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Invited Review Article

DNA based pre-implantation diagnosis

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Introduction

Gametes are the carrier of genetic information from one generation to the next. If these (sperm and egg forming cells) are affected either by intrinsic or extrinsic factors, the next generation may be produced with various genetic defects. These defects may express at any time during developing embryo to mid adulthood (Srivastava and Olson, 2000). However, now it is possible to have pre-implantation genetic diagnosis (PGD) performed before implantation stage (Pellestor, 1995). More than 1,200 genetic disorders have been identified. Most of us carry a few defective genes with no signs of disease and many of these can only contribute to susceptibility. In addition to hereditary disorders, lifestyle choices and environmental factors also raise or lower the risk of diseases. Molecular basis of a vast majority of these diseases is not yet clear (Delhanty and Handyside, 1995) (Magli *et al*., 1998).

Since 1989, when first successful attempt was made to diagnose genetic defects before embryo implantation in human, this diagnostic technique gained lot of importance (Rhoads *et al*., 1989). The technique is performed by removing one or two cells from the 8-16 cell embryo. Researches have shown that it is possible to remove one or two cells from an 8-10 celled embryo three days after fertilization without detrimental effects to the embryo for further development (Grifo *et al*., 1994 ; Handyside *et al*., 1992). Embryos were sexed on the basis of the presence or absence of a DNA fragment specific for the Y chromosome (Lo *et al*., 1989) ; in 1990 two sets of twin girls were born to five couples at risk of passing on an X linked disorder. Subsequently, a number of babies have been born after the pre-implantation genetic diagnosis has ruled out cystic fibrosis (Handyside *et al*., 1992 ; 1997), Tay Sachs disease (Gibbons *et al*., 1995), Lesch-Nyhan syndrome, Duchenne muscular dystrophy and for diseases carried on the X chromosome. Sexing the embryo to avoid X-linked diseases remain the commonest reason for pre-implantation diagnosis, now optimally carried out by the molecular cytogenetic technique of FISH (fluorescent *in situ* hybridization) with DNA probes derived from the X and/or Y chromosomes (Griffin, 1992).

Genetic defects are tested for by PCR (polymerase chain reaction) and chromosomal abnormalities by FISH techniques (Handyside *et al*., 1990 ; Griffin *et al*., 1994). In addition to these, many more techniques are developed in order to test even monogenic defects in the developing embryo. For those defects in which cytogenetics, biochemical or molecular basis is known, prenatal diagnosis and selective termination of affected fetuses is possible. These diagnostic techniques are more effective in IVF (*in vitro* fertilization). After IVF, on the 3rd day, the 8-cell embryo is biopsed to obtain blastomeres (single cells) for molecular diagnosis (Handyside *et al*., 1992 ; International Working Group, 1999). An embryo biopsy is done using micromanipulators under the visual control provided by an inverted tissue culture microscope. The embryo is held in position using a holding pipette, while a glass needle is used to drill a hole through the zona pellucida using a laser. A single cell is then removed by gentle suction. The cell is then available for genetic diagnosis. Analysis of DNA from a single cell is performed by either FISH or PCR technique. FISH utilizes fluorescent probes, which are specific for a given chromosome, and therefore allows one to screen for chromosomal abnormality (Griffin *et al*., 1994). PCR allows to amplify a selected DNA sequence of interest (Saiki *et al*., 1988), so that it can be analyzed. After the biopsy and DNA analysis on the single cell, the embryos are kept in culture and allowed to divide further. Once the appropriate molecular diagnosis is made, unaffected embryos can be transferred back into the uterus in the IVF cycle. PGD (Pre-implantation Genetic Diagnosis) is now also being used in order to increase pregnancy rates for older infertile women (Reubinoff and Shushan, 1996 ; Munne *et al*., 1997). One of the reasons older women have a

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poorer pregnancy rate is because their embryos are often chromosomally abnormal, because of the fact they have older eggs (which may have genetic defects). PGD allows the doctor to select only the chromosomally normal embryos, so that only these can be transferred back into the uterus, resulting in a higher, healthy and term pregnancy rates (Mancuso *et al.*, 1996) in older women.

