Cystic fibrosis, Duchenne muscular dystrophy and preimplantation genetic diagnosis

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\textbf{Cystic fibrosis (CF) is the most common genetic disease among Caucasians. Duchenne muscular dystrophy (DMD) is one of the most common X-linked genetic disorders. The CF and DMD genes were discovered a few years ago, which provided the possibility for prenatal diagnosis and preimplantation diagnosis of CF and DMD by using specific DNA analysis. In this article, CF, DMD, the process of the identification of the genes of CF and DMD and preimplantation genetic diagnosis are briefly described.}

\textbf{Key words: cystic fibrosis/Duchenne muscular dystrophy/preimplantation diagnosis}

\textbf{Cystic fibrosis}

Cystic fibrosis (CF) is the most common genetic disorder among Caucasians. It is inherited as an autosomal recessive disease affecting between 1/2000 and 1/2500 children (Boat \textit{et al.}, 1995). Parents who are carriers have a 1 in 4 risk of having an affected child. It is estimated that ~30,000 people have the disease today. The disease is caused by mutations in the \textit{CFTR} (cystic fibrosis transmembrane conductance regulator) gene, producing a deficient protein leading to inadequate ion transport (Riordan \textit{et al.}, 1989; Rommens \textit{et al.}, 1989). The major clinical manifestations of CF are chronic pulmonary disease and pancreatic enzyme insufficiency. The patients also show increased sweat electrolyte concentrations which, although of little clinical consequence, have long been and still are a very reliable diagnostic test for the disease (sweat test; Gibson and Cooke, 1959; Hodson \textit{et al.}, 1983). All clinical symptoms are consistent with CF being an exocrine disorder. In males with CF, there is also an abnormality of the epididymis and vas deferens. In CF patients, these tubes end in blind channels instead of connecting through to the urethra. Approximately 97% of CF males have this problem from birth and, as a result, most of them are sterile, although they have normal spermatogenesis. There are no equivalent changes in the female reproductive tract but, occasionally, reduced quantities of the mucus that normally lubricates the female tract may be a cause of infertility in female CF patients.

Although the treatment of CF patients has considerably improved considerably over the past 30 years, most will die before the age of 30 years. Before the end of 1985, and despite extensive research efforts, very little was known about the basic defect causing CF. It became clear that the metabolic and biochemical abnormalities observed in epithelial cells of CF patients were secondary consequences of the primary defect. A first hint as to the identification of the CF gene was given by the demonstration of genetic linkage between CF and a polymorphic protein marker (Eiberg \textit{et al.}, 1985).

\textbf{The identification of the cystic fibrosis gene}

The idea of genetic linkage analysis to identify unknown genes by following the cosegregation between restriction-fragment-length polymorphisms (RFLP) recognized by cloned DNA sequences (probes) and the incidence of the disease in small nuclear families with two or more affected children was first proposed by Botstein...
The position of the CF gene was further refined by the discovery of two additional linked markers, \textit{MET} and \textit{D7S8} (Wainwright 	extit{et al.}, 1985; White 	extit{et al.}, 1985, Knowlton 	extit{et al.}, 1985). These markers were found to flank the CF gene, and the genetic distance between them was estimated to be \(1.6 \times 10^6\) bp. This region was further narrowed by the discovery of an expressed gene \(\sim 600\) kb from \textit{MET} and between \textit{MET} and \textit{D7S8} (Estivill 	extit{et al.}, 1987). This gene, \textit{IRP} (int-1 related protein), was originally proposed as a candidate for the CF gene, but it soon became clear that it was distinct, coding for a protein that is related to the murine oncogene \textit{int-1} and to the \textit{Drosophila} segment polarity gene \textit{wingless} (Wainwright 	extit{et al.}, 1988). Several DNA sequences have been subcloned from \textit{IRP} and the surrounding genomic sequences, all recognizing frequent RFLP. These RFLPs were in strong linkage disequilibrium with CF, but several recombinations between \textit{IRP} and CF were found (Farral 	extit{et al.}, 1988). The distance between \textit{IRP} and \textit{D7S8} (\(\sim 1 \times 10^6\) bp) was still too large to use standard chromosome-walking techniques through identification of overlapping cloned fragments of DNA. The gene was finally found by a combination of chromosome walking and chromosome jumping starting from \textit{D7S8} and from two newly discovered markers, \textit{D7S122} and \textit{D7S340}, localized between \textit{MET} and \textit{IRP} (Rommens 	extit{et al.}, 1989). In all, \(\sim 2.5 \times 10^5\) bp of genomic sequence were cloned before the 5' end of the CF gene was found. The gene codes for a transcript of \(\sim 6.5\) kb that is expressed in sweat glands, lungs and the pancreas (Kerem 	extit{et al.}, 1989; Riordan 	extit{et al.}, 1989). It contains 27 exons spread over a genomic DNA region of almost 250 kb. The amino acid sequence (1480 amino acids) was derived from the cDNA and its sequence gave a strong indication of the biochemical nature of the presumed protein, called CFTR.

