Overview of genetic causes of recurrent miscarriage and the diagnostic approach

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Abstract: Recurring miscarriage (RM) is a frustrating reproductive complication with variable etiology. Numerous genetic defects have been known to play a crucial role in the etiology of RM. Chromosomal abnormalities are frequently detected, while other genetic defects cannot be diagnosed through routine research, such as cryptic chromosomal anomalies, single nucleotide polymorphism, single-gene defect, and gene copy number variation. Diagnostic laboratories have recently used variable advanced techniques to detect potential genetic abnormalities in couples with RM and/or in products of conception. Here we aim to summarize the known genetic causes of RM, with a focus on the new diagnostic techniques. Knowledge of the genetic profile of miscarriages is important for prognosis and potential counseling planning, as well as the prenatal diagnostic strategy in subsequent pregnancies.

Introduction

Human reproduction is a most frustrating process where ~70% of human conceptions fail to achieve viability, and about 50% of all pregnancies are lost before the expected menses (McCoy et al., 2015). Recurrent miscarriage (RM) is a common reproductive complication defined by the World Health Organization (WHO) as three or more consecutive fetal losses before the 20th week of gestation, whereas the American College of Obstetricians and Gynecologists defined it as two consecutive miscarriages (Toth et al., 2018). The risk of abortion in successive pregnancies is related to the previous outcome, as the risk of first abortion is estimated at ~10% for the first pregnancy, ~24% for the second, 26% for the third, and 32% for the fourth pregnancy. It is also suggested that 40% of women with three abortions and 50% of women with four abortions expect a fetal loss in their upcoming pregnancy. Despite thorough diagnostic techniques, in about 50% of affected women, the specific cause of RM remains unexplained (El-Hachem et al., 2017).

RM etiology is variable, where genetic disorders account for approximately 25% of known causes, and the incidence may be higher in unexplained cases (Molazadeh et al., 2014). These disorders could involve the genetic profile of the parent or the fetus, so it is most informative to investigate both the parents and the products of conception. Chromosomal abnormalities represent a large proportion of these genetic disorders, yet researchers reported other significant genetic defects involved in RM etiology, but could not be detected through routine investigation, Fig. 1 summarises the common applicable genetics causes of RM. These include cryptic chromosomal anomalies, gene copy numbers variation, single gene defect, single nucleotide polymorphism, abnormal micro-RNA expression, and many other genetic and epigenetic factors (van den Berg et al., 2012).

Chromosomal Disorders

The most common cause of RM was fetal chromosomal abnormalities. It accounts for 50% of all cases in the first trimester, where the majority is de novo (Romero et al., 2015). However, one partner carries a balanced chromosomal rearrangement in 5% of couples with RM; and the frequency may be higher in with non-viable children (Ly et al., 2011). Most autosomal chromosomal aneuploidies are often due to meiotic non-disjunction in oogenesis, while most sex chromosome aneuploidies are due to meiotic spermatogenesis disorders (Szczygiet and Kurpisz, 2001). The sperm

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chromosomal analysis revealed a wide range of abnormalities among healthy individuals, in which numerical anomalies are frequently observed (Chatziparasidou et al., 2015).

Embryos with abnormal karyotypes are more likely to be lost due to either implantation failure or other mechanisms interfering with normal placental growth (Koot et al., 2012). It has been noted that anomalies of chromosomes can affect placental morphology and histology, as well as certain hormones and protein secretions. In addition, the placenta of chromosomally abnormal pregnancies shows decreased cell proliferation in the vascular smooth muscles, with increased apoptosis, particularly in the villus stromal tissues. This will result in hypoplasia and dysmorphic placental structure with impaired vascular development, resulting in severe retardation of intrauterine fetal growth and subsequent miscarriage (Qumsiyeh et al., 2000). In a proportion of couples with RM, subtle chromosomal rearrangements were reported. In these cases, during meiosis, chromosomes fail to pair or segregate properly, leading to gametes that contain an unbalanced chromosomal component that may be lethal to the embryo (Daughtry and Chavez, 2016).

The Spectrum of Chromosomal Abnormalities

Chromosomal aneuploidy
A diploid embryo formation requires a sperm and an ovum containing a haploid set of chromosomes, i.e., one copy of each chromosome. This is produced by a specialized cell division that is known as meiosis. The meiotic errors associated with defective chromosome segregation ensued with sperms or ova result in chromosome aneuploidies. Most of these aneuploidies result mainly from errors in female meiosis, whereas < 5% result from errors in male meiosis (Hassold and Hunt, 2009). In vitro fertilization (IVF) studies have suggested that in combination with advanced maternal age, maternal non-disjunctions and premature centromere divisions in meiosis 1 lead to oocyte aneuploidy. In addition, oocytes in advanced maternal age are more susceptible to meiotic spindle disorders or changes in chiasma, resulting in increased non-disjunction incidence. It has also been suggested by meiotic studies that sex chromosomes and chromosome 21 are more susceptible to non-disjunction (Chiang et al., 2012).

