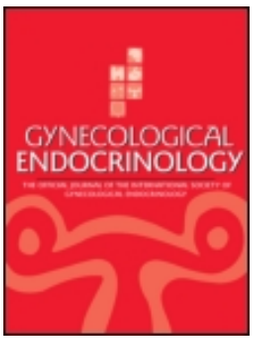

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ORIGINAL ARTICLE

Effects of subtelomeric copy number variations in miscarriages

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Abstract

Purpose: This study was performed on miscarriage samples for chromosome analysis to detect copy number variations (CNVs) related to subtelomeric regions, and with these results we aimed to adapt multiplex ligation-dependent probe amplification (MLPA) method for prenatal diagnosis.

Materials and methods: The cell cultures and DNA isolations were performed on 60 miscarriage samples. For maternal contamination analysis, DNA isolations and quantitative fluorescent polymerase chain reactions were done using peripheric blood of mothers who had miscarriages. We compared short tandem repeat peak profiles of miscarriage samples and mothers. The subtelomeric regions of the chromosomes were assessed using the MLPA method.

Results: Of 43 miscarriage samples, 19 had normal karyotype (44.2%), 10 had numerical abnormalities (23.3%), and 2 had structural abnormalities (4.7%). Subtelomeric 16q duplication was determined in 2 of the 30 miscarriage samples investigated with MLPA method (6.6%).

Conclusion: There is no statistically significant difference between two groups ($p > 0.05$). However, the fact that the 6.6% subtelomeric CNV found in miscarriage samples was not found in controls, showed that further studies are required. We recommend that the miscarriage samples of the couples with recurrent miscarriage should be analyzed in terms of subtelomeric CNV after the exclusion of other clinical reasons.

Keywords

Infertility, pregnancy, urogenital system

History

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Introduction

Miscarriage is defined as the spontaneous loss of a pregnancy before viability [1,2]. In other words, miscarriages include loss of pregnancy until the maximum of 24 weeks of gestation [2–4]. Recurrent miscarriage has been defined as the loss of two or more consecutive pregnancies in the guidelines of the American Society of Reproductive Medicine [5]. While 15% of clinically recognized pregnancies result with a spontaneous miscarriage, it is stated that ~5% of all couples have recurrent miscarriage [2]. 70% of all pregnancies fail to go to term, in which 50–60% are lost within the first month of pregnancy [6]. Many factors negatively affecting the reproductive physiology may also cause miscarriages. Genetic and uterine abnormalities, endocrine and immunological factors, infectious and environmental agents may lead to miscarriages [6,7]. Chromosomal abnormalities are one of the genetic factors causing miscarriages [6,8] and are responsible for 50–70% of miscarriages [7,9]. The chromosomal abnormality rates consist of 30% trisomy, 10% triploid or tetraploid, 9% monosomy X constitution and 2% structural rearrangements, including translocation, deletion, duplication and inversion [10].

Microdeletions and microduplications that can be determined with molecular genetic techniques are called as submicroscopic chromosomal abnormalities. The submicroscopic chromosomal changes are also termed DNA copy number variants (CNVs). And, it is specified that the rates of submicroscopic chromosomal abnormalities are, respectively, 10–20% and 1–13% in patients with development delay and miscarriages samples [9,11]. Detection of these submicroscopic changes could elucidate some of the still unexplained miscarriages [11]. Also, it is suggested that the submicroscopic abnormalities of telomeric or subtelomeric regions might be involved in the development of miscarriages or infertility [12–18].

The telomeric regions have high-degree sequence similarity within itself and show differences from other DNA sequences in terms of structure and function [16,19,20]. The subtelomeric regions contain some genes that are transcriptionally active. The frequency of genetic recombination increases toward the telomeres and because of the repeat motifs in these regions, they may be prone to rearrangements [21]. Though some studies have expressed that the copy number variations of telomeric and subtelomeric regions can cause miscarriages [12–18], adequate studies have not been performed to study the relationship with miscarriage and telomeric and subtelomeric abnormalities. Also, while conventional cytogenetic techniques are allowed in the detection of chromosomal abnormalities that are >5 Mb, they do not detect chromosomal abnormalities smaller than 5 Mb [16]. It is reported that the use of fluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe hybridization (MLPA) and comparative genomic hybridization (CGH) methods are required in the investigation of these abnormalities [4,22]. This study was performed to detect subtelomeric chromosomal abnormalities that cannot be identified by conventional cytogenetic methods, to determine frequencies of these abnormalities in

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miscarriages samples and to adapt prenatal diagnostic applications of MLPA method in miscarriage samples.