CFTR shows considerable organizational and sequential similarities with a family of ATP-dependent transport systems, of which \(\sim 30\) were known to exist at the time of the cloning of the CF gene (Hyde 	extit{et al.}, 1990). This superfamily includes (i) the well-characterized periplasmic binding protein-dependent uptake systems of prokaryotes, bacterial exporters and eukaryotic proteins, including the P-glycoprotein associated with multidrug resistance (MDR) in tumours, (ii) the \textit{STE6} gene product that mediates export of yeast \(\alpha\)-factor mating pheromone, (iii) MDR that is implicated in chloroquine resistance of the malarial parasite and (iv) CFTR. In prokaryotes, the basic membrane complex consists in general of four protein subunits, two of which are highly hydrophobic and span the membrane five or six times; the other two are hydrophilic and are associated with the cytoplasmic site of the membrane. The hydrophilic subunits share considerable amino acid sequence similarity over much of their length and the conserved sequences include a potential ATP-binding site. The eukaryotic counterparts to these bacterial transporters all differ from them in that the individual protein subunits are combined into a single multi-domain polypeptide. However, the overall organizational and amino acid sequences are strongly conserved. CFTR has two hydrophobic domains, which comprise potential membrane-spanning helices, and two ATP-binding domains (Riordan 	extit{et al.}, 1989). Furthermore, CFTR includes an additional, large cytoplasmic domain (R-domain or regulatory domain) which, based on consensus phosphorylation sites for protein kinase C and cAMP-dependent protein kinase, probably serves a regulatory role. Although much work remains to be done in order to characterize CFTR more completely, it has been shown that it is a membrane-associated glycoprotein showing CF-channel activity that is regulated by phosphorylation of the R-domain by a cAMP-dependent protein kinase. Once phosphorylated, the channels require cytosolic ATP to open, and transport activation occurs by a protein-kinase-independent mechanism. The commonest mutation found in CF (\textit{AF508}, see below) was shown to have two major effects on the fixation of the CFTR CF-channel: the majority of the protein is not localized in the membrane, as it is for the wild-type protein, and the protein that reaches the membrane is mostly in a closed configuration (Li 	extit{et al.}, 1988; Chen 	extit{et al.}, 1989; Rich 	extit{et al.}, 1990; Anderson 	extit{et al.}, 1991; Cheng 	extit{et al.}, 1991; Dalemans 	extit{et al.}, 1991; Clarke 	extit{et al.}, 1992; Collins, 1992).

\emph{Mutation analysis}

The final proof that \textit{CFTR} was the gene associated with CF came from the identification of mutations that distinguish normal from affected individuals. In a first mutation study, a 3 bp deletion (CTT), predicting the loss of a phenylalanine residue at position 508 of the CFTR
polypeptide (ΔF508), was identified in exon 10 of the gene in almost 70% of CF alleles (Riordan et al., 1989). It was never found in known normal alleles, identified through linkage analysis in CF families. To date, >40 000 CF chromosomes have been analysed worldwide, and >66% of them carry the ΔF508 mutation (Cystic Fibrosis Genetic Analysis Consortium, 1990 and 1994, unpublished data).

