphases (Viersbach et al., 1994; Müller-Navia et al., 1995; Engelen et al., 1996). Another approach combines chromosome isolation by fluorescence activated cell sorting (FACS) with the DOP-PCR technique (Blennow et al., 1992; Carter et al., 1992). Secondly, a new technique,

Table 2.6 FISH for confirmation and further characterization of a cytogenetically detected structural chromosome rearrangement

Case	Cytogenetic result	Probes[1]	FISH result
1	i(Xq)	pBamX5	ISH idic(X)(p10)(pBamX5×2)
2	i(X)	WCPX, pBamX5, cAL24, Arcos2.4	ISH idicX(p11.4)(WCPX+,cAL24-,pBamX5×2,Arcos2,4×2)
3	i(Yp) and del(Y)	pDP105, pDP97, LOR2.6.4, p49f, RPN1305X	ISH idic(Y)(q11)(pDP105×2,pDP97×2,LOR2.6.4-, p49f-,RPN1305X-)[14]/del(Y)(pDP105×1,pDP97×1,LOR2,6,4-, p49f-,RPN1305X-)[16][2]
4	i(1)(q10)	WCP1	ISH i(1)(q10)(WCP1+)
5	idic(9)	WCP9, pHuR98	ISH idic(9)(q12)(WCP9+, pHuR98+)
6	inv(9)	WCP 9, pHuR98	ISH inv(9)(p24q12)(WCP9+,pHuR98×2)
7	inv(Y)	pDP105, LOR2.6.4, RPN1305X	ISH inv(Y)(p11.2q11.2)(pDP105st,LOR2.6.4mv,RPN1305Xst)
8	t(1;2)	WCP1, WCP2, pUC1.77	ISH t(1;2)(p11.2;q11.2)(WCP2+,pUC1.77+;pUC1.77-,WCP1+)
9	t(1;13)	WCP1, r521	ISH t(1;13)(q31;p12)(WCP1+,r521+;WCP1+,r521+)
10	t(5;13)	WCP5, WCP13	ISH t(5;13)(q13;q12)(WCP13+;WCP5+)
11	inv(9)(p11.2q13)t(9;10) (p10;q10)	WCP9, WCP10, pHuR98, pH8	ISH inv(9)(p11.2q13)t(9;10)(q21.32;p11.23) (pHuR98+,WCP10+;WCP9+,pH8+)
12	t(11;22)(q25;q13.1)	WCP11, WCP22, P190.22, M51, 58B12, M69	ISH t(11;22)(q23.3;q11.2)(WCP22+,M69+,58B12+; WCP11+,P190.22+M51+)
13	der(21;21)(q10;q10)	WCP21, CB21c1, cCMP21.a	ISH der(21;21)(q10;q10)(WCP21+,CB21c1×2, cCMP21.a×2)
14	der(16p)	WCP16, WCP 9	ISH t(9;16)(q32;p13.1)(WCP16+;WCP9+)
15	del(7)	WCP7	ISH del(7)(WCP7+)
16	del(Y)	pDP105, pDP97, LOR2.6.4, p49f, RPN1305X	ISH del(Y)(q11.2)(pDP105+, pDP97+, LOR2.6.4+, p49f-, RPN1305X-)
17	r(18)	WCP18, L1.84	ISH r(18)(WCP18+, L1.84+)[3]
18	monosomy 21	WCP21	ISH 21(WCP21×1)[4]

Note.- [1] see table 2.1 for chromosomal localization and references of the probes; [2] see in 't Veld et al. (1995); [3] see appendix publication II; [4] see Joosten et al. (1997).

developed in the field of cancer cytogenetics, and called *spectral karyotyping or SKY*, involves the hybridization of 24 fluorescently labelled chromosome painting probes allowing the simultaneous and differential colour display of all human chromosomes (Schröck et al., 1996; Veldman et al., 1997). Thirdly, another revolutionary technique is *comparative genomic hybridization (CGH)* (Kallioniemi et al., 1992), which allows to screen the entire genome for genetic losses and gains (in contrast to conventional molecular techniques). It has already been succesfully applied for cytogenetic analysis of tumors (Steenman et al., 1997). CGH involves the isolation of DNA from a test sample and a normal control sample, followed by a simultaneous hybridization of both differentially labelled genomic complements with normal metaphases. The analysis of fluorescence intensity ratios along the target chromosomes by a digital image analysis system reflects the ratio between tested and reference genomes, and can detect gains and losses of chromosome material in the test sample. This technology is especially useful if metaphases are not available or are difficult to obtain

3.1.2 Determination of the breakpoints of a rearranged chromosome and rapid confirmation of an uncertain cytogenetic result (table 2.6)

During four years, we used FISH for identification of the breakpoints of an isochromosome (cases 1-3, 5), an inversion (cases 6, and 7), a translocation (cases 8-12), or a deletion (case 16). In cases 11 and 12, the translocation breakpoints showed to be different from what was suspected on the basis of cytogenetic analysis. FISH results in case 9, involving a t(1;13)(q31;p12), are shown in figure 2.3. In cases 4, 13, and 15, FISH was used for verification of the cytogenetic result, which was uncertain due to the poor quality of the metaphases. The benefits of FISH are clearly illustrated by case 14, in which chromosome analysis was only suggestive of an abnormal chromosome 16, with all other chromosomes in the karyotype showing a normal banding pattern. FISH revealed the presence of a balanced translocation of chromosomes 9 and 16, with breakpoints in 9q32, and 16p13.1. The derivative chromosome 9 was not distinguishable from the normal chromosome 9 on the basis of banding pattern alone (figure 2.4). In case 17, FISH confirmed the chromosome 18 origin of a ring chromosome, which is described and extensively discussed in appendix publication II. FISH studies performed in the rare case of monosomy 21 (case 18) showed that pure monosomy 21 does exist indeed (Joosten et al., 1997).
In most of these cases, FISH provided us with a better understanding of the banding pattern of the abnormal chromosome(s), and, therefore, it improved genetic counselling. In cases of poor chromosome quality, FISH showed to be a powerful tool for rapid verification of what was suspected on the basis of traditional chromosome analysis.

3.1.3 Detection of subtle familial chromosome rearrangements (table 2.7)

The analysis of subtle familial chromosome rearrangements poses a problem in PD. Carriers of such a chromosome aberration are at high risk for unbalanced offspring, making an early PD in CVS important. However, these chromosome abnormalities are difficult to detect with chromosome banding techniques, due to the small size or similar banding pattern of the exchanged chromosome fragments, especially in metaphases of lesser quality as those directly prepared from CVS. During the time period 1993-1996, we sucessfully investigated seven cases of subtle familial chromosome rearrangments in semi-direct CV preparations using

Figure 2.3 t(1;13)(q31;p12). A) partial karyotype of AF cells. B) FISH signals on der(1) (arrowhead) and der(13) (arrow) after hybridization with r521, detecting NORs in p-arm of the acrocentric chromosomes, revealing the breakpoint in 13p12.

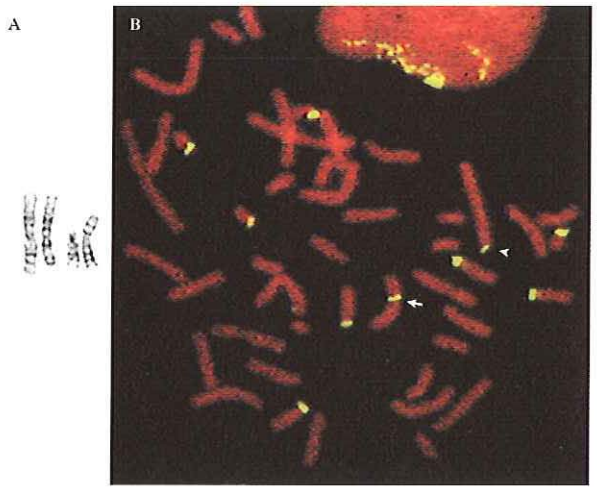
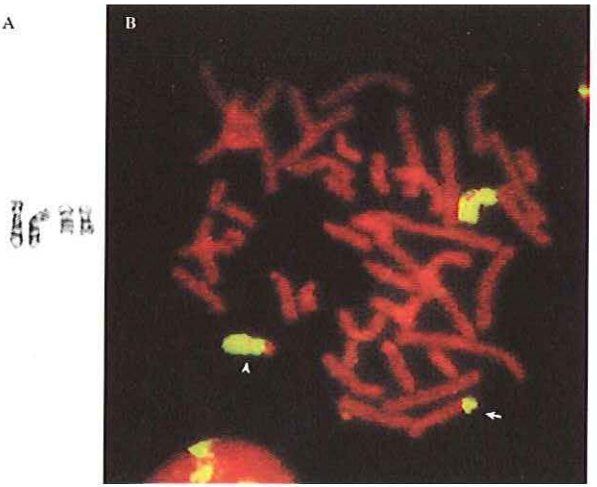


Figure 2.4 t(9;16)(q32;p13.1). A) partial karyotype of AF cells. B) FISH signals on normal chromosome 16, der(16) (arrowhead), and der(9) (arrow) after hybridization with WCP16, showing a balanced t(9;16).



FISH (table 2.7). In three cases (cases 3, 5, and 7), FISH showed the presence of an unbalanced karyotype, resulting in a partial trisomy 12, 11, and 16, respectively. FISH results in case 3 are shown in figure 2.5. In the remaining four cases, FISH results were indicative for a normal karyotype (cases 1 and 4), and for a balanced translocation (cases 2 and 6).

Table 2.7 FISH for detection of subtle familial chromosome rearrangements

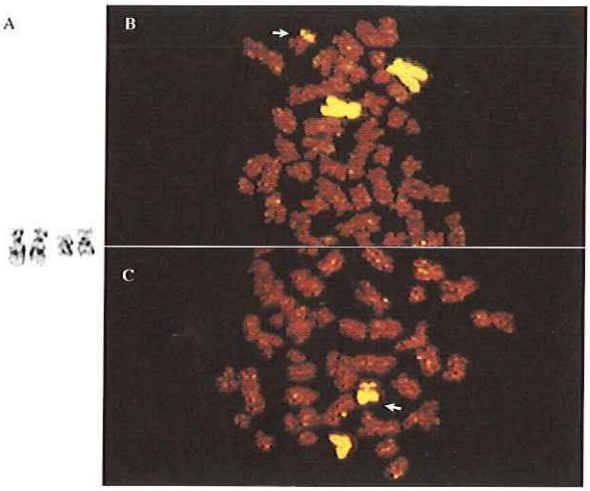
Case	Familial chromosome aberration	Probes[1]	FISH result
1	inv ins(18;5) (q21.3;p14p13.1)pat	WCP5, WCP18	ISH 5(WCP5+)18(WCP18+)
2	t(1;22)(q24.1;q13.1)mat	WCP1, WCP22	ISH t(1;22)(q24.1;q13.1)mat(WCP22+;WCP1+)
3	ins(18;12)(p11.3;q13q15)mat	WCP12, WCP18	ISH der(18)ins(18;12)(p11.3;q13q15)mat(WCP12+)
4	t(8;11)(q12p23;q21)mat	WCP8, WCP11	ISH 8(WCP8+)11(WCP11+)
5	inv ins(5;11)(p14;q24q14)mat	WCP5, WCP11	ISH der(5)inv ins(5;11)(p14;q24q14)mat(WCP11+)
6	t(12;18)(p12.3;q21.2)pat	WCP12, WCP18	ISH t(12;18)(p12.3;q21.2)pat(WCP18+;WCP12+)
7	t(16;18)(p13.3;p11.23)mat	WCP16, WCP18	ISH der(18)t(16;18)(p13.3;p11.23)mat(WCP16+)

Note.- [1] See table 2.1 for chromosomal localization and references of the probes

In all these cases, FISH enabled a rapid PD in first trimester CV semi-direct slides, despite the poor quality of these metaphases, allowing a pregnancy termination in the first trimester of pregnancy in case of an unbalanced karyotype (Speleman et al., 1992; Mangelschots et al., 1992; Fuster et al., 1997). Hybridization of the relevant WCPs to metaphases of the carrier of the structural rearrangement before PD, especially when small chromosomal fragments are involved, showed to be essential in terms of testing whether FISH is able to visualize the involved chromosome aberration.

FISH may sometimes not be possible if relevant probes are not available. In such cases, polymerase chain reaction (PCR) amplification of polymorphic microsatellite repeats (Weber and May, 1989) may be a powerful tool for further characterization of an unbalanced structural chromosome aberration, as shown in appendix publication II. A de novo ring chromosome 18 [r(18)] was detected in AF cells of a 39-year-old pregnant woman. Ultrasound (US) investigations showed a slightly abnormal facial profile. FISH with WCP18 and 18 centromere probe confirmed the chromosome 18 origin of the ring chromosome, but could not reveal any deletions of 18p or 18q material in r(18).

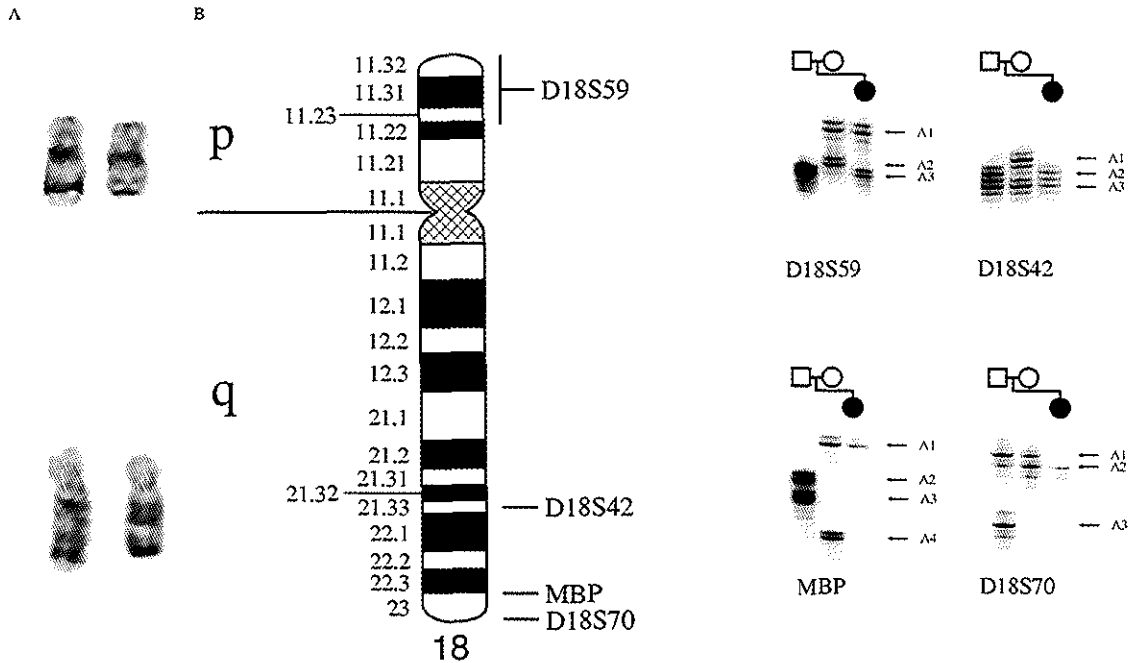
Figure 2.5 ins (18;12)(p11.3;q13q15). A) partial karyotype of CV. B) FISH signals on normal chromosomes 12 and der(18) (arrow) after hybridization with WCP12, and C) FISH signals on normal chromosome 18 and der(18) (arrow) after hybridization w ith WCP18, both showing an unbalanced ins(18;12), resulting in a partial trisomy 12.



Subtelomeric probes were not yet available, and, therefore, we performed PCR analysis of various microsatellite markers on chromosome 18 to establish potential deletions and determine the parent of origin.These DNA investigations indicated that r(18) was of paternal origin and displayed a small 18p deletion of undetermined size, together with a large 18q deletion, at least del(18)(q21.33). These DNA data were important for genetic counselling, as well as for getting more insight into the relationship between genotype and phenotype.
Another example is shown in figure 2.6. Amniocentesis was performed at 20 weeks of gestation because of abnormal maternal serum serum markers. Chromosome analysis was suspicious for a terminal deletion of 18q. DNA studies were performed in order to unambiguously confirm the subtle cytogenetic result. PCR products of some microsatellite repeats on chromosome 18 (D18S59, D18S42, MBP, and D18S70) all showed an informative pattern, and revealed a paternal deletion of at least 18q23, on the basis of which the parents decided to terminate the pregnancy.

In conclusion, PCR amplification of DNA polymorphisms may sometimes be a useful adjunctive tool for further characterization of de novo subtle structural chromosome rearrangements, if cytogenetic results remain undefined and relevant FISH probes are not available. Today, we would prefer to use FISH with chromosome specific subtelomeric probes, although this would not elucidate the parent of origin.

Figure 2.6 Cytogenetic and DNA analysis of del(18)(q23). A) partial karyotype of AF cells. B) ideogram of chromosome 18 with localization of the tested microsatellite repeats. PCR analysis of D18S59, D18S42, MBP, and D18S70 shows absence of a paternal allele at loci MBP, and D18S70 in AF cells, indicating a deletion of at least 18q22.3



3.2 Interphase FISH for the rapid detection of chromosome aberrations in uncultured AF cells

3.2.1 Ultrasound abnormalities

In appendix publication III, we describe our first succesful investigations of uncultured AF cells from 20 high-risk pregnancies by using FISH with a chromosome X, Y, 18, and 21 specific probe. Within 24 hours after sampling, a triploidy was detected in one sample, showing that FISH is a valuable additional tool for PD, specifically for pregnancies at high genetic risk, in which a quick result is important.

Between 1993 and the end of 1996 we received a total of 622 AF samples for karyotyping because of ultrasound (US) abnormalities, and FISH was applied in 196 cases, which were selected on the basis of fetal anomalies, which had to fit one of the most common chromosome aberrations (trisomy 13, 18, 21, triploidy, sex chromosomal aneuploidy), and on the basis of gestational age. We screened the cells with a chromosome X, Y, 13, 18, and 21 specific probe (the US probe set, see table 2.2). This focus is based on the observation that aneuploidies involving these chromosomes are reported to account for the great majority of all chromosome aberrations found in chromosomally abnormal fetuses showing sonographically determined anomalies (Wladimiroff et al., 1995). For interpretation of the FISH results we used the statistical approach as suggested by Lomax et al. (1994), since it provides a method for establishing the level of tissue mosaicism that can be detected using FISH, whereas other analytical approaches are only suitable for detection of non-mosaic aneuploidies (Klinger et al., 1992; Ward et al., 1993).

FISH results in 196 cases that we investigated are shown in table 2.8. Among 196 AF samples that we selected, 56 (29 %) showed a chromosome abnormality of which 46 (82 %) were theoretically detectable with FISH, since they involved chromosomal parts identified by the probes. In 43 of these 46 cases, abnormal signal distributions, consistent with aneuploidy of one or more chromosomes, were indeed found (table 2.9). In addition to the common aneuploidies (trisomy 13, 18, 21, 45,X, triploidy, and sex-chromosomal aneuploidies) (figure 2.7), we were able to detect two unbalanced Robertsonian translocations [der(13;14)(q10;q10), and der(14;21)(q10;q10)], and a rare mosaic case involving a cell line with an extra dicentric chromosome 21 and a cell line with an extra dicentric and deleted chromosome 21. The latter case as well as three cases of trisomy 21 are included in appendix publication IV. One of the two triploidy cases is described in publication III.

Signal distributions in abnormal cases were clearly distinct from those of diploid controls (table 2.9). However, in two cases of full trisomy 18, hybridization patterns were suggestive of a mosaic trisomy 18 as only 55 % and 29 % of the analysed cells showed three signals (95 % confidence interval (CI) of the one-sided upper reference limit (I): 3%-4%). Maternal cell contamination (MCC) of the AF sample may be an explanation. However, hybridization of L1.84 to metaphases in both cases revealed the presence of a very small signal on one of the three chromosomes 18, probably due to polymorphism. This small signal might have been

Table 2.8. FISH results in cases with ultrasound abnormalities that were investigated during four years (1993-1996)

Year	N	FISH results		
		Normal (false negative)	Abnormal (false positive)	Non-informative
1993	46	37 (0)	9 (4)	0
1994	29	18 (1[1])	9 (2)	2[2]
1995	73	51 (0)	20 (1)	2[3]
1996	48	34 (0)	12 (0)	2[4]
Total	196	140 (1)	50 (7)	6

Note.- [1] trisomy 13, not detected with L1.26; [2] 45,X, and trisomy 21 ; [3] both normal karyotypes; [4] low mosaic -13/r(13), and one normal karyotype

overlooked in the uncultured AF cells, explaining the relatively small proportion of 3-signal containing nuclei in both cases. In the nine non-mosaic 45,X cases the percentage of nuclei with two signals varied between 0-10 %. These nuclei may represent maternal cells, which leak into the AF at the time of the amniocentesis procedure, as was suggested by Winsor et al. (1996). They found a strong correlation between visible detection of blood contamination and the presence of female nuclei in the karyotypically male samples. The samples we receive in our laboratory are only rarely contaminated with blood, and an exceptional blood stained sample is excluded from FISH analysis. In this way we minimize the risk of a false negative result due to MCC.

Three detectable chromosome aberrations were missed. We had one *false negative result* with L1.26, which failed to detect a trisomy 13. In two other cases (a case of 45,X, and one of trisomy 21), results were uninformative due to technical failures (hybridization failure of Y-probe in control 46,XYsample, and weak hybridization signals of 21-probe, respectively).

In seven cases we had a *false positive result* (table 2.10). Two cases involved the 13/21 centromere probe, L1.26 (see Materials and methods, section 2.2). In case 6, a false positive result was achieved with the X centromere probe, indicating the presence of 45,X. FISH was also applied to cultured AF cells, revealing the presence of a very weak signal on one of the two X chromosomes, which was not visible in uncultured cells (figure 2.8). Of the seven false positive results, six were encountered during the first two years of our investigations, and one during the last two years. Continuous adjustment of the US probe set, with substitution of unsuitable probes (see Materials and methods, section 2.2), led to a decrease in false positive results.

Non-informative results in six cases, including two detectable chromosome aberrations (45,X and trisomy 21), one non-detectable abnormality (45,XY,-13/46,XY,r(13)), and three normal karyotypes, originated from technical problems, such as weak or absent hybridization signals, increased background fluorescence, and hybridization with less than 50 scorable nuclei.

Table 2.9 FISH signal distributions in uncultured amniocytes in cases with ultrasound abnormalities showing a chromosome aberration

Chromosome abnormality	N	Probe	Mean % (range) of nuclei with 1->3 signals			
			1	2	3	> 3
Trisomy 18	13	L1.84	2 (0-10)	25 (7-58)	73 (29-93)[1]	1 (0-5)
Trisomy 21	12	CB21c1	1 (0-6)	17 (7-30)	80 (64-93)	2 (0-9)
45,X	9	pBamX5	97 (90-100)[2]	3 (0-10)		
69,XXX	2	pBamX5		6 (0-12)	94 (88-100)	
		L1.84		16 (2-30)	84 (70-98)	
		CB21c1		24 (15-33)	76 (67-85)	
68,XX	1	pBamX5	8	92		
		L1.84		14	86	
		CB21c1		10	90	
		cCMP21.a		12	88	
69,XXY	1	pBamX5	2	97	1	
		RPN1305X	100			
		L1.84	1	16	83	
		CB21c1	5	9	86	
		cCMP21.a		2	98	
Trisomy 13	1	cFK2,6	1	14	84	2
47,XXY	1	CEPX/CEPY		1	99[3]	
+ der(13;14)(q10;q10)	1	cFK2,6	1	7	92	
+ der(14;21)(q10;q10)	1	cCMP21.a		15	85	
48,XY,+dic(21)(q11),+del(21)(q11)[54])/ 47,XY,+dic(21)(q11)[21]/46,XY[25]	1	CB21c1		23	35	43[4]

Note.- [1] In two cases, the % of nuclei with 3 signals was 29% and 55%, indicative for a mosaic trisomy 18. Karyotyping revealed a full trisomy 18. [2] Screening with the Y-specific probe was negative in all cases.[3] Nuclei with 3 signals showed two red (X) and one green (Y) signal. The one nucleus with two signals showed one green (Y) and one red (X) signal. [4]19, 11, and 13 % of nuclei showed 4, 5, and 6 signals, respectively.

Figure 2.7 Trisomy 18. A) metaphase spread and B) partial karyotype of cultured AF cells, and C) interphase nuclei of uncultured AF cells, showing three chromosome 18 signals after hybridization with LI.84 (18 centomere probe).

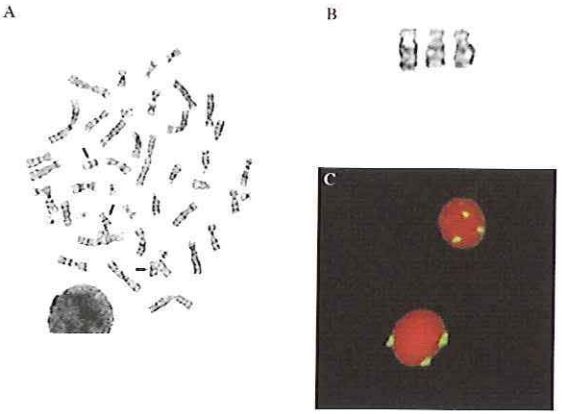


Figure 2.8 FISH results in case 6 after hybridization of pBamX5 (X centromere probe) to normal 46,XX AF cells (uncultured and cultured), showing that the repeated sequence detected by pBamX5 is a polymorphic trait. A) uncultured AF cells showing one chromosome X signal. B) interphase nuclei of cultured AF cells, showing one bright and one small chromosome X signal. C) metaphase spread with one X chromosome showing a bright signal, and one with a small, weak signal (arrow).

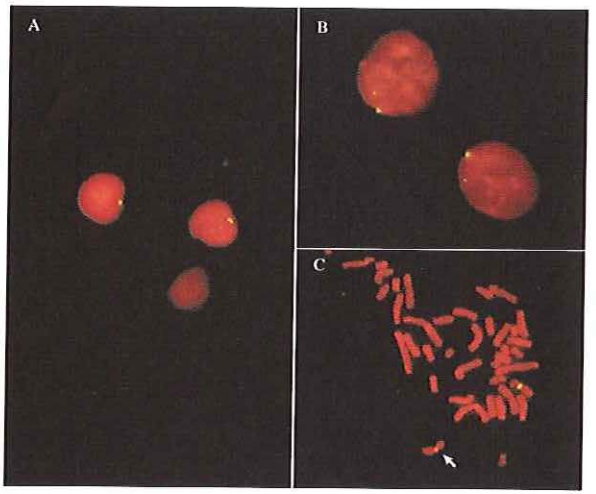


Table 2.10 False positive FISH results

Case	Probe	% of nuclei with 1-6 signals							Karyotype
	(chromosome)	0	1	2	3	4	5	6	
1	LI.26 (13/21)	-	0	2	22	37	**35**	**2**	46,XX
2	LI.26 (13/21)	-	0	0	3	13	**47**	**37**	47,XX,+i(12)(q10)[17]/ 47,XX,+del(12)(q10)[6]/46,XX[1]
3	RPN1305X (Y)	0	35	**65**	0	0	0	0	46,XY
4	RPN1305X (Y)	**88**	**2**	**10**	0	0	0	0	46,XX
5	CB21c1 (21)	-	2	76	**18**	2	0	0	46,XY
6	pBamX5 (X)	-	**91**	9	0	0	0	0	46,XX
7	pBamX5 (X)	-	**20**	**78**	2	0	0	0	46,XX

The first major prospective clinical study comparing FISH analysis of uncultured AF cells with classical cytogenetics of the cell cultures was published in 1993 (Ward et al., 1993). They investigated a total of 4500 samples, mainly of pregnancies of advanced gestational age, and they found 146 aneuploidies of which 107 were identified with FISH. They had seven false negative results (5 %, compared to 2 % in our series) and in the remaining 32 abnormal cases results were non-informative (22 %, compared to 4 % in our series). This relatively large number of non-informative abnormal specimens is the consequence of stringent reporting criteria they designed, in order to minimize the risk of false positive and false negative results. In eight of the 43 abnormal cases we found (three cases of trisomy 21, one triploidy, and four cases of trisomy 18) hybridization patterns did not meet these reporting criteria. However, we believe that the combination of abnormal ultrasound findings and abnormal FISH results, which fitted the fetal anomalies, justified the report of an abnormal result in these eight cases. Moreover, their stringent criteria do not allow a diagnosis of mosaicism. The rare mosaic case with the dicentric and deleted chromosome 21 of our series would, therefore, have gone undetected.

In earlier years, cordocentesis was the preferred method for PD in cases of ultrasound abnormalities in our laboratory, as chromosome analysis of fetal lymphocytes can be completed within one week. However, fetal blood can only be used to study the karyotype of the fetus, but is unsuitable for metabolic studies which sometimes need to be performed. In addition to some obstetric contra-indications, such as small-for-gestational-age fetuses and umbilical cord obstructions, cordocentesis may fail in case of oligohydramnios or polyhydramnios. Moreover, fetal blood can not be stored for potential future studies, and it does not always accurately reflect the fetal karyotype in cases of fetal mosaicism. Therefore, FISH on uncultured AF cells became the prefered method for rapid fetal chromosome analysis during the last years. The number of cordocenteses in cases of ultrasound abnormalities decreased from 53 in 1993 to only 18 in 1996.

We conclude that FISH on uncultured AF cells provides a rapid and accurate method for

prenatal identification of chromosomal aneuploidies in pregnancies complicated by fetal anomalies. However, the reliability, and therefore, the clinical utility, will stand or fall with specificity and hybridization efficiency of the probes. A few probe changes were necessary to reduce the number of false positive and false negative results that were encountered during the first two years of our investigations. The US probe set that we use today has proven to be highly reliable. Therefore, we believe that abnormal FISH findings justify the report of an abnormal result to the parents in pregnancies with US abnormalities. However, normal FISH results should always be complemented by a full cytogenetic analysis of the cell cultures, since 18 % of chromosome aberrations in the group of US abnormalities can not be detected with probes for chromosomes X, Y, 13, 18, and 21, as they involve structural chromosome aberrations, such as marker chromosomes and translocations, as well as aneuploidies of other chromosomes than those detected by the US probe set. Therefore, FISH can not be considered to be a stand-alone diagnostic test.

