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Double trisomy in spontaneous miscarriages: cytogenetic and molecular approach

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BACKGROUND: Although single trisomy is the most common chromosomal abnormality observed within first trimester spontaneous abortions (SA) (>50%), double trisomy (DT) ranges from 0.21 to 2.8% in the literature. Since little is known about mechanisms underlying DT, we report the results of our experience with 517 SA, establishing parental origin and cell stage of non-disjunction when possible in DT cases, and making a revision of those previously reported. METHODS: Cytogenetic analysis was performed in all aborted specimens. Quantitative fluorescent PCR (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) were performed in DT cases in order to assess parental origin and stage of error of aneuploidy in addition to its reliability in detecting aneuploidies. RESULTS: Karyotyping was successful in 321 miscarriages; the rate of DT was 2.18%. Among the seven DT cases reported, three new combinations were found. Maternal origin was established for all DT SA analysed. Meiotic stage of error was presumed meiosis I (MI) for 48,XX+15+22 and 48,XX+8+21, meiosis II (MII) for 48,XXX+18, and MII and MI respectively for 48,XY+18+22. Molecular results agreed with cytogenetic results. CONCLUSIONS: Similar maternal age-related mechanisms could be implicated in both single and double trisomy. Molecular techniques could be useful in diagnosing not only single but multiple aneuploidy and determining its origin. This will improve our knowledge about mechanisms underlying human aneuploidy, and enable appropiate genetic counselling.

Key words: aneuploidy/double trisomy/MLPA/QF-PCR/spontaneous miscarriage

Introduction

Although single trisomy is the most common chromosomal abnormality observed within first trimester spontaneous miscarriages (>50%) (Hassold *et al*., 1980; Nagaishi *et al*., 2004), double trisomy ranges from 0.21 to 2.8% among karyotyped spontaneous abortions (SA) in the literature (Carr, 1967; Creasy *et al*., 1976; Lauritsen, 1976; Takahara *et al*., 1977; Kajii *et al*., 1980; Hassold *et al*., 1980; Lin *et al*., 1985; Eiben *et al*., 1990; Zhou, 1990; Ohno *et al*., 1991; Warburton *et al*., 1991; Dejmek *et al*., 1992; Gardo and Bajnoczky, 1992; Kalousek *et al*., 1993; Reddy, 1997; Li *et al*., 2005). Around 65% of all conceptions and 10% of all clinically recognized pregnancies end in fetal loss (Miller *et al*., 1980; Warburton *et al*., 1980; Santalo *et al*., 1987). This suggests that frequency of double trisomy might be higher than the observed one. Mean gestational age has been described to be significantly lower for double trisomy cases than that reported for single trisomy ones (Reddy, 1997). However, some cases of double trisomies involving chromosomes 8, 13, 18, 21, X and Y have been observed in liveborns, suggesting that lethality of the abnormality depends on which chromosomes are involved in the aneuploidy.

Several chromosome-specific patterns for missegregation have been studied, such as those for autosomal trisomies involving chromosomes 8, 13–16, 18, 21 and 22 (Robinson *et al*., 1993; Zaragoza *et al*., 1994; Nicolaidis and Petersen, 1998). Despite the fact that maternal meiosis I non-disjunction seems to be the major cause of the whole single trisomy cases, chromosome-specific patterns do exist and a possible mitotic origin must also be considered.

Since little is known about biological mechanisms underlying double trisomy, we report the results of our experience with a collection of 517 spontaneous abortions (SA), establishing the parental origin and cell stage of non-disjunction when possible in double trisomy cases. Three new combinations are reported and new approaches to the screening of single and multiple aneuploidy are proposed. Even though karyotyping is still the preferred technique for the study of miscarriages, molecular techniques are beginning to appear as reliable methods in the diagnosis of numerical chromosome anomalies, which comprise the vast majority of chromosomal causes of fetal loss. As far as we know, only 178 complete double trisomy cases including this survey have been reported so far among miscarriages and liveborns. Moreover, only one study

concerning the origin of the additional chromosomes in double trisomy SA has been published to date (Li *et al*., 2005).