Chromosomal aneuploidy is commonly seen in the products of conception, while only 0.6% of newborns are aneuploid (Hassold and Hunt, 2009). Aneuploidy etiology is correlated mainly with advanced maternal age, whereas pathogenesis is variable, and there may be prezygotic (sperm or oocyte) or postzygotic (mitotic or meiotic) errors. However, in normal individuals, 2-3% of sperms and about 20% of oocytes show chromosomal aneuploidy (Čulić et al., 2011). Autosomal trisomies are frequently detected and account for up to 60% of all aneuploidies. Trisomy 16 followed by trisomy 22 are, however, more common, while trisomy 1 is rarely detected in the products of conception (Sheth et al., 2013). In addition, the only non-mosaic autosomal aneuploidies compatible with life are trisomies 21, 13, and 18. On the other hand, in live-born babies, gonosomal trisomies (XXX, XXY, and XYY) are less commonly observed (Staessen et al., 2003). Chromosomal monosomies, on the other hand, are relatively rare, and the only type compatible with life is X-chromosome monosomy. X-chromosome monosomy, however, represents 13% of all miscarried aneuploidies, while it is detected in 0.3% of live-born babies. Indeed, conventional karyotyping can easily detect numerical
Chromosomal polyploidy

Polyploidy occurs as a triploid (3n = 69) or as a tetraploid (4n = 92). Triploidy is relatively more common than tetraploidy, accounting for about 10% of the early pregnancy loss. Polyploid embryo occurs irrespective of maternal age and may result from an extra haploid set, either maternal or paternal (Filges et al., 2015). Dispermy is usually the common cause of triploidy, but it may be due to diploid oocytes caused by maternal meiotic errors. Tetraploidy, on the other hand, accounts for ~2% of early pregnancy loss and is usually caused by post-fertilization errors (Hardy and Hardy, 2015). Studies have revealed a relationship between the origin of chromosomal polyploidy and the feto-placental outcome. A paternally derived extra chromosome set is usually associated with a normally growing fetus and a large cystic placenta, while the maternal origin of polyploidy is associated with severe intrauterine fetal growth retardation (Goddijn and Leschot, 2000).

Structural chromosome anomalies

Structural chromosome anomalies could be balanced if there is no loss or gain of genetic material, or imbalanced. In several human disorders, including recurrent miscarriages, more than 1000 types of constitutional chromosome anomalies have been clinically recognized. Structural chromosome anomalies may disrupt the structure of the gene(s) or alter the gene(s) copy number, resulting in the affected region’s genes being haploinsufficient. This, in turn, could affect the normal function(s) of other genes supporting the continuation of pregnancy (Theisen and Shaffer, 2010). Structural chromosome anomalies were detected in approximately 5% of couples with RM, 6% of products of conception, and 0.1% of live births (Priya et al., 2018; Pylyp et al., 2018). Structural chromosome anomalies could be deletions, duplications, translocations, or inversions, of which only translocations and inversions play a significant role in RM. However, during gametogenesis, most structural chromosomal anomalies were developed de novo, while a small proportion was inherited from one parent who had a balanced rearrangement (Goddijn and Leschot, 2000). Conventional karyotyping does not reveal all structural chromosomal anomalies, as cryptic subtelomeric as well as microdeletion and microduplication rearrangements that were noticed in patients with intellectual disabilities (Dawson et al., 2002) and couples with RM (Stephenson et al., 2002). Y-chromosome microdeletion has also been detected in a proportion of male partners of women experiencing RM (Ghorbian et al., 2012). In addition, in some products of conception karyotyped as 46, XX, the Y-chromosomal DNA material was detected by a PCR and cannot be recognized by conventional karyotyping (Bell et al., 1999).

Single Nucleotide Polymorphism

Single nucleotide polymorphism (SNP) is the most common diffuse genomic variation where a single nucleotide is replaced at a specific position in the DNA. SNPs are the result of mutations that produce base-pair differences between DNA sequences. There are at least 10 million SNPs in the human genome, where they can occur in the coding and non-coding regions, or in between genes. Although the majority of these variations do not alter cellular function and thus have no effect, some SNPs have been discovered to contribute to the development of several diseases, including RM (Robert and Pelletier, 2018). However, advanced genome sequencing technology, such as chromosome microarray and next-generation sequencing, allows simultaneous genome-wide screening for SNPs and helps predict pathological forms (Salk et al., 2018). Tab. 1 summarizes some studies in which SNPs in specific genes are strongly associated with RM, where the frequency of SNPs in RM cases is significantly higher than in normal cases.