3.2.2 Cytogenetic abnormality in previous chorionic villus sample (CVS)

If a chromosome abnormality, other than non-mosaic trisomy 21, triploidy, 47,XXY, 47,XYY or 47,XXX, is detected in CV semi-direct preparations, in the absence of early US abnormalities, it may represent confined placental mosaicism (CPM). Therefore, follow-up investigations in AF cells for verification of the fetal karyotype and of potential uniparental disomy (UPD) in case of confined placental trisomy, may be necessary (see section 1.4.2 of chapter I).

Table 2.11 Reasons for not performing amniocentesis after uncertain abnormal results in CVS

Reason	N
US abnormalities + TOP	11
FISH on semi-direct CV preparations normal	10
TOP on parents request, without further investigations	7
Continuation of pregnancy, without further investigations	2
Structural chromosome aberration of familial origin[1]	5
IUD before amniocentesis	2
Total	37

Note.- US: ultrasound; TOP: termination of pregnancy; IUD: intrauterine death;[1] two balanced translocations, two inversions, and one marker chromosome

In appendix publication VII we describe UPD studies in AF cells in cases of confined placental trisomy found during a four-year period (1992-1995) (see chapter III). We used FISH on uncultured AF cells for discrimination between CPM and generalized mosaicism in order 1) to get a quick result, and 2) to preclude generalized mosaicism concealed in cell

Table 2.12 Cases of (mosaic) aneuploidy, detected in CVS, in which confirmatory FISH studies were performed on subsequent uncultured AF cells

Abnormal cell line	Cases of non-mosaicism	Cases of high level mosaicism (> 33.3 %)	Cases of low level mosaicism (≤33.3 %)	Total
1.Common aneuploidies				
trisomy 13	0	0	1	1
trisomy 18	2	2	3	7
trisomy 21	0	1	0	1
45,X	3	1	8	12
47,XXY	0	1	0	1
2.Unusual trisomies				
trisomy 3	0	1	2	3
trisomy 7	1	1	4	6
trisomy 9	1	0	0	1
trisomy 11	0	0	1	1
trisomy 15	0	0	2	2
trisomy 16	3	0	1	4
trisomy 22	1	0	1	2
trisomy X	0	0	1	1
trisomy 7, 13, 20, 21	0	0	1	1
trisomy 13, 20	0	0	1	1
3.Structural aberrations				
+ der(21;21)(q10;q10)	1	0	0	1
+ der(13;13)(q10;q10)	0	1	0	1
4.Tetraploidy	2	4	0	6
Total	14	12	26	52

cultures. FISH results were in agreement with cytogenetic results in 13 out of 14 cases that were investigated. In one case of full trisomy 16 in CVS, a discrepancy was found with 26 % of uncultured AF cells showing three chromosome 16 signals, whereas the cell cultures showed a normal karyotype. Postmortem examination of the fetus after intrauterine death at 33 weeks of gestation, revealing several congenital malformations fitting a mosaic trisomy 16 phenotype, and postnatal FISH studies, support the presence of generalized mosaicism. On the basis of these results we believe that FISH is a reliable method for rapid differentiation between CPM and generalized mosaicism, and that it may even better reflect the actual fetal

karyotype than chromosome analysis of the cultured cells, since uncultured cells are not affected by culture induced selection mechanisms.

Between 1993 and the end of 1996 a total of **110** cases of potential CPM were detected among 3499 CV samples. In **37** cases a follow-up amniocentesis was not performed for various reasons (table 2.11); in 10 out of these 37 cases [nine cases of low-mosaic 45,X (two out of 30 metaphases), and one case of low mosaic tetraploidy], FISH on CV semi-direct preparations did not confirm the presence of mosaicism, and normal results were reported to the parents without follow-up investigations (see section 3.3.2). In the remaining **73** cases a follow-up amniocentesis was performed: in **21** cases, AF cells were only studied cytogenetically, because of the involvement of a structural chromosome aberration which was not detectable with FISH, and in **52** cases, FISH was applied to uncultured AF cells for rapid verification of the fetal karyotype. The chromosome aberrations initially found in CVS and the number of cases are shown in table 2.12.

FISH results in 52 cases are summarized in table 2.13. In 48 out of 52 cases, FISH results were in agreement with cytogenetic results, including 12 abnormal (generalized mosaicism) and 36 normal (confined placental mosaicism) cases. Inconsistent FISH and cytogenetic results were encountered in three cases, and in one case, showing a normal karyotype, FISH results were not informative.

Table 2.13. FISH on uncultured AF cells for verification of a chromosome abnormality detected in a previous CVS: number of cases investigated from 1993 until 1996, and FISH results

Year	N	FISH results		
		Normal (false negative)	Abnormal (false positive)	Non-informative
1993	7	3 (0)	3 (1)	1
1994	15	10 (0)	5 (1)	0
1995	14	10 (1)	4 (0)	0
1996	16	14 (0)	2 (0)	0
Total	52	37 (1)	14 (2)	1

The twelve cases of generalized mosaicism, encountered by both methods, are shown in table 2.14. In 11 of these 12 cases, FISH correctly identified the presence of a non-mosaic ór mosaic chromosome aberration; only in case 10, FISH results were suggestive of a mosaic trisomy 21, whereas the cell cultures revealed a full trisomy 21. Maternal cell contamination of the AF sample may be an explanation for the relatively high proportion of disomic uncultured AF cells.

The *two false positive FISH results*, and the *false negative FISH finding* are shown in table 2.15. In cases 1 and 2, FISH on uncultured AF cells confirmed the presence of an abnormal cell line, whereas the cell cultures showed a normal karyotype in seven and 15 cell colonies,

Table 2.14 Cases of generalized mosaicism: karyotypes in CVS and cultured AF cells, and FISH signal distributions in uncultured AF cells

Case	Karyotype in CVS [number of cells]	Probe	% of nuclei with 1-4 signals					Karyotype in cultured AF cells [number of cell colonies]
			N	1	2	3	4	
1	47,XX,+18[18]	L1.84	100	1	15	**84**	0	47,XX,+18[1]
2	47,XY,+18[30]	L1.84	145	2	30	**63**	5	47,XY,+18[4]
3	45,X[31]	X+Y[1]	200	**16**	84	1	0	45,X[3]/46,XY[20]
4	45,X[30]	X+Y	304	**98**	2	0	0	45,X[13]
5	47,XX,+9[30]	pHuR98	363	2	64	**34**	0	47,XX,+9[7]/46,XX[30]
6	47,XY,+22[17]	58B12	100	0	10	**90**	0	47,XY,+22[5]
7	46,XX,der(21;21)(q10;q10)[21]	CB21c1	25	4	8	**88**	0	46,XX,der(21;21)(q10;q10)[2]
8	47,XY,+18[18]/48,XY, +18,+20[13]/46,XY[3]	L1.84 Tel 20p	173 95	0 0	39 100	**61** **0**	0 0	47,XY,+18[5]
9	47,XY,+18[27]/46,XY[3]	L1.84	175	1	7	**92**	0	47,XY,+18[21]
10	47,XX,+21[24]/46,XX[6]	CB21c1	50	8	38	**53**	2	47,XX,+21[19]
11	47,XXY[6]/46,XY[2]	pBamX5	200	83	**17**	0	0	47,XXY[3]/46,XY[6]
12	45,X[6]/46,XX[24]	pBamX5	200	**24**	76	0	0	45,X[1]/46,XX[15]

Note.- [1] X+Y: mixture of pBamX5 and RPN1305X

respectively, excluding a mosaic of 35% and 19% with 95% confidence, respectively (Hook,1977). It is possible that the abnormal cells in both cases had a proliferative disadvantage as compared to normal cells, which may explain their absence in the cell cultures, as was shown for trisomy 9 mosaicism (van den Berg et al., 1997). On the other hand, the FISH results may represent true false positive results due to aspecific hybridization of the 16-probe to another chromosome, and hybridization failure of the X centromere probe in the other case. However, whether the FISH results in the trisomy 16 case were false positive indeed is questionable, considering the pregnancy outcome, postmortem examination of the fetus, and postnatal FISH studies in this case (see appendix publication VII).

Table 2.15 Discrepant FISH and cytogenetic results: karyotypes in CVS and cultured AF cells, FISH signal distributions in uncultured AF cells, and pregnancy outcome

Case	Karyotype in CVS [number of cells]	Probe	% of nuclei with 1-4 signals				Karyotype in cultured AF cells [number of cell colonies]	Pregnancy outcome
			1	2	3	4		
1	47,XX,+16 [30]	pHuR195	6	65	**26**	3	46,XX[7]	IUD 33 wks., 845g, MCA[1]
2	45,X[15]/46,XX[7]	pBamX5	**21**	79	0	0	46,XX[15]	healthy ♀
3	45,X[30]	pBamX5	9	91	0	0	45,X[5]/46,XX[34]	healthy ♀[2]

Note.- IUD: intra-uterine death; MCA: multiple congenital abnormalities;[1] see appendix publication VII;[2] mosaicism neonatally confirmed in lymphocytes: 45,X[4]/46,XX[46]

In addition to two false positive results, we had *one false negative FISH finding*. In case three of table 2.15 a non-mosaic 45,X was found in CVS. FISH analysis in uncultured AF cells showed normal signal distributions with the X centromere probe indicative for a normal female karyotype. Cultured cells showed a low mosaic 45,X[5]/46,XX[34], which was postnatally confirmed in lymphocytes. In general, it will be difficult to detect low level mosaicism due to the broad ranges of nuclei exhibiting one, two, and three signals in normal samples. Therefore, the sensitivity of FISH for detection of small subpopulations of monosomic or trisomic cells is limited by the frequency at which they occur in normal control samples (Eastmond and Pinkel, 1989). On the other hand, we were able to detect two other cases of low level mosaicism (13 % and 7 %) involving a 45,X cell line, with 16 % and 24 % of the interphase nuclei showing one X signal, respectively (cases 3 and 12 in table 2.14). On the basis of these results we believe that the distribution of normal and abnormal cells in AF cell cultures does not necessarily reflect the pattern found in uncultured AF cells (Kromer et al., 1996; Bryndorf et al., 1997), which may be explained by selection in favour of normal or abnormal cells during cell culturing.

In conclusion, FISH on uncultured AF cells allows a rapid differentiation between CPM and generalized mosaicism, shortening the reporting time of the fetal karyotype to the parents. However, discrepancies between FISH (uncultured cells) and cytogenetic (cell cultures) results may occur, and may cause an interpretation dilemma. Firstly, *false positive FISH findings* may sometimes be the result of technical failure of the probe. However, selection

against abnormal cells in the cell cultures may be another explanation, with cytogenetic results, in fact, representing false negative results. Generally, it is believed that in mosaic cases uncultured cells better reflect the fetal chromosome constitution than cultured cells, due to selective in vitro growth (Lomax et al., 1994; Bryndorf et al., 1997). Secondly, *false negative FISH results* may occur if the level of mosaicism is below the detection level of interphase FISH.

3.2.3 Other indications

Other indications for performing FISH on uncultured AF cells are:

1) previous AF sample abnormal
2) abnormal triple test (TT) results (risk of Down syndrome ≥ 1/20)
3) advanced maternal age (≥ 44 years)
4) twin pregnancy of advanced gestational age (± 16 weeks)
5) X-linked mental retardation or biochemical disease

In all these cases a quick result is important because of an increased risk for a chromosome aberration (indications 1-3), or for pregnancy complications in case of selective termination after 17 weeks of gestation (indication 4). For indication 5, a rapid verification of fetal sex is necessary, as further prenatal biochemical or DNA investigations are only initiated when the fetus is male.

Indication 1. Between 1993 and the end of 1996, we applied FISH to uncultured AF cells of a second AF sample in **seven cases**, because one or a few cell colonies were found to show a chromosome abnormality in the first sample, and the total number of cell colonies was too small to exclude true mosaicism, according to Hsu et al. (1992). Cytogenetic and FISH results are shown in table 2.16. FISH results were concordant with cytogenetic results in all seven cases. FISH confirmed the presence of a mosaic chromosome aberration in two cases (cases 3 and 4). Subsequent chromosome analysis confirmed the FISH result in case 4, although it revealed a higher mosaic trisomy 18 (66 %) than was found with FISH (18 %). This is in contrast with the more common observation of a larger proportion of abnormal cells in an uncultured specimen as compared to the cultured tissue, most probably due to a proliferative advantage of normal cells over abnormal cells (Lomax et al., 1994). In case 3, chromosome analysis was not performed, but FISH was simultaneously applied to cells of different compartments (AF cells, umbilical cord lymphocytes, and CV), all revealing a considerable number of trisomic cells (lymphocytes and CV results not shown), which, together with slightly abnormal US findings, made a diagosis of generalized true mosaicism for trisomy 9 certain (van den Berg et al., 1997). After termination of the pregnancy, the prenatal findings were confirmed in different fetal and placental tissues in both cases. In the other five cases, FISH on the second sample did not show the presence of a significant proportion of abnormal cells, which was confirmed by chromosome analysis of the cell cultures.

Table 2.16 FISH on uncultured cells of a second amniotic fluid sample (AF2) for verification of a chromosome aberration in a previous AF sample (AF1)

Case	Karyotype AF1	Probe	% of nuclei with 1-3 signals in AF2				Karyotype AF2
			N	1	2	3	
1	47,XX,+8[1]/46,XX[10]	αp8	123	13	87	0	46,XX[25]
2	47,XY,+9[1]/46,XY[5][1]	pHuR98	100	11	85	4	46,XY[17]
3	47,XY,+9[4]/46,XY[26][2]	pHuR98	170	6	71	**24**	---[3]
4	47,XX,+18[3]/47,XX,+16[1]/46,XX[14]	L1.84	295	6	76	**18**	47,XX,+18[19]/46,XX[8]
		pHuR195	200	3	97	0	
5	45,X[1]/46,XX[7]	pBamX5	303	8	92	0	46,XX[24]
6	46,XX[13]/46,XY[2]	CEPX/CEPY	200	1	99[4]	0	46,XX[15]
7	92,XXYY[1]/46,XY[9]	L1.84	99	10	86	4	46,XY[16]
		pHuR98	151	6	88	6	

Note.- [1] and [2]: cases 5 and 3, respectively, in van den Berg et al. (1997); [3] karyotyping was not performed; [4] 99 % of AF cells showed two red (X) signals

Indications 2-5. Between 1993 and the end of 1996, **25 cases** were investigated within indication groups 2-4. Cells were screened with the US probe set (see materials and methods section 2.2, and table 2.2). This revealed two chromosome aberrations (trisomy 21 and 47, XXY), and 23 normal results by both methods. Additionally, **14 cases** were investigated with a chromosome X and Y specific probe for determination of fetal gender (indication 5). No discrepancies were found between FISH and chromosome analysis.

3.3 Interphase FISH for studying chromosomal mosaicism

3.3.1 Mosaicism in amniotic fluid (AF) cell cultures

Several prenatal cases of presumed pseudomosaicism in AF cell cultures have resulted in the birth of children affected with true mosaicism (Camurri et al., 1988; Cheung et al., 1988; Vockley et al., 1991). Differentiation between both phenomena is therefore essential, and requires the analysis of a large number of cells (Hsu et al., 1992).

Between 1993 and the end of 1996, we used FISH in nine cases of pseudomosaicism in AF cell cultures, all involving one abnormal colony, in order to test the reliability of interphase FISH for discrimination between pseudomosaicism and true mosaicism (table 2.17). FISH was applied to cells that were left on the bottom of the "affected" culture dish (dish with abnormal colony), so excluding the abnormal colony from analysis, and/or to remaining cells of other culture dishes of the same sample. These cells were trypsinized and seeded on glass coverslips before FISH analysis.

In all cases, except for case 4, FISH did not reveal the presence of a trisomic cell line, confirming the cytogenetic diagnosis of pseudomosaicism. A diagnosis of pseudomosaicism type A could be made in case 3 (partial abnormal single colony), whereas the others showed a pseudomosaicism type B (abnormal single colony) (Boué et al., 1979). In case 4, involving one trisomy 9 cell colony and five colonies with a normal karyotype, FISH applied to cells of the "affected" dish revealed a significant proportion of trisomic cells. This implicates that at least one additional colony in the affected dish showed a trisomy 9 karyotype. FISH signal distributions in the two other dishes were normal, establishing a diagnosis of pseudomosaicism type C (more than one abnormal colony restricted to one culture dish). In all nine cases FISH results were in agreement with the cytogenetic diagnosis of pseudomosaicism. Pseudomosaicism was confirmed by follow-up investigations in a repeat AF sample in two cases (cases 2 and 4. See table 2.16, cases 1 and 2, respectively), and by the birth of healthy children in the others.

In conclusion, these results suggest that whenever the number of analysable cell colonies is too small to exclude true mosaicism, interphase FISH can be used to determine whether any additional cell colonies have an abnormal karyotype, so that a second amniocentesis for confirmatory studies can be obviated. In all cases, FISH was applied to trypsinized cell colonies left on the bottom of the culture dishes after removal of the coverslip carrying the

Table 2.17 Differentiation between pseudomosaicism and true mosaicism in AF cell cultures using interphase FISH analysis

Case	Karyotype	Probe	Dish[1]	% of nuclei with 1-4 signals					Prenatal diagnosis
				N	1	2	3	4	
1	47,XY,+8[1]/46,XY[33]	αp8	A	200	3	91	5	1	pseudomosaicism type B
2	47,XX+8[1]/46,XX[10]	αp8	N	800	0	99	1	0	pseudomosaicism type B
3	47,XX,+9[1][2]/46,XX[18][3]	pHuR98	N	200	5	90	5	0	pseudomosaicism type A
4	47,XY,+9[1]/46,XY[5][4]	pHuR98	A	200	3	59	27	11	pseudomosaicism type C
			N	203	5	91	2	2	
			N	205	5	88	5	2	
5	47,XY,+16[1]/46,XY[17]	pHuR195	A	200	4	96	1	1	pseudomosaicism type B
			N	200	3	96	1	2	
6	47,XX,+17[1]/46,XX[16]	p17H8	A	200	1	99	0	0	pseudomosaicism type B
			N	200	1	97	2	0	
			N	200	1	99	0	0	
7	47,XY,+18[1]/46,XY[28]	L1.84	A	320	5	92	2	0	pseudomosaicism type B
			N	400	8	92	0	0	
8	47,XY,+21[1]/46,XY[23]	cCMP21.a	A	204	5	87	4	4	pseudomosaicism type B
			N	200	3	91	5	1	
			N	200	3	91	2	4	
9	47,XX,+21[1]/46,XX[23]	CB21c1	N	200	8	84	6	2	pseudomosaicism type B

Note.- [1] A= dish with abnormal cell colony (the abnormal colony was NOT included in the FISH slide, and hence in the scoring), and N= dish with normal cell colonies; [2] colony includes normal and abnormal cells; [3], [4]: cases 4 and 5, respectively, reported by van den Berg et al. (1997)

cell colonies that are used for cytogenetic analysis. Nowadays, we prefer to destain previously trypsin-Giemsa stained slides prepared for chromosome analysis (one normal slide of each AF container, and the "affected slide"), and use them for FISH analysis as well, in order to augment the number of actually investigated cell colonies with those not suitable for chromosome analysis.

3.3.2 Mosaicism in semi-direct chorionic villus preparations

Interphase FISH on semi-direct CV preparations may add to the cytogenetic data in two instances:

1) if non-mosaic or mosaic trisomy 18 is encountered
2) if low level mosaicism (≤33.3 % abnormal cells) is detected

1) In appendix publication V we showed that if trisomy 18 is found in CV semi-direct slides, interphase FISH may contribute to the results of classical chromosome analysis in terms of predicting the fetal chromosome constitution. Mosaic and non-mosaic trisomy 18 may be confined to the placenta and may not represent the fetal karyotype (see section 1.4.2). Therefore, confirmatory studies in CV long term cultures and AF cells may be necessary. Since CV cultures were not established during the study period (1993-1996), we investigated the use of interphase FISH as a potential tool of rapid verification. Thirty cases of trisomy 18 (22 non-mosaic and 8 mosaic cases), encountered during an 8-year period (1985-1992) were, retrospectively, investigated. The only non-confirmed case of full trisomy 18 had a significantly smaller number of interphase nuclei displaying three signals in semi-direct preparations than the real, confirmed cases of trisomy 18. In cases of mosaic trisomy 18, the application of FISH also contributed to the results of traditional metaphase analysis: higher levels of three signal containing nuclei were found in the three confirmed mosaic cases as compared with the four non-confirmed cases. If the percentage of nuclei with three signals was smaller than 66 %, trisomy 18 was not confirmed in fetal cells. In general, the FISH data were better able to predict the fetal chromosome constitution than cytogenetic analysis of CV semi-direct slides, although FISH yielded ambivalent results in some cases.

Between 1993 and the end of 1996, another eight cases of trisomy 18 (four non-mosaic and four mosaic cases) were studied with FISH (table 2.18). In seven out of eight cases, FISH results were suggestive for trisomy 18 in the fetus in five cases (cases 1, 2, 3, 5 and 6) and CPM in two cases (cases 7 and 8), which was confirmed by follow-up investigations. In one case of non-mosaic trisomy 18 (case 4), ambivalent FISH results were achieved, which could correspond to either situation of non-mosaic trisomy 18 in the fetus, generalized mosaicism, or CPM. Fetal karyotyping after termination of the pregnancy, on the basis of US abnormalities and cytogenetic results, confirmed a non-mosaic trisomy 18 in the fetus.

In conclusion, interphase FISH results on CV semi-direct slides of (non)-mosaic trisomy 18 cases can aid in the counselling procedures, although a final result can only be achieved by (FISH or cytogenetic) analysis of a subsequent AF sample.

Table 2.18 FISH on CV semi-direct preparations in cases of (non)-mosaic trisomy 18, and pregnancy outcome

Case	Karyotype in CVS [number of cells]	% of nuclei with 1-3 signals			Follow-up and pregnancy outcome
		1	2	3	
1	47,XX,+18[18]	0	9	**91**	TOP after amnioc. (case 1 in table 2.14)
2	47,X-,+18[18]	3	4	**93**	TOP (US abnormalities); tris. 18 confirmed in fetal skin
3	47,XY,+18[30]	2	4	**94**	TOP after amnioc. (case 2 in table 2.14)
4	47,X-,+18[18]	0	22	**79**	TOP (US abnormalities); tris. 18 confirmed in fetal skin
5	47,XY,+18[27]/46,XY[3]	0	18	**82**	TOP after amnioc. (case 9 in table 2.14)
6	47,XY,+18[18]/48,XY,+18,+20[13]/46,XY[3]	1	11	**88**	TOP after amnioc. (case 8 in table 2.14)
7	47,XX,+18[2]/46,XX[28]	5	95	0	Continuation after amnioc.; healthy ♀, SGA
8	47,XX,+18[2]/46,XX[28]	5	93	2	Continuation after amnioc.; healthy ♀

Note.- TOP= termination of pregnancy; US= ultrasound; SGA= small for gestational age

2) If a chromosome abnormality is encountered in CV semi-direct preparations, in the absence of early US abnormalities, it may represent CPM and follow-up investigations, such as amniocentesis, may be necessary for verification of the fetal karyotype. Between 1993 and the end of 1996 we encountered 110 cases of potential CPM (3,1 %) among 3499 CV samples, and amniocentesis was performed in 73 cases, representing a second invasive procedure in 2,1 % of women undergoing CVS. Low level mosaicism (≤ 33.3 % abnormal cells) was encountered in 37 (34 %) cases, with ≤ 10 % abnormal cells in 25 of these cases. The question is whether these cases represent mosaicism, justifying follow-up studies in a second sample, or wether the abnormal cells are the consequence of a local mitotic division error.

In order to discriminate between both phenomena, we performed FISH in these 37 cases; FISH confirmed the presence of mosaicism in 13 cases (two out of 25 cases with ≤ 10 % abnormal cells, and 11 out of 12 cases with > 10 % abnormal cells) (table 2.19), and normal results were found in the remaining 24 cases. Since FISH analysis involves the scoring of a few hundreds of cells, we believe that it better reflects the real level of mosaicism than traditional chromosome analysis. Therefore, it is likely that the few abnormal metaphases in the 24 cases with normal FISH results represented local mitotic errors instead of mosaicism.

Table 2.19 Abnormal FISH distributions in CV semi-direct slides in cases of low level mosaicism (≤ 33.3 % abnormal cells)

Case No.	Abnormal cell line	% abnormal cells	Probe	% of nuclei with 1-4 signals			
				1	2	3	4
	1.Common aneuploidies						
1	**45,X**(45,X/46,XX)	20	pBamX5	**24**	76	0	0
2		23		**57**	41	2	0
3		7		**24**	73	2	1
4		17		**28**	72	0	0
5	**45,X**(45,X/46,XY)	13	CEPX/CEPY	**20**[1]	80	0	0
6	47,XXX	30	pBamX5	4	66	**30**	0
	2. Unusual trisomies						
7	trisomy 3	17	pα3.5	0	70	**30**	0
8		24		0	79	**20**	1
9	trisomy 7	16	pα7t1	7	63	**30**	0
10		10		4	77	**19**	0
11		20		1	92	7	0
12		29		0	60	**40**	0
13	trisomy 11	17	pLC11.A	0	63	**36**	1

Note.- [1] 20 % of the nuclei showing only one red (X) signal, and 80 % showing one red (X) and one green (Y) signal.

In 12 of these cases, a mosaic 45,X/46,XX or 45,X/46,XY was involved. The two 45,X cells (on a total of 30 metaphases) in all these cases may also have been incomplete normal cells, rather than monosomy X cells, since incomplete metaphases are often observed in CV semi-direct preparations. However, low level mosaicism, below the detection level of interphase FISH, can not definitely be excluded in these cases. When FISH is applied to normal diploid control samples, a small percentage of cells will exhibit only one signal, due to technical factors such as inefficient probe hybridization, an inefficient detection of hybridized probe, or overlapping signals. Another small proportion will show three signals, due to aspecific probe hybridization or the so-called "split spots" caused by DNA replication in G2 cells (Eastmond et al., 1995). Therefore, the sensitivity of interphase FISH is limited by the frequency of one- and three signal containing cells in normal samples (Eastmond and Pinkel, 1989) (see table 2.2).

During the first years of our investigations, follow-up studies in AF cells were performed in mosaic cases with normal FISH results. However, after recurrent normal findings in AF cells and the birth of healthy children, an amniocentesis was no longer performed in 12 out of the 24 cases with normal FISH findings, all of which resulted in the birth of healthy children.

In conclusion, FISH adds to the cytogenetic result in CV semi-direct slides in cases of low level mosaicism , since it enables a rapid differentiation between real mosaicism and local mitotic errors, apparently without clinical significance, for which follow-up investigations, such as amniocentesis can be omitted.

3.4 FISH for detection of microdeletions

In appendix publication VI, we report on the first PD by FISH of a familial 22q11 microdeletion, associated with a spectrum of malformations (Cardiac defects, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcaemia) covered by the acronym CATCH 22 (Wilson et al., 1993). The deletion was known in a previous child with symptoms of the classical DiGeorge syndrome, who died two weeks after birth, and turned out to be present in the physically normal father, who showed some psychiatric problems. The deletions were not visible cytogenetically. FISH with a probe from the DiGeorge syndrome critical region (Mulder et al., 1995) showed to be a reliable and rapid method for their detection.

Between 1993 and the end of 1996, a total of 48 prenatal cases at risk for a microdeletion syndrome were studied with FISH. The syndromes involved were CATCH22 (chromosome 22q11), Miller-Dieker syndrome (chromosome 17p13.3), Williams syndrome (chromosome 7q11.23), and Tuberous Sclerosis Complex 2 (TSC2) (chromosome 16p13.3). Such microdeletions are difficult to detect or cannot be detected at all with classical banding techniques, even with high resolution banding. The isolation of FISH probes from the critical region of these microdeletion syndromes enabled their rapid and reliable detection (Van Tuinen et al., 1988; Kuwano et al., 1991; Desmaze et al., 1993; Ewart et al., 1993; European Chromosome 16 Tuberous Sclerosis Consortium, 1993).
In most cases the indication for PD was a previous child with the deletion and/or the

associated syndrome. FISH was performed irrespective of the FISH results on the parents' lymphocytes, taking into account the small possibility of gonadal mosaicism. In all but one case (described in publication VI), normal FISH results were achieved, as could be expected.

Chapter III

Fetal uniparental disomy (UPD) with and without confined placental mosaicism (CPM)

1. Aim of the experimental work

Confined placental mosaicism (CPM) involving a trisomy may be associated with fetal uniparental disomy (UPD), i.e. both chromosomes of a chromosome pair derived from one parent only, as was described in section 1.3.2 of chapter I. Briefly, in such situation, the trisomy in the placenta has a meiotic origin, and fetal UPD arises through loss of one particular copy of the set of three chromosomes (namely the chromosome from the parent contributing only one copy) in embryonic progenitor cells, so that the two chromosomes left are from one parent only. The process of removal of one chromosome from the trisomic conception is called "trisomic zygote rescue". The aim of the experimental work described in this chapter was to investigate the incidence of fetal UPD in a consecutive series of chorionic villi (CV) samples collected during four years (1992-1995), in order to get more insight into the origin of placenta confined trisomy, and in the mechanism of trisomic zygote rescue.

