Well-devised quantification analysis for duplication mutation of Duchenne muscular dystrophy aimed at preimplantation genetic diagnosis

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Abstract *Purpose:* Preimplantation genetic diagnosis (PGD) has been performed for deletion and point mutation type of Duchenne muscular dystrophy (DMD). Our aim was to develop a PGD technique, not yet established, to directly detect duplication mutation instead of substitute diagnosis similar to gender determination.

Methods: Our method is based on comparative quantification using conventional duplex PCR, real-time PCR and gender determination. We evaluated this method in single lymphocytes from a duplication type of DMD patient and a normal male.

Results: There was a significant difference in the mean values of the ratios (the mutation locus/a normal reference): mean value $\pm$ SE was 1.84 ± 0.15 in the duplication patient, and 1.00 ± 0.09 in the normal male ($p < 0.001$).

Conclusion: It is suggested that our comparative quantification method could be a new option in PGD for carriers with duplication mutation who wish to have an unaffected son.

Keywords DMD · A single cell · Duplication · Real-time PCR · Quantification

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Introduction

Duchenne muscular dystrophy (DMD) (NIM #310200) is a lethal X-linked recessive disorder affecting 1 in 3,500 male births [1]. Few efficient treatments have been developed so far for DMD, and genetic treatment research continues to be performed [2, 3]. Genetic counseling and molecular diagnosis that allow the identification of carriers are therefore the only solutions offered to DMD families. DMD is caused by mutations in the dystrophin gene located at Xp21.2, which encodes protein of the membrane cytoskeleton in skeletal muscle (dystrophin) [4]. The dystrophin gene is the largest encoding gene found in nature to date, measuring 2.4 Mb and containing 79 exons that encode 14 kb mRNA, and probably accounts for the high frequency and high proportion of de-novo mutations [5]. Two-thirds of DMD mutations are deletions, with the remaining mutations consisting of large duplications and point mutations [6].

Preimplantation genetic diagnosis (PGD) is an evolving technique that provides a practical alternative to prenatal diagnosis and termination of pregnancy for couples who are at substantial risk of transmitting a serious genetic disorder to their offspring [7]. Samples for genetic testing are obtained from oocytes or cleaving embryos after in vitro fertilization (IVF) [7]. Only embryos that are shown to be free of the genetic disorders are made available for replacement in the uterus, in the hope of establishing a pregnancy [7]. In the mid-1980s, the advent of polymerase chain reaction (PCR) provided a far superior method for genetic testing, making it possible to carry out a diagnostic test on highly concentrated and relatively pure amplified PCR fragments that spanned the appropriate genetic mutation [8]. Substantial groundwork for the clinical application of PGD to various conditions was

undertaken in the late 1980s. The first pregnancies that resulted from the transfer of embryos that had been genotyped as female were reported in 1990 [9], and the first live birth was reported following PGD for cystic fibrosis in 1992 [10]. However, the majority of PGD for heterozygous female patients have so far consisted of the positive selection of female embryos [11]. The main disadvantage of this approach is that half of the discarded male embryos are unaffected. Accordingly, allelic diagnosis by direct detection of a mutant gene has also been performed in the course of PGD [12].

