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First-trimester euploid miscarriages analysed by array-CGH

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Abstract It is estimated that 10–15 % of all clinically recognised pregnancies results in a miscarriage, most of which occur during the first trimester. Large-scale chromosomal abnormalities have been found in up to 50 % of first-trimester spontaneous abortions and, for several decades, standard cytogenetic analysis has been used for their identification. Recent studies have proven that array comparative genomic hybridisation (array-CGH) is a useful tool for the detection of genome imbalances in miscarriages, showing a higher resolution, a significantly higher detection rate and overcoming problems of culture failures, maternal contamination and poor chromosome morphology. In this study, we investigated the possibility that submicroscopic chromosomal changes, not detectable by conventional cytogenetic analysis, exist in euploid miscarriages and could be causative for the spontaneous abortion. We analysed with array-CGH technology 40 foetal tissue samples derived by first-trimester miscarriages with a normal karyotype. A whole-genome microarray with a 100-Kb resolution was used for the analysis. Forty-five copy number variants (CNVs), ranging in size between 120 Kb and 4.3 Mb, were identified in 31 samples (24 gains and 21 losses). Ten samples (10/31, 32 %) have more than one CNV. Thirty-one CNVs (68 %) were defined as common CNVs and 14 were classified as unique. Six genes and five microRNAs contained within these CNVs will be discussed. This study shows that array-CGH is useful for detecting submicroscopic CNVs and identifying candidate genes which could account for euploid miscarriages.

Keywords Array comparative genomic hybridisation · Miscarriages · Copy number variants (CNVs)

C. D. Viaggi · S. Cavani · M. Malacarne · F. Floriddia · G. Zerega · C. Baldo · M. Mogni · M. Castagnetta · G. Piombo · D. A. Coviello · M. Pierluigi
S.C. Laboratorio di Genetica Umana, E.O. Ospedali Galliera, Genova, Italy

F. Camandona · D. Lijoi
S.C. Ostetricia e Ginecologia, E.O. Ospedali Galliera, Genova, Italy

W. Insegno
S.C. Ostetricia e Ginecologia, S.S. Diagnostica Prenatale, Ospedale Evangelico Internazionale, Genova, Italy

M. Traversa
S.C. Ostetricia e Ginecologia, Ospedale San Paolo, Savona, Italy

C. D. Viaggi (✉)
Laboratory of Genetics, Galliera Hospital, Via A. Volta 6, 16128 Genova, Italy
e-mail: chiara.viaggi@galliera.it

Introduction

Approximately 10–15 % of all clinically recognised pregnancies ends in a spontaneous abortion, most of which occur within the first trimester of pregnancy. Of these first-trimester miscarriages, about 50 % are due to the presence of large-scale chromosome abnormalities (Hassold et al. 1980; Jacobs and Hassold 1995): aneuploidies or polyploidies (86 %), structural rearrangements (6 %) and others (8 %) (Goddijn and Leschot 2000).

Understanding the cause for the foetal loss may be invaluable, as it may eliminate further testing and provide a better recurrence risk estimation for the couple involved. Clinical information is routinely obtained by pathology and cytogenetic analysis of the miscarriage tissues. Classical cytogenetic investigations, however, require viable tissue and up to 40 % of cases undergoes culture failure (Lomax et al. 2000). Maternal cell contamination and suboptimal quality of chromosome preparations are adjunctive limiting factors to karyotype analysis (Bell et al. 1999; Menasha et al. 2005). Moreover, the possibility that miscarriages could be due to submicroscopic chromosomal aberrations has been raised (Philipp et al. 2003; Schaeffer et al. 2004; Rajcan-Separovic

