Studies of Mosaicism for Chromosomal Aneuploidy in Fetal Loss Using Fluorescence In Situ Hybridization on Several Tissue Types

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Abstract

The main objective of this study was to determine the presence and level of mosaicism for chromosomal aneuploidy in several cases of fetal demise that had previous indications of chromosome aberrations. The presence of aneuploidy was determined using fluorescence in situ hybridization (FISH) probes. Techniques that were utilized in attempts to prepare several tissue types for FISH are described. The analyses made with FISH provided useful data on the chromosomal makeup of two of the cases studied. In one case FISH results showed that the product of conception (POC) did not contain the trisomy 18 that is present at low levels in the mother's blood cells. In the other case the miscarried fetus was previously identified as being mosaic for trisomy X. In this research project the presence of varying levels of trisomy X in both embryonic and placental tissue was confirmed.

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INTRODUCTION

In a normal human somatic cell there are two sets of 23 chromosomes, making a total of 46 chromosomes per cell. The term aneuploidy describes a cell that does not contain a multiple of 23 normal chromosomes in the nucleus. Aneuploid cells can have an excess or lack of one or more chromosomes. The origin of aneuploidy is typically due to the action of nondisjunction during meiosis or mitosis. Nondisjunction is the lack of separation of either two homologous chromosomes during meiosis I or two sister chromatids during meiosis II or mitosis. In meiosis, the result is one or more gametes containing two copies of the chromosome and one gamete lacking a copy altogether. Upon fertilization, the former will produce a trisomic zygote and the latter, a monosomic zygote. In mitosis the outcome is one daughter cell lacking a copy of the chromosome and the other daughter cell containing an extra copy. In trisomies, maternal meiosis I nondisjunction is the most common. However, mitotic, or postzygotic, nondisjunction is responsible for 5-15% of trisomies 15, 18, and 21 (Nicolaidis and Petersen, 1998).

Another cause of aneuploidy, which occurs in both mitosis and meiosis, is termed anaphase lag. This occurs when the chromosomes or chromatids succeed in disjoining but one of the pair fails to be bound by the spindle fiber and is therefore left at the metaphase plate and is not incorporated into the new cell (Barch et al., 1997).

Two common aneuploidies are monosomy, which describes a cell with only one copy of a certain chromosome, and trisomy, in which the abnormal cell has three copies of the chromosome. Monosomies are usually associated with more detrimental phenotypic abnormalities than trisomies. As Jorde et al. (1999) state, "the body can tolerate excess

genetic material more readily than it can tolerate a deficit of genetic material". For this reason there are very few cases of fetuses with autosomal monosomy surviving to birth.

Trisomy

Around half of all clinical miscarriages have a chromosome abnormality. Of these, about 60% are trisomies (Gardner and Sutherland, 1996). There are several well-characterized syndromes associated with trisomies. The most common autosomal aneuploidy is trisomy 21, which leads to Down's syndrome. It has a frequency of about 1 in 800 live births (Jorde et al., 1999). The second most common autosomal trisomy is trisomy 18, also known as Edward's syndrome, with a frequency of 1 in around 6000 live births. Another common autosomal trisomy is that of chromosome 13, also termed Patau syndrome. This occurs in about 1 in 10,000 births.

In this research project one case study involves trisomy 18. Of pregnancies affected by trisomy 18, 95% abort spontaneously (Fisher et al., 1993). Some of the physical disorders associated with trisomy 18 are intrauterine growth retardation, and structural defects, including ventricular septal defects, omphalocele, diaphragmatic hernia, and spina bifida. In a study by Baty et al. (1994), cardiopulmonary arrest accounts for 68% of deaths in trisomy 18 babies. In addition, 11% died of congenital heart disease and another 11% from pneumonia. Jorde et al. (1999) state that around half of trisomy 18 babies die within the first month and 10% survive the first year. However, Root and Carey (1994) have shown that these survival rates are significantly lower.

The physical and mental results of most sex chromosome aneuploidies are not as detrimental as those associated with autosomal aneuploidy. A case examined in this

project involves trisomy for the X chromosome in an aborted fetus. The frequency of trisomy X is about 1 in 1000 females, with most affected persons having a normal life span (Jaruratanasirikul and Jinorose, 1994). This karyotype is not usually associated with severe physical problems. However, reproductive and mental deficiencies are sometimes seen. The lack of major physical problems being associated with an extra X chromosome is associated with the inactivation of all but one X chromosome as seen in normal females.

Mosaicism

The major focus of this research is on the presence and level of mosaicism for the aneuploidies described above, in various tissues. Mosaicism is defined as the presence of more than one genetically distinct cell line within the body. Mosaicism may be derived from "chromosomal abnormalities (missing or extra chromosomes or parts of chromosomes), from single-gene mutations, or possibly from the incorporation of extrachromosomal DNA" (Hall, 1988). Thus, trisomic individuals may not be uniform for the condition in all cells. On close analysis of the different tissues, mosaicism may be discovered in certain individuals. Kalousek and Barret (1994) state that "chromosomal mosaicism is the result of postzygotic error(s) giving rise to a viable cell line(s)". This can appear at any stage in development after conception. In terms of the aneuploidies mentioned above, mosaicism is found in 1-3% of trisomy 21 live births and less than 5% of trisomy 18 individuals (Jorde et al., 1999). In a study by Baty et al. (1994) those children studied "with trisomy 18 mosaicism . . . had a phenotype distinct from full trisomy 18, including the ability to walk, and higher skills in many areas".

The stage of development at which the abnormality appears determines the level of mosaicism (Barch et al., 1997). If the error in division occurs early in development then it is possible that the majority of the cells could be abnormal. But, if the abnormality originates late in development it is impossible to completely eliminate the presence of mosaicism without determining the karyotype of every cell in the body. The three main cell types in the body are ectodermal, endodermal, and mesodermal. If the nondisjunction or anaphase lag occurs after differentiation into these three cell types, the abnormal cell line could be isolated to only one cell type or possibly, only one organ. Thus, the phenotypic effect of mosaicism for a particular chromosomal abnormality depends on the number and type of cells or organs effected by the misdivision and the problems incurred by the excess or shortage of genetic material.

There are several types of mosaicism including gonadal (also called germ-line mosaicism), somatic, and confined placental mosaicism. Gonadal mosaicism occurs when the abnormality appears in a number of germ-line cells of a phenotypically normal individual, and it can be conferred to offspring. This parent "may transmit several gametes that are clonal descendants of a single progenitor cell in which a *de novo* mutation occurred during [their] early development"(Hall, 1988). The possibility of germ-line mosaicism must be considered when parents are unaffected by a certain disorder but have multiple affected offspring (Bernards and Gusella, 1994). Bernards and Gusella (1994) also point out that it is unlikely that a mosaic will have the same chromosome anomaly in both their germ-line and somatic cells unless the mutation occurs very soon after the first postzygotic divisions. Somatic mosaicism is that which occurs in the somatic cells of the body and can be seen in a segmental distribution

dependent on the point in development when the misdivision occurs. Confined placental mosaicism (CPM) is a form of tissue specific mosaicism in which only the placenta is affected and the fetus proper is not affected (Kalousek and Vekemans, 1996). CPM occurs postzygotically in two manners. A trisomic conceptus undergoing mitosis may lose the extra chromosome in the cells destined to become the embryo and retain it in the cells destined to form the placenta. The other mechanism involves the gain of an extra chromosome by the cells destined to become the placenta that had previously lacked a copy of one chromosome.