PGD for DMD has also been performed by gender determination when no specific PCR test was available, but recently techniques have been developed that can be used for direct detection of mutation. The first specific PGD for DMD was performed for a family carrier of a deletion encompassing exon 17 of the dystrophin gene, through the study of this exon in single blastomeres [12]. There are several reports that describe a single cell multiplex PCR protocol allowing the analysis of dystrophin gene combined with gender determination [11, 13, 14]. More recently, Girardet et al. [15] clinically applied this approach to PGD for a family displaying a large deletion extending from exon 21 to exon 34 of the dystrophin gene. Deletions in the dystrophin gene that account for approximately 60% of mutations responsible for DMD are diagnosed by single cell multiplex PCR. The remaining cases are due to point mutation (25–30%) [16] or duplications (5–10%) [5, 17], but there have been no reports of PGD for DMD to the best of our knowledge. PGD for point mutation was performed for familial amyloidotic polyneuropathy (FAP) by nested PCR and single-stranded conformational polymorphism (SSCP) analysis [18], and for β -thalassaemia using sequencing of single cell PCR products [19]. Meanwhile, only a few reports exist concerning PGD for duplications [20, 21], of which the detection is commonly possible using DNA probes located in the duplicated region (FISH or Southern blotting method). Pregnancy after PGD for Charcot-Marie-Tooth disease type 1A caused by a duplication of a 1.5 Mb region was first reported in 1998, in which mutations were detected by fluorescent PCR with a polymorphic (CA)_n marker localized within the duplication [22]. In cases where polymorphic (CA)_n markers are localized out of the duplication, however, diagnosis has to rely on linkage analysis that does not involve direct detection of mutations.

The ethical committee of the Japanese Society of Obstetrics and Gynecology (JSOG) has been carefully considering the clinical enforcement of PGD, especially for DMD within the context of Japanese history and society. From an ethical aspect, normal embryos should not be discarded on the basis of gender due to the fact that embryos are considered as precious form of life. From a technical aspect, there is a need not only for high diagnostic accuracy but also, as a consequence, for a specific method to clarify gene mutation.

Table 1 Normal reference in comparative quantification: the ratio between the duplicated exon and the normal reference

Normal reference	A normal male XY (1)*	A DMD patient XY (2)*	A normal female XX (2)*	A DMD carrier XX (3)*
Autosome	0.5(1/2)	1(2/2)	1(2/2)	1.5(3/2)
X chromosome	1(1/1)	2(2/1)	1(2/2)	1.5(3/2)
Y chromosome	1(1/1)	2(2/1)	Y(–)	Y(–)

*Number of duplicated exon copy.

X: the X chromosome with the duplicated exon.

In previous deliberations of the JSOG, the substitute diagnosis including gender determination for X-linked genetic disorders has been dismissed. In addition, each PGD case requires JSOG approval. It will be necessary to overcome the formidable hurdle that this decision represents, even for duplication mutation for PGD. We aimed to develop a PGD technique for direct detection of duplication mutation.

Quantitative real-time PCR has evolved over the last few years as a valuable tool in molecular diagnosis. We examined whether a single cell specimen can be quantified by using real-time PCR, and also tested a comparative quantification method: conventional multiplex PCR followed by quantitative real-time PCR using the unspecific dsDNA dye SYBR green 1. At the same time, we used gender determination to simplify discrimination of the normal, the carrier, and the affected, because comparison within the dystrophin gene on X chromosome is more complicated than comparison between the mutant locus on X chromosome and a normal reference on Y chromosome (Table 1). This approach for DMD patients with a large duplication in the dystrophin gene was technically improved in this study to the clinically available level of PGD for the carrier of duplication mutation. This study has obtained the approval of the institutional ethical committee of Keio University School of Medicine.

Materials and methods

Sampling of single lymphocytes

Venous blood samples from a normal male, and a DMD patient who was 27 years old and was proved to have mutation of exon 8–9 duplication by Southern blot analysis, were collected into heparinized tubes. Blood from the DMD patient was drawn from the internal carotid vein after informed consent was obtained at their bedside because their limb muscles and peripheral vessels were atrophic and made it difficult to collect blood samples. Blood was then diluted 2-fold with Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL, France), layered over Lymphoprep (NYCOMED PHARMA