Materials and methods

Patients and biological samples

A total of 517 miscarriage cases have been collected so far. Products of conception were provided to the Genetics Service of Fundación Jiménez Díaz (Madrid, Spain) since 1996 by the Obstetrics and Gynecology Service of the hospital and some private clinics to perform cytogenetic studies. Since may 2005, the entire curettage products after surgical interventions performed in our hospital have been processed in both genetic and anatomopathological studies (a total of 43 different serial samples). Miscarriage samples consisted of chorionic villi, embryo and/or placental tissue obtained after surgical evacuation between weeks 4 and 24 of gestation. To avoid maternal blood contamination, repeated washing of the sample in a saline solution (NaCl 0.9%) was performed prior to culturing. Selected material was examined to exclude maternal decidua remains and then cultivated in 3 ml of BIO-AMF-1 basal medium plus supplement (Biological Industries, Israel) containing HEPES 1 mol/l, penicillin–streptomycin and G-glutamine 200 mmol/l (Gibco Invitrogen, CA, USA) following standard protocols. Metaphase spread slides were G-banded using trypsin–Giemsa for karyotyping. From 2003 onwards, a piece from the majority of the specimens was stored frozen at –20°C for DNA extraction in order to perform molecular studies as described in detail elsewhere (Diego-Alvarez *et al*., 2005). Briefly, after dissection of 100–150 mg of tissue and chemical digestion with proteinase K at 56°C during 48–72 h, DNA was isolated with the Tissue Kit reagents and the BioRobot EZ1 (QUIAGEN, Hilden, Germany) following supplied protocols. Although DNA could be obtained from the majority of specimens to perform molecular studies, only abortions in which karyotype was available were included in this study.

Conceptional history of the seven couples implicated in the pregnancy losses was obtained so that genetic counselling could then be offered in most cases. All studied subjects were from Caucasian unrelated couples and five out of the seven couples had had at least one previous miscarriage (details are given in Table I). No information about the previous fetal losses except for the time and gestational age at which they occurred was available. After signed informed consent, blood samples were collected from both parents in order to determine the parental and cell stage origin of trisomies. DNA samples from four couples were extracted from 5 ml of peripheral blood in tubes with EDTA and the DNA blood 350 µl extraction Kit for BioRobot EZ1 (QIAGEN).

The present study has been performed according to the 1964 'Declaration of Helsinki' (World Medical Organization, 1996).

Double trisomy studies

Conventional GTG-banded karyotype was performed in all the miscarriage samples in order to detect a possible chromosomal anomaly. A minimum of 10 metaphases were routinely analysed per specimen. When abnormal metaphases were found, a few more metaphases were counted before concluding a cytogenetic result.

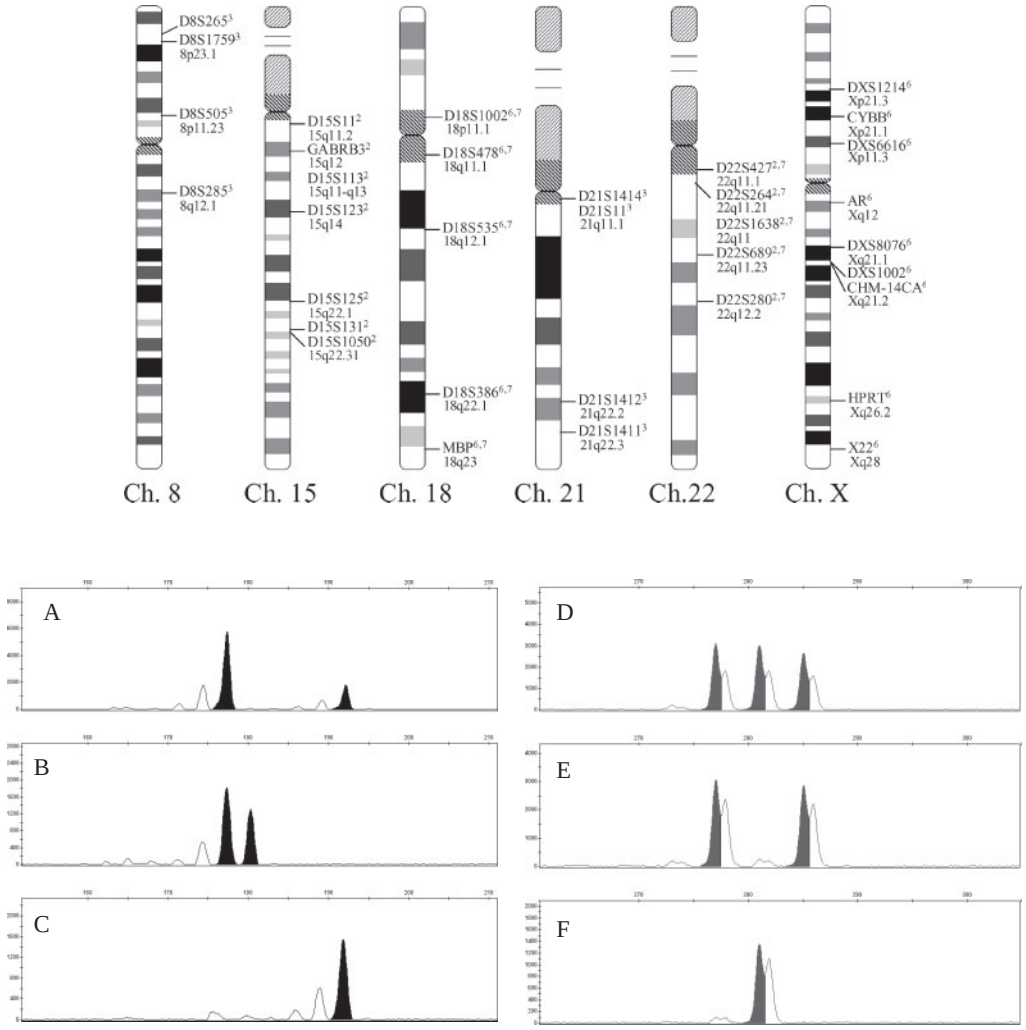
In order to confirm cytogenetic results and verify the reliability of molecular techniques to detect numerical chromosome anomalies, quantitative fluorescent (QF) PCR and multiplex ligation-dependent probe amplification (MLPA) with subtelomeric probes were performed as blinded assays in those cases in which DNA was available.

