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Cytogenetic Analysis of Spontaneous Miscarriage

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1. Introduction

Approximately 15% of all clinically recognized pregnancies end in spontaneous miscarriage. The most frequent cause of spontaneous miscarriage is fetal chromosome abnormalities such as autosomal trisomy, monosomy X and polyploidy. In this chapter, cytogenetic abnormalities associated with spontaneous miscarriage are reviewed based on the latest studies. Molecular cytogenetic technique has been introduced in the genetic analysis of miscarriages in addition to the conventional karyotyping and provides new insights into this field.

2. Cytogenetic abnormalities of miscarriage

The considerable proportion of all conceptions fails to reach a live birth in humans. Approximately 15% of all clinically recognized pregnancies end up with miscarriage, and the total pregnancy loss is estimated to be 30-50% of all conceptions (Rai & Regan, 2006; Stephenson & Kutteh, 2007). The most important etiology of pregnancy loss is cytogenetic factor, namely chromosome abnormality. About 50-60% of spontaneous miscarriages are etiologically attributed to chromosome abnormalities (Kajii, et al., 1980; Rai & Regan, 2006; Stephenson & Kutteh, 2007; Simpson, 2007; The ESHRE Capri Workshop Group [ESHRE], 2008). The proportion of chromosome abnormality in chemical abortion, defined as demise before clinical recognition by ultrasound examination, is unclear, but the proportion is expected to be higher than clinical miscarriage, as the incidence of chromosome abnormality is reported to be inversely proportional to gestation (Stephenson & Kutteh, 2007; Simpson, 2007).

The most common chromosome abnormality in miscarriage is autosomal trisomy, followed by ployploidy such as triploidy or tetraploidy, monosomy X and structural abnormality (Stephenson & Kutteh, 2007). Trisomies are generally the results of meiotic errors leading to the appearance of chromosomally abnormal gametes, which is strongly associated with maternal age (Simpson, 2007). All autosomal trisomies except trisomies 13, 18, 21 miscarry at early stage of gestation (ESHRE, 2008). Although there should be a corresponding monosomy for each trisomy, monosomy is rarely detected in clinical miscarriage except chromosome X, suggesting that autosomal monosomies are unlikely to be compatible with survival (ESHRE, 2008). Polyploidy mainly originates from fertilization by polyspermy or...
postzygotic division error (Simpson, 2007). In monosomy X, the lack of X chromosome mostly derives from paternal meiotic division error of sex chromosomes (Simpson, 2007). Structural rearrangements and chromosomal mosaicism due to postzygotic errors are occasionally detected in miscarriages. In case of balanced structural rearrangements, either parent usually has the same rearrangement and the cytogenetic cause for miscarriage is deniable, whereas de novo occurrence of balanced structural rearrangements might be associated with abnormal phenotype owing to possible gene interruption (Bui, et al., 2011). On the other hand, unbalanced structural rearrangements are always connected with abnormal phenotype, and naturally selected as miscarriage when the imbalance is too severe for embryos to survive. Mosaicism occurs as the result of postzygotic or mitotic errors. If abnormal cell lines persist during the preimplantation stage, embryos will be candidates for fetal or confined placental mosaicism, leading to miscarriage or impaired fetal development (Bielanska, et al., 2002; Vorsanova, et al., 2005). Although the consequence is unclear, the high incidence of mosaicism is demonstrated in miscarriage specimens (Vorsanova, et al., 2005).

3. Cytogenetic investigation of miscarriage

The identification of genetic cause of miscarriage is not necessarily performed as routine clinical work, as most of them are untreatable and unavoidable due to sporadic occurrence. However, it is highly recommended to identify karyotype and establish the cause of miscarriage in cases with recurrent miscarriages (RM) (Stephenson, et al., 2002). RM affects 1-5% of women, and the etiologies are multiple and at times even multifactorial (Rai & Regan, 2006; Stephenson & Kutteh, 2007). The estimated causes of RM include genetic, anatomical, endocrinologic, immunologic and thrombophilic disorders (Rai & Regan, 2006; Stephenson & Kutteh, 2007), although most of them have not yet be fully clarified. In fact, no credible explanation can be given for more than half of the cases, and about half of miscarriages are attributed to fetal chromosome abnormalities even in RM (Stephenson, et al., 2002).