Techniques Used to Study Chromosomal Mosaicism

The technical aspect of this honors thesis research involved mastering one technique currently used to identify aneuploid cells. This process involved two main steps: the preparation of tissue on microscope slides, and the use of fluorescence in situ hybridization (FISH) in order to probe the tissue on these slides for chromosomal aneuploidy.

Sample tissue used to study chromosome aberrations can be obtained through several different procedures and can come from many different sites of the body. Sample tissue is obtained from sources such as amniocentesis, chorionic villus sampling (CVS), blood samples, biopsy, fetal pathology, and placenta. Fresh tissue can either be prepared then analyzed directly, or preserved and archived for study at a later date (see Figure 1).

Archiving allows researchers to store many tissue samples, which can later be processed and analyzed. This makes it possible to study many samples at the same time for a particular disorder. Methods for archiving tissue must preserve DNA structure and

cell morphology. Some typical methods for archiving are freezing, formalin fixation, and formalin fixation with subsequent paraffin embedding (see Figure 1). Frozen tissue must be kept frozen until it can be fixed and analyzed. Premature thawing can cause lysis of cells and cellular components, which makes further analysis impossible. Paraffin and formalin archiving make room temperature storage possible. The ability to analyze formalin-fixed tissues makes the "retrospective study of archived tissue possible" (Kuchinka et al., 1995).

One means of obtaining a large population of sample cells is cell culturing. This can be done on fresh or frozen tissue. Cells are provided with a media containing growth factors and antibiotics. Cells can either be grown on a solid substrate such as a microscope slide or coverslip or they can be cultured while suspended in solution.

There are certain procedures that must be completed in order to prepare cells for analysis. Cells or tissue that have been cultured or frozen must be fixed before standard genetic analysis (G-banding) or FISH can be performed. Fixation functions in permeabilizing cells and cross-linking their macromolecules so that structures are set in position. In G-banding the cells are also immersed in a hypotonic solution in order to cause swelling of the nuclei and also lysis of the cell membrane making analysis of individual chromosomes possible.

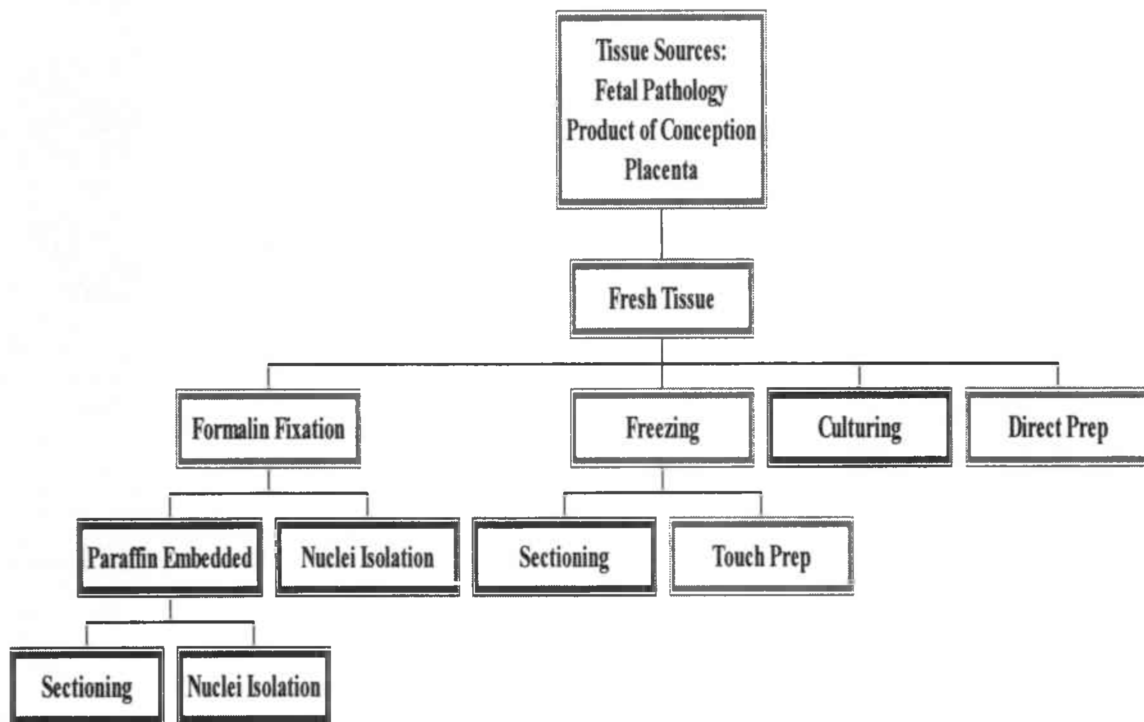

Figure 1. **Tissue Preparation Chart**: Procedures attempted in this study are bordered in color. Those that provide useful slides for FISH are bordered in green and those that were unsuccessful are bordered in red.

In many cases it is necessary to disaggregate tissue so that individual cells can be readily identified and studied without being in contact with other cells or tissue. As Van Sedum (1998) states, " the success of FISH is directly dependent on the accessibility of the target DNA within the cell nucleus". This requires removal of connective tissue and release of cells from connections with other cells and the extracellular matrix. Disaggregation is accomplished using proteolytic enzymes. The cells recovered from this procedure and all tissue preparations for FISH must have intact nuclei. Cells must not be emaciated and there must be a sufficient abundance of cells for analysis.

FISH is a molecular labeling technique used in various clinical and research realms of the biomedical sciences. FISH is an improvement over earlier developed types of in situ hybridization in that it does not utilize radioactive isotopes as labels. In the study of cytogenetics, FISH is used to identify specific segments of DNA on chromosomes in metaphase or interphase cells. A FISH probe consists of an oligonucleotide DNA segment that is complementary to the target segment of chromosomal DNA, and a fluorophore component, which can be visualized using an epifluorescence microscope and documented with photography or imaging equipment. When excited by the light source each specific fluorophore fluoresces at certain wavelengths producing a specific color such as red (rhodamine) or green (fluorescein). This light is then passed through a set of filters, which makes viewing one two or three colors possible, depending on the combination of filters used. In addition to labeling specific target DNA with the FISH probes, all of the chromatin in the nucleus is stained with DAPI (4',6-Diamidino-2-phenylindole) to provide a blue background that contrasts the probe colors.

The mechanism for labeling chromosomal DNA with FISH involves five major steps: denaturation of both the probe and sample DNA, hybridization of the probe to the sample, removing probes that did not directly hybridized to target DNA, counterstaining the nucleus, and viewing under a epifluorescence microscope. This mechanism is depicted in Figure 2.

Probes can be synthesized by first producing a large number of the oligonucleotide specific for the chromosome or sequence under analysis. This DNA is then directly or indirectly labeled with fluorophores. Direct-labeled probes are available commercially from Vysis, Inc. and other companies. When bound indirectly, the DNA is singly or doubly labeled through a series of biochemical reactions involving biotin, digoxigenin, and antibody-bound fluorophores. In many instances, "home-made" FISH probes can be synthesized and utilized as an alternative to commercially prepared probes.

