Application of touch FISH in the study of mosaic tetraploidy and maternal cell contamination in pregnancy losses

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Abstract

Purpose Karyotype is a well established technique in the study of spontaneous miscarriages but is associated with selective overgrowth of maternal cells and other culture artefact (spp) such as tetraploidy, which could mask the true karyotype of the conceptus.

Methods 328 cases of pregnancy losses were studied by karyotype and Multiplex Ligation Dependent Probe Amplification technique. Touch FISH performed in non-cultured cells was used to evaluate the ploidy complement and sex

discrepancies using centromeric probes for chromosomes X, Y and 18.

Results Touch FISH confirmed 13 cases of maternal contamination, identified a triploidy and a monosomy X. True tetraploidy was confirmed in 7/14 cases studied.

Conclusion Touch FISH protocol is extremely accurate in the distinction of genuine mosaicism from tissue culture artifacts namely in cases with suspicion of tetraploidy and can be used to confirm maternal cell contamination in cases with sex discrepancy between karyotype and MLPA.

Capsule Touch FISH as an effective approach for the study of pregnancy losses with maternal cell contamination or with mosaic tetraploid cells present in the karyotype.

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Keywords Touch FISH · Spontaneous miscarriages · Foetal death · Tetraploidy · Maternal contamination · Mosaicism

Introduction

Approximately 15% of all clinically recognizable pregnancies are genetically abnormal ending in miscarriage and about half of these are attributable to detectable chromosome abnormalities [1–4]. The most common abnormality is trisomy (namely of the chromosomes 16, 15, 22, 21, 13 and 18). Sex chromosome monosomy (45,X), polyploidy (more frequent triploidy) or mosaicism (presence of more than one different chromosomal complement in the same individual) account for the majority of the remaining chromosome abnormalities found [1, 2, 5, 6]. The methodology of cell culture and analysis of chromosomes is a well established procedure but this approach could be associated with selective overgrowth of maternally derived cells that could mask the true karyotype of the conceptus [3, 4]. In fact, most clinical laboratories report an excess of normal female over normal male karyotypes and a number of studies have confirmed that maternal cell overgrowth is not uncommon [5, 6].

The presence of mosaic tetraploid cells, characterized by four complete sets of chromosomes ($4n=92$) in cultures from amniotic fluid or from pregnancies losses is frequently associated with culture artifacts. Although about 1,3% of first trimester abortions cases are true tetraploids, distinction between culture artifact and true mosaicism should be performed [1, 7].

Interphase Fluorescence *in situ* Hybridization (FISH) is an efficient approach to screen for maternal cell contamination, at least in male foetus cases [8]. In a previous study, our group used a modified interphase FISH (which was designated by Touch-FISH) in the study of miscarriages or foetal deaths with no karyotype due to culture failure [1]. This technique uses samples from frozen tissues touched on a slide and allows the study of ploidy changes and identification of the Y chromosome in male cases with maternal contamination.

The importance of karyotyping the aborted material is consensual in the literature. In cases with culture failure other techniques (that are not culture depend), such as Comparative Genomic Hybridization (CGH) or Multiplex Ligation-dependent Probe Amplification (MLPA) for subtelomeric regions could be used to overcome this problem [1, 9–12]. Nevertheless, polyploidy changes (that could explained about 10% of all spontaneous miscarriages) are not detected by these two techniques, and an additional technique such as interphase FISH should be performed [1, 11].

The aim of the present study was to investigate mosaic tetraploid cells observed in the karyotype of pregnancy losses samples, using Touch-FISH, an interphase FISH technique that allows the study of noncultured cells and the distinction of true mosaicism from culture artifacts. Additionally, Touch FISH was also applied in cases with suspicion of maternal contamination, which means a discordant result between a female karyotype and a male profile achieved by MLPA technique.

Material and methods

Tissue preparation

Ovular fragments (5–10 weeks of gestation), foetal or placental specimens (11–39 weeks of gestation) from 328 women were successfully cultivated to perform karyotype and analyzed by MLPA technique. Spontaneous miscarriages or an intrauterine fetal death specimens were collected in a sterile tube containing RPMI 1640 medium (PAA, Haidmannweg, Austria) supplemented with antibiotics. After gross examination and clearing of blood clots and mucus, each tissue sample was divided in fragments, one part was cultured for cytogenetic

analysis and the other was frozen and stored at -80°C . Informed consent was obtained from all participants (Genetic Testing and Personal Data Protection National Laws).

Cytogenetic analysis

Tissues were sliced into small pieces and digested with collagenase type I (Gibco, Grand Island, New York), during 3–4 h at 37°C . Cells were cultured in AmnioMax™ C100 Basal medium with Supplement (Gibco). Preparation of chromosome slides and G-banding using Leishman stain were performed according to standard methods [13].

