




# Cytogenetic Analysis of Spontaneous Miscarriages Using Long-Term Culturing of Chorionic Villi

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**Abstract** Spontaneous miscarriages are strongly associated with fetal chromosomal abnormalities. We sought to validate the occurrence of chromosomal abnormalities in Japanese patients with early spontaneous miscarriages using a conventional long-term culture method. One hundred and thirty-two patients were studied after excluding 7 cases with culture failure. Chromosomal analysis of 132 cases was performed using a long-term culture method of the villous placental tissue. Patients' age, number of previous spontaneous abortions, fetal data from ultrasonography, and the method of conception (ART or spontaneous pregnancy) were obtained from the electronic medical records. The association between the patient's background and the cytogenetic data was analyzed using a Chi squared test. The average maternal age of the 132 cases of early spontaneous abortion was  $34.2 \pm 4.7$  year (range: 24–43). Chromosomal abnormalities were detected in 89 cases (67.4%), among which 52.8% represented trisomy, 1.1% represented double trisomy, 10.1% represented polyploidy, 7.9% represented monosomy, 12.4% represented structural chromosomal abnormality, and 15.7% represented mosaic chromosome abnormalities. Women older than 35 year had

a higher rate of chromosomal abnormalities than younger women (76.6% vs. 58.8%;  $P = 0.015$ ). Twenty-five of the 132 pregnancies were achieved with ART and were associated with a higher rate of trisomy (64.0% vs. 37.4%;  $P = 0.008$ ). G-banding identified a rare form of polyploidy 94,XXYYYY. Our results confirmed previous findings that older Japanese women with spontaneous miscarriages have a higher rate of chromosomal abnormalities.

**Keywords** Chromosomal abnormality · Cytogenetic analysis · Spontaneous miscarriage · Karyotyping

## Introduction

Early spontaneous miscarriage occurs in 10–15% of all pregnancies, and may be caused by maternal disorders, such as diabetes and antiphospholipid syndrome, [1] antibiotic consumption, [2] body mass index, [3] etc. Chromosomal abnormalities are observed in 50–70% of spontaneous miscarriages and are reported to be the most common cause of spontaneous miscarriage [4–6]. These studies indicate that cytogenetic analysis of chorionic villi is crucial in understanding the cause of fetal loss and assess the risk of recurrence [6].

The incidence of fetal chromosomal abnormalities is known to increase with increasing maternal age [7–9]. The influence of other factors, such as assisted reproduction techniques (ART) and the number of previous miscarriages, has also been investigated, but the results are inconsistent [10–13]. While Bonduelle and colleagues [10] reported a higher incidence of chromosomal abnormalities in women undergoing ART, a recent study by Wu and colleagues [11] found no such association.

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Similarly, contradictory reports exist on the influence of previous miscarriages on miscarriage recurrence. Ogasawara et al. showed that the incidence of miscarriages increased with the number of previous spontaneous abortions; [12] however, a recent study by Goldstein et al. contradicted the existence of such a correlation [13].

Studies by Jenderny et al. and Menasha et al. examined chromosomal aberrations in large scale cohorts of spontaneous miscarriages [14, 15]. In this study, we corroborated these previous findings in a small-scale Japanese cohort. We also examined the relationship between the patient's background and the occurrence and type of chromosomal abnormality.

## Materials and Methods

### Patient Information

Pregnant women who experienced a spontaneous abortion within the first 12 gestational weeks (as confirmed by clinical ultrasonography) were enrolled in the study. One hundred and six cases from January 2001 to December 2009 at the Hyogo College of Medicine and 33 cases from July 2012 to March 2018 at the Jichi Medical University Saitama Medical Center who underwent chorionic villous chromosome examination were included in this study. The study was performed prior to elective dilation and curettage procedures. The protocol was approved by the Ethics Committee and all participants provided written informed consent in opt-out documents prior to the study.

Patients were included as per the following criteria: (1) gestational sac empty diameter  $\geq 25$  mm, (2) crown-rump lengths  $\geq 7$  mm for embryos with no cardiac activity, or (3) no cardiac activity upon the initial scan and post 7 days scan for embryos with a crown-rump length  $< 7$  mm.

A miscarriage was diagnosed following international guidelines [16]. Cases were limited to a single fetus with no evidence of progressing abortion or villus contamination to avoid culture failure. Information regarding patients' age, number of previous spontaneous abortions, fetal data from ultrasonography, and method of conception (ART or spontaneous pregnancy) were obtained from the electronic medical records. Information about the fetal sex ratio was also collected.