There are three main categories of FISH probes produced by Vysis for cytogenetics. Probes used to identify the presence of a certain gene or DNA sequence are called locus specific identifiers (LSI). Whole chromosome paint probes (WCP) are used to label the entire length of a chromosome. Chromosomes enumeration probes (CEP) are used to rapidly determine the presence of a specific chromosome or chromosomes within nuclei.

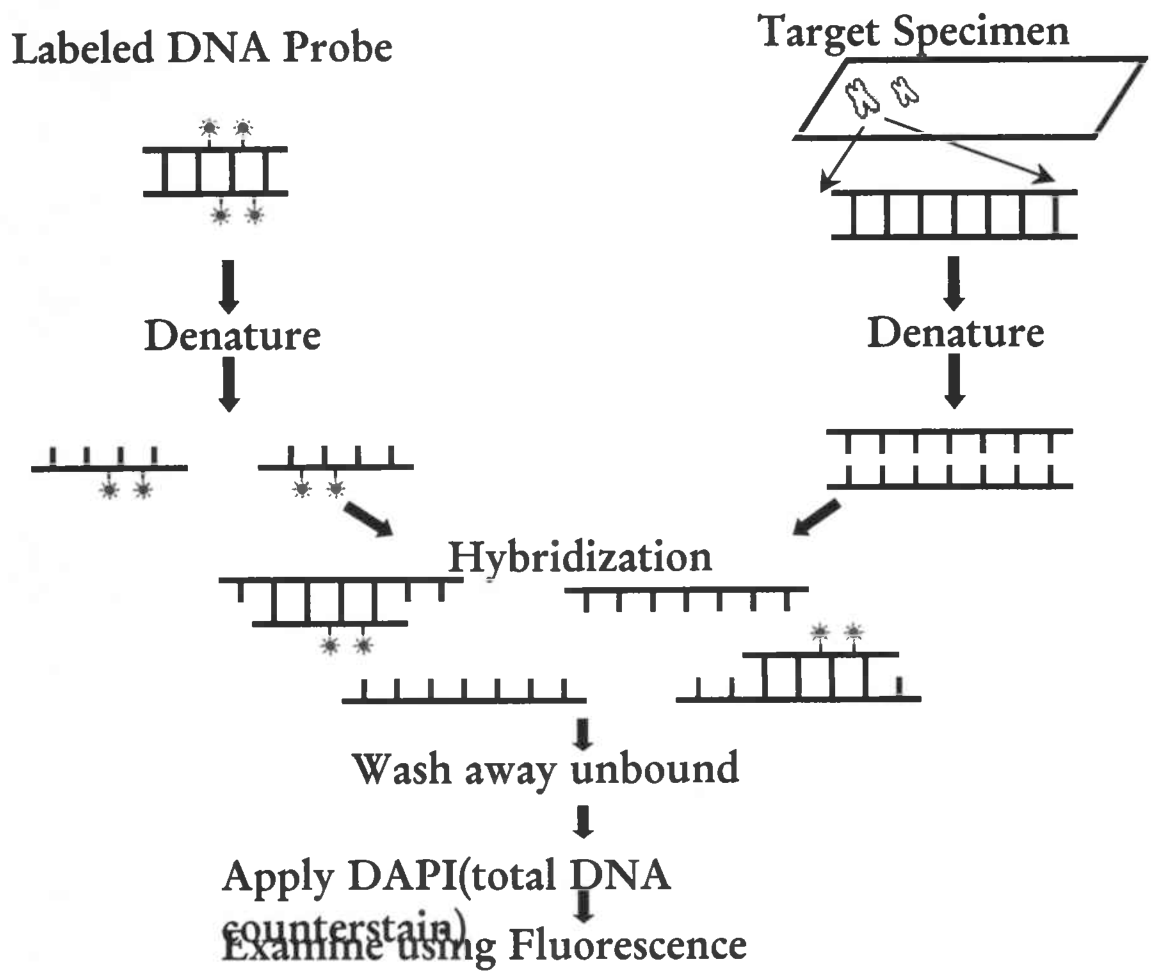

Figure 2. **Fluorescence in situ hybridization (FISH) mechanism.**

MATERIALS AND METHODS

Case Studies

One of the cases studied in this project involved a spontaneously aborted fetus, which was suspected of having trisomy 18 (Case1). In previous studies the mother was characterized as having trisomy 18 in about 4% of the peripheral blood cells that were tested. Phenotypically, the mother had normal intelligence and was physically normal, except for early ovarian failure. The miscarriage occurred early in the pregnancy and the abortus, or products of conception (POC), was archived for later study.

Another case included in this research project involves trisomy X (Case 2). The mother is phenotypically and genotypically normal. In this case the mother miscarried the fetus and placental samples were taken. Some studies of fetal tissue from this case include analysis of fetal lymphocytes and amniocytes. Standard chromosome (G-banding) analysis of cultured fetal cord blood cells showed that out of 100 metaphase spreads 65% were XXX and 35% were XX. Cells cultured from an amniotic fluid sample contained 61% XXX, by G-banding analysis.

Attempts were also made to do FISH studies on four other cases. Case 3 involved a miscarried fetus with unknown karyotype. Case 4 involved a fetus with suspected monosomy X, or Turners Syndrome, and in Case 5 placental tissue from a pregnancy in which the cause of fetal demise is unknown was studied. In Case 6 tissue from a miscarried, chromosomally normal fetus that was used as a control.

In analyzing the cases introduced above several methods of tissue preparation were utilized. In the trisomy 18 case (Case 1) the POC had been fixed in formalin then

embedded in paraffin. In addition, several sections had been cut from the POC block and mounted on slides while other sections were left unmounted. All of this preparation was completed before the tissue arrived at the Shodair Genetics Lab

In the trisomy X case (Case 2), prior to this research project, tissue from five sample sites of the placental had been cultured on coverslips and mounted on slides but were not stained for standard cytogenetic analysis. Some of the placental samples were frozen without being cultured. In addition, a sample of fetal cord blood cells had been obtained and cultured, then frozen for storage.

In cases 3-6 the tissue had been fixed and stored in formalin for varying lengths of time for archiving. In cases 3 and 4 the tissue was from the fetal lung. In cases 5 and 6 the tissues were from the placenta and the fetal liver, respectively.

The Vysis probes used in studying Cases 1 were CEP 18 and CEP Y. Ideograms of the chromosomes localized by each probe and the target region on chromosomes are illustrated in Figure 3. The CEP 18 probe consists of DNA sequences specific to the alpha satellite DNA (D18Z1) contained within the centromeric region (18p11.1-q11.1) of chromosome 18. The CEP Y probe contains alpha satellite sequences specific to the DYZ3 centromere region (Yp11.1-q11.1) of chromosome Y. In studying Case 2 Vysis CEP X and CEP 7 were used. The CEP X probe contains alpha satellite sequences specific to the centromere region Xp11.1-q11.1 of chromosome X. The CEP 7 probe consists of sequences specific to the alpha satellite DNA (D7Z1) contained within the centromeric region (7p11.1-q11.1) of chromosome 7.

