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Identification of copy number variants in miscarriages from couples with idiopathic recurrent pregnancy loss

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BACKGROUND: Recurrent pregnancy loss (RPL), defined as two or more miscarriages, affects 3–5% of couples trying to establish a family. Despite extensive evaluation, no factor is identified in ~40% of cases. In this study, we investigated the possibility that submicroscopic chromosomal changes, not detectable by conventional cytogenetic analysis, exist in miscarriages with normal karyotypes (46,XY or 46,XX) from couples with idiopathic RPL.

METHODS: Array comparative genomic hybridization (array-CGH) was used to assess for DNA copy number variants (CNVs) in 26 miscarriages with normal karyotypes. Parental array-CGH analysis was performed to determine if miscarriage CNVs were *de novo* or inherited.

RESULTS: There were 11 unique (previously not described) CNVs, all inherited, identified in 13 miscarriages from 8 couples. The maternal origin of two CNVs was of interest as they involved the imprinted genes *TIMP2* and *CTNNA3*, which are only normally expressed from the maternal copy in the placenta. Two additional cohorts, consisting of 282 women with recurrent miscarriage (RM) and 61 fertile women, were screened for these two CNVs using a Quantitative Multiplex Fluorescent PCR of Short Fragments assay. One woman with RM, but none of the fertile women, carried the *CTNNA3*-associated CNV.

CONCLUSIONS: This preliminary study shows that array-CGH is useful for detecting CNVs in cases of RPL. Further investigations of CNVs, particularly those involving genes that are imprinted in placenta, in women with RPL could be worthwhile.

Key words: recurrent pregnancy loss / copy number variants (CNVs) / miscarriage / *TIMP2* / *CTNNA3*

Introduction

Miscarriage is a common outcome of pregnancy, especially with advancing maternal age, primarily because of an increasing frequency of trisomy (Hassold and Chiu, 1985). Conventional cytogenetic analysis has been used for decades to identify miscarriages which occurred due to numeric chromosome errors, such as trisomy, monosomy or polyploidy, or due to unbalanced structural chromosome rearrangements. Approximately 50–70% of miscarriages have such lethal chromosome errors (Ohno *et al.*, 1991; Jacobs and Hassold, 1995).

Recurrent pregnancy loss (RPL), defined as two or more miscarriages, affects 3–5% of couples trying to establish a family (Stephenson and Kutteh, 2007). Classic factors associated with RPL include parental chromosome translocations, and uterine, endocrine and autoimmune factors (Stephenson, 1996). Despite extensive evaluation, ~40% of couples with a history of RPL have no identifiable factors, therefore, they are classified as having idiopathic RPL (Stephenson and Kutteh, 2007).

The possibility that miscarriages are due to lethal submicroscopic chromosomal changes has been raised (Philipp *et al.*, 2003). With the advancement of new DNA technologies, the evaluation of the whole genome at resolutions much higher than with conventional cytogenetic analysis is possible (Bejjani and Shaffer, 2008). Array comparative genomic hybridization (array-CGH) is an example of a

genome-screening method now widely used clinically for identification of chromosomal microdeletions and microduplications in human disorders, such as intellectual disability (Sagoo *et al.*, 2009). These submicroscopic chromosomal changes are also termed DNA copy number variants (CNVs) and can be detected in both clinically normal and abnormal populations. Pathogenic CNVs tend to be differentiated from benign CNVs based on their *de novo* origin, larger size and presence of gene(s) that have been associated with disease.

The application of array-CGH to study CNVs in miscarriages has been limited to ~500 miscarriages worldwide (Schaeffer *et al.*, 2004; Benkhalifa *et al.*, 2005; Shimokawa *et al.*, 2006; Menten *et al.*, 2009; Robberecht *et al.*, 2009; Zhang *et al.*, 2009; Rajcan-Separovic *et al.*, 2010). These initial studies indicate that small chromosomal changes exist in 1–13% of miscarriages. However, the origin of the chromosomal changes, either *de novo* or inherited, has not been addressed, and the size of the CNVs or their gene content remains largely unknown because the majority of the miscarriages have been studied with targeted and low resolution (1 Mb) whole genome arrays. In addition, detailed obstetrical histories and pathology findings usually have not been provided. One small study using higher resolution arrays reported that the unique CNVs detected in sporadic miscarriages were all <0.25 Mb and predominantly inherited, although ~10% of the CNVs were *de novo* CNVs (Rajcan-Separovic *et al.*, 2010).

The present study was initiated to evaluate CNVs, using high-resolution array-CGH, in miscarriages with normal karyotypes from couples with a history of idiopathic RPL. Our goal was to identify candidate genes within the CNVs which could be causative for the specific miscarriage and, perhaps for the RPL.

Materials and Methods

Case ascertainment

There were 23 couples recruited based on the following criteria: (i) a history of idiopathic RPL, based on a negative evaluation, as previously described (Stephenson, 1996) and (ii) at least one miscarriage with a normal karyotype (46,XY or 46,XX). All couples were evaluated by the author (M.D.S.) in the University of Chicago Recurrent Pregnancy Loss Program. Institutional review board approval was obtained from both the University of Chicago and University of British Columbia. All couples gave written consent.