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et al. 2010a, b) and recent studies (Dória et al. 2009; Menten et al. 2009; Rajcan-Separovic et al. 2010a, b) demonstrated that array comparative genomic hybridisation (array-CGH) of DNA extracted from uncultured foetal tissue is a very useful tool to detect these submicroscopic changes not visible by routine cytogenetic analysis. Molecular karyotyping is fast, does not require tissue culture and allows the screening of the entire genome at high resolution. Submicroscopic aberrations were found in 8 % of non-euploid spontaneous abortions and 4–13 % of euploid miscarriages (Schaeffer et al. 2004; Shimokawa et al. 2006; Zhang et al. 2009). In addition array-CGH analysis of miscarriages that failed to grow in culture revealed chromosomal abnormalities in 29–58 % of cases, 6 % of which were submicroscopic (Benkhalifa et al. 2005; Zhang et al. 2009). The application of array-CGH in miscarriages has been limited to about 530 miscarriages worldwide, the majority of which were studied with targeted and low-resolution (1-Mb) arrays that failed to report the origin, the exact size of the copy number variants (CNVs) or their gene content (Schaeffer et al. 2004; Benkhalifa et al. 2005; Shimokawa et al. 2006; Menten et al. 2009; Robberecht et al. 2009; Zhang et al. 2009). These studies indicated that small CNVs were present in 1–13 % of samples. Two recent studies with high-resolution arrays (60 Kb) (Rajcan-Separovic et al. 2010a, b) reported potential pathogenic submicroscopic CNVs in 6–12 % of cases, all <0.25 Mb. Thus, the study of spontaneous abortions with normal karyotype have the potential to identify CNVs that could lead to developmental failure.

The present study was designed to identify and evaluate possible miscarriage-associated CNVs using high-resolution array-CGH in euploid spontaneous abortions occurring between the 7th and 11th weeks of gestation. Our goal was to assess the presence and prevalence of CNVs and identify possible candidate genes within the CNVs which could be involved in early embryonic development and could be causative for miscarriage.

Materials and methods

Samples

Forty first-trimester miscarriages with a normal karyotype were investigated by array-CGH using a 100-Kb resolution platform. All samples were sent to the Laboratory of Human Genetics, Galliera Hospital, Italy, for chromosome analysis. Ethical approval for this study was obtained through the Galliera Hospital Ethics Committee. All the mothers gave consent for the execution of the molecular cytogenetic investigation on the miscarriage sample. No sample from the parents could be retrieved. Specimens were generally collected by curettage. In all cases, the chorionic villi were separated from maternal deciduas and blood clots. Each tissue sample was divided into fragments: part of them was used for the routine cytogenetic test and part was frozen and stored at −80 °C for subsequent DNA extraction and array-CGH analysis. Conventional Q-banding karyotype on uncultured and cultured foetal tissue samples was performed according to standard procedures. Chorionic villi were selected for array-CGH analysis.

Array-CGH

DNA was isolated from tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Array-CGH was performed on the miscarriage DNA and the sex-matched Human Genomic DNA (Promega, Madison, WI, USA), as controls, using a Human Genome CGH Microarray Kit 8x60K (Agilent, Santa Clara, CA, USA), with a resolution of 100 Kb. Experiments were performed according to the protocol provided by the company (version 4.0, June 2006). Array slides were analysed with am Agilent G2505B Microarray Scanner. Image analyses were carried out with Feature Extraction 9.5.1 (Agilent, Santa Clara, CA, USA). The results were visualised with Genomic Workbench Standard Edition 5.0.14 (Agilent, Santa Clara, CA, USA), annotated against build NCBI 37 (human genome assembly UCSC hg19, Feb. 2009). An ADM-2 algorithm (cut-off 5.1), followed by a filter to select regions with four or more adjacent probes and a minimum average log2 ratio ±0.25, was used for the analyses. All results were confirmed by repeating the experiments.

CNVs were compared to those present in the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation), Decipher (http://decipher.sanger.ac.uk) and the Database of Human CNVs (http://gvarianti.homelinux.net/gvarianti/index.php) to differentiate between benign CNVs and CNVs that are likely to be pathogenic. Common CNVs were defined as those that overlapped completely with CNVs reported in at least a control population survey catalogued in the DGV with more than 100 subjects studied and a frequency equal to or greater than 1 %. Unique CNVs were defined as those that showed no or incomplete overlap with CNVs published in the DGV or which are reported in the DGV in surveys with less than 100 subjects studied and/or a frequency less than 1 %.

