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The impact of parental unaffected allele combination on the diagnostic outcome in the preimplantation genetic testing for myotonic dystrophy type 1 in Japanese ancestry

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Abstract

Purpose: The objective is to clarify the practical problem of the preimplantation genetic testing (PGT) for myotonic dystrophy type 1 (DM1) in Japanese subjects.

Methods: For the 32 couples who consented to participate in PGT for DM1, CTG repeats number on the unaffected alleles was analyzed. Based on the allele combination, they were classified into 3 groups by the number of diagnostic allelic pattern; "full informative," "semi informative," and "noninformative." According to the Japan Society of Obstetrics and Gynecology (JSOG) principle, PGT was performed using the direct diagnosis to the 288 embryos from the 17 couples who received the ethical approval from both our institution and JSOG.

Results: In the 32 couples, the frequency of CTG repeats on the unaffected alleles showed bimodal distribution. The "full informative," "semi informative," and "noninformative" couples accounted for 46.9% (15/32 couples), 46.9% (15/32 couples) and 6.2% (2/32 couples), respectively. The transferable embryos accounted for 28.9% (33/114 embryos) in the "full informative" couples, although it was limited to 12.6% (22/174 embryos) in the "semi informative" couples.

Conclusion: The loss of unaffected embryos which cannot be diagnosed as transferable was a clinically major problem and implied an increase in oocyte retrieval, especially for "semi informative" couples.

KEYWORDS

CTG repeats number, direct diagnosis, myotonic dystrophy type 1, polymerase chain reaction, preimplantation genetic testing

1 | INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a progressive muscular dystrophy with autosomal dominant inheritance. It has the highest prevalence among adult muscular dystrophies, which is estimated to be 9.13-10.4/100 000.[1,2] The responsible gene is *DMPK* (19q13.3), and the mutation is an excessive extension of CTG repeats present in the untranslated region. Depending on the time of onset and

severity, DM1 is classified into congenital, childhood, adult, and mild form (Table 1).[3] The pathognomonic phenomenon is, known as "anticipation," that when the affected allele is passed down from generation to generation, the mutation is further elongated as it is inherited; this causes the onset to occur at a younger age and follows the more severe clinical course in offspring than in parents.[4] Based on the gravity of these conditions, preimplantation genetic testing (PGT) has been applied to couples with DM1.[5]

PGT is a highly advanced technique for early prenatal diagnosis of embryo, which requires a series of complex procedures for the accurate diagnosis. As for the PGT for DM1, whole-genome amplification (WGA) is performed on the genomic DNA extracted through an embryo biopsy, and the WGA products are subjected to polymerase chain reaction (PCR) for the amplification of the nucleotide sequence of the CTG repeats region, which is followed by capillary electrophoresis using genetic analyzer with Genescan®. On the basis of the electrophoresis analysis on the sequence, the genetic status of the embryo is determined (direct diagnosis). On the principle of the Japan Society of Obstetrics and Gynecology (JSOG), PGT for monogenic disorder is performed based on the direct diagnosis for the disease responsible gene.

The challenges of PGT for DM1 are as follows:

1. because the gene mutation of DM1 is an excessive extension of CTG repeats, the binding between G and C nucleotides on complementary strands is likely to occur, and the gene amplification efficiency is likely to decrease [6];
2. in the gene amplification process, amplification errors such as allele dropout (ADO) will occur at a certain level of probability and pose a risk of misdiagnosis[7,8];
3. there is a limit to the base length which can be amplified by conventional PCR method, and the reaction fails to occur when the gene mutations cause CTG repeats extensions of 1000 or greater in base length. As a result, the affected alleles are difficult to detect on the capillary electrophoresis, and the analysis of affected embryos can detect only one peak from the unaffected allele;
4. the Southern blotting method, which is conventionally used for the diagnosis of the gene mutation is not capable for the analysis of rare DNAs (in extremely small quantities of a few picograms) extracted from a single cell;
5. in unaffected embryos as well, if the CTG repeats number on the alleles is identical or close to each other, the peaks in the capillary electrophoresis overlap (only one peak can be detected), and consequently, the analysis results is the same as those of embryos with ADO or affected embryos[8];
6. DM1 is an autosomal dominant inherited disorder, and embryos cannot be diagnosed as "transferable" unless the unaffected alleles are proven to be inherited from both the parents. In other words, the embryo diagnosed as "transferable" in PGT are those which shows two distinguishable peaks in the capillary electrophoresis.