The endothelial nitric oxide synthase (eNOS) gene plays a key role in the uterine and placental angiogenesis and is known to be expressed in the syncytiotrophoblasts and villus blood vessels during pregnancy. SNP of this gene could alter the gene expression resulting in RM (Zhao et al., 2019; Azani et al., 2017). DNA methylation is important for gametogenesis and pregnancy continuation. An SNP of the DNA methyltransferase 3B (DNMT3B) rs1569686 gene and DNMT3A-448A > G polymorphism of the 3A promoter region were commonly detected in RM (Barišić et al., 2017; Liu et al., 2017). In addition, the matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) genes are essential for cell proliferation, apoptosis, and angiogenesis. However, the expression and proper activation of these genes in the decidua and extravillous trophoblasts appears to be important for a normal pregnancy. Multiple SNPs have been detected in the promoter regions of the matrix
### TABLE 1

Summary of prevalent SNPs studies involving particular genes related to the etiology of RM

| Cases | Control | Site of SNP | Gene involved | Author |
|-------|---------|-------------|---------------|--------|
| 120   | 120     | IL-6 G634G  | Interleukin-6 gene | Nasiri and Rasti, 2016 |
|       |         | CTLA-4 A 49G | Cytotoxic T-lymphocyte associated protein-4 gene | |
| 350   | 200     | IL-10 C592A | Interleukin 10 (IL-10) | Zammiti et al., 2006 |
|       |         | IL-10 C819T |               | |
| 106   | 74      | TLR9 G2848A | Toll-like receptors-9 (TLR9) | Razdalbiedina et al., 2018 |
|       |         | IL-10 C592A | Interleukin 10 (IL-10) | |
| 200   | 300     | Leptin C 2549A (rs7799039) | Leptin | Parveen et al., 2012 |
|       |         |               | Tumor necrosis factor-alpha | |
| 300   | 500     | CTLA-4 (AT)n 3'UTR | Cytotoxic T-lymphocyte associated protein-4 gene | Gupta et al., 2012 |
|       |         |               | Tumor necrosis factor-alpha | |
| 192   | 201     | eNOS G894T   | Endothelial nitric oxide synthase gene | Zhao et al., 2019; Azani et al., 2017 |
| 204   | 248     | TNF-a T1031C | Tumor necrosis factor-α | Finan et al., 2010 |
| 129   | 116     | MMP2 - C1306 T | Matrix metalloproteinases | Lee et al., 2018 |
|       |         | MMP9 - C1562 T |               | |
| 120   | 120     | IL-6 G634G  | Interleukin-6 gene | Nasiri and Rasti, 2016 |
|       |         | CTLA-4 A 49G | Cytotoxic T-lymphocyte associated protein-4 gene | |
| 350   | 200     | IL-10 C592A | Interleukin 10 (IL-10) | Zammiti et al., 2006 |
|       |         | IL-10 C819T |               | |
| 106   | 74      | TLR9 G2848A | Toll-like receptors-9 (TLR9) | Razdalbiedina et al., 2018 |
|       |         | IL-10 C592A | Interleukin 10 (IL-10) | |
| 200   | 300     | Leptin C 2549A (rs7799039) | Leptin | Parveen et al., 2012 |
|       |         |               | Tumor necrosis factor-alpha | |
| 300   | 500     | CTLA-4 (AT)n 3'UTR | Cytotoxic T-lymphocyte associated protein-4 gene | Gupta et al., 2012 |
|       |         |               | Tumor necrosis factor-alpha | |
| 192   | 201     | eNOS G894T   | Endothelial nitric oxide synthase gene | Zhao et al., 2019; Azani et al., 2017 |
| 204   | 248     | TNF-a T1031C | Tumor necrosis factor-α | Finan et al., 2010 |
| 129   | 116     | MMP2 - C1306 T | Matrix metalloproteinases | Lee et al., 2018 |
metalloproteinases genes in association with RM (Li et al., 2018a). The cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) gene has a regulatory function of the immune tolerance of the maternal-fetal interface. SNP of the promoter region and exon-1 is usually associated with abnormal expression of this protein may be the key factor for RM (Li et al., 2018b). Anti-inflammatory cytokines such as interleukin-10 (IL-10) and Toll-like receptors are important for embryonic implantation and development. SNPs of these genes may affect their expression and therefore may affect fetal implantation and development resulting in fetal loss (Zammiti et al., 2006; Razdaibiedina et al., 2018). Additionally, tumor necrosis factor-alpha (TNF-α) is an apoptosis-inducing factor in tumor cells. It is also a primary inflammatory cytokine that helps to maintain the balance of several body processes including coagulation, angiogenesis, and endothelial functions. However, several studies have shown that polymorphisms of the promoter region of the (TNF-α) gene are a genetic risk factor for recurrent fetal loss (Finan et al., 2010; Gupta et al., 2012; Parveen et al., 2012).