### Chromosomal Examination

Chromosomal examination was performed on the collected villous placental tissue using a long-term culture method [5]. Briefly, 50–100 mg of villous tissue was harvested and immediately cleaned to remove maternal blood. Subsequently, the tissue was rinsed 5 times with a solution

containing alfa-MEM (Sigma-Aldrich), penicillin, and streptomycin. The specimen was then transferred to a 35-mm petri dish in 5 mL of Chang's medium (Irvine Scientific) and incubated at 37 °C, 5% CO<sub>2</sub> for 1–2 weeks. The medium was changed 2–3 times per week. Following confirmation of adequate cell growth of the tissue explants, colcemid (1 µg/mL; Adipo Gen) was added to the medium for 2–3 h. The medium was subsequently removed, and the dishes were washed for 3–4 min in phosphate buffered saline and 0.5% trypsin–EDTA solution (Sigma-Aldrich). Finally, the cells were aspirated, centrifuged, treated with 0.075 M hypotonic KCl for 15 min, and fixed in 3 changes of Carnoy's fixative. Chromosome preparations were made by transferring the cell suspensions onto clean slides and allowing them to air-dry at room temperature (25–27 °C). Coverslips were G-banded following standard laboratory techniques [17].

### Ultrasonography

Transvaginal ultrasonography was performed by experienced sonographers using a 5–9 MHz vaginal probe (GE Voluson E8/730, GE Tech Co., Ltd., New York, America). All patients received at least two ultrasonography examinations at our hospital between the 6th and 12th weeks of gestation to diagnose spontaneous miscarriage.

### Statistical Analysis

Data were analyzed for potential associations between patients' background and the presence of fetal chromosomal abnormalities using a Chi squared test.  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using SPSS (version 19.0, SPSS Inc, Chicago, IL, USA) software.

## Results

Among the 139 cases of spontaneous abortion, chromosome analysis was successfully performed for 132 cases (culture success rate = 95.0%). Seven cases were excluded due to contamination of the culture.

The mean age of the patients was  $34.2 \pm 4.7$  y (range: 24–43), the mean number of gestational weeks was  $8.8 \pm 1.2$  weeks (range: 6–11), and the mean number of previous spontaneous abortions was  $1.1 \pm 1.4$  (range: 0–6).

Chromosomal analysis revealed a normal karyotype in 43 cases (32.6%) and abnormalities in 89 (67.4%) cases. The results of the cytogenetic analyses are summarized in Table 1 and Supplementary Table 1. The most commonly found abnormality was trisomies (52.8%). Other

**Table 1** Cytogenetic analysis of 132 cases of spontaneous miscarriage

Karyotype	Number of cases (n)	% of abnormalities
Trisomy	47	52.8%
+ 2	4	
+ 4	1	
+ 12	2	
+ 13	4	
+ 14	1	
+ 15	8	
+ 16	12	
+ 17	1	
+ 20	1	
+ 21	3	
+ 22	10	
Double trisomy		
+ 10 + 21	1	1.1%
Polyploidy	9	10.1%
Triploidy	5	5.6%
Tetraploidy	4 (including one case: 94,XXYYYY)	4.5%
Monosomy	7	7.9%
Structural abnormalities	11	12.4%
Mosaic	14	15.7%
Total	89	67.4%

**Table 2** Incidence of an abnormal karyotype according to previous miscarriage history

Number of previous miscarriages	Number of cases (n)	Abnormal karyotype (n,%)
0–2	113	77 (68.1%)
≥ 3	19	12 (63.2%)

Results are presented as n or n (%); *P* = 0.334

abnormalities included double trisomy (1.1%), polyploidy (triploidy: 5.6%; tetraploidy: 4.5%), monosomy (45,X) (7.9%), structural abnormality (12.4%), and mosaicism (15.7%) (Table 1). The types of mosaic and structural abnormalities found are described in Supplementary Table 1.

**Table 3** Occurrence of an abnormal karyotype according to the method of conception

Method of conception	Number of cases (n)	Maternal age (y) (mean ± SD)	Abnormal karyotype (n,%)	Trisomy (n,%)
Non-ART	107	33.5 ± 4.6 <sup>a</sup>	70 (65.4%)	40 (37.4%) <sup>b</sup>
ART	25	37.5 ± 4.1 <sup>a</sup>	19 (76.0%)	16 (64.0%) <sup>b</sup>