TABLE 1 Clinical classification of myotonic dystrophy type 1

Type	CTG repeats	Clinical features
(unaffected)	≦37	—
Mild	50-150	Weakness and cataract
Adult	100-1000	Myotonia, muscle weakness, and cataract
Childhood	500-2000	Hypotonia and developmental disorder
Congenital	1000-	Hydramnios, respiratory distress, severe myotonia, and developmental disorder

We report the diagnostic outcomes of PGT for DM1 in our center and clarify the clinical problems in Japanese subjects.

2 | MATERIALS AND METHOD

From among couples in which either spouse was diagnosed with DM1, who opted for PGT and attended the Center for Medical Genetics (Keio University Hospital) for counseling between June 2006 and March 2017, our study was conducted on 32 couples who had received genetic counseling and provided consent to participate in the study. PGT was carried out on the couples who had been approved by the ethics committee of both our institution and JSOG; and 288 embryos retrieved from 17 couples over 43 PGT cycles were subjected for analysis.

Peripheral blood was collected from each couple, cells were lysed by adding an alkaline lyses buffer (50 mmol/L dithiothreitol/200 mmol/L NaOH), followed by 10 minutes incubation at 65°C. Gene amplification was performed on the extracted genomic DNA by conventional PCR method, using primers labeled with fluorescein (FAM). Primer design was as follows: forward primer 5′-(FAM)-GAA-CGG-GGC-TCG-AAG-GGT-CCT-TGT-AGC-3′, reverse primer 5′-CTT-CCC-AGG-CCT-GCA-GTT-TGC-CCA-TC-3′, and PCR conditions were as follows: 94°C for 2 minutes (denaturation), followed by 35 cycles under the condition of 94°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute (amplification), and finally one cycle of 72°C for 5 minutes (final extension). For capillary electrophoresis of the amplification products, 3500 Genetic Analyzer (Applied Biosystems®) was used. The capillary length was 50 cm, the electrophoresis and size separation were carried out using a POP-6 polymer, and the results were analyzed using Genescan®. The number of expanded CTG repeats on the affected alleles of the couples and their referrals were analyzed using the Southern blotting method. To ensure the accuracy and safety of the diagnosis, 2 or more difference in CTG repeats number was considered as a criterion for determining whether CTG repeats number can be clearly distinguished on the results of the electrophoretic analysis. Based on the allele combination of the unaffected alleles, the couples were classified into three groups; "full

informative" who have no indistinguishable allele pattern, "semi informative" who have 1 indistinguishable pattern and "noninformative" who have no distinguishable allele pattern.[9]

Oocyte retrieval was conducted after controlled ovarian stimulation (COS), which were subjected to micro fertilization, and we conducted on biopsy of 3-5 trophectoderm cells from blastocyst. Using the multiple displacement amplification (MDA) method, WGA was carried out on the genomic DNA extracted from each cell; the amplification products were subjected to conventional PCR under the conditions expressed above, and analyses were carried out using the capillary electrophoresis and Genescan®. The results of the electrophoretic analysis were classified according to whether the number of detected peak from the unaffected allele was 2, 1, or 0; and depending on the number, the embryo was diagnosed as "transferable," "indeterminable," or "inconclusive," respectively.

Statistical analysis was carried out using a χ^2 test or Fisher's exact test, $P < .05$ was considered as the criterion for significance.