Trisomy can be detected by amplifying highly polymorphic regions of the DNA specific for a chromosome such as short tandem repeats (STR) by QF-PCR (Adinolfi *et al*., 1997). Fluorescence-labelled amplification products are shown as different peaks or alleles varying in intensity (peak area) and size (in base pairs) after capillary electrophoresis in a Genetic Analyzer. As the amount of DNA produced is proportional to the quantity of the initial target, trisomy can be presented either in a triallelic form with relative peak area doses near to 1:1:1 or as a diallelic form (2:1) (Figure 1). An average of five highly polymorphic STR markers specific for each chromosome involved in the aneuploidy were analysed by QF-PCR in both parental and miscarriage DNA samples when possible. Parental origin and cell stage of error of the aneuploidy can be inferred by comparing the inherited alleles and their relative doses. Pericentromeric markers were preferentially employed. Although pericentromeric markers are in some cases by themselves inefficient for determining meiotic stage of error, the efficiency can be considerably increased if parental origin is known with certainty (Chakravarti, 1989). Parental origin was established when at least two different markers studied for each chromosome were informative (Robinson *et al*., 1999). Meiotic origin of the aneuploidy was ensured when three distinct alleles were amplified in the abortion DNA sample. The meiotic division error could be inferred as meiosis I (MI) or meiosis II (MII) on the basis of non-reduction/reduction to homozygosity at the pericentromeric STR markers (Figure 1) (Chakravarti and Slaugenhaupt, 1987). On the other hand, a mitotic (post-zygotic) non-disjunction origin of trisomy is presumed when a trisomic diallelic pattern exists for chromosome-specific STR markers and the lack of observed recombination along the entire chromosome. Nonetheless, definitive proof of a somatic origin is impossible to establish because recombination events can cause large regions of a chromosome to be reduced to homozygosity even in

Table I. Chromosome constitution and origin of aneuploidy in double trisomy abortions

Case no.	Karyotype	Gestational age (weeks)	Pregnancy history	Parental origin	Stage of error of aneuploidy	Maternal age (years)	Paternal age (years)
1	48,XX,+9+21	10	G6/P2/A4	ND	ND	45	36
2	48,XX,+15+22	7	G3/P2/A1	Maternal	MI	37	39
3	48,XX,+8+21	8	G4/P2/A2	Maternal	MI	40	49
4	48,XY,+2+8	9	G3/P1/A2	ND	ND	36	35
5	48,XX,+20+22	11	G1/P0/A1	ND	ND	40	42
6	48,XXX,+18	13	G3/P1/A2	Maternal	MII	37	43
7	48,XY,+18+22	8	G4/P2/A2	Maternal	MI (22)/MII (18)	43	60
Mean ± *SD*		9.4 ± 2.1				39.7 ± 3.4	43.4 ± 8.7

G = gravida; P = para; A = abortion; ND = not determined; MI = meiosis I; MII = meiosis II.



Case 2	MI	Case 3	MI	Case 6	MII	Case 7*	MII/MI
D15S11	NI	D8S265	NR	D18S1002	R	D18S1002	R
GABRB3	NR	D8S1759	NR	D18S478	NI	D18S478	NI
D15S113	NR	D8S505	NR	D18S535	R	D18S535	NR
D15S123	NI	D8S285	NR	D18S386	NR	D18S386	NR
D15S125	NI	D21S1414	NR	MBP	R	MBP	NR
D15S131	NR	D21S11	NR	DXS1214	R	D22S427	NR
D15S1050	NR	D21S1412	NR	CYBB	R	D22S264	NI
D22S427	NR	D21S1411	NR	DXS6616	R	D22S1638	NR
D22S264	NR			AR	R	D22S689	NR
D22S1638	NR			DXS8076	R	D22S280	NI
D22S689	NI			DXS1002	R		
D22S280	NR			CHM-14CA	R		
				HPRT	NR		
				X22	NR		