Statistical analysis

The CNVs identified in this study were compared with similar CNVs reported in phenotypically normal individuals from control population studies present in the DGV. Control populations published in this database are usually used as references to determine CNV frequencies and include healthy women and men from different ethnic origins (Caucasian, Asian and African). The prevalence of common CNVs (gains or losses) in our cases was calculated. For each of these

chromosomal regions, we compared the prevalence of CNVs (i.e. the percentage of samples with loss and the percentage of samples with gain, respectively) in our samples to the prevalence of CNVs in control populations using χ^2 or Fisher's exact test when appropriate. When more than one control study for a specific CNV was present, the median prevalence was calculated.

Results

Whole-genome array-CGH with a 100-Kb resolution was performed on 40 first-trimester euploid miscarriages (<12 weeks). Forty-five CNVs, ranging in size between 120 Kb and 4.3 Mb and corresponding to 22 different CNVs, were identified in 31 samples (31/40, 77.5 %). Nine samples showed no CNVs. The complete CNVs list, reporting size, location, gene content and functions is shown in Table 1. Nineteen CNVs ranged between 120 Kb and 200 Kb, six between 200 Kb and 500 Kb, three between 500 Kb and 1 Mb, and 17 between 1 Mb and 4.3 Mb. Forty-three CNVs were located on autosomes and two on chromosome X. Twenty-four CNVs were submicroscopic genomic gains and 21 losses. Ten samples (10/31, 32 %) had more than one CNV.

Thirty-one out of 45 CNVs (68 %), present in 24 cases (24/31, 77 %), were defined as common CNVs. Fourteen CNVs were classified as unique: seven of them overlapped only partially with CNVs published in the DGV, and seven, all gains, had never been previously reported (Table 1; 22q13.33; 7q11.22; 7p22.2; 11p11.12p11.11; 2p21; 14q24.1; 10q24.31). The common CNV in the 15q11.21 region resulted in being the most frequent (17/45, 37.8 %), followed by common CNVs in the 2q37.3 and 2p11.2 regions (4/45, 8.9 %). Four common CNVs showed no gene content (1q21.2; 2p11.2; 2q37.3; 14q11.2).

Comparing the prevalence of the CNVs identified in our samples with that found in control population studies published in the DGV, seven CNVs frequencies resulted in being statistically different from that calculated in control populations (Table 2). Among them, three were observed as gains (8q12.1; 15q11.11q11.2; Xq22.2) and four as losses (1p36.13; 2p11.2; 3q29; 13q12.11). CNVs 13q12.11, 15q11.1q11.2, 2p11.2 and 8q12.1 resulted in being more frequent in our samples than in control populations, whereas CNVs 1p36.13, 3q29 and Xq22.2 were less frequent.

Discussion

Array-CGH studies of spontaneous abortions with normal karyotype have the potential to identify CNVs that could lead to developmental failure (Rajcan-Separovic et al. 2010a). Studies reported in the literature identified numerous genes required for proper development of the placenta (Adams et al. 2000; Hemberger 2007) and the disruption of many of these genes, including growth factors, transcription factors, extra-cellular matrix proteins and proteins involved in cell signalling, leads to embryonic lethality (Cross et al. 2003). Numerous genes are up- or down-regulated in the course of differentiation and several functional genes were involved in angiogenesis, morphogenesis, organogenesis, cell-cycle control, cellular transport, growth and maintenance (Gheorghe et al. 2010). Microarray analyses have provided insights into the genetic mechanism of development, cell growth, stress response and numerous other processes (Gasa et al. 2004).

We applied array-CGH to study 40 euploid first-trimester miscarriages to assess the presence and prevalence of CNVs, and identify possible candidate genes which could be involved in early embryonic development and could be causative for miscarriage. Forty-five CNVs, ranging in size between 120 Kb and 4.3 Mb, were identified in 31 samples. Thirty-one CNVs resulted in being common, whereas 14 CNVs were classified as unique: seven overlapped only partially with CNVs published in the DGV, and seven, all gains, had never been reported previously (Table 1).