<sup>a</sup>*P* < 0.01; <sup>b</sup>*P* = 0.008

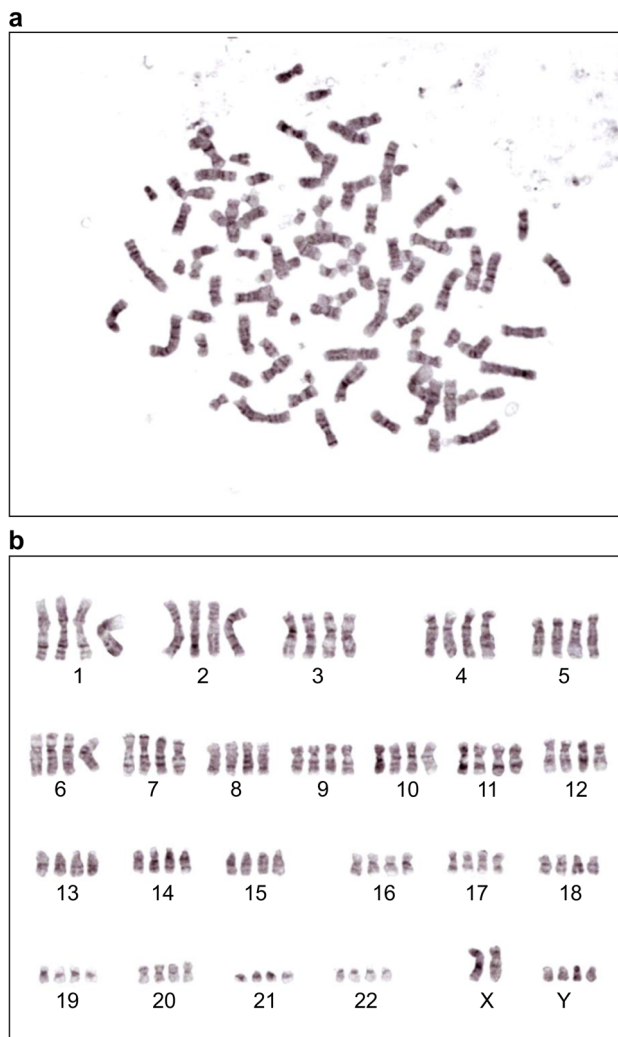
Chromosomal abnormalities were significantly associated with maternal age. Women with abnormal karyotypes tended to be older than women with a normal karyotype (abnormal karyotype: 89 (67.4%), age: 34.8 ± 4.8 year vs. normal karyotype: 43 (32.6%), age: 33.1 ± 4.5 year; *P* = 0.034) (Supplementary Table 2). To further explore the effect of maternal age, patients were grouped according to age (< 35 or ≥ 35 year of age) and the incidence of chromosomal abnormalities was analyzed (Supplementary Table 3). Women older than 35 year were found to have a significantly higher rate of chromosomal abnormalities (58.8% for women < 35 year old vs. 76.6% for women ≥ 35 year old; *P* = 0.015). These women also had a significantly higher rate of trisomy (35.3% for women < 35 year old vs. 50.0% for women ≥ 35 year old; *P* = 0.044).

Trisomy cases included mosaic cases (normal karyotype/trisomy and normal karyotype/double trisomy) and double trisomy cases. Only one case (normal karyotype/trisomy/tetraploidy) was not counted as a trisomy as the number of trisomy cells was small.

The association between the number of previous spontaneous abortions and the frequency of chromosomal abnormalities is presented in Table 2. The number of previous spontaneous abortions did not significantly affect the frequency of chromosomal abnormalities (68.1% for 0–2 vs. 63.2% for ≥ 3; *P* = 0.334).

Of the 132 patients included in the study, ART treatment resulted in 25 pregnancies, which include conventional IVF (n = 14) and intracytoplasmic sperm injection (n = 11). Women who underwent ART treatment were older than those who conceived spontaneously (37.4 ± 4.1 year for ART vs. 33.5 ± 4.6 year for non-ART; *P* < 0.01) (Table 3). Although, the overall occurrence of chromosomal abnormalities did not differ significantly between ART and non-ART treated women (*P* = 0.154), the number of trisomies was significantly higher in ART treated women (64.0% for ART vs. 37.4% for non-ART; *P* = 0.008).

Regarding the sex ratio of fetuses in all 132 cases, the Y chromosome was detected in 49 cases (37.1%) and not detected in 83 cases (62.9%). In the 89 cases with an abnormal karyotype, the Y chromosome was detected in 37 cases (41.6%) and absent in 52 cases (58.4%). In the 43 cases with a normal karyotype, 12 cases were 46,XY (27.9%) and 31 cases were 46,XX (72.1%).



**Fig. 1** G-band staining showing rare chromosome case of 94,XXYYYY in (a) and (b)

G-banding identified a rare form of polyploidy 94,XXYYYY as shown in Fig. 1.

## Discussion

Here, we investigated chromosomal abnormalities in 132 cases of spontaneous miscarriage diagnosed at our clinic. Our study found that 67.4% of all cases had chromosomal abnormalities that included trisomies, polyploidy, monosomy, structural abnormalities, and mosaicism. The percentage of chromosomal abnormalities increased with increasing maternal age (women  $\geq 35$  year old) but was not affected by previous spontaneous abortions. Women who conceived using ART had a higher percentage of trisomies. Our study is a confirmatory investigation and adds to the literature on this topic in the Japanese population [14, 15, 18].