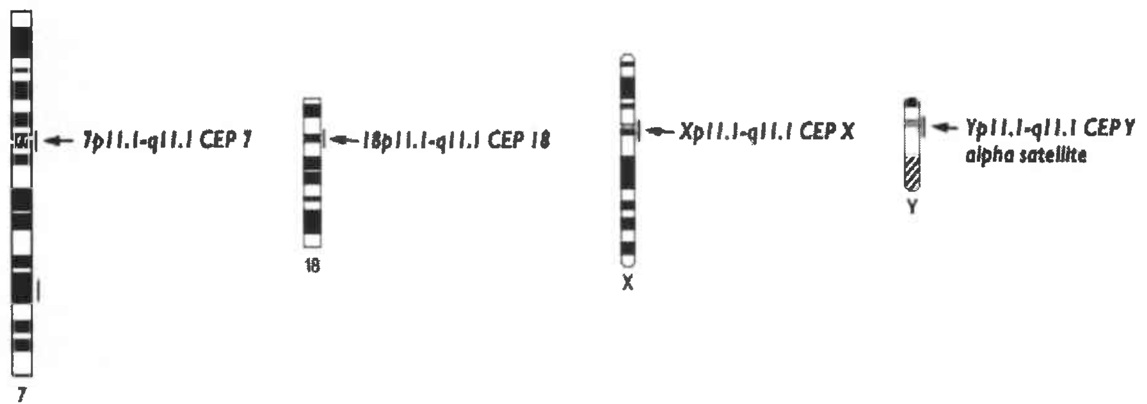

Figure 3. **Ideograms on CEP probe specific regions of the chromosomes analyzed in this study.**

Procedures used to study paraffin-embedded samples

The preparation process for the slide mounted, paraffin-embedded tissue sections from the POC in case 1 involves deparaffinization then digestion of the sample tissue. The procedures followed for this preparation were adapted from a protocol published by Vysis Inc. (Vysis, 1997). The initial step removes paraffin from the tissue section. Immersing the slides three times in xylene for 10 minutes each does this. Next, the slides are dehydrated twice in 100% ethanol (ETOH) at room temperature for five minutes each, then air dried. Next, the slides are incubated in 2X SSC (Salt and Sodium Citrate) at 75°C for ten minutes. Then the tissue on the slides is digested in 4mg/ml pepsin (Sigma P-7012, in 0.9% NaCl, adjusted to pH 1.5 using HCL) for ten to fifteen minutes at 37°C. Finally, the slides are dipped in distilled water and rinsed in 2X SSC for 2 minutes at room temperature.

After the preparation delineated above, FISH is performed on the mounted POC tissue sections from case 1 according to the general description illustrated in Figure 2 of the introduction. First, the DNA of the cells in the tissue is denatured using a 70% formamide, 2X SSC solution at 75°C for three to five minutes. The tissue is then dehydrated for two minutes in 85% ETOH and two minutes in 100% ETOH, both at room temperature. The slides are then air dried. In order to prepare the Vysis probe mixture 1 μL CEP Y probe (Spectrum Green), 1 μL CEP 18 probe (Spectrum Orange), 2 μL purified H_2O, and 7 μL CEP Hybridization Buffer are combined in a microcentrifuge tube. This tube is then spun momentarily in a microcentrifuge to mix. The probe mixture is denatured at 73±1°C for 5 minutes and mixed again. The probe mixture is placed on

the denatured tissue section (approximately 22 x 22 mm area), covered with a glass coverslip, and sealed with rubber cement. The slide is then incubated at 37°C in a humid chamber overnight. After incubation the coverslip and rubber cement are carefully removed. The slides are washed for two minutes each in the following 45°C wash solutions: 50% Formamide/2X SSC three times, then one wash each in 2X SSC and 2X SSC/0.1% NP-40 (Nonident P-40, Fluka 74385). Next, the slides are washed once in 2X SSC/0.1% NP-40 at room temperature for two minutes, then air dried. The slides are counterstained with 10 μL of 2:1 DAPI:Antifade. Finally, the slides are viewed using an epifluorescence microscope.

The process for isolation of nuclei from the unmounted, paraffin-embedded tissue sections of case 1 is adapted from a protocol written by Rizwan Naaem and published by Vysis Inc. (Naaem, 1997). Again, the first step is deparaffinizing the section in xylene three times, ten minutes each. The tissue is then dehydrated in 100% ETOH two times, for five minutes each. Next, the tissue is incubated in room temperature distilled water two times, for five minutes each. The tissue is digested in 4mg/ml pepsin (Sigma P-7012, in 0.9% NaCl pH 1.5) for two hours at 37°C. During this digestion the tubes containing the tissue are vortexed every thirty minutes. Subsequently, the tissue is filtered through a 40 μm nylon mesh then centrifuged at 800 rpm in a standard tabletop centrifuge for five minutes. The pellet is washed in Hank's Balanced Salt Solution (HBSS) and spun twice more at 800 rpm for five minutes each. Then the isolated nuclei are resuspended in a minimal amount (about 1 ml) of HBSS and vortexed. The nuclei are spread on slides, dried at 65°C for ten minutes, then rinsed in HBSS. These slides are incubated in 0.01%

Triton X-100 (Sigma) in HBSS for one minute and thirty seconds. Next the slides are rinsed in HBSS three times for three minutes each. The slides are incubated in 0.3 mg/ml pronase (Proteinase type XIV, Sigma P5147) in 50 mM Tris/Cl pH 7.6, 5 mM EDTA at room temperature for five minutes. Then the slides are incubated three times in 2 mg/ml glycine in HBSS at room temperature for three minutes each. After that, the slides are incubated in 4% parafomaldehyde in HBSS at room temperature for five minutes. The slides are incubated in 2mg/ml glycine in HBSS again, this time for three minutes. Then they are dehydrated in a series of 30%, 60%, 80%, 95%, and 100% ETOH at room temperature for three minutes each. Finally, the slides are air dried.

Following the preparation of slides containing isolated nuclei FISH is performed. The Vysis probe mixture is prepared as described above. To each sample slide, 10 µL of the Vysis probe mixture is applied to an approximately 22 x 22 mm area. A coverslip is placed on the sample are and sealed with rubber cement. The probe and sample DNA are denatured simultaneously by placing the slide in a 90 °C oven for one to three minutes. Hybridization occurs when the slide is then incubated in a 37°C humid chamber overnight. After hybridization the rubber cement and coverslip are removed. The excess probe mixture is removed in the following wash steps. The slides are washed for one minute each in the following 45°C wash solutions: 50% Formamide/2X SSC three times, then one wash each in 2X SSC and 2X SSC/0.1% NP-40 (Nonident P-40, Fluka 74385). Next the slides are washed once in 2X SSC/0.1% NP-40 at room temperature for two minutes, then air dried. The slides are counterstained as above. Finally, the slides are viewed using an epifluorescence microscope.