AS, Norway) and centrifuged at 1600 rpm for 10 min. The interface containing the mononuclear cell fraction was collected and cells were washed three times with PBS. The cells were then resuspended in 1 ml Roswell Park Memorial Institute (RPMI1640) medium (Invitron, France) containing 20% fetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO)(Sigma) and frozen at -80°C until further processing. At the same time, Epstein-Barr-transformed lymphoblast cell lines were established to avoid the repeated drawing of blood. For isolation of single lymphocytes, an aliquot of the cell suspension was thawed and $1\text{ }\mu\text{l}$ was added to 4 ml of 90% RPMI1640 supplemented with 10% FCS in a culture plate. Individual lymphocytes were deposited in $4\text{ }\mu\text{l}$ of lysis solution 1 (0.2% sarcosyl, TE containing 10 mM EDTA) [23], with a mouth-controlled glass microcapillary under an inverted microscope. The cells were then denatured at 65°C for 10 min and kept frozen at -20°C until the PCR procedure.

Single cell multiplex PCR

A multiplex PCR strategy was designed so that the ratio between the test exon (mutation locus) and a normal reference could be compared. We chose exon 8 of the dystrophin gene as the test exon and the SRY gene of the Y chromosome as the normal reference (both are single copy genes). The primers of exon 8 have been previously described [24, 25] on the Leiden Muscular Dystrophy pages (<http://www.dmd.nl/>), and the primers of the SRY gene have been previously described [26] (Table 2). The first PCR was performed by a duplex PCR of exon 8 and the SRY gene for preparation of the next quantification and gender determination procedure.

The first round PCR reaction contained $4\text{ }\mu\text{l}$ of lysis solution 1, $6\text{ }\mu\text{l}$ of lysis solution 2 (50% glycosyl, 10 mM Tris-Hcl, pH 8.0, 1 mM EDTA, pH 8.0, 10 ng/ μl poly A, 10 mM ascorbic acid), $5\text{ }\mu\text{l}$ of 25.0 mM MgCl_2 solution, $5\text{ }\mu\text{l}$ of 8 mM dNTP mix, $2.5\text{ }\mu\text{l}$ (10 pM) each of the two pairs of primers, 2.5 U AmpliTaq Gold and water in a total

volume of $50\text{ }\mu\text{l}$. PCR was carried out on a GeneAmp 9700 thermocycler (Applied Biosystems, USA) using the following program: 10 min denaturing at 95°C , then 25 cycles of 30 s at 95°C , 30 s at 60°C , 30 s at 72°C reactions, followed by an extension phase at 72°C for 7 min.

Real-time quantitative PCR

One microliter of the first round PCR products was then aliquoted into two separate tubes for each of the individual second round real-time PCR. Primers were designed according to the manufacturers' recommendations with the Primer Express software version 2.0 (Applied Biosystems, USA) within the first PCR products (Table 2). All primers were conformed so as not to form primer dimer and hair-pin structure.

The PCR reaction mix consisted of SYBR Green PCR Master Mix (Applied Biosystems, USA) ($25\text{ }\mu\text{l}$), 900 nM ($4.5\text{ }\mu\text{l}$) forward primer, and 900 nM ($4.5\text{ }\mu\text{l}$) reverse primer. One microliter of the first round PCR products was transferred to this PCR reaction mix, and individual real-time PCR was carried out using the ABI PrismTM 7000 sequence detection system and 96-well MicroAmpTM optical plates (Applied Biosystems, USA). The pre-run thermal cycling conditions were 10 min at 95°C , the thermal cycling conditions were 40 cycles at 95°C for 15s and 65°C for 1 min.

Comparative Ct method, as previously described by Livak [27], detected relative gene expression, or, in our case, the relative gene number. The fractional cycle number (Ct) at which the amount of amplified target reaches a fixed threshold is directly related to the amount of starting target. The ratio of the amount of the first PCR products is determined by measuring their Ct values. The Ct value was analyzed using the Sequence Detection System (SDS version 1.1, Applied Biosystems, USA). The following formula can be used to show the gene copy number: $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{the patient male}) - \Delta\text{Ct}(\text{a normal male})$, and $\Delta\text{Ct} = \text{Ct}(\text{the test exon}) - \text{Ct}(\text{a normal reference})$. The ratio between the DMD patient and the normal male was determined by calculating the expression $2^{-(\Delta\Delta\text{Ct})}$. This formula was based on the assumption that the rate of Ct change versus the rate of target DNA copy change was identical for both genes, and also that doubling of the target gene copy number resulted in a 1-cycle decrease in the measured Ct. Subsequently, the ratios between the test exon (the mutation locus) and the normal reference were calculated: the ratios of 1.0, 1.5, and 2.0 theoretically indicate a normal male/female, a carrier, and a patient with duplication, respectively. The mean values and distribution of the patient male and the normal male were statistically analyzed with Student's t-tests.