Figure 1. Determination of parental origin and cell stage of error of aneuploidy based on non-reduction/reduction to homozygosity of microsatellite markers by QF-PCR. Ideograms show chromosomal location of STR markers analysed in double trisomy miscarriages (primer information available at www.gdb.org). Superscript type indicates the case number in which a specific microsatellite marker has been employed. Electropherograms illustrate reduction (*left*) and non-reduction (*right*) to homozygosity of two different STR markers (AR and HPRT respectively) in case 6. *A, D*: abortion; *B, E*: mother; *C, F*: father; *NI*: not informative; *R*: reduction; *NR*: non-reduction. Trisomy is evidenced by trisomic diallelic pattern (2:1) for AR and trisomic triallelic pattern (1:1:1) for HPRT in abortion DNA sample. By comparing inherited alleles from both parents, maternal origin in the first meiotic division (MI) is considered for the additional chromosome X in the miscarriage.
*As reduction to homozygosity occurs for D18S1002 (the most pericentromeric marker) in case 7, second meiotic division error is presumed for trisomy 18, although posterior recombination events may have lead to non-reduction to homozygosity of informative markers studied.

a meiotic error (Robinson *et al.*, 1999). STR markers were separately PCR-amplified as previously described (Diego-Alvarez *et al*., 2005). Briefly, PCR assays were carried out in a total volume of 15 µl containing 40–100 µg of genomic DNA, 125 µmol/l dNTP (Invitrogen Corporation, USA), 10 pmol of each primer, 1×Taq polymerase buffer (15 mmol/l $MgCl_2$) and 0.6 IU of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). After denaturation at 95°C for 12 min, hot-start PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems) for 10 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and 15 cycles at 89°C for 30 s, 55°C for 30 s and 72°C for 90 s, with a final extension time of 30 min at 72°C. One microlitre of fluorescence-labelled PCR product was mixed with 12 µl of deionized formamide and 0.5 µl of 400HD Size Standard (Applied Biosystems). Mixed samples were electrophoresed in an ABI Prism 3100 Genetic Analyzer and analysed with the GeneMapper 3.5 software package (Applied Biosystems).

Recently, a new molecular technique based on the amplification of specific probes previously hybridized onto target DNA sequences was described by Schouten *et al.* (2002). MLPA permits the relative quantification of up to 48 different DNA sequences in a single reaction requiring only 20 ng of sample DNA. Each probe consists of two oligonucleotides that hybridize to adjacent sites of the target sequence in the DNA. Once hybridized probe oligonucleotides are ligated, amplification by PCR with a unique primer pair (all ligated probes contain identical end sequences) is performed. As the amount of specific probes that are amplified depends on the quantity of target sequences present in the DNA, it allows the relative quantification of such sequences, enabling the detection of copy number changes such as deletions, duplications or whole chromosome aneuploidies. We have currently applied the MLPA SALSA-P070 Probe-mix (MRC-Holland, Amsterdam, The Netherlands) in miscarriage samples, which contains specific probes for subtelomeres of both arms for every chromosome, in order to detect such numerical anomalies. Trisomy may be suspected if subtelomeric probes for both arms of a chromosome appear duplicated. Duplication or deletion of a targeted sequence will be respectively shown as an ~1.5-fold increase or 0.5 decrease in relative peak area of the amplified fragment compared with that of a control sample after normalization of data by dividing each probe's signal strength by the average signal strength of the sample, and then by dividing this normalized peak pattern by the average peak pattern of all the samples in the same experiment. Although it can be relatively easy to detect duplications or deletions of targeted regions by analysing electropherograms if few samples are simultaneously shown, data should be normalized before finalizing conclusions. MLPA reactions were performed as recommended by the manufacturer's protocol with minor modifications. Briefly, 100–150 ng of abortion genomic DNA were diluted in 5 µl of distilled water and denatured at 98°C for 5 min. After lowering the temperature to 25°C, 1.5 µl of SALSA Probe-Mix and 1.5 µl of Probe-Mix Buffer were added to the eluted DNA sample. After denaturation at 95°C for 1 min, hybridization of probes was achieved by incubation at 60°C for 16 h. Thirty-two microlitres of Ligase-Mix containing 25 µl of distilled water, 3 µl of Buffer A, 3 µl of Buffer B and 1 µl of Ligase-65 were added at 54°C. After mixing by repeated pipetting, ligation of hybridized probes was performed at 54°C for 15 min. After heating at 98°C for 5 min, samples were stored at 4°C until amplification reaction was performed. PCR was carried out in a total volume of 25 µl containing 5 µl of ligation reaction product, 2 µl of PCR buffer, 13 µl of distilled water and 5 µl of Polymerase Mix (1 µl of primers, 1 µl of enzyme dilution Buffer, 2.75 µl of distilled water and 0.25 µl of Polymerase). Polymerase Mix was added when the sample was at 60°C. PCR reaction was performed for 35 cycles at 95°C for 30 s, 60°C at 30 s and 72°C for 60 s, with a final extension of 20 min at 72°C. All the reagents are provided in kit form by MRC-Holland. One microlitre of sample was mixed with 12 µl of deionized formamide and 0.5 µl of 500 LIZ Size Standard, electrophoresed in an ABI Prism 3100 Genetic Analyzer and analysed with the GeneMapper 3.5 software package (Applied Biosystems).