Materials and methods

Study group and biologic samples

This study was performed on 60 (study group) miscarriage samples and 20 (control group) healthy persons who have healthy children and no history of miscarriage. Miscarriage samples were referred for cytogenetic analysis by the Department of Gynecology and Obstetrics, Department of Medical Biology, Section of Medical Genetics of Ondokuz Mayis University. Informed consent was in accordance with the study protocol, approved by the ethics committee of Faculty of Medicine. All individuals signed a written consent form after being informed about the details of the study. All studied subjects were from Caucasian origin and the average age of mothers who had miscarriage were 30.68 ± 5.74. The average of miscarriage was 2.2 ± 1.4 in the study group. Miscarriage samples consisted fetal remains obtained after surgical evacuation from clinical abortions between the 4th and 24th weeks of gestation (average 9.15 ± 4.17 weeks of gestation). The cell cultures were performed on chorionic villi received from 60 miscarriage samples sent to laboratory for investigation. Pieces of samples were stored by freezing at $-80\,^{\circ}C$ for DNA extraction to perform molecular studies. DNA was extracted from uncultured miscarriage samples, peripheric blood of mothers who had miscarriages and control group using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany).

Cytogenetic analysis

Cytogenetic analysis was performed on 60 miscarriage samples of the study group. Owing to the frequency of maternal cell contamination (MCC) in long-term cultured villi (LTC-villi) was generally very low [23,24], LTC method was preferred as culture procedure. The miscarriage samples were carefully washed and estimated under a stereo microscope. Chorionic villi fragments were isolated from maternal tissue and blood clots. Cells were cultured in 5% CO_2 and $37\,^{\circ}C$ incubator (Thermo Scientific, Waltham, MA) and harvested *in situ* with trypsin-EDTA treatment after 2–3 weeks. Chromosome analyses were performed after GTG-banding (Trypsin and Giemsa) using Giemsa stain. A minimum of 20 cells were analyzed from each miscarriage sample.

Multiplex quantitative fluorescent polymerase chain reaction

Quantitative fluorescent polymerase chain reaction (QF-PCR) method was performed for investigating MCC from miscarriage samples. Miscarriage samples of mothers who do not want to give blood for maternal contamination were excluded from the study. Therefore, maternal contamination analyses were conducted on DNA of 52 miscarriage sample and 52 mothers who had miscarriages. Maternal contamination analysis was made by comparing the QF-PCR provider 19 short tandem repeat (STR) peak profiles (on the 13, 18, 21 and sex chromosomes) and two gene region peak profiles (SRY and Amelogenin) on the sex chromosomes of miscarriage samples and mothers who had miscarriage (http://www.aneufast.com) (Figure 1). The highly polymorphic STR regions are informative for both dosage ratios of STR and parental origin of DNA when analyzing with the parental DNA. Fluorescence-labeled PCR products were electrophoresed in ABI Prism 3130 Genetic Analyzer and analyzed with the GeneMapper software version 4.1 package (Applied Biosystems, Waltham, MA).

Subtelomeric MLPA

The subtelomeric regions of the chromosomes in 30 of the 43 miscarriage samples without maternal contamination and 20 healthy persons who had healthy children and without history of miscarriage were assessed by the MLPA method. The MLPA assay was performed using the Salsa P070 human telomere probe mix kit (MRC-Holland, The Netherlands) that contains subtelomeric probes for p and q arm of all chromosomes. MLPA method was carried out as recommended by the manufacturer. Each sample was run in duplicate and compared with the control samples obtained from the Section of Medical Genetic. PCR products were electrophoresed in an ABI Prism 3130 Genetic Analyser and analyzed with the GeneMapper software version 4.1 package using LIZ 500 size standard (Applied Biosystems, Waltham, MA). Normalization of peak area values was achieved as recommended by the manufacturer (Coffalyser analysis program, www.mlpa.com, MRC-Holland, The Netherlands).