The finding that one particular mutation was responsible for such a high percentage of all CF mutations suggested that there may have been some heterozygote selection or a strong founder effect for this mutation. Therefore, the original expectation was that only a few other mutations might account for the remainder of CF alleles. At present, >500 mutations have been described in CF patients. Most of them are rather rare, many having been identified in only a single individual; only 11 of them are represented by >100 mutated alleles (Cystic Fibrosis Genetic Analysis Consortium, 1994, unpublished data). Approximately half of the mutations in the CFTR are single amino acid substitutions (missense mutations). They are distributed all over the gene. The majority of the remaining mutations consists of nonsense mutations, frameshift mutations (insertion or deletion of a small number of nucleotides from the coding region of the gene) and splice-site mutations; these three classes of mutations are more or less equally present. Large deletions spanning multiple exons or the whole gene are probably very rare (Tsui, 1992a,b).

Almost 100 sequence variations within the coding region of the gene or within introns have also been identified (Tsui, 1992a,b). These polymorphisms might be useful for segregation studies in CF families.

**Congenital bilateral absence of the vas deferens and cystic fibrosis**

Congenital bilateral absence of the vas deferens (CBAVD) is an important cause of male infertility (Holsclaw et al., 1971). Since absence of the vas deferens is also noted in almost all CF males, the hypothesis that CBAVD males represent a mild or incomplete form or a genital form of CF without the characteristic lung and pancreatic symptoms has been proposed (Holsclaw et al., 1971). With the identification of the CF gene, it has become possible to study CBAVD patients for the presence of mutations in this gene. In all studies conducted so far (Dumur et al., 1990; Anguino et al., 1992; Gervais et al., 1993; Osborne et al., 1993; Williams et al., 1993; Chillon et al., 1995), the frequency of the detection of at least one CF mutation in CBAVD patients is significantly higher than expected on the basis of the carrier frequency of CF. In some of the patients, two mutations on different chromosomes have been found (compound heterozygote); these patients carry one mutation that is known to result in severe disease in CF patients and one mutation that is known to result in mild disease. This group represents ≤25% of CBAVD patients. In the remaining patients, either one mutation (≤40%) or no CF mutation could be found, although the whole coding region of the gene was studied. A defect in chloride conductance of the CFTR across the nasal epithelium was found in CBAVD patients, but this defect was intermediary between CF and normal. On the other hand, normal sodium transport was found (Osborne et al., 1993). These results indicate that the CFTR is probably involved in CBAVD, but other genes might also be implicated.

The fact that CBAVD men are often carriers of at least one CF mutation (or of an as yet unknown mutation in the gene outside the coding region but affecting the CFTR) has, however, important reproductive consequences, especially since the development of microsurgical sperm aspiration (MESA; Silber et al., 1987), in-vitro fertilization (IVF) and, more recently, intracytoplasmic sperm injection (ICSI; Palermo et al., 1992; Van Steirteghem et al., 1993a,b). Indeed, spermatogenesis is known to be normal in men with CBAVD and spermatozoa can be removed by MESA from the epididymal remnant. Couples in which the men have CBAVD and the women carry a CF mutation might have children presenting CF. Mutation screening in both partners and counselling about their risk of having a CF or CBAVD child is mandatory for these couples. When both partners are carriers of CF mutations, preimplantation diagnosis can eventually be carried out in order to transfer unaffected or carrier embryos (Handyside et al., 1992; Liu et al., 1994).

**Duchenne muscular dystrophy**

Duchenne muscular dystrophy (DMD) was first described over a century ago by Duchenne in his studies published in 1861 and 1868. Duchenne muscular dystrophy belongs to a group of muscular dystrophies which include X-linked DMD and Becker muscular dystrophy (BMD), Emery Dreifuss muscular dystrophies (also inherited on the X chromosome) and the autosomal dominant myotonic and facioscapulohumeral dystrophies. This disease shows an X-linked recessive pattern of inheritance, affecting ~1 in 3500 to 1 in 4000 male births (Harper, 1989).

