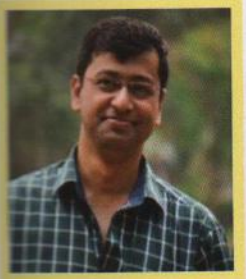
Indian Society for Assisted Reproduction

ROLE OF GENETIC TESTING IN RECURRENT PREGNANCY LOSS: What, How & Why?



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Spontaneous abortion or miscarriage is defined as the involuntary termination of pregnancy before 20 weeks of gestation (dated from the last menstrual period) or below a fetal weight of 500gm. ASRM has defined RPL as *a distinct disor der defined by two or more failed clinical pregnancies* . Here pregnancy is defined as a clinical pregnancy documented by ultrasonography or histopathological examination. The vast majority of all early pregnancy losses result from chromosomal abnormalities arising in the egg, the sperm, or during early embryonic development and are random events. Even repeated miscarriages can occur by chance alone, but at least some affected couples have a predisposing factor. Among all the factors that have been implicated, the only undisputed causes of recurrent pregnancy loss are **1)** Genetic [balanced chromosomal translocation in either partner, maternal age-related increase in prevalence of aneuploid oocytes] **2)** Anatomic [congenital and acquired uterine abnormalities], **3)** Immunologic [the thrombotic complications of anti-phospholipid syndrome]

Approximately 12%-15% of all clinically recognized pregnancies end in miscarriage, but the true incidence of miscarriage ,including unrecognized early pregnancy losses, is two to four times higher (30%-60%). Among all the causes of RPL the data till date available says that 2% -5% loss is due to genetic abnormality of parents (?) and majority 40%-50% is still unexplained with the available scientific gadgets (e.g. karyotyping, FISH). This is the area where if we use the newer techniques (e.g. Microarray or NGS), we will be able to find out many genetically abnormal Product of Conception (POC) which were previously missed by scientifically inferior techniques and help us explore the more unknown genetic causes of abortus.

The first case of abnormal POC was reported by Penrose and Delhanty 1961. It was a triploidy in spontaneous abortion. It took several years before cytogenetic analysis of miscarriage became an option in laboratories, reason being difficulties in culturing the fetal tissue. Today we have mapped out the possible *numerical* genetic abnormality of all the 23 pairs of chromosomes by the conventional Karyotyping method. But is this the only type of abnormality a POC possesses?

What are the other possible types of abnormality a POC may have? And how to diagnose them?

Genetic abnormalities may be divided into **1]** Numerical (aneuploidy, polyploidy) **2]** Structural (inversion,insertion,deletion, translocation) **3]** Point mutation or **4]** Polygenic.

Genetic abnormalities of the POC may occur via two reasons. Either the abnormality is derived from the known parental chromosomal abnormalities or embryonic errors that arise de novo in an apparently chromosomally normal parent. Of the de novo fetal genetic abnormalities 90% is numerical (aneuploidy, polyploidy); the remainders are split between structural abnormalities (translocation, inversion) and mosaicism. Overall autosomal trisomies are most common.

Without genetic evaluation, women who repeatedly miscarry generally are assumed to be losing normal pregnancies; when, in fact, most are not. There are two school of thoughts; one who thinks genetic evaluation of POC is a crucially important investigation & the other thinks this is an unnecessary expensive luxurious investigation. Now, one important question is when to start genetic evaluation of a couple of RPL? In ideal situation, every healthy fetus in utero should be delivered and should go home. If not then even the POC of first miscarriage should have genetic evaluation done. But the evaluation of healthy

woman after a single loss is usually not recommended because this may be a common sporadic event. Literature says the risk of pregnancy loss after 2 miscarriage is only slightly lower (24%-29%), than that of women with 3 or more spontaneous abortion (31%-33%). So Evaluation should be started after second pregnancy losses.

But the real practical problem starts the moment one start prescribing a particular test. The points that one should keep in mind before advising a particular genetic evaluation are

1) What information I want to get from the particular test?
2) What are the tests available at present to give me that information?
3) Is the information provided to me by a particular test is sufficient? If notthen
4) Out of these tests, which one is more specific? More sensitive? More accurate?
5) Any new test available recently?
6) What are sample requirements? How are samples transported to the laboratory? Are shipping/courier services available?
7) What is the test turnaround time?
8) Are results clear and concise (easily understandable)? How are abnormal results communicated to the ordering physician?
9) What are the advantages/disadvantages of a particular test procedure?
10) Will results have implications for other family members?

