Detection of chromosome abnormalities in chorionic villi of ectopic pregnancies by multiplex fluorescent polymerase chain reaction and fluorescence in situ hybridization

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Submitted for publication
Background  In ectopic pregnancies, the frequency of chromosome abnormalities appears to be low, but conventional cytogenetic techniques fail in a high percentage. The question therefore is whether molecular cytogenetic techniques can detect chromosome abnormalities more reliably.

Methods  70 chorionic villi samples of ectopic pregnancies were studied by multiplex fluorescent polymerase chain reaction (MF-PCR). Probes for chromosomes 16, 21, X and Y in chorionic villi were evaluated. FISH was performed where results of MF-PCR showed aneuploidy, in case of uninterpretable MF-PCR results, and in ten cases with normal MF-PCR results. FISH was performed without prior knowledge of the MF-PCR results.

Results  MF-PCR gave a result in 53 (76%) cases of ectopic pregnancy. Fifty-one (73%) of these results were normal and two (3%) were abnormal, one trisomy 16 and one triploidy. The trisomy 16 could be confirmed with fluorescence in situ hybridization. In ten control cases MF-PCR diagnosis was confirmed by FISH. In seventeen cases (24%) inconclusive findings, partial results or no results were found with MF-PCR. In five of these cases (7%) a result could be obtained with FISH.

Conclusions  In ectopic pregnancies, MF-PCR and/or FISH techniques can establish the chromosomal status for chromosomes 16, 21, X and Y, but the technical failure rate is still considerable.

Key words  ectopic pregnancy/quantitative fluorescent polymerase chain reaction/STR/trisomy/FISH/aneuploid


Introduction

The role of chromosome abnormalities as a cause of ectopic pregnancies is still controversial. In about 30% percent of ectopic pregnancies no maternal risk can be identified\(^1\). Although a high percentage of chromosome abnormalities in ectopic pregnancies was originally reported \((47/60=78\%)^2\), this could not be confirmed in three more recent studies, with respectively 3/21 (14\%), 1/22 (5\%) and 3/62 (5\%) chromosome abnormalities\(^3\)-\(^5\). In respectively 1/22 (5\%), 8/30 (27\%) and 32/94 (34\%) of cases no chromosomal diagnosis could be established, due to the failure of conventional cytogenetic techniques\(^3\)-\(^5\). The main reason that these techniques are often not successful in ectopic pregnancies is non-viability or lack of fetal tissue.

It has been suggested that multiplex fluorescent polymerase chain reaction (MF-PCR) can be performed on single cells, and it might thus be of use in ectopic pregnancies\(^6\). Thus far, the technique has been used effectively in prenatal diagnosis –amniotic fluid samples\(^7\)-\(^9\), fetal tissues and blood\(^10\).

We report our first experiences using MF-PCR to determine the chromosomal status for chromosomes 16, 21, X and Y of chorionic villi in ectopic pregnancies. These chromosomes were chosen, as trisomy 16 is the most common trisomy in miscarriages. Other frequently noted chromosome abnormalities are trisomy 13, 18, 21 and 22, sex chromosome monosomy (45,X) and tri- and tetraploidy\(^11\)-\(^12\). Fluorescence in situ hybridization (FISH) was used to confirm chromosomal outcome of MF-PCR.

Materials and methods

Specimens

Between January 1996 and July 2001 surgically removed material of 128 consecutive ectopic pregnancies was sent in Hank's Balanced Salt Solution (HBSS) to the department of Clinical Genetics. All tissues were examined under a dissecting microscope, freed of maternal tubal tissues if necessary, and washed clean of blood in HBSS. Only in 71 cases (55\%), a sufficient amount of chorionic villi (2.5 mg or more) was found. Chorionic villi were dried and stored at -20°C for later DNA extraction. The remainder of the samples was sent for histological examination.
**MF-PCR preparation and analysis**