Statistical analysis

Analysis of the data was performed using the computer software SPSS 15.0 (SPSS, Chicago, IL). Continuous data were given as mean $\pm$ SD (standard deviation) and (min–max). The subtelomeric abnormality rates of the study group and control group were compared by the Fisher-exact test.

Results

Demographic variables and baseline characteristics of mothers who suffer from miscarriages are given in Table 1. Based on the result of maternal contamination analysis conducted on 52 miscarriage sample, it is determined that maternal contamination rates are 17.3% (9/52). Cytogenetic analysis results of miscarriages samples without maternal contamination are given in Table 2. Also, the culture of 15 of the 60 miscarriage samples could not be achieved, so the rate of culture failure was estimated as 20% in this study. It is determined that maternal contamination is present in the 3 of 15 miscarriage samples, which are culture failure. The chromosomal constitutions of miscarriage samples without maternal contamination and culture failure were evaluated with QF-PCR and MLPA methods. The QF-PCR peak profile samples of miscarriage samples and mothers who had miscarriages are given in Figure 1. Beside cytogenetic methods, the subtelomeric regions of 30 miscarriage samples without maternal contamination analyzed with MLPA method in terms of subtelomeric abnormality are microdeletion and microduplication. The subtelomeric MLPA results of miscarriage samples are given in Table 3. The analyses showed that 14 of 24 miscarriage samples have duplication in subtelomeric region of chromosome 16. and, it was determined that the abnormality rates related to the subtelomeric regions of miscarriage samples are 6.6% (2/30). The identified subtelomeric abnormality is duplication in which GAS8 gene is localized in 16q subtelomeric region. However, it was seen that the subtelomeric MLPA results of 20 healthy controls having healthy children and no history of miscarriage were completely normal and did not have subtelomeric abnormality (the subtelomeric abnormality rates 0%). There was no statistical difference between the subtelomeric abnormality rates of the study group and control group ($p > 0.05$). The MLPA peak profile samples obtained from miscarriage samples are given in Figure 2.

Discussion

Embryonic development is a complex process that involves a balanced interaction of environmental and genetic factors. The genetic abnormalities may cause abnormal embryonic