Procedures used to study formalin-fixed samples

In attempting to form an effective protocol for FISH on formalin-fixed tissue the sample from case 3 was processed first. Upon viewing the results from case 3 an alternate protocol was attempted on cases 4-6. The protocol followed in attempts to isolate nuclei from formalin-fixed tissue follows the methods described by Kuchinka et al. (1995). An approximately 1 cm square piece of tissue is minced into fine pieces using a scalpel. This minced tissue is then placed in a 15 ml centrifuge tube and rehydrated in and room temperature series of 70%, 50%, and 30% ETOH. Between each solution change in this procedure the tissue is spun down at 800 rpm, in a standard tabletop centrifuge for three to five minutes. After the rehydration steps the ETOH is removed and sterile distilled water is added and the tube is stored in the refrigerator (4°C) overnight. The tissue is then washed in HBSS at room temperature two times for five minutes each. Next, the tissue is digested in 1 mg/mL Collagenase XI (Sigma) for 1.5-2 hours at 37°C. During this digestion the tube is vortexed every ten minutes. The tissue is again washed twice in HBSS as above. The tissue is treated with a 0.05% Trypsin/EDTA (Gibco) solution for one hour or longer. This digestion should be monitored frequently under a dissecting scope. When there is a high concentration of single cells, the tissue is vortexed and the cells are allowed to settle for two to three minutes. Then the supernatant is decanted or pipetted off. This supernatant is spun down for three to five minutes in order to concentrate the cells. With a Pasteur pipette, cells are then dropped onto a clean

slide. These slides are air dried overnight and baked for 1-2 hours at 60°C. The sample slides are passed through a series of 70%, 80%, 90%, and 100% ETOH solutions at room temperature for two minutes each in order to dehydrate the sample. Then the slides are treated with 30% sodium bisulfite for fifteen minutes and Proteinase K (250μg/mL) for fifteen to thirty minutes, both at room temperature. Upon viewing results from the above procedure, additional steps were undertaken in order to remove excess tissue. These included filtering the cells through a 40 μm nylon mesh and digesting tissue off of slides with a 4 mg/ml Pepsin, 37 °C treatment for 2 hours.

After preparing slides that had sufficient numbers of isolated nuclei from case 3, FISH was executed. The following protocol is the standard protocol used to perform FISH with Vysis CEP probes on most samples, except the paraffin-embedded samples. It will be referred to in some of the following FISH procedures on other tissues. Slides are first pretreated in 2X SSC/0.1% NP-40 for thirty minutes at 37°C. They are then dehydrated at room temperature in 70%, 80%, and 95% ETOH for two minutes each, air dried, and placed on the slide warmer. DNA of the sample cells is then denatured by placing the slides in 70% formamide/2X SSC at 73±1°C for five minutes. The slides are dehydrated again in 70%, 80%, and 95% cold ETOH for two minutes each in order to maintain denatured DNA strands in the specimen. The Vysis probe mixture is prepared by combining 1 μL CEP probe, 2 μL purified H_2O, and 7 μL CEP Hybridization Buffer in a microcentrifuge tube. When a combination of probes is used, 1μL of each probe and the same amount of the other two components as listed above are added. In case 3 the two probes used were CEP 21 and CEP Y. The tube is then spun momentarily in a

microcentrifuge to mix the components. The probe mixture is then denatured at 73±1°C for 5 minutes and mixed again. Then 10 μL of the probe mixture is applied to the denatured sample on the slide(approximately 22 x 22 mm area), covered with a glass coverslip, and sealed with rubber cement. The CEP probe DNA is then hybridized to the target specimen at 37°C in a humid chamber overnight. After incubation the coverslip and rubber cement are carefully removed. A rapid wash is executed in which the slide is washed in 0.4X SSC/0.3% NP-40 at 73±1°C for two minutes followed by a 2X SSC/0.1% NP-40 wash at room temperature for thirty seconds to one minute. The slide is air dried and 10 μL counterstain (DAPI/Antifade) is applied.

In response to obtaining unsatisfactory results from the protocol used on the formalin-fixed tissue in case 3 described above, the protocol used to isolate nuclei from paraffin-embedded tissues in case 1 was adapted for the formalin-fixed tissue in cases 4,5, and 6. This procedure omitted the deparaffinization step but followed the rest of the nuclei isolation protocol from case 1 exactly. FISH was executed, according to the standard protocol described above, on cases 4 and 6 only, due to poor results from the preparation of case 5. The probes used to FISH cases 4 and 6 were CEP 21 and CEP X.

Procedures used to study frozen samples

The tissue sample used in developing techniques for FISH on frozen tissue was placental tissue from Case 2. The sample was from chorionic plate site #2. In preparing frozen tissue sections the sample must first be placed in a material that can provide structural support during sectioning. This is accomplished by covering the sample with

room temperature Optimal Cutting Temperature (OCT) Embedding Medium. OCT surrounds the tissue without penetrating it, in order to provide support to the sample tissue. The medium is then frozen in the cryostat, which is set at -15°C. The block containing the sample is then faced, or cut so that the microtome blade cuts mostly sample tissue. The block is placed in the microtome and sections 10 μm in thickness are cut. These sections are placed on cold, saline coated sections. Then the sample slides are fixed in 3:1 methanol:acetic acid fixative at -20°C for 20 minutes. This step is repeated twice more with fresh fixative. The slides are stored at 4 °C until FISH procedures can be initiated.

After the frozen tissue is sectioned and fixed the tissue sections are digested in order to make cells more accessible to FISH probes. The slides are treated with a 0.1 mg/mL Collagenase XI solution for forty five minutes at 37°C. If this treatment is insufficient then a more concentrated enzyme solution can be used or treatment time can be extended. In order to determine if intact nuclei are present 10 μL of DAPI is placed on the slides. If nuclei are sufficient for FISH, then the standard FISH procedure for Vysis CEP probes, as described above, is completed with CEP X and CEP 17 probes.

Procedures used to study cultured cells

Case 2 also involved two tissue samples that had been cultured previous to this study. Placental cells had been grown on coverslips and fetal cord blood cells were cultured in solution. There were five sample sites on the placenta and from each of these sites a

piece of chorionic membrane and a piece of the chorionic plate were cultured, except site five, in which only the chorionic plate was sampled.

After culturing the placental cells, the coverslips were glued cell-side-up on slides in preparation for FISH. In order to prepare the cultured fetal cord blood cells for FISH the cells were fixed three times in 3:1 methanol:acetic acid for five minutes. They were then resuspended in a minimal volume of fixative, dropped on slides, and air dried.

The FISH procedures for cultured cells follow the standard protocol described above. Some of the cultured placental slides were probed with the Vysis CEP X probe while others were probed with the Oncor CEP X probe. The cord blood cell slides were probed with the Vysis CEP X and CEP 7 probes. Here the CEP 7 probe was used as a control in order to determine if FISH hybridization was effective.

Analysis of FISH slides

After tissue had been prepared on slides and FISH had been completed the slides were analyzed using an epifluorescence microscope. Analysis involves counting the number and type of fluorescent probe signals found within each nuclei scored. The number of nuclei scored depends on the desired level of confidence for the presence of the aberrant chromosome number.

RESULTS

Trisomy 18 (Case 1)

In this case the presence of trisomy 18 in a miscarried POC from a low level mosaic trisomy 18 mother was investigated using CEP FISH probes. Slides prepared from the mounted sections of the paraffin-embedded POC produced inadequate results. Most of the nuclei on these slides were buried in tissue or clumped with other nuclei. This made analysis difficult in that FISH signals from the nuclei surrounded in tissue were hard to resolve and often it was impossible to determine which nuclei contained a specific signal. Attempts were made to determine the location of the majority of the nuclei containing Y signals, thus localizing the male fetus. Male cells were indeed found but they were not concentrated in any distinct area.

In order to view the hybridized FISH probes more clearly, nuclei were isolated from unmounted POC sections. The isolation of nuclei from paraffin-embedded sections of the POC yielded satisfactory FISH results. The sections came from three different planes of the POC block (A,B, & C). Section A was prepared twice in order to confirm the variation in this section as compared to the other two sections. As Table 1 shows, very few cells were trisomic in any of the sections. Sections B and C show a high percentage of maternal (female) cells while section A shows similar percentages for male and female cells. In Figure 4, two cells normal for chromosome 18 are shown. The CEP 18 probe tends to split and this explains the two small orange signals seen in both cells.