Twelve fertile males and females, with stored anonymized DNA samples, served as array-CGH controls. In addition, anonymized DNA samples from 282 women with a history of recurrent miscarriage (RM) (≥3 consecutive miscarriages) and 61 fertile women with no history of infertility, who delivered after the age of 37 years, were used for molecular screening of array-CGH-detected CNVs. The RM and fertile cohorts used for molecular screening are described in prior publications (Beever *et al.*, 2003; Hanna *et al.*, 2009).

Chromosome testing of miscarriages

The miscarriage tissue was separated from the maternal decidua, cleaned with preservative-free saline and sent for cytogenetic analysis in the Department of Pathology (Cytogenetics) at the University of Chicago. If the miscarriage yielded a 46,XX result, DNA from the cultured cells was extracted and sent for microsatellite analysis. If maternal cell contamination was ruled out, the 46,XX miscarriage specimen was included. In addition, DNA was extracted from paraffin blocks from prior miscarriages of <20 weeks gestation, with known normal karyotype results.

Whole genome array CGH analysis

Genomic DNA was extracted from parental peripheral blood using PUREGENE DNA Isolation Kits (Gentra, Minneapolis, MN, USA). Genomic DNA from miscarriage cultured cells or tissues was obtained using standard protocols for extracting DNA from cultured cells, cryopreserved or paraffin-fixed tissues (Qiagen, Valencia, CA, USA). Diploid male and female reference DNA (Promega, Madison, WI, USA) was used as controls.

The DNA of miscarriages in 3/23 recruited couples was of insufficient quality or quantity for array-CGH testing. Thus, array studies of miscarriages were only completed for 20/23 couples (Table I). Only one miscarriage with a normal karyotype was initially studied per couple. When a unique CNV was detected, additional miscarriage specimens from the same couple, if available, were studied. An additional six miscarriages with normal karyotypes and one trisomic miscarriage were included, giving a total of 27 miscarriages. Also, when a unique miscarriage CNV was found, parental array-CGH was performed. A total of 22 partners, 14 female and 8 male, were studied.

Agilent 105 K oligonucleotide array-CGH was performed, according to the protocol provided by the company (version 4.0, June 2006, Agilent Technologies, CA, USA) and reported by Fan *et al.* (2007). Feature Extraction software (version 9.5.1, Agilent Technologies) rendered image analysis annotated against build NCBI 36 (human genome assembly UCSC hg18, March 2006). CNV selection was done in CGH Analytics (version 3.5.14, Agilent Technologies), using the ADM-2 algorithm (cutoff 6.0), followed by a filter to select regions with three or more adjacent probes and a minimum average log2 ratio ± 0.25 (Fan *et al.*, 2007).

CNVs identified in miscarriages and/or partners were classified as either common or unique CNVs. Common CNVs were defined as those overlapping with >50% of their length with CNVs detected in controls from at least two independent studies catalogued in the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation). The unique CNVs were defined as those not present or partially overlapping (<50% of length) with CNVs in the controls from DGV.

Follow up of whole genome array-CGH results

Custom CGH arrays were designed using eArray (Agilent technologies) as described previously (Rajcan-Separovic *et al.*, 2010). The ADM-2 algorithm was used to confirm the parental origin and breakpoints for two smaller CNVs (5q12 and 14q13) which showed ambiguous results on whole genome array in miscarriages from families 5 and 12. In addition, a custom array was designed for two larger X-linked CNVs from Xp22.2 and Xp22.31 from Families 9 and 12 to determine if there were breakpoint differences between the miscarriage and the carrier parent. Agilent-optimized probes were selected from the *H. sapiens* (UCSC hg18) probe set and searches were done using the standard high density probe search for user defined genomic intervals.

Fluorescent *in situ* hybridization (FISH) was performed using DNA probes from the CNVs detected in the miscarriages and couples. They were labeled by nick translation and hybridized to interphase/metaphase cells following previously published protocols (Rajcan-Separovic *et al.*, 2007).

X chromosome inactivation was performed by assaying methylation at the Androgen Receptor *(AR)* (Allen *et al.*, 1992) or *FMR-1* (Hecimovic *et al.*, 1997) locus using a PCR-based protocol as detailed previously (Beever *et al.*, 2003). X chromosome inactivation was expressed in terms of the percent inactivation of the smaller *AR* allele.

Table I Demographics and obstetrical outcomes (n = 20 couples).