The clinical features of DMD are age-related. In the preclinical stage of DMD, before there are any symptoms of the disease, muscle abnormality can only be found by histological examination. There are no obvious clinical features of DMD in infants. There is a delay in learning to walk: in more than half the affected boys, walking was delayed until at least 18 months of age (Emery, 1987).
Some affected boys show a more general delay in development, including speech. The onset of clinical symptoms of DMD are usually observed before school age (about 4 or 5 years of age). The age at onset is difficult to assess accurately because it is dependent on the description given by the parents. Muscle weakness can be noticed by the parents after an affected boy is able to walk. The pattern of muscle involvement results in several physical features associated with the DMD. By the age of 4 or 5 years, the typical waddling gait which is one of the clinical characteristics of DMD can be observed. (Not everybody that waddles has muscular dystrophy, for other conditions can also produce this type of gait, for example, spinal muscular atrophy.) Classical Gowers’ manoeuvre is another feature of DMD, which is due to the weakness of the knee and hip extensors. Gradually, the motor functions decline and by the age of 12 the children are wheelchair-bound. The age at which they become confined to a wheelchair has been found to be correlated significantly with age at death: generally, the earlier a boy becomes confined to a wheelchair, the poorer the prognosis (Emery, 1987). At the later stage, as the disease progresses and muscle weakness becomes more severe, movements of the elbows, knees, hips, shoulders and wrists become limited. Respiratory problems increase with the development of weakness of the intercostal muscles. The cardiac muscle can be also involved in advanced DMD. Cardiorespiratory problems, such as pneumonia, respiratory failure and cardiac arrhythmias, are the principal direct causes of death. The mean age of death of affected boys is ~16 years (Emery, 1987; Harper, 1989).

The diagnosis of DMD is based on the clinical symptoms, elevated concentrations of serum creatine kinase, muscle biopsy and DNA analysis. Unfortunately, no efficient treatment has so far been developed to cure DMD. Many efforts, such as exercise, physiotherapy, surgical correction and drug therapy, are made to try to improve the general condition or quality of life of affected boys.

Localization and identification of the Duchenne muscular dystrophy gene

Fifteen years ago, our knowledge concerning the localization of the DMD gene was limited to the fact that it was on the X chromosome, an observation based on the inheritance pattern of the disease. The first indication that the gene was on the short arm of the X chromosome came from reports of a small number of girls with a disorder similar to DMD (Greenstein et al., 1977; Verellen et al., 1977; Canki et al., 1979; Lindenbaum et al., 1979). In contrast to DMD boys who show no cytogenetic abnormalities, the DMD girls had a balanced translocation between the X chromosome and an autosome. The common features of the translocations were that the autosomal breakpoints were variable but that the X chromosome breakpoints were always located at band p21 on the short arm of the chromosome. Moreover, as no female cases of DMD were reported, it was also apparent that these cases were de novo, with no other patients in the family. These findings suggested that the X chromosome breakpoints were related to the function of the DMD gene and that the site of the breakpoints reflected the location of the gene (Verellen-Dumoulin et al., 1984; Ray et al., 1985; Boyd et al., 1986).

With the development of techniques such as flow cytometry to sort chromosomes according to their size and staining properties (Young, 1986), the construction of human–rodent hybrid cell lines, and the preparation of DMD libraries from them, it has become possible to isolate DMD fragments from the X chromosome. The first two probes isolated were RL8 (from short arm sequences of X chromosome; Murray et al., 1982) and L128 (from the total human DNA library; Maniatis et al., 1978), and these were used to detect linkage with the DMD gene. When these two probes were used in BMD families, clear evidence of linkage was also found, with distances comparable to those found for DMD (Kingston et al., 1983). These results suggested that BMD and DMD are allelic diseases that are caused by defects in the same gene or, alternatively, that the genes causing both diseases are close together. In the following years, many probes were produced covering the short arm of the X chromosome. Unfortunately, linkage analysis in DMD families demonstrated that the linked markers showed a relatively high frequency of recombination with the gene. This suggested that the existing markers were still too distant from the DMD locus and were not suitable as a starting point for the identification of the gene.