At present the methods available to genetic status of POC are **1]** Karyotype **2]** fluorescent in situ hybridization (FISH) **3]** Microarray technique are of two types {i}single nucleotide polymorphism (SNP) & {ii}array comparative genomic hybridization (aCGH) **4]** next generation sequencing (NGS). Traditionally miscarriage specimens were evaluated till yesterday in most of the places by ***karyotyping*** or ***FISH*** method in an attempt to find out the aneuploidy status. But karyotyping of POC has its own challenges. ***Limitations of karyotyping are*** **1]** the process requires culture of actively dividing metaphase 2 cells. But in most cases, by the time the patient presents to the doctor and the diagnosis of embryo demise is made and the D&E is done, the tissues were all dead and results in culture failure (10% - 40%). **2]** Even if the tissues were alive while collecting the sample if precautions are not taken (vagina should not be cleaned by betadine or other antiseptic solutions before collecting the sample, sample shouldn t touch the vaginal walls or any other structure before dipping into the sample collecting media to prevent contamination) **3]** During collecting the sample utmost precautions should me made not to collect maternal cell (decidual cell) along with POC in order to prevent maternal cell contamination (MCC) & growth of the same. Because of MCC, with a 46,XX G banded Karyotype one can t be certain that the analyzed cells are fetal or maternal in origin and therefore of little clinical value. **4]** The detection threshold level is up to 5-10 Mb (mega base) only. This causes inability to detect submicroscopic deletion & duplications of clinical significance. **5]** Test result comes out within 7-14 **days**. **6]** Detection of mosaicism : The mosaic rate of miscarriage derived from the culture of villi from POC varies, but is considered to be less than 1%. The mosaic rate of miscarriage derived from placental tissue is considerably higher. It is possible that karyotyping underestimates mosaicism because the standard analysis of 1015 cells may not detect low-level (<1520%) mosaicism. Because of these limitations, FISH gradually replaced the Karyotyping procedure. ***Advantages of FISH*** : **1]** It is possible to analyze interphase chromosomes with FISH, as well as the metaphase chromosomes used in karyotyping, which eliminates the requirement for cell culture. **2]** It is possible to simultaneously monitor multiple sites if the hybridization probes have been labeled with different flurophores.

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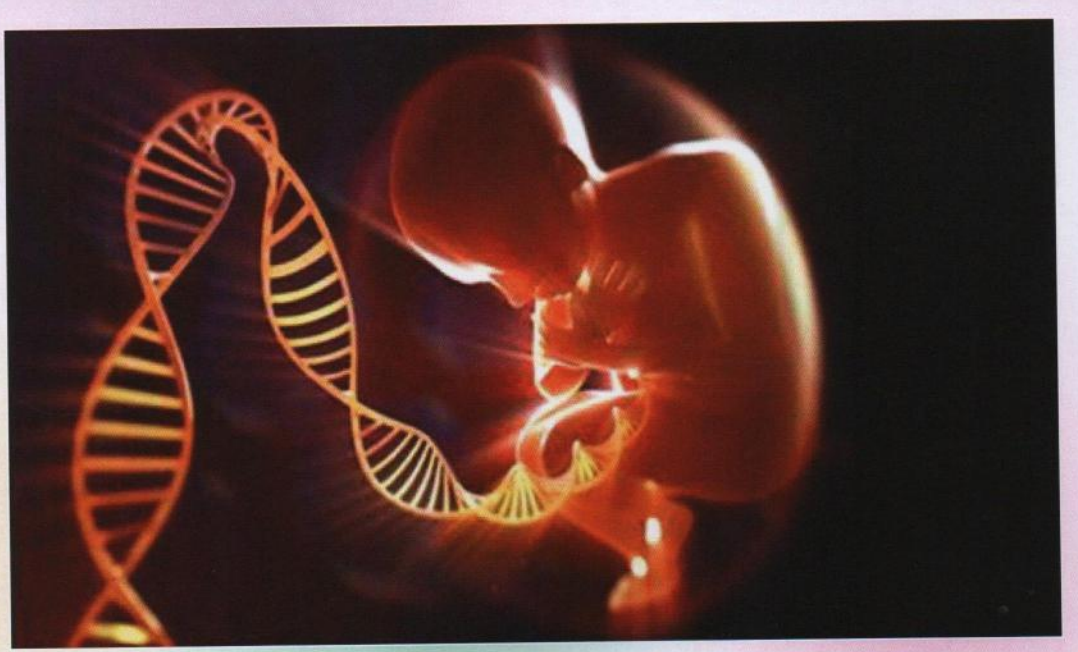
Limitations* of *FISH procedure are **1]** it typically evaluates between 3 and 5 rather than all 23 chromosome pairs. **2]** The ability of FISH to detect chromosomal anomalies is limited by the number of probes selected. **3]** Manual scoring and signal enumeration may be difficult, because of the truncation of nuclei on tissue sections and overlapping of nuclei. Special training in cytogenetic laboratory techniques is required.**4]** FISH cannot detect some structural chromosomal abnormalities (e.g. inversions, balanced translocations) 5] Test result comes out within 7-10 **days.**