Couple	Ethnicity	Obstetrical history	Subsequent pregnancy outcomes
1	Cauc	Misc <10 weeks (31 years); Misc <10 weeks (32 years); Anemb misc (32 years); **1-3A Emb misc 46,XX** (32 years)	Healthy term female 3799 gm (33 years)
2	Cauc	Biochem misc (27 years); Anemb misc (30 years); Emb misc 46,XX (31 years); Biochem misc (31 years); Biochem misc (33 years)	**2-3A Emb misc 46,XX (34 years)**; Biochem misc (35 years); Anemb misc (37 years)
3	Cauc	Preterm male 1928 gm (20 years); Neonatal female demise (26 years); Termination (27 years); Emb misc (28 years); Misc <10 weeks (28 years); Misc <10 weeks (31 years)	Fetal demise (32 years); **3-3A Emb misc 46,XY (33 years)**; Anemb misc 92,XXYY (34 years)
4	Cauc	Emb misc (27 years); **4-3A Emb misc 46,XY (28 years)**	**4-3B Emb misc 46,XY (29 years)**; On-going pregnancy, EDC 9/24/10 (30 years)
5	Cauc	Emb misc (37 years); Emb misc (37 years); Emb misc (38 years); Biochem misc (39 years)	**5-3A Emb misc 46,XY (39 years)**; Term male, 3062 gm (41 years)
6*	Cauc	Fetal demise (34 years) *multiple placental infarcts*; Fetal demise 46,XX (35 years) *multiple placental infarcts*; Fetal Demise (35 years); Fetal demise 46,XY (36 years) *decidual infartcs 50%*	**6-3D Yolk sac misc 47,XX,+16 (37 years)**; Biochem misc (37 years); **6-3A Emb misc 46,XX (37 years)** ***marked perivillous fibrin deposition involving >90% of villi*****; 6-3B Emb misc 46,XY (38 years)** ***marked perivillous fibrin involving 80% of villi*****; 6-3C Emb misc 46,XX (38 years)** ***extensive perivillous fibrin with villous fibrosis*****; 6-3E Emb misc 46,XY (39 years)** ***multifocal perivillous fibrin with villous fibrosis***
7	Cauc	Anemb misc (29 years); Anemb misc (30 years); Biochem misc (30 years); Anemb misc (31 years); Anemb misc (31 years); Anemb misc (32 years); Emb misc (32 years); Biochem misc (32 years); Yolk sac misc (33 years); Emb misc (33 years); Twins: anemb and yolk sac misc (33 years)	**7-3A Emb misc 46,XX (34 years)**; Gestational surrogacy term twins 3345, 3487 gm, both 46,XY (35 years)
8	AA	Misc <6 weeks (35 years); Emb misc (41 years); Yolk sac misc (41 years); Emb misc (41 years)	**8-3A Anemb misc 46,XY (42 years)**; Term female 3033 gm (43 years)
9	Cauc	Misc <6 weeks (30 years); Emb misc 46,XY (31 years); Misc <6 weeks (31 years); Emb misc 46,XY (32 years)	**9-3A Emb misc 46,XY (33 years); 9-3B Emb misc 46,XX (33 years)**
10*	Cauc	Term male 2807 gm (31 years); Fetal Demise 46,XY (32 years) *focal fibrinoid necrosis of decidual arterioles*; Fetal Demise 46,XY (34 years) *acute inflammation of villi +/− decidual necrosis*; Emb misc (34 years)	**10-3A Fetal demise 46,XX (35 years)** ***uneven accelerated maturation or size of villi***
11	Cauc	Misc <6 weeks (37 years); Emb misc 48,XX, +13, +16 (38 years)	**11-3A Emb misc 46,XX (39 years)**; Term male, 4445 gm (40 years)
12	Cauc	**12-3A Emb misc: 46,XX (37 years**); Biochem misc (38 years); Biochem misc (38 years)	**12-3B Emb misc: 46,XY (39 years)**; ectopic (40 years)
13	Cauc	Termination (30 years); Biochem misc (34 years); Biochem misc (34 years)	**13-3A Emb misc 46,XX (34 years)**
14	AA	Misc <10 weeks (20 years); Misc <10 weeks (22 years); Yolk sac misc (28 years); **14-3A Emb misc 46,XY (33 years)**	
15	AA	Emb misc (27 years); **15-3A Emb misc 46,XX (28 years)**	Term male 3115 gm (29 years)
16	Cauc	Emb misc 46,XY (27 years): Emb misc 46,XX (27 years); Emb misc (28 years); Emb misc 46,XY (28 years); Emb misc 46,XX (29 years)	Biochem misc (33 years); **16-3A Emb misc 46,XY (33 years)**
17	Cauc	Biochem misc (29 years); Anemb misc 46,XX (29 years)	**17-3A Yolk sac misc 46,XY (30 years)**
18	Cauc	Term male 4224 gm (31 years); **18-3A Fetal demise 46,XY (35 years)**; Fetal demise 46,XX (36 years)	Emb misc 47,XY, +14 (36 years); Fetal demise 46,XX (37 years)
19	Cauc	Emb misc 47,XX,+6 (34 years); Biochem misc (34 years); Biochem misc (34 years)	**19-3A Emb misc 46,XX (35 years)**; On-going pregnancy, EDC 09/05/10 (36 years)
20	Cauc	Term male 3289 gm (31 years); **20-3A Emb misc 46,XX (33 years)**; Biochem misc (33 years)	Term male, 3810 gm (33 years)

Bolded miscarriages were studied by array-CGH; the specimen number is provided. Biochem misc: decreasing hCGs <1500 mIU/ml; Anemb misc: anembryonic, empty gestational sac. Yolk sac misc: gestational sac with yolk sac only; Emb misc: embryonic, crown rump length between 5 and 33 mm without cardiac activity; Misc <6 or <10 weeks: positive hCG, no ultrasound performed, passage of tissue at <6 or 10 weeks; Fetal demise: crown rump length ≥33 mm without cardiac activity; EDC, estimated date of confinement; Cauc, caucasian; AA, African American.

*Recurrent placental pathology.

Table II CNVs identified in miscarriages and parents.