2. Results and discussion

2.1 Incidence of uniparental disomy associated with confined placental mosaicism (appendix publication VII)

Among 3958 CV samples that we investigated cytogenetically during four years using the semi-direct preparation technique, 69 cases (1,7 %) of CPM (type I or type III) were found. Of these 69 cases, 29 cases (42 %) involved a trisomy. Parental origin studies could be performed in 23 of these cases, revealing UPD (maternal heterodisomy 16) in only one case.

There are three factors that seem to be important in predicting the disomic state of the fetus:

(1) the chromosome involved
(2) the level of mosaicism
(3) the type of CPM

The incidence of UPD associated with CPM is expected to be one in three, if CPM is the result of trisomic zygote rescue, and loss or removal of one of the three chromosomes is a random process (see figure 1.6). This theoretical figure has been established for chromosomes 16 and 22 (Kalousek et al., 1993; Wolstenholme, 1995, 1996), but not for some other chromosomes, such as chromosomes 2, 3, 7, and 8 (Robinson et al., 1995; Kalousek et al., 1996; Shaffer et al., 1996; Wolstenholme, 1996; Robinson et al., 1997). A different origin of the placenta confined trisomies involving these chromosomes, being predominantly meiotic for chromosomes 16 and 22, but mitotic for the other chromosomes, explains the difference in frequency of UPD depending on the chromosome involved (Robinson et al., 1997). Likewise, a high level of mosaicism in both placental cell lineages (cytotrophoblast and mesenchymal core) was expected and shown to be significantly correlated with a meiotic origin of the trisomy, and therefore with fetal UPD (Wolstenholme, 1996; Robinson et al., 1997). The low

incidence (one in 23) that we found, could therefore be expected as in most cases low level mosaicism was found in semi-direct CV preparations, most probably having a mitotic origin (Crane and Cheung, 1988; Wolstenholme, 1996; Robinson et al., 1997).

One other group investigated the incidence of UPD in a series of 94 cases of CPM and found UPD in 17 cases, including 13 cases of UPD 16 (Robinson et al., 1997). However, their study population might not be considered a random sample of CPM cases encountered during prenatal diagnosis (PD), because of inclusion of postnatal cases ascertained through intrauterine growth retardation noted at birth, and because of a large number of trisomy 16 cases, since they were the initial focus of their research. Both factors may contribute to an overestimation of the incidence of UPD.

In conclusion, the incidence of UPD associated with CPM (type I or type III), in a consecutive series of CV samples collected during four years, is very low, indicating that in most cases the trisomic cell line most probably originates from somatic duplication, which is supported by the low level of mosaicism in most of these cases.

The normal pregnancy outcome of the UPD 16 case we found, further supports the hypothesis that the impaired fetal growth, encountered in most cases of UPD 16, may not be the result of the UPD itself, but is rather due to a malfunctioning placenta, caused by high levels of trisomic cells in the placenta (Kalousek et al., 1993; Kalousek and Barrett, 1994; Wolstenholme, 1995; Brandenburg et al., 1996; Robinson et al., 1997). However, a dysfunctional placenta can not explain the fetal congenital malformations observed in some cases of UPD 16. We hypothesize that in symptomatic cases, trisomy 16 cells are in fact not confined to the placenta, but that a mosaic trisomy 16 is also present in the fetus, despite a normal karyotype in amniotic fluid (AF) cell cultures. This is illustrated by ultrasound and pathological observations of the fetus, and FISH studies, indicating a mosaic trisomy 16 in uncultured AF cells, performed in a CPM 16 case without UPD of our series. We advise the analysis of uncultured AF cells for verification of (mosaic) trisomy 16 in fetal cells, since the trisomic cell line may be completely lost in AF cell cultures due to selection. Finally, we showed that the obstetrical complications, found in seven out of 23 CPM cases of the present series, are not the consequence of fetal UPD.

2.2 Uniparental disomy without confined placental mosaicism: a model for trisomic zygote rescue (appendix publication VIII)

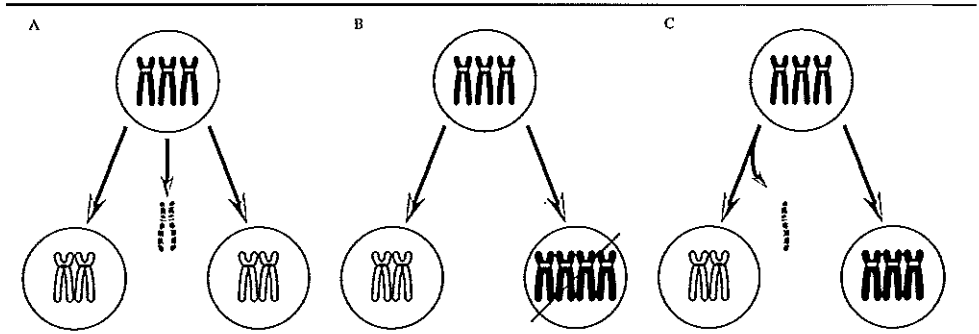
Although fetal UPD is prenatally mainly suspected and observed in cases of confined placental trisomy, the incidence of UPD associated with CPM, investigated during a four-year period (1992-1995), showed to be low (one in 23 cases of confined placental trisomy). Surprisingly, during that same time period, we encountered two cases of UPD which were both associated with a normal karyotype in semi-direct CV preparations: one (UPD 15) was only discovered postnatally after birth of a child with Prader-Willi syndrome, and the other (UPD 16) was prenatally encountered in the course of prenatal DNA analysis of the tuberous sclerosis complex 2 region at 16p13.3. These findings suggest that a normal karyotype in CV

might not be an exception in cases of UPD.

Of all mechanisms potentially leading to UPD, that have been proposed by Engel (1993), gamete complementation and trisomic zygote rescue seem to be the most likely causes of UPD in these two cases. Engel (1980) calculated the potential incidence of UPD, originating from gamete complementation, i.e., union of a disomic gamete with a gamete nullisomic for the homologue, based on data from cytogenetic studies of spontaneous abortions. The expected incidence was calculated as 2,8 in 10.000 conceptions. However, so far, no convincing proof of UPD by gamete complementation has been reported. Therefore, trisomic zygote rescue seems to be the most likely cause.

The precise mechanism of trisomic rescue is not known. Anaphase lagging (AL) and non-disjunction (ND) in an early postzygotic cell division have been proposed (figure 3.1) (Kalousek, 1994) . However, both seem not to be perfect: anaphase lagging will give rise to one disomic and one trisomic daughter cell, and non-disjunction will produce one disomic and a lethal quadrisomic cell, reducing the number of blastomeres significantly, especially when trisomic zygote rescue takes place in the first two cell divisions, which may hamper further normal development (Tarín et al., 1992). Therefore, we propose an alternative correction mode, which we call chromosome demolition (CD), and which involves the destruction and removal of one of the set of three chromosomes during cell division resulting in two disomic daughter cells (figure 3.1).

Figure 3.1 Mechanisms of trisomic zygote rescue: A) chromosome demolition, B) non-disjunction, and C) anaphase lagging



We would like to present a model for the arising of the various combinations of karyotypes in semi-direct CV preparations (cytotrophoblast of CV), long-term cultured CV (LTC CV) (mesenchymal core of CV), and the fetus from trisomic zygote rescue by these three different correction modes (figures 3.2). Taking into account the embryogenic models proposed by Crane and Cheung (1988) and Bianchi et al. (1993) (see figure 1.5), we further assume the correction to take place in the first four cell divisions, with a subsequently unknown

distribution of normal and abnormal cells among the progenitor cells of trophoblast and inner cell mass (ICM) until the 16-cell stage. In case of a reduced number of blastomeres by ND correction, we assume that compensatory reallocation may occur between trophoblast and ICM. From the 16-cell stage onwards, at which the separation between trophoblast and ICM occurs, we assume that cells within the ICM can still allocate their daughter cells freely to the compartment of the fetus, to that of the extraembryonic mesoderm (EEM), or one daughter cell to each compartment.

As shown in figures 3.2 and 3.3, trisomic zygote rescue by CD and ND can explain all combinations of karyotypes in trophoblast, EEM, and fetus that have been described in cases of fetal UPD associated with CPM or normal karyotypes in both placental cell lineages, as well as in cases of generalized mosaicism with UPD in the disomic cell line. During the first two cell divisions we believe that CD is the preferred method for correction, since ND will critically reduce the number of blastomeres, and AL can not produce fetal UPD in combination with normal karyotypes in all compartments. From the third cell division onwards, their is not much difference between the three types of correction. The frequently observed combination of a full trisomy in both placental cell lineages and a normal karyotype but UPD in the fetus can only be produced by ND or AL in the fourth cell division. Of all theoretical combinations of karyotypes in the various compartments as described by Pittalis et al. (1994), CPM II (abnormal cells confined to LTC CV) does not occur in this hypothetical model. This is in agreement with the observation that, up till now, fetal UPD has never been described in association with this type of CPM. Therefore, CPM type II most probably has a mitotic origin, as suggested by Wolstenholme (1996). Most published cases of UPD showed to be associated with CPM type III (abnormal cells in both semi-direct CV preparations and LTC CV). However, theoretically, it may be found in cases of CPM type I (abnormal cells confined to semi-direct CV preparations), involving either a 100 % trisomy, or high level mosaicism (> 67 %). This was actually shown by two cases (one of UPD 10, and one of UPD 9), described by Jones et al. (1995) and Wilkinson et al. (1996), respectively. Robinson et al. (1997) found a significant correlation between a meiotic origin of the trisomy and high levels of trisomic cells in both placental cell lineages, especially in the trophoblast. Our model shows that, at least theoretically, trisomic zygote rescue in the first two cell divisions through CD may result in low level mosaicism (about 33 %) in the cytotrophoblast.

Figure 3.2 Theoretical distributions of trisomic and disomic cells at the 2-, 4-, 8-, and 16-cell stage after trisomic zygote rescue through chromosome demolition (CD) and anaphase lagging (AL). The resulting karyotypes in long-term cultured CV (LTC-CV) and fetus, originating from the ICM, and in semi-direct CV preparations (short-term cultured CV=STC-CV), originating from trophoblast, are shown. A= abnormal, N= normal, M= mosaic.

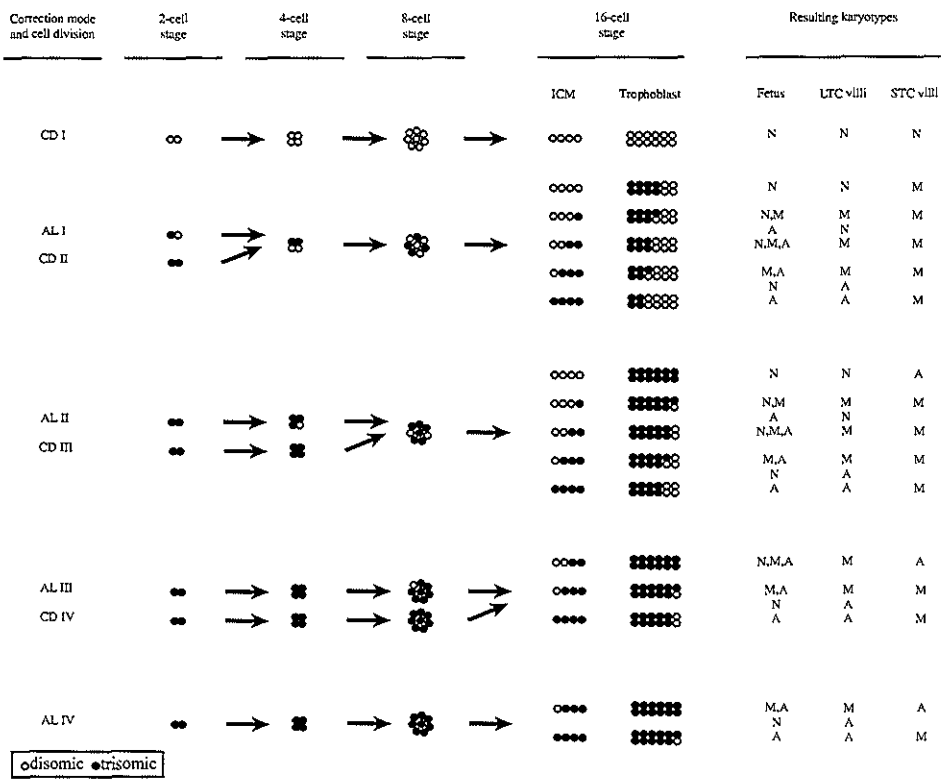
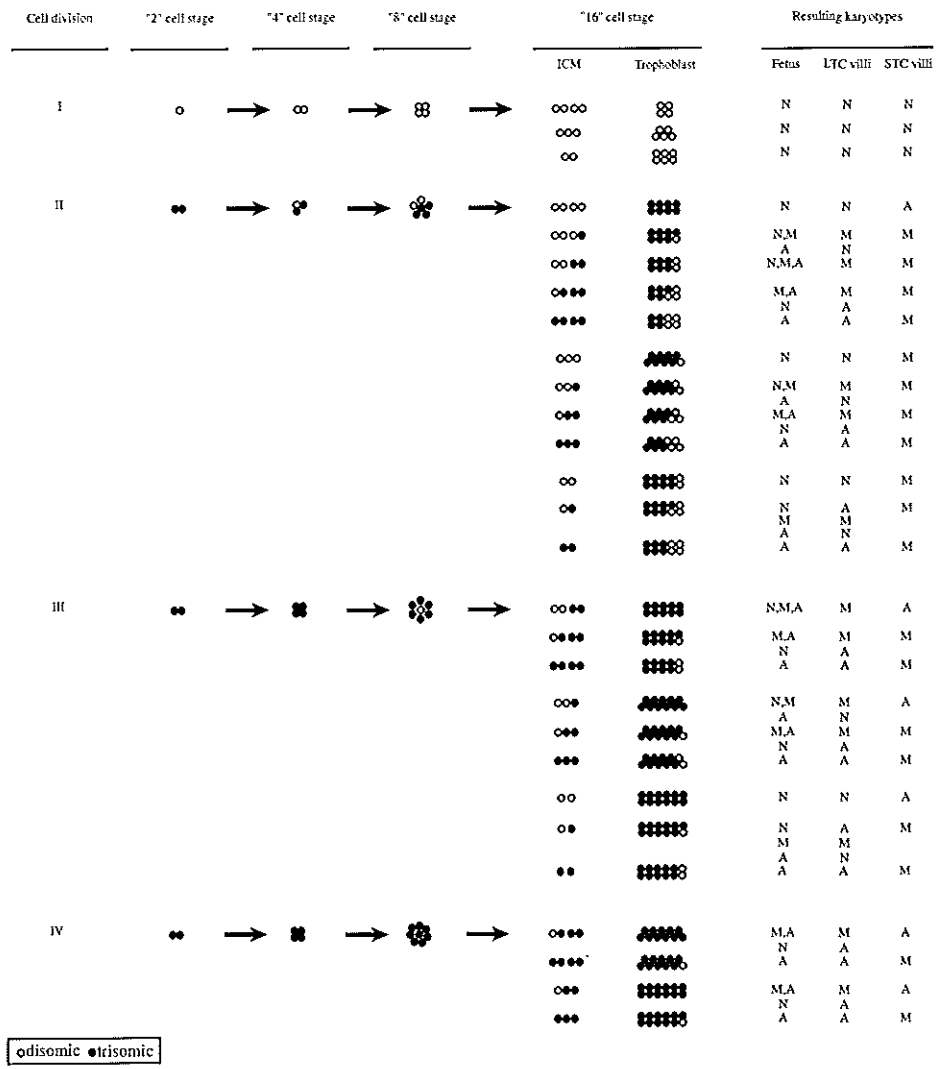


Figure 3.3. Theoretical distributions of trisomic and disomic cells at the 2-, 4-, 8-, and 16-cell stage after trisomic zygote rescue through non-disjunction (ND), with and without compensatory reallocation of the cells between inner cell mass (ICM) and trophoblast. The resulting karyotypes in long-term cultured CV (LTC-CV) and fetus, originating from the ICM, and in semi-direct CV preparations (short-term cultured CV=STC-CV), originating from trophoblast, are shown. A=abnormal, N= normal , M= mosaic.



Concluding remarks and future prospects

In our efforts to overcome limitations of traditional cytogenetic studies in amniotic fluid and chorionic villi cells, the FISH technique proved to be an indispensable tool for prenatal diagnosis. We use FISH as an adjunctive tool in about 8 % (about 200 samples per year) of all prenatal samples that we receive in our laboratory. In addition to the four indications discussed in this thesis, we now also use FISH for cytogenetic confirmation of all prenatally detected (non)-mosaic aneuploidies in uncultured fetal and/or placental tissues after termination of pregnancy.

Continuous development of new FISH probes, such as, recently, that of chromosome-specific subtelomeric probes (National Institutes of Health and Institute of Molecular Medicine Collaboration, 1996), will further improve the detection rate of chromosome aberrations with FISH at metaphase as well as interphase level. Comparative genomic hybridization (CGH) (Kallioniemi et al., 1992) may significantly add to interphase cytogenetics, as a technique for screening the entire genome for losses and gains of DNA. It may theoretically improve the detection rate of chromosome aberrations in uncultured amniotic fluid cells of pregnancies at high genetic risk (Bryndorf et al., 1995), and may allow the cytogenetic investigation of early cleavage embryos, which will be important for understanding the arising of chromosomal mosaicism during early embryogenesis (Delhanty et al., 1997). The development of new techniques, such as SKY (Schröck et al., 1996; Veldman et al., 1997) and micro-FISH (Viersbach et al., 1994; Engelen et al., 1996) will further improve the efficiency and accuracy of chromosome identification.

The introduction of FISH also added to preimplantation diagnosis (PID) of genetic diseases. The first applications of PID were to avoid X-linked diseases by selection of female embryos, and embryo gender was determined by using PCR amplification of X and Y chromosome specific sequences (Handyside et al., 1990). However, at the beginning of the 1990s, FISH with chromosome X and Y specific probes became the method of choice for embryo sexing (Griffin et al., 1992), since FISH is not affected by sample contamination and it allows to detect the chromosome X copy number as well, avoiding the transfer of embryos with sex chromosomal aneuploidy. In addition to further improvement of the diagnosis of single gene defects and extension of the range of diseases amenable to specific diagnosis, PID research is currently focusing on the diagnosis of chromosomal aneuploidy in preimplantation embryos of infertile women of advanced maternal age undergoing in vitro fertilization (IVF) in order to improve delivery rates. Multicolor FISH has already been used for screening of IVF embryos (Munné et al., 1993; 1995a), as well as of first and second polar bodies, as an alternative approach to PID (Verlinsky et al., 1996). However, several difficulties must still be addressed before PID of chromosome aberrations can routinely be applied in women of advanced reproductive age undergoing IVF (Reubinoff and Shushan, 1996).

FISH also opened the possibility to non-invasive prenatal diagnosis. Efforts have been made to develop such technology, since amniocentesis and chorionoic villus sampling are both associated with a low risk of fetal mortality and morbidity. A potential future non-invasive technique may be the analysis of fetal cells recovered from the maternal circulation. Several

groups were already successful in detecting fetal aneuploidy in preparations of maternal blood by using FISH with chromosome specific DNA probes (Price et al., 1992; Bianchi et al., 1992; Gänshirt-Ahlert et al., 1993; Jansen et al., 1997). Since fetal cells are extremely rare in maternal blood, the development of this technology has mainly been hampered by problems in isolation and enrichment of these cells. Therefore, introduction of this technique into clinical practice is still some way off.

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Summary

Amniocentesis and chorionic villus (CV) sampling are two widely used invasive techniques for prenatal diagnosis (PD). Their possibilities and limitations for cytogenetic studies are discussed in chapter I. The main limitations of classical cytogenetic analysis in amniotic fluid (AF) and CV cells are:

- the correct interpretation of chromosome aberrations with indistinct banding patterns (marker chromosomes, de novo structural chromosome aberrations)

- the time-consuming AF cell culturing, with a result about the fetal karyotype only available two to three weeks after sampling

- the detection of a (non)-mosaic chromosome aberration in CV, which is potentially confined to the placenta [confined placental mosaicism (CPM)] and not present in the fetus, and which may require follow-up investigations in AF cells, delaying the reporting time

- the interpretation of low level mosaicism in AF and CV cells, which may represent true mosaicism, but also pseudomosaicism (culture artefacts without clinical significance) or local mitotic division errors, respectively

- the limited resolution of the light microscope, with some chromosome rearrangements, such as microdeletions, going undetected

- the possibility of maternal cell contamination of the sample

In 1993 we introduced the fluorescent in situ hybridization (FISH) technique in our laboratory as an adjunctive tool to classical chromosome analysis in order to overcome some of these limitations. The main scope of this thesis is to evaluate the merits and demerits of this technique after implementation for diagnostic purposes during four years in our laboratory.

We have four main indications for FISH, which are discussed separately in chapter II. Indication I (section 3.1) involves the application of FISH for identification of structural chromosome aberrations. We experienced that FISH may be a useful tool for characterization of the breakpoints of cytogenetically detected structural chromosome rearrangements. This may be important for understanding the banding pattern of the abnormal chromosome. Moreover, we used FISH for rapid verification of an uncertain cytogenetic result (because of poor quality of the chromosomes), and for fast detection of subtle familial chromosome aberrations in the first trimester of pregnancy. However, FISH with chromosome-specific probes may be a time-consuming technique for identification of extra chromosomal material, such as marker chromosomes and de novo unbalanced chromosome rearrangements. Recently, new FISH techniques (micro-FISH, SKY, CGH) have been developped, which will enable a much more efficient and accurate identification than traditional FISH in the near future. PCR analysis of polymorphic microsatellite repeats showed to be a powerful tool for determination of the extent of a deleted chromosomal segment, if relevant FISH probes are not available.

Indication 2 (section 3.2) involves the rapid detection of numerical chromosome aberrations in uncultured AF cells in pregnancies at high genetic risk in which a fast result is important. If fetal anomalies, suspicious for one of the most common chromosome aberrations (trisomy 13, 18, 21, triploidy, 45,X) are detected by ultrasound (US), a rapid result is necessary when gestational age comes close to 24 weeks, after which termination of pregnancy is prohibited by law, or when impending birth demands decisions concerning obstetric and perinatal policy (section 3.2.1). We screened uncultured AF cells with a set of chromosome X, Y, 13, 18, and 21 specific probes (the so-called US probe set) in 196 cases and we were able to detect 43 (77 %) of 56 chromosome aberrations found among these samples. Thirteen chromosome aberrations went undetected, because other chromosomes than X, Y, 13, 18, or 21 were involved (10 cases), or because of technical problems (3 cases). We conclude that FISH on uncultured AF cells is a fast (within two days after sampling) and highly reliable method for rapid detection of chromosome aberrations if appropriate probes are used. False positive (7 cases) and false negative results (one case) were mainly encountered during the first two years of our investigations, and could further be prevented by improving the technique. Since in 18 % of the chromosome aberrations in this study other chromosomes than X, Y, 13, 18, and 21 were involved, a normal FISH result should always be complemented by cytogenetic analysis of the cell cultures.

If a chromosome aberration other than full trisomy 21, triploidy, 47,XXX, 47,XXY, or 47,XYY, is detected in semi-direct CV preparations, in the absence of US abnormalities, it might be confined to the placenta and a follow-up amniocentesis for verification of the fetal karyotype may be necessary, especially if cultured CV are not available. This severely prolongs the reporting time. FISH on uncultured AF cells for rapid differentiation between CPM and generalized mosaicism (section 3.2.2), was found to be an accurate method, since in 48 out of 51 cases FISH correctly identified the fetal karyotype. However, false positive (two cases) and false negative (one case) results may occur. They may occasionally be the result of inefficient or unspecific probe hybridization, although we found evidence that false positive FISH findings may arise from selection against abnormal cells in the cell cultures, with cytogenetic results in fact representing false negative results.

Application of FISH on uncultured AF cells in some other situations requiring a rapid result (low level mosaicism detected in a previous AF sample, maternal age ≥ 44 years, triple test results indicative for a high risk (≥ 5 %) of Downs syndrome, twin pregnancies of advanced gestational age, sex determination in pregnancies at risk for X linked diseases) (section 3.2.3) showed to be highly reliable, since no discrepancies were found between FISH and cytogenetic results in all 46 cases that we investigated.

In section 3.3 we describe our experience with FISH for studying low level mosaicism in AF cell cultures and CV semi direct preparations in order to discriminate between pseudomosaicism (in AF cultures) or local mitotic division errors (in CV) and mosaicism (indication 3). Differentiation between both phenomena requires the analysis of a large number of cells which may be difficult due to a limited number of metaphases. Interphase FISH on cultured AF cells of samples showing one abnormal colony was found to be an efficient and accurate tool for determination whether any additional cells in the different culture vessels of one sample showed the chromosome abnormality. When pseudomosaicism

is established, a second amniocentesis for confirmatory studies can be obviated.

If chromosomal mosaicism is encountered in semi-direct CV preparations, a follow-up amniocentesis may be necessary for verification of the fetal karyotype. This meant a second invasive procedure for 2,1 % of women undergoing CVS during the time period 1993-1996. However, the question is whether a follow-up amniocentesis is really always necessary, since in many cases low level mosaicism (≤ 33.3 % abnormal cells) is encountered which may represent low level mosaicism, although the few abnormal cells may also result from local errors during mitosis. We used interphase FISH in 37 cases of low level mosaicism: 13 cases showed abnormal and 24 cases showed normal FISH results, indicating local mitotic division errors. In 12 of these 24 cases, a follow-up amniocentesis was performed, all with normal results. Healthy children were born in all 24 cases. We believe that FISH results better reflect the real level of mosaicism than traditional cytogenetic analysis, since FISH analysis involves the scoring of a few hundreds of cells. This means that a normal karyotype can be reported to the parents when FISH results are normal and follow-up investigations can be omitted in these cases.

If non-mosaic or mosaic trisomy 18 is encountered in semi-direct CV slides, follow-up investigations in cultured CV and/or AF cells are necessary, since the trisomy may be confined to the placenta. Since CV cultures were not performed in our laboratory during many years, we investigated the use of FISH on semi-direct CV preparations as a potential tool of rapid verification. We found that interphase FISH may add to the cytogenetic data, in terms of predicting the fetal karyotype, since a higher percentage of three signal containing nuclei were found in the confirmed cases as compared to the non-confirmed cases. However, since FISH yielded ambivalent results in some cases, we conclude that a final result can only be achieved by analysis of a subsequent AF sample.

In section 3.4, we describe our experience with FISH for the detection of microdeletions associated with specific syndromes (indication 4). These microdeletions can not be identified with conventional banding techniques, and FISH showed to be an accurate method for their detection, irrespective of the quality of the chromosomes. Among 48 cases that we investigated, one 22q11 deletion was encountered, which was known to be present in a previous child with the classical DiGeorge syndrome. After the prenatal findings, the deletion was also found in the father, confirming that this syndrome may be sporadically transmitted as an autosomal dominant trait. FISH results in all other cases were normal.

Chapter III deals with the phenomenon of CPM involving a trisomy which may potentially be associated with fetal uniparental disomy (UPD) (both chromosomes of a chromosome pair derived from one parent only), if the trisomy has a meiotic origin and one particular copy of the set of three chromosomes is lost (trisomic zygote rescue). Fetal UPD may explain the perinatal complications occasionally found in cases of CPM. We investigated the incidence of UPD associated with CPM in a consecutive series of CV samples collected during four years in order to get more insight into the origin of CPM, and into the mechanism of trisomic zygote rescue. Twenty-nine cases of CPM involving a trisomy were found between 1992 and the end of 1995, and DNA studies were performed in 23 cases. Only one case of fetal UPD (UPD 16) was found, indicating that in most cases the trisomic cell line most probably

originates from somatic duplication. This is supported by the low level of mosaicism in most of these cases. Furthermore, the normal pregnancy outcome in this case further supports the hypothesis that the impaired fetal growth encountered in most cases of UPD 16, and the congenital maformations found in some of them, are probably not the result of the UPD itself. Moreover, the obstetrical complications encountered in seven out of 23 CPM cases showed not to be the consequence of fetal UPD.

During the same study period, another two cases of UPD (one case of UPD 15 and one of UPD 16), both associated with a normal karyotype in CV, were accidently found. Despite the absence of trisomic cells in CV, trisomic zygote rescue might be the cause of UPD in these cases. The mechanism of trisomic zygote rescue is not known, but anaphase lagging (AL) as well as non-disjunction (ND) in an early postzygotic cell division have been proposed. However, AL can not explain the combination of fetal UPD and a normal CV karyotype, and ND will significantly reduce the number of blastomeres of the early embryo, which may hamper its normal development. Therefore, we propose an alternative correction mode, chromosome demolition (CD), which involves the destruction and removal of one of the set of three chromosomes, resulting in two disomic daughter cells. We present a model for the arising of the various combinations of karyotypes in semi-direct CV slides, cultured CV, and fetus from trisomic zygote rescue with each of the different correction modes. All cases of UPD associated with CPM or with a normal karyotype in CV semi-direct preparations fit this model. It further shows that trisomic zygote rescue does not necessarily result in a 100 % trisomy or high level mosaicism in semi-direct CV preparations, as was suggested by some investigators, but it may theoretically result in a low level of mosaicism.