Since CNV differences in the normal population have recently gained considerable interest as a source of genetic diversity likely to play a role in functional variation (Henrichsen et al. 2009), influencing transcriptional and translational levels of overlapping or nearby genes (Perry et al. 2008), we investigated the potential biological consequences of the CNVs identified in our study, comparing their prevalence to that of similar CNVs reported in phenotypically normal individuals in the DGV. We evaluated the frequencies of both the common CNVs and the unique CNVs that overlapped partially with CNVs published in the DGV. Not being able to access parental samples, the de novo or inherited origin of the CNVs could not be assessed. However, also, CNVs that are inherited from a healthy carrier parent could potentially lead to miscarriage if: the CNV contains imprinted genes; a gene(s) relevant for embryonic/placenta growth is present in the CNV and has a mutation in the other allele; or a gene(s) in the CNV is variably expressed. As shown in Table 2, seven CNVs showed statistical differences from control populations. Among them, three were observed as gains (8q12.1; 15q11.11q11.2; Xq22.2) and four as losses (1p36.13; 2p11.2; 3q29; 13q12.11). CNVs 13q12.11, 15q11.1q11.2, 2p11.2 and 8q12.1 resulted in being more frequent in our samples than in control populations, whereas CNVs 1p36.13, 3q29 and Xq22.2 were less frequent. Studies in both human and model organisms revealed that genes in CNV regions are expressed at more variable levels than genes mapping elsewhere and, also, that CNVs not only affect the expression of genes inside them, and, thus, varying in copy number, but can also influence the transcriptome (Henrichsen et al. 2009). Both

Table 1 Copy number variants (CNVs) in the studied miscarriages. Common CNVs are highlighted in *grey*; CNVs never reported before are in **bold**

Sample ID	CNV ID	Locus	Genomic position (start/end, bp)	Type of CNV	Size (Kb)	Genes involved	Functions
1/11	U1	22q13.33	50.662.989-51.178.264	Gain	515.3	HDAC10,MAPK11, MAPK12	HDAC10 histone deacetylase 10; MAPK11-12 extracellular signalling stress-activated transduction
10/11	U2	7q11.22	70.356.011-71.708.048	Gain	1352.0	CALN1, WBSCR17, MIR3914-1	CALN1 negative regulation Golgi-to-plasma membrane trafficking; WBSCR17 N-acetylgalactosaminyltransferase; MIR3914-1 microRNA 3914-1
13/11	U3	13q12.11	20.285.281-20.405.620	Gain	120.4	PSPC1	PSPC1 androgen receptor-mediated transcriptional regulation in Sertoli
14/11	U4	7p22.2	3.347.012-4.017.883	Loss	670.9	SDK1	SDK1 Cell adesion regulation in developing neurons
	U3	13q12.11	20.285.281-20.405.620	Gain	120.4	PSPC1	PSPC1 androgen receptor-mediated transcriptional regulation in Sertoli
15/11	C1	15q11.1q11.2	20.481.759-22.432.687	Loss	1950.9	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
16/11	U5	7q11.21	64.691.936-65.070.919	Loss	378.9	ZNF92,INTS4L1	ZNF92 trascriptional regulation INTS4L1 Regulation apoptosis, neuroplasticity, citoscheleton
	C1	15q11.1q11.2	20.102.541-22.486.999	Gain		CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
19/11	C2	2p11.2	90.012.337-90.234.023	Loss	221.7		
21/11	U6	11p11.12p11.11	51.432.683-55.710.526	Gain	4277.8	TRIM48,OR, SPRYD5	OR family Olfactory receptor
	C1	15q11.1q11.2	20.102.541-22.509.254	Gain	2406.7	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
22/11	U7	2p21	45.063.376-45.186.612	Gain	123.2	SIX3	SIX3 Holoprosencephaly-2
	C2	2p11.2	90.012.337-90.234.023	Loss	221.7		
	C3	2q37.3	242.886.386-243.007.300	Loss	120.9		
23/11	U8	8q12.1	56.776.665-56.922.601	Gain	145.9	LYN	LYN Regulation of Trophoblast giant cell differentiation
	C3	2q37.3	242.886.386-243.007.300	Loss	120.9		
	C4	11p11.12	50.032.746-50.378.802	Gain	346.1		
	C1	15q11.1q11.2	20.481.702-22.509.254	Gain	2027.5	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
24/11	C1	15q11.1q11.2	20.102.541-22.509.254	Gain	2406.7	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
25/11	C5	1q21.2	149.079.747-149.224.043	Gain	144.3		
28/11	C1	15q11.1q11.2	20.575.646-22.509.254	Loss	1933.6		
30/11	C1	15q11.1q11.2	20.481.702-22.318.656	Loss	1836.9	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
34/11	U9	1p36.13	16.840.487-17.231.817	Loss	391.3	MIR3675	MIR3675 MicroRNA 3675
36/11	U10	Xq22.2	103.220.412-103.376.914	Gain	156.5	MIR1256, H2BFXP, H2BFWT, H2BFM	H2B histone family; MIR1256 microRNA 1256
37/11	C1	15q11.1q11.2	20.102.541-22.378.143	Gain	2275.6	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
38/11	C1	15q11.2	20.575.646-22.432.687	Loss	1857.0		
39/11	C2	2p11.2	90.012.337-90.234.023	Loss	221.6		
	C1	15q11.2	20.481.702-22.378.143	Gain	1896.4		
40/11	U11	14q24.1	68.466.776-68.609.006	Loss	142.2	RAD51B	RAD51B homologous recombination repair (HRR) pathway of double-stranded DNA breaks
41/11	C1	15q11.2q11.1	20.481.702-22.509.254	Gain	20207.5		
42/11	U10	Xq22.2	103.220.412-103.376.914	Gain	156.5	MIR125, H2BFXP, H2BFWT, H2BFM	H2B histone family; MIR1256 microRNA 1256
	C1	15q11.2	22.318.597-22.509.254	Gain	190.7		
44/11	C1	15q11.1q11.2	20.481.702-22.509.254	Loss	2027.5		
46/11	C6	6p21.32	32.485.173-32.604.038	Loss	118.9		
47/11	C1	15q11.1q11.2	20.481.702-22.486.999	Gain	2027.5	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
48/11	C1	15q11.1q11.2	20.481.702-22.509.254	Gian	2027.5	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
49/11	C7	3q29	195.340.844-195.459.538	Gain	118.7	MIR570	MIR570 microRNA 570
50/11	C1	15q11.2	22.318.597-22.509.254	Loss	190.7		
51/11	C5	1q21.2	149.079.747-149.224.043	Gain	144.3		
	C8	14q11.2	22.387.418-23.016.598	Gain	629.2		
52/11	U12	10q24.31	102.777.838-102.897.500	Loss	119.7		
	C1	15q11.1q11.2	20.481.702-22.509.195	Gain	2027.5	CHEK2P2	CHEK2P2 Homo sapiens checkpoint kinase 2 pseudogene 2
53/11	C2	2p11.2	90.012.337-91.234.023	Loss	122.2		
	C7	3q29	195.340.844-195.459.589	Gain	118.7	MIR570	MIR570 microRNA 570
	C6	6p21.32	32.485.173-32.604.038	Loss	118.9		