Another approach was used by Kunkel and colleagues (Francke et al., 1985; Kunkel et al., 1985) and was based on the study of the very few male patients that had been detected with visible cytogenetic deletions. One of these patients ‘BB’ has been studied in great detail. This patient had a visible deletion in the p21 region and besides DMD had mental retardation, chronic granulomatous disease, the McLeod red blood cell phenotype and retinitis pigmentosa. This combination of DMD and several other diseases suggested that they were determined by a series of closely linked genes that had been lost in this particular patient. He also provided the basis for the detailed molecular investigation of the deletion region. The importance of such patients is now recognized to be crucial in the fine mapping of many genetic diseases. In the study of patient BB, a method called phenol-enhanced reassociation technique (pPERT) was used. In this way, it was possible to obtain normal DNA sequences that were absent in patient BB. In
one of the DMD clones, pERT87, it was shown that ~7% of DMD patients had this region deleted, though with no obvious correlation between this deletion and the clinical features of the patients (Monaco et al., 1985). Interestingly, 2% of BMD patients also showed this deletion. However, pERT87 deletion was not detected in normal males.

Another DNA clone, XJ, isolated by the group of Worton et al. (1984), was also found to map physically in the p21 region and also to be deleted in some of the DMD patients. The XJ locus was found to be located centromeric to pERT87 and thus represented a separate point in the DMD gene. These studies, with the cloning of the pERT and XJ sequences, gave us a first idea of the DMD locus at the molecular level, and confirmed that DMD and BMD were determined by the same locus.

From 1984 to 1987, a series of molecular studies were successfully carried out in DMD patients or in manifesting carriers with X autosome translocation. An initial image of the DMD gene started to appear: the DMD gene was located on the short arm of the X chromosome at region p21; the length of DNA in which a genetic change caused DMD was extremely large, possibly ~2 × 10^6 bp. The remaining questions were whether there was one exceptionally large DMD gene, or several separate genes, or whether the function of the gene was susceptible to changes in neighbouring DMD regions. These questions were not answered until 1987. In that year, Kunkel’s group reported their results of cloning and sequencing a 14 kb human DMD cDNA, which was constructed using fetal muscle mRNA and corresponded to a complete copy of the fetal skeletal muscle transcript (Koening et al., 1987, 1988). In the same year, the product of this cDNA was found, a protein corresponding to a muscle cell membrane protein, called dystrophin, which plays a key role in maintaining the integrity of myofibrillar structure and function (Hoffman et al., 1987). Now, it is known that the gene defect responsible for DMD is located at Xp21; the gene itself spans ~2.4 Mb of DNA (Den Dunnen et al., 1989; Boyce et al., 1991), contains at least 75 exons of average size 200 bp and has introns of average size 35 000 bp (Koening et al., 1988; Love and Davies, 1989). So far, the DMD gene is known to be the largest human gene and is ~0.001% of the total human genome. DMD shows a high mutation rate, which is due to the remarkably large size of the DMD gene itself. In ~70% of DMD patients rather large gene deletions or duplications are responsible for their condition. Point mutations have also been described, but they are of course not easy to detect (Prior et al., 1993). In most instances, prenatal diagnosis can be offered to couples at risk. Either the deletion can be detected or, if not, the X chromosome bearing the mutation can be identified through linkage analysis (Feener et al., 1991; Clemens et al., 1991).

Research on the association between the type of DMD gene deletions observed and the clinical phenotype is continuing. Today, DNA analysis is regarded as an essential part of DMD diagnosis. DNA analysis is based on specific DNA probes to detect dystrophin gene deletion. More than 70% of affected males show partial DMD gene deletions, including several exons. Once a DMD gene deletion is found in a male DMD patient, the segregation of the deletion can be followed in his family for carrier detection and for prenatal diagnosis in carrier females using polymorphisms located in and close to the DMD gene (Chamberlain et al., 1988; Beggs et al., 1990; Clemens et al., 1991). The method of linkage analysis can be carried out to detect carriers throughout a family. In women carrying a known mutation, specific prenatal diagnosis can be provided in order to prevent the birth of an affected boy.