Table 2 Primer sequences

Name	Sequence	Size
Exon 8 of the dystrophin gene		
Outer forward	GGCCTCATTCTCATGTTCTAATTAG	360
Outer reverse	GTCCTTTACACACTTTACCTGTTGAG	
Inner forward	CACTCTTCCAAGTTTTGCCTCAA	63
Inner reverse	GCAACATTTCCACTTCCTGGAT	
The SRY gene		
Outer forward	GTTGTCCAGTTGCACTTCGCTGCA	351
Outer reverse	CAGTGTGAAACGGGAGAAAACAGT	
Inner forward	AACGCATTCATCGTGTGGTC	109
Inner reverse	GCATTTTCCACTGGTATCCCA	

Results

Duplex PCR from single cells

Conventional duplex PCR from single lymphocytes was performed with two pairs of primers: one pair for the mutation locus (exon 8), and the other for the normal reference locus and gender determination (SRY gene). A single lymphocyte is taken to contain 4–10 pg of DNA and is considered to be insufficient for quantification in real-time PCR alone. Single lymphocyte must first be amplified by conventional PCR before quantification, and the product amount should be within the dynamic range of the next real-time PCR (1–1000 ng). First of all, we optimized the PCR condition in the number of cycles. Because plateau of amplified DNA product exists in the latter phase of PCR amplification cycles, the first PCR must be completed in the exponential phase before it reaches plateau. As a preliminary experiment, we examined the amplification curve in real-time PCR using single lymphocytes. Due to instability in amplification curve from a single lymphocyte alone, we analyzed 10 lymphocytes together in one tube. It revealed that the PCR reaction was characterized by an exponential increase in the amount of the PCR product below 35 cycles (data not shown). Too much amplification in the first PCR resulted in PCR plateau, and too little resulted in sample insufficiency in the next real-time PCR. The product amount was about 100–500 ng after 25 cycle amplification in multiplex PCR of exon 8 and the SRY gene.

Real-time quantitative PCR

After the first round PCR optimization, we examined real-time PCR as nested PCR and quantification system to detect duplication mutation from the amplified products of single lymphocytes. Three detection methods were tested: fluorescently labeled specific hybridization probes (TaqMan probe) (Applied Biosystems, USA), the fluorogenic primers (LUX Primer) (Invitrogen, USA), and the unspecific dsDNA-dye SYBR green (Applied Biosystems, USA). Discrimination between the patient male and the control male was unclear from single cells with both the TaqMan probe and the LUX Primer, but relatively clear with the SYBR green dye. Therefore, we used the unspecific dsDNA-dye SYBR green that was simple to design and cost-effective. The first round PCR products were amplified with a pair of primers for the test exon (exon 8), and then for the normal reference and gender determination (SRY gene) in separate reactions (Fig. 1(a), (b)).