DNA was extracted from all frozen samples using a Masterpure™ DNA Purification Kit. Four DNA markers were used for chromosome 21, and ten DNA markers for chromosome 16. For sexing, the AMELXY probe was used. Thirteen probes for the X chromosome were used. All probes are listed in Table 1. As positive controls, cell lines containing trisomy 21 (n=5), and a triploidy, 69,XXY (n=1) of chorionic villi material were used. Extracted DNA samples ranging from 18 ng to 500 ng (in a volume of 1.2 μl) were amplified in a reaction mixture comprising 0.2 to 0.75 μl of each primer, 1.5 μl (1.25 mM) deoxyribonucleoside triphosphate (dNTP) (Roche, Mannheim), 0.08 μl AmpliTaq Gold® DNA Polymerase (Applied Biosystems/Roche, Mannheim), 0.75 μl GeneAmp PCR Gold Buffer (Roche, Mannheim), 0.72 μl MilliQ, and 25 mM MgCl2. After denaturation at 94°C for seven minutes, 10 cycles at 95°C, 55°C, 72°C for resp. 15,15 and 30 seconds, and 20 cycles at 89°C, 55°C, 72°C for resp. 15,15 and 30 seconds, and 72°C for ten minutes, were performed in a Gene Amp® PCR system 9600/9700. The entire procedure took 1.7 hour.

**Table 1** Markers used for chromosomes 16, 21, X and Y

<table>
<thead>
<tr>
<th>Chromosome 16</th>
<th>Chromosome 21</th>
<th>X chromosome</th>
<th>Y chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>D16S503</td>
<td>D21S263</td>
<td>DXS5987</td>
<td>AMELX</td>
</tr>
<tr>
<td>D16S155</td>
<td>D21S266</td>
<td>DXS5990</td>
<td></td>
</tr>
<tr>
<td>D16S20</td>
<td>D21S1914</td>
<td>DXS5991</td>
<td></td>
</tr>
<tr>
<td>D16S3068</td>
<td>D21S1252</td>
<td>DXS5993</td>
<td></td>
</tr>
<tr>
<td>D16S3075</td>
<td></td>
<td>DXS1001</td>
<td></td>
</tr>
<tr>
<td>D16S3091</td>
<td></td>
<td>DXS1060</td>
<td></td>
</tr>
<tr>
<td>D16S3046</td>
<td></td>
<td>DXS1068</td>
<td></td>
</tr>
<tr>
<td>D16S3136</td>
<td></td>
<td>DXS1073</td>
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<td></td>
<td></td>
<td>DXS1106</td>
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<td></td>
<td></td>
<td>DXS1227</td>
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<tr>
<td></td>
<td></td>
<td>DXS8043</td>
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<tr>
<td></td>
<td></td>
<td>DXS8051</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DXS8091</td>
<td></td>
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</tbody>
</table>

One to two microliters of PCR product was mixed with 1.7 μl formamide/Rox mixture and amplification products were sized using capillary electrophoresis on an ABI-PRISM™ 310 Genetic Analyzer. MF-PCR reactions producing inconclusive results were repeated to clarify
the result. Positive and consistent results from at least two informative markers for each chromosome were required before a conclusion could be drawn.

**Slide preparation and FISH analysis**

Prior to FISH analysis, tissues were thawed, washed in HBSS, then Collagenase dissociation of the thawed specimens was performed, followed by fixation in methanol/acetic acid (3:1) for at least 15 min, and the tissue was suspended in a few drops of 70% acetic acid. The dispersed single cells were dropped onto clean slides, dried at 37°C and placed in an incubator at 60°C overnight. Two slides were prepared for each sample. FISH was performed using two sets of probe mixtures. Probe mix 1 identified chromosomes 16 and 21. Probe mix 2 identified chromosomes X and Y.

In each mixture the probes were directly-labelled with different fluorophores, which could be visualized with appropriate filter combinations. Slides were analysed with a Zeiss epifluorescence microscope, equipped with selective filters. Three-colour analysis with green (fluorescein isothiocyanate=FITC/SpectrumGreen), red (SpectrumOrange/SpectrumRed) and blue (4,6-diamidino-2-phenylindole = DAPI for counterstaining of the nuclei) was performed. The number of hybridization signals was counted in at least 50 nuclei. FISH was performed without prior knowledge of the MF-PCR results. Aneuploidy was diagnosed when >20% of the cells showed an abnormal number of signals.

