Evaluation of array comparative genomic hybridization in recurrent miscarriage

Chromosomal analysis of a failed pregnancy following the diagnosis of recurrent miscarriage is not standard practice yet an abnormal karyotype is one of the commonest causes. Array comparative genomic hybridization heralds a new and improved era of cytogenetics, which may prevent unnecessary interventions for patients.

Miscarriage is the most common pregnancy complication. Approximately 15% of established pregnancies result in pregnancy loss and 1% of women experience recurrent miscarriage (more than three consecutive miscarriages) (Jauniaux et al, 2006). Miscarriage before 6 weeks' gestation has been estimated at 30–50% (Wilcox et al, 1999). Although there are recognized factors such as thrombophilia and autoimmune disorders, the majority of cases remain unexplained (idiopathic recurrent miscarriage). Within this knowledge vacuum of recurrent miscarriage aetiology, patients and clinicians sometimes feel pressurized to treat despite robust evidence exposing the futility of unproven treatment regimens in the management of idiopathic recurrent miscarriage (Clark et al, 2010; Kaandorp et al, 2010).

Chromosomal abnormalities are found in approximately 50% of sporadic first trimester losses. The majority consist of gross numerical imbalances including trisomy, monosomy and polyploidy (86%) with a small proportion containing structural chromosome anomalies (6%) or mosaic imbalances (8%) (Goddijn and Leschot, 2000). Conventional cytogenetic analysis using meta-phase chromosomes via traditional cell culture has been the gold standard test for over 50 years. Important limitations of analysing pregnancy tissue include the presence of maternal cell contamination, problems of appropriate sampling and degeneration of DNA within fetal tissue.

Complementary molecular cytogenetic techniques, such as fluorescence in situ hybridization (FISH), are frequently used but offer assessment of a limited spectrum of the total chromosomal complement. By contrast, array comparative genomic hybridization combines whole genome analysis with increased sensitivity thus providing a superior alternative to karyotype and FISH analysis (Robberecht et al, 2009; Zhang et al, 2009).

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Considering the frequency of chromosomal abnormalities in miscarriage, it is surprising that cytogenetic analysis is not routinely performed after failed medical intervention to prevent recurrent miscarriage. There is a debate as to whether the success or failure of a treatment can ever be defined without accurate cytogenetic analysis. Should cytogenetic testing be implemented as standard to fully ascertain treatment outcomes in the randomized controlled trial setting? The chromosomal analysis of a recent miscarriage can be an important and informative diagnostic tool for patient counselling and subsequent pregnancy management.

Array comparative genomic hybridization

Array comparative genomic hybridization is a technique that bridges the gap between molecular genetics and cytogenetics (Kallioniemi et al, 1992). When performing cytogenetic investigation of miscarriage, miscarriage tissue is obtained from surgical management via aspiration and transported to the laboratory in appropriate media. Maternal decidua is removed and a biopsy of the remaining tissue is taken for DNA extraction. The sample DNA, in conjunction with a control DNA, is amplified separately using a whole genome approach. The individual DNA is labelled with a fluorochrome, i.e. green for sample DNA and red for control DNA. Both DNAs are then mixed together in equal proportions and compete to hybridize onto an array platform containing small pieces of chromosome (Harper and SenGupta, 2012).

Well-defined genomic clones are arrayed onto a slide and these may consist of thousands of clones covering the entire genome (Wessendorf et al, 2002). Analysis is automated, usually within 24 hours, through computerized software and laser scanning. *Figure 1* shows a normal genome. Every 'dot' is a sequence of the genome. A duplication of a chromosome would rise to the green line and a deletion would fall to the red line. In *Figure 2*, an abnormal profile is evident. A 'shift' in the DNA targets on chromosome 2 has increased to the green line indicating duplication.

Array comparative genomic hybridization does have some disadvantages. The process fails to detect polyploidies (e.g. triploidy) since there is no cumulative loss of DNA content. Specific gene mutations or single gene

defects are not detected unless there is a difference in genetic material. The test cannot identify balanced translocations or inversions as the amount of DNA within the sample does not differ from the control DNA (Harper and SenGupta, 2012).