Samenvatting

Vruchtwaterpunctie (amniocentese) en vlokkentest (chorion villus biopsie) zijn twee wijdverspreide invasieve technieken voor prenatale diagnostiek. De mogelijkheden en beperkingen van cytogenetisch onderzoek in vruchtwater (VW) en chorion villi (CV) cellen worden besproken in hoofdstuk I. De belangrijkste beperkingen van het klassieke chromosoomonderzoek in VW en CV cellen zijn:

- de moeilijke interpretatie van chromosoomafwijkingen in geval van een onduidelijk bandenpatroon (marker chromosomen, de novo structurele chromosoomafwijkingen)

- de tijdrovende VW celkweek, waardoor het foetale karyotype pas twee à drie weken na de punctie bekend is

- de detectie van een chromosoomafwijking in CV die beperkt kan zijn tot de placenta [confined placental mosaicism (CPM)] en dus niet aanwezig is in de foetus; dit vereist eventueel een vervolgonderzoek in VW cellen, wat de wachttijd voor een definitieve uitslag aanzienlijk verlengt

- de juiste interpretatie van een laag mozaïek chromosoomafwijking, als een mozaïek of een pseudomozaïek (kweekartefact zonder klinische betekenis) in VW cellen, dan wel een mozaïek of een locale mitotische delingsfout in CV cellen

- de beperkte resolutie van de lichtmicroscoop, waardoor sommige chromosoomveranderingen, zoals microdeleties, kunnen gemist worden

- de mogelijkheid van maternale cel contaminatie van VW en CV specimen

In 1993 hebben we de 'fluorescentie in situ hybridisatie' (FISH) techniek geintroduceerd in ons prenataal cytogenetisch laboratorium als een aanvullende test op het klassieke chromosomen onderzoek, met het doel een aantal van de bovenvermelde beperkingen te overbruggen. De belangrijkste doelstelling van dit proefschrift is de evaluatie van de voor- en nadelen van FISH, nadat we deze techniek gedurende vier jaren hebben gebruikt voor diagnostische doeleinden.

We onderscheiden vier indicaties voor FISH die afzonderlijk worden besproken in hoofdstuk II. Indicatie 1 (paragraaf 3.1) betreft de toepassing van FISH voor identificatie van structurele chromosoomafwijkingen. Onze ervaring heeft geleerd dat FISH een nuttige techniek kan zijn voor het vaststellen van breukpunten van deze chromosoomafwijkingen, wat van belang kan zijn om het banderingspatroon van het afwijkende chromosoom beter te interpreteren. Tevens gebruiken we FISH voor de snelle bevestiging van een onzeker cytogenetisch resultaat (onzeker vanwege een onvoldoende chromosoomkwaliteit), en voor snelle detectie van subtiele familiaire chromosoomafwijkingen in het eerste trimester van de zwangerschap. Echter, FISH met chromosoom-specifieke probes is potentieel een zeer tijdrovende techniek voor de identificatie van extra chromosomaal materiaal, zoals marker chromosomen en de novo ongebalanceerde chromosoomafwijkingen. Recent zijn nieuwe FISH technieken (micro-

FISH, spectral karyotyping, comparative genomic hybridization) ontwikkeld, die in de nabije toekomst een meer efficiente en accurate identificatie mogelijk zullen maken. Indien relevante FISH probes niet beschikbaar zijn , bleek PCR analyse van polymorfe microsatelliet repeats een nuttige aanvullende techniek te zijn voor het bepalen van de grootte van een gedeleteerd chromosomaal segment.

Indicatie 2 (paragraaf 3.2) betreft de snelle detectie van numerieke chromosoomafwijkingen in ongekweekte VW cellen van zwangerschappen met een groot risico op een chromosoom-afwijking indien een snel resultaat belangrijk is. Dit is het geval wanneer echografisch vastgestelde foetale afwijkingen verdacht zijn voor een van de meest voorkomende chromosoom-afwijkingen (trisomie 13, 18, 21, triploidie, 45,X) en de zwangerschapsduur 24 weken nadert of een spoedige geboorte wordt verwacht. Zwangerschapsterminering na 24 weken is wettelijk verboden. Indien een geboorte dreigt zijn snelle beslissingen omtrent obstetrisch en perinataal beleid nodig (paragraaf 3.2.1). We onderzochten 196 ongekweekte VW monsters met een set van chromosoom X, Y, 13, 18, en 21 specifieke probes (de zogenoemde US (ultrasound) probe set) en we waren in staat om 43 (77 %) van de 56 chromosoomafwijkingen op te sporen. Dertien afwijkingen werden niet gedetecteerd, omdat er andere chromosomen dan X, Y, 13, 18, of 21 bij waren betrokken (10 casus) of omwille van technische problemen (3 casus). We concluderen dat FISH op ongekweekte VW cellen een snelle (binnen twee dagen na VW punctie) en zeer betrouwbare methode is voor snelle detectie van chromosoomafwijkingen indien geschikte probes worden gebruikt. Fout positieve (7 casus) en fout negatieve (1 casus) resultaten werden voornamelijk gevonden tijdens de eerste twee jaren van het onderzoek en konden verder worden voorkomen door voortdurende verbetering van de techniek. Aangezien in 18 % van de chromosoomafwijkingen in deze studie andere chromosomen waren betrokken dan X, Y, 13, 18, en 21, zal een normaal FISH resultaat steeds moet worden aangevuld met karyotypering van gekweekte cellen.

Indien een chromosoomafwijking anders dan trisomie 21, triploidie, 47,XXX, 47,XXY, of 47,XYY wordt gevonden in semi-directe CV preparaten, kan vervolgonderzoek in VW cellen noodzakelijk zijn voor verificatie van het foetale karyotype, vooral als gekweekte CV niet beschikbaar zijn. Uiteraard verlengt dit de wachttijd voor een definitief resultaat aanzienlijk. Het gebruik van FISH op ongekweekte VW cellen na eerdere afwijkende bevindingen in vlokken bleek een accurate methode te zijn voor een snelle differentiatie tussen CPM en gegeneraliseerd mozaïek (paragraaf 3.2.2). In 48 van de 51 onderzochte casus werd het foetale karyotype correct geidentificeerd middels FISH. Echter, fout positieve (2 casus) en fout negatieve (1 casus) resultaten kwamen voor. Zij kunnen af en toe het resultaat zijn van inefficiente of niet-specifieke probe hybridisatie, alhoewel wij aanwijzingen vonden dat fout positieve FISH bevindingen ook het gevolg kunnen zijn van selectie ten voordele van normale cellen in de celkweken, waardoor het cytogenetische resultaat in gekweekte cellen in feite een fout negatieve bevinding is.

Het gebruik van FISH op ongekweekte VW cellen in nog enkele andere situaties die een snelle uitslag vereisen (laag mozaïek in een voorgaand VW sample, maternale leeftijd ≥44 jaar, afwijkende triple test met een ≥ 5 % risico op Down syndroom, tweeling zwanger-schappen indien de zwangerschapsduur ongeveer 16 weken bedraagt, geslachtsbepaling in

zwangerschappen met een verhoogd risico op een geslachtsgebonden ziekte) (paragraaf 3.2.3) bleek zeer betrouwbaar te zijn , aangezien er geen discrepanties werden gevonden tussen FISH resultaten en karyotype in alle 46 casus die we onderzochten.

In paragraaf 3.3 beschrijven we onze ervaring met de toepassing van FISH voor het bestuderen van laag mozaïeken in VW celkweken en CV semi-directe preparaten, teneinde een onderscheid te maken tussen pseudomozaïeken of locale delingsfouten en mozaïeken (indicatie 3). Deze differentiatie vereist analyse van een groot aantal cellen/clonen, wat soms bemoeilijkt wordt door de aanwezigheid van slechts een beperkt aantal analyseerbare metafasen. Interfase FISH op gekweekte VW cellen in geval van één chromosomaal afwijkende clone, bleek een zeer efficient en accuraat middel te zijn om na te gaan of in de verschillende kweekbakjes nog meer cellen met dezelfde chromosoomafwijking aanwezig waren. Op deze manier kan, bij bevestiging van de aanwezigheid van een pseudomozaïek, een eventueel vervolgonderzoek in een tweede vruchtwatermonster worden voorkomen.

Indien een mozaïek chromosoompatroon wordt gevonden in semi-directe CV preparaten, is vervolgonderzoek in vruchtwater soms nodig voor verificatie van het foetale karyotype. In de periode 1993-1996 betekende dit een tweede invasieve ingreep voor 2,1 % van alle zwangeren die een vlokkentest ondergingen. De vraag is of dit vervolgonderzoek ook echt altijd nodig is, aangezien er vaak een laag mozaïek (≤ 33 % abnormale cellen) aanwezig is, wat echt een CPM kan betekenen, doch mogelijk ook het gevolg kan zijn van een locale mitotische delingsfout in de CV. We gebruikten interfase FISH in 37 casus met een laag mozaïek, en vonden in 13 casus een afwijkend FISH resultaat, maar in de overige 24 casus bleken de FISH resultaten normaal te zijn. In 12 van deze 24 casus werd nog een vervolgonderzoek gedaan in vruchtwater, en in alle gevallen werd een normaal karyotype gevonden. Alle 24 zwangerschappen met een normaal FISH resultaat eindigden met de geboorte van een gezond kind. Omdat interfase FISH het onderzoek betreft van enkele honderden cellen, geven de FISH resultaten een beter beeld van de werkelijke hoogte van het mozaïek dan traditioneel cytogenetische resultaten. Dit betekent dat als FISH resultaten normaal zijn, een vervolgonderzoek in VW cellen achterwege kan blijven.

Als (mozaïek) trisomie 18 wordt gevonden in semi-directe CV preparaten, is vervolg-onderzoek in gekweekte CV, en eventueel in VW cellen, noodzakelijk, omdat de afwijking beperkt kan zijn tot de placenta. We onderzochten de bruikbaarheid van interfase FISH op semi-directe CV preparaten als potentieel middel voor snelle verificatie van het foetale karyotype, vooral omdat CV kweken niet voorhanden waren gedurende de studieperiode. We vonden dat FISH resultaten een predictieve waarde hadden voor het foetale karyotype, in die zin dat een hoger percentage cellen met drie signalen werd gevonden in die casus waarbij de trisomie 18 werd bevestigd in foetale cellen in vergelijking met de niet-bevestigde casus. Echter, wegens het af en toe voorkomen van ambivalente FISH resultaten, moeten we concluderen dat een definitieve uitslag slechts kan worden verkregen door een vervolg-vruchtwateronderzoek.

In paragraaf 3.4 beschrijven we onze ervaring met FISH voor de detectie van microdeleties die geassocieerd zijn met specifieke syndromen (indicatie 4). Deze microdeleties kunnen niet worden geidentificeerd met behulp van conventionele banderingstechnieken. We

onderzochten 48 casus met behulp van FISH en vonden één 22q11 deletie, waarbij reeds eerder eenzelfde deletie was aangetoond in een voorgaand kind met het klassieke DiGeorge fenotype. De deletie werd na de prenatale bevindingen ook gevonden bij de vader, wat bevestigt dat dit syndroom soms familiair voorkomt.

Hoofdstuk III behandelt het fenomeen van trisomie CPM, wat geassocieerd kan zijn met foetale uniparentele disomie (UPD). UPD betekent dat beide chromosomen van een chromosoompaar afkomstig zijn van één en dezelfde ouder. Dit verschijnsel kan zich voordoen als de trisomie een meiotische oorsprong heeft, en het chromosoom afkomstig van de ouder die er één bijdraagt, verloren gaat. Deze reductie van trisomie naar disomie noemt men 'trisomic zygote rescue'. Foetale UPD is één van de mogelijke verklaringen voor de perinatale complicaties die af en toe optreden in zwangerschappen met CPM. We onderzochten de frequentie van foetale UPD geassocieerd met trisomie CPM gedurende vier jaren, met de bedoeling meer inzicht te krijgen in het ontstaan van CPM, en in het mechanisme van trisomic zygote rescue. In totaal werden 29 casus met trisomie CPM gevonden, waarvan in 23 DNA studies konden worden verricht. Slechts één casus met UPD 16 werd gevonden, wat aangeeft dat in de meeste gevallen de trisomie cellijn hoogstwaarschijnlijk ontstaat als gevolg van somatische duplicatie. Dit wordt ondersteund door de aanwezigheid van een laag mozaïek in de cytotrophoblast in de meeste van deze gevallen. De normale zwangerschapsuitkomst in de UPD 16 casus ondersteunt de hypothese dat de foetale groeiachterstand die optreedt in de meeste, en de congenitale afwijkingen die worden gezien in sommige casus met UPD 16, waarschijnlijk niet worden veroorzaakt door de UPD 16 zelf. Bovendien kunnen we concluderen dat de obstetrische complicaties die zich voordeden in zeven van de 23 onderzochte zwangerschappen met CPM niet het gevolg waren van UPD.

Gedurende dezelfde studieperiode werden nog twee andere casus met UPD (één met UPD 16 en één met UPD 15) gevonden. Beiden waren geassocieerd met een normaal karyotype in CV. Niettegenstaande de afwezigheid van trisome cellen in CV lijkt trisomic zygote rescue toch de meest waarschijnlijke oorzaak van UPD in deze casus. De manier waarop het extra chromosoom verloren gaat, is niet bekend, maar 'anaphase lagging' (AL) en 'non-disjunctie' (ND) tijdens één van de eerste postzygotische celdelingen werden reeds als mogelijke mechanismen voorgesteld. Echter, AL kan niet de combinatie foetale UPD en normaal CV karyotype verklaren, en ND leidt tot een significante reductie van het aantal blastomeren in het vroege embryo, wat een verdere normale ontwikkeling kan bemoeilijken. Daarom stellen wij een alternatieve correctiemethode voor, 'chromosome demolition' (CD), wat inhoudt dat één van de drie chromosomen wordt verwijderd, resulterend in twee disome dochtercellen. We presenteren een model voor het ontstaan van alle mogelijke combinaties van karyotypes in cytotrophoblast cellen, gekweekte CV cellen, en foetus als gevolg van trisomic zygote rescue middels de drie bovengenoemde correctiemethoden. Alle UPD casus geassocieerd met CPM of met een normaal CV karyotype passen in dit model. Het toont verder aan dat trisomic zygote rescue niet noodzakelijk resulteert in een 100 % of hoog mozaïek trisomie in semi-directe CV preparaten, zoals eerder werd gesuggereerd door sommige onderzoekers, maar dat het theoretisch ook kan leiden tot veel lagere mozaïeken in de cytotrophoblast.

Abbreviations

AF	amniotic fluid
AL	anaphase lagging
CCD	charge-coupled device
CD	chromosome demolition
CGH	comparative genomic hybridization
CPM	confined placental mosaicism
CV	chorionic villi
CVS	chorionic villi sampling
DNA	deoxyribonucleic acid
EEM	extraembryonic mesoderm
FISH	fluorescent in situ hybridization
ICM	inner cell mass
ISH	in situ hybridization
IUGR	intrauterine growth retardation
IVF	in vitro fertilization
MCC	maternal cell contamination
ND	non-disjunction
PCR	polymerase chain reaction
PD	prenatal diagnosis
SKY	spectral karyotyping
UPD	uniparental disomy
US	ultrasound
VW	vruchtwater
WCP	whole chromosome paint

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Dankwoord

Eindelijk is het dan zover! Het boekje is klaar. Graag maak ik van de gelegenheid gebruik om een aantal mensen te bedanken zonder wiens hulp en steun dit proefschrift er misschien nooit zou zijn geweest.
Op de eerste plaats Prof. Dr. E.S. Sachs. U ben ik zeer erkentelijk voor de kans die U mij destijds heeft geboden om naast het dagelijkse labwerk ook wat onderzoek te doen, waarvan de resultaten leidden tot mijn eerste publicaties.
Frans, zonder jouw enthousiaste begeleiding, jouw vertrouwen in mijn praktische en theoretische kennis, je bewonderenswaardige belezenheid, en heldere inzicht in bepaalde prenatale vraagstukken, zou dit boekje er op zijn minst minder fraai hebben uitgezien. Bedankt voor je onvoorwaardelijke steun in de afgelopen maanden. Ik ben ontzettend blij onze samenwerking te kunnen continueren.
Mijn promotor Prof. Dr. H. Galjaard ben ik zeer dankbaar voor het vertrouwen dat hij steeds in mij heeft gesteld en voor de kansen die hij mij heeft geboden om mij als analist verder te ontplooien. Evenals de overige leden van de promotie-commissie, Prof. Dr. J.W. Wladimiroff, Prof. Dr. N.J. Leschot en Prof. Dr. P.J. Willems, ben ik hem zeer erkentelijk voor het snelle beoordelen van het manuscript en voor de nuttige adviezen t.a.v. inhoud en stijl waardoor het uiteindelijk beter werd.

Alle stafleden en hun vele medewerkers van de 24e verdieping wil ik bedanken voor de plezierige sfeer waarin ik de afgelopen jaren heb mogen werken. Jan, jou wil ik in het bijzonder bedanken voor je morele steun, die ik, vooral in de beginperiode, af en toe nodig had, en uiteraard ook voor al je wetenschappelijke hulp bij o.a. het schrijven. Peter (in 't Veld), dank je wel voor jou bijdrage aan mijn onderzoek en voor je hulp bij mijn publicaties. Ik wens je veel succes in het diabetes onderzoek! Dicky, Ans en Ben, bedankt voor jullie gastvrijheid in de tijd dat we nog geen FISH-lab hadden en we dankbaar gebruik konden maken van jullie ruimte, apparatuur en andere spullen. Dicky en Ans, bedankt voor jullie bereidheid om steeds hulp en advies te bieden als DNA technieken nodig zijn voor het oplossen van een cytogenetisch probleem. Dit heeft reeds geleid tot een aantal publicaties waarvan drie in dit proefschrift. Wim, bedankt voor het ter beschikking stellen van de ruimte die we nodig hadden voor het inrichten van een FISH lab.

Dit proefschrift kon alleen maar tot stand komen dank zij de tomeloze inzet en de steun van mijn collega's van het prenataal cytogenetisch laboratorium. Dit is ook jullie boekje! Cardi, jou ben ik veel dank verschuldigd voor het vertrouwen dat je de afgelopen jaren steeds in mij hebt getoond, en voor je enorme steun als ik het even niet meer zag zitten. Jij hebt mij destijds de chromosomen leren kennen en de liefde voor het vak bijgebracht. Ik heb grote bewondering voor de manier waarop jij je als hoofdanalist inzet voor het lab (zowel de mensen als de werkzaamheden), en ben er trots op dat je mijn paranimf wilde zijn. Armando (ABart), bedankt voor alle prachtige dia's, foto's, posters die je doorheen de jaren voor mij hebt gemaakt en voor alle adviezen t.a.v. lay-out e.d. (volgens mij heb je je roeping echt gemist). Ook al was ik niet altijd je gemakkelijkste klant ("veel te veel voor één dia", "veel te weinig tijd"), toch maakte je er steeds weer iets moois van. In het bijzonder bedankt voor alle prachtige illustraties in dit boekje! Petra ("Peppi"), wij zijn tien jaar geleden ongeveer gelijktijdig begonnen bij de vlokken. Verschillende collega's die kort na ons kwamen, zagen we snel weer vertrekken. Ik ben blij dat jij bleef! Annemarie (AMS)

bedankt voor de vrolijke noot waarmee je ons dagelijks verblijdt (al zou ik af en toe die knop wel eens willen omdraaien!). Joke, jij bent denk ik de meest veelzijdige werkkracht bij prenataal. Of het nu vlokken zijn, vruchtwaters, AFP's of FISH, in alles ben je zeer bedreven en toegewijd. Dank zij jouw flexibele houding kunnen we daar ook dankbaar gebruik van maken. Jacqueline, jij hebt zo je eigen stijl. Heel erg bedankt voor al je inzet. Els en Nicole, jullie zijn al lang meer dan collega's. Ik hoop dat onze periodieke etentjes (samen met Loes) nog lang blijven voortduren, zodat we weer eens lekker kunnen bijkletsen over "van alles en nog wat". Gerda, samen met Joke een oude rot in het vak. Mensen met jouw ervaring zijn onmisbaar voor het goed draaiende houden van een lab. En je bent echt niet de enige zonder vlokkenervaring, hoor. Simone, je inzet voor een gezonde werkomgeving en goede voorlichting van patient en arts vind ik zeer te bewonderen. Hopelijk komt die brochure er nu eindelijk eens, en zijn je inspanningen niet tevergeefs geweest. Ellen, behalve naar chromosomen gaat jouw hart ook uit naar dieren. Bedankt nog voor de goede zorgen die je Cujo gaf. Jammer dat hij zo snel al het loodje legde. Japie (de Mosselman), ik ken niemand die zo lekker mosselen kan koken als jij! Wanneer doen we dat nog een keer over ? Bedankt voor je goede humeur. Martin (Flatfish), bedankt voor alle bestellingen die de afgelopen jaren via jou liepen. Ongeloofelijk dat die ronde glaasjes toch steeds weer aankwamen, ook al leek het er even op dat de firma niet bestond. Constant, Monique en Ragonda, ik heb nog weinig gelegenheid gehad om jullie echt goed te leren kennen. Dat jullie alle drie een aanwinst zijn voor het lab staat buiten kijf. Robert-Jan, ik weet dat de combinatie research en prenatale diagnostiek je soms zwaar valt, en dat je af en toe wat meer tijd zou willen hebben voor experimenten. Daarom waardeer ik je inzet en interesse voor de labactiviteiten en kijk ik ernaar uit onze samenwerking voort te zetten. Mieke (jij hoort uiteindelijk ook bij prenataal), bedankt voor de plezierige en nuttige werk/privé discussies die we de afgelopen jaren hebben gevoerd, en voor je hulp en vooral geduld bij het efficient in orde brengen van mijn referentielijst (ik besef dat ik een slechte leerling was).

Mijn collega's van postnatale cytogenetica o.l.v. Jan en Cokkie ben ik zeer dankbaar voor de prettige samenwerking. Alhoewel we de afgelopen tien jaren fysiek wel uit elkaar zijn gegroeid, vereisen onze werkzaamheden gelukkig een nauw contact, waarin ik mijzelf steeds heel prettig heb gevoeld. Bert, jij bracht mij de grondbeginselen bij van de FISH techniek, maar je bent doorheen de jaren vooral een zeer stimulerende en inspirerende bron geweest. Ik bewonder je tomeloze energie, je aanstekelijke enthousiasme, je betrokkenheid, brede interesse en grote kennis van zaken, en ben dan ook heel blij dat je mijn paranimf wilde zijn. Annet, jou wil ik in het bijzonder bedanken voor je collegialiteit en je vriendschap. Soms verlang ik nog wel eens terug naar de tijd dat we slechts met drieën "FISHten", en communicatiestoornissen nog een zeldzaamheid waren.
De collega's van DNA diagnostiek o.l.v. Dicky en Ans wil ik bedanken voor de gastvrije en gezellige werksfeer en voor de hulp die ik mocht ontvangen bij het PCR' en. Carola, bedankt voor de precieze manier waarop je mij de techniek hebt geleerd. Wout, dank je wel voor alle wijze raad, en Sarvan, bedankt voor al het werk dat je van mij overnam toen ik tijdelijk het RA-lab niet in kon.

Alle medewerkers van de sector prenatale diagnostiek en verloskunde, afdeling gynecologie/verloskunde van het Academisch Ziekenhuis Rotterdam, m.n. Prof. Dr. J.W. Wladimiroff, Dr. M.G.J. Jahoda, Helen, Hajo, Titia, Nicolette, Ernst, en Marja en onze Dordse connectie Dr. Leen Pijpers van de afdeling gynecologie en verloskunde van het Merwede

Ziekenhuis in Dordrecht wil ik bedanken voor al hun bijdragen aan het onderzoek.

Ook alle mensen van wie we in de loop der jaren FISH-probes en -adviezen ontvingen (teveel om op te noemen) ben ik zeer erkentelijk. Zonder anderen te kort te willen doen, wil ik een speciaal woord van dank richten aan Dr. J. G. Wauters (U.I.A, Antwerpen). Jij leverde ons de zo fel begeerde 13-probe (naast anderen), waarvan we nog steeds wekelijks dankbaar gebruik maken. En dank zij jouw nuttige tip voor het zwellen van celkernen konden we onze resultaten verder verbeteren. Heel erg bedankt voor je vriendelijkheid!

Dan zijn er nog een aantal mensen die indirect hun bijdrage leverden en die ik hier wil bedanken. Eerst en vooral de secretaressen Jaqueline, Jolanda, Hermine, Cilesta en Jeannette, voor al jullie hulp bij het faxen, post versturen, tekstverwerking, en oplossen van kopieerapparaat- en printer-problemen, en voor de gezellige babbel(s). Peter van Vuuren voor je hulp als de PC weer eens kuren vertoonde (of ligt het aan de gebruiker !?). Ruud, Tom en Mirko voor het fotografische werk dat jullie gedurende al die jaren voor mij hebben verzet. Melle, Rein en Mieke voor alle bestellingen, en Jopie, Joke, Elly, Rob, Wilma en Louise voor alle keukenactiviteiten.

Naast mijn werkkring wil ik ook vrienden en familie bedanken voor hun niet aflatende interesse voor mijn werkzaamheden (al blijft het toch moeilijk voor te stellen wat ik nu feitelijk doe).
Pa en ma, bedankt voor de kans die jullie me gaven om te studeren, voor het tijdig bijsturen, en vooral voor jullie vertrouwen en steun bij alle keuzes die ik moest maken. Ik weet dat jullie ontzettend trots op mij zijn. Besef dat dit wederzijds is.
John, zonder jouw flexibiliteit en grote loyaliteit was dit boekje er echt nooit geweest. Lieve Jimi, ik ben blij om samen met jou af en toe weer eens kind te kunnen zijn, om zo het "serieuze" werk even te vergeten.

Appendix publications I-VIII

Appendix publication I

Prenat. Diagn., 13, 825-832 (1993)

Application of Fluorescent In Situ Hybridization for "de-novo" anomalies in Prenatal Diagnosis

D. Van Opstal, H.J. Eussen, J.O. Van Hemel, E.S. Sachs

Summary

Fluorescent In Situ Hybridization (FISH) was carried out for 3 cases of abnormal karyotypes in prenatal studies. Two concerned de-novo structural anomalies and the 3rd a marker chromosome. The origin of the extra material could be defined in all 3 cases which gives better insight into the relationship between genotype and phenotype and makes more adequate genetic counseling possible.

Introduction

Prenatal diagnosis has its limitations. The fetus can only be seen on ultrasound and physical examination is not possible. Cytogenetic problems are caused by de novo structural anomalies such as deletions, inversions and partial trisomies. The possibility of Fluorescent In Situ Hybridization (FISH) studies have made a more accurate diagnosis of these structural anomalies possible which results in better counseling of the prospective parents (Callen et al., 1992, Jauch et al., 1990, Stetten et al., 1992). We report on the identification of three structural chromosome anomalies in prenatal diagnosis. FISH showed the origin of a de-novo partial trisomy of chromosome 11 and 18, and a partial trisomy of chromosome 15 respectively. The data provided us a better understanding of the fetal anomalies.

Material and methods

Cell preparations

Cultivation and chromosome preparations of amniotic fluid cells, skin fibroblasts and lymphocytes were performed according to standard techniques. Amniotic fluid cells were cultured with the in-situ method on glass-coverslips. Trypsin-Giemsa staining was used routinely. Additional staining techniques such as DA-DAPI-, NOR- and C-banding were applied in one case. Unstained preparations used the same day for FISH were put on a hot plate (65°C) for 2 hours. The slides were incubated in 70% acetic acid for 1 min. After washing in PBS, the cells were treated with RNase (Pharmacia) (100 µg/ml in 2 x SSC) at 37°C for 1 hr, followed by a pepsin (Serva) (100 µg/ml in 0,01N HCl) treatment at 37°C for 10 min and finally fixed in 3.7% formaldehyde (Merck) in PBS/50 mM $MgCl_2$ for 10 min. Dehydration in three ethanol solutions (70%, 90% and 100%) followed before the hybridisation procedure.

DNA-probes and labelling

Whole chromosome libraries pBS-4, pBS-11, pBS-15 and pBS-18 were a gift from Dr. J.W. Gray (Lawrence Livermore National Laboratory, California), (Collins et al., 1991). LI.26 (Devilee et al., 1986) and p22/1:2.1 (Mc Dermid et al., 1986) are alphoid DNA probes localized in the pericentromeric region of chromosomes 13 and 21, and chromosome 22 respectively. CRN189-1 is a cosmid DNA-probe which maps to 15q11.2-q12. (Donlon et al., 1986; Tantravahi et al., 1989).

Fluorescent In Situ Hybridization (FISH) and probe detection

Chromosomal in situ suppression (CISS) hybridization for chromosome specific libraries was based on the methods described by Pinkel et al., (1988). The hybridization mixture, 10 µl total volume consisting of 50% formamide (Merck)/ 2 x SSC, 10% dextran sulphate (Pharmacia), 5 µg salmon sperm DNA, 5 µg Cot-1 DNA (BRL) and 100 ng biotinylated probe DNA, was denatured at 90°C for 5 min and immediately put on ice, followed by 1 to 3 hrs preannealing at 37°C. Target DNA was denatured by immersion in 70% formamide /2 x SSC for 3 min at 80 °C and dehydrated in an ethanol series. The hybridization reaction was performed at 37°C for 40 hrs.
In case of CRN189-1, 40 ng of biotinylated probe was precipitated with 5 µg salmon sperm DNA and 2 µg of Cot-1 DNA and dissolved in 10 µl 50% formamide/2 x SSC/10% dextran sulphate. Denaturation of probe and target DNA was performed as described above. The hybridization reaction took place overnight at 37°C.
The centromere probes LI.26 and p22/1:2.1 (40 ng in 10 µl 60% formamide/2 x SSC) and target DNA were denatured simultaneously for 3 min at 80°C. Hybridization was allowed to proceed overnight at 37°C. After hybridization the slides were washed 3 times in 50% formamide/2 x SSC at 42°C for 5 min, followed by 3 changes of 2 x SSC, twice at 42°C and once at 65°C respectively. For the centromere probes, the last washing step (2 x SSC at 65°C) was replaced by 0,1 x SSC at 65°C.
The probe was detected by alternating layers of fluoresceinated avidin and biotinylated goat anti-avidin (Vector Lab, Burlingame, USA). The slides were mounted in anti-fade medium with propidium iodide (Sigma) for counter-staining of the chromosomes and examined under a Leitz Aristoplan fluorescence microscope.