Results

Karyotyping was attempted on 517 miscarriages, being successful in 321 of them. The rest (196) either failed to grow in culture or were infected. One hundred and twenty-nine out of 321 karyotypes (40.2%) were chromosomally abnormal. Complete single trisomy accounted for 61.24% of abnormal karyotypes, this frequency being similar to that from previous reports (Hassold and Jacobs, 1984; Robinson *et al*., 2001; Nagaishi *et al*., 2004). Seven out of 321 (2.18%) SA studied carried a double trisomy. Table I lists karyotype of the double trisomy abortions, gestational age in weeks, parental ages at the time of miscarriage, pregnancy history of the patient after having the double trisomy abortion and origin of trisomies when determined. Three new double trisomy combinations were found in this study (48,XX+9+21, 48,XY+2+8 and 48,XX+20+22). Mean gestational age was 9.4 ± 2.1 weeks, ranging from 7 to 13 weeks of gestation. Mean maternal and paternal ages were 39.7 ± 3.4 and 43.4 ± 8.7 respectively. Although slightly increased, mean maternal age for double trisomy cases (39.7 ± 3.4) was not significantly different ($P = 0.076$) from that for single trisomy cases in our cohort of patients (36.6 ± 4.3; $n = 66$). Mosaicism and triple aneuploidy could be discarded in the seven cases as a large number of metaphases could be analysed. Parental karyotypes were only studied for case 6, resulting in apparently normal 46,XX and 46,XY respectively, at a resolution level of 550 bands.

Material for DNA extraction was not available for cases 1, 4 and 5. Cases 2, 3, 6 and 7 could be studied by molecular techniques [additional Figures of multiplex QF-PCR for cases 2 and 6 have been published elsewhere (Diego-Alvarez *et al*., 2005), available upon request]. Parental DNA was also available for those cases, so QF-PCR with STR markers was performed in order to determine both parental and cell stage origin of the additional chromosomes involved in the double aneuploidy by comparing the inherited alleles and their relative doses. Maternal origin could be established in cases 2, 3, 6 and 7, MI being inferred as the meiotic stage of error for cases 2 and 3, and MII for case 6. In case 7, MI was presumed for trisomy 22 and MII for trisomy 18 (Figure 1). Mitotic origin could be discarded for the trisomies as a triallelic pattern was present for at least one marker of the studied chromosomes.

MLPA with the SALSA-P070 Probe-mix was performed in cases 2, 3, 6 and 7 (Figure 2). Each trisomy was detected as a ≥1.3-fold increase in relative peak area of both of the amplified probes (one for each chromosomal subtelomere) compared with those of the control sample. No false negative results were obtained, being in accordance with cytogenetic results.

Discussion

We report seven cases of double trisomy among 321 karyotyped SA (2.18%). The reported frequency for double trisomy cases