Figure 2 shows the mean values of exon 8/SRY gene ratio in the DMD duplication patient and the normal male. Mean value $\pm$ SE of the ratio was 1.85 ± 0.15 in the duplication patient (29 measurements) and 1.00 ± 0.09 in the normal male (29 measurements) respectively, and it was significantly

different (p -value of <0.001). The distributions of the DMD patient and the normal male are shown in Fig. 3; the range of 25–75% was differentiated, but overlap was observed. Two overlapping distributions of results are transected by a cut-off line. The region beneath the distribution of results for the DMD patient that lies above the cut-off line corresponds to the test's true positive (i.e. sensitivity); the region that lies below the cut-off line corresponds to the false negative. For the distribution of results for the normal male, these two regions correspond to the false positive and the true negative (i.e. specificity). For the two overlapping distributions, moving the cut-off line affects the sensitivity and specificity, but in opposite directions. Sensitivity and specificity cannot both be improved by changing the cut-off line, but rather only by changing the distributions such that less overlap occurs (i.e. by improving the discrimination level of the test itself). We examined diagnosis accuracy and efficiency assuming PGD by calculating the false negative rate (leading to a misdiagnosis) and transferable male embryo rate (below cut-off line) on the assumption that the cut-off line was set at 1.75, 1.20, 0.88, and 0.70 of the distributions (corresponding to upper 10, 25, 50, and 75% of the control male) (Table 3). The false negative rate was too high to be performed clinically with single cells, but was decreased by analyzing single cells in duplicate and only using those which gave identical results (Table 4). In theory, half of male embryos are normal, and all female embryos are unaffected (normal or carrier). When the cut-off line is set at 0.88, 12.2% of male embryos (24.4% of healthy male) can be transferred. Although all male embryos were discarded in PGD by previously performed gender determination, about half of normal male embryos can be rescued by this method.

Amplification rate

A total of 200 single lymphocytes including preliminary tests were analyzed using this conventional duplex PCR followed by separate nested real-time PCR with co-amplification of exon 8 and one of the following loci (exon 12, 45, and SRY

Table 3 Calculated false negative rate and transferable male embryo rate based on our results—1 blastomere diagnosis

Cut-off line	False negative rate	Transferable male embryo rate
1.75 (upper 10%)	35.7% (15/42)	72.4% (21/29)
1.20 (upper 25%)	24.1% (7/29)	50.0% (14.5/29)
0.88 (upper 50%)	17.6% (3/17)	29.3% (8.5/29)
0.70 (upper 75%)	12.5% (1/8)	14.8% (4/29)

The false negative rate is that of affected male embryos identified as normal, which leads to a dangerous misdiagnosis in PGD. The transferable embryo rate is the proportion of male embryos identified as normal. In theory, 50% of male embryos are normal, while 100% of female embryos are unaffected (normal or carrier).

Fig. 1 AB: Amplification curve of exon 8 (a) and SRY gene (b) from the same first round duplex PCR product. Amplification curve of exon 8 in the DMD patient shifted to the left due to the extra exon (the duplicated exon), resulting in smaller Ct value.

gene). The efficiency of the individual loci (exon 8, 12, 45, and SRY gene) was 188/200 (94.0%), 60/60 (100%), 61/70 (87.1%), and 67/70 (95.7%) respectively. The overall

amplification rate was 376/400 (94.0%) and the failure rate was 6.0%.

Table 4 Calculated false negative rate and transferable male embryo rate based on the results—2 blastomere diagnosis

Cut-off line	False negative rate	Transferable male embryo rate
1.75 (upper 10%)	23.6% (225/954)	56.7% (477/841)
1.20 (upper 25%)	9.2% (49/533)	31.6% (266/841)
0.88 (upper 50%)	4.4% (9/205)	12.2% (103/841)
0.70 (upper 75%)	2.0% (1/50)	3.0% (25/841)

The false negative rate was too high to allow PGD to be performed clinically with single cells, but was reduced by analyzing single cells in duplicate and only using those which gave identical results. However, the transferable male embryo rate was reduced simultaneously (12.2% of the transferable male embryos is equal to 24.4% of the healthy male embryos).