Results

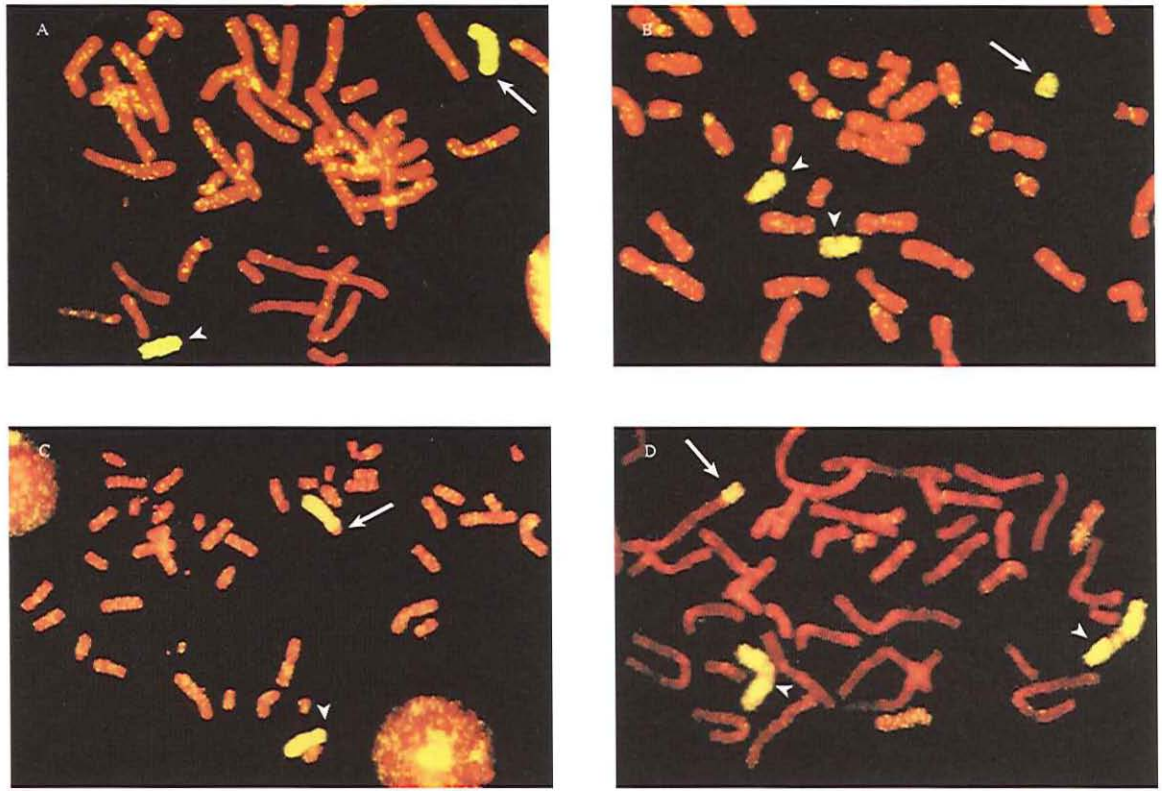
Case 1.

Amniotic fluid was sampled in the 16th week of the first pregnancy of a 31-year old woman because of a recurrence risk for neural tube defects. The α-fetoprotein level was within normal range.

Cytogenetic and molecular studies

The amniotic fluid cultures showed a chromosome 18q+ in a female fetus in 8 clones.

Figure 1. FISH signals on normal chromosomes (arrowhead) and abnormal chromosomes (arrow). (A) Case 1 with dup (18)(q12.3 - q21.3) hybridized with PBS-18. (B) Case 3 with deleted chromosome 15 (PBS.15). (C) Case 2 hybridized with PBS-4, and(d) with PBS-11, both showing that 4p+ material is derived from chromosome 11.



Subsequent karyotyping of both parents gave normal karyotypes, 46,XX and 46,XY, respectively. Chromosome painting was applied to identify the 18q+ chromosome. The 18 library showed that the extra material in 18q+ was derived from chromosome 18 (fig. 1A).
On the basis of the G-banding pattern and chromosome painting, we described the karyotype of the fetus as 46,XX, inv dup(18) (pter → q22 ::q21.3 → q12.3::q22 → qter) (fig. 2A).
Ultrasound screening showed a positional abnormality of one hand. The parents were counseled about the expected anomalies corresponding to trisomy 18q (de Grouchy, 1984) and elected to terminate the pregnancy.
A female fetus was born with external dysmorphic signs. The open eyes showed strabismus, the base of the nose was broad, the nose flat. Micrognathia, low-set ears and a large tongue were present. The 2nd fingers were crossed over the 3rd and there were clubfeet. There were no internal anomalies present. This phenotype showed most signs of trisomy 18q. There was no cell growth of fetal skin.

Case 2.

Fetal blood was obtained by cordocentesis from a 28-year old woman in her first pregnancy in the 28th week. Ultrasound examination had shown the absence of amniotic fluid caused by bilateral renal agenesis.

Cytogenetic and molecular studies.

Karyotyping of fetal blood showed extra material on the short arm of chromosome 4. Subsequent karyotyping of both parents gave normal karyotypes (46,XX and 46,XY respectively).
Chromosome painting with the 4 library left a small terminal segment unstained on 4 p (fig. 1C). The origin of this extra 4p material was determined by painting with the 11 library (fig. 1D). Some other probes could be excluded: pBS-6, 7, 8, 9, 12 and 17 respectively. On the basis of the G-pattern and chromosome painting the fetal karyotype was estimated to be 46,XY,-4,+ der (4), t(4;11)(p16;q22.2) (fig. 2B).
Because of the abnormalities diagnosed by ultrasound and the de-novo unbalanced fetal karyotype, the parents decided to terminate the pregnancy at 34 weeks. Autopsy was performed on the stillborn neonatus.
The stillborn had a birthweight of 1232 g (below 10th percentile) and a height of 34 cm. The diagnosis of renal agenesia with lung hypoplasia was confirmed while external anomalies of the head and extremities were caused by the oligohydramnion. Death had occurred because of respiratory insufficiency. Skin cell cultures confirmed the fetal karyotype.

Case 3

Amniotic fluid was sampled in the 35th week of the pregnancy of a 25-year old woman because the Dandy Walker syndrome had been diagnosed by ultrasound.

Cytogenetic and molecular studies

Eight amniotic fluid cell clones were analysed and showed in all metaphase spreads a female

Figure 2. Schematic presentation of the mutated chromosomes of case 1 (a) and case 2 (b).

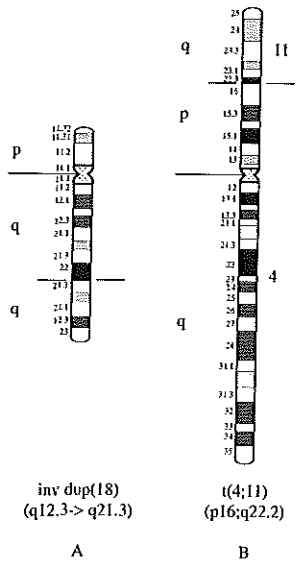
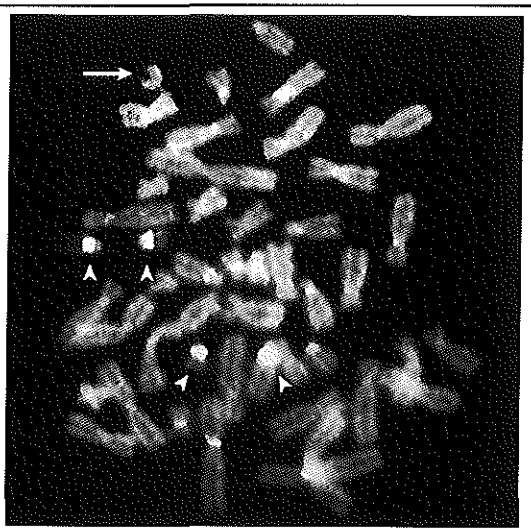


Figure 3. Amniotic metaphase of case 3 showing FISH signals with probe LI.26 on the centromeric regions of chromosome 13 (large arrowhead) and of chromosome 21 (small arrowhead). The marker chromosome (arrow) is without hybridization signal.



karyotype with an extra chromosome. The size of this marker chromosome was comparable to the length of a no. 20. Additional staining methods, consisting of C-,DA-DAPI- and NOR-banding, were negative. The karyotype of the mother was 46,XX. Blood of the biological father from a previous relationship was not available.
After FISH with the 15 library, which painted the normal chromosomes 15, but also showed cross-hybridization to the centromeric regions of the other acrocentric chromosomes (Collins et al., 1991), we found the marker to be totally fluorescent (fig. 1B). We excluded the origin of a centromere of chromosomes 13, 14, 21 and 22 on the extra chromosome by FISH with LI.26 and p22/1:2.1, because LI.26 showed signals on the centromeres of chromosomes 13 and 21, but not on the marker chromosome (fig. 3), and after hybridization with p22/1:2.1, we found strong signals on the centromeres of both chromosomes 22 and a weaker signal on a number of other chromosomes, such as 14, but no signal was seen on the marker.
On the basis of these results we expected the marker to be a deleted chromosome 15, with the deletion most probably covering 15q11.2-12, because of the absence of CRN189-1 on the marker in about 15 metaphases in which the normal chromosome 15 showed 100% hybridization. The karyotype of the fetus therefore results in a partial trisomy 15.
A female child was born spontaneously within a week after amniocentesis and died the same day. Birthweight was 2460 g, height 44 cm. The Dandy Walker syndrome was confirmed by the autopsy results. The head had greatly increased (39 cm) and the vermix cerebelli was replaced by a cyst of 4 cm. There were no other anomalies.

Discussion

Cytogenetic problems in prenatal diagnosis arise when de-novo structural chromosome anomalies or a marker chromosome are present. Conventional cytogenetic methods, like NOR-banding, centromere and DA-DAPI staining are important in cases of marker chromosomes (Sachs et al., 1987), but cannot always give a definite diagnosis. FISH (Fluorescent In Situ Hybridization) has made further identification of small de-novo structural anomalies and marker chromosomes possible. The use of FISH in prenatal diagnosis for the identification of de-novo structural anomalies as in our cases one and two, has to our knowledge not been described before. The prenatal cases in the literature concerned known familial translocations (Jauch et al., 1990; Klever et al., 1992; Rosenberg et al., 1992; Speleman et al., 1992) whilst the de-novo cases were of liveborns (Sachs et al., 1992; Van Hemel et al., 1992; Hulten at al., 1991; Jauch et al., 1990; Speleman et al., 1991).
FISH studies for the identification of marker chromosomes have been described for pre- and postnatal cases by Callen et al., (1992) and Stetten et al., (1992). For the further identification of the marker der (15) in case 3 it would be necessary to use FISH with single copy probes mapping to chromosomal subregions. However, the occurrence of Dandy Walker syndrome makes a breakpoint at 15q22.3 likely since Ieshima et al., (1985) described a patient with Dandy Walker malformations caused by partial trisomy of 15q22.3 → qter. The autopsy results of case one were in accordance with partial trisomy 18q. Two cases of partial trisomy 11 with renal agenesia as in our case 2 have been described by Francke et al., (1972) and Los et al., (1992). Cytogenetic studies followed by FISH studies can therefore give better insight into the

relationship between genotype and phenotype which may improve genetic counseling and decision making for prenatal diagnosis.

Acknowledgements

We acknowledge Dr. L. Pijpers, Merwede Hospital Dordrecht, for referral of the patient in case 1. We wish to thank Ruud Koppenol and Mirco Kuit for fotographic work and Jacqueline du Parant for typing the manuscript.

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Appendix publication II

Am. J. Med. Genet., 66, 216-220 (1996)

Short Report: Ring Chromosome 18 in a Fetus with only Facial Anomalies

Frans J. Los, Cardi van den Berg, Armando P.G. Braat, Firas K. Cha'ban,
Johan M. Kros, and Diane Van Opstal

Abstract

A prenatally detected case of ring chromosome 18 [46,XX,r(18)] in amniotic fluid cells of a fetus displaying an abnormal facial profile on ultrasound as the only malformation is reported. The chromosome 18 origin of the ring chromosome, of a supernumerary marker chromosome in some cells, and of micronuclei was demonstrated by fluorescent in situ hybridization with a whole chromosome 18 paint (Cambio) and 18 centromere probe L1.84. DNA investigations showed deletions of 18p as well as 18q material of r(18), which turned out to be of paternal origin. Autopsy of the fetus after termination of pregnancy at 20 weeks of gestation showed no additional malformations, in agreement with the previous ultrasound findings.

Introduction

Some cases of prenatally detected structural chromosome 18 abnormalities have been reported; isochromosome 18q [i(18q)] [Froster-Iskenius et al., 1984; Wurster-Hill et al., 1991], mosaic i(18p) [Göcke et al., 1986], 18p- [Göcke et al., 1988], mosaicism of deletion (18)(p11)/i(18q) [Sutton & Ridler, 1986], and ring chromosome 18 [r(18)] [Eiben et al., 1992]. We describe the prenatal detection of a 46,XX,r(18) karyotype in amniotic fluid cells investigated with conventional cytogenetic techniques and fluorescent in situ hybridization (FISH). DNA investigations for the establishment of the parent of origin as well as potential deletions of 18p and 18q material of the r(18) were carried out. Furthermore, the prenatal ultrasound findings and a detailed clinical description are presented.

Clinical Report

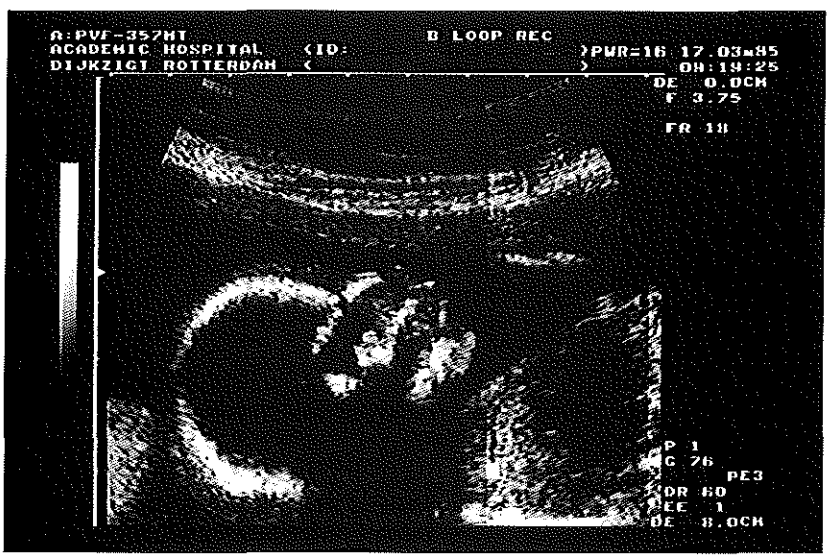
A 39 year-old pregnant woman (G4, P2, Ab1) asked for prenatal diagnosis because of advanced maternal age. In two previous pregnancies prenatal diagnosis had also been performed with normal results and favourable pregnancy outcome. Her family history showed a sister with Down syndrome; the family history of her husband (40 years of age) was unremarkable. Amniocentesis was performed at a gestational age of 16 weeks. No abnormalities were noted on ultrasound investigation. After the finding of a r(18), detailed ultrasonography was performed at 19 weeks of gestation; no structural malformations were seen in a fetus with normal biometry (Table 1). Intracranial anatomy was normal. The only

Table 1. Fetal ultrasonographical biometry at 19 weeks and body measurements after termination of pregnancy at 20 weeks of the fetus with 46,XX,r(18)*

Parameter[1]	Ultrasound biometry				Body measurements			
	fetus (mm)	reference values (mm) 5%	50%	95%	fetus (mm)	reference values (mm) mean ± 1.SD		
CHL	-	-	-	-	248	261	±	17
HC	151	141	152	164	170	175	±	12
DBP	41	39	43	47	-	-		-
IOD/OCD	11.5	8	11	14	12	13.6	±	1.8
OOD/OCD	27.5	26	30	34	34	35.9	±	3.4
Femur Length	27	24	27	30	-	-		-
AC	133	114	128	144	-	-		-
handlenght	-	-	-	-	24	29.5	±	2.9
middlefinger lenght	-	-	-	-	10	12.2	±	1.5
foot lenght	-	-	-	-	29[2]	35	±	2.5
IND	-	-	-	-	35	34.8	±	3.4

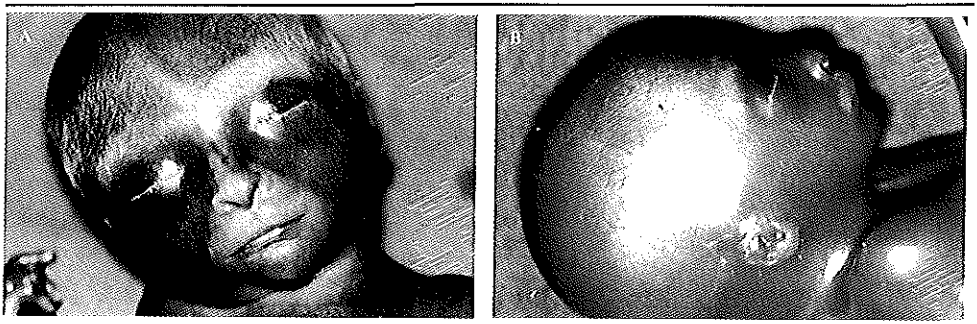
*Ultrasonographical reference values according to Snijders & Nicolaides, 1994, and Trout et al., 1994; body measurement reference values according to Chambers et al., 1993.
[1] CHL = crown to heel length, HC = head circumference, DBP = distantia biparietalis, IOD/ICD = inter orbital distance/inner canthal distance, OOD/OCD = outer orbital distance/outer canthal distance, AC = abdominal circumference, IND = inter nipple distance. [2] Measurement outside mean ± 2. SD-area.

Figure 1. Ultrasonographical image of the fetal facial profile at 19 weeks, showing the receding forehead, rethrognathia and pronounced upper lip/philtrum.



remarkable finding was a slightly abnormal facial profile with a receding forehead, pronounced upper lip/philtrum and rethrognathia (Fig. 1), which might fit the fetal phenotype of the 18p- syndrome [Göcke et al., 1988]. Since the r(18) turned out to have arisen de-novo and the chances for a normal phenotype were counselled low [Schinzel, 1984], the parents opted for termination of pregnancy. At 20 weeks of gestation, labour was induced by intravenously administered prostaglandin. A female fetus was born of 270 gr (Mean-1x S.D) [Chambers et al., 1993] with normal body-measurements (Table 1). A receding forehead, pronounced convex philtrum, micro- and rethrognathia, a broad neck, hypoplastic alae nasi, and dysplastic ears were noted (Fig. 2). Autopsy did not demonstrate any internal malformation; all organs showed a normal weight and development for gestational age. The brain had developed normally with normal midline structures.

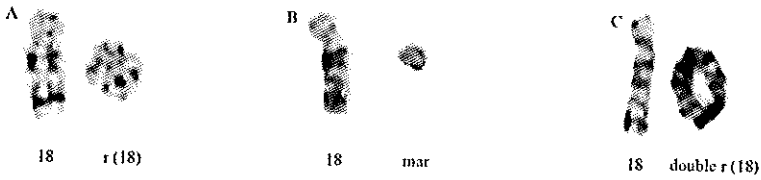
Figure 2. (A) Frontal and (B) lateral view of the fetus at 20 weeks, showing the receding forehead, hypoplastic alae nasi, pronounced convex philtrum and icro/rethrognathia.



Cytogenetic and DNA Studies

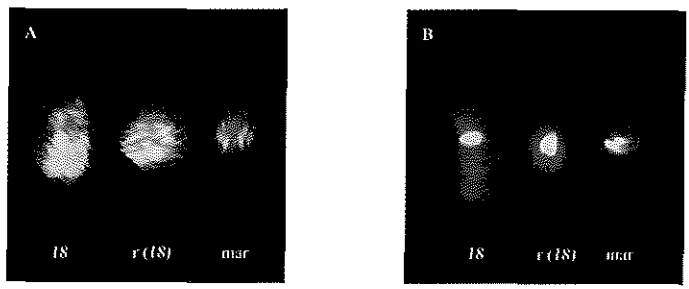
Amniotic fluid cells were cultured by the in-situ method on glass coverslips. Trypsin-Giemsa staining was used. The karyotype was 46,XX,r(18) in the majority of investigated clones (21 out of 27). In 5 clones a mosaicism of 45,XX,-18/46,XX,r(18) was encountered and one clone showed a mosaicism of 45,XX,-18/ 46,XX,r(18)/ 47,XX,r(18),+ marker chromosome (Fig. 3). In one cell, a double-sized (dicentric) ring chromosome was found (Fig. 3).

Figure 3. Partial karyotype of cultured amniotic fluid cells (Trypsin-Giemsa staining). A. r(18). B. marker chromosome (mar).C. double r(18), each accompanied by the normal chromosome 18.



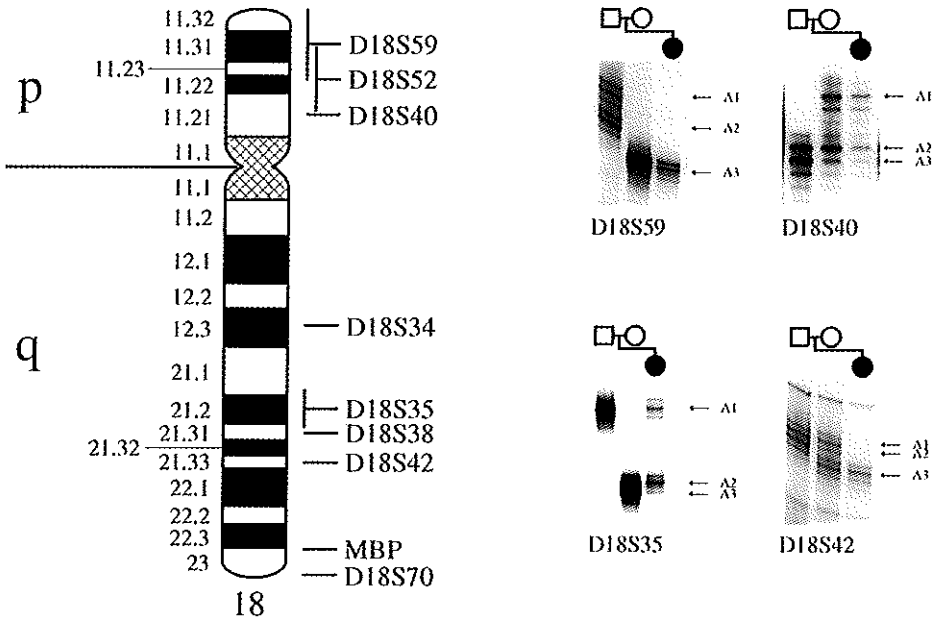
In fetal fibroblasts, only the 46,XX,r(18) line was found in 16 investigated cells. Karyotypes of the parents were normal 46,XY and 46,XX, respectively.
FISH was performed on unstained slides of cultured amniotic fluid cells with a whole chromosome 18 paint (Cambio Ltd., Cambridge, U.K.) and chromosome 18 centromere probe L1.84 [Devilee et al., 1986]. Hybridization with the chromosome 18 paint was performed according to the procedure recommended by the manufacturer. FISH with L1.84 was done as described before [Van Opstal et al., 1995]. Slides were examined with a Leitz aristoplan fluorescence microscope and images were captured by the Genetiscan Probe Master System (Perceptive Scientific Instruments Ltd., Chester, U.K.). Hybridization with the whole chromosome 18 paint resulted in a fluorescent staining of both ring and marker chromosome (Fig. 4A), as well as of micronuclei found in the vicinity of some interphase nuclei, indicating a chromosome 18 origin. Hybridization with L1.84 yielded strong signals on both ring and marker chromosome (Fig. 4B), also demonstrating a chromosome 18 origin.

Figure 4. FISH signals on normal chromosome 18, r(18) and marker chromosome (mar) after in situ hybridization with A. whole chromosome 18 paint (Cambio) and B. 18 centromere probe L1.84 to cultured amniotic fluid cell metaphases.



DNA isolated from cultured amniotic fluid cells and blood of both parents was investigated by performing PCR analysis of various microsatellite markers on chromosome 18 to establish potential deletions of 18p and 18q material of r(18) and determine the parent of origin (Fig. 5). The PCR products of D18S59 and D18S40, located on 18p, and of D18S34, D18S35, D18S42, MBP and D18S70, located on 18q [Le Beau et al., 1993; Geurts van Kessel et al., 1994] showed an informative pattern. Unfortunately, 18p marker D18S52 and 18q marker D18S38 [Le Beau et al., 1993; Geurts van Kessel et al., 1994] turned out to be non-informative. PCR analysis demonstrated the absence of the paternal alleles in fetal cells at the loci D18S59, D18S70, MBP, and D18S42 whilst a paternal as well as a maternal allele were present at the loci D18S35, D18S34, and D18S40 (Fig. 5). These DNA investigations indicated that r(18) was of paternal origin and displayed an 18p deletion of undetermined size together with a large 18q deletion, at least del(18)(q21.33).

Figure 5. Ideogram of chromosome 18 with the localization of the tested microsatellite markers. PCR analysis of D18S59, D18S40, D18S35 and D18S42 shows the absence of paternal alleles A_1 or A_2 at loci D18S59 and D18S42 in fetal cells.



Discussion

A de-novo r(18) was established prenatally in amniotic fluid cells with cytogenetic evidence for mitotic instability such as intra-clonal mosaicism [Rocchi et al., 1984; Kosztolányi, 1987a], the presence of a supernumerary marker chromosome [Koulischer et al., 1980; MacDermot et al., 1990], dicentric ring formation [Kosztolányi, 1987a; 1987b], and the occurrence of micronuclei [Kosztolányi, 1987a]. FISH showed the ring and marker chromosome to be chromosome 18-derived and the micronuclei to contain chromosome 18 material.

DNA investigations demonstrated a deletion of 18p and 18q material of r(18), which was of paternal origin. These investigations were performed on DNA isolated from amniotic fluid cells after termination of pregnancy, but they could have been performed prenatally. Especially in cases of de-novo ring chromosomes without any ultrasound abnormality, in which the fetus might only be affected with the "ring syndrome" [Kosztolányi, 1987b; Pezzolo et al., 1993], DNA data concerning subtelomeric deletions are important for genetic counselling.

In contrast to the expected concomitance of 18p and 18q deletions with serious fetal malformations, the ultrasound findings were surprisingly normal, apart from the facial profile. This case confirms the importance of the interpretation of fetal facial profile abnormalities on ultrasound, which turned out to be in agreement with clinical observations of the fetus after

termination of pregnancy.
The fetus had no malformations of the internal organs, but only facial anomalies, which is in contrast with an earlier prenatal diagnosis of de-novo r(18) [Eiben et al., 1992]. However, the facial anomalies resembled those of a reported prenatal case with 18p- syndrome [Göcke et al., 1988], and, remarkably, also those of cases with tetrasomy 18p [Göcke et al., 1986] and mosaic monosomy 18p/trisomy 18q [Sutton & Ridler, 1986]. The hypoplastic alae nasi together with some other reported anomalies of the 18p-/r(18) phenotype as absent permanent teeth, hypothyroidism, diabetes mellitus and anorectal malformations [Schinzel, 1984] suggest similarities between this phenotype and the autosomal recessive inherited Johanson Blizzard syndrome (JBS)[phenotype 243800; McKusick 1994]. Some cases of JBS might be recurrences of chromosome 18 microdeletions, transmitted through parental germ line mosaicism, a well-known alternative explanation for autosomal recessive inheritance [Petrella et al., 1993]. The phenotypic overlap between the r(18)/18p- syndrome and JBS leads to the assumption that the 18p region is a candidate area for a potential JBS gene.

Acknowledgements

We like to thank Mrs. J. du Parant and Mrs. J. van Deursen for preparing the manuscript and Professor M.F. Niermeijer for advise.

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Appendix publication III

Lancet, 342, 802 (1993)

Fetal aneuploidy diagnosed by fluorescence in-situ hybridisation within 24 hours after amniocentesis.

D. Van Opstal, J.O. Van Hemel, E.S. Sachs

Sir,-Results of amniocentesis at 16 weeks are available, at the very earliest, after 8-12 days because of time-consuming cell culture. Quicker results would cause less anxiety in pregnant women and also be an improvement in cases of serious fetal anomalies when termination is considered. The most common fetal chromosome aneuploidies are trisomy 21 and 18.
We have used fluorescence in-situ hybridisation (FISH) with probes specific for chromosomes 18, 21, X and Y on uncultured amniocytes[1,2] of 20 pregnancies at high genetic risk because of advanced maternal age ($\geq$ 40 years) or in cases of fetal anomaly detected on ultrasound. Gestational age varied between 16 and 34 weeks. Direct preparations for FISH were made from 2 ml of each sample. The samples were centrifuged for 5 min at 150 g, the cell pellet was resuspended in 2 mL 1% sodium citrate, and kept at 37°C for 20 min. The cells were centrifuged, resuspended in 2 mL methanol/acetic-acid (3/1), kept at -20°C for 20 min, and spun. The supernatant was discarded and at least two slides were made from each cell suspension. The probes used are defined in the table. About 50 cells per probe were monitored for each sample.

Probes	FISH*		
	1	2	3
X (pBamX5)	10 (96%)	9 (93%)	1 (88%)
Y (Amprobe RPN1305X)	10 (97%)	0	0
18 (L1.84)	0	19 (93%)	1 (70%)
21 (C0755 + B11128 + B02134)	0	19 (86%)	1 (67%)

*No of pregnancies and (mean %) of nuclei with 1-3 signals. pBAMX5, Willard H.F., et al., Nucleic Acids Res 1983; 11: 2017-33. Amprobe RPN1305X, Amersham. L1.84, Devilee P, et al. Cytogenet Cell Genet 1986; 41: 193-200. C0755 + B11128 + B02134 provided by Dr. H. Lehrach, London.