Array comparative genomic hybridization has a limited use in prenatal diagnosis, mainly because copy number variations detected in patients with uncharacterized genetic syndromes cannot be clearly classified as benign or pathogenic (Evangelidou et al, 2010). Even though the high resolution of analysis allows the identification of smaller genetic imbalances, the probability of identifying a benign variant is increased, thus imposing challenges for the interpretation of results and genetic counselling (Shaffer et al, 2007). This can also be an issue in the recurrent miscarriage population when copy number variations are detected in miscarriage products. The clinician may not be able to determine whether the genetic variant is benign and, therefore, may possibly not be the cause of the miscarriage.

Recurrent miscarriage and cytogenetics

Patients with idiopathic recurrent miscarriage may seek more than early pregnancy surveillance and ultrasound reassurance. In particular, several unproven treatment strategies have been used without knowledge of cytogenetic evaluation when pregnancy loss is repeated.

With the appearance of new DNA technologies such as array comparative genomic hybridization, evaluating the whole genome at resolutions far higher than with conventional cytogenetic analysis is now possible. With standard cytogenetic testing, diagnosis can be hindered because of a relatively high rate of tissue culture failure (10–40%) and overgrowth of maternally derived cells (maternal cell contamination) (Lomax et al, 2000). A case can be made for cytogenetic analysis to be cost effective (Wolf and Horger, 1995). From the patient perspective, results provide useful support in counselling couples who feel that treatment failure of recurrent miscarriage is the primary cause rather than a lethal cytogenetic abnormality.

Figure 1. Illustration of a normal genome using Agilent DNA Microarray Scanner at a 10 micron resolution. Scanned image and data analysis using BlueFuse Multi Software version 2.2 (BlueGnome).

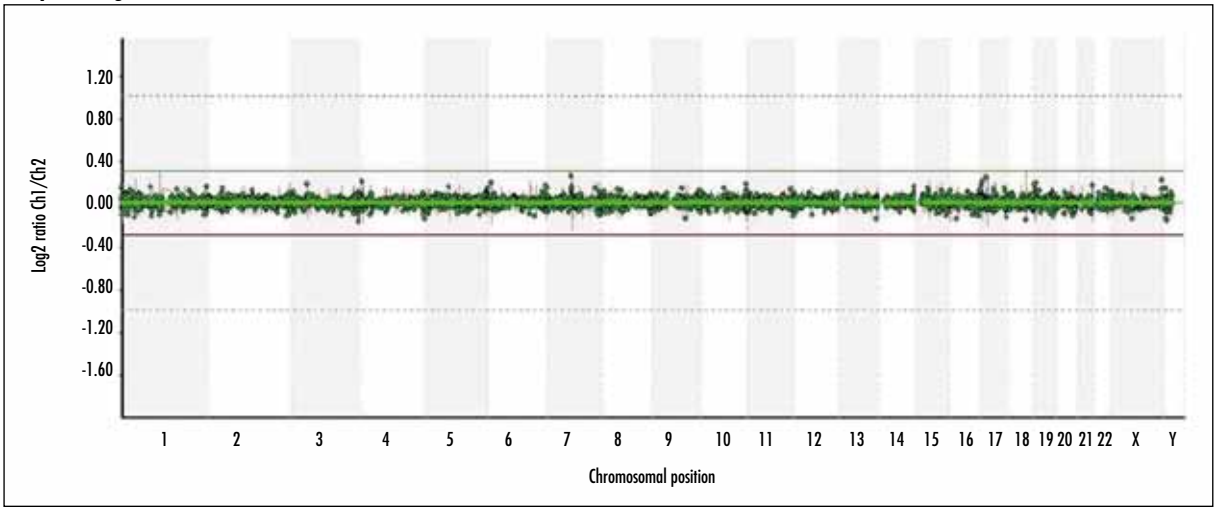
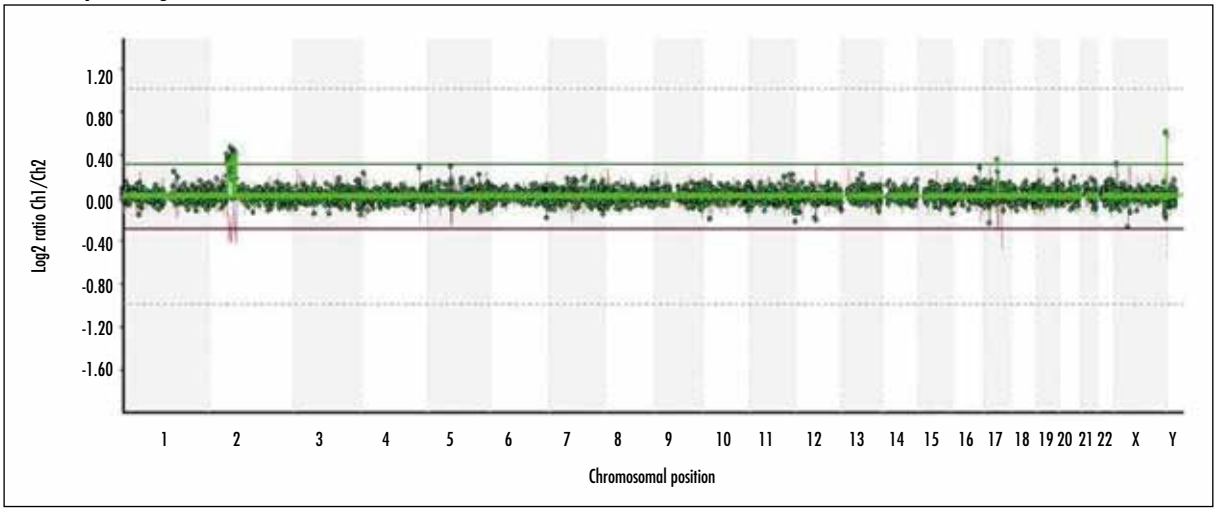