**Manifesting carriers**

Though rare, DMD has been diagnosed in females. In general, female carriers do not manifest clinical symptoms. Their serum creatine kinase concentrations, however, are increased. Manifesting carriers of X-linked recessive diseases may be the result of non-random X inactivation. This was the case in one of the two DMD girls who have contributed to the mapping and identification of the DMD gene (Verellen et al., 1977). In fact, both girls carried a X autosome translocation at the Xp21 locus. In these cases, most probably the non-translocated X chromosome is preferentially inactivated, and the translocated X chromosome carrying a disrupted DMD gene is expressed, which explains the clinical symptoms in these patients. The most common features of DMD in females are (i) the onset of symptoms usually occurs in adult life (later than males); (ii) manifesting carriers tend to show progression of muscle weakness (though progression is generally slow) and present greatly elevated amounts of serum creatine kinase; significant muscle weakness has been found in ~10% of adult female carriers.

**Preimplantation genetic diagnosis**

Preimplantation genetic diagnosis (PGD) is the technique whereby genetic diagnosis of an oocyte or an early cleavage-stage embryo is carried out before implantation. Preimplantation genetic diagnosis is a novel and still experimental procedure with only about 5 years’ history of clinical applications. There are still relatively few centres
where PGD is performed clinically. The first girls were born in 1990 after PGD by gender determination based on the presence or absence of a Y signal in couples at risk of having children with a variety of X-linked recessive diseases affecting only boys (Handyside et al., 1990). The first child after PGD of a single gene defect was born to a couple at risk for the CFΔF508 mutation (Handyside et al., 1992). Recently, at the Vth meeting of the International Working Group on Preimplantation Genetics, the current results of PGD were summarized (Harper, 1996). The data show that so far 197 PGD cycles have been carried out, and 50 pregnancies established in the world. Among these 50 pregnancies, three misdiagnoses have been reported from three centres, one after gender determination based on the presence or absence of a Y signal and two in cases of compound heterozygous embryos for unexplained reasons.

PGD can be applied to human embryos obtained through standard IVF but also through ICSI, which was introduced in 1991 at our Centre and has proved to be highly successful in the alleviation of severe male-factor infertility (Palermo et al., 1992; Van Steirteghem et al., 1993a,b). ICSI can be used with ejaculated, epididymal and testicular spermatozoa (Devroey et al., 1994; Tounaye et al., 1994). If PGD is to be carried out in a couple with male-factor infertility, ICSI should be used (Liu et al., 1994). This is certainly the case in patients with CBAVD, given that the majority of men with CBAVD carry CF mutations (Dumur et al., 1990; Anguino et al., 1992; Gervais et al., 1993; Williams et al., 1993). ICSI has another advantage for PGD since the oocyte is not incubated with several thousands of spermatozoa. The presence of spermatozoa on or in the zona pellucida may produce a false signal if, at the time of biopsy, some sperm DNA is removed together with the blastomere. Therefore, the policy at our Centre is now to carry out the ICSI technique on all patients requiring PGD.

So far, most clinical experience has been gained in diagnosis on blastomeres from a cleaving embryo. There are no reports concerning pregnancies after PGD on blastocyst-stage human embryos. The first polar body or second polar body has been biopsied for PGD, but the results are disappointing, since a rather low pregnancy rate has been observed. The main disadvantage of the use of blastomeres biopsied from early cleavage-stage embryos (usually 6-cell to 10-cell stage) is that only limited material, i.e. one or two cells, is available for diagnosis. Theoretically, blastomere analysis can be used for diagnosis of all kinds of genetic diseases and with different methods, such as polymerase chain reaction (PCR), fluorescent in-situ hybridization (FISH), or even a combination of PCR and FISH. The combination of PCR and FISH in single cells, called ‘cell recycling’, may enhance the accuracy of PGD since the method of cell recycling provides two opportunities of diagnosis in the same cell (Thorhill et al., 1994; Muggleton-Harris et al., 1995; Thornhill and Monk, 1996). It may also add more information for PGD, such as detection of the sex of embryos, sex chromosome or autosome aneuploidies and gene defects from single cells. If some technical problems, such as DNA contamination and cell loss are overcome, cell recycling may have great potential in PGD.