When abnormal karyotype is the cause of miscarriage in RM patients, it is possible to avoid unnecessary testing or treatments for RM (Stephenson, et al., 2002), and abnormal karyotype results reportedly have a better prognosis for future pregnancies (Ogasawara, et al., 2000), although there might be an increased recurrence risk for another trisomy pregnancy in cases with gonadal mosaicism or genetic tendency to non-disjunction (ESHRE, 2008). On the other hand, the further investigation and alteration of current treatments would become necessary in case of miscarriage with normal karyotype in RM patients. When unbalanced structural abnormality is detected in miscarriage, ascertainment of carrier status in the parents is important to offer accurate information about the possibility of having another miscarriage or abnormal offspring and preimplantation genetic diagnosis (PGD) should be considered for the carrier couples (ESHRE, 2008). Thus, cytogenetic investigation of miscarriage specimens is crucial for the management of RM patients and provides valuable information for future pregnancies (Stephenson, et al., 2002; Diego-Alvarez, et al., 2005; ESHRE, 2008; Jobanputra et al., 2011). In addition, the identification of the possible cause of miscarriage is generally very comforting for RM patients, as they usually have psychological distress such as self-blame, anxiety, depression and grief (Nikcevic, et al., 1999).
4. Cytogenetic analytic methods for miscarriage specimens

4.1 Classical cytogenetics

Traditionally, cytogenetic analysis of miscarriage has been performed by G-banding method for metaphase spread after culture of villous cells (Kajii, et al., 1980). This standard cytogenetic methodology needs living cells to culture and the quality of metaphase is crucial for analysis. Thus, the fresh miscarriage specimens are desirable for analysis (Stephenson, et al., 2002). In fact, the success rates for culture and karyotyping of miscarriages vary among laboratories, ranging from 60 to 90% (Kajii, et al., 1980; Stephenson, et al., 2002; Jobanputra, et al., 2011). It has also been speculated that conventional karyotyping may detect only abnormal karyotypes that permit cell proliferation in vitro and the miscarriage specimens that fail to grow might have the rare abnormalities that do not sustain culture growth (Benkhalifa, et al., 2005; Jobanputra, et al., 2011). Another serious concern on classical cytogenetics is that overgrowth of maternally-derived cells or microorganisms contaminated in the specimen is not uncommon (Bell, et al., 1999; Jarrett, et al., 2001; Vorsanova, et al., 2005; Jobanputra, et al., 2011), because the complete removal of maternal decidua/blood cell is not always possible. As a result, karyotype could be falsely categorized as normal female, and skewed sex ratio in favor of females is often recognized in karyotype analysis of miscarriage specimens. In addition, incorrect interpretation such as tetraploidy could occur by tissue culture artifact (Doria, et al., 2010). Subtle abnormalities such as microdeletion are also overlooked due to the limited resolution in banding. Furthermore, microscopic chromosome analysis of cultured cells is time-consuming and labor intensive.

In recent years, several new genetic methods have been introduced in cytogenetic analysis of miscarriage to overcome the above-mentioned drawbacks of conventional karyotyping. In classical banding method, direct or semidirect analysis is attempted to reduce tissue culture effect by minimizing culture time. This technique allows a rapid analysis of all kinds of chromosome abnormalities (Morales, et al., 2008), although there is still a possibility of culture failure.