In our study, chromosomal abnormalities were detected in 67.4% of the analyzed samples, which falls within the previously reported range of 50–70% [6, 19, 20]. It has been suggested that chorionic villous cells with chromosomal abnormalities may have impaired proliferative capacity in long-term in vitro cultures, leading to the underestimation of the abnormality rate [21]. In fact, several studies have reported higher rates of fetal chromosomal abnormalities [22–24]. It is possible that the lower abnormality rate in our study resulted from reduced proliferative capacity of villous cells with abnormal chromosomes.

Nevertheless, our results indicated that women over 35 year of age present a higher occurrence of chromosomal abnormalities than those younger than 35 year. This result is consistent with findings that report an increase in the chromosomal abnormality rate with increasing maternal age [19, 25, 26].

In terms of abnormality types, we found that the highest proportion corresponded to trisomy (52.8%), followed by structural abnormalities (12.4%), polyploidy (10.1%) and monosomy (7.9%). Mosaics of the normal karyotype and chromosomal aberrations were observed in 15.7% of the cases, and double trisomy was observed in 1.1% of the cases. Comparable proportions have been reported in previous studies [27, 28].

The most frequent trisomy detected in our study was trisomy 16, which is similar to a past report by Soler et al. [6] We only found one case of trisomy 17, which is known to be rare [29]. Several important genes are present on chromosome 17 and its abnormality may be lethal at a very early stage in embryonic development, leading to a lower incidence of this trisomy in our study [30]. Similar to past reports, polyploidies were not uncommon in this study, but one rare case of 94,XXYYYY was detected. This karyotype is known to be associated with sperm chromosome abnormality [31]. In our study, this karyotype was found in a recurrent miscarriage case with no paternal chromosomal abnormalities. Therefore, it is possible that this karyotype resulted from meiosis errors during spermatogenesis.

Most cases of trisomy derive from errors in maternal meiosis (meiotic non-disjunction), in which advanced maternal age is a risk factor [19]. In line with this, the trisomy rate was found to be significantly higher in women older than 35 year compared to younger women in our study.

Although a study has reported an increased likelihood of a future pregnancy loss in cases with a history of spontaneous miscarriages, [32] the relationship between previous miscarriages and the occurrence of fetal chromosomal abnormalities remains controversial [12, 13]. We found no significant difference in the occurrence of chromosomal abnormalities between women with 3 or more previous miscarriages and those with less than 2 miscarriages.



Similarly, reports about the influence of ART on the occurrence of fetal chromosomal abnormalities are contradictory [10, 11]. In this study, the overall occurrence of fetal chromosomal abnormalities was not significantly different between ART and non-ART patients. However, the proportion of trisomy was higher in ART patients. In addition, the age of ART women was significantly higher than that of non-ART mothers. This may imply that the higher rate of trisomy may be an indirect consequence of higher age, rather than a direct consequence of the ART technique. It has been reported that the age of Japanese women seeking ART is increasing [33].

The Y chromosome was less frequently detected in patients with a normal karyotype than those with an abnormal karyotype. This could result from maternal cell contamination [34]. It is possible to identify this using quantitative fluorescent polymerase chain reaction (QF-PCR); [35] however, this was not performed as the patient did not agree to incur the additional cost. Although there was only a slight increase in the percentage of the Y chromosome in patients with an abnormal karyotype, more studies are required to rule out maternal cell contamination.

Karyotyping has been used as a conventional method for genetic analysis of products of conception [36]. However, the need to culture the cells and the possibility of maternal cell contamination lead to a high percentage of sample loss (approx. 20–40%). In a genetic analysis by Sahoo et al. [36] 91% of samples were successfully analyzed using chromosomal microarray analysis. Although we did not compare the results of our method with more sensitive methods like microarray analysis, we successfully analyzed 95% (132/139) of the samples using a long-term culturing method. Future studies comparing the success rate of both methods in large sample cohorts will be useful.

This study is limited by the small sample size and the use of only one method for genetic analysis. The effect of maternal cell contamination on the sex ratio was not determined using additional techniques like QF-PCR. Future studies addressing these issues are warranted.

In conclusion, our study confirmed previous reports of chromosomal abnormalities in patients with spontaneous miscarriages in a Japanese cohort. Future large-scale prospective studies comparing the different analytical methods are required.

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#### Compliance with Ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical Approval Statements** The protocol was approved by the Ethics Committee of Jichi Medical University, Japan (Ethics Committee Approval Number Rin-S17-100), and all participants provided written informed consent in opt-out documents prior to the study.

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