In this review we have described various PGD techniques which are used currently for improving chances of conception, defect free, live births and reducing chances of abortions in human and farm animals. Certain techniques which are not yet perfected as routine for diagnostic purposes in humans but used frequently for farm and laboratory animals and plants have also been described, however, newly developed RNA based diagnostic techniques were not included in this review.

Chromosomal analysis

Human somatic cells contain 46 chromosomes ; these comprise 22 homologous pairs of autosomes and two sex chromosomes, XX in the female and XY in the male (Tjio and Levan, 1956). Human lymphocytes, the cells which are easy to obtain but they do not divide, are generally used for chromosomal analysis. On PHA (phytohemoaglutanin) stimulation, they divide and treatment of colcemide, arrest them in metaphase. In a metaphase spread, the individual chromosome takes the form of two chromatids connected at the centromere. Individual chromosome has distinctive and reliable pattern of alternating light and dark bands along its length. C band contains tandemly repeated DNA ; G-band contains late-replicating AT-rich heterochromatin ; Q-band contains some housekeeping genes and tissue-specific regulatory regions ; and R-band contains early-replicating GC-rich regions. A normal male karyotype with G-banding technique detects approximately 300 to 500 bands per haploid set (Federman, 1987 ; Biesecker, 2001). Recently, the banding techniques have been dramatically improved by obtaining the cells in prophase. The individual chromosomes appear markedly elongated and up to 1,500 bands per karyotype are likely to be recognized, if prophase chromosomes are used for the technique (Yunis *et al.*, 1978) (Ikeuchi, 1984).

Chromosomal anomalies occur in 0.4% of live births. Chromosomal anomaly is an important cause of mental retardation and congenital defects (See Tables 1 and 2). Chromosomal anomalies are present in much higher frequencies among spontaneous abortions and stillbirths. The phenotypic anomalies that result from chromosomal aberrations are mainly due to imbalance of genetic information.

Normal chromosome count is expressed as 46, XX for the female and 46, XY for the male. However if an error occurs in meiosis or mitosis, which cell acquires a chromosome complement that is not an exact multiple of 23. For example, when nondisjunction occurs during gametogenesis, the gametes will have either an extra chromosome (n+1) or one less chromosome (n−1). Fertilization of such gametes by normal gametes would result in two types of zygotes—trisomic (2n+1) or monosomic (2n−1). The trisomy or monosomy involving the sex chromosomes is usually associated with variable degrees of phenotypic abnormalities. The most common abnormalities of chromosome number are trisomies. Most individuals with trisomy exhibit a consistent and specific phenotype. These phenotypes depend on chromosome involved (Table 1).

The most frequent and best-known trisomy in human is Down's syndrome (trisomy 21) (Valenti *et al.*, 1968).

Table 1

Syndrome	Incidence	Clinical Manifestations
Trisomy 13 *Patau syndrome*	1/10,000 births	Cleft lip often midline, Flexed fingers with poliydactyly, Ocular hypodactyly, Bulbous nose, Low-set malformed ears, Small abnormal skull, Cerebral malformation, (holoprosencephaly) Microphthalmia, Cardiac malformation, Scalp defects, Hypoplastic or absent ribs, Visceral and genital anomalies
Trisomy 18 *Edwards syndrome*	1/6,000 births	Low birthweight, Closed fists with index finger overlapping the 3rd digit and the 5th digit overlapping the 4th Narrow hips with limited abduction, Short sternum, Rocker-bottom foot, Microcephaly, Prominent occiput Micrognathia, Cardiac and renal malformations and mental retardation 95% of cases are lethal in 1st year
Trisomy 21 *Down's syndrome*	1/600~800 births	Hypotonia, flat face,Upward and slanted palpebral fissures and epicanthic folds, Brushfield spots, Various degrees of mental and growth retardation, Dysplasia of the pelvis, Cardiac malformation, Short, broad hands, hypoplasia of middle phalanx of 5th finger, Intestinal atresia, High arched palate