Touch fluorescence *in situ* hybridization (touch FISH)

Protocol for Touch FISH was previously described [1]. Briefly, small frozen pieces of tissue ($2\text{--}3\text{ mm}^3$) were cut and gently touched onto a poly-L-lysine coated slide (Menzel-Glaser, Braunschweig, Germany) and then fixed in methanol and fresh fixative (3:1 methanol/acetic acid). Subsequently, slides were incubated in a series of solutions: 2xSSC (pH 7.0), formaldehyde solution (2 ml 37% formaldehyde, 0,36 g Mg_2Cl_2 and 78 ml 1xPBS), pepsin solution (112,5 mg Pepsin and 75 ml 0,01 N HCl) and rinsed in 1xPBS. Dehydration was performed in ethanol series (70%, 96% and 100%).

Alpha satellite probes for the centromeric regions (CEP probes for chromosomes X, Y and 18) or a locus specific probe (LSI for chromosome 21) were prepared according to manufacturer instructions (Abbott, Wiesbaden, Germany). Slides were counterstained with DAPI/Vectashield (Vector Laboratories, California, USA) and analyzed in a epifluorescence microscope (Nikon; Tokyo, Japan) equipped with a charge-coupled device camera (Sony, Tokyo, Japan) and appropriate software (Applied Imaging International, Sunderland, UK).

Multiplex ligation-dependent probe amplification (MLPA)

DNA extraction was performed using the ReadyAmp kit Genomic DNA Purification System (Promega Corporation, Madison, USA). The probe mix included in the MLPA kit (SALSA P095, MRC Holland) contains eight probes for human chromosome X, four for Y target sequences, as well as 8 probes specific for each of chromosome 13, 18 and 21 sequences. After ligation and PCR (Polymerase Chain Reaction), the multiplex-fluorescent products were denatured and capillary electrophoresis was carried out in an ABI PRISM 310. Analysis of results and calculations of peak areas of the target sequences (chromosomes 13, 18, 21, X, Y) was performed using GeneScan Software (Applied Biosystems).

Results

A successful karyotype was performed in 328 samples from spontaneous miscarriages or foetal deaths, 87 of which were abnormal and distributed as follows: 49/87 cases showed trisomies, 14/87 cases showed full or mosaic tetraploidy, 11/87 cases showed triploidy, 7/87 showed monosomy X and 6/87 showed structural abnormalities. An apparent normal karyotype was found in the remaining, 181 female karyotypes and 60 male karyotypes. MLPA was performed in all 328 cases and found 24 abnormal profiles, 216 female and 88 male normal profiles. In 12 cases sex discrepancy between karyotype and MLPA profile was observed. Touch FISH was performed in all these 12 cases to evaluate the presence of foetal cells (male cells) in the original non-cultured tissue (Table 1). Additionally, one case with a slightly decrease of X chromosome ratio in the MLPA profile, was also investigated by Touch FISH (Table 1).

Touch FISH approach confirmed all suspected cases of maternal cell contamination due to sex discrepancy between karyotype and MLPA, including one case with trisomy 21 (Table 1, Fig. 1a). A MLPA male profile (Table 1, case 71752) was found in a case with a female chromosomal balanced rearrangement (Robertsonian translocation). Touch FISH confirmed the presence of cells with Y chromosome so the balanced Robertsonian translocation was interpreted as being mother's karyotype (maternal contamination). Additionally, Touch FISH allow the detection of a triploid case (Table 1, case 73931; Fig. 1b) not detected either by the karyotype (only maternal 46,

XX cells were present) or by MLPA approach. In the case with a MLPA result showing a slight decrease of X chromosome (Table 1, case 75616), Touch FISH identified cells with monosomy of the chromosome X and cells with normal XX complement which could correspond to maternal contamination.

From the 328 cases karyotyped, 14 showed the presence of tetraploid cells in different slides performed from distinct cultures. These cases were selected to Touch FISH analysis (Table 2).

Confirmation of a true tetraploidy was achieved in half of the cases (7/14) studied by Touch FISH (Table 2, Fig. 1c). Cases without mosaicism, in which all cells observed were tetraploid, were confirmed as true tetraploid cases (Table 2).

Discussion

Among the many complicated causes of early pregnancy losses, chromosomal abnormalities are considered the most common etiological factor [11, 14–16]. Finding an abnormal karyotype in the products of conceptions provides an obvious explanation for the miscarriage, which avoids unnecessary testings and treatments namely in recurrent cases [17]. Nevertheless, the value of the karyotype is limited in cases of maternal cell contamination or culture artifacts and genetic counseling is obviously compromised.