	No Y Signal		Y Signal Present		
	Number of 18 Signals/ Nucleus		Number of 18 Signals/ Nucleus		
Section	2	3	2	3	Total Cells Counted
A	104(52%)	1(0.5%)	92(46%)	3(1.5%)	200
A(trial 2)	53(53%)	3(3%)	43(43%)	1(1%)	100
B	162(81%)	1(0.5%)	36(18%)	1(0.5%)	200
C	168(84%)	2(1%)	30(15%)	0	200

Table 1. **FISH results for isolated nuclei from Case 1**: Cells were hybridized with CEP Y and CEP 18 probes.

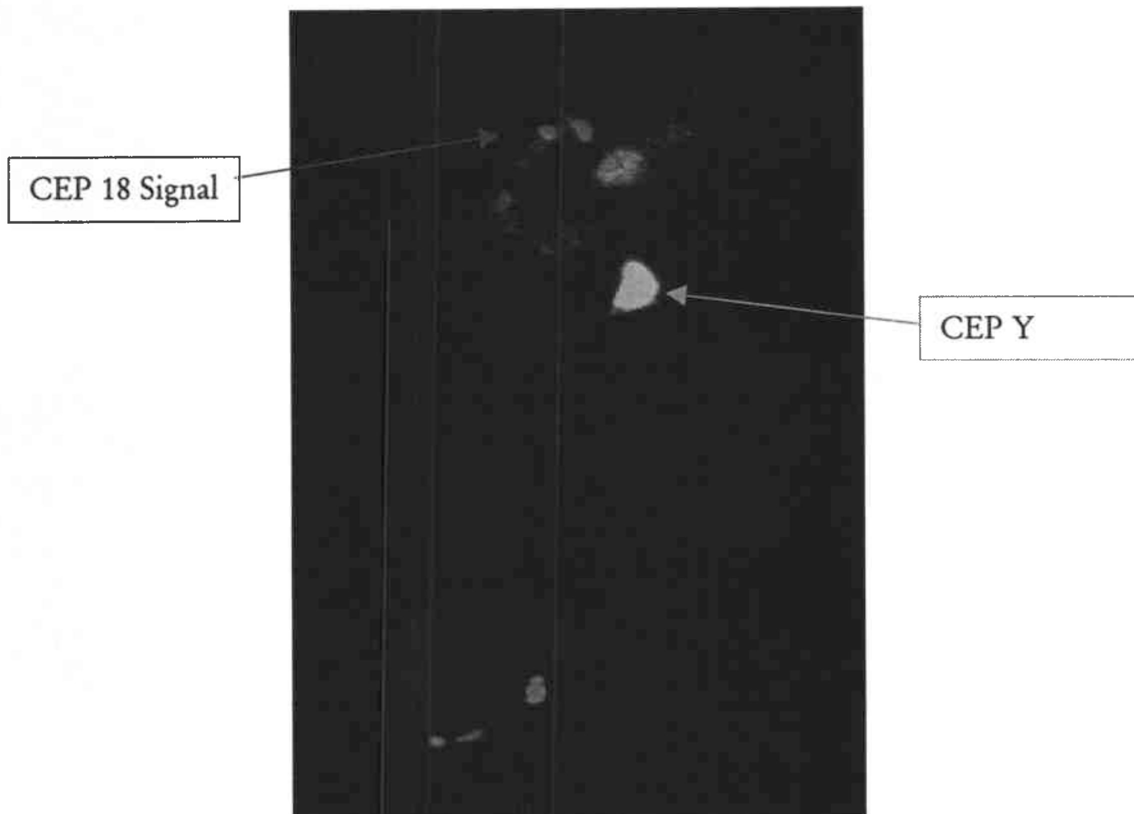

Figure 4. **Micrograph of two isolated nuclei from Case 1**: The upper nucleus is a normal male with two 18 signals and one Y signal. The lower nucleus is a normal female nucleus with two 18 signals and no Y signal.

Trisomy X (Case 2)

In Case 2 the presence of trisomy X was examined in cultured placental cells, fetal cord blood cells, and uncultured frozen placental tissue from a miscarried fetus that was identified as being a mosaic for trisomy X in previous studies. Analysis was accomplished using CEP FISH probes specific for chromosome X. Three different analyses were performed on Case 2, one for each of the three tissue sample types. Slides from two of these tissue samples (placenta and fetal cord blood) produced satisfactory FISH results.

The culturing of cells sampled from the five sites on the placenta produced a large number of cells that hybridized well with the CEP X FISH probes. In Table 2 the FISH results are shown; on each slide cells with 2 and 3 X's were scored.

These FISH scores indicate that the majority of the cells are, in fact, trisomic for the X chromosome. There seems to be no difference in the percentage of trisomic versus euploid cells between the slides hybridized with Vysis CEP X and Oncor CEP X probes. There are consistently higher numbers of trisomic cells in the chorionic plate as compared to the chorionic membrane slides. These results will later be compared to those from analyses of the other tissues from Case 2.

Analysis was also done on cells from the miscarried fetuses cord blood. These cells were cultured, pelleted, and frozen. The slides prepared with this sample had a high concentration of cells and produced good hybridization with Vysis CEP X probes. As Table 3 shows, there are more than twice as many trisomy X cells as euploid cells in the fetal cord blood. Figure 5 shows several cultured fetal cord blood cells with hyridized CEP probes.

		Cells with 2 X Signals		Cells with 3 X Signals		
Sample Site	Tissue	Number	% of total	Number	% of total	n
1	Membrane	33	16.5	167	83.5	200
1	Ch. Pl.	26	13	174	87	200
2	Membrane	34	17	166	83	200
2	Ch. Plate	20	10	180	90	200
3	Membrane	65	32.5	135	67.5	200
3*	Ch. Plate	27	13.5	173	86.5	200
4*	Membrane	45	22.5	155	77.5	200
4*	Ch. Plate	40	20	160	80	200
5	Ch. Plate	14	7	186	93	200
		Avg. % =	17	avg. % =	83	

Table 2. **FISH results for cultured placental cells from Case 2**: Cells were hybridized with CEP X probes. "*" indicates slides which were hybridized with Oncor probes. "Ch. Pl." indicates Chorionic Plate tissue.

Cells with 2 X Signals		Cells with 3 X signals		
Number	% of total	Number	% of total	n
60	30	135	67.5	200

Table 3. **FISH results from cultured fetal cord blood cells of Case 2**: Cells were hybridized with CEP X and CEP 7 probes. Only scores from CEP X are shown.