Discussion

The aim of this study was to develop PGD technique for direct detection of DMD duplication by quantification method, which is a common diagnostic method in duplication mutation. Although many duplication mutations differing in their localization and extent have been described so far in DMD patients (the Leiden muscular dystrophy pages), a versatile and effective detecting method for PGD has not been developed and mutation diagnosis at PGD is not available for about 10% of DMD patients with duplication mutation. In recent years, techniques for the detection of duplications have multiplied (reviewed in Armour et al. [28]), and very few protocols have been published proposing quantitative real-time

Fig. 2 Mean values of the ratio between the test exon and the normal reference. The ratios between exon 8 and SRY gene were calculated from the difference in Ct values. Mean $\pm$ SE was 1.84 ± 0.15 in single lymphocytes of the DMD patient (29 measurements), and 1.00 ± 0.09 in single lymphocytes of the normal male (29 measurements). Their mean values were significantly different (p -value of < 0.001).

Fig. 3 Distributions of the ratio between the test exon and the normal reference. Box plot of the ratios obtained for a DMD patient and a normal male (29 measurements, respectively); box: 25th–75th percentile; error bars: 10th–90th percentile. Two overlapping distributions of results are transected by a cut-off line.

PCR for measuring copy number changes in patients with duplications spanning one or more exons [29]. Conventional PCR strategies only analyze final PCR products without visualizing the kinetics of the reaction and are not adequate for quantification due to their inability to indicate the exponential phase. In spite of this demerit of conventional PCR, we have succeeded in quantification analysis of single cells by coordinating the cycle numbers of nested conventional PCR and using capillary electrophoresis (ABI Prism™ 310 GeneScan Analysis: Applied Biosystems, USA) (data not shown). This approach allowed duplication mutation in the dystrophin gene to be distinguished from normal status, but the optimized course was complicated and time-consuming. Unlike endpoint quantitative PCR, real-time PCR is suitable

for quantification because the quantity is defined by a point threshold cycle (Ct) when PCR amplification is still in the exponential phase [30].

We presented a method of conventional duplex PCR followed by a rapid real-time PCR using SYBR-green dye for monitoring product formation. Discrimination of single cells between the DMD patient and the normal male was achieved under the optimum conditions by quantification and subsequent comparison of test exons (exon 8 of dystrophin gene) and a normal reference (SRY gene). Real-time PCR is suitable for quantification because of its robustness, rapidness, and high reproducibility. Its rapid analysis ability is essential for time-constraints related to PGD. Due to a large dynamic range of quantification (1–1000 ng) and a very small amount of starting material in real-time PCR, an unknown sample can be easily quantified. DNA from a single cell is insufficient (4–10 pg) to be analyzed and must be amplified to the dynamic range prior to quantification. Single cells can be amplified by conventional PCR, of which the cycle number should be adjusted in the exponential phase so that the initial dose rate of two samples could be maintained. In addition, at insufficient cycles the amount of the PCR products did not reach the detection range for the following real-time PCR analysis. Therefore, determining an appropriate number of cycles is important. Secondly, making or selecting primers is important at the first round duplex PCR, in which the T_m value of the primers and product sizes should be adjusted. Decreasing the T_m difference increases the efficiency of the duplex PCR. The closer the two product sizes, the more similar the two product amounts are, and this makes the following quantification easier. Furthermore, proper detection method of the mutation in real-time PCR is essential for precise quantification. Because exons of the dystrophin gene are short (about 100 bp), the selection of primers and probes is restricted and difficult to make. Although fluorescence-labeled TaqMan-probes or LUX-primers are a little complicated and expensive to synthesize, SYBR-green dye is cost-effective and reliable. In addition, Joncourt et al. [29] have described that fluorescence signals of the hybridization probes decreased over time even when probes were stored as stock solutions at a constant temperature of -20°C and the stability of the probes was difficult to control. We also experienced instability of quantification analysis using fluorescence-labeled TaqMan-probes and LUX-primers. Compared with other gene dosage techniques, quantification with SYBR-green dye has many advantages in terms of low costs, less labor, and short preparation time.