Samples	Family	Specimen number and karyotype	Locus	Breakpoints	Size (Kb)	Type of CNV and origin	Gene involvement	Additional studies
Miscarriages	3	03-3A (46,XY)	6q26	162 126 633–162 271 770	145	Loss-U-pat	*PARK2*: loss involves part of exon and intron	na
	4	04-3B (46,XY)	16q23.1	75 252 974–75 407 549	155	Loss-U-mat	no genes; *CNTNAP4* is 30kb from CNV	Deletion confirmed in female carrier using FISH probe RP11-96P7
	5	5-3A (46,XY)	5q12.1	60 407 026–60 464 658	58	Loss-U-pat	*NDUFA12L* loss involves exon 3	Custom array determined CNV size: 37 635 in miscarriage (37 416 in parent)
	6	06-3A (46,XX) 06-3B (46,XY) 06-3C (46,XX) 06-3D (47,XX,+16)	17q25.3	74 381 287–74 466 887	86	Gain-U-mat Gain-U-mat Gain-U-mat Gain-U-mat	*TIMP2*: gain involves exon 1, 2	QMPSF confirmed *TIMP2* duplication in mother and miscarriages, screening of females with RM and controls did not detect additional cases with this CNV
		06-3E (46,XY)	15q22.1	56 487 120–56 562 873	76	Loss-U-pat	*LIPC*: loss of exon 1	na
	7	7-3A (46,XX)	10q21.3	67 992 425–68 064 617	72	Loss-C-mat	*CTNNA3*: loss involves exon 11	FISH confirmed maternal deletion (RP11-206F24); deletion also confirmed by QMPSF; screening of females with RM and controls identified the same CNV in one additional RM case
	9	09-3B (46,XX)	Xp22.2	13 415 099–13 745 233	330	Gain-U-mat	*GPM6B* gain of first 6 exons, *RAB9A, TRPPC2, OFD1, GPM6B* complete gain	FISH confirmed Xp22.2 gain on maternal chromosome (probe RP11-150F14); random X inactivation was demonstrated for 9-3B; X inactivation test not informative for carrier female partner 9-1; custom array determined the size of the CNV in miscarriage 367 281 bp (367 281 bp in carrier parent)
	10	10-3A (46,XX)	5q23.3	129 388 119–129 441 487	53	Loss-U-pat	*CSS3*: loss involves intron	na
			11p15.1	20 442 396–20 559 837	117	Gain-U-pat	*PRMT3*; gain involves exon 9-11	na
			7p14.1	39 470 588–39 647 671	177	Gain-U-mat	*RALA* gain involves first exon; *POU6F*-gain involves last exon, *C7orf36* is completely gained	na
	12	12-3B (46,XY)	Xp22.31	6 498 521–8 091 951	1593	Gain-C-mat; CNV not seen in 12-3A	*HDHD1A; STS;VCX;PNPLA4* all genes show complete gain	Custom array determined CNV size: 1 626 570 in 12-3B (1 633 749 in parent);real-time qPCR confirmed *GST2 (PNPLA4)* duplication in female partner and miscarriage; X inactivation in carrier female partner showed 84% skewing
		12-3B (46,XY) 12-3A (46,XX)	14q13.1	32 706 548–32 733 815	27	Loss-U-pat Loss-U-pat	*NPAS3* loss involves intron	Custom array determined CNV size: 62 259 in 12-3A; 61 949 in 12-3B (62 259 in carrier parent)
	14	14-3A (46,XY)	19q13.41	57 668 023–58 150 321	482	Gain-U-pat	Zink Finger family of genes, complete gain of most genes, break in *ZNF518* and *ZNF816A*	na

Parents	1	1-1 (46,XX)	6p21.31	34 842 144–35 010 514	168	Gain in parent only-U-unknown	*UHRF1BP1*-partial gain of 3 exons; *ANKS1A*-partial gain of exon 1; complete gain of *SNRPC* and *TAF1 1*	na
	10	10-1 (46,XX)	14q12	29 081 313–29 178 022	96	Gain in parent only-U-unknown	*PRKD1*; gain involves 12 exons	na
	14	14-1 (46,XX)	12 q24.33	130 060 706–130 430 847	370	Gain in parent only-U-unknown	*GPR133* gain involves 11 exons; *LOC116437* gain involves 6 exons	na

U, unique CNV; C, common CNV which could be a potential risk factor for miscarriage; pat, patemal; mat, maternal.

Real-time quantitative PCR (RT-qPCR) was used for confirmation of the Xp22.31 duplication and was performed as described previously (Tyson *et al*., 2005). The test probe was designed within exon 1 of the *GS2* gene. The primers/probe sequence for *GS2*_Ex1 are as follows: *GS2* ex1 forward probe AAAATGAGGATTATTAAAGGTCAGTTGCT, *GS2* ex1 reverse probe GGCGTTGTAATCCTTACGTGTCTT, and *GS2* exon1 MGB probe (VIC) AACGCTACTTGGCCCTT. The control *FAM* primer and probe set, TaqMan® Gene Expression Assay Hs99999170_s1 (*GJA5*, *CX40*), was ordered from Applied Biosystems.

A quantitative multiplex PCR of short fluorescent fragments (QMPSF) assay was designed to assess the copy number status of two candidate genes, specifically, *TIMP2* and *CTNNA3*, which showed partial gain and loss, respectively, by array-CGH. The screening assay was performed on the anonymized stored DNA from the 282 RM and 61 fertile controls, as described above. Four genes *AMELX, BBS9*, *PRMT3* and *CAV1* were used as normal copy number control genes. QMPSF assay was designed as reported in the literature (Charbonnier *et al*., 2000). Primer sequences for tested and control regions (two sites per region) included in the assay are detailed in the Supplementary data, Table S1. Gene deletion or duplication was considered when a 35–50% variation of the relative peak area of both gene-specific sequences compared with those from a control pool was obtained after normalization. Intra-assay reproducibility of the QMPSF assay was assessed by amplifying duplicated samples (mean correlation $R^2 = 0.98$; $n = 10$).