CNV code: *U* unique; *C* common

deletion and duplication can be associated with the same phenotype, suggesting that the simple presence of a structural change at a given position may disturb a certain pathway, regardless of gene dosage (Henrichsen et al. 2009). Considering this, we analysed

Table 2 CNVs identified in our samples compared to CNVs in control populations published in the Database of Genomic Variants (DGV)

	Gain			Loss		
	Samples[a]	Controls[b]	*P*-value	Samples[a]	Controls[b]	*P*-value
13q12.11				2	2/2,026	**0.0024**[c]
15q11.1q11.2	12	115/4,825	**<0.001**[c]	5	65/983	0.2029
7p22.2				1	2/776	0.1430
2p11.2				4	17/730	**0.0249**[c]
2q37.3				2	12/1,089	0.0907
8q12.1	1	2/2,026	**<0.001**[c]			
1q21.2	2	46/10,402	0.6978			
1p36.13				1	17/30	**<0.001**[c]
Xq22.2	2	1/270	**0.0484**[c]			
6p21.32				2	5/30	0.2349
3q29	2	188/270	**<0.001**[c]			
14q11.2	1	82/1,190	0.5116			

[a] Number of miscarriage samples identified with CNVs

[b] Number of subjects with CNVs in control populations

[c] $P<0.05$

the gene content of these seven statistically significant regions and that of the seven unique CNVs described for the first time and identified potential genes that could be considered as candidate 'miscarriage' genes.