4.2 Interphase fluorescence in-situ hybridization (FISH)

Fluorescence in-situ hybridization (FISH) on the interphase nucleus of uncultured cells has been performed not only for prenatal samples obtained by amniocentesis or chorionic villus sampling (Shaffer & Bui, 2007), but also spontaneous miscarriage samples (Jobanputra, et al., 2002; Vorsanova, et al., 2005; Doria, et al., 2010; Jobanputra, et al., 2011). This procedure enables rapid identification of common aneuploidies using relatively small amounts of cells, avoiding major drawbacks of classical cytogenetics such as culture failure, overgrowth of maternal cells and tissue culture artifact (Vorsanova, et al., 2005; Doria, et al., 2010; Jobanputra, et al., 2011). The accurate diagnosis of polyploidy (Fig. 1) or the frequency of abnormal cell line in low-grade mosaicism cases is possible only by this method. Multiplex probe sets for analysis are selected based on knowledge about the frequencies of autosomal trisomies detected in spontaneous miscarriage and the availability of commercial probe sets (Jobanputra, et al., 2002). The limitation of this technique is that information of chromosomes not included in probe sets is lacking and structural abnormality is undetectable unless specific probes are applied. In addition, this relatively labor-intensive technique may have technical problems such as hybridization failure or cross-hybridization of probes to different chromosomes.
Fig. 1. The representative results of FISH analysis.
FISH analysis was performed by AneuVysion Prenatal Set (Abbott Japan) for the villous cells dispersed from the miscarriage specimens. Green fluorescence; DNA probe corresponding to the RB1 gene (13q14) labeled with SpectrumGreenTM, orange fluorescence; DNA probe corresponding to loci D21S529, D21S341 and D21S342 (21q22.13-q22.2) labeled with SpectrumOrangeTM. A; Diploid, B; Triploid, C; Tetraploid.

4.3 Molecular cytogenetic methods

With the development of new molecular techniques for chromosome analysis (Shaffer & Bui, 2007; Bui, et al., 2011), DNA-based analysis has been introduced in cytogenetic analysis of miscarriage. DNA-based analysis is divided into two groups; PCR-based analysis such as quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA), and microarray-based comparative genomic hybridization (aCGH). Compared to conventional karyotyping, these methods require only a few amounts of specimens, especially PCR-related methods, avoiding analysis failure due to inadequate amounts of samples. In addition, they are applicable for non-dividing or non-viable cells that fail to grow in vitro or archived tissue such as formalin-fixed or paraffin-embedded tissues, enabling retrospective investigation when the need for karyotype analysis is recognized later.

4.3.1 Quantitative fluorescent polymerase chain reaction (QF-PCR)

Polymorphism markers are used widely in molecular cytogenetic studies as well as forensic medicine. In recent years, the diagnostic efficacy of quantitative fluorescent polymerase chain reaction (QF-PCR) assay has been demonstrated in prenatal testing using fetal DNA derived from amniocentesis or chorionic villus sampling (Shaffer & Bui, 2007). Also in miscarriage analysis, several reports have indicated that QF-PCR assay is a rapid, low-cost and reliable tool to diagnose aneuploidies (Diego-Alvarez, et al., 2005; Diego-Alvarez, et al., 2006; Zou, et al., 2008). Besides, it can provide information about both parental and meiotic origin of aneuploidy and detect uniparental disomy (UPD) by additional parental testing (Diego-Alvarez, et al., 2005). Generally, short tandem repeats (STR) markers in the chromosomes where aneuploidies are commonly found in miscarriages, namely 13, 15, 16, 18, 21, 22 and sex chromosomes, are used. The PCR products amplified using the labeled primers with a fluorescent dye are visualized by capillary electrophoresis and fluorescent intensity (peak area/height) and size of the amplified products are quantitatively evaluated. In normal heterozygous pattern, two peaks of fluorescent activities are observed with the ratio of 1:1 (disomic diallelic), whereas only one fluorescent peak is detected in homozygous pattern. In a trisomic case, the STR markers can be detected either as three fluorescent peaks, 1:1:1 (complete heterozygote), or two fluorescent peaks, 2:1 (trisomic diallelic) (Diego-
Alvarez, et al., 2005; Zou, et al., 2008). Triploidy is also detected when all markers present trisomic patterns (Fig. 2), while postzygotic tetraploidy is difficult to detect. Low grade of mosaicism could also be recognized. On the other hand, the cytogenetic analysis could result in uninformative results when all STR markers of a chromosome show monoallelic patterns, and the aneuploidies not associated with the chromosomes examined are not detectable. Balanced chromosome rearrangements are also missed.