Results were obtained in all patients within 24 h after amniocentesis (table). Of 20 patients, 19 had normal signal distribution. 9 female fetuses showed two X-signals and 10 male fetus one X-signal and one Y-signal, respectively. These 19 samples also showed two signals for the 18 and 21 probes. Trisomy for the X, 18 and 21 probes was seen in one sample from the first pregnancy of a 26-year-old woman obtained at 27 weeks because of fetal anomalies on ultrasound (spina bifida, hydrocephalus, and left rocker-bottom foot). All normal findings and the trisomy were confirmed by karyotyping of amniotic-fluid cell cultures.
Our results show that FISH with probes specific for chromosomes X, Y, 18, and 21 on

uncultured amniocytes can rapidly detect the most frequent chromosome aneuploidies, and is therefore a valuable additional tool for prenatal diagnosis, specifically for pregnancies at high genetic risk.

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Appendix publication IV

Prenat Diagn, 15, 705-711 (1995)

A chromosome 21-specific cosmid cocktail for the detection of chromosome 21 aberrations in interphase nuclei

Diane Van Opstal, Jan O. Van Hemel, Bert H.J. Eussen, Annet van der Heide,
Cardi van den Berg, Peter A. In 't Veld, Frans J. Los

Summary

Fluorescent in situ hybridization (FISH) with a 21q11-specific probe (CB21c1), consisting of three non-overlapping cosmids, has been applied to interphase amniocytes of pregnancies at increased risk for fetal aneuploidy (N=78) and to interphase lymphocytes, cultured and uncultured, of patients referred for Down syndrome (N=19, and 28, respectively). In the uncultured amniocytes six chromosome aberrations were detected: three cases of trisomy 21, a triploidy, a de-novo 46,XX,t(21q21q), and a mosaic 46,XY/47,XY,+dic(21)(q11)/48,XY,+dic(21)(q11),+del(21)(q11). In 15 cultured and 20 uncultured blood samples, FISH correctly diagnosed a trisomy 21 (full or mosaic) at the interphase level, which was confirmed in all cases by subsequent karyotyping. Because of specific and strong signals in interphase nuclei, CB21c1 appeares to be a useful tool for rapid detection of chromosome 21 abnormalities.

Introduction

Fluorescent in situ hybridization (FISH) has proven to be a powerful method for the rapid detection of chromosomal aneuploidies in uncultured amniocytes (Klinger et al., 1992; Van Opstal et al., 1993), in uncultured mesenchymal chorionic villus cells (Bryndorf et al., 1994), in fetal cells circulating in maternal blood (Gänshirt-Ahlert et al., 1993), and in pre-embryonic blastomeres (Munné et al., 1994). The reliability of this technique depends highly on the specificity and the hybridization efficiency of the probes. Centromeric repetitive alphoid DNA probes are often applied for interphase cytogenetics as they produce strong signals. However, for the detection of the most frequently encountered chromosome abnormality in prenatal diagnosis, trisomy 21, only the probe L1.26 (Devilee et al., 1986) is available. This probe has been reported to be succesfull for detection of trisomy 21 by several authors (Zahed et al., 1992; Lebo et al., 1992; Rao et al., 1993). However, others showed that this probe was not reliable, as the copy number of the sequence recognized by the probe is a highly polymorphic trait and sometimes appears to be too small to produce any signal which resulted in false negative outcomes (Verma et al., 1992; Mizouno and Young, 1992; Seres-Santamaria et al., 1993; Cacheux et al., 1994). Moreover, L1.26 cross-hybridizes to the centromeric region of chromosome 13 which does not allow distinction between trisomy 21 and trisomy 13. For both reasons, we investigated the utility of the present 21q11 cosmid cocktail, CB21c1, for its applicability in pre- and postnatal diagnosis.

Materials and methods

Cell preparations

Amniocentesis was performed because of advanced maternal age (≥40 yrs.) (N=14), ultrasound abnormalities (N=61), and confirmation of trisomy 21 mosaicism (N=2) and of a de-novo translocation (21q21q) in previous direct chorionic villi. From routine amniotic fluid samples one slide was prepared directly (Van Opstal et al., 1993) for hybridization with the present 21-probe and the remainder was cultured by the in-situ method for cytogenetic analysis. Metaphase spreads of lymphocytes were prepared according to standard techniques. Interphase nuclei of uncultured lymphocytes were obtained by incubating the cells in 0.075M KCL at 37°C for 18 min. Subsequently, the cells were fixed by three changes of methanol-acetic acid (3:1) and dropped onto slides. Routine trypsin-giemsa banding was applied for cytogenetic analysis of the cultured amniotic fluid cells and the lymphocytes.

The pretreatment of the slides consisted of a RNase (Pharmacia) treatment (100 µg/ml in 2*SSC) at 37°C for one hour, followed by a pepsin (Serva or Sigma) treatment (4 mg/ml for amniocytes and 100 µg/ml for lymphocytes) in 0.01N HCL at 37°C for 15 min. Uncultured amniotic fluid preparations were postfixed in 3.7 per cent formaldehyde (Merck) in PBS for 10 min.

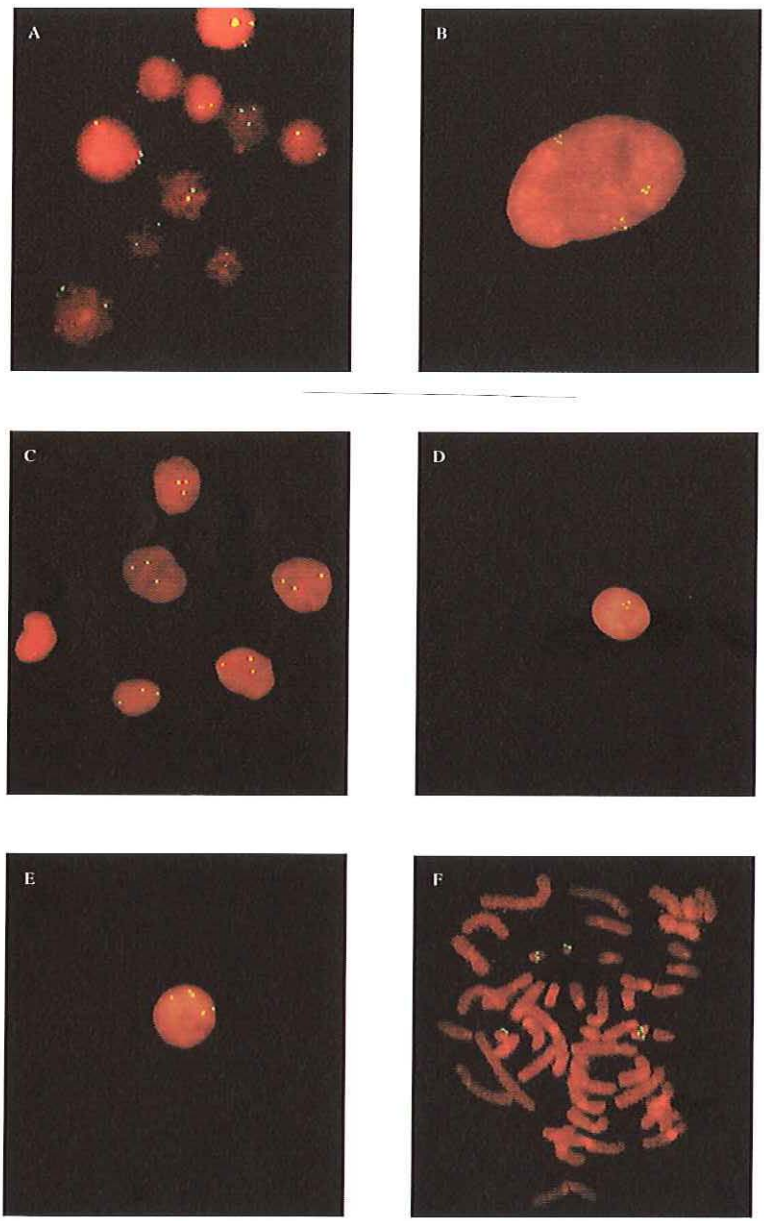
DNA probe and labelling

The 21 cosmid cocktail, CB21c1, consists of three non-overlapping cosmids, ICRFc102B02134, ICRFc102B11128, and ICRFc102C0755, which map to the D21S13 locus at 21q11 (Stinissen et al., 1990; 1991). The probes were labeled with biotin-11-dUTP by nick translation with the BioNick system (Gibco BRL).

Probe detection and signal analysis

The hybridization mixture, consisting of 50 per cent formamide (Merck)/2*SSC, 10 per cent dextran sulphate (Pharmacia), one per cent Tween-20 (Bio Rad), 5 µg salmon sperm DNA (Sigma), 5 µg tRNA (Gibco BRL), 1 µg Cot-1 DNA (Gibco BRL), and 32 ng of each of the three biotinylated probes was denatured at 90°C for 5 min and immediately put on ice, followed by one hour preannealing at 37°C. Target DNA on the slides was denatured by immersion in 70 per cent formamide/2*SSC for 3 min at 80°C and dehydrated in an ethanol series. The hybridization reaction was performed overnight at 37°C.
After hybridization, the slides were washed three times in 50 per cent formamide/2*SSC at 42-45°C for 5 min, followed by three changes of 2*SSC, twice at 42-45°C and once at 60-65°C, respectively. They were treated with alternating layers of fluoresceinated avidin and biotinylated goat anti-avidin (Vector Lab), and finally the slides were mounted in 0.2M Tris-HCL/glycerol (1:9 v/v, pH 7.5) containing 2 per cent DABCO (Sigma) and the fluorescent counterstains propidium iodide (0.5 µg/ml) and DAPI (0.25 µg/ml). Coded slides were examined under a Leica Aristoplan epifluorescence equipped microscope and images were captured with the Genetiscan ProbeMaster system (Perceptive Scientific International Ltd. (PSI), Chester, U.K.) including a Xybion cooled CCD 24-bit color camera. One hundred

Figure 1. FISH with the 21q11-specific probe CB21c1. (A) Interphase lymphocytes of a mosaic trisomy 21 case showing two and three signals. (B) Interphase lymphocyte showing three signals, each consisting of three spots produced by the individual cosmids. (C) Uncultured amniocytes showing three signals in a case of full trisomy 21 and (D) a de-novo unbalanced translocation (21q21q). (E) Uncultured amniocyte and (F) metaphase showing five signals in a case with two normal chromosomes 21 and an extra del(21)(q11) and dic(21)(q11).



lymphocytes and a mean of 41 (range 10-84) amniocytes were counted in each case. Nuclei without signals were not included in the data.

Results

FISH was performed on interphase nuclei of cultured (N=19) and uncultured (N=28) blood samples and of uncultured amniotic fluid samples (N=78). The signal distributions in cultured and uncultured lymphocytes are shown in table 1 and 2, respectively. More than 99 per cent of the nuclei showed compact and bright signals (Figure 1A). Separate spots per signal, produced by each of the three cosmids in the cocktail, were sometimes noticed in the largest cultured cells (Figure 1B). The signal distributions allowed a clear discrimination between normal samples and samples with a trisomy 21 (full as well as mosaic).

Table 1. Signal distribution after FISH with CB21c1 in 19 samples of cultured lymphocytes

No. of cases	Mean percentage (range) of nuclei showing 1-4 signals				Karyotype
	1	2	3	4	
4	3 (2-4)	89 (83-95)	8 (3-12)	0	46,--*
13	2 (0-6)	12 (94-24)	84 (68-94)	2 (0-6)	47,--*,+21
1	3	48	47	1	Mos 46,XX/47,XX,+21(21/79)
1	0	79	21	0	Mos 46,XX/47,XX,+21(82/18)

* = XX or XY

Table 2. Signal distribution after FISH with CB21c1 in 28 samples of cultured lymphocytes

No. of cases	Mean percentage (range) of nuclei showing 1-4 signals				Karyotype
	1	2	3	4	
8	9 (1-18)	85 (78-99)	5 (0-8)	1 (0-4)	46,--*
18	2 (0-4)	15 (4-35)	81 (61-93)	3 (0-10)	47,--*,+21
1	5	52	43	0	Mos 46,XX/47,XX,+21(64/36)
1	0	21	79	0	Mos 46,XX/47,XX,+21(40/60)

* = XX or XY

Although the number of analysable nuclei varied among the uncultured amniotic fluid samples (mean 41; range 10-84), their distributions did suggest six chromosome 21

Table 3. Signal distribution after FISH with CB21c1 in 78 samples of uncultured amniotic fluid samples

N cases	Mean percentage (range) of nuclei showing 1-4 signals						Karyotype
	1	2	3	4	5	6	
72	8 (0-30)	89 (70-100)	5 (0-18)	0	0	0	Disomy 21[1]
3	4 (0-8)	26 (10-38)	68 (52-86)	2 (0-4)	0	0	47,--[2],+21
1	0	33	67	0	0	0	69,XXX
1	4	8	88	0	0	0	46,XX,t(21q21q)
1	0	23	35	19	11	13	Mos 46,XY/47,XY,+dic(21)(q11)/48,XY, +dic(21)(q11),+del(21)(q11)(25/21/54)

[1]=Aneuploidy of other chromosomes not mentioned, [2]= XX or XY

aberrations (Table 3). Karyotyping of the cultured cells revealed full trisomy 21 in three cases (Figure 1C), a triploidy, and a de-novo unbalanced translocation (21q21q) (Figure 1D). In the sixth case with 23 per cent of the uncultured cells showing two signals, and 77 per cent three to six signals (Figure 1E), subsequent chromosome analysis of the cell cultures revealed different karyotypes with marker (mar) chromosomes;
46,XY/47,XY,+mar1/48,XY,+mar1,+mar2 (25%/21%/54%). FISH on metaphases of this case identified mar1 as a dicentric chromosome 21 (dic(21)(pter->q11::q11->pter)) showing two signals with CB21c1, and mar2 as a deleted chromosome 21 (del(21)(q11->qter)) showing only one signal (Figure 1f).

Discussion

Several types of chromosome 21-specific probes have been investigated for interphase cytogenetics; chromosome 21-specific libraries (Pinkel et al., 1988; Lichter et al., 1988; Kuo et al., 1991), plasmid clones (Lichter et al., 1988), YAC's (Bryndorf et al., 1992), Alu-PCR YAC's (Romana et al., 1993; Bryndorf et al., 1994), single cosmids (Lichter et al., 1989), and cosmid contigs (Klinger et al., 1992; Ried et al., 1992; Ward et al., 1993; Spathas et al., 1994).
We investigated the utility of the present 21-probe CB21c1, consisting of three non-overlapping cosmids, for interphase cytogenetics. We preferred to use a mixture of these probes because their joint signal was brighter than that produced by the individual cosmids. Separate spots per signal produced by each of the three non-overlapping cosmids were only occasionally encountered in the largest cultured lymphocytes (Figure 1B), but were never observed in uncultured cells.
Zheng et al (1992), using a cosmid contig on uncultured amniocytes, described that more than 80 per cent of the cells in amniotic fluid are degenerate squamous epithelial cells which are unsuitable for FISH analysis. According to Spathas et al (1994) this problem may be solved by coating the slides with 3'-aminopropyltriethoxysilane which results in high quality preparations with the majority of cells showing hybridization signals. However, they also

mention that gestation time may influence the quality of the slides. This is in agreement with our results as a large proportion of amniocytes in most samples of relatively late gestational age did not show any signals. However, in our experience this problem is mainly restricted to cosmid probes and in a lesser degree to centromeric probes.

With CB21c1 we correctly diagnosed all cases of trisomy 21. Moreover, despite its localization in 21q11 instead of in the Down specific region (21q22), we detected a Robertsonian translocation (21q21q) in uncultured amniocytes in which two of the three signals were situated in close proximity in the interphase nucleus. However, these paired spots could easily be distinguised from the twin spots due to DNA replication in G2 cells. As free trisomy 21 and Robertsonian translocations of chromosome 21 make up 97 per cent and 3 per cent respectively of all cases of Down syndrome investigated during a 10 year period in our postnatal cytogenetic laboratory, CB21c1 is able to detect all cases of Down syndrome at the interphase level. Only an exceptional case showing an unbalanced reciprocal translocation of chromosome 21 with breakpoints distal to 21q11, can not be identified with the present probe but only with a probe from the Down region. CB21c1 could also detect an extra dicentric and deleted chromosome 21 in a rare prenatal mosaic case. Both marker chromosomes lacked the Down specific region and would have gone undetected if a probe was applied mapping to 21q22 such as used by other investigators (Zheng et al., 1992; Klinger et al., 1992; Ried et al., 1993; Ward et al., 1993; Spathas et al., 1994). In order to minimize the risk of a misdiagnosis in further studies, we suggest the hybridization of both CB21c1 as well as a probe derived from the Down syndrome region.

In five cases of uncultured amniocytes most nuclei showed three signals indicating the presence of three copies of chromosome 21 in the fetus. In two of these cases a differential diagnosis of triploidy and of an unbalanced translocation (21q21q) could be made. In the first case a triploidy was expected since in addition to CB21c1 chromosome 18-, X-, and Y-specific probes were routinely applied to all amniotic fluid samples, and three signals were found in the majority of the cells after hybridization with CB21c1 as well as with the 18- and X-specific probes. In the second case FISH was performed for verification of a de-novo t(21q21q) in previous direct chorionic villi. The presence of three signals in most nuclei, with two signals in close proximity, confirmed the presence of the t(21q21q) in fetal cells.

Five mosaic cases involving trisomy 21 in lymphocytes and tetrasomy/pentasomy of the 21q11 region in uncultured amniocytes could be identified with the present probe. However, the level of mosaicism in interphase nuclei did not always match that in cultured cells, which in some cases might be explained by selection during cell culturing. Theoretically, detection of a low level of mosaicism with FISH on interphase nuclei will be difficult because of the broad range of nuclei exhibiting two and three signals.

FISH on uncultured amniocytes has the potential for a rapid prenatal diagnosis of the most common chromosome abnormalities. However, it does not allow the detection of other chromosome aberrations than those detected by the probes that are commonly used (in our laboratory for chromosomes 18, 21, X, and Y). At this moment we use the technique as an adjunct test in two prenatal instances: firstly, for the verification in amniotic fluid cells of a specific chromosome aberration previously detected in chorionic villi and potentially confined to the placenta, and secondly, for the rapid detection of the most common chromosome abnormalities (trisomy 21, trisomy 18, triploidy, and 45,X) in pregnancies complicated by fetal anomalies detected by ultrasound. A normal FISH result in these particular cases is always complemented by a full cytogenetic analysis of the cultured cells. An abnormal FISH

result only can not be the basis for irreversible therapeutic action. However, interphase FISH can complement ultrasound findings and previously detected abnormalities in chorionic villi and provide help in counselling procedures.
In our experience, probe CB21c1 which could detect aneuploidies as well as some unbalanced structural rearrangements of chromosome 21, contributes to the detection of the most common abnormality in clinical cytogenetics.

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Appendix publication V

Prenat Diagn, 15, 51-55 (1995)

Retrospective study of trisomy 18 in chorionic villi with fluorescent in situ hybridization on archival direct preparations

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Summary

Trisomy 18 in direct chorionic villus preparations needs further investigation since the chromosome abnormality may be confined to the placenta and may not represent the actual fetal karyotype. We performed, retrospectively, fluorescent in situ hybridization (FISH) with the chromosome 18 centromere probe on interphase nuclei of destained slides of all cases of full trisomy 18 (n=22) and mosaic trisomy 18 (n=8), detected among 7600 first trimester chorionic villi samples during an eight year period (1985-1992). More nuclei displaying three signals were encountered in cases of full and mosaic trisomy 18 confirmed in fetal tissue than in non-confirmed cases. FISH can be useful for the verification of trisomy 18 in direct chorionic villi preparations.

Introduction

Trisomy 18 in non-mosaic as well as mosaic appearance in direct preparations of placental chorionic villi may not represent the chromosomal status of the fetus (Simoni et al., 1985; Wirtz et al., 1991). Confirmatory studies of long-term villi cultures are used as one of the means of verification. However, the culturing of chorionic villi adds significantly to the reporting time, while contamination of the sample with maternal tissue can interfere with the accurate interpretation of the results (Vejerslev and Mikkelsen, 1989; Kalousek et al., 1992). Moreover, discrepancies between the karyotype of cultured villi and fetal tissue have been reported (Hogge et al., 1986; Wang et al., 1993). We performed, retrospectively, fluorescent in situ hybridization (FISH) with a chromosome 18-specific probe on interphase nuclei in destained archival direct villus preparations of 30 trisomy 18 cases, to investigate whether this technique can be used as a possible quick and accurate method of verification of trisomy 18 in chorionic villi direct preparations.

Materials and methods

Chorionic villi samples and slide preparations

Thirty cases of trisomy 18 (eight mosaic and 22 non-mosaic) were encountered in 7600 consecutive first trimester chorionic villi samples over an eight year period (1985-1992).

Sampling was performed transcervically in 10 cases and transabdominally in the remaining cases as described previously (Jahoda et al., 1989; 1990). Indications for prenatal diagnosis were advanced maternal age and/or ultrasound abnormalities. Karyotyping was performed on trypsin-giemsa stained direct preparations (Simoni et al., 1983; Gibas et al., 1987). A mean of 20 metaphases (range 5-50) in the non-mosaic and 29 (range 9-50) in the mosaic cases was analysed. Preparations of long-term villi cultures (Smidt-Jensen et al., 1989) were karyotyped in some mosaic cases.
Giemsa stained archival direct villi preparations of the 30 trisomy 18 cases and of 30 control cases with a normal karyotype, matched for maternal age, gestational age, fetal gender and storage-time, were destained prior to hybridization (Klever et al., 1991).

DNA-probe and labelling

The 18 centromere-probe L1.84 (Devilee et al., 1986) was used for detection of the chromosome 18 copy number in metaphases and interphase nuclei. The probe was labelled with biotin-11-dUTP by nick translation with the BioNick system (BRL, Gaithersburg, USA).

Fluorescent In Situ Hybridization (FISH) and probe detection

The centromere probe L1.84 (40 ng in 10 µl 60% formamide/2xSSC) and chromosomal DNA were denatured simultaneously for three min at 80°C. Hybridization was allowed to proceed overnight at 37°C. After hybridization the slides were washed three times in 50% formamide/2xSSC at 42°C for five min, followed by three changes of 2xSSC, twice at 42°C and once at 65°C, respectively. The probe was visualized by alternating layers of fluoresceinated avidin and biotinylated goat anti-avidin (Vector Lab, Burlingame,USA). The slides were mounted in anti-fade medium containing the fluorescent counterstains propidium iodide (0,06µg/ml) and DAPI (0,6µg/ml). Slides were examined under a Leitz Aristoplan fluorescence microscope and cells were photographed on Kodak Ektachrome 400 ASA daylight film.
Samples were analysed in a blind fashion on coded slides. For each case the number of fluorescent spots was counted in 200 hybridized intact non-overlapping and non-clumped interphase nuclei. The specificity of probe hybridization was checked in metaphases present on each slide.

Results

Cytogenetic analysis

Twenty-two of the 30 cases with a trisomy 18 in direct villus preparations revealed a non-mosaic trisomy 18 karyotype. The pregnancies were terminated at the parents request; one pregnancy resulted in an intra-uterine fetal death within a week after sampling. The diagnosis of trisomy 18 was confirmed in the fetus by karyotyping skin fibroblasts in 15 of the 22 cases. In one early case we could not confirm the trisomy 18 in fetal cells, which showed a normal 46,XY karyotype. In the six remaining cases cytogenetic confirmatory studies could not be carried out or failed.
Eight of the 30 cases with a trisomy 18 displayed a mosaic trisomy 18 (Table 1). Five

pregnancies were terminated at the parents request and the trisomy 18 was confirmed in fetal tissue in only three instances. In case No.26, showing a double trisomy of chromosomes 18 (mosaic) and 21 (non-mosaic) in chorionic villi, only a full trisomy 21 was recovered in skin fibroblasts and the cytogenetic confirmation in case No.23 failed. Three pregnancies continued after extensive follow-up studies, including a long-term villus culture (LTC) and amniocentesis. They resulted in the birth of healthy children. In two out of five mosaic cases, in which LTC was performed, the culture showed a mosaic trisomy 18 while fetal skin fibroblasts did not exhibit this chromosome aberration (Nos. 26 and 30).

Table 1. Eight cases of mosaic trisomy 18 in first-trimester chorionic villi

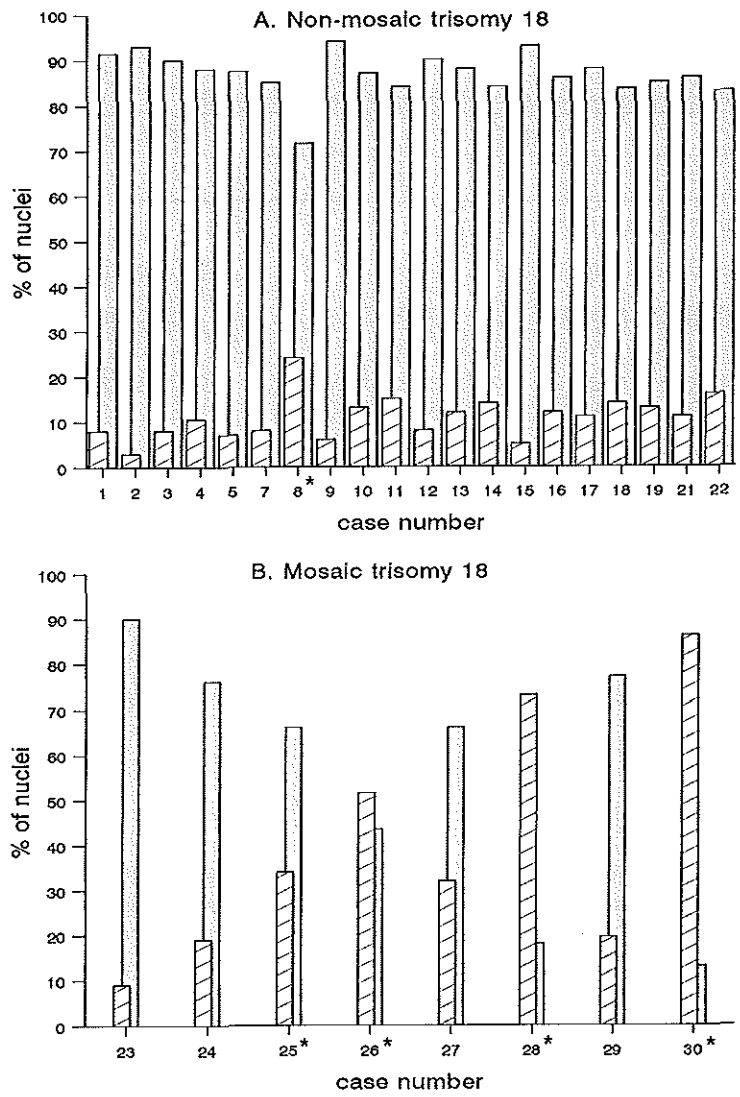
Case no.	CVS		Follow-up	Outcome
	DP	LTC		
23	46,XX/47,XX,+18(2/7)	-	-	TOP
24	46,XX/47,XX,+18(3/47)	-	F: 47,XX,+18(45)	TOP
25	46,XX/47,XX,+18(1/31)	46,XX(7)	A: 46,XX(15)	Healthy ♀ 3200gr
26	47,XX,+21//48,XX, +18,+21(1/19)	47,XX,+21//48,XX, +18,+21(28/23)	F: 47,XX,+21(16)	TOP
27	46,XY/47,XY,+18(12/23)	47,XY,+18(32)	F: 46,XY/47,XY,+18(2/36)	TOP
28	46,XX/47,XX,+18(19/19)	46,XX(27)	A: 46,XX(8)	Healthy ♀ 3375gr
29	46,XX/47,XX,+18(1/15)	-	F: 47,XX,+18(16)	TOP
30	46,XX/47,XX,+18(28/2)	46,XX/47,XX,+18(17/13)	A: 46,XX(19)	Healthy ♀ 2750gr

CVS=Chorionic villus sample, DP=direct villus preparation, LTC=long-term villus culture, A=amniocentesis, F=fetal fibroblast culture, TOP=termination of pregnancy. The numbers in parentheses denote the number of cells analysed.

FISH interphase analysis

Fluorescent in situ hybridization (FISH) with the 18 centromere probe L1.84 was succesfully applied to destained archival slides of all but two of the trisomy 18 cases and to the corresponding normal controls. The mean percentage of nuclei showing one, two and three signals in the 30 normal cases was 7%, 92% and 1%, respectively. Figure 1 shows the percentage of nuclei with two and three signals in individual cases of full- and mosaic trisomy 18. The mean percentage of nuclei showing one signal was 2% (range 0%-4,5%) in the non-mosaic and 3% (range 0%-9%) in the mosaic trisomy 18 cases. In the series with a non-mosaic trisomy 18, the FISH results on interphase nuclei closely matched the cytogenetic findings in the direct preparations (Figure 1A). The confirmed cases were found to express three fluorescent signals in more than 83% of their nuclei (mean 87,5%). In the only non-confirmed case (No. 8), the percentage of nuclei with three signals was 72%, which is far outside the 95% confidence

Figure 1. Percentage of interphase nuclei showing three (closed bars) and two (hatched bars) fluorescent signals after in situ hybridization with a 18-specific probe on direct villus preparations of (A) cases with non-mosaic and (B) mosaic trisomy 18. (* = not-confirmed case)



interval of the confirmed cases. In the series with a mosaic trisomy 18, a broad range of signal distributions was found which did not always match the cytogenetic analysis of the direct preparations (Figure 1B); four mosaic cases (Nos. 24, 25, 26 and 29) with more than 90% of abnormal cells in GTG metaphase analysis showed 3 fluorescent signals in 76%, 66%, 44% and 77% of the interphase nuclei, respectively. In general, cases that were confirmed as being trisomy 18 in amniotic fluid and/or fetal cells (Nos. 24, 27 and 29) showed a higher percentage of 3 signal-containing nuclei (76%, 66% and 77%, respectively) than did the non-confirmed cases Nos. 25, 26, 28 and 30 (66%, 44%, 18% and 13%, respectively).