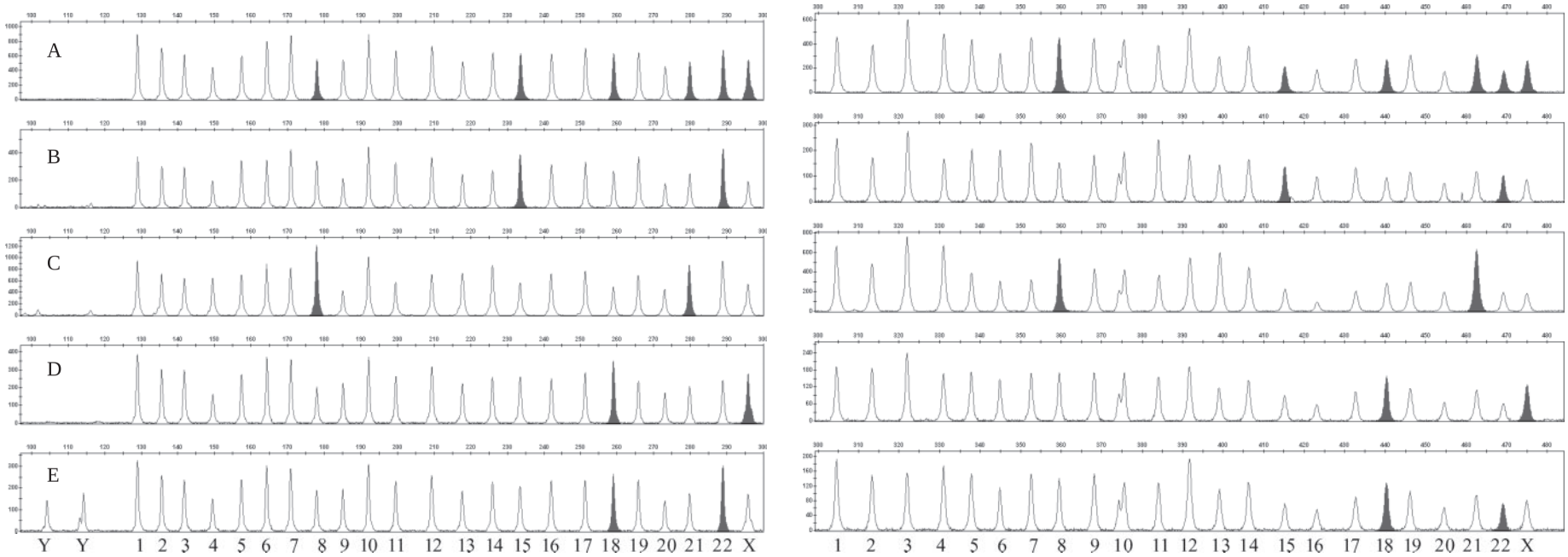


Figure 2. Detection of double trisomies by multiplex ligation-dependent probe amplification (SALSA-P070 Probe-mix). Electropherograms show amplified probes specific for subtelomeric regions of both arms (q, *left*; p, *right*) of each chromosome indicated below (probe numbers, detected genes and chromosomal positions available at www.mrc-holland.com). The *x*-axis shows length of PCR products in base pairs as determined by the use of internal lane standard; *y*-axis shows the fluorescence intensity in arbitrary units. Normal female control (46,XX) (**A**), case 2 (**B**), case 3 (**C**), case 6 (**D**) and case 7 (**E**). Shadowed peaks indicate chromosomes involved in the double trisomy. Trisomy is shown as a ≥1.3-fold increase in relative peak area of the amplified probe compared with the control samples (numerical data not shown).

ranges from 0.21 to 2.8% in studies not restricted to first trimester abortions. Our frequency is therefore within the reported range in other studies. A survey performed by Guerneri *et al.* (1987) of 202 first trimester-only loss villus samples found double trisomy in 5.8% of the cases. In the present study, gestational age of karyotyped miscarriages ranged from 4 to 24 weeks. The mean gestational age for double trisomy cases was 9.4 ± 2.1. The pooled mean based on other studies is 8.7 ± 2.2 (Carr, 1967; Lauritsen, 1976; Creasy *et al*., 1976; Takahara *et al*., 1977; Kajii *et al*., 1980; Hassold *et al*., 1980; Lin *et al*., 1985; Eiben *et al*., 1990; Zhou, 1990; Ohno *et al*., 1991; Warburton *et al*., 1991; Dejmek *et al*., 1992; Gardo and Bajnoczky, 1992; Kalousek *et al*., 1993; Reddy, 1997). The slight increase on the mean gestational age observed in this study might be due to the low number of miscarriages included. It has also been proven that double trisomies are aborted significantly earlier than single trisomies, excluding those that go to term (Reddy, 1997). If it is considered that ~65% of all conceptions and 10% of clinically recognized pregnancies end in fetal loss (Miller *et al*., 1980; Warburton *et al*., 1980; Santalo *et al*., 1987), this would suggest that the expected frequency of double trisomy among very early SA might be higher than the observed one, as demonstrated by Guerneri *et al.* (1987). Moreover, some authors have suggested that double aneuploidy might be more frequent than expected by multiplying the individual frequencies (Mikkelsen *et al.*, 1976), contrary to what is expected by chance. This possibly increased frequency might be explained by an individual tendency to non-disjunction.

Trisomy for every chromosome has been observed in aborted embryos, either constitutional, confined to placental tissues or in a mosaic state in the embryo. The most frequently reported are 13–16, 18, 21 and 22 ones. No double trisomies involving chromosomes 1, 3 and 19 have been reported (see Table II) and only tetrasomy for chromosome 21 has been observed. Acrocentric chromosomes appear frequently in multiple cases with a particular double trisomy and also partner a wide range of chromosomes (Reddy, 1997). Several combinations do appear more frequently than others, such as those

Table II. Chromosomal combinations in the reported double trisomies[a] (modified from Reddy, 1997)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X/Y
1																							
2																							
3																							
4																							
5		1																					
6																							
7		1		1																			
8		1			2																		
9		1						1															
10							1																
11																							
12							1																
13				1				1															
14				2				1															
15								1					1										
16		6			1	2		3		1		1	3	1	5								
17																							
18		3											1			1							
19																							
20								1					1		1	2	2						
21		1				1		2	1		1		3	2	3	6		6			2		
22		1					1	2	1				1	1	2	4		2		1	4		
X/Y				1			2	3	1				6			1		31			34		
#	0	15	0	5	4	3	7	18	5	2	1	2	18	7	13	37	2	44	0	8	66	20	79

Total number of cases: 178. The total number of cases with double trisomy is 178 (number of reports of each combination is given in the grid). *x*- and *y*-axes represent chromosome number.