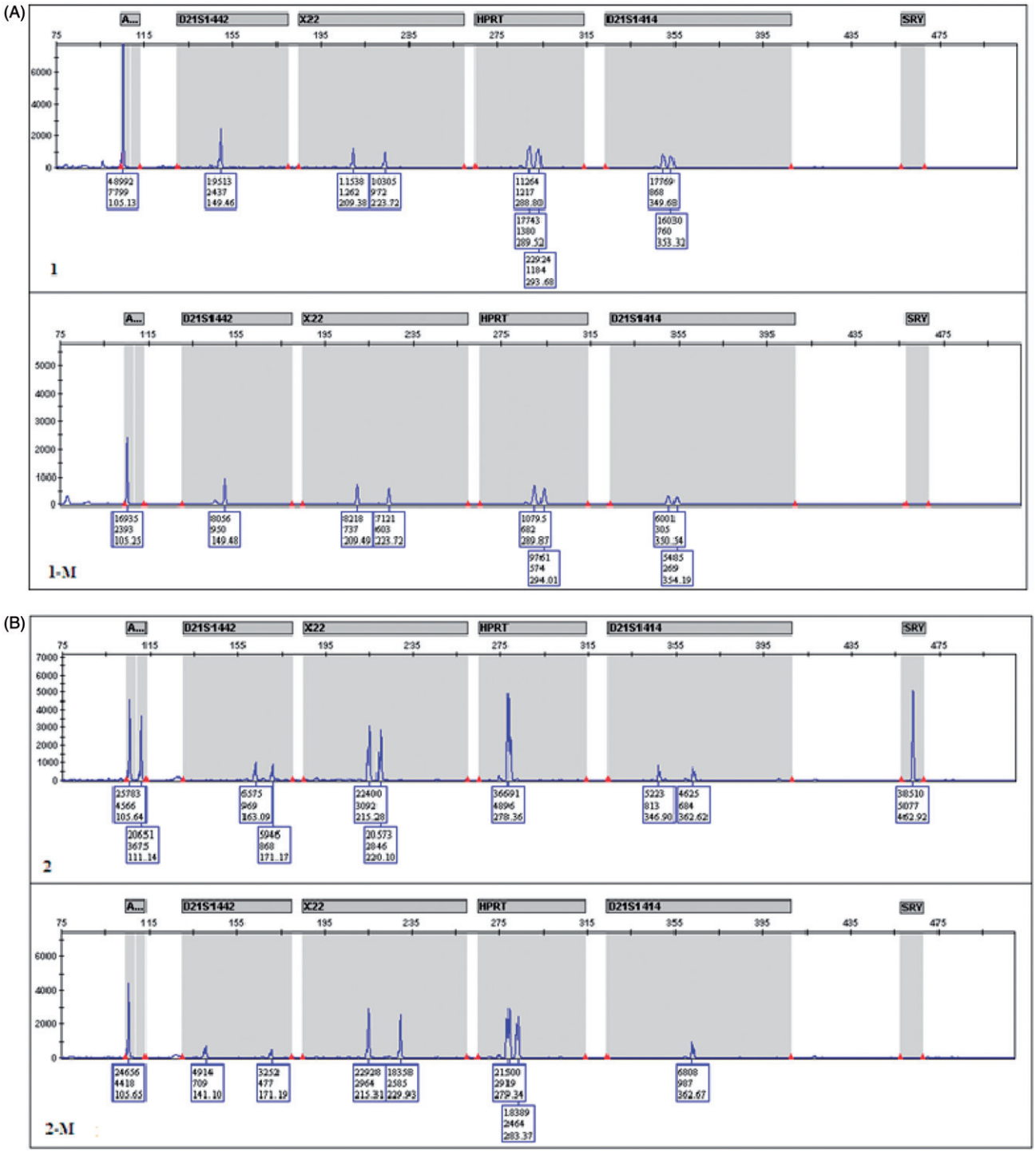

Figure 1. (A) The image of a miscarriage sample with maternal contamination. 1: QF-PCR peak profile of miscarriage sample. 1-M: QF-PCR peak profile of mother who had miscarriage. (B) The image of a miscarriage sample without maternal contamination. 2: QF-PCR peak profile of miscarriage sample. 2-M: QF-PCR peak profile of mother who had miscarriage.

development and miscarriages [4]. One of the primary causes of miscarriages is cytogenetic defects. The analysis of aneuploidies, translocations and other gross structural aberrations help to explain some cases of recurrent miscarriages, but cytogenetic test fails to render a complete lighting on the genetic etiologies of miscarriages [8].

This study was performed to determine frequencies of chromosomal abnormalities in miscarriages samples, to detect subtelomeric chromosomal abnormalities that cannot be identified by conventional cytogenetic methods. It has been stated that 50% of miscarriages are based on chromosomal abnormalities [9,25,26]. Fabro et al. [5] reported that the chromosome abnormality rate was 39% in miscarriage samples and the most common anomalies were autosomal trisomies. So, it is observed that there is a concordance between our data and literature in terms of numerical chromosomal abnormality rates. Also, the rate of structural abnormality that was determined as 4.7% in this study is concordant with other studies [27–29]. Kano et al. reported that chromosomal abnormality was not frequent (14.6%) in the miscarriage samples of the patients with recurrent miscarriages, but they stated that it did not completely exclude the possibility of maternal contamination [30]. The evaluation of

the miscarriage samples without maternal contamination analysis may lead to incorrect results. Therefore, the maternal contamination analyses are important in terms of achieving the correct results in miscarriage samples. In our study, it is determined that maternal contamination rates are 17.3%. The evaluation of miscarriage samples with QF-PCR method excluded the possibility of maternal contamination causing false-negative and false-positive results, and provided a significant contribution for determining the karyotype of miscarriage samples. Additionally, the culture failure is important problem for cytogenetic analysis. The analysis with molecular techniques such as CGH or MLPA of the subtelomeric regions could be used to overcome culture failure problem [31]. In our study, the chromosomal constitutions of miscarriage samples without maternal contamination, which are culture failure, were successfully evaluated with QF-PCR and MLPA methods. The molecular analysis with combined QF-PCR and MLPA of miscarriage samples excluded the possibility of maternal contamination and analysis of miscarriage samples to result in culture failure.