Table 2

Deletion	Clinical Abnormalities
4p-of chromosomal part	*Wolf-Hirschhorn syndrome.* The main features are a typical "Greek helmet" facies with ocular hypertelorism, prominent glabella and frontal bossing. microcephaly, dolichocephaly, hypoplasia of the eye socket, ptosis, strabismus, nystagmus, bilateral epicanrhic fold, cleft lip and palate, beaked nose with prominent bridge, hypospadias, cardiac malformations, and mental retardation.
5p-	*Cri-du-chat syndrome.* The main features are hypotonia, short stature, characteristic cry, microcephaly, with protruding metopic suture, moonlike face, hypertelorism, bilateral epicanthic folds, high arched palate, wide and flat nasal bridge, and mental retardation.
9p-	The main features are craniofacial dysmorphology with trigonocephaly, slanted palpebral fissures, discrete exophthalmos, arched eyebrows, flat and wide nasal bridge, short neck with pterygium colli, genital anomalies, long fingers and toes, cardiac malformations and mental retardation.
13q-	The main features are low birthweight, failure to thrive, and severe mental retardation. Facial features include microcephaly, flat wide nasal bridge, hypertelorism, ptosis, and micrognathia. Ocular malformations are common. The hands have hypoplastic or absent thumbs and syndactyly.
18p-	A few patients (15%) are severely affected and have cephalic and ocular malformations, cleft lip and palate, and varying degrees of mental retardation. Most (80%) have only minor malformation and mild mental retardation.
18q-	The main features are hypotonia with "froglike" position with the legs flexed, externally rotated, and in hyperabduction. The face is characteristic with depressed midface and apparent protrusion of the mandible, deep-set eyes, short upper lip, everted lower lip ("carplike mouth"). Antihelix of the ears is very prominent. Varying degrees of mental retardation and belligerent personality.
21q-	The main features are hypertonia, Microcephaly, downward-slanting palpebral fissures, high palate, prominent nasal bridge, large low-set ears, micrognathia, and varying degrees of mental retardation. They may have skeletal malformation.

The occurrence of Down's syndrome as well as other autosomal trisomies increase with advancing maternal age (Munne *et al.*, 1995), however about 4.5% trisomy 21 cases are not due to age (Antonarakis *et al.*, 1993). The increased risk of this syndrome in woman older than 35 years warrants for PGD. Fetal chromosome analysis is a reliable way to detect fetal Down syndrome. In woman younger than 35 years, maternal serum testing (triple screen) can be effective in prenatal screening for Down syndrome. Low levels of alpha-fetoprotein, unconjugated estriol and human chorionic gonadotropin in maternal serum are indicators of Down's syndrome (Brambati and Simoni, 1983).

The Klinefelter syndrome is also well known as one kind of trisomy, but not autosomal trisomies. These individuals have a male karyotype with an extra X chromosome, 47, XXY, and phenotype is male (Abramsky and Chapple, 1997). The patient tends to be tall, slim, underweight and have relatively long legs. The testis tends to be small for the age, but this sign may become apparent only after puberty, when normal testicular growth fails to occur. Pubertal development may be delayed. Some degree of androgen deficiency is usually detected, although some children may undergo almost normal appearances. Most males with this condition go through life undiagnosed. The chromosomes should be examined in all patients suspected of having Klinefelter syndrome.

The Phenotypic medical examinations of monosomies (2n−1) are shown in Table 2. In humans, (Robinson *et al.*, 1992; Schrurs *et al.*, 1993; Verlinsky *et al.*, 1995) all complete autosomal monosomies appear to be lethal early in development and only survive in mosaic forms (Evsikov and Verlinsky, 1998; Delhanty *et al.*, 1993). Partial monosomies are usually the offspring of a translocation carrier (Scriven *et al.*, 1998).