Comparative quantification using a normal reference is necessary due to various DNA quantities in single cells (4–10 pg). The number of duplicated exon copy is 1, 2, 2, and 3 in a normal male, a duplication patient, a normal female, and a carrier female respectively. When the normal reference is a gene on the X chromosome, the expected ratio

between exons located in the mutation region and the normal reference is 1:2:1:1.5 in a normal male, a duplication patient, a normal female, and a carrier female respectively (Table 1). In a preliminary experiment, we examined exon 8/45 ratio on the dystrophin gene. The results showed significantly different mean values, but also showed overlapping distributions. False negative results which lead to a misdiagnosis arose due to two overlapping distributions. It is important to detect affected male embryos in PGD, and discrimination is easier (discrimination of 1:2) by using the SRY gene on the Y chromosome as the normal reference (Table 1). Consequently, comparison of mutation locus (exon 8) and Y chromosome-related single copy gene (SRY gene) can provide quantification and gender determination simultaneously. All female embryos without the SRY gene and male embryos with the SRY gene in which the ratio of exon 8/SRY gene is below the cut-off line can be transferred. As performing PGD, two blastomeres can be examined from one embryo at the same time, providing a double check for the results. It was reported that pregnancy and implantation rates did not appear to be significantly affected in spite of the biopsy of the two cells from embryos with 7 or more cells [31]. When the results of both blastomeres are identically normal, there is a high probability that the embryo will also be normal. Female embryos are all unaffected and can be transferred. Male embryos could be diagnosed using this method with a high degree of accuracy by examining the two blastomeres respectively, although the number of transferable male embryos decreases (Tables 3 and 4). Transferable embryos identified as unaffected should be selected in descending order of embryo quality. A low cut-off line reduces the false negative rate, but it also reduces the transferable embryo rate. The cut-off line should be decided in advance with the client's opinion after informed consents.

The main reason for the overlapping distributions is the limitation of detection ability with this method. The distribution is wider in patient's lymphocytes: some of the patient's lymphocytes were categorized as normal contrary to expectations. These results may be due to PCR amplification failure in duplicated exons in the same way as the phenomenon that results in the failure to amplify one allele of a heterozygous locus, commonly called allele drop-out, of which the rate ranges from 0 to 12.9% with the average rate taken to be 7.5% [31]. Amplification failure, which is a dangerous risk in PCR from single cells, can lead to a misdiagnosis, especially in gender determination. If the SRY in an affected male embryo doesn't amplify, the embryo will be look as a female embryo and transferred. With our method, the amplification failure rate of the SRY gene was found to be 4.3%. Therefore, the possibility of misdiagnosis regarding gender determination would be very low by analyzing two single cells respectively.

In conclusion, we performed DNA quantification from single cells of a normal male and a DMD patient, and there was a significant difference in the mean values between the two groups. Duplication mutation differs from type to type in localization and extent, and its primers and probes must be carefully matched in each case. The simplicity of this protocol will allow its easy implementation in diagnostic laboratories. Linkage analysis through repetitive micro satellite measurement may be a good method in PGD in terms of accuracy. However, as it is a substitute method, it doesn't meet the ethical committee's requirements. The location detection and sequencing of the junctional locus are very difficult in DMD duplication mutation due to the enormous size of the dystrophin gene. In addition, unaffected male embryos discarded by mere gender determination can be successfully transferred by our quantification method. In this respect, this method ideally meets the JSOG committee's requirements which stipulate that the indication of PGD should be strictly restricted and that clarifying gene mutation is a must. The problem lies with the distribution overlap, and the fact that false negative results can not be avoided, although the rate can be reduced to 5% or lower. This problem can be dealt with through informed consent based on adequate explanation and subsequently, prenatal diagnosis. We propose that this comparative quantification method, with gender determination using two blastomeres, can be a choice for DMD carriers who wish to have unaffected sons, or live in strictly restricted countries for PGD, due to the fact that there is no other PGD technique of direct detection of duplication mutation currently available.

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