[a]Lehmann and Forssman (1960); Uchida *et al.* (1962); Carr (1967); Lauritsen *et al.* (1972); Creasy *et al.* (1976); Takahara *et al*. (1977); Hassold *et al*. (1980); Kajii *et al*. (1980); Byrne *et al*. (1985); Lin *et al*. (1985); Guerneri *et al*. (1987); Kalousek (1987); Eiben *et al*. (1990); Zatti (1990); Zhou (1990); Lorda-Sanchez *et al*. (1991); Ohno *et al*. (1991); Warburton *et al*. (1991); Dejmek *et al*. (1992); Gardo and Bajnoczky (1992); Kalousek *et al.* (1993); Park *et al*. (1995); Hanna *et al*. (1996); Reddy (1997); Swarna *et al*. (1998); Robinson *et al*. (2001); van Huizen *et al*. (2004); Iliopoulos *et al*. (2004); Li *et al*. (2004 (2005); Rossino and Nucaro (2005); the present study. Additional considered cases appear and are referenced in the review by Li *et al*. (2004).

#: number of times that each chromosome appears in double trisomy (sex chromosomes X and Y have not been computed separately).

involving chromosomes 2 and 16, 16 and 21, 18 and 21 (1.7% each) and those found in liveborns involving a 13, 18 or 21 autosome and a sexual chromosome (1.7%, 9.0% and 9.9% respectively). This would suggest that non-viability of conceptions carrying a double trisomy would depend on which chromosomes are involved in the aneuploidy, either because of a more or less severe gene dosage imbalance or due to epigenetic factors such as genomic imprinting (differential expression of genes depending on parental origin).

Morphological examination of double trisomic specimens tends to reveal empty sacs (Reddy, 1997) but occasionally some of these develop into embryos with localized defects (Creasy *et al*., 1976; Takahara *et al*., 1977; Byrne *et al*., 1985; Kalousek *et al*., 1993). Cases 1–5 of our study were accompanied by no detail of their morphology. Case 6 was an anembryonic gestation. Case 7 (48,XXX+18) was diagnosed as hydrops fetalis. Unfortunately, no more clinical manifestations were studied in this case.

Abnormal chromosome complements are found in ~20% of unfertilized oocytes and in 2–5% of spermatocytes (Martin *et al*., 1991). Despite maternal MI non-disjunction seeming to be the major cause of the whole single trisomy cases, chromosome-specific patterns do exist and a possible mitotic origin should also be considered. While most non-mosaic single trisomies involving any of the acrocentric chromosomes (13–15, 21 and 22) and chromosome 16 can be attributed to maternal MI errors (Hassold *et al*., 1996; Koehler *et al*., 1996), trisomy 18 is mostly associated with those occurring in maternal MII (MacDonald *et al*., 1994; Fisher *et al*., 1995). Among maternal origin cases, only 5–15% of trisomy 15, 18 and 21 cases seem to be of mitotic origin. Complete trisomy 8 of meiotic origin has been found only in SA and does not seem compatible with a continuing pregnancy. In contrast, a mitotic origin for trisomy 8 has been documented in one complete trisomic liveborn (James and Jacobs, 1996) and in 12 of 13 mosaic liveborn cases (DeBrasi *et al*., 1995; Robinson *et al*., 1995; James and Jacobs, 1996; Seghezzi *et al*., 1996). Paternal errors of meiotic origin are relatively rare and comprise ~10% of the total meiotic errors involving acrocentric chromosomes (Robinson *et al*., 1999). Moreover, paternal meiotic errors have never been observed for trisomy 16 (Hassold *et al*., 1995) and only rarely for trisomy 18 (Eggermann *et al*., 1996; Bugge *et al*., 1998). Our results are consistent with previous findings for those cases in which origin of the additional chromosomes could be determined. Maternal MI errors are presumed for cases 2 (48,XX+15+22) and 3 (48,XX+8+21), while maternal MII seems to be responsible for case 6 (48,XXX+18). Regarding case 7 (48,XY+18+22) and according to molecular results (Figure 1), a maternal error is responsible for double trisomy, but non-disjunction of homologues (MI) might have occurred for chromosome 22, and non-disjunction of chromatids (MII) could have occurred for chromosome 18. This case shows that two non-disjunction events involving two different chromosomes could occur in different cell divisions forming the gametes, following the same chromosome-specific patterns for missegregation described for single trisomy. These results suggest that a common maternal age-related mechanism could be implicated in both single and double trisomy.

