PGD for dystrophin gene deletions using fluorescence in situ hybridization

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Duchenne muscular dystrophy and Becker muscular dystrophy (DMD and BMD) are caused by mutations in the dystrophin gene (Xp21). In two-thirds of DMD/BMD cases, the mutation is a large deletion of one or several exons. We have established PGD for DMD/BMD using interphase fluorescence in situ hybridization (FISH) analysis on single nuclei from blastomeres for the detection of deletions of specific exons in the dystrophin gene. We performed PGD for two carrier females; one had a deletion of exons 45–50 (DMD), and the other had a deletion of exons 45–48 (BMD). An exon 45-specific probe was used in combination with probes for the X and Y centromeres. Using this straightforward approach, we can distinguish affected and unaffected male embryos as well as carrier female and normal female embryos. Three cycles were performed for each patient, which resulted in a pregnancy and the birth of a healthy girl. To the best of our knowledge, this approach for PGD has not been previously reported. The use of interphase FISH is an attractive alternative to sexing or PCR-based mutation detection for PGD patients with known deletions of the dystrophin gene.

Key words: Becker muscular dystrophy/Duchenne muscular dystrophy/dystrophin gene/fluorescence in situ hybridization/PGD

Introduction

Duchenne muscular dystrophy and Becker muscular dystrophy (DMD and BMD) are caused by mutations in the dystrophin gene located on the short arm of the X chromosome (Xp21). The gene is one of the largest in the genome and encompasses 2.6 Mb, including 79 exons. The encoded protein, dystrophin, has a major structural role in muscles and links the internal cytoskeleton to the extracellular matrix. DMD is the most common myopathy in children and affects 1 in 3500 boys. It is caused by a lack of dystrophin, which leads to progressive degeneration of the muscles. The clinical onset is usually before school age (4–6 years), and the disease progresses with the loss of ability to walk around 10–12 years of age, when the patient becomes wheelchair bound. Respiratory insufficiency later results in death around 25–30 years of age.

BMD is a milder form of the disease and is caused by a reduction in the amount or an altered dystrophin protein. The onset is between 2 and 20 years of age, and the disease progression is usually slower. However, the clinical picture varies, and some BMD patients resemble DMD patients, whereas others are still able to walk at the age of 60. In two-thirds of DMD/BMD cases, the mutation is a large deletion of one or several exons. In the remaining one-third, point mutations or small deletions or duplications can be found. In one-third of the cases with DMD, the mutation occurs de novo, whereas in two-thirds of the cases, the mother of the affected male is a heterozygous carrier.

Female carriers of DMD/BMD mutations have a 50% risk of transmitting the mutation to their offspring, of which boys will be affected and girls healthy carriers. Consequently, the total risk of having an affected child is 25%. Prenatal diagnosis after chorionic villus sampling may be performed by mutation or linkage analysis (Beggs et al., 1990; Hentemann et al., 1990; Kim et al., 2002). In doubtful cases, fetal biopsies have been conducted. As an alternative, PGD for X-linked disorders was introduced in 1989 (Handyside et al., 1990). The sex of the embryos was first detected using PCR, but this method has now almost exclusively been replaced by interphase fluorescence in situ hybridization (FISH) detection of the sex chromosomes, which is a quick and reliable method with less risk of misdiagnosis. Embryo sexing for different sex-linked disorders, including DMD/BMD, is now being offered at most PGD centres worldwide. However, couples who choose PGD and sexing will only be able to have girls, of which half will be carriers. PGD with mutation analysis using PCR for the detection of deleted exons has previously been performed in a few cases (Hussey et al., 1999; Ray et al., 2001; Girardet et al., 2003).

We have for the first time used interphase FISH analysis on single nuclei from blastomeres for the deletion detection of specific exons in the dystrophin gene in two patients at risk of having children with DMD and a severe form of BMD, respectively. Six PGD cycles were performed, which resulted in one singleton pregnancy and the birth of a healthy girl.

Materials and methods

The study was approved by the Research Ethics Committee of Karolinska Institutet.

Case reports

DMD patient

A 30-year-old woman had an elder brother who was diagnosed with DMD and died when he was 20 years old. The brother had a deletion of exons 45–50 in...
the dystrophin gene, and his sister was diagnosed as a carrier of the same dele-
tion. She and her husband were in need of IVF because of unexplained infertil-
ity and wanted to include PGD in the IVF procedure to minimize the need for
pregnancy termination if she became pregnant.

**BMD patient**

A 37-year-old woman had in a previous marriage, two sons that were diag-
nosed with severe, early-onset BMD. Genetic investigation of the boys showed
the deletion of exons 45–48 in the dystrophin gene, which makes their mother
an obligate carrier. Owing to several years of infertility in the present marriage,
the couple requested IVF and PGD.