3 | RESULTS

The proportion of affected males and females in the 32 couples was as follows: 3 males and 29 females. The affected individuals' mean age at diagnosis was 31.2(17-38) years; the number of affected individuals who had already developed clinical symptoms such as myotonia was 27; and the number of couples who had family history diagnosed with DM1 accounted for 17. A total of 14 couples had pregnancy and delivery history, and 18 couples had been attending a hospital for counseling before pregnancy.

In the analysis of the CTG repeats number on the unaffected alleles in the 96 chromosomes from the 64 individuals, the minimum number of CTG repeats was 3 and the maximum was 24. The frequency of CTG repeats on the unaffected alleles showed bimodal distribution; the peaks lied in $(CTG)_{4\text{-}5}$ and $(CTG)_{12\text{-}13}$ allele groups and the proportion of the allele groups was 22.9% and 43.7%, respectively (Figure 1). Meanwhile, the number of CTG repeats in the affected alleles ranged from 73 to 900, and the numbers showed no accumulation of specific repeats. The proportion of the "full informative," "semi informative," and "noninformative" couples was 46.9% (15/32 couples), 46.9% (15/32 couples), and 6.2% (2/32 couples), respectively. The PGT for the 2 "noninformative" couples had not been approved by our institution's ethics committee.

In total, 288 embryos were collected from 17 couples during 43 PGT cycles. In the 17 couples, the number of the "full informative" couple was 9 (21 PGT cycles, 114 embryos) and the "semi informative" was 8 (22 PGT cycles, 174 embryos). The "transferable" "indeterminable" and "inconclusive" embryos accounted for 19.1% (55/288 embryos), 52.8% (152/288 embryos), and 28.1% (81/288 embryos), respectively. Analysis about the results of the "full informative" and "semi informative" couples showed that the proportion of the "transferable" embryos accounted for 28.9% (33/114 embryos) and 12.6% (22/174 embryos), and the "indeterminable" embryos were 40.3% (46/114 embryos) and 60.9% (106/174 embryos), respectively. There were significant differences in the results between the couples (Table 2, χ^2 test, $P < .05$). Meanwhile, the "inconclusive" embryos accounted for 30.7% (35/114 embryos) and 26.4% (46/174 embryos) and there was no significant difference ($P < .05$).