Figure 2. Illustration of a duplication of chromosome 2 using Agilent DNA Microarray Scanner at a 10 micron resolution. Scanned image and data analysis using BlueFuse Multi Software version 2.2 (BlueGnome).



Evaluation of array comparative genomic hybridization *vs* conventional cytogenetics

The continuing advance of cytogenetic testing with array comparative genomic hybridization and its potential impact on recurrent miscarriage has been considered since 1998 (Daniely et al, 1998; Lomax et al, 2000; Bell et al, 2001; Stephenson et al, 2002, 2010; Rai and Regan, 2006; Rajcan-Separovic et al, 2010). Array comparative genomic hybridization appears to overcome the persistent problem of maternal cell contamination manifested as discordance in conventional cytogenetic results. Advantages and disadvantages of array comparative genomic hybridization *vs* conventional cytogenetic analysis are summarized in *Table 1*.

Table 1. Advantages and disadvantages of array comparative genomic hybridization *vs* conventional cytogenetic testing

Array comparative genomic hybridization	Advantages	Whole genome scanning (possible to use targeted arrays, which are the equivalent of multiple independent FISH (fluorescence in situ hybridization) analyses on a single chip)
		High resolution (up to 1Kb)
		Detection of constitutional chromosomal abberations
		Whole chromosome aneuploidies
		Deletions and duplications, sub-microscopic deletions
		Telomeric or cryptic rearrangements
		Automated, objective analysis, quality control
		Fast turnaround time
		Avoidance of culture contamination
		Non-dividing cells can be tested and a smaller sample size can be used
	Disadvantages	Fail to detect balanced inversions or translocations as no cumulative loss in DNA content
		Expensive
		The detection of benign copy number variants and the process of determining their clinical significance. Necessary input from genetic counselling and anxiety for patients
		Unlikely to detect mosaicism below 20% (Stankiewicz and Beaudet, 2007)
		Uncertainty over optimal array (genome wide *vs* targeted and optimal resolution)
Conventional cytogenetic testing	Advantages	American College of Obstetricians and Gynecologists recommend conventional karyotyping to remain the principal cytogenetic tool in prenatal diagnosis (Zuffardi et al, 2011)
		Cost: karyotyping £117.00 compared to £442.00 for array comparative genomic hybridization (Wordsworth et al, 2007)
		Accessible to health-care professionals and established protocols in place
	Disadvantages	FISH on metaphase or interphase cells is limited by the number of probes that can be used simultaneously
		The resolution of the current conventional cytogenetic analyses lies in the range of 3–10 Mb
		Fail to detect common microdeletion or duplication syndromes
		Fail to identify origin of small supernumerary marker chromosomes
		Fail to identify subtle rearrangement of subtelomeric regions
		The obstacle of maternal cell contamination in order to obtain accurate results
		Requires dividing cells