The Turner syndrome is one of the most common monosomies in liveborn humans. The chromosome finding in Turner syndrome is a loss of part or whole of one sex chromosome (45, X) and the resultant phenotype is female (Munne *et al.*, 1993). Many patients with Turner syndrome are recognizable at birth, because of its characteristic edema of the dorsa of the hands and feet and loose skin folds at nape of the neck. Low birthweight and decreased height are common features. Clinical manifestation in childhood includes webbing of the neck, a low posterior hairline, small mandible, prominent ears, epicanthal folds, high arched

palate, a broad chest presenting the illusion of widely spaced nipples, cubitus valgus, and hyperconvex fingernails. The diagnosis is often first suspected at puberty when sexual maturation fails to occur. The mean adult height is 143～144 cm in United States and most of northern Europe, but 140 cm in Argentina and 147 cm in Scandinavia. The height is well correlated with the average of the parents' height. Specific growth curves have been developed for girls with Turner syndrome. Sexual maturation usually fails to occur, but 10～20% of girls have spontaneous breast development, and a small percentage may have menstrual periods. Chromosomal analysis must be considered in all short girls.

Chromosomal abnormalities include not only the chromosome number but also chromosome structure, these are as follows:

1. *Deletion*: an aberration in which a segment of a chromosome is missing; this may be a segment large enough to be detected under the microscope (macrodeletion) or so small that only sophisticated methods can detect it (microdeletion).
2. *Duplication*: an aberration in which a segment of a chromosome is repeated and thus is present in more than one copy within the chromosome.
3. *Rearrangement*: an aberration in which a segment of a chromosome is shifted within the same chromosome, sometimes turned upside-down (inversion) or transferred to another chromosome; the latter kind of rearrangement is called a translocation.
4. *Translocation*: an aberration in which a chromosome segment from one chromosome is transferred to another chromosome.

The karyotyping and banding techniques using human lymphocytes are basic tool to detect abnormalities of chromosome number and structure. Because these techniques permit identification of each chromosome as well as delineation of precise breakpoints and other subtle alternations. Recently new fluorescent chromosome banding technique that uses methyl green, a nonfluorescent AT-sensitive dye that efficiently absorbs green fluorescence, and YOYO-1 dye, a green fluorescent dimeric cyanine dye that stains chromosomes without significant sequence specificity are used. When stained with both of these dyes, chromosomes show a distinct fluorescent banding pattern in which G band appears dark against green signal. The use of these banding techniques permits identification of each chromosome as well as delineation of precise breakpoints and other subtle alternations.

A woman known to be carrying an X-linked disease with a 50% risk of an affected male in each pregnancy. In addition, her daughters have a 50% risk of being carriers, but are unlikely to be clinically affected. She may not wish to become pregnant if she has to make decisions about an affected child in a viable pregnancy. However, she would become pregnant if she knew she had conceived a daughter, and with pre-implantation diagnosis this possibility becomes a reality. PGD thus eliminates the need for possible pregnancy termination after prenatal diagnosis of a genetically-affected fetus.

Molecular diagnosis of genetic diseases

Identification of bands on chromosome still may not pinpoint the monogenic disorders and therefore more precise molecular methods were developed for diagnosis. The following methods are capable of locating gene defects and can give even the change in DNA sequence. In contrast to the earlier crude methods for detecting genetic abnormalities, FISH is a newer molecular biology technique that can give more precise information about the actual location of alterations or mutations on the DNA molecules for deletions and transpositions. This technique employs a DNA probe, which is a defined DNA sequence that binds to highly specific site on target chromosomal DNA by hybridization. Whole chromosomal probes are also available for detection of abnormal structure on chromosomes. These DNA probes are labeled with a fluorescent dye so as to visualize the particular sites along the chromosome where DNA probe enables to bind (Sansom, 2000). A growing number of laboratories that conduct diagnostic cytological analysis, at least among research hospitals and specialty clinics, are offering FISH-based tests as part of their standard repertoires and several companies are developing standardized FISH kits for detecting genetic abnormalities associated with specific diseases including pre-implantation genetics (Munne *et al.*, 1996).

PRinted *in situ* labeling (PRINS) technique, a primer, in which thymidine is replaced by dUTP labeled with aminomethyl coumarim acetic acid (AMAC) or fluorescein isothiocyaanate (FITC) or, amino actyl fluorine (AAF) or digoxigenin, anneals to a specific chromosomal locus where it is extended by *Taq* polymerase (Pellestor *et al.*, 1996). This process is repeated for numerous cycles, and resultant fluorescent signal can be detected under a fluorescence microscope. It is potentially more sensitive than FISH, especially for detecting unique sequences of less than 3-5 Kb, because multiple cycles of primer extension can be performed in this technique. In addition to this, PRINS technique can reduce the reaction time and background, and preserve the chromosome structure. And above all, this technique made it possible to study somatic cell chromosome. In 1994, PRINS has been shown to provide genetic information from single blastomeres (Gosden and Lawson, 1994).