The aim of the present study was to determine the value of the interphase FISH technique using touch preparations (Touch FISH) from ovular fragments, frozen foetus or placental tissues as an efficient approach to be used in cases

Table 1 Cases of pregnancy losses with suspicion of maternal cell contamination

Cases	Trimester	Type of tissue	Karyotype	MLPA	Touch FISH	Final Result
71752	1T	OF	45,XX,rob(14;15)	Normal XY	XY,18,18/XX,18,18	Normal male
71912	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
73060	2T	PL	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
73931	1T	OF	46,XX	Normal XY	XXY,18,18,18/XX,18,18	Male with triploidy
74103	1T	OF	46,XX	Trisomy 21, XY	XY,21,21,21/XX,21,21	Male with trisomy 21
75082	3T	PL	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
75530	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
75616	1T	OF	46,XX	Normal XX ^a	X,18,18/XX,18,18	Female with monosomy X
75734	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
75862	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
76762	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
82789	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male
82860	1T	OF	46,XX	Normal XY	XY,18,18/XX,18,18	Normal male

1T, first trimester; 2T, second trimester; 3T, third trimester; OF, Ovular Fragments; PL, Placenta

^a in this case, a slightly decrease of X

chromosome ratio was observed, but with no criteria to be validate as monosomy X

Fig. 1 Touch FISH. Centromeric probes to chromosomes X (green), Y (red) and 18 (blue). **a** Case 82860 - cells with an XX complement confirm maternal cell contamination in a male fetus; **b** Case 73931 - triploidy XXY; **c** Case 83524 - tetraploidy XXXX

with suspicion of maternal contamination or with high grade mosaic tetraploid cells present in the karyotype. Touch FISH has the advantage of analyzing cells directly from the tissue sample without cells being exposed to culture, overcoming the problem of overgrowth of maternal cells or culture artifacts.

We found only 87 (27%) chromosomal abnormal cases in 328 samples from pregnancy losses. The majority of literature refers that at least 50% of all spontaneous abortions have abnormal chromosomes, but this is particularly true for early cases, namely before 12 weeks [17–19]. The lower incidence in our series could be explained by an excess of normal female karyotypes due to maternal contamination. In fact, other authors concluded that as many as 30–40% of the 46,XX karyotypes from cultured samples may represent mater-

nally derived cells [20, 21]. Other reason could be the inclusion of samples from all three trimesters of pregnancy that will lower the probability of detection of a chromosomal abnormality.

Maternal cell overgrowth was confirmed in all 13 cases studied by Touch FISH with suspicion of maternal cell contamination, showing the presence of XY cells (male fetus) mixed with XX cells (maternal cells). Additionally, was able to detect one triploid case and a case with monosomy X. This case was not detected by MLPA due to an excess of maternal cells present in the sample. The SALSA MLPA P095 kit can be used easily to detect aberrant copy numbers of the human chromosomes 13, 18, 21, X and Y and enables the identification of maternal contamination in male cases detecting the Y chromosome. Notwithstanding MLPA, itself, could

Table 2 Cases of pregnancy losses with tetraploid in the karyotype

Cases	Trimester	Type of tissue	Karyotype	MLPA	Touch FISH	Final Result
71271	1T	OF	92,XXYY/46,XY	Normal XY	XY,18,18	Normal male
71675	3T	PL	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
71754	1T	OF	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
72938	1T	OF	92,XXXX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
72247	3T	PL	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
72268	1T	OF	92,XXXX/46,XX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
73398	1T	OF	92,XXXX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
74077	1T	OF	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
76049	1T	OF	92,XXXX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
76904	1T	OF	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
77178	1T	OF	92,XXXX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
77613	1T	OF	92,XXXX/46,XX	Normal XX	XX,18,18	Normal female
82977	1T	OF	92,XXXX/46,XX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy
83524	1T	OF	92,XXXX/46,XX	Normal XX	XXXX,18,18,18,18	Female with tetraploidy

1T, first trimester; 2T, second trimester; 3T, third trimester; OF, Ovular Fragments; PL, placenta

detect misclassification of female normal karyotype (in male samples) due to maternal cell overgrowth in the culture. It is important to stress that Touch FISH, even though being a less cost-effective technique than MLPA, is highly efficient in detecting low-grade mosaicism. This allows identification of abnormalities not always detected by MLPA analysis due to a high grade of maternal cells present in the sample, justifying the result obtained in the case of monosomy X (case 75616, Table 1). Additionally, Touch FISH could detect polyploidies (triploidies and tetraploidies) which is an added value of this technique.

Maternal cell contamination was not the only drawback observed. In fact, other authors described high levels of tetraploidy or full tetraploidy in long-term cultures from spontaneous abortions [3, 5, 22]. In chorionic villi or amniotic cells the presence of tetraploid cells is generally considered as a false positive result due to confined placental mosaicism or placental culture artifacts [23, 24]. Although, not common, true tetraploidy in foetuses with multiple congenital defects were found [23, 25]. About 1.3% of first trimester miscarriages are true tetraploid which means that the presence of tetraploid cells should be carefully analyzed and confirmed. From the 14 cases with a high proportion of tetraploid cells, we were able to demonstrate it as a true condition in 7 cases. These tetraploids cells were seen in different flasks from independent cultures. We think that Touch FISH approach performed directly in foetal or placental tissue with no interference of an *in vitro* culture could be an efficient assay to prove the existence of a true tetraploidy.

In conclusion, Touch FISH protocol is an efficient approach to be used as a tool to confirm maternal cell contamination in cases with sex discrepancy between karyotype and MLPA, allowing also the identification of new cytogenetic abnormalities that could be present and also being extremely accurate in the distinction of genuine mosaicism from tissue culture artifacts.

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