The DGV (http://projects.tcag.ca/variation/) was used to assess the presence of CNVs in controls; http://www.geneimprint.org/site/genes-by-species and http://igc.otago.ac.nz/home.html were used to determine the gene imprinting status (if known) and BioGPS (http://biogps.gnf.org) was consulted for gene expression status in human cell lines.

Results

A total of 20 couples with idiopathic RPL were included in this study, as shown in Table I. Seventeen of the couples were Caucasian and three were African-American. The cohort had a total of 118 pregnancies, consisting of 46 preclinical miscarriages (<6 weeks), 44 embryonic miscarriages, 11 fetal demise (≥10 weeks), 10 term and 1 preterm (a set of twins) deliveries, 1 neonatal death, 1 ectopic and 2 elective terminations. In addition, there were two ongoing pregnancies. The mean number of miscarriages <20 weeks of gestation per couple was 5 (range 2–12). The study cohort is described in more details in Supplementary data, Table S3.

Array-CGH analysis was performed on 27 miscarriages and 22 partners from 20 couples with RPL. It revealed unique and common CNVs described in Table II and Supplementary data, Table S2.

Miscarriage CNVs

A total of 11 unique, previously unreported, CNVs were identified in 13 miscarriages from 8 couples and all were inherited from one of the partners (Table II). Thus, there were no *de novo* unique CNVs identified. These unique inherited CNVs mapped to one X-linked and 10 autosomal sites and included multiple genes as described in Supplementary data, Table S2. Two additional common CNVs (duplication of Xp22.31 and deletion of 10q21.3) were classified in our study as a potential risk factors for miscarriage (Table II, Supplementary data, Table S2).

The two CNVs from Xp22.2 and Xp22.31, were transmitted from the female partners in Families 9 and 12 to miscarriages 9-3B (female)

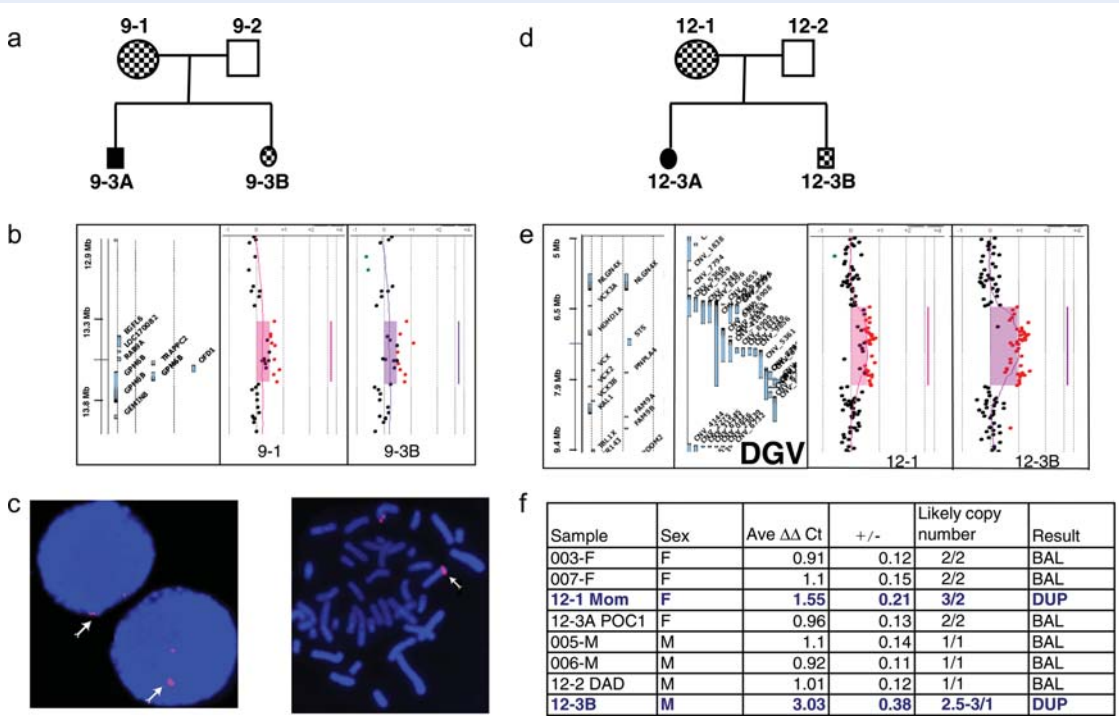
and 12-3B (male), as shown in Fig. 1. X chromosome inactivation was random (57%) in miscarriage 9-3B; testing for female partner 9-1 was non-informative. X chromosome inactivation was moderately skewed (84%) in female partner 12-1. Custom array analysis of the two X-linked CNVs (Xp22.2 and Xp22.31) in the miscarriages and corresponding carriers showed the CNVs were comparable in size (Table II).