Advanced maternal age remains the ultimate demonstrated risk factor for trisomic pregnancies (Hassold and Chiu, 1985). It has also been demonstrated that maternal age in double trisomy cases is significantly higher than that for single trisomy cases (Reddy, 1997; Li *et al*., 2005). This may mean that errors in meiosis leading to multiple aneuploidy are more prone to occur in oocytes as women age. In fact, the relatively high frequency of double trisomy cases in this study (2.18%) may be a consequence in part of the advanced age of our cohort of patients bearing a double trisomic pregnancy [39.7 ± 3.4 compared to the pooled values of 34.1 ± 5.7 in Reddy's (1997) study]. The fact that maternal age for double trisomy cases is not significantly different from that for single trisomy cases in our study ($P = 0.076$) might be due to the reduced size of the sample.

Prognosis for patients after having a trisomic miscarriage is favourable; nevertheless, the risk of trisomic pregnancies with advanced maternal age should be considered (Warburton *et al*., 1987). In contrast, a poor prognosis is found in patients having had an euploid miscarriage, suggesting that alternative causes of miscarriage may exist, such as variants in proteins affecting DNA methylation or meiotic segregation (Robinson *et al*., 2001), gonadal mosaicism for a chromosome abnormality or balanced translocations (Sugiura-Ogasawara *et al*., 2004; Lorda-Sanchez *et al*., 2005). In our study, five out of the seven couples had had at least one previous miscarriage before the double trisomy miscarriage, but unfortunately, no information about the previous fetal losses was available. Information about pregnancies after the double trisomic miscarriage could be obtained in cases 2 and 5. In case 2, a pregnancy loss at 8 weeks of gestation 1 year after the double trisomic miscarriage was followed by the delivery of a healthy newborn 2 years later. In case 5, one pregnancy ended in a healthy child 1 year later and two more fetal losses occurred 3 and 4 years later respectively after that. Although no conclusions can be drawn in this respect, because no karyotype was available, fetal losses could be theoretically attributable to the increased risk of having a trisomic conceptus owing to the advanced maternal age (>37 years in both cases). Parental karyotypes were only available for case 6, which were normal at a resolution level of 550 bands. Nevertheless, a similar counselling could be offered to those couples having a single trisomic conception.

Cytogenetic study of miscarriages entails a high rate of culture failure because of yeast or bacterial infection or due to the poor growth of macerated specimens in culture. Moreover, when maternal cell contamination is present in the sample it often leads to wrong normal female karyotypes (Bell *et al*., 1999). The main advantage of molecular techniques is that DNA is usually easy to obtain from products of conception rather than the need for live cells. Comparative genomic hybridization (CGH) has been demonstrated to detect numerical chromosome imbalances even in paraffin-embedded miscarriage tissues (Bell *et al*., 2001; Fritz *et al*., 2001; Tabet *et al*., 2001), and moreover at a resolution level of 10–100 kb (Schaeffer *et al*., 2004). QF-PCR ensures the fetal origin of the collected tissue and also permits determination of the parental and cell stage origin of the aneuploidy, studying the inherited chromosomes by comparing parental and fetal DNA STR markers. A recent study conducted by Benkhalifa *et al.* (2005)

has been performed on 26 SA that failed to grow in culture. Array CGH revealed chromosomal abnormalities uncommonly seen by classic cytogenetics, including high rates of double aneuploidy and autosomal monosomy. They suggest that conventional cytogenetics may yield normal karyotypes or only those abnormal karyotypes that permit cell proliferation *in vitro*. Regarding the MLPA technique, it has been proven to detect the most common aneuploidies on amniotic fluid cells and chrorionic villi in prenatal diagnosis (Slater *et al*., 2003; Gerdes *et al*., 2004), but no reports of its application in SA have been published until now. The main advantage of this technique is that aneuploidy for every chromosome can be screened in a single experiment requiring only 20 ng of genomic DNA. Moreover, its costs are relatively low compared to the high and still unaffordable costs of array CGH for the majority of diagnostic laboratories. This makes the technique ideal for the detection of single and multiple aneuploidy among very early SA in which very little material is obtained or in those specimens which failed to grow in culture. In our study, MLPA results agree with those obtained by karyotyping and QF-PCR. Nonetheless, reliability of this technique for the detection of numerical chromosome imbalances in spontaneous miscarriages will be further evaluated elsewhere (Diego-Alvarez D *et al*., manuscript in preparation).

Results in our study suggest that a common maternal age-related mechanism could be implicated in both single and double trisomy cases, and that meiotic errors could cause similar chromosome-specific patterns for missegregation that occur in single trisomy. Considering the fact that different prognoses have been found in patients after having a trisomic or an euploid abortion, we recommend performing both cytogenetic and molecular studies even in the case of the first spontaneous abortion because of the importance at that time of offering appropriate genetic counselling. Moreover, determination of parental origin and stage of error of the aneuploidy by molecular techniques will be helpful not only for this purpose, but also to better understand the aetiology and biological mechanisms underlying human aneuploidy.

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