Discussion

The diagnosis of trisomy 18 (mosaic and non-mosaic) in direct chorionic villi preparations in the first trimester of pregnancy is complicated by the occurence of false positive (Sachs et al., 1990; Breed et al., 1990; Ledbetter et al., 1992) and false-negative results (Leschot et al., 1988; Kalousek et al., 1989). Confirmatory studies of long-term villi cultures have been proposed as a means of verification, as mesenchymal cells in the villus core are suggested to have a closer ontogenetic relation to the fetal cells than the trophoblast cells (Crane and Cheung, 1988). Our own results as well as various earlier reports argue against the use of long-term villus cultures (LTC) as sole and sufficient independent confirmation (Wirtz et al., 1991; Miny et al., 1991; Ledbetter et al., 1992). Cytogenetic analysis of a subsequent amniotic fluid sample seems the most reliable procedure for verification of trisomy 18 in chorionic villi. We studied the usefulness of interphase FISH as a possible quick and accurate method of further investigation of trisomy 18 in chorionic villi direct preparations. It was shown that FISH with a chromosome 18-specific probe, applied to interphase nuclei in direct villus preparations of non-mosaic trisomy 18 cases, has a strong predictive value for the chromosomal status of the fetus and contributes significantly to the results of the classical cytogenetic metaphase-analysis. The non-confirmed case of full trisomy 18 had a significantly lower number of interphase nuclei displaying three signals than the real, confirmed cases of trisomy 18. In cases of mosaic trisomy 18 the application of FISH adds probably also to the classical cytogenetic analysis. Higher levels of three signal containing nuclei were found in the three confirmed cases as compared to the four non-confirmed cases. If the percentage of nuclei with 3 signals turned out to be lower than than 66%, the trisomy 18 was not confirmed in fetal cells. The FISH data were better able to predict the fetal outcome than the cytogenetic data from the direct preparations. However, they yielded ambivalent results in some cases; an intermediate level (66%-83% in our series) of three signal nuclei could either correspond to a true mosaic, a non-mosaic trisomy 18 in the fetus, or a false positive result.

We showed that the application of FISH on chorionic villi direct preparations with a trisomy 18 karyotype, which is frequently found to be confined to the placenta, has a predictive value for the fetal chromosome constitution and therefore can aid in the counselling procedures. However, a definite result can only be achieved by karyotyping a subsequent amniotic fluid sample.

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Appendix publication VI

J Med Genet, 32, 657-658.

Recurrence of DiGeorge syndrome: antenatal detection by FISH of a molecular 22q11 deletion

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Abstract

We report on an antenatal diagnosis by FISH of a familial 22q11 deletion associated with DiGeorge syndrome (DGS). The deletion was seen in the proband with symptoms of full DGS, in the physically normal father and in a subsequent pregnancy. After birth this child showed hypocalcaemia, a T-cell deficit and a rightsided arcus aortae.

The DGS involves conotruncal heart defects, hypoplastic or absent thymus and parathyroids, and facial dysmorphisms (McKusick index # 188400). Most likely, a deletion of a gene or a group of contiguous genes from 22q11 is the cause of this disorder. This is supported by the detection in DGS children of unbalanced chromosomal translocations[1] or microdeletions[2], mostly involving 22q11. Deletions of specific DNA sequences have also been described[3,4]. Approximately 8% of index patients with DGS show familial transmission of the 22q11 deletion[4]. Noticeably, most of the parents with deletions have only minor symptoms or just signs of learning disability or mild mental retardation. Antenatal diagnosis applying fluorescence in situ hybridisation (FISH) with 22q11 specific probes has been performed[4], with normal outcome.

We offered genetic counseling to a couple whose second child had died two weeks after birth. This girl had the typical symptoms of complete DGS. She had a truncus arteriosus communis type II with atrio-ventricular septum defect and a rightsided arcus aortae. Other symptoms were neonatal hypocalcaemia and T-cell disturbances. At necropsy only one parathyroid gland and a small hypoplastic thymus were present. High resolution cytogenetic studies performed on her fibroblasts (cell line F92-31; 93RD59) showed a deletion at 22q11, which could be confirmed by applying FISH[5] with probe M51, that detects a molecular deletion in the DGS critical region of chromosome 22 (Mulder et al., submitted) (Fig.1). Both parents were physically normal, although they both had mild learning disabilities; especially the father. In childhood he had recurrent upper airway-infections and was frequently hospitalized. However no data could be obtained about possible immunological disturbances. The father had a tendency to depression and alcohol abuse. In lymphocyte metaphases of both parents no microdeletions were visible. After the mother became pregnant, she asked for antenatal diagnosis. FISH on lymphocyte metaphases from both parents and fetal amniotic cells was done with M51. The molecular deletion was detected in the male fetus and in its father. Cytogenetically these deletions were not visible.

The parents were informed about the risk for DGS, velo-cardio-facial syndrome (VCFS), cardiac defects, and mental retardation[6,7]. The parents decided to continue the pregnancy. Ultrasound studies in the 20th and 23rd week of pregnancy did not show cardiac or other

anomalies. The boy was born at term and developed hypocalcaemia with low parathyroid hormone levels. T-cell function studies indicated a moderate T-cell deficit. Echocardiography revealed a rightsided arcus aortae without intracardial anomalies. Now at six months of age, his clinical condition is stable.

Figure 1. Part of a metaphase after FISH with cosmid probe M51, region 22q11 (arrow) and cosmid M69, region 22q13.3 (arrowheads), the latter used for recognition of chromosome 22. Only one chromosome 22 shows the M51 signals, pointing to a deletion in the homologous 22.

This is the first reported prenatal diagnosis of a molecular deletion in the DGS critical region of chromosome 22. Such a deletion is associated with a spectrum of malformations covered by the acronym CATCH 22[8]. The anomalies seen in this family show the phenotypic variability of the M51 deletion. The anomalies of the youngest boy are less severe than those of the proband, with psychiatric problems in the father. A variety of psychiatric problems have been described in VCFS patients[9]. After detection of the 22q deletion in the father additional inverstigations were performed. Serum calcium and immunological screening, including IgG, IgA, IgM, IgG-subclasses and complement reactions (CH50) were all normal. Intra-cardiac abnormalities were excluded by echocardiography; the only abnormality was a right sided aorta descendens detected by X-ray of the chest.

In this family and others the range of phenotypes associated with the molecular deletions detected with probe M51 complicates genetic counseling. The classical DGS syndrome is usually sporadic but may be transmitted as an autosomal dominant trait; Shprintzen syndrome is usually autosomal dominant. We propose that parents with a proven molecular deletion are counseled as having an increased risk for cardiac defects, DGS, immunological disturbance, and cleft palate, occurring as single abnormalities or in syndromic forms. Further studies are

needed to determine the potential of probe M51 to differentiate between isolated and familial forms of congenital heart defects with a very low and a 50% recurrence risk, respectively.
For the antenatal diagnosis of microdeletions associated syndromes like DGS, FISH appears to be a rapid and reliable method[10]. It may also be reliably applied in chorionic villus mitoses which usually give a lesser quality of their chromosomes. An early first trimester diagnosis is important as an option during genetic counseling when there is a 50% recurrence risk of familial DGS[7], after careful explanation to the parents of the substantial variability of this disorder.

Acknowledgements

For skilfull laboratory work we owe to Bert Eussen and Annet van der Heiden, and Profs. H. Galjaard and J.C. Molenaar for their continuous support.

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Appendix publication VII

Prenat Diagn, in press

Prospective prenatal investigations on potential uniparental disomy in cases of confined placental trisomy

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Summary

In most reported cases of uniparental disomy (UPD) associated with confined placental mosaicism (CPM), a high level of mosaicism or a full trisomy was found in chorionic villi (CV). At the time we started our investigations it was not quite clear whether fetal UPD also existed in the more frequently occuring low levels of mosaicism. During a four-year period a follow-up amniocentesis was performed in all cases of mosaic or non-mosaic trisomy detected in CV semi-direct preparations and suspected to be confined to the placenta. We performed fluorescent in situ hybridization (FISH) on uncultured amniotic fluid cells to differentiate between generalized mosaicism and CPM. We found 29 cases of CPM and we determined the incidence of UPD in 23 of these cases. Normal biparental chromosome contributions were found in 22 cases. In one case we detected a maternal heterodisomy for chromosome 16. UPD appeared to be a rare phenomenon in the cases of CPM (type I and/or III) that we encountered in 3958 consecutively investigated CV samples, and is not the cause of the pregnancy complications found in seven out of 23 cases with CPM.

Introduction

In 1980, Engel introduced the concept of uniparental disomy (UPD) i.e. the presence of a chromosome pair derived from one parent in a diploid offspring. Eight years later, Spence et al. (1988) reported the first example of UPD in a 16-year-old girl with short stature and cystic fibrosis; she had inherited two copies of the maternal chromosome 7 with a CF mutation. In 1992, the first case of UPD associated with confined placental mosaicism (CPM) was documented; a patient with Prader-Willi syndrome, caused by maternal UPD for chromosome 15, was born after prenatal detection of a trisomy 15 in chorionic villi (CV), and a subsequent normal karyotype in amniotic fluid (AF) cells (Purvis-Smith et al., 1992). This case supported the hypothesis that the loss of an extra chromosome from an initially trisomic zygote is one of the mechanisms that could lead to fetal UPD (Engel and DeLozier-Blanchet, 1991). Bennett et al. (1992) studied two cases of trisomy 16 confined to the placenta and found maternal UPD in one of them with a paternal chromosome 16 present in chorionic villi but being lost in the fetus, proving that a trisomic zygote can indeed undergo postzygotic loss of a supernumerary chromosome resulting in a diploid fetus. Various cases of UPD associated with CPM have been published uptill now (Kalousek and Barrett, 1994). The type of CPM as well as the

chromosome involved, and the origin of the trisomy (meiotic or mitotic), which itself is correlated with the level of mosaicism, all seem to be associated with the incidence of UPD (Wolstenholme, 1996; Robinson et al., 1997). UPD can affect human development through imprinting or homozygosity for recessive traits, and may be related to pregnancy and perinatal complications occasionally found in cases of CPM such as intra-uterine growth retardation (IUGR) (Kalousek et al., 1991), fetal loss (Goldberg et al., 1990), or poor perinatal outcome (Johnson et al., 1990). However, the presence of cytogenetically abnormal cells in the extraembryonic tissues may have a direct effect on placental function with subsequently these complications of pregnancy (Kalousek and Barrett, 1994).
The purpose of our study, which started in 1992, was to determine the incidence of UPD associated with CPM in a consecutive series of CV samples that we recieved in our laboratory during four years. Twenty-nine cases of trisomy CPM were found, and in 23 cases we studied extensively the level of mosaicism in CV with interphase fluorescent in situ hybridization (FISH), and determined the parental origin of the particular chromosome pair in cultured AF cells. Additionally, we investigated uncultured AF cells for the presence of trisomic cells to preclude generalised mosaicism, concealed in cultured cells.

Materials and methods

Patients and procedures

Twenty-nine cases of confined placental (mosaic) trisomy (type I and/or III), with at least two trisomic cells on a total of 30 metaphases in CV semi-direct preparations, were encountered in 3958 consecutively investigated CV during a four-year period (1992-1995). In all these cases follow-up amniocentesis was offered to the parents for cytogenetic studies and UPD investigations. For the determination of the level and extent of the mosaicism, we applied interphase FISH on CV and uncultured AF cells. In six out of 29 cases, UPD studies could not be performed because blood of the parents was not available. So, the present series comprises the remaining 23 cases.
Five to 35 mg (mean 14 mg) of CV were sampled transabdominally at 11 to 14 weeks of gestation as described previously (Jahoda et al., 1990). Indications for prenatal diagnosis were advanced maternal age (≥ 36 years) (n=20), risk of X-linked mental retardation, recurrence risk of Niemann Pick type A, and risk of X-linked adrenomyeloneuropathy (each n=1). At 16 weeks of gestation, 18 to 20 ml of AF was sampled in all but one case. In two cases a fetal skin biopsy was taken after intra-uterine death at 15 and 33 weeks of gestation.

Cytogenetic investigations

CV were incubated overnight using fluorodeoxyuridine (FdU) synchronization (Gibas et al., 1987). Karyotyping was performed on Trypsin-Giemsa stained semi-direct preparations. A mean of 32 (range 19-50) metaphases were analysed. Culturing of CV samples was not performed during the study period.
AF-cells were cultured according to standard techniques by the in situ method on glass coverslips. Trypsin-Giemsa staining was routinely used. A mean of 15 clones (range 7-26) of cultured AF cells were analysed.

Table 1. Chromosome specific probes used for FISH

Chromosome	Probe	Reference
3	pα3.5	Waye and Willard, 1989
7	pα7t1	Waye et al., 1987
12	pα12H8	Looyenga et al., 1990
16	pHuR195	Moyzis et al., 1987
18	L1.84	Devilee et al., 1986
22	M51	Mulder et al., 1995
X	pBamX5	Willard et al., 1983

Interphase FISH analysis

FISH was performed on CV semi-direct preparations and on 2 ml of uncultured AF cells as described previously (Van Opstal et al., 1993; 1995). The probes used in this study are listed in table 1. For each case the number of fluorescent spots was counted in a mean of 185 (range 100-300) hybridized CV interphase nuclei, and a mean of 154 (range 50-505) hybridized uncultured AF cells. For interpretation of the FISH results, the same probes were applied to a series of normal CV and AF cell samples. Statistical analysis of data obtained from these control samples was used to determine the 95 % confidence interval of the one-sided upper reference limit (97,5%) for the proportion of cells with three signals for each of the probes used, according to Lomax et al. (1994) (Table 2). This cut-off level was used to discriminate the normal non-mosaic state from the lowest detectable level of mosaicism.

DNA analysis

DNA was extracted from blood of both parents and in all but one case from cultured AF cells according to standard techniques. In one case the fetal DNA source consisted of cultured skin fibroblasts. In three cases Trypsin-Giemsa stained CV chromosome preparations, which had been stored at room temperature for two months to two years, were also used for DNA extraction. Briefly, cells of one slide were collected in 10 mM NaCl/10 mM EDTA and spun down for 15 sec at 12000 rpm. The cell pellet was resuspended in 50 mM NaOH and boiled for 20 min. After neutralization with 1 M Tris.HCl, the suspension was spun down to remove cell debris and 0.5-2 µl of the supernatant was used for PCR analysis.
Molecular analysis of the parental origin of both chromosomes of a chromosome pair in AF cells (and skin fibroblasts in one case), and of the extra chromosome in CV, was performed with polymerase chain reaction (PCR) amplification of polymorphic microsatellite repeats. Loci that were examined for each of the chromosomes involved, are listed in table 3. Information on primer sequence and map location is available from the Genome Database.
Sixty ng of fresh DNA and 0.5 to 2 µl of archival DNA solution (CV-slide extracted) was amplified in a total volume of 15 µl containing 60 ng of each primer, 0.1 µl 100 mM dNTP-

Table 2. One-sided upper reference limit (97,5 %) and corresponding 95 % confidence interval (CI) for the proportion of CV and AF cells with three FISH signals obtained from a series of diploid controls

Probe (number of controls)	Upper reference (97,5 %) limit (95 % CI) CV	AF
pα3.5 (5 CV, 4 AF)	1,84 (1,09;2,6)	1,57 (0,53;2,6)
pα7t1 (12 CV, 11 AF)	3,78 (2,43;5,13)	5,35 (3,18;7,53)
pα12H8 (4 CV)	3,27 (1,29;5,25)	---
pHuR195 (16 CV, 10 AF)	6,04 (4,21;7,87)	5,44 (3,41;7,48)
L1.84 (12 CV, 38 AF)	2,37 (1,95;3,51)	7,37 (5,75;9,0)
M51 (5 CV)	6,69 (4,02;9,37)	---
pBamX5 (5 CV, 16 AF)	3,36 (1,29;5,43)	4,08 (2,63;5,54)

Note. CV= chorionic villi semi-direct preparations; AF= uncultured amniotic fluid cells.

Table 3. List of microsatellite loci used for parent of origin studies

Chromosome	Markers *
2	D2S73, MHC/CD, D2S103, D2S72, CTLA4, D2S102, D2S125
3	D3S1270, D3S1304, D3S100, D3S1360, D3S11, GLUT2, D3S1232
7	D7S531, D7S472, D7S488, D7S471, D7S473, D7S504, D7S495, D7S483, D7S550
9	D9S104, D9S52, D9S43, D9S51, D9S177
12	D12S62, D12S61, D12S43, D12S64, D12S60
13	D13S175, D13S220, D13S170, D13S159, D13S174, D13S158, D13S173
16	HBAP1, D16S407, D16S298, D16S285, D16S308, D16S261, D16S419, D16S301, D16S266, D16S305
18	D18S59, D18S52, D18S452, MFD80, D18S40, D18S34, D18S35, D18S42, D18S43, MBP, D18S70
20	D20S66, D20S48, D20S102, D20S120
22	D22S257, D22S156, D22S120, D22S315, IL2RB, CYP2D
X	MAOA, DXS1003, DXS426, DXS453, DXS454

Note. * in order pter--> qter

mix with a lower dCTP concentration, 0.45 µl 50 mM $MgCl.6H_2O$ (BRL, Gaithersburg, USA), 1.5 µl 10XPCR buffer (BRL, Gaithersburg, USA), 0.3 µl of 25 mM spermidine, 0.75 µl 1% W1 (BRL, Gaithersburg, USA), 0.1µl α^{32}P-CTP, and 0.1 µl Taq-polymerase (5U/µl) (BRL, Gaithersburg, USA). Samples were processed through 25 cycles for fresh and 40 cycles for archival DNA samples, each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min extension at 72°. The amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler 9600. The alleles were separated by electrophoresis on a 6 % denaturing polyacrylamide gel. Gels were fixed with 10 % acetic acid/ 10 % methanol and dried. They were exposed to X-ray film overnight at room temperature.
Conclusions about parental origin of a particular chromosome required at least two informative markers.

Results

Cytogenetic analysis and FISH studies

On a total of 3958 CV samples that were investigated cytogenetically during a four-year period, 69 cases (1,7 %) of CPM (type I and/or III) were detected. In 29 cases (42 %) a trisomy was involved, of which 23 cases comprises the present series. Cytogenetic results of CV are presented in table 4; normal karyotypes were present in amniotic fluid cells or fetal skin fibroblasts (case 22).
In three cases (cases 16, 17, 18) a non-mosaic trisomy 16, and in 18 cases a mosaic trisomy involving the chromosomes 2, 3, 7, 12, 13, 16, 18, 22, and X was found in CV. In the remaining two cases a mosaic double trisomy of chromosomes 7 and 9 (case 7), and of chromosomes 13 and 20 (case 14) was present in the sample. The proportion of abnormal cells in the mosaic cases varied between 7% and 98%.
FISH results on interphase nuclei in semi-direct CV and uncultured AF cell preparations are shown in table 4. Where no results are shown, appropriate probes were not available at the time of investigation or no results were obtained due to inefficient hybridization. In case 22, AF was not available because of intra-uterine death and termination of pregnancy at 15 weeks of gestation.
In three of the 19 cases in which FISH results were obtained on CV slides, a discordance was found between standard cytogenetic and FISH analysis: FISH did not confirm the cytogenetic presence of mosaicism.
In one of the 14 cases, in which FISH results were obtained on uncultured AF cells, a discrepancy was found between FISH analysis of uncultured cells and cytogenetic analysis of the cell cultures (case 16); 26 % of the uncultured cells showed three chromosome 16 signals, whereas the cell cultures showed a normal karyotype. In all other cases, neither classical cytogenetic nor FISH analysis revealed the existence of a trisomic cell line in AF cells.

DNA studies

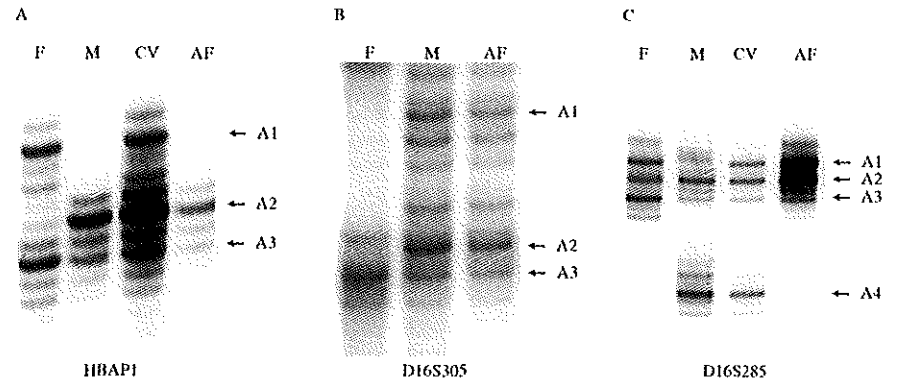
Parent of origin studies in AF cells (or skin fibroblasts in case 22) of both homologues of the chromosome that was found to be trisomic in CV, revealed a normal biparental chromosome contribution in 21 of the 23 cases, and an abnormal result in two cases (cases 13 and 17).

Table 4. Karyotypes, interphase FISH results, and pregnancy outcomes in 23 cases of CPM

Case No.	Karyotype in CV* (number of cells)	% of trisomic cells	Interphase FISH results										Pregnancy outcome (gestational age, birth weight, percentile)
			N		% of nuclei with 1-4 signals								
					1		2		3		4		
			CV	AF	CV	AF	CV	AF	CV	AF	CV	AF	
1	47,XX,+2[4]/46,XX[25]	14	--	--	--	--	--	--	--	--	--	--	41,5 wks., 4180g, P90-95
2	47,XY,+3[6]/46,XY[29]	17	200	100	0	0	70	100	30	0	0	0	41 wks., 3900g, P50-75
3	47,XY,+3[7]/46,XY[22]	24	200	100	0	1	79	98	20	1	1	0	38 wks., 3600g, P75
4	47,XY,+3[32]/46,XY[1]	97	105	100	0	6	15	93	85	1	0	0	39 wks., 4050g, P95-97.7
5	47,XX,+7[3]/46,XX[30]	9	300	200	5	4	89	95	6	1	0	0	36 wks., 2660g, P25-50
6	47,XX,+7[3]/46,XX[26]	10	200	50	4	6	77	94	19	0	0	0	40 wks., 3920g, P75-90
7	48,XY,+7,+9[2]/46,XY[17]	11	--	--	--	--	--	--	--	--	--	--	41 wks., 3060g, P10-25
8	47,XX,+7[5]/46,XX[26]	16	100	--	7	--	63	--	30	--	0	--	40,5 wks., 3500g, P50
9	47,XY,+7[6]/46,XY[24]	20	300	200	1	5	92	92	7	2	0	1	41 wks., 4000g, P75-90
10	47,XY,+7[12]/46,XY[18]	40	200	179	0	6	65	92	35	2	0	0	41 wks., 3810g, P50-75
11	47,XX,+12[2]/46,XX[28]	7	100	--	15	--	84	--	1	--	0	--	39,5 wks., 2115g, <P2.3
12	47,XX,+13[2]/46,XX[28]	7	--	--	--	--	--	--	--	--	--	--	41 wks., 3450g, P25-50
13	47,XY,+13[2]/46,XY[28]	7	--	--	--	--	--	--	--	--	--	--	40 wks., 3850g, P75-90
14	48,XY,+13,+20[3]/46,XY[27]	10	--	--	--	--	--	--	--	--	--	--	33 wks., 1775g, P10-25 solutio placenta
15	47,XX,+16[2]/46,XX[28]	7	200	50	4	16	94	80	2	4	0	0	40 wks., 4000g, P75-90
16	47,XX,+16[30]	100	200	100	1	6	6	65	92	26	1	3	IUD at 33 wks., 845g, <<P2.3, MCA
17	47,XX,+16[37]	100	200	86	0	10	19	84	79	6	2	0	38 wks., 2880g, P25
18	47,XY,+16[32]	100	200	137	0	4	13	95	87	1	0	0	36 wks., 2850g, P50, neonatal infection, and wet lung
19	47,XX,+18[2]/46,XX[28]	7	200	300	1	4	86	91	13	3	0	2	38 wks., 2750g, P10-25
20	47,XX+18[2]/46,XX[28]	7	200	50	5	6	95	94	0	0	0	0	41 wks., 2970g, P5-10
21	47,XX,+18[19]/46,XX[19]	50	200	--	9	--	73	--	18	--	0	--	39 wks., 3375g, P50
22	47,XX,+22[49]/46,XX[1]	98	200	--	1	--	7	--	92	--	0	--	IUD at 15 wks., 14 g, <<P2.3, MCA
23	47,XXX[9]/46,XX[21]	30	200	505	4	15	66	84	30	1	0	0	42 wks., 4100g, P75-90

Note. *: karyotypes in amniotic fluid cell cultures were normal in all cases. N= number of interphase nuclei; CV: chorionic villi semi-direct preparations; AF= uncultured amniotic fluid cells; -= not tested or non-informative results; "gray box" denotes discordance between cytogenetic and FISH results; IUD= intra-uterine death; MCA= multiple congenital abnormalities; P= percentiles of Dutch neonates (Kloosterman, 1970)

Figure 1: Autoradiograms of polymorphic dinucleotide repeat markers (HBAP1, D16S305, and D16S285) on chromosome 16 in cases 17 (A and B) and 18 (C) (F= father, M= mother, CV= chorionic villi, AF= amniotic fluid). (A) shows loss of the paternal allele A1 in AF cells of case 17 leading to maternal UPD. (B) shows the presence of two different maternal alleles (A1 and A2), and absence of a paternal allele (A3), consistent with maternal heterodisomy, in AFC of case 17. (C) demonstrates presence of two maternal (A2 and A4) and one paternal allele (A1) in CV, and subsequent loss of an extra maternal allele (A4) in AF cells, leading to biparental inheritance of chromosomes 16 in case 18.



In case 17, showing a non-mosaic trisomy 16 in CV, UPD for chromosome 16 was found in AF cells, with four of the tested markers, two located on 16p (HBAP1 and D16S298), and two on 16q (D16S261 and D16S305), showing an informative pattern. The fetal pattern showed a maternal heterodisomy as mother and fetus were both heterozygous for the same alleles of D16S285, D16S298, D16S308, and D16S305 (Fig. 1). Non-paternity and maternal cell contamination (MCC) were excluded by analysis of three highly informative markers, FABP, THO1, and HPRT on chromosomes 4, 11, and X, respectively.

In case 13 no paternal and only one maternal allele could be detected in AF cells for two of the tested markers (D13S170, D13S174). Amplification of polymorphic markers located on other chromosomes (D21S120 on chromosome 21, and D9S43 on chromosome 9) also revealed the presence of a maternal but absence of a paternal allele which proved non-paternity in this case.

In the three non-mosaic trisomy 16 cases (cases 16, 17, and 18) we studied the parental origin of the supernumerary chromosome in CV. PCR amplification of DNA extracted from CV slides revealed the presence of two maternal and one paternal allele for at least one polymorphic marker in all three cases (fig. 1). MCC of the slides of cases 16 and 18 was excluded by PCR amplification of markers on some other chromosomes (D1S158 and D21S156 in case 16; D20S120, D18S40, D3S11, D13S159, and D17S250b in case 18).

Pregnancy outcome (Table 4)

In 16 cases, the pregnancies were uneventful and resulted in the birth of infants with birth weights beyond the 10th centile (Kloosterman, 1970). In seven cases pregnancy complications were observed. There were two cases of intra-uterine growth retardation (IUGR) (cases 11 and

20), and one case of preterm delivery at 33 weeks because of solutio placenta (case 14). In case 17 maternal hypertension and deteriorating renal function were diagnosed at 34 weeks of gestation. The mother recovered rapidly after the delivery of a 2880 g female infant at 38 weeks of gestation. Inta-uterine death and multiple congenital abnormalities were observed in cases 16 and 22. In case 16, the abnormalities involved severe IUGR, facial dysmorphisms, simian crease right, atrial septal defect, hypoplastic truncus pulmonalis with valvular atresia, ventricular septal defect, right lung with one lobe, and left lung with two lobes. In case 22, severe IUGR, facial dysmorphisms, malrotation of the intestine, asplenia and atrial septal defect were the major malformations. In one further case (case 18) transient perinatal complications were encountered (wet lung and infection).

Discussion

The incidence of UPD in a diploid fetus supported by a trisomic placenta is theoretically 1/3, if the conception originally was trisomic and loss of one copy of the trisomic chromosome in the embryonic progenitor cells during cleavage occurred randomly and resulted in CPM (Engel and Deloizier-Blanchet, 1991). This theoretical figure has actually been established for chromosome 16 and 22 (Kalousek et al., 1993; Wolstenholme, 1995), since these trisomies are predominantly of meiotic origin, and a significant correlation was found between the presence of UPD and a meiotic origin of the trisomy (Robinson et al., 1997). Trisomies of many other chromosomes, such as 2, 3, 7, 8, and 9, seem to be primarily the result of somatic duplication (Kalousek et al., 1996; Shaffer et al. 1996; Wolstenholme, 1996; Robinson et al., 1997). Robinson et al. (1997) found fetal UPD in 17 out of 94 cases of trisomy CPM, including 13 cases of UPD 16. However, their study population might not be considered a random sample of CPM cases found during prenatal diagnosis, because of inclusion of postnatal cases ascertained through IUGR noted at birth, and because of a high number of trisomy 16 cases, since they were the initial focus of their research, which both may be responsible for an overestimation of the frequency of UPD. The purpose of our study was to determine the incidence of UPD associated with CPM in a series of consecutively investigated CV samples received during a four-year period. As a consequence of our approach to cytogenetic investigations of CVS, UPD studies were only performed in cases of CPM type I (abnormal cells confined to cytotrophoblast) and undetermined CPM type III (abnormal cells in cytotrophoblast as well as extraembryonic mesenchyme). Of 29 cases of trisomy CPM that we found during four years, 23 cases could be investigated, and the incidence of UPD showed to be 1 in 23.