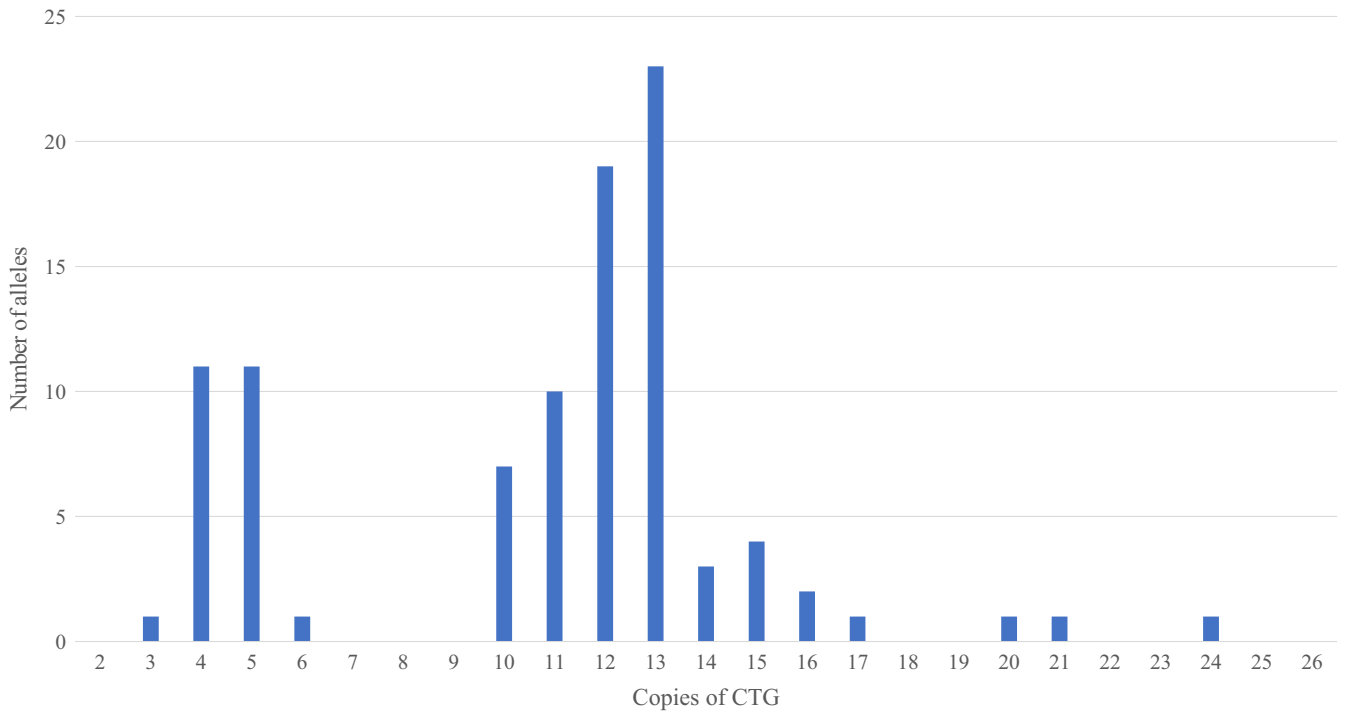

FIGURE 1 Frequency distribution of the DM1 associated CTG repeats in the 32 couples. In the 96 unaffected alleles of the 32 couples, the allele frequency showed bimodal distribution. $(CTG)_{12\text{-}13}$ alleles was the most common allele group

TABLE 2 Comparison and χ^2 analysis of PGT results from the "full informative" and "semi informative" couples

Analysis result of PGT	Number of embryo (%) in "full informative" couples	Number of embryo (%) in "semi informative" couples	χ^2 test
Transferable	33 (28.9%)	22 (12.6%)	P = 7.524e-8
Indeterminable	46 (40.3%)	106 (60.9%)	P = 1.705e-5
Inconclusive	35 (30.7%)	46 (26.4%)	P = .4619
Total	114	174	

Note: There were significant differences in PGT analysis result, the number of "transferable" and "indeterminable" embryos, between the "full informative" and "semi informative" couples (P < .05).

4 | DISCUSSION

Among single-gene disorders experienced in our center, DM1 is the second most disorder after Duchenne muscular dystrophy. The couples opting for PGT were diverse in terms of family history, obstetric history, and psychosocial background. For some of the couples, the proband was only their own child who developed congenital DM1, suggesting the impact of anticipation.

Previous reports on the distribution of "unaffected" CTG repeats (≤37) have shown that, although there is interpopulation variation in the frequencies of CTG repeats, $(CTG)_5$ allele and $(CTG)_{11\text{-}14}$ allele group are major in all classes of CTG repeats arrays.[10-12] It is also reported that there is a significant difference in the frequency of the major alleles between Japanese and European population, with a smaller frequency of $(CTG)_5$ and greater frequency of $(CTG)_{11\text{-}14}$ on Japanese subjects.[10] On the other hand, there is no significant difference between Malay and Japan.[11] In our study, the CTG repeats number on the unaffected alleles from the 32 couples showed similar distribution to previous reports (Table 3, Fisher's exact test, P < .05).[10] Our data may imply the practical problem of PGT for DM1 in Japan, due to the allele combination.