Fig. 2. The representative result of STR analysis. STRs were analyzed by GenomeLab Human STR Primer Set (Beckman Coulter) for the miscarriage specimen. The PCR product has three distinct peaks or two peaks with the ratio of 1:2, suggesting triploidy.

4.3.2 Multiplex Ligation-Dependent Probe Amplification (MLPA)

Multiplex Ligation-Dependent Probe Amplification (MLPA) is an efficient genetic diagnostic technique based on polymerase chain reaction (PCR) amplification to detect copy-number changes (Schouten, et al., 2002). MLPA permits the relative quantification of more than 40 sequences in a single multiplex assay using only 20ng of sample DNA (Diego-Alvarez, et al., 2006). Denatured genomic DNA is hybridized with a set of two probes, which consist of a target specific sequence and a universal forward or reverse PCR primer binding site (Schouten, et al., 2002). One probe has a stuffer sequence to generate various PCR products with different sizes (Fig. 3). After ligation of the two parts of hybridized probes, the products are amplified by PCR using only one fluorescent-labeled primer pair. The multiplex-fluorescent products are separated by capillary electrophoresis and the peak height/areas are quantified. The relative amounts of amplified products depend on the quantity of target DNA present in the sample (Schouten, et al., 2002; Slater, et al., 2003), enabling the detection of copy number changes such as deletion, duplication or whole chromosome aneuploidy. In prenatal testing, MLPA has been carried out as a rapid, flexible, sensitive and robust assay to screen aneuploidy such as trisomy 13, 18, 21 in a single experiment using a small amount of genomic DNA (Slater, et al., 2003; Shaffer & Bui, 2007).
A set of two probes are used for MLPA analysis. Each probe has a target specific sequence and a universal forward or reverse PCR primer binding site. One probe has a stuffer sequence to generate various PCR products with different sizes (Schouten, et al., 2002).

Since almost all of cytogenetic abnormalities of miscarriage involve gains or/and losses of subtelomere copy numbers, MLPA targeted for every subtelomere region is applicable for miscarriage analysis (Bruno, et al., 2006; Diego-Alvarez, et al., 2007; Donaghue, et al., 2010; Carvalho. et al., 2010). Whole chromosome aneuploidies are indicated if the increased or decreased copy number dosages at both arms of one individual chromosome are recognized (Fig. 4). The increased or decreased dosage of only one chromosome end indicates a segmental aneuploidy, and unbalanced structural abnormalities are indicated when the dosage change of another chromosome end is also present. The favorable aspect of this method in the clinical setting is low cost and reduced turn round time for analysis as well as high accuracy and robustness (Bruno, et al., 2006; Diego-Alvarez, et al., 2007; Donaghue, et al., 2010; Carvalho. et al., 2010). It can be easily implemented in standard laboratories and facilitate laboratory work by simultaneous analysis of large number of samples or automated system.

MLPA assay was performed using SALSA MLPA KIT P036-E1 (MRC-Holland). Both arms of chromosome 21 showed increased copy number dosages, suggesting trisomy 21.
Since aneuploidies are diagnosed by the results of only one or a few PCR products, the discordant results attributed to the inherent copy number polymorphism could occur when two probe sets are utilized (Ahn, et al., 2007). Moreover, this method has a limitation in miscarriage analysis in that polyplody or balanced structural abnormalities remain undetectable (Bruno, et al., 2006; Diego-Alvarez, et al., 2007; Donaghue, et al., 2010; Carvalho. et al., 2010).. Unbalanced Robertsonian translocation could be misdiagnosed as single chromosome aneuploidy. The detection of mosaicism would also be limited depending on the proportion of aneuploid cell line.