**Statistical analysis**

Prior to MF-PCR processing, it was decided to perform FISH analysis when MF-PCR results showed aneuploidy, in case of partial or uninterpretable MF-PCR results, and in ten cases with a normal MF-PCR result. If these ten cases with a normal MF-PCR result could all be confirmed with FISH, they are supposed to reflect a 60% chance on a true error rate of 5%. Mean values of maternal age, gestational age, and median of hCG values were calculated, using the Statistical Package for Social Sciences 10.0 (SPSS Inc., Chicago, IL, USA).
Results

MF-PCR data

Of the frozen samples, in one case, no DNA could be extracted (1/71 = 1%). In the remaining 70 samples MF-PCR processing was performed. The results obtained with MF-PCR are summarized in Table 2.

<table>
<thead>
<tr>
<th>MF-PCR</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>%</td>
</tr>
<tr>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Abnormalities</td>
<td></td>
</tr>
<tr>
<td>Trisomy 16</td>
<td></td>
</tr>
<tr>
<td>Triploidy</td>
<td></td>
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<tr>
<td>Inconclusive findings</td>
<td></td>
</tr>
<tr>
<td>Possible trisomy 16</td>
<td></td>
</tr>
<tr>
<td>Partial result</td>
<td></td>
</tr>
<tr>
<td>No result</td>
<td></td>
</tr>
<tr>
<td>Tested/Confirmed</td>
<td></td>
</tr>
<tr>
<td>Tested/Result</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Results in 70 MF-PCR processed ectopic pregnancies for chromosomes 16, 21, X and Y, and eventual FISH results

In seven cases, only one marker for chromosome 21 gave result and in two cases, only one marker for chromosome 16 gave result.

Definite MF-PCR results were obtained in 53 cases (53/70 = 76%), consisting of 51 normal results (51/53 = 96%), and two abnormal results (2/53 = 4%), one trisomy 16 and one triploidy. In Figure 1 MF-PCR amplification products are shown for a normal case, showing disomy 16, and the trisomy 16 case.

In the remaining 17 cases no definite MF-PCR results were obtained. In four cases there were inconclusive findings (4/70 = 6%), in nine cases partial results (9/70 = 13%), and in four cases there was no amplification (4/70 = 6%). For the respective probes used, different results were obtained.

Establishing MF-PCR results was possible in 63 (90%) cases for chromosomes X and Y. In 33 cases a normal male gender (XY) was established, in 29 cases a normal female gender (XX), and one triple X (XXX) in the triploid case. In one female karyotype, results were derived from only one marker.
For chromosome 16, results were available in 65 (93%) cases, in 60 cases a normal disomy was established, in two cases a trisomy 16 (one in the triploid case), twice a possible trisomy 16, and there was one inconclusive result. In six cases, results were derived from only one marker. For chromosome 21, results were available in 57 (81%) cases, in 55 cases a normal disomy was established, twice a possible trisomy 21, and there was one inconclusive result. In nine cases, results were derived from only one marker. The triploid case failed to show amplification for chromosome 21.

**Figure 1  Multiplex fluorescence PCR figure showing peaks consistent with a triallelic trisomy 16 (a) and a normal disomy 16 (b)**

*The x axis shows the length of the PCR products. The y axis shows fluorescent intensities in arbitrary units. Probe D16S3091.*

![PCR Peaks](image)

**FISH data**

Of the 51 samples with normal MF-PCR outcome, ten samples were at random verified by FISH method. In all samples, the probes used confirmed normal results, and matching gender. One abnormal MF-PCR result, a trisomy 16 was confirmed by FISH method (see Figure 2). In the case with a MF-PCR result showing triploidy no material was available for FISH confirmation. FISH confirmation in four other samples, with possible abnormalities did not succeed in three cases, due to problems in slide preparation. In one case, for which trisomy 21 could not be ruled out with MF-PCR, FISH showed normal results for all chromosomes tested.
Figure 2  FISH results consistent with trisomy 16 and disomy 21 (a) and a normal disomy 16 and 21 (b).

- = chromosome 16 specific probe
- = chromosome 21 specific probe

A full colour image of figure 2a is available on the inside back cover.

Clinical data

In 70 cases out of 128 (55%) ectopic pregnancy samples, MF-PCR processing was done. In this group of women, the mean maternal age was 31.3 years (range 19 to 46). Thirty-nine women were multiparous, 31 women were nulliparous. The mean gestational age was 7½ weeks (range 4½ to 11½). The median level of hCG at day of surgery was 4,400 U/L (range 490 – 84,600). Altogether, the women had had 134 pregnancies before the current ectopic pregnancy. Of these pregnancies, 25 had resulted in a miscarriage (19%) and 20 in an ectopic pregnancy (15%).