Two unique autosomal CNVs were found in multiple miscarriages from two couples. One CNV involved a gain in exons 1 and 2 of the tissue inhibitor of metalloproteinases-2 (*TIMP2*) gene which has a role in placenta development (see Discussion). This CNV was detected in four of the five tested miscarriages from Family 6 (four miscarriages with normal karyotypes and one with trisomy 16), as well as in the female partner (Fig. 2). The other CNV involved the intron of the basic Helix-Loop-Helix gene, *NPAS3* (Supplementary data, Table S2), from 14q13.1, and was transmitted from the male partner to both miscarriages in Family 12. Custom array analysis of this CNV in the male partner (12-2) and the two miscarriages (12-3A and 12-3B) showed that the CNVs were comparable in size (Table II). In addition to the unique CNVs, there were 66 common CNVs detected in our cohort of miscarriages, which are observed in apparently healthy adults from at least two studies reported in the DGV.

Parental CNVs

The array-CGH screening identified 3 unique parental CNVs that were not transmitted to the miscarriages (Table II), 13 CNVs transmitted to miscarriages from one of the parents (as described above) and 57 common CNVs in our cohort of 22 partners with idiopathic RPL studied by array-CGH. No potential RPL candidate genes of interest were identified in the three non-transmitted unique parental CNVs (Supplementary data, Table S2).

As the obstetric history for controls in the DGV is generally unknown, we evaluated the genes from common CNVs, detected in RPL couples but not fertile controls, in respect to their role in uterine function, placenta development, hormonal or immune response in pregnancy. A literature review identified one common CNV from the 10q21.3 region which included the *CTNNA3*



f

Sample	Sex	Ave ΔΔ Ct	+/-	Likely copy number	Result
003-F	F	0.91	0.12	2/2	BAL
007-F	F	1.1	0.15	2/2	BAL
12-1 Mom	**F**	**1.55**	**0.21**	**3/2**	**DUP**
12-3A POC1	F	0.96	0.13	2/2	BAL
005-M	M	1.1	0.14	1/1	BAL
006-M	M	0.92	0.11	1/1	BAL
12-2 DAD	M	1.01	0.12	1/1	BAL
12-3B	**M**	**3.03**	**0.38**	**2.5-3/1**	**DUP**

Figure 1 Array detected chromosome X duplications in Family 9 (Xp22.2) and 12 (Xp22.31) and confirmation by FISH and RT-qPCR. (**a**) Subjects from Family 9 with RPL studied by array CGH. In 9-1, the skewed X inactivation test was not informative, in 9-3B there was no skewing (57%). (**b**) Whole genome array profile in female partner 9-1 and her miscarriage 9-3B showing comparable gain of Xp22.2. CNVs completely overlapping with the Xp22.2 gain were not noted in DGV. (**c**) FISH confirmation of the Xp22.2 duplication in 9-1. Arrow points to the duplicated chromosome in metaphase and interphase cells. (**d**) Subjects from Family 12 with RPL studied by array CGH. X inactivation ratio of 84% was detected for female 12-1. (**e**) Whole genome array profile in 12-1 and her miscarriage 12-3B showing comparable gain of Xp22.31. One CNV from DGV overlapped completely with Xp22.31 gain, the other CNV overlaps were partial. (**f**) RT-qPCR confirmation of a gain of *GS2* gene within Xp22.31 duplication in 12-1 female partner and her miscarriage 12-3B. *GS2* was compared with an endogenous two copy control gene, connexin 40 and normalization based on the appropriate sex-matched controls. The final copy number result is based on an average of the copy numbers from two controls and shows a confirmation of the Xp22.31 gain in 12-1 female partner and her miscarriage 12-3B. Checkered square represents male carrier of CNV and checkered circles represent female carriers of CNV. 003-F and 007-F control females; 005-M and 006-M control males. Higher resolution image of Figure 1 is provided in the Supplementary data.