DNA Chips: The newest and most promising tech-

nology for the accurate identification of DNA sequence alterations is the DNA chip. DNA chips are part of a new wave of miniaturized devices that offer extraordinary analytic versatility and sensitivity- in part, because they enable investigators to perform thousands of separate tests on the surfaces of silicon, glass or plastic chips that may be a mere one centimeter square. At the core of this technology is reliance on DNA probes-short-chain, single-stranded DNA fragments similar to those used in FISH. But in this case, hundreds or thousands of different DNA probes are synthesized and arrayed in a discrete regular grid on silicon chip surface, which are made with the help of techniques borrowed from the electronics industry (Wells and Delhanti, 2001). Many different kinds of DNA chips are now being made which are of particular use in fundamental research for exploring functioning of single gene or sets of genes in living cells. Others are being developed to suit specific applications, including those required for practical medical diagnosis (Dhiman *et al*., 2001). In most cases, however, the underlying principle remains same. DNA chips can detect mutations at the molecular level-that is, changes in DNA sequence- with exquisite sensitivity.

Southern blotting: One of the traditional techniques utilizing molecular hybridization is called Southern blotting. DNA is first digested with a restriction endonuclease and then separated according to the size of DNA fragments by electrophoresis through an agarose gel. Small fragments are able to migrate more quickly through the gel than long fragments. Most restriction enzymes will digest human DNA into about a million fragments. The DNA is first denatured to make single stranded by treatment with NaOH. It is then transferred to the surface of a nylon filter by blotting. And then, the fragments in filter are exposed by probe, which incorporating a label into a fragment of DNA from the target sequence. The label may be either a radioactive isotope (often ^{32}P) or a chemical hapten such as biotin. The single stranded probe is annealed to its target sequence which is then stringently washed to remove any probe which has bound other than by perfect formation of a long run of complementary hydrogen bonds. Finally, the position of the annealed probe is determined. This blotting technique is a fast way of analyzing a small number of DNA fragments, which may be present in a complex mixture. And it is not only the presence, but the size of the fragments is also detected.

Restriction Fragment Length Polymorphism (RFLP) was the first method developed to perform to produce different sized DNA fragments and, now it has been superseded by newly designed methods *e.g*. PCR, it still has important applications for some types of analysis. This procedure depends on two factors; the annealing of the probe which defines the locus, and the positions of the restriction enzyme recognition sites which produce the polymorphism. RFLP probes can be transferred from one cross to another and still mark the same locus, even though the polymorphism may be different. For this reason these types of markers have an important role to play as 'anchoring' markers to link different maps together. For example, if a particular RFLP is usually associated with a particular genetic disease, then the presence or absence of that RFLP can be used to counsel people about their risk of developing or transmitting the disease. The assumption is that the gene they are really interested in is located so close to the RFLP that the presence of the RFLP can serve as a surrogate for the disease gene itself. But people wanting to be tested cannot simply walk in off the street. Because of crossing over, a particular RFLP might be associated with the mutant gene in some people, with its healthy allele in others. Thus it is essential to examine not only the patient but as many members of the patient's family as possible.

Polymarase Chain Reaction (PCR): Another technique called PCR has become critically important to molecular genetics. This method permits one to enzymatically amplify a DNA sequence, using short synthetic DNA probes or primers. Hybridization is the process in which the labeled probe is allowed to anneal with the single stranded DNA on the filter. However the longer probe sequences increase both the time required and allow for a greater degree of discrimination. PCR primers are designed to be pretty much 100% specific and anneal in seconds, whereas hybridization probes require much longer, but can reveal similarity in sequence rather than complete homology.