Abnormal Micro-RNA Expression

MicroRNAs (miRNAs) are a group of endogenous, short noncoding molecules that play a role in gene expression. Most of the miRNAs are transcribed from DNA sequences into primary, then precursor, and finally mature, miRNAs (O’Brien et al., 2018). It has been reported that a single miRNA can regulate the expression of multiple genes, while different miRNAs can regulate a particular gene (Barchitta et al., 2017). Several studies have revealed that miRNAs are involved in variable regulatory pathways associated with various biological and pathological conditions, such as cell growth and differentiation, development, and many human diseases (Wahid et al., 2010). Numerous placenta-specific levels of miRNA expression have been identified and their levels of expression could be crucial for fetal growth and development (Hosseini et al., 2018). Dysregulated expression of some miRNAs has been reported from compromised pregnancies in the maternal circulating blood and placenta, suggesting that miRNA profiling may be associated with early RM. Tang et al. (2016) found 155 miRNAs expressed differently in placental tissues from RM cases, where 98 genes were up-regulated, and 57 genes were down-regulated. Genes of these miRNAs were found to be involved in B-cell receptor, T-cell receptor, and tumor-associated signaling pathways. These genes of differentially expressed miRNAs may help to understand the mechanism of early RM etiology and pathophysiology. In addition, over-expressed miRNAs (microRNA-575) were detected in cases with RM in conjunction with increased villus apoptosis and reduced placental angiogenesis (Xia et al., 2017). Several studies also reported differential miRNA expression in adverse pregnancy outcomes (Amin-Beidokhti et al., 2017; Yang et al., 2018) and concluded that differential miRNA expression levels represent a promising diagnostic biomarker for unexplained RM (Qin et al., 2016).

Diagnostic Approach

Karyotyping

Most cytogenetic diagnostic centers routinely use conventional karyotyping to diagnose chromosomal rearrangements in couples with RM or in products of conception. Conventional karyotyping is good for aneuploidy, translocation, large deletion, and duplication; however, cryptic rearrangements of less than two mega-bases (Mb) cannot be detected. Therefore, using another high-resolution technique, from a few kilo-bases (kb) to multiple Mb, such as chromosomal microarray (CMA), can add additional information beyond the scope of conventional G-banding (Dhillon et al., 2014). Fig. 2 summarizes the common diagnostic procedures applied to MR cases.

![FIGURE 2. Summary of the diagnostic genetic tests often conducted in RM cases.](image-url)
Chromosomal microarray

Recently, diagnostic centers have started using CMA to evaluate couples with RM, or to analyze the products of conception for an accurate diagnosis. However, two different CMA platforms currently used to diagnose micro-deletion and/or micro-duplication; the array comparative genomic hybridization (aCGH) and the single nucleotide variation (SNV) arrays. CMA increases the diagnostic yield over karyotyping in these conditions and may help in impact clinical management decisions (Sahoo et al., 2017; Popescu et al., 2018). Array-CGH can detect variants of copy numbers by hybridizing a reference genomic sequence with patient (unknown) sequences labeled with different fluorescent tags to a microarray of DNA fragments; Fig. 3 illustrates the basics of chromosomal microarray technology. If the unknown sample results in a cryptic deletion or duplication of genetic material, the sequence imbalance can easily be detected as a difference in fluorescence intensity (Hayes et al., 2013). Array CGH can also detect variants of copy numbers for larger deletions and duplications, including trisomy or monosomy. But, because they are not associated with a fluorescence intensity change, they cannot detect balanced translocations, polyploidy, or sequence inversions (Ahn et al., 2016). Single nucleotide polymorphism or variation (SNV) is a variation in the DNA sequence, where a single nucleotide differs in an individual between populations or paired chromosomes. SNV is the most common form of genetic variation in the human genome and could or could not be associated with diseases (Haraksingh and Snyder, 2013). Unlike aCGH, the genomic sample of the patient is directly hybridized to the array platform containing thousands of SNVs in the SNV-based CMA test. The variants of the DNA sequence are diagnosed.