Table 1. Demographic variables and baseline characteristics of mothers who suffer from miscarriages and control group.

Characteristics	
Average age of mothers who had miscarriages ($n = 60$)	30.68 ± 5.74
Average age of control group ($n = 20$)	33.55 ± 5.56
Gestational week rate of miscarriages	9.15 ± 4.17
Average of miscarriage	2.2 ± 1.4
Miscarriage rates of first trimester (%)	94.9
Missed abortion (%)	56.7
Habitual abortion (%)	35
Anembryonic (%)	6.7

Table 2. Cytogenetic analysis results of miscarriage samples without maternal contamination.

Groups	Subgroups	*n* (%)	Total *n* (%)
Normal karyotype	46, XX	13 (68.4)	19 (44.2)
	46, XY	6 (31.5)	
Numeric abnormalities	45, X	2 (20)	10 (23.3)
	Trisomy	3 (30)	
	Triploidy	4 (40)	
	Tetraploidy	1 (10)	
Structural abnormalities	Translocations		2 (4.7)
Culture failure			12
Total			43

We determined that the abnormality rates related to subtelomeric regions of miscarriage samples are 6.6%, while the subtelomeric MLPA results of control group are completely normal without any subtelomeric abnormality. Albeit there was no statistical difference between the subtelomeric abnormality rates of the study group and control group, the 6.6% subtelomeric abnormalities found in miscarriage samples that were not found in control group is remarkable. Many studies investigated whether MLPA method is alternative to conventional cytogenetic techniques and the reason for success rate in detection of genetic abnormalities [32–38]. It is expressed that MLPA assays are widely used for copy number measurement [32]. Yakut et al. determined a cryptic translocation between chromosome 3 and 10 in one couple with recurrent miscarriages. And, they proposed that the miscarriage sample of cases with recurrent miscarriages should be analyzed with subtelomeric methods such as FISH [39]. Additionally, Diego-Alvarez et al. [33] proposed the use of subtelomeric MLPA to detect aneuploidy and unbalanced terminal chromosomal rearrangements in miscarriages. Bruno et al.

Table 3. Subtelomeric MLPA analysis results of miscarriage samples.

No	Age	GPA	Gestation	PTT	PT/INR	Cytogenetic results	MLPA results
01	30	G2P0A2	9	31.7	0.98	46, XX	Normal
02	38	G2P1A1	9	23.6	1.06	46, XY, der(1), t(1;2)	1p.del
03	28	G1P0D1	8 + 6	23.8	0.89	C.F.	Normal
04	27	G1P0A1	11	38	1.15	46, XX	Normal
05	34	G4P1A3	9 + 1	27	1.15	46, XX	Normal
06	31	G1P0A1	10 + 1	23.66	0.99	46, XY	Normal
07	19	G2P0A2	14	25.42	0.98	C.F.	Xp.del. Xq.del.
08	25	G5P1A4	8 + 1	23	0.99	C.F.	Normal
09	31	G3P1A2	9 + 5	–	–	46, XY	Normal
10	29	G3P0A3	10	23.49	0.89	46, XX	Normal
11	31	G3P0A3	10	–	–	46, XX, der(1), t(1;8)	1p.del. 8p.dup.
12	32	G2P0A2	9	22.1	0.91	46, XX	Normal
13	37	G3P1A2	6	–	–	C.F.	9p.dup. 9q.dup.
14	27	G3P0A3	9	24.34	0.96	46, XX	16q.dup
15	29	G1P0A1	6	20	1.08	46, XX	Normal
16	36	G6P0A6	8	27.35	0.9	C.F.	Normal
17	27	G1P0A1	8	26.48	0.89	46, XX	Normal
18	26	G1P0A1	8	25.15	0.97	46, XX	Normal
19	25	G1P0A1	8	21.08	0.97	C.F.	Normal
20	28	G1P0A1	9	–	–	46, XX	Normal
21	40	G2P1A1	13	23.21	0.87	C.F.	Normal
22	30	G3P1A2	9	–	–	46, XX	Normal
23	30	G1P0A1	9 + 4	25.53	0.88	C.F.	Normal
24	25	G1P0A1	7	29.7	1.1	C.F.	16q.dup
25	35	G3P1A2	8	–	–	C.F.	16p.dup. 16q.dup
26	35	G2P1A1	5	29.2	0.98	46, XX	Normal
27	21	G4P1A3	7	27.7	1.09	46, XX	Normal
28	31	G1P0A1	8	21.98	0.98	46, XY	Normal
29	39	G5P1A4	8	27.67	0.89	46, XY	Normal
30	28	G3P2A1	6	28.27	1.03	46, XY	Normal