The increased incidence of trisomy with advancing maternal age is well documented and is especially relevant in the modern age where the average maternal age at first birth has increased to 29 years in the UK. The Practice Committee of the American Society for Reproductive Medicine (2006) quote the miscarriage rate among women under 35 years of age as 14% compared with 40% for women over 40 years old. This emphasizes the importance of cytogenetic testing to avoid unnecessary investigations and avoids the use of non-evidence-based treatments. If a miscarriage remains unexplained, women will be more likely to expose themselves to unproven and potentially unsafe therapies.

The type of array used for analysis of miscarriage tissue may vary and can use high resolution oligo array comparative genomic hybridization on miscarriage samples (Rajcan-Separovic et al, 2010). The resolution of array comparative genomic hybridization is limited only by the size and spacing of its probes and the resolution of a 1 Mb array surpasses that of conventional banding (5–10 Mb) by 5–10 fold (Ledbetter, 2008). Choice of array platform depends upon the type and size of imbalance that is looked for and the clinical relevance. In the authors' experience, evaluation using a 1 Mb bacterial artificial chromosome array has successfully shown the effectiveness of this platform for use in recurrent miscarriage to detect sub-microscopic alterations beyond the level of the light microscopic resolution in addition to detecting numerical and large structural alterations not necessarily evident by traditional karyotyping. The abnormality detection rate is also attributable to the sensitivity of the array to detect low level fetal DNA of approximately 10%.

Where cytogenetic services allow, miscarriage tissue can be sent to the laboratory and prepared for testing by three different techniques. These include FISH (Wolff and Schwartz, 2005), cell culture or karyotype (Bayani and Squire, 2004) and array comparative genomic hybridization where DNA samples are hybridized to whole-genome bacterial artificial chromosome microarrays (CytoChip Focus Constitutional; BlueGnome Cambridge, UK) and samples processed according to the manufacturer's protocol (www.cytochip.com).

A local audit comparing conventional cytogenetics with array comparative genomic hybridization in the detection of chromosomal abnormalities in failed pregnancy from recurrent miscarriage patients revealed interesting data compatible with enabling change in investigation practice. Of 50 consecutive samples, four results

(8%) showed triploidy based on FISH analysis and therefore were not processed for array comparative genomic hybridization. FISH analysis is still needed to identify balanced translocations, triploid and tetraploid cases (Stephenson et al, 2002). Including the triploidy results, the overall rate of chromosomal abnormalities was 54% (*n*=27). The other 23 samples showed a normal cytogenetic result (*n*=19) or suffered failed tissue conventional culture (*n*=2) or result discordance as a result of overwhelming maternal cell contamination (*n*=2) where <5% of available cells were fetal in origin. Aneuploidy was mostly numerical (*n*=19) (*Table 2*) and in addition there were four unbalanced structural chromosomes abnormalities (*Table 3*). There was discordance between conventional cytogenetic testing and array comparative genomic hybridization in 35% of samples (*n*=8). The average age of a patient with a normal cytogenetic result on miscarriage analysis was 33 years compared to 37 years in patients with a chromosomal anomaly.

There was a larger proportion of aneuploid samples in the idiopathic recurrent miscarriage cohort (45%) compared to those with a positive diagnosis for recurrent miscarriage (33%). A previous recurrent miscarriage case-control study by Stephenson et al (2002) found no difference in aneuploid rates in the recurrent miscarriage cohort compared to the general population. By contrast, a study which declined to reveal their culture failure rate found a high aneuploid rate of 78% in the recurrent miscarriage cohort compared to 70% in sporadic miscarriage in women over 35 years (Marquard et al, 2010). Embryoscopy as an adjunct to array comparative genomic hybridization is a novel method of observing the failed embryo in utero hence reducing damage in obtaining tissue (Philipp et al, 2003). A study of 221 miscarriage samples utilising array comparative genomic hybridization, karyotype and embryoscopy discovered a 75% abnormality rate. A total of 61% were trisomic followed by monosomy X (22%) (Philipp et al, 2003).