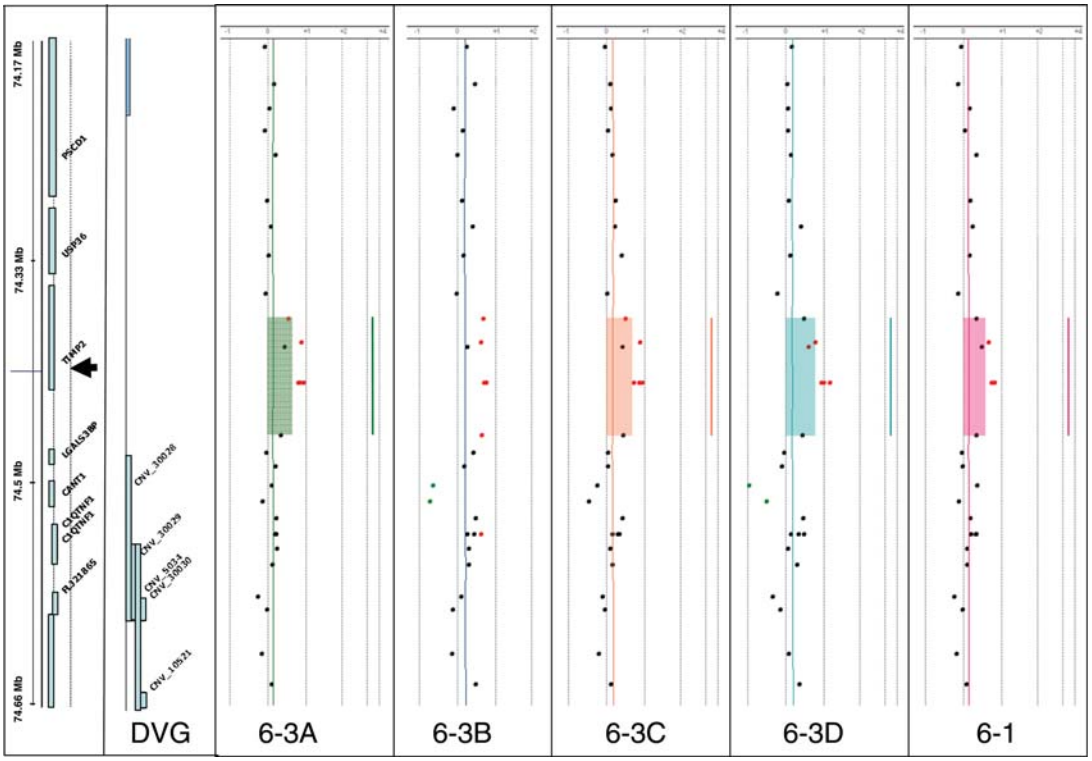


Figure 2 Array detected chromosome 17q25.3 duplication in female partner 6-1 and her 4 pregnancies (6-3A, 6-3B, 6-3C, 6-3D). This CNV disrupts *TIMP2* gene (arrowhead). No CNVs overlapping with this duplication have been reported in the DGV. In 6-3B, the shift of probes on the profile indicates a gain, although the array algorithm did not mark the gain of *TIMP2*.

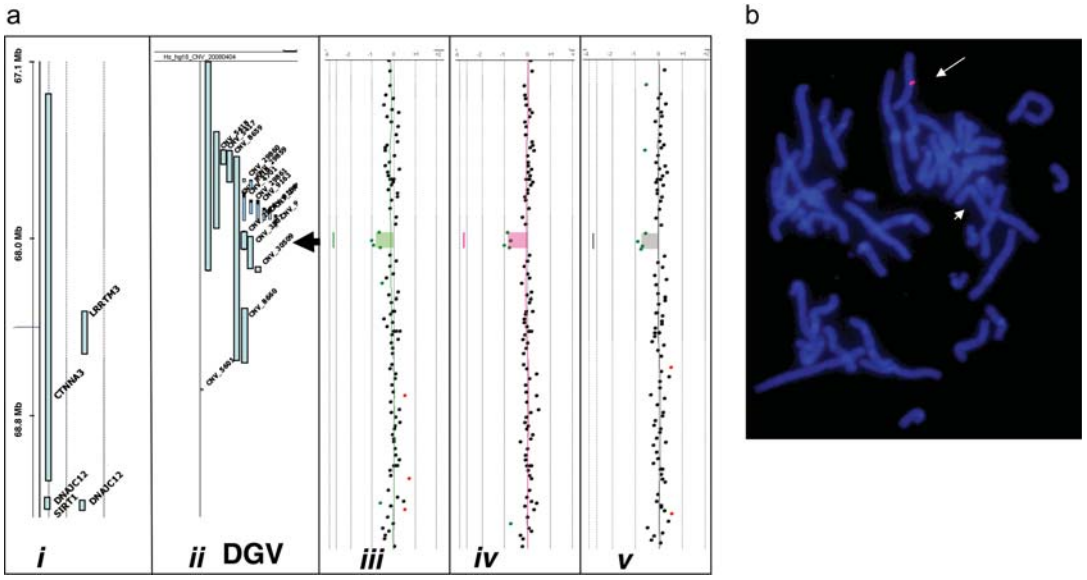


Figure 3 (**a**) Array detected chromosome 10q21.3 deletion: (i) the *CTNNA3* gene region and (ii) DGV view of the *CTNNA3* gene region. Arrowhead indicates overlap of >2 CNVs reported in DGV with the 10q21.3 deletion detected in female partner 7-1 (iii) and her miscarriage 7-3A (iv). In (v), the array profile shows the same *CTNNA3* deletion in unrelated female with RM (RM-77) identified by QMPSF screening. (**b**) Confirmation of *CTNNA3* gene deletion by FISH in female carrier 7-1: only one signal is visible for *CTNNA3* containing FISH probe (arrow). Deleted chromosome 10 is marked with an arrowhead. Higher resolution image of Figure 3 is provided in the Supplementary data.