Two samples (ab13/11 and ab14/11) showed the same 120.3-Kb gain at band 13q12.11 that contains gene *PSPC1* (Fig. 1a), expressed in different tissues but especially in the placenta. PSPC1 codifies for paraspeckle protein 1, a critical protein for the nuclear RNA retention, a novel mechanism of gene expression involved in many cellular processes, such as stress responses (Fox and Lamond 2010). A perturbation in the level of its expression due to the presence in the detected CNV may have affected placenta development.

The *Lyn* gene was present in the 145.9-Kb CNV gain at band 8q12.1 (ab23/11, Fig. 1b). *Lyn*, codifying for an Src-related non-receptor tyrosine kinase, is activated during trophoblast cell proliferation and differentiation, and is involved in tyrosine kinase signalling pathways. Ligand-tyrosine kinase receptor systems are established activators of the PI3-K/Akt pathway and regulate the trophoblast giant cell differentiation. Each branch of the trophoblast lineage develops specialised functions required for successful pregnancy. Disruptions in trophoblast development can lead to early pregnancy loss, intrauterine growth retardation and tumorigenesis. The Src family tyrosine kinases activate the MAPK pathway, leading to changes in transcription, cellular division and differentiation (Kamei et al. 2002). Interestingly, MAPK11 and MAPK12 genes, belonging to the MAPK family, were found in the CNV gain at region 22q13.33 (ab1/11, Fig. 1c). These