Newer molecular techniques like **SNP microarray or aCGH** offer several advantages. SNP arrays directly evaluate ploidy by genotyping alleles on a dense chip of approximately 300000 genetic markers. aCGH by contrast evaluate far fewer genetic markers and determine ploidy by comparing the clinical DNA sample with male and female reference DNA samples. The ***advantages*** are **1]** No cell culture is required, which avoids culture failure, contamination, overgrowth of maternal cells & selection against mosaic cell lines. **2]** Test results are typically available within 8 -10 **hours** and the results are not dependent on user variability. **3]**These techniques provide much higher resolution and allow detection of microduplication and microdeletions below the traditional 10MB resolution of G-banding which were otherwise missed by Karyotyping and FISH and results would come as normal. **4]** Array based techniques allow detection of MCC. **5]** These techniques can be applied to archival tissue from prior miscarriages stored in formaldehyde-fixed, paraffin-embedded blocks. Such *r escue karyotyping* can thus be performed on curettage specimens that were never sent for karyotypic analysis or to allow reanalysis of specimens that were called normal by conventional G- banding. **6]** SNP arrays can identify loss of heterozygosity, consanguinity, uniparental disomy and the parental origin of aneuploidy.

Next-generation sequencing (NGS):

NGS involves multiple parallel sequencing of millions of small fragments of DNA. By means of advanced bioinformatics, individual readings of the sequenced DNA fragments are mapped to the human reference genome, which enables the delivery of accurate data, even on small DNA variations in entire genomes. Unlike microarrays which are considered a *closed system* because they can only account for sequences that are targeted by probes on the array, NGS technologies represent an *open system* suitable for cataloguing gene diversity (including discovery of novel gene diversity), without a priori sequence information. NGS can t detect balanced translocation and the results are available within 10-14 **days.**



Advantages :**1]** The strength of NGS platforms lies in their ability to lay millions of DNA fragments on a single chip and then simultaneously process the fragments. **2]** This increases throughput and permits a larger dynamic range of input data. **3]** NGS technologies have greater sensitivity, specificity and accuracy than microarrays. **4]** Ability to sequence hundreds to thousands of genes or gene regions simultaneously. **5]** Comprehensive genomic coverage **6]** Lower limit of detection (between 3-10 MB) **7]** No cell culture is required, which avoids culture failure, contamination, overgrowth of maternal cells & selection against mosaic cell lines.**8]** Multiple dissections made to detect or rule out maternal cell contamination (MCC) with > 99% accuracy. **9]** It can detect partial aneuploidy also. **10]** The sample is also not required to be shipped within 24 hours from the collection centre to the processing laboratory since it doesn t require live cells. **11]** A tiny (nanogram) quantity of tissue is sufficient for NGS, eliminating the reliance on polymerase chain reaction (PCR) amplification.

CONCLUSIONS

Genetic variables appear to play a complex role in the efficiency of human reproduction. Classically, high rates of chromosomal errors have been among the leading etiologies for fetal loss and more recent studies have begun to highlight the important role that specific single gene defects may play in pregnancy maintenance. To help aid couples struggling with RPL, limited and focused genetic testing is recommended as part of the diagnostic approach. European Society of Human Reproduction and Embryology (**ESHRE**) currently recommend aCGH should be the method of choice for analyzing POC & future is for NGS. The material for testing should be properly prepared. Knowledge of the pathogenesis of a miscarriage, as well as advantages and limitations of diagnostic methods, is necessary for appropriate genetic counseling.

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