PCR can be used to detect the presence of unwanted genetic material, as in the case of a bacterial or viral infection. Conventional diagnostic tests that involve the culture of microorganisms or use of antibodies can take weeks to complete or be tedious to perform. PCR offers a fast and simple alternative. For example, in the diagnosis of AIDS, PCR can be used to detect the small percentage of cells infected by the human immunodeficiency virus (HIV). DNA isolated from peripheral blood cells is added to a PCR reaction containing primers complementary to DNA sequences specific to HIV. Following amplification and gel electrophoresis, the presence of an appropriate-sized PCR product indicates the presence of HIV sequence and therefore, HIV infection. This technique is also being used for infertility testing.

The Ligase Chain Reaction (LCR): The point mutations are the most difficult to detect of all the genetic alterations. Nevertheless, there are quite a few techniques capable of recognizing the presence of single base changes in mammalian genes. The LCR is one technique that allows detection of single point mutations

in diseased genes. The technique utilizes a thermostable DNA ligase to ligate together perfectly adjacent oligos. Two sets of oligos are designed to anneal to one strand of the gene at the site of the mutation, a second set of two oligos anneals to the other strand. The oligos are designed such that they will only completely anneal to the wild-type sequences. For example, in the sickle-cell mutation, the 3' nucleotide of one oligo in each pair is mismatched. This mismatch prevents the annealing of the oligos directly adjacent to each other. Therefore, DNA ligase will not ligate the two oligos of each pair together. With the wild-type sequence the oligo pairs that are ligated together become targets for annealing the oligos and, therefore, result in an exponential amplification of the wild-type target. However prior sequence knowledge is required in order to detect point mutations in diseased genes, the LCR technique is utilized for the diagnosis of the presence of a mutant allele in high-risk patients.

Allele-specific oligonucleotide (ASO) hybridization: When the DNA sequence of a specific gene or mRNA is known, it can be used to synthesize an oligonucleotide probe that is only a few nucleotides long such as allele-specific oligonucleotide (ASO) probes. Hybridization with radioactively labeled ASO probe has been applied directly to the detection of specific point mutations. ASO hybridization is one of the direct methods for screening for a mutation. These probes can be used for any disorders in which the nucleotide sequence of the mutant and normal alleles are known. Under the right conditions (known as hybridization stringency), a complementary oligonucleotide probe that exactly matches the mutated sequence will anneal with that sequence, while a probe for the normal sequence will not. Heterozygotes for a particular mutation can be identified because both probes anneal with the DNA to an equal extent.

DNA Sequencing: Sequencing of DNA can be accomplished by either chemical or enzymatic means. The original technique for sequencing (Maxam and Gilbert, 1977), relies on the nucleotide-specific chemical cleavage of DNA. The enzymatic technique, Sanger sequencing (Sanger and Coulson, 1978), involves the use of dideoxynucleotides (2′, 3′-dideoxy) that terminate DNA synthesis and is, therefore, also called dideoxy chain termination sequencing. The Sanger DNA sequencing protocol utilizes dideoxynucleotides (ddNTPs) to terminate chain elongation during the *in vitro* synthesis of DNA from a cloned template. Synthesis is initiated using a specific oligonucleotide primer. During the synthesis reaction a radioactive nucleotide (usually dATP) is incorporated into the elongating strands. Four separate reactions are carried out simultaneously, each of which contains all 4 dNTPs and a single ddNTP. The higher the concentration of ddNTP the more frequently chain elongation will terminate. Therefore, one can regulate the extent of sequence information obtainable by varying the dNTP/ddNTP ratio. Following the extension reactions the products are resolved by electrophoresis in a denaturing (urea) polyacrylamide gel. The results are obtained when the gel is dried and exposed to x-ray film. Bands near the bottom of the gel represent short reaction products (ie closest to the 3′-end of the primer) and those near the top the longest products.

Conclusion

With the advancement of technology it is now possible to recover fetal or early embryonic cells for genetic analysis. Many sensitive techniques like FISH, PCR and other molecular biological techniques which can detect even signal abberations. With the gene replacement technology if this can be corrected then it is possible to produce a healthy baby. By now with the employment of new technologies babies having genetic disorders were treated and normal healthy babies were produced. However it would take time to put these techniques for general use.

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