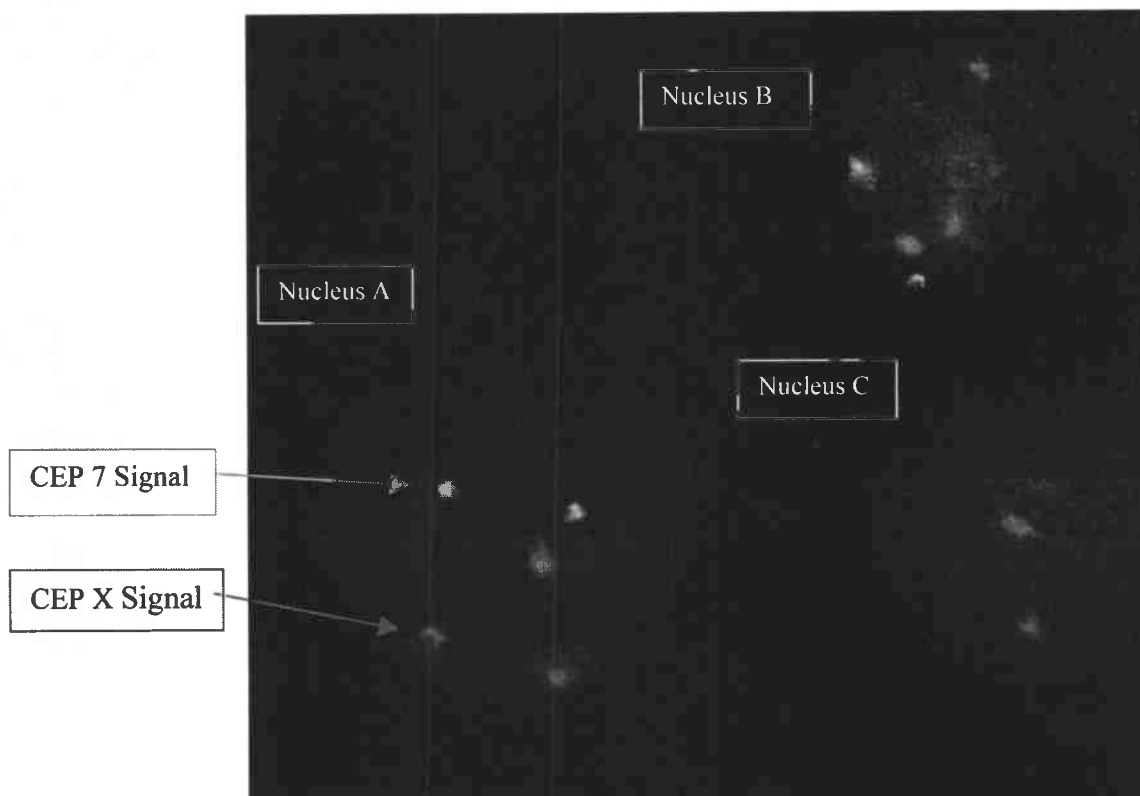

Figure 5. **Micrograph from FISH on cultured fetal cord blood nuclei of Case 2**: Nucleus A is trisomy X, as is Nucleus B. Nucleus C is not assessable from this micrograph because the whole nuclei is not visible.

Slides were also prepared from sections of a frozen sample of chorionic plate site #2 of Case 2. These slides did not produce useful results. Cells on these slides failed to hybridize to the CEP X and CEP 17 probes or the DAPI counterstain. Cells were emaciated and there were no visible, round, nuclei.

Other Cases

In addition to the study of the paraffin-embedded tissue, cultured cells, and frozen tissue in Cases 1 and 2, attempts were made to prepare and analyze tissue from several other cases using FISH. The tissue in all of these cases had been fixed and stored in formalin prior to slide preparation. In all four cases the slides produced in this study were insufficient for reliable FISH analysis. The initial attempt to isolate and perform FISH on the nuclei from the formalin-fixed lung tissue of Case 3 produced slides that had nuclei that were excessively clumped together. In addition, no true, interpretable FISH signals were visible on these slides. In an effort to find a better way to isolate nuclei, cells were filtered through a nylon mesh as in the isolation of paraffin-embedded nuclei. The nuclei made from filtering tissue from Case 3 through the nylon mesh contained inadequate signals as well as being too small and sparse to evaluate.

A modified protocol for isolating nuclei from paraffin-embedded tissue was then attempted on formalin-fixed tissue from Cases 4 through 6. After filtering the degraded tissue from Case 5 it was found that very few cells remained in the filtrate and this preparation was terminated. Slides made from Cases 4 and 6 had sufficient, although poor-quality, nuclei on which to execute FISH. The slides from Case 4 had ragged inconsistent outlines with very little DAPI staining and no visible hybridized probes.

Slides from Case 6 contained small, oval nuclei with some DAPI staining but no distinct FISH probe signals.

DISCUSSION AND CONCLUSIONS

Discussion of Methods

The methods in this thesis were used to prepare various tissue samples for a highly specific chromosome localization and enumeration technique. In preparing the different types of tissue in this study it became necessary to modify and attempt to optimize some of the published methods.

The methods applied to the paraffin-embedded tissue and the previously cultured cells were carried out in a straightforward manner and yielded useful results. In contrast, the methods used in preparing formalin-fixed and frozen tissue required the modification and adaptation of different protocols in an attempt to obtain slides that had visible, intact nuclei that hybridized well with the applied probe. Unfortunately, the slides produced from formalin-fixed tissue were not useful for FISH. As mentioned earlier, this tissue is readily available for retrospective study and FISH on this type of tissue is described by Kuchinka et al. (1995) as being "especially beneficial in follow-up studies of cases involving termination [of pregnancy] after prenatal diagnosis or patients with a malignant disease where previous cytogenetics established the chromosomal aneuploidy". The greatest difficulty in preparing the formalin-fixed tissue was found in attempting to obtain a reasonable concentration of nuclei on the slides. Numerous attempts were made at this, but neither filtering the partially digested tissue nor digesting the tissue further with an enzyme with a broader specificity produced useful slides. In addition, the nuclei that were visible on these slides did not hybridize well with the FISH probes. This could be

due to structural modifications of the DNA from the fixation or the inability of the probes to enter the nuclei and reach the chromosomal DNA.

It has been previously established that the isolation and FISH of formalin-fixed nuclei provides a more accurate reproduction of the true karyotype of the sample than does FISH on paraffin-embedded sections of the same tissue (Kuchinka et al., 1995). The proposed problem with sectioning the paraffin-embedded tissue is that many nuclei are sliced and this results in "the underestimation of ploidy due to the unavoidable truncation of nuclei". However, that study did not include the isolation and FISH of nuclei from paraffin-embedded tissue. In my study, FISH on the isolated nuclei was far more informative than FISH on the sections of paraffin-embedded tissue. This is because the isolated nuclei were clearly visible, readily accepted DAPI staining, and hybridized well with probes. In contrast, the nuclei on the paraffin sections were hidden in undissolved tissue and the signals that were present were not localizable. The morphology was somewhat distinguishable in these sections but extensive comparison to other histology slides would be necessary in order to make sense of the section. Thus, I propose that FISH on isolated nuclei from paraffin-embedded tissue should be considered as another valuable method for studying a large number of archived specimens.

The results from the sectioning of frozen placental tissue in Case 2 were disappointing in that no useful information could be obtained. As describe above, there was no hybridization with FISH probes and the nuclei were deteriorated. Reasons behind these problems could be freeze-thaw effects, insufficient fixation or digestion, and nuclei bisection. Modifications that might produce better results include: assuring that tissue

remains frozen throughout handling, varying section width, fixing for longer intervals, or digesting with a more potent enzyme.

The slides from Case 2 that contained cultured cells produced useful and informative results after FISH analysis. Due to culturing, there were a large number of cells and these cells readily hybridized with FISH probes. Signals in the nuclei from these cells were clear and distinct, making scoring of these signals simple. As cultured cells are available in large numbers in most cytogenetic labs, they provide a good resource for FISH analysis.