This low frequency of UPD is in agreement with the correlation of fetal UPD with high levels of trisomic cells in the trophoblast (Robinson et al., 1997). In 18 out of 23 cases a low mosaic trisomy was present in semi-direct CV preparations, with less than 50% of abnormal cells, and in half of the cases even less than 10%. The trisomies in all these cases most probably originated from a postzygotic mitotic non-disjunction (Crane and Cheung, 1988; Wolstenholme, 1996; Robinson et al., 1997). In five cases we found a high mosaic or full trisomy in semi-direct CV preparations. In three cases of non-mosaic trisomy 16 we showed that the trisomy arose as a result of a maternal meiotic error, as could be expected from earlier reports (Hassold et al., 1995). Trisomic zygote rescue resulted in CPM in these cases and caused UPD for chromosome 16 in one of them by eliminating the paternal chromosome 16.

Although most reported cases of UPD were found to be associated with CPM type III, with high levels of trisomic cells in both placental lineages, a few cases were found to be associated with CPM type I (Jones et al., 1995; Wilkinson et al., 1996). Robinson et al. (1997) showed that CPM type II (abnormal cells confined to the extraembryonic mesenchyme) may sometimes have a meiotic origin with a risk for UPD, although Wolstenholme (1996) stated, on the basis of observations and theoretical considerations, that meiotic errors are not assumed to be associated with CPM type II.

Some 21 cases of CPM and maternal UPD for chromosome 16 have been described previously (Bennett et al., 1992; Dworniczak et al., 1992; Kalousek et al., 1993; Sutcliffe et al., 1993; Miny et al., 1994; Vaughan et al., 1994; Kalousek and Barrett, 1994; Whiteford et al., 1995; Schneider et al., 1996; O'Riordan et al., 1996; Kalousek and Vekemans, 1996; Robinson et al., 1997). In most cases IUGR has been observed, and in some cases congenital malformations were found as well (imperforate anus, two-vessel umbilical cord , club-foot, inguinal hernia, hypospadias, clinodactyly, and atrioventricular septal defect). However, in a few cases, including the present case, UPD16 was found to be associated with a normal outcome (Kalousek and Barrett, 1994; Robinson et al., 1997). This further supports the hypothesis that the impaired fetal growth in cases of UPD16 may not be the result of the fetal UPD itself, but rather due to a malfunctioning placenta, caused by high levels of trisomic cells in the placenta (Kalousek and Barrett, 1994; Wolstenholme, 1995; Brandenburg et al., 1996), or by imprinting effects limited to placental tissues and in utero growth (Robinson et al., 1997). The maternal hypertension and deteriorating renal function in the present UPD 16 case further supports this hypothesis. Moreover, there appears to be an association between CPM for chromosome 16 and an unexplained abnormal profile of maternal serum markers (Vaughan et al., 1994; Zimmerman et al., 1995; Tantravahi et al., 1996), also pointing in the direction of a dysfunctional placenta. However, this can not explain the presence of fetal congenital malformations in some cases of UPD 16. Therefore, another possible explanation might be that in symptomatic cases trisomy 16 cells are in fact not confined to the placenta, but a mosaic trisomy 16 is also present in the fetus or infant. Clinical observation of the fetus and FISH studies in case 16 of our series supports this hypothesis. FISH revealed a raised proportion of trisomic cells in uncultured AF, although cytogenetic as well as FISH analysis of the AF cell cultures only revealed disomic cells (FISH data on cell cultures not shown). These discrepant FISH results between uncultured and cultured tissue may be explained by a proliferative advantage of normal cells in the cell cultures. Postmortem examination of the fetus after intra-uterine death at 33 weeks of gestation revealed several congenital malformations which fitted a mosaic trisomy 16 phenotype (Devi et al., 1993). Postnatal cytogenetic and FISH studies of the fetus were only succesfull in cultured ovary tissue, revealing disomic cells, and in fetal lymphocytes with 5 % of interphase nuclei showing three chromosome 16 signals with FISH (normal up to 3% in a series of ten control samples). In the two other cases of full trisomy 16 in the present series the FISH results in AF were normal. Based on these results, we believe that the detection of trisomy 16 in uncultured AF cells with FISH might have a predictive value for the actual fetal chromosomal constitution and outcome.

In general, we showed that FISH is a reliable method for rapid differentiation between generalized mosaicism and CPM, as in 13 out of 14 cases FISH results were in agreement with cytogenetic results, and they could be achieved within two days after sampling in all cases. The only case with discrepant results is discussed above.

In conclusion, the incidence of UPD in a series of CPM (type I and/or III) cases, collected over a four-year-period in our laboratory, is very low, indicating that in most cases the trisomic cell line in CV originates from somatic duplication, which is supported by the low level of mosaicism in most of these cases. Furthermore, it indicates that the obstetrical complications as IUGR and IUD, found in the present series, are not associated with UPD.

Acknowledgements

We wish to thank Carola Jansen for expert technical assistence, Armando Braat for generating figure 1, and the Rotterdam Foundation of Clinical Genetics for its financial support.

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Appendix publication VIII

Prenat Diagn, in press

Uniparental disomy with and without confined placental mosaicism: a model for trisomic zygote rescue.

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Summary

In the population of children born after prenatal cytogenetic investigation in chorionic villi at our department from 1992 to 1995 (N=3940), three are known to us with uniparental disomy. One case of maternal heterodisomy 16 was prenatally discovered because of trisomy 16 in direct chorionic villi with subsequently normal amniotic fluid cells. The other two had normal karyotypes in chorionic villi. Maternal heterodisomy 15 was postnatally detected in one of them because of Prader-Willi syndrome. Maternal hetero/isodisomy 16 was accidentally encountered in the other case in the course of prenatal DNA analysis of the tuberous sclerosis complex 2 region at 16p13.3. A model is presented for the understanding of the various combinations of karyotypes in direct chorionic villi, cultured chorionic villi and the fetus in case of successful and unsuccessful trisomic zygote rescue.

Introduction

Uniparental disomy (UPD) is prenatally mainly observed or suspected in case of (mosaic) trisomy in (semi)-direct (short term culture; STC) and cultured (long term culture; LTC) chorionic villi with subsequently normal amniotic fluid cells (Kalousek & Barrett, 1994; Ledbetter & Engel, 1995; Wolstenholme, 1996). The process of the loss or removal of one of the three chromosomes from the trisomic conception, at least from the cells that will form the fetus proper is known as trisomic zygote rescue, and the three situations of abnormal karyotypes in STC villi, LTC villi, or both with a normal karyotype in the fetus are designated confined placental mosaicism (CPM) type, 1, 2 and 3, respectively (Kalousek & Barrett, 1994; Kalousek & Vekemans, 1996; Wolstenholme, 1996).

Various cases of CPM (type 1 or 3) and fetal UPD have been documented (Purvis-Smith et al., 1992; Cassidy et al., 1992; Bennet et al., 1992; Kalousek et al., 1993; 1996; Webb et al., 1995; 1996; Langlois et al., 1995; Jones et al., 1995; Wilkinson et al, 1996). Furthermore, cases of generalized mosaicism with UPD in the disomic cell line are known (Sirchia et al. 1994; Harrison et al., 1995; Christian et al., 1995; De Pater et al., 1997; Van den Berg et al., 1997).

We would like to present three cases of UPD in which prenatal cytogenetic investigations were carried out in chorionic villi at our department; two showed normal karyotypes in STC villi and maternal UPD 15 and 16, respectively, the third displayed CPM trisomy 16 in STC villi and maternal UPD 16. A model for trisomic zygote rescue, based on the embryogenic

model originally described by Crane and Cheung (1988) and modified by Bianchi et al. (1993), is presented for the explanation of the possible combinations of karyotypes in the various compartments in fetal UPD with or without CPM or UPD in the disomic cell line in case of generalized mosaicism.

Material and Methods

During the years 1992-1995, 3940 prenatal cytogenetic investigations were carried out in chorionic villi at our department. The majority of investigations (N=3731) was performed for cytogenetic reasons (advanced maternal age, ultrasound abnormalities, parental carriership of chromosomal rearrangement, recurrence risk for fetal aneuploidy). In the remaining cases (N=209), karyotyping was performed in addition to DNA or metabolic investigations. Chorionic villus sampling (CVS) was carried out transabdominally in all cases as described before (Jahoda et al., 1990). In case of any mosaic trisomy or full unusual trisomy (trisomy other than 13, 18 or 21) in chorionic villi, follow-up amniocentesis and DNA analysis were offered for the differentiation between generalized chromosomal abnormality and CPM, and for the establishment of potential UPD (Van Opstal et al., 1997a).

Preparation of STC chorionic villi slides was done according to Gibas et al (1987). We did not perform LTC villi preparations in this period. Routine Trypsin Giemsa staining was used. Normally, 16 cells were investigated, but in case of mosaicism we investigated at least 30 metaphases. Amniotic fluid cells were cultured according to standard techniques with the in situ method on glass coverslips.

Slide preparation for fluorescence in situ hybridization (FISH) on first trimester STC chorionic villi, and on STC and LTC placental villi were carried out as described before (Van Opstal et al., 1995). For the detection of the copy number of chromosome 15 or 16, the probes pHuR195 for chromosome 16 (Moyzis et al., 1987) and pTRA-20 for chromosome 15 (Choo et al., 1990) were used. The probes CW9D and CW23, located at 16p13.3 were used for the investigation of deletions in the tuberous sclerosis complex (TSC)2 region (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). Probe labelling, detection, and visualization were carried out as described before (Van Opstal et al., 1995; Van den Berg et al., 1997). Two hundred non-overlapping and non-clumbed interphase nuclei of each sample were investigated. Metaphase analysis was carried out on 10 cells.

Fetal DNA was isolated from fresh chorionic villi, uncultured as well as cultured amniotic fluid cells, and cells scraped from stored STC villi slides (Van Opstal et al, 1997b). Postnatal DNA was isolated from peripheral blood according to standard techniques. In a pregnancy at risk for TSC (McKusick phenotype 191092; McKusick, 1994) from a family showing linkage to the TSC2 region on 16p13.3, DNA analysis was performed with the flanking markers 3'HVR, KG8, and 16AC2.5 (D16S291) (Janssen et al. 1994). DNA analysis for Prader Willi syndrome (PWS) was carried out with probe PW71B and the microsatellite markers LS6-1 and GABRβ3 (Van den Ouweland et al., 1995). Prenatal or postnatal investigations on the parent of origin of the chromosomes 15 and 16 were carried out with analysis of various polymorphic microsatellite markers on chromosome 15 (CYP19, D15S87 and ACTC) and on chromosome 16 (HBA1, D16S404, D16S298, D16S261, D16S285, D16S415, D16S320, D16S503, D16S515, D16S422 and D16S305). Data on localisation and primer sequences were derived from the Genome Data Base.

Clinical data on the course of pregnancy, delivery and baby were collected in all three cases.

Results

Two cases of UPD were found among the women who underwent CVS on cytogenetic indications, one with a normal karyotype (case 1) and the other with a trisomy 16 (case 2) in STC villi. The third case of UPD, again with a normal karyotype in STC villi, occurred in a women who underwent CVS on a DNA indication (case 3).

Table 1. Cytogenetic and FISH data of the three UPD cases (UPD 15 in case 1, UPD 16 in cases 2 and 3)

Case	karyotype in STC villi [no. of cells]	FISH on STC villi: nuclei with 1-4 signals(%) [control range (%)]*					Karyotype in AF cells or lymphocytes
		Probe	1	2	3	4	
1	46,XY [16]	pTRA-20	1 [0-2]	97.5 [97-100]	1.5 [0-3]	0 [0-1]	46,XY
2	47,XX,+16[37]	pHuR195	0 [1.5-15]	19 [80-96]	79 [0-10]	2 [0-7]	46,XX
3	46,XY[40]	pHuR195	3 [1.5-15]	94 [80-96]	3 [0-10]	0 [0-7]	46,XY

AF = amniotic fluid; * The control range consisted out of 3 normal cases for pTRA-20 and 15 for pHuR-195.

Case 1.

A boy of 2735 g (25th percentile) was born after an uncomplicated pregnancy at 37 weeks by cesarean section due to malposition. His mother underwent CVS on the indication of advanced maternal age (39 years) with normal cytogenetic results (Table 1). The boy displayed congenital hypotonia, transient feeding difficulties, bitemporal narrowing of the head, short palpebral fissures and hypogenitalism, suspect for PWS. The diagnosis of PWS was ascertained by demonstrating the absence of a paternal fragment with probe PW71B and maternal heterodisomy 15 with various microsatellite markers (Table 2). Additional retrospective FISH investigation on a stored STC villi slide confirmed the absence of trisomy 15 cells (Table 1). Analysis of DNA from another stored STC villi slide confirmed maternal heterodisomy 15 in the first trimester chorionic villi (Table 2).

Case 2.

With the follow-up protocol for unusual trisomy in STC villi, case 2 was prenatally identified. This case has been extensively reported by Van Opstal et al. (1997a). In short: after prenatal diagnosis because of advanced maternal age (40 years) a CPM of non-mosaic trisomy 16 was established with maternal heterodisomy 16 in amniotic fluid cells (Table 1 and 2). A normal girl was born at term with a normal birthweight.

Table 2. Alleles of the investigated polymorphic microsatellite markers in the three UPD cases

Case	Marker	Localisation	Father	Mother	CV	AF cells/ lymphocytes	UPD	
1	LS6-1	15q11-q12	2,2	1,3	-	1,3	Mat	H
	GABRβ3	15q11-q12	1,2	2,3	-	2,3	?	
	CYP19	15q21.1	1,2	3,3	3,3	3,3	Mat	?
	ACTC	15q13-qter	2,3	1,4	1,4	1,4	Mat	H
	D15S87	15q25-qter	1,3	1,2	1,2	1,2	?	
2	HBA1	16p13.3	1,3	2,2	2,2,3	2,2	Mat	?
	D16S298	16p12.1	1,2	3,4	-	3,4	Mat	H
	D16S261	16q12.1	2,3	1,1	1,1,2	1,1	Mat	?
	D16S285	16q12.1	1,3	1,2	1,2,3	1,2	?	
	D16S308	16q12.1	1,2	2,3	-	2,3	?	
	D16S305	16q24.3	3,3	1,2	1,2,3	1,2	Mat	H
3	3'HVR	16p13.3	1,4	2,3	3,3	-	Mat	I
	KG8	16p13.3	1,3	2,3	2,2	2,2	Mat	I
	D16S291	16p13.3	1,2	3,4	4,4	4,4	Mat	I
	HBA1	16p13.3	1,4	2,3	2,2	-	Mat	I
	D16S404	16p13.1	1,4	2,3	3,3	-	Mat	I
	D16S285	16q12.1	1,4	2,3	2,3	-	Mat	H
	D16S415	16q12.1	1,3	2,4	2,4	-	Mat	H
	D16S320	16q13	2,2	1,3	1,3	-	Mat	H
	D16S503	16q21	3,3	1,2	1,2	-	Mat	H
	D16S515	16q22.3-q23.1	1,4	2,3	2,3	-	Mat	H
	D16S422	16q24.2	2,3	1,2	1,1	-	Mat	I
	D16S305	16q24.3	1,3	2,4	2,2	-	Mat	I

CV = chorionic villi; AF = amniotic fluid; Mat = maternal; H = heterodisomy; I = isodisomy; ? = inconclusive.

Case 3.

In the third case, maternal iso/heterodisomy 16 was accidentally encountered during prenatal DNA analysis in a pregnancy of a TSC affected mother. Marker analysis showed the abscence of paternal alleles and the presence of one type of maternal alleles only (Table 2). A deletion of the 16p13.3 region was excluded with FISH by showing the presence of signals of the probes CW9D and CW23 on both chromosomes 16. Extended chromosome 16 marker analysis showed alternate regions of maternal iso- and heterodisomy; fortunately, the isodisomic parts comprised the normal region of 16p13.3, leaving the fetus unaffected with TSC (Table 2). The karyotype in STC villi was 46,XY; additional FISH studies revealed a signal distribution compatible with disomy 16 (Table 1). Ultrasound investigation at 19 weeks of gestation did not show fetal abnormalities. However, the second half of pregnancy was complicated by intrauterine growth retardation and pregnancy induced hypertension. At 37 weeks, a boy was delivered by cesarean section with a birthweight of 1850 g (< 5th percentile) without congenital malformations. After birth the placenta was received for further investigation. FISH on STC and LTC villi of 10 different placental biopsies showed signal distributions of the 16 centromere probe pHuR195 within the range of normal control samples.

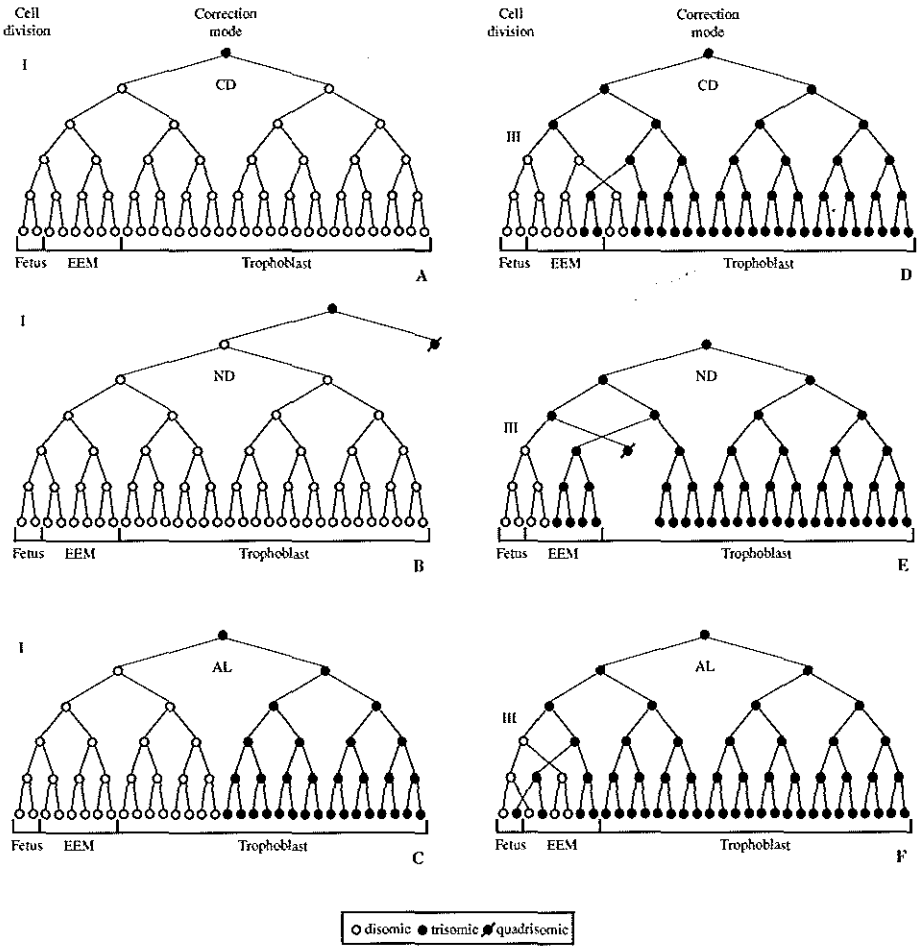
Discussion

UPD can be purely heterodisomic, combined hetero/isodisomic or purely isodisomic, dependent on the meiotic division in which the non-disjunctional error occurred and the extent of cross-over between the homologues of the chromosome pair involved (Engel, 1980; Engel & DeLozier-Blanchet, 1991). Heterodisomy can be without consequences, but isodisomy of (parts of) chromosomes may lead to autosomal recessive disorders (Engel, 1993; Ledbetter & Engel, 1995). However, heterodisomy results in autsomal dominant disease when the parent contributing both chromosomes is affected. In our third case, the fetus was saved from being affected with TSC because the TSC2 locus of the normal chromosome 16 was contained in a region of isodisomy. Irrespective of the heterodisomic and isodisomic component, UPD causes developmental disturbances when imprinted regions present on some chromosomes are involved (Bennet et al., 1992; Engel, 1993; Ledbetter & Engel, 1995).

It is believed that trisomic zygote rescue resulting in fetal UPD is associated with CPM, especially CPM type 1 and 3 involving non-mosaic trisomies (Ledbetter & Engel, 1995; Wolstenholme, 1996). In our three cases of UPD, prenatal cytogenetic investigations in chorionic villi revealed only one case with CPM and two cases with completely normal karyotypes, suggesting that a normal karyotype in chorionic villi might not be an exception in UPD. Prenatal cases of generalized mosaicism of various trisomies with UPD in the disomic cell lines (Harrison et al., 1995; Christian et al., 1995; Van den Berg et al., 1997) as well as postnatally established mosaic cases of autosomal and sex chromosomal aneuploidy in which the mosaicism is due to loss of one of the chromosomes (Niikawa & Kajii, 1984; Robinson et al., 1995) further suggest that trisomic zygote rescue might not always be a successful event. The very high rate of mosaic aneuploidy in 2- to 8-cell stage human embryo's (Munné et al., 1994; Almeida & Bolton, 1996; Kligman et al., 1996) indicates that trisomic zygote rescue might be a rather common phenomenon.

The exact mechanism of trisomic zygote rescue is not known; anaphase lagging or non-disjunction in an early postzygotic cell division has been proposed (Kalousek et al., 1991). Since correction by anaphase lagging (AL) will result in one disomic and one trisomic daughter cell, this type of trisomic correction seems not to be perfect. Correction by non-disjunction (ND) will result in one viable disomic and one lethal quadrisomic cell and reduce the number of cells in the embryo which might delay normal development (Tarin et al., 1992). Therefore, we propose an alternative correction mode, chromosome demolition (CD), as a process of deliberate fragmentation and/or removal of one of the set of three chromosomes during metaphase or anaphase resulting in two disomic daughter cells. With each of these correction modes (AL, ND and CD), we would like to present a model for the arising of the various combinations of karyotypes in STC villi, LTC villi, and fetus from trisomic zygote rescue. We consider trisomic zygote rescue to consist out of one correction event in the first to fourth postzygotic cell division with a subsequently unknown (random or non-random) distribution of trisomic and disomic cells among the progenitor cells of the inner cell mass (ICM) and throphoblast compartment untill the 16-cell stage (fig. 1). In order to show all possible combinations of karyotypes, the data are presented under a random distribution (fig. 2 and 3). In their embryogenic model, Crane and Cheung (1988) and Bianchi et al. (1993) assume cells to lose their omnipotentiality after the 8-cell stage, and the ICM to contain 16 cells at the 64-cell (blastocyst) stage; four cells will form the fetus proper and 12 cells the extra-embryonic mesoderm (EEM).

Figure 1.Trisomic zygote rescue by chromosome demolition (CD), non-disjunction (ND), and anaphase lagging (AL). One example of a distribution of trisomic and disomic cells among fetus, extra-embryonic mesoderm (EEM), and trophoblast is shown for each correction mode in the 1st and 3rd cell division.
(A) CD correction, 1st cell division; (B) ND correction, 1 st cell division; (C) AL correction, 1st cell division; (D) CD correction, 3rd cell division; (E) ND correction, 3rd cell division; (F) AL correction, 3rd cell division.



Besides these assumptions, we assume that within the ICM, cells can allocate one or both daughter cells freely to the compartment of the fetus proper and/or that of the EEM after the irreversible separation of ICM and trophoblast compartment at the 16-cell stage. Furthermore we assume that in case of a reduced number of cells by ND correction, compensatory reallocation may occur between the trophoblast and ICM compartment (untill the 16-cell stage), comparable to the situation after preimplantation diagnosis (Tarín et al., 1992).

Figure 2. Theoretical distributions of trisomic and disomic cells among the inner cell mass (ICM) and trophoblast compartment at the 16-cell stage after trisomic zygote rescue by chromosome demolition (CD) or anaphase lagging (AL). The resulting karyotypes in fetus and cultured (LTC) villi, originating from the ICM, and in (semi-) direct (STC) villi, originating from the trophoblast, are shown. EEM = extra-embryonic mesoderm; N = normal; Abn = abnormal; Mos = mosaicism. [1] = example shown in figure 1A; [2] = example shown in figure 1C; [3] = example shown in figure 1D; [4] = example shown in figure 1F.

Cell division	Correction mode	ICM (fetus + EEM) cells (at 16-cell stage)	Resulting karyotypes Fetus	Resulting karyotypes LTC villi	Trophoblast cells (at 16-cell stage)	Resulting karyotype STC villi
I	CD	○○○	N	N	○○○○○○ ○○○○○○	N[1]
I	AL	○○○	N	N	●●●●●●	Mos[2]
		○○●	N,Mos Abn	Mos N	●●●●●●	Mos
II	CD	○●●	N,Mos,Abn	Mos	●●●●●●	Mos
		●●●	Mos,Abn N	Mos Abn	●●●●●●	Mos
		●●●	Abn	Abn	●●●●●●	Mos
II	AL	○○○	N	N	●●●●●●	Abn
		○○●	N,Mos Abn	Mos N	●●●●●●	Mos[3]
III	CD	○●●	N,Mos,Abn	Mos	●●●●●●	Mos
		●●●	Mos,Abn N	Mos Abn	●●●●●●	Mos
		●●●	Abn	Abn	●●●●●●	Mos
III	AL	○○●	N,Mos,Abn	Mos	●●●●●●	Abn[4]
IV	CD	○●●	Mos,Abn N	Mos Abn	●●●●●●	Mos
		●●●	Abn	Abn	●●●●●●	Mos
IV	AL	○●●	Mos,Abn N	Mos Abn	●●●●●●	Abn
		●●●	Abn	Abn	●●●●●●	Mos

○ disomic ● trisomic

Trisomic zygote rescue by CD and ND correction can explain all prenatally encountered combinations of karyotypes in the trophoblast, EEM, and fetal compartment in fetal UPD or UPD in the disomic cell line in case of generalized mosaicism. AL correction cannot produce the important combination of UPD with normal karyotypes in all compartments, and seems not to be the mechanism of first choice in trisomic zygote rescue. In our opinion, CD correction is the preferable mechanism at least in the first two cell divisions since it will not result in a critical diminished number of cells in the embryo. From the third cell division onwards, there is not much difference between the three types of correction. Among the theoretical combinations of karyotypes in the various compartments described by Pittalis et al (1994), CPM type 2 does not occur in our model, confirming other statements with respect to the absence of an association between CPM type 2 and UPD (Bianchi et al., 1993;

Figure 3. Theoretical distributions of trisomic and disomic cells among the inner cell mass (ICM) and trophoblast compartment at the 16-cell stage after trisomic zygote rescue by non-disjunction (ND) with and without compensatory reallocation. The resulting karyotypes in fetus and cultured (LTC) villi, originating from the ICM, and (semi-) direct (STC) villi, originating from the trophoblast are shown. EEM = extra-embryonic mesoderm; N = normal; Abn = abnormal; Mos = mosaicism. [1] = example shown in figure 1B; [2] = example shown in figure 1E.

Cell division	Correction mode	ICM (fetus + EEM) cells (at 16-cell stage)	Resulting karyotypes Fetus	Resulting karyotypes LTC villi	Trophoblast cells (at 16-cell stage)	Resulting karyotype STC villi
I	ND	○○○	N	N	○○○○○○ ○○○○○○	N[1]
II	ND	○○○	N	N	●●●●	Abn
		○○●	N,Mos Abn	Mos N	●●●●	Mos
		○●●	N,Mos,Abn	Mos	●●●●	Mos
		○●●	Mos,Abn N	Mos Abn	●●●●	Mos
		●●●	Abn	Abn	●●●●	Mos
III	ND	○○○	N	N	●●●●●	Mos
		○○●	N,Mos Abn	Mos N	●●●●●	Mos
		○●●	Mos,Abn N	Mos Abn	●●●●●	Mos
		●●●	Abn	Abn	●●●●●	Mos
III	ND	○○	N	N	●●●●●	Mos
		○●	N Mos Abn	Abn Mos N	●●●●●	Mos
		●●	Abn	Abn	●●●●●	Mos
III	ND	○○●●	N,Mos,Abn	Mos	●●●●●●	Abn[2]
		○●●●	Mos,Abn N	Mos Abn	●●●●●●	Mos
		●●●●	Abn	Abn	●●●●●	Mos
III	ND	○○●	N,Mos Abn	Mos N	●●●●●●	Abn
		○●●	Mos,Abn N	Mos Abn	●●●●●●	Mos
		●●●	Abn	Abn	●●●●●●	Mos
III	ND	○○	N	N	●●●●●●	Abn
		○●	N Mos Abn	Abn Mos N	●●●●●●	Mos
		●●	Abn	Abn	●●●●●●	Mos
IV	ND	○●●	Mos,Abn N	Mos Abn	●●●●●●	Abn
		●●●	Abn	Abn	●●●●●●	Mos
		○●●●	Mos,Abn N	Mos Abn	●●●●●●	Abn
○ disomic ● trisomic		●●●●	Abn	Abn	●●●●●●	Mos

Wolstenholme, 1996). The opposite situation of abnormal karyotypes in fetus and STC villi with a normal karyotype in LTC villi, designated generalized mosaicism confined culture normality (GMDC) does occur in our model. In their series, GMDC was not observed and considered to be very rare (Pittalis et al., 1994). So, the distribution of chromosomally normal and abnormal cells among the various compartments after trisomic zygote rescue seems not to be random but directed towards a rankorder in the compartmentalization of the fetal, EEM, and trophoblast compartments, respectively, with the maximal number of normal cells. Correction in the first cell division with normal karyotypes in all compartments and biparental inheritance of all chromsomes is the best result of trisomic zygote rescue and is unnoticed.

Acknowledgements

We are grateful to Professor H. Galjaard for his support and to Mrs. J. du Parant for preparing the manuscript.

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