Conclusions

The potential use of array comparative genomic hybridization in the recurrent miscarriage population has yet to be fully explored. While analysis provides an opportunity to offer more accurate and less failure-prone diagnoses, conventional cytogenetic testing would still have a role to detect balanced rearrangements on all normal array comparative genomic hybridization results. A more robust method of cytogenetic technology may assist the clinician when selecting patients for thorough recurrent miscarriage investigation screening. A patient with a history of two consecutive euploid

Table 2. Numerical aneuploid results obtained from miscarriage tissue by standard cytogenetics and array comparative genomic hybridization (*n*=19)

Array result	Array sex	Karyotype	Concordant results
Loss of X	Female	45,X	Yes
Loss of X	Female	45,X	Yes
Loss of X	Female	45,X	Yes
Gain of 8	Female	46,XX.MCC	No
Gain of 10	Male/female	47,XY,+10	Yes
Gain of 10	Male/female	46,XX.MCC	No
Gain of 13	Male/female	47,XY,+13	Yes
Gain of 13	Female	47,XX,+13	Yes
Gain of 14	Female	45,XX,der(13;14)(q10;q10)/46,XX,+14,der(13;14)(q10;q10)	Yes
Gain of 15	Male/female	47,XY,+15	Yes
Gain of 15	Male/female	46,XX.MCC	No
Gain of 15	Male	47,XY,+15	Yes
Gain of 16	Female	47,XX,+16	Yes
Gain of 16	Male	47,XY,+16	Yes
Gain of 16	Male	46,XX	No
Gain of 16	Female	46,XX,t(1;16)(q3?2;p?11.2).MCC	No
Gain of 21	Female	47,XX,+21	Yes
Gain of 21	Male/female	47,XY,+21	Yes
Gain of 22	Male/female	46,XX.MCC	No

MCC = maternal cell contamination

Table 3. Structural chromosomal rearrangements obtained from miscarriage products by standard cytogenetics and array comparative genomic hybridization

Array result	Array sex	Karyotype	Concordant results
27.54–28.75 Mb deletion of 14q31.1-q32.33	Female	46,XX.MCC	No
0.85–6.42 Mb duplication of 22q11.21	Male/female	46,XX.MCC	No
84.0–85.1 Mb deletion of 13q12.3-q34	Male/female	46,XY,-13,MCC	Yes
65.05–65.99 Mb deletion of 13q14.2-q34	Female	46,XX,del(13)(q14.2-q34)	Yes

MCC = maternal cell contamination

miscarriages may warrant further testing for a non-cytogenetic cause of recurrent miscarriage rather than three miscarriages without chromosomal analysis. Array comparative genomic hybridization may be of financial benefit to health-care trusts when compared to the several recurrent miscarriage investigations and treatments currently in practice. A formal cost-effectiveness analysis would be welcome. **BJHM**

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KEY POINTS

- Recurrent miscarriage affects 1% of the reproductive population and in the majority of cases the cause remains unknown.
- Cytogenetic abnormalities are thought to be responsible for over half of miscarriages in the first trimester with trisomy being the most frequent followed by polyploidy and monosomy X.
- Routine cytogenetic analysis is not commonplace in the recurrent miscarriage setting. Conventional techniques may fail to find a diagnosis as a result of cell culture failure or maternal cell contamination.
- Array comparative genomic hybridization examines the whole genome with one test and is standardized with modern computer software.
- Polyploidy, balanced translocation and inversions will not be identified by array comparative genomic hybridization.
- Conventional cytogenetics failed to identify 35% of aneuploid samples in the recurrent miscarriage population. These women may be more prone to experiment with non-evidence-based, possibly dangerous treatment to prevent a further miscarriage.
- Array comparative genomic hybridization could be performed in conjunction with conventional cytogenetics to elucidate a possible chromosomal cause of a miscarriage. Potentially, this could impact the decision for further investigation and treatment into recurrent miscarriage.
- Although proven cost effective in some clinical settings, the economic value of array comparative genomic hybridization not been fully evaluated in recurrent miscarriage population.