4.3.3 Microarray-based comparative genomic hybridization (aCGH)

Microarray-based comparative genomic hybridization (aCGH) is a powerful genetic tool for the comprehensive analysis of DNA copy number gains and losses throughout the whole genome at high resolution in a single experiment. In recent years, this technique has been vigorously applied in the clinical setting such as investigation of mental retardation, developmental delay and dysmorphism, especially in cases with normal karyotypes (Hayashi, et al., 2011). In addition, the identification of pathogenic copy number variations could lead to the discovery of genes responsible for various conditions/disease and the elucidation of specific gene function (Inzawa, et al., 2004; de Ravel, et al., 2007; Hayashi, et al., 2011). Array platforms are composed of a large number of genomic DNA clones such as bacterial artificial chromosomes or oligonucleotides. Test genomic DNA and reference genomic DNA are differently labeled with different fluorescent dye, and mixed together with blocking DNA to intercept repetitive sequences in the genome. After hybridization of this mixture to an array of genomic clones, the fluorescence ratio of two fluorochromes is measured for each spot (Snijders, et al., 2003; de Ravel, et al., 2007). of arrayed clones (Fig. 5) (Snijders, et al., 2003; de Ravel, et al., 2007).

![Schematic diagram of aCGH technique.](https://www.intechopen.com)

Test and reference genomic DNA are differently labeled with different fluorescent dye, and mixed together with blocking DNA. This mixture is hybridized to an array of genomic clones, and the fluorescence ratio of two fluorochromes is measured for each spot.

Although structural status of chromosome aberrations is not recognized, DNA copy-number changes are detected at high-throughput and high-resolution manner. Since most
of all observed chromosome abnormalities in spontaneous miscarriage involve copy-number changes in one or more subtelomere regions, this technique can apply miscarriage analysis, like MLPA with subtelomere probe sets (Schaeffer, et al., 2004). The increased or decreased copy number changes of all spots of any individual chromosome indicate a whole chromosome aneuploidy. The increased or decreased dosages of not all spots of a chromosome indicate a segmental aneuploidy, and unbalanced structural abnormalities are indicated when the terminal dosage change is present in two different chromosomes (Fig. 6).

As DNA-based analysis, aCGH can overcome some drawbacks of conventional karyotyping including culture failure, overgrowth of maternal cells and tissue culture effect (Schaeffer, et al., 2004). In addition, the spectrum of cytogenetic abnormalities detected is broader compared to MLPA and the resolution of analysis is more detailed than conventional karyotyping, allowing the detection of cryptic deletion or duplication (Schaeffer, et al., 2004; Shimokawa, et al., 2006), which might lead to the identification of new regions or genes that play a role in early embryonic development or demise. In fact, the recent studies have shown the involvement of submicroscopic abnormalities in miscarriages (Table 1), although it is unclear whether these subtle imbalances could cause miscarriage.

<table>
<thead>
<tr>
<th>Time of miscarriage</th>
<th>Submicroscopic abnormalities (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schaeffer et al. (2004)</td>
<td>≤20 wks del 9p21, dup 15q11-q13, dup 10qtel</td>
</tr>
<tr>
<td>Benkhalifa et al. (2005)*</td>
<td>9-11 wks del 22q13, dup 1pter</td>
</tr>
<tr>
<td>Shimokawa et al. (2006)</td>
<td>5-12 wks del 3p26.2-p26.3 (1.4 Mb)</td>
</tr>
<tr>
<td>Robberecht et al. (2009)</td>
<td>- del Xp22.3 (787.5 kb)</td>
</tr>
<tr>
<td>Warren et al. (2009)</td>
<td>10-20 wks dup Xp22.31 (289 kb), del 13q33.3 (115 kb), dup 5p15.33 (93 kb)</td>
</tr>
<tr>
<td>Menten et al. (2009)</td>
<td>- del 7q36qter, del Xq28qter</td>
</tr>
</tbody>
</table>

*The specimens that failed to grow in culture were analyzed.

Table 1. Submicroscopic abnormalities detected by aCGH in spontaneous miscarriage specimens (Schaeffer, et al., 2004; Benkhalifa, et al., 2005; Shimokawa, et al., 2006; Robberecht, et al., 2009; Warren, et al., 2009; Menten, et al., 2009).