**PGD**

**IVF**

Controlled ovarian hyperstimulation (COH) was performed with recombinant
FSH (rFSH) in a long-protocol GnRH agonist (GnRHa) cycle. When at least
two follicles ≥18 mm were seen on ultrasound scan, 10 000 IU of hCG was
injected subcutaneously, and 37 h later, oocyte retrieval was performed. Two
days after oocyte retrieval, vaginal micronized progesterone (1200 mg daily)
was added and continued until pregnancy test 18 days later.

Oocytes were fertilized; the zygotes were transferred into sequential culture
media (G1.2, VitroLife, Gothenburg, Sweden) or BlastAssist 1 (MediCult,
Jyllinge, Denmark) and individually cultured in microdroplets under oil. Cumu-
lus cells were removed by hyaluronidase treatment (Vitrolife) before ICSI.

**Blastomere biopsy**

Biopsies were performed on 6–10 cell embryos, early on day 3 after oocyte
retrieval, using a single-needle approach (Inzunza et al., 1998). If possible, two
cells were removed from each embryo. After the biopsy, the embryos were
kept in culture until the results of the genetic analysis were evaluated. Embryo
transfer, of up to two embryos, if available, was performed on day 4 after
oocyte retrieval. For details regarding fertilization, embryo culture and biopsy,
see Iwarsson et al. (1998).

**Blastomere spreading**

After biopsy, blastomeres were washed in phosphate-buffered saline (PBS)
and transferred into a drop of spreading solution on a poly-L-lysine-coated
slide (Sigma, St Louis, MO, USA). The blastomere was observed under an
inverted microscope until the nucleus was free of cytoplasm. The slides were
left to dry, then washed in PBS and dehydrated through an alcohol series.

**FISH analysis**

PGD was performed by FISH using a cosmid probe specific to exon 45 in the
dystrophin gene (cPT1 ex45), in combination with probes for the X and Y cen-
tromeres. Several exon-specific probes from the dystrophin gene were kindly
provided by Dr den Dunnen, Faculty of Medicine, Department of Genetics,
Leiden, The Netherlands. The exon 45 probe cPT1 ex45 was chosen as the
optimal probe to detect the deletion in both patients. The probe was labelled
with fluorescein isothiocyanate (FITC) by nick translation and after precipita-
tion dissolved in 50% formamide, 2× SSC, 50 mM phosphate buffer pH 7.0 at
a probe concentration of 4–5 ng/μl. In addition, 2–3 μg of Cot-1 DNA (Invitro-
gen, Lidingö, Sweden) was added to the probe mixture. After denaturation
at 73°C for 5 min, the probe mixture was left to prehybridize at 37°C for 1 h.
After prehybridization, denatured probes CEP X (Spectrum Aqua) and centro-
mere Y (Spectrum Orange) were added (Vysis, Downers Groove, IL, USA).
The slides with blastomeres were incubated for 20 min in 100 ml of 0.01 M
HCl containing 10 μg of pepsin and then fixed in 1% formamide. After dehy-
dration, the slides were denatured at 73°C for 5 min, and the probe mixture was
applied to the slides. Hybridization was performed in a moist chamber at 37°C
overnight, after which the slides were washed in 2× SSC at 72°C for 5 min.
After dehydration, the slides were mounted in glycerol containing 1.4-
diazabicyclo-(2,2,2)-octane (DABCO) as antifade and 4,6-diamino-2-phenyl-
indole (DAPI) at 0.5 μg/ml as counterstain. The signal was visualized using a
Zeiss Axioskop fluorescence microscope equipped with a cooled CCD-camera
(Cool SNAP HQ, Photometrics, Tucson, AZ, USA), controlled by a Macintosh
Quadra 950 computer. Grey scale images were captured, pseudocoloured and
merged using the SmartCapture software (Digital Scientific, Cambridge, UK).

Metaphase slides from lymphocyte cultures of both parents were analysed to
evaluate the likelihood of mis-scoring carrier females and affected males. The
hybridization efficiency of the exon 45 probe was 94%. The risk of diagnosing
an affected male as unaffected or a carrier female as a non-carrier was 4% when
only one cell was examined and 0.16% when two cells were examined.
Therefore, to minimize the risk of misdiagnosis, we performed two-cell biopsy
when possible.