Besides cell recycling, the methods of primer extension preamplification and multiplex PCR, by which several gene sequences can be analysed from a single cell, will expand the possibility for PGD (Zhang et al., 1992; Avner et al., 1994; Kristjansson et al., 1994; Findlay et al., 1995a; Scobie et al., 1996). Primer extension preamplification is particularly useful for those inherited diseases caused by heterogeneous mutations in the genes involved, as it allows amplification of combinations of closely linked markers or amplification of multiple target sequences from a single cell.

It may also be the case that, during the early stage of human embryo development, embryos are not genetically uniform. Embryos can be mosaics with varying ploidy, trisomies or monosomies, and the same embryo can also contain some anucleate or some multinucleate blastomeres. Another problem is allele-specific non-amplification (Handyside, 1991). We observed discordant results from blastomeres of one embryo using the PCR assay for the analysis of the CF ΔF508 mutation (Liu et al., 1993). This might be due to failure of amplification of one of the parental alleles or failure to amplify both alleles correctly. This phenomenon is now called allele dropout (ADO). The exact reason for ADO is not clear. Several measures have been tested to try to eliminate this problem in single-cell PCR (Findlay et al., 1995b; Gitlin et al., 1996; Kontogianni et al., 1996; Ray et al., 1996). Therefore, it is recommended that two blastomeres are analysed per embryo in PGD using PCR assays. Only unaffected, or possibly carrier embryos for which the PCR assay results of two blastomeres are concordant, should be transferred. In this way, the chance of misdiagnosis can be greatly reduced.

Both PCR and FISH are used clinically for gender determination of embryos in PGD for prevention of X-linked recessive diseases. For PGD of X-linked recessive diseases by PCR, co-amplification of X-chromosome- and Y-chromosome-specific (X- or Y-specific) sequences is recommended and may prevent misdiagnosis resulting from the analysis of only Y-chromosome-linked repetitive or unique sequences from single cells (Kontogianni et al., 1991; Strom et al., 1991; Chong et al., 1993; Harper and
Handyside, 1994). However, the main drawback to using a strategy based on the co-amplification of X- or Y-specific sequences is the existence of aneuploidies which cannot be detected by PCR (Delhanty et al., 1993; Harper and Handyside, 1994; Munné et al., 1994). It seems that using the FISH technique to detect X and Y chromosomes is better than using PCR because FISH will supply additional information about sex chromosome aneuploidies (i.e. XXY or monosomy X); also, using FISH, the problem of DNA contamination is avoided (Delhanty et al., 1993; Munné et al., 1994). These advantages of FISH cause it to be favoured in the PGD of X-linked diseases if there is no known specific mutation available. It also has considerable potential for use in PGD of several chromosomal abnormalities, including screening for trisomies in older women who undergo IVF treatment. Recently, the FISH technique has been shortened, and the results can be obtained in a few hours (Coonen et al., 1994), which is very useful for PGD since timing is important. In women carrying a known DMD gene deletion, specific PGD using PCR can now be carried out and allows the transfer of unaffected male embryos as well as normal and carrier female embryos (Kristjansson et al., 1994; Liu et al., 1995). The first child after PGD for a DMD gene deletion was born in 1994 (Liu et al., 1995). However, when no DMD gene deletion can be detected in carrier females, gender determination of the fetus or preimplantation embryos may be the only means of prenatal diagnosis in order to avoid having an affected boy.

Since PGD requires the techniques of micromanipulation, assisted reproductive technology and molecular genetic diagnosis, which are specialized and expensive, PGD of specific genetic disorders has so far been applied only to a limited number of couples, particularly to those who oppose abortion or have already had an affected fetus or child, or to those who also have an infertility problem. Because only very limited cell numbers are available for PGD, precautions have to be taken in order to obtain a reliable result. The data also teach us that PGD based on the genetic analysis of one or two cells biopsied from cleavage-stage embryos may lead to misdiagnosis. Conventional prenatal diagnosis is therefore still necessary to confirm the PGD results. Furthermore, it is very important to follow up carefully the children born after PGD. Considering the technical difficulties and the current status of PGD, fundamental research is still needed, and it will be some time before PGD can be used widely in routine clinical practice.

References


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