**FIGURE 3.** Illustration of chromosome micro-array, where a normal genomic DNA and the test DNA are labelled with different fluorescent dye color. Equal amounts of the two DNA samples are mixed, hybridized, and tested for gain or loss of the genetic material (Dugoff et al., 2016).

**FIGURE 4.** Illustration of the whole genome, whole exome and targeted gene/s sequencing. First, the genomic DNA is extracted, fragmented into small pieces, cloned, amplified and sequenced separately. After that, assemble ordered sequencing (GenomixLAB, 2016; Commins et al., 2016).
directly by the signal intensity (Cheung and Bi, 2018). SNV-based CMA can detect other genomic aberrations rather than cryptic rearrangements, such as genomic homozygosity and uniparental disomy. Generally speaking, CMA has higher analytical accuracy to detect copy number variants in more than 95% of cases (Papenhausen et al., 2011).

Next-generation DNA sequencing
The human genome includes approximately 20 000 genes, representing about 2% of the entire genome. The remaining DNA sequences, however, can regulate these genes' function or expression through different pathways, and their screening is challenging (Chi, 2016). Advances in DNA sequencing technology have made it possible to screen a large scale of DNA that extends to the entire genome sequencing, with low cost, greater confidence, and potential advantages over the classic approaches in which genes are screened individually. This will help identify numerous genetic mutations to provide the diagnosis of several unexplained disorders. This strategy offers genomic sequencing-based tests such as next-generation DNA sequences (NGS), with its subsequent variation that includes whole exomes (all exons), whole genomes, and analysis of multigene panels (Liu et al., 2012; Quintero-Ronderos et al., 2017). Fig. 4 demonstrated the basics of the whole genome, whole exome, and the targeted genes/DNA sequences. Not only can NGS diagnose submicroscopic chromosomal rearrangement that cannot be detected by aCGH, but it can also detect variations in the DNA and RNA sequence. NGS can also detect epigenetic variants that contribute to genomic expression or regulations that may affect the outcome of pregnancy (Haraksingh and Snyder, 2013).

Whole exome sequencing (WES) determines with high accuracy the order of nucleotides in the coding DNA sequences. WES analyzes the exons or coding regions of thousands of genes simultaneously, approximately 18 000 exons transcribed into mature RNA, the element of interpretable mutations associated with clinical disorders (Schwarze et al., 2018). Numerous studies have used WES to investigate families with RM and the product of conceptions. Studies have found several deleterious gene mutations, copy number variations, SNVs, and small insertions and deletions that can affect the motive pathways of placental functions, embryo implantation, and coagulation (Bao et al., 2014; Qiao et al., 2016). On the other hand, whole genome sequencing (WGS) determines the entire genome sequences including sequences of coding and non-coding regions. This will help detect variable genetic anomalies such as single nucleotide variants, deletions, insertions, and copy number variants (Ekblom and Wolf, 2014). WGS, however, misses some sequential genomic regions, such as regions with high GC content, highly repetitive sequences, centromeres, and telomeres (Quintero-Ronderos and Laissue, 2019). WGS has become an established method for studying human genetic variation in various diseases, including RM (Nagirnaja et al., 2014).

Conclusion and Summary
Knowledge of pathogenesis of miscarriage is important for the prognosis and planning of prenatal diagnosis in subsequent pregnancies, as well as for appropriate genetic counseling. Karyotyping is routinely used to investigate the product of conception and RM cases, but it cannot detect cryptic or submicroscopic genetic aberrations. However, new diagnostic methods such as chromosomal microarray and next-generation DNA sequencing allow accurate genetic diagnosis, especially in unexplained cases with RM. The technique of chromosomal microarray allows simultaneous investigation for submicroscopic chromosome anomalies, SNP, and gene copy number variation. Whereas next-generation DNA sequencing allows sequencing of coding DNA regions (whole exome sequence) or even sequencing of coding and non-coding DNA regions (whole genome sequence). Whole exome sequencing may be an efficient diagnostic technique for unexplained cases of RM. Indeed, some non-coding regions of the genome can play significant functions in gene expression, so after using exome sequencing, still a proportion of patients without a diagnosis. This will increase the value of the whole genome sequencing as a diagnostic method. A debate has arisen about the benefits of each technique as a whole genome and exome sequencing as a better molecular diagnostic method in research and clinical practice. Researchers, however, prefer the whole genome, while clinicians are more interested in exome sequences due to the time-consuming, the cost, and difficulties with result interpretation.

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