In 34 cases (49%), one or more maternal risk factors for ectopic pregnancy, including histological signs of salpingitis at time of surgery, were present. Recorded were a history of salpingitis (n=20, 29%), tubal pathology (n=18, 26%), histological signs of salpingitis post-surgery (n=10, 14%), appendectomy (n=7, 10%), previous tubal surgery (n=8, 11%), in-utero DES exposure (n=2, 2.8%), previous laparotomy (n=1, 1.4%), and current IUD use (n=0). In ten cases of all ectopic pregnancies (10/70=14%) at least a part of an embryo was found. In six of these cases, an intact fetus was seen.
The MF-PCR established that trisomy 16 occurred in the ectopic pregnancy of a woman aged 32 years and at gestational age of 5\(\frac{4}{7}\) weeks. She was known with one risk factor, i.e. a previous salpingitis. In the triploid case, the women was aged 36 years, and the ectopic pregnancy occurred in her second pregnancy, at a gestational age of 7\(\frac{4}{7}\) weeks. She had had a single miscarriage before, and no risk factors were present.

**Discussion**

The current study was initiated in order to evaluate the use of MF-PCR technique on frozen samples of chorionic villi of ectopic pregnancies. FISH was performed to confirm test results when they showed aneuploidy, in case of uninterpretable MF-PCR results, and in ten random cases with normal MF-PCR results. To our knowledge, these are the first data on MF-PCR results performed in chorionic villi of ectopic pregnancies.

In 53 (76\%) cases of ectopic pregnancy conclusive results from MF-PCR were available, using probes for chromosomes 16, 21, X and Y. Fifty-one (51/53-96\%) of these results were normal, and 2 (2/53-4\%) were abnormal, one trisomy 16, and one triploidy. FISH confirmation was performed in ten random cases with normal MF-PCR results, one case with inconclusive findings, three with partial results, and one case without amplification. FISH confirmed the findings of MF-PCR and furthermore was able to provide conclusive results in the five cases with no, partial or inconclusive results from MF-PCR.

The group under study represents patients with vital ectopic pregnancies, as reflected by the median hCG level 4,400 U/L (range 490 – 84,600) at day of surgery. In 49\% of cases a maternal risk factor was present. Due to the low number of chromosome abnormalities found, it was not relevant to correlate chromosome abnormalities with maternal risk factors.

MF-PCR has been mainly used in case of prenatal diagnosis (amniotic fluid samples). It proved to be a reliable technique for the detection of trisomy 13, 18 and 21, and for triploidy\(^{7-9,15}\). The advantage of the technique, is its feasibility in cases where very small volumes are available. Our study proved that determining disomy or trisomy 16 on chorionic villi of ectopic pregnancies is feasible.

In the samples processed by MF-PCR, a failure rate of 24\% was found, lowered to 17\% after performing FISH. Recently reported failure rates of conventional techniques in ectopic pregnancies are 5\%, 27\% and 34\% in respectively 22, 30 and 94 cases\(^{3-5}\). Most probably, the number of results obtained after the use of fresh material by MF-PCR method would be higher.
The small tissue volume and non-viability of the tissue probably further increased the failure rate. During this study it was found that a minimum of approximately 1 mg of fresh chorionic villi was needed to perform MF-PCR and approximately 5 mg to perform FISH. We cannot rule out the possibility that the samples with a chorionic villus amount of <2.5 mg, not tested by MF-PCR, could be the ones with a chromosome abnormality. Maybe in the future with better probes for chromosome 21, MF-PCR alone may suffice for a reliable diagnosis. Microarrays for the genome-wide measurement of DNA copy numbers may become another alternative. The disadvantage of this technique however is the high cost in terms of materials and equipment which makes it unlikely to be applicable on a large scale.

In summary, the use of molecular biological techniques, like multiplex fluorescent polymerase chain reaction and fluorescence in situ hybridization on frozen chorionic villi of ectopic pregnancies is feasible, but the technical failure rate is still considerable.

Acknowledgements

We thank our colleagues at the department of Obstetrics and Gynecology for their help in collecting ectopic pregnancy specimens, SKM van Daalen and CM Korver for FISH analysis, JMN Hoovers and GJ Bonsel for their valuable comments.
References