It would not have been possible to perform standard karyotypic analysis on the paraffin-embedded, and formalin-fixed tissues. Thus, FISH provides a useful tool for analyzing archived specimens that otherwise would not be accessible for study. As Schad et al. (1996) state, "the introduction of fluorescence in situ hybridization (FISH) and chromosome specific DNA probes have greatly enhanced the sensitivity of the detection of . . . chromosomes from interphase cells of a variety of tissues ranging from bone marrow, buccal cells, sperm, amniocytes, and chorionic villi". In addition, FISH does not require culturing so analyses can be completed faster that standard karyotypic analysis. Eiben et al. (1998) characterize FISH as being useful in "identifying unknown marker chromosomes, clarifying complicated chromosomal rearrangements and detecting abnormalities in tumour cytogenetics . . . [and] the detection of aneuploidies without cell culture". The fact that cell culture is not required for FISH removes a possible bias for dividing cells in cell culture. In cell culture it is possible that cells which have an abnormality will divide at a different rate as compared to normal cells. If the aberrant cell divides more slowly then the normal cells than the incidence of the abnormality will be

falsely low. If the abnormal cells divide rapidly there will be an overestimate of the frequency of the abnormality. Also, the analysis of cultured cells with standard cytogenetic methods restricts analysis to only cells that are in metaphase. In contrast, FISH analysis can be made on both interphase and metaphase cells. Another advantage of FISH is that it costs about one third of the cost of standard chromosome analysis (Schad et al., 1996). Lomax et al. (1994) state that "the combination of FISH with methods of isolating nuclei, such as the digestion of tissue or touch preparations, will eliminate tissue culture derived bias and provide a more accurate assessment of the in vivo karyotypic status of the specimen". However, when FISH is performed alone, without a complete routine chromosome analysis (G-banding), knowledge of the whole chromosomal makeup of the sample is impossible. In summary, the use of interphase FISH on archived tissues provided an effective means of determining the presence and level of chromosomal aneuploidy in the paraffin-embedded and cell cultured specimens.

Discussion of FISH Results from Cases 1 and 2

Studies on two cases proved highly successful and conclusions concerning the mosaic nature of the tissues were attainable. Through the use of interphase FISH it was determined that the miscarried POC from the pregnancy in Case 1 was not, in fact trisomic for chromosome 18. In the POC the level of maternal and fetal cells varied, yet neither maternal nor fetal cells had a level of trisomy 18 greater than 1.5% of the 200 cells counted per slide. The enumeration of at least 200 nuclei per target specimen is generally accepted as sufficient when studying specimens in which mosaicism is

suspected. Thus, the cause of miscarriage is not due to an extra chromosome 18 in the fetal or maternal cells of the products of conception.

There are very few cases of mosaicism for trisomy 18. As of 1996, ten previous cases of mosaicism for trisomy 18 with normal intelligence had been reported (Satge et al., 1996). The previous cases of this abnormality were reported because they were parents of children with trisomy 18 or because of multiple miscarriages. In the case studied by Graham et al. the patient had complete trisomy 18 in lymphocytes. This "near-normal phenotype . . . shows that trisomy 18 in lymphocytes can have little functional importance when it exists with a majority of normal cells in other tissues" (Graham et al., 1992). This is similar to Case 1 in that the impact in most other tissues is minimal.

Satge et al. (1996) suggest that hypofertility is a typical occurrence in trisomy 18 mosaicism and that "one or several genes situated on chromosomes 18 may be important for normal ovarian development and function". The early ovarian failure of the mother in Case 1, therefore, probably originates from the extra chromosome 18, even if the extra chromosome is not found in her germ cells and most of her other tissues. This varied effect of mosaicism is described by Hall's statement that "some chromosomal anomalies and single-gene mutations may be lethal to the cells and others may be tolerated if they do not have a severe effect on that tissue"(Hall, 1988). In short, Case 1 represents an additional example of an otherwise phenotypically normal person with a low level of trisomy 18 mosaicism and subfertility. Satge et al. (1996) warn that "any aneuploid germ cells present are at high risk for trisomy recurrence". Thus, people with low levels of trisomy 18 in their germ cells and other tissues should be informed of the risks of recurrence of trisomy 18.

The results from FISH analysis on the cultured placental and fetal cord blood cells from Case 2 indicated a mosaic trisomy X pregnancy. Mean values of 83% XXX and 67.5% XXX, respectively, were observed. The levels of mosaicism from studying these tissues with FISH agree with previous clinical studies on this case. Studies of the cultured placenta using standard karyotypic analysis determined that 65% of the cells were XXX. This corresponds exactly with the results obtained using FISH on the same tissue. Standard karyotypic analysis of cells cultured from amniocentesis found that 61% of the cells were trisomic.

This is not a case of confined placental mosaicism because the supernumerary chromosome was found in both the embryo and the extraembryonic structures, including the chorionic membrane and plate (Kalousek and Vekemans, 1996). This indicates that the origin of the extra chromosome in Case 2 occurred prior to the division of the zygote into the embryonic progenitor cells and the cells that give rise to the placenta. In contrast to some cases of confined placental mosaicism in which the presence of a "normal cell line in the placenta may . . . explain why a small minority of fetuses affected with specific chromosome abnormality are able to survive to term" (Hall, 1988), Case 2 involved a placenta that was also chromosomally abnormal. This abnormality in the placenta may have been the reason that the fetus failed to survive to term. However, pathology reports on the placenta were not completed. So, the cause of the fetal demise is not clear.

A possible origin of mosaicism for trisomy in Case 2 is that the zygote was chromosomally normal and a somatic duplication occurred before the blastocyte stage and differentiation of the trophoblast (Robinson et al., 1997). The other possible origin is that the conceptus was actually trisomic and subsequently a loss of the extra chromosome

occurred in a cell whose descendants were present in both the embryo proper and the placenta.

The variation in the level of trisomy X mosaicism between the sample specimens in Case 2 is indicative of tissue level mosaicism. The highest level of trisomy X was 93%, found in the chorionic plate site #5 of the cultured placental cells. The lowest level was found in the previous study of the amniocytes, with a trisomy X level of 61%. Thus, it appears that there are higher trisomy levels in the placenta than in the embryo proper. This variation is possible because "a great discrepancy between the level of trisomy can occur with either early loss of the extra chromosome . . . or selection against the trisomic cell line" in either the embryo or the placenta (Robinson et al., 1997). However, it is possible that the samples were contaminated with maternal cells. In Case 2, this contamination would lead to an underestimate of the level of trisomy X in the sample because the normal maternal cells would be scored as normal fetal cells, lowering the percentage of trisomic cells.

In summary, the analyses made with FISH provided useful data on the chromosomal makeup of two of the cases studied here. In one case a fetus miscarried due to unknown cause and FISH showed that the POC did not contain the trisomy 18 that is present at low levels in the mothers blood cells. In the other case a mother miscarried a fetus that was previously identified as being mosaic for trisomy X. In this research project the presence of varying levels of trisomy X in both the embryo and placenta was confirmed using FISH. In conclusion, this project was successful in analyzing two cases with previous indications of chromosome abnormality for the presence of mosaicism for chromosomal

aneuploidy. In doing so several techniques in tissue preparation were utilized and adapted with varying success.

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