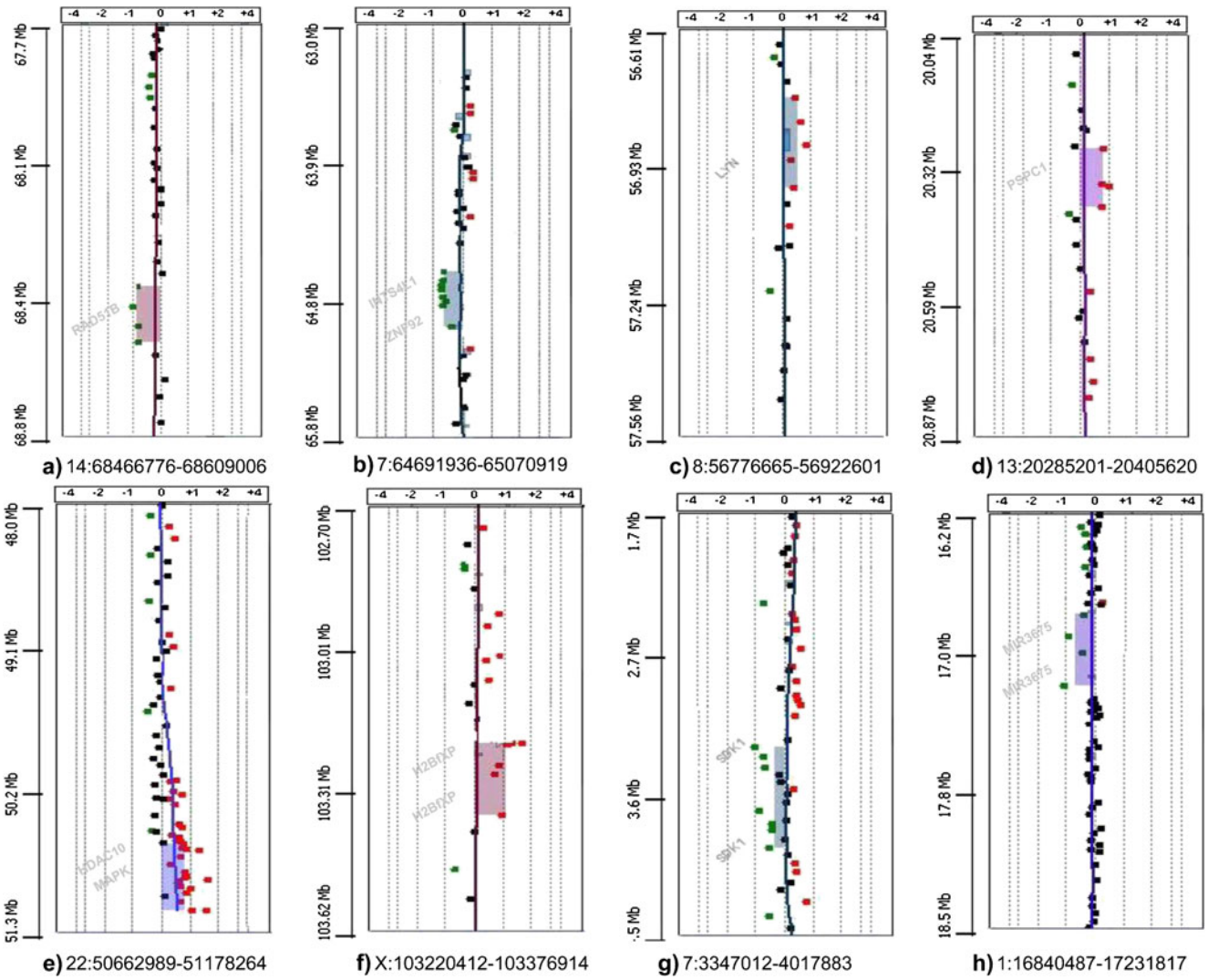

Fig. 1 **a**–**h** Array comparative genomic hybridisation (array-CGH) results (GRCh37-Feb 2009): **a** 14q24.1(68.466.776-68.609.006)x1 (ab40/11), **b** 7q11.21(64.691.936-65.070.919)x1 (ab16/11), **c** 8q12.1(56.776.665-56.922.601)x3 (23/11), **d** 13q12.11(20.285.281-20.405.620)x3 (ab13/11;ab14/11), **e** 22q13.33(50.662.989-51.178.264)x3 (ab1/11), **f** Xq22.2(103.220.412-103.376.914)x3 (ab36/11;ab42/11), **g** 7p22.2(3.347.012-4.017.883)x1 (ab14/11), **h** 1p36.13(16.840.487-17.231.817)x1 (ab34/11)

proteins, especially MAPK11, are involved in the apoptotic pathway under stress response. Another interesting gene present in the same region is *HDAC10*, a histone deacetylase. Embryonic development is based on a complex pattern of methylation/demethylation: during gastrulation, methylation levels are high and decrease during differentiation (Klose et al. 2006; Dodd et al. 2007). Enzymes, such as HDAC10, are critically associated with these epigenetic modifications and their altered expression may lead to a disruption of the epigenetic state.

Genes *H2BFXP*, *H2BFWT* and *H2BFM*, belonging to the H2B histone family, were included in the CNV at region Xq22.2 (ab36/11, ab42/11, Fig. 1d). This region also contained a microRNA, MIR1256. MicroRNAs (miRNA) have emerged as important players in DNA methylation and post-transcriptional gene regulation, since they are capable of base pairing with mRNA and can tune gene expression during development and differentiation in a sequence-specific manner (Saito et al. 2006; Lujambio et al. 2007). miRNAs are also included in CNVs at regions 7q11.22 (MIR3914-1, MIR3914-2), 1p36.13 (MIR3675) and 3q29 (MIR570).

Another interesting gene is *CHEK2P2*, contained in the 15q11.1q11.2 region, which is essential in responding to hypoxia and reoxygenation and regulates downstream targets responsible for G_2 arrest and DNA repair (Freiberg et al. 2006). Hypoxia has been identified as a major stress in development, and is believed to be a contributing cause to placental pathology (Gheorghe et al. 2010).

In conclusion, this study, performed on spontaneous miscarriages with high-resolution array-CGH, has shown the possibility to identify submicroscopic chromosome abnormalities, not detected by conventional cytogenetic analysis, in 15 % of cases. We have reported pathways and genes that may be interesting for the study of early human development and pregnancy effects. Although the role of these CNVs remain uncertain, the gene content showed an involvement in transcription, cellular trafficking, cell cycle regulation, apoptosis, DNA methylation or histone modification, and cellular differentiation.

This study, together with previous investigations, provides emerging information on CNVs and data on their potential association with human disease, emphasising the importance of assessing CNVs in spontaneous abortion. The accumulation of such data can lead to the identification of pathogenic CNVs and 'miscarriage' genes. This would be of interest in order to better understand early human development and improved management of couples with recurrent pregnancy loss.

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