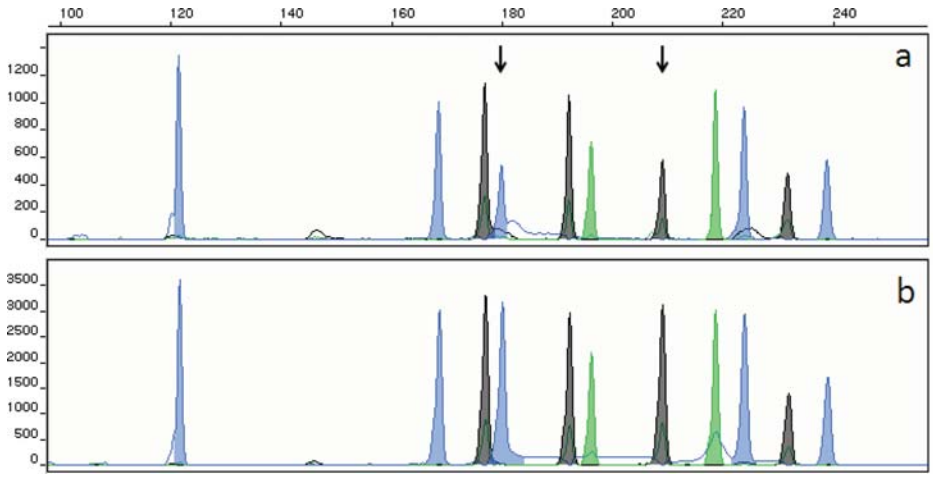


Figure 4 Deletion of *CTNNA3* gene identified by QMPSF screening of women with RPL. Electropherograms show amplification products of the QMPSF assay in (**a**) a RM sample (RM-77) and (**b**) a control. The *x*-axis shows length of PCR products in base pairs as determined by the use of an internal lane standard; the *y*-axis shows the fluorescence intensity in arbitrary units. Peaks correspond to the following set of primers indicated in increasing length order: *AMELX*, *TIMP2*_a, *PRMT3*_a, *CTNNA3*_a, *PRMT3*_b, *TIMP2*_b, *CTNNA3*_b, *BBS9*_a, *BBS9*_b, *CAV1*_a, *CAV1*_b. Arrows indicate the deletion pattern for two *CTNNA3* gene PCR products in RM-77 female (a) compared with the control (b).

(α-catenin) gene, which could be a potential miscarriage gene (see discussion). It was identified in the female partner and the corresponding miscarriage in Family 7, as shown in Fig. 3. Using publicly available information on known or suspected imprinted genes and regions, as specified in the methods section, we did not find that any of the genes in the other common CNVs show evidence of imprinting.

Additional follow-up studies

Screening for CNVs containing the candidate miscarriage genes *CTNNA3* and *TIMP2*, using QMPSF in the RM and fertile controls identified one additional female (RM-77) who had a deletion of *CTNNA3* gene, as shown in Fig. 4. This finding was confirmed using whole genome array-CGH (Fig. 3); the size of the unique CNV containing *CTNNA3* in RM-77 was exactly the same as in the female 7-1. *TIMP2* CNVs were not found in either of the cohorts screened by QMPSF.

Discussion

We applied array CGH to study miscarriages from a clinically well defined cohort of couples with RPL. The selection of RPL couples with at least one miscarriage with a normal karyotype appears to have identified couples with a lower likelihood of having a successful pregnancy outcome (23% in this study) compared with the 75% successful outcome estimated by Rai *et al.* in couples with idiopathic RM (Rai and Regan, 2006).

Array-CGH studies of miscarriages with normal karyotypes have the potential to identify CNVs that could lead to developmental failure ('miscarriage' CNVs). Those CNVs that are *de novo* in miscarriage are not expected to recur, similar to autosomal trisomy leading to miscarriage. CNVs that are inherited from a carrier parent could lead to RPL if (i) the CNV contains imprinted genes (expressed from only one parent in pregnancy tissues); (ii) a gene(s) relevant for embryonic/placenta growth is present in the CNV and has a mutation in the other allele; or (iii) a gene(s) from the CNV is variably expressed (Lee *et al.*, 2007). As we limited the present study to couples with a history of RPL, it is not surprising that no *de novo* unique CNVs were identified in miscarriages with a normal karyotype. However, we did identify inherited CNVs, some of which contain genes that impact early pregnancy and thus can be considered candidate miscarriage genes.

Interesting candidate genes identified in the present study include *TIMP2* and *CTNNA3*, both of which act as inhibitors of trophoblast invasion and are expressed from only the maternal allele in the placenta (Okamoto *et al.*, 2002; Li *et al.*, 2003; Oudejans *et al.*, 2004; Seval *et al.*, 2004; van Dijk *et al.*, 2004). They have not yet been associated with RPL in humans, however, overexpression of *TIMP2* was detected in a mouse model of RPL (Dixon *et al.*, 2006). The *TIMP2* disruption in miscarriages in Family 6 may have affected the placental development, but the possibility remains that maternal disruption of *TIMP2* may contribute to RPL by impairing the remodeling of the endometrium in early pregnancy. It has been previously reported that *TIMP2* is up-regulated and highly expressed at the fetal maternal interface and in maternal decidual cells (Salamonsen and Nie, 2002; Ledgard *et al.*, 2009).

The two X-linked unique CNVs from Xp22.2 and Xp22.31 were also maternally transmitted (Fig. 1) and contain genes *EGFL6* (Xp22.2) and *STS* (Xp22.31) which show strong expression in the placenta in normal pregnancies. They also have roles in maternal reproductive tissues at the time of pregnancy, such as uterine receptivity and estrogen production, respectively (Yeung *et al.*, 1999; Bowen and Hunt, 2000; Leon *et al.*, 2008).

Further research is needed, with detailed demographics and obstetrical histories, miscarriage karyotype and pathologies, and

gene expression studies, to determine the frequency and pathophysiology of *de novo* and inherited CNVs in human sporadic and RPL. Distinguishing causative from harmless CNVs is challenging because of potential parental and pregnancy effects; it is similar to the challenges associated with interpreting the transmission of unique CNVs from an unaffected parent to a child with developmental delay. Furthermore, CNVs detected in couples with RPL should be carefully evaluated, and their presence in controls from the DGV should not be assumed as sufficient evidence of their benign nature, as the obstetric history of the controls in this database is unknown. Our preliminary results suggest that large scale, research based, mutational and CNV screening for the *TIMP2* and *CTNNA3* genes is warranted in women with RPL. Wider application of array CGH in miscarriages and couples with RPL may lead to identification of additional RPL-associated CNVs and genes, better understanding of early human development and improved management of couples with RPL.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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