**Results**

We have performed PGD for two patients who were both carriers of a
deletion (including exons 45–48) in the dystrophin gene. Both women
were in need of IVF and opted for PGD to avoid the birth of affected
sons. To identify a suitable probe for the detection of the deletions, we
used FISH probes specific to exon 45 and exons 46 and 47 in the dys-
trophin gene, hybridized to metaphase chromosomes of the carrier
females (Figure 1). For both carriers, the exon 45-specific probe
showed a strong, specific signal that was deleted on one of the X chro-
mosomes. The probe was used for PGD, in combination with probes for
centromeres X and Y.

The DMD patient went through three treatment cycles. During the
first cycle, 10 embryos were biopsied, and results were obtained for
eight embryos—two affected males, two unaffected males, three carri-
er females and one non-carrier female (Figure 2). Two of the una-
affected embryos were transferred on day 4, but no pregnancy was
established. The other unaffected embryos were not frozen because of
bad quality on day 4. During the second cycle, no embryos were avail-
able for biopsy. During the third cycle, only two embryos underwent
biopsy. One embryo was transferred on day 4 after oocyte retrieval,
resulting in a pregnancy and the birth of a healthy girl.

The BMD patient went through three PGD cycles. Although there
were generally very few embryos for biopsy, she got at least one
embryo to transfer each cycle. During the first cycle, five embryos
were biopsied, and three of these were diagnosed as healthy—two car-
rrier females and one healthy male. Two of these were transferred, but
no pregnancy was established. During the second cycle, one embryo
was biopsied and diagnosed as healthy and suitable for transfer, but
no pregnancy was established. During the third cycle, two embryos were
diagnosed and one healthy embryo was possible to transfer, but no
pregnancy was established.
Discussion

We have performed PGD for DMD/BMD with FISH for the detection of deletions in the dystrophin gene. To the best of our knowledge, this has not been previously reported. As for many other sex-linked disorders, the most simple and straightforward way to avoid a pregnancy with an affected boy is to perform sex determination of the embryos and to transfer solely female embryos. It is a rapid and reliable test that is easy to perform without the need to establish mutation detection in single cells and can be offered even if the mutation has not been possible to identify in the female. The main disadvantage of that procedure is that half of the male embryos that are discarded are in fact healthy, and as a result, the patient will only be able to have girls, of which half are carriers.

The establishment of a reliable PGD assay for DMD/BMD with the detection of the specific mutation is delicate and time consuming. However, multiplex PCR amplification of different exons in the dystrophin gene in combination with gender determination has been performed for PGD of DMD (Hussey et al., 1999; Ray et al., 2001; Girardet et al., 2003). The assay developed by Ray et al. has the advantage of detecting most deletions (70%), and thus the same assay could be used for a large number of patients. However, PCR methods suffer from the disadvantage of having a risk for misdiagnosis because of contamination or allelic dropout. The introduction of linked polymorphic markers in combination with exon amplification may facilitate the detection of contamination and confirms the diagnosis (Findlay et al., 1995). Indirect linkage analysis has also been used for DMD (Lee et al., 1998) and could also be an alternative for PGD.

Another method, which has been used to screen for both deletions and duplications in the dystrophin gene, is multiplex ligation-dependent probe assay (MLPA) (Gatta et al., 2005). However, this method has not been applicable in single-cell analysis and will probably require a whole-genome amplification prior to MLPA analysis before it could be used for PGD.

We have established deletion detection by FISH for the diagnosis of DMD/BMD by using exon-specific probes. This approach allows the detection of both deletions and duplications (Rosenberg et al., 1998; Ligon et al., 2000), as well as the identification of healthy carriers, carrier females, affected males and unaffected males. It is a reliable and straightforward PGD strategy that can be used for carriers of DMD/BMD with an identified deletion. The advantage is that unaffected male embryos may be transferred, a lower risk for misdiagnosis as compared with deletion detection by PCR, and in addition, the carrier status of the female embryos can be determined without the need for quantitative PCR. However, not all carrier females can be offered PGD by this strategy because of deletions that are not possible to detect by FISH or point mutations in the gene. One advantage of the method may also be an ethical disadvantage. The detection of female carrier embryos may pose an ethical dilemma. A carrier will be just as healthy as a non-carrier but will, on the contrary, have the same risk of having affected sons as her carrier mother. The detection of a female carrier during traditional prenatal diagnosis would in most cases not result in a termination of the pregnancy. If we decide not to transfer these embryos, we will therefore take a further step down the ‘slippery slope’ and increase the selection. In our two cases, the parents opted to make no difference between the female embryos, and only affected male embryos were not considered for transfer. The choice as to which of the remaining embryos should be transferred was made on morphological grounds by the embryologist.

In conclusion, PGD with FISH for the detection of deletions in the dystrophin gene is an attractive alternative to sex selection or PCR-based mutation analysis. This is the first time it has been reported in clinical practice.

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References


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