A potential drawback of this technique is inability to detect balanced structural abnormalities such as reciprocal/Robertsonian translocations and inversions. The change in ploidy is also not detectable, since the amount of sample DNA is adjusted to the same extent as the reference in the assay process (Lomax, et al., 2000). Another great concern of this technique is the detection of copy number variations (CNV) of unknown or uncertain clinical significance (de Ravel, et al., 2007; Bui, et al., 2011). CNVs extend across the whole chromosomes more frequently than previously expected and clinical interpretation of CNVs is difficult in case of lack of available information. Therefore, targeted arrays for aneuploidies and known microdeletion/duplication syndromes may be a current option in miscarriage analysis as a clinical use until the pathogenicity of CNVs assayed becomes elucidated.
Fig. 6. The representative results of aCGH using Genome Disorder array. Genome Disorder (GD) array analysis was performed by Aizu Y, Ph.D. at Division of Advanced Technology & Development, BML, Inc., Kawagoe, Japan. GD array was developed in Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Japan (Inazawa, et al., 2004; Udaka, et al., 2007; Hayashi, et al., 2011). This BAC array covers every subtelomeric region except p-arms of acrocentric chromosomes as well as responsible regions for microdeletion syndromes. Clones are horizontally ordered from chromosome 1 to 22. Thresholds of copy-number ratios are 1.25 for gain and 0.75 for loss, respectively. A; Normal, B; gains at 15q11.2-13.1, 15q26.3 corresponding to trisomy 15, C; gain at 7p22.3-22.2 and loss at 6q27 corresponding to unbalanced reciprocal translocation between chromosome 6q and 7p.
4.4 Combined methods

Since every cytogenetic method has some drawbacks in miscarriage analysis, the combination assays have been encouraged in recent years. In MLPA and aCGH assays, ancillary FISH, microsatellite analysis, or flow-cytometry is performed to diagnose polyploidy in the event of normal assay results (Lomox, et al., 2000; Bruno, et al., 2006; Robberecht, et al., 2009; Menten, et al., 2009). On the other hand, aCGH assay could be additionally attempted to detect microabnormalities in normal karyotype cases judged by conventional analysis (Shimokawa, et al., 2006). Besides, FISH or polymorphism marker has been applied to exclude maternal contamination (Bell, et al., 1999; Jarrett, et al., 2001; Robberecht, et al., 2009), which is a troublesome issue in both classical karyotyping and DNA-based analysis of miscarriage. In FISH analysis, the demonstration of Y chromosome in cases with normal female karyotypes suggests maternal contamination, whereas it is impossible to distinguish maternal contamination in normal female fetus. Molecular approaches using microsatellite markers can evaluate maternal contamination irrespective of fetal sex by comparing maternal and putative fetal DNA polymorphism if both DNA are available (Jarrett, et al., 2001).

Recently, non-surgical managements have been performed for selected miscarriage cases to reduce patients' discomfort and avoid surgical complications such as uterine perforation, uterine adhesion, cervical trauma, hemorrhage and infection (Griebel, et al., 2005). It is also reported that the patients with miscarriages should be given the opportunity to choose a treatment option for their health-related quality of life (Wieringa-De Waard, et al., 2002). Since the specimens obtained by non-surgical managements are inappropriate for classical cytogenetics because of extensive degeneration and possible maternal contamination (Stephenson, et al., 2002). DNA-based analysis is a feasible strategy in patients who desire expectant management and cytogenetic analysis of miscarriage.

5. Conclusions

Cytogenetic study of miscarriage is of great significance for the management of RM patients as well as reproductive genetic research. As mentioned above, the currently-performed classical karyotyping has some drawbacks, possibly leading to failure of analysis or misdiagnosis. The introduction of new genetic techniques into miscarriage analysis could offer valuable information to RM patients and clinicians through more refined and complete diagnosis, and elucidate the genetic mechanism of early fetal development or demise as well as the precise incidence of genetic abnormalities associated with miscarriage.

6. References


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Human embryology is now rapidly moving to a new phase due to recent innovation and advances of life science including ES and iPS technology. This new era also directs a difficult challenge for scientists in terms of technological and ethical issues for future human embryology. However, human embryology is difficult to research due to ethics involved in the collection of human materials. This book traces the early history and provides knowledge on demonstration of principles from ancient to the most recent embryo studies amidst the unresolved scientific and ethical issues. We hope this book will help the readers to understand human embryo development better.

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