M.A., missed abortion; A.E., anembryonic pregnancy; H.A., habitual abortion; C.F., culture failure; G, gravid; P, parity; A, abortion; PTT, partial thromboplastin time; PT/INR, prothrombin time/international normalized ratio.

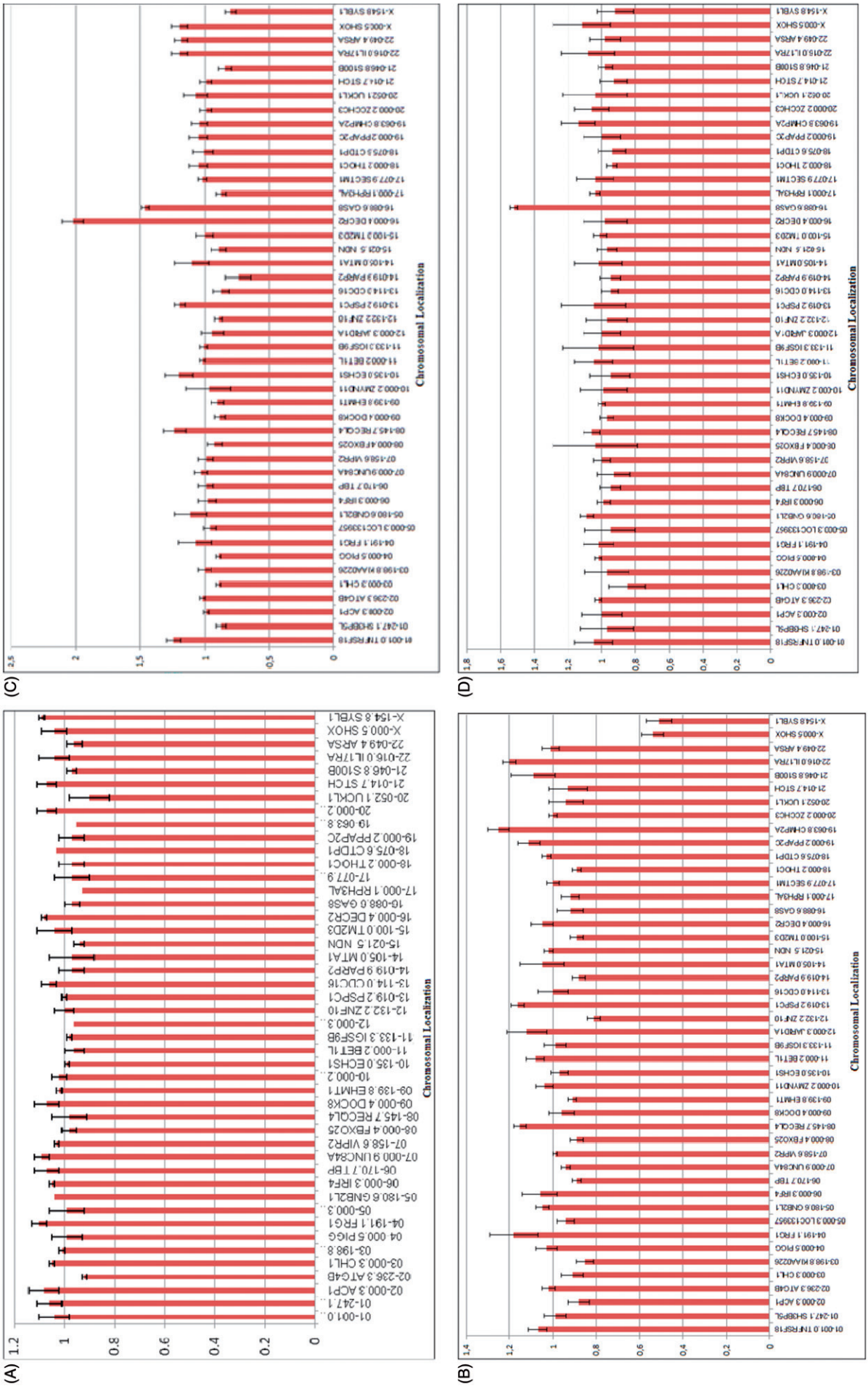

Figure 2. The MLPA peak profiles. (A) The normal sample (numbered 23). (B) The compatible with monosomy X of sample (numbered 07). (C) The compatible with trisomy 16 sample (numbered 25). (D) The compatible with subtelomeric duplication of chromosome 16 sample (numbered 14).

expressed that there were no subtelomere test failures compared to karyotype failures and the subtelomeric MLPA method is successful in detection of subtelomeric abnormalities [32]. Ahn et al. [40] stated that MLPA is a highly efficient technique for medium throughput screening for subtelomere imbalance. The subtelomeric abnormalities are clinically associated with fetal malformations and intrauterine growth restriction (IUGR). Mademont-Soler et al. showed that the rate of cryptic subtelomeric imbalances in pregnancies with ultrasound findings and normal karyotype is 1.3%. Also, they stated that MLPA is very useful for the characterization of unbalanced karyotypes [41]. Donaghue et al. [37] reported that subtelomeric abnormality rate is 1.8% in miscarriage samples. In this study, the clinically relevant cryptic subtelomeric imbalances rates are different to that found in the studies of Mademont-Soler et al. (1.3%) and Donaghue et al. (1.8%). Our results showed that the MLPA method is successful in detecting subtelomeric chromosomal abnormalities that cannot be identified by conventional cytogenetic methods and our results seem to support the results of Diego-Alvarez et al., Bruno et al. and Ahn et al. [32,33,40]. In this study, the identified subtelomeric abnormality is a duplication of GAS8 gene localized in 16q subtelomeric region, and it is interesting that the same subtelomeric abnormality has been observed in two miscarriage samples (6.6%). GAS8 is a microtubule-binding protein localized to regions of dynein regulation in mammalian cells (http://omim.org). Ahn et al. [40] identified a case with subtelomeric duplication in MLPA 16q probe region, and they expressed that this abnormality is caused to developmental delay, cleft palate and hearing loss. Ahn et al.'s study results seem to support our results. Besides the results associated with MLPA method, we think that the of rate and characteristic of the subtelomeric abnormality determined in our study is remarkable.

Conclusion

We conclude that culture failure of miscarriage samples can be evaluated with combined QF-PCR and MLPA methods. Also, the evaluation of miscarriage samples with QF-PCR method excluded the factor that maternal contamination causes false-negative and false-positive results and provided a significant contribution for determining the karyotype of miscarriage samples. Our results showed that the MLPA method is successful in detecting subtelomeric chromosomal abnormalities that cannot be identified with conventional cytogenetic methods. We think that rate and characteristic of the subtelomeric CNV determined in our study are remarkable. Additionally, we recommend that the miscarriage samples of the couples with recurrent miscarriage and without any cytogenetic abnormality should be analyzed in terms of subtelomeric abnormalities after the exclusion of other clinical reasons.

Declaration of interest

The authors declare no conflict of interest. This study was supported by Ondokuz Mayis University Research Foundation (PYO.TIP.10043).

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