As for the allele combination of the 32 couples, the proportion of "semi informative" and "noninformative" couples accounted for 53.1% (17/32 couples) and this result showed that the unaffected embryos with identical or close number of CTG repeats, diagnosed as "indeterminable" through the direct diagnosis, were easy to arise. Because DM1 follows an autosomal dominant pattern of inheritance, the incidence of unaffected embryos is theoretically 50%, but in practice, the "transferable" embryos accounted for only 19.0% (55/288 embryos), and the "indeterminable" embryos accounted for 52.7% (152/288 embryos), which was the largest percentage. However, considering the limitation of the direct diagnosis that cannot distinguish unaffected embryo from "indeterminable" embryo, the incidence of the "transferable" embryos in the "semi informative" couples decreases to 25%. As a result, the percentage of the "transferable" embryos was 28.9% (33/114 embryos) in the "full informative" couples and 12.6% (22/174 embryos) in the "semi informative" couples, which values were lower than expected. This might be due to amplification errors of extracted DNA such as amplification failure (AF) and ADO. AF is a phenomenon of total failure of amplification, which incidence is reported as 5%-10% of single cell subjected to PCR.[7] The loss of isolated cell during transfer, cell degeneration or cell lysis failure are considered as causes of AF.[7] On the other hand, ADO is a failure of PCR to amplify one allele of the cell. The incidence of ADO is reported as 5%-15%.[7] ADO is explained by technical failures due to broken sequence of the targeted DNA during lysis of single cells, inefficient or no priming of the PCR primers, chromosomal mosaicism, and aneuploidy of embryos due to mitotic nondisjunction or anaphase lagging.[8,13] Further, the proportion of 12.6% in the "semi informative" couples was approximately half the expected value, suggesting that they needed to be burdened with a larger number of oocyte retrieval in order to detect "transferable" embryos. In fact, couples from whom the "transferable" embryo could not be collected in one oocyte retrieval trial accounted for 11.1% (1/9 couples) in the "full informative" couples and 62.5% (5/8 couples) in the "semi informative" couples.

In addition, as for the two "noninformative" couples, the direct diagnosis theoretically cannot detect "transferable" embryos. This finding emphasizes the limitation of PGT for DM1. To compensate this limitation, the techniques using linkage analysis and triplet repeat primed PCR (TP-PCR) analysis have been applied to PGT for DM1. The linkage analysis, known as the indirect analysis, uses polymorphic microsatellite markers to detect not the gene mutation but the inherited haplotype. For the linkage analysis, both DNAs of parents-child trios and 2 or more informative markers are imperative because of genetic recombination.[8,14] However, the informative markers are limited

TABLE 3 Comparison of the frequency of CTG repeats in unaffected alleles of Japanese from 1992 (Davies) and current study

CTG repeats	Number of alleles from current study (%)	Number of alleles from Davies (%)
5	11 (11.4)	21 (19.8)
11	10 (10.4)	9 (8.4)
12	19 (19.7)	25 (23.5)
13	23 (23.9)	29 (27.3)
14	3 (3.1)	1 (0.9)
Others	30 (31.2)	21 (19.8)
Total	96	106

Note: In Davies' study, 37 unaffected and 32 affecteed Japanese were subjected.[10] Fisher's exact test was performed to compare the frequency of CTG repeats in unaffected alleles and showed negative correlation (P < .05).

in the PGT for DM1[15-17] and, as mentioned above, PGT for monogenic disorder should be based on the direct diagnosis for the disease responsible gene. Analysis by the indirect diagnosis is supportive but not used as basis of diagnosis. TP-PCR is a PCR technique using multiple primers to detect expanded allele qualitatively. This technique is reported to be efficient in screening affected embryos in PGT for DM1,[9] but it is inevitable ADO will occur at a certain level of probability resulting in the false-negative result, which leads to misdiagnosis. The haplotype analysis using single nucleotide polymorphism (SNP) microarray could have possibility of new diagnostic method, but the accuracy, safety, and limitation should be validated.[18]

In our study, the frequency of the CTG repeats in the unaffected alleles in 32 Japanese couples showed bimodal distribution, which was consistent of previous reports. The "semi/noninformative" couples accounted for approximately half of the couples, and the allele combination had significant impact on the diagnostic outcomes of PGT for DM1. This might contribute to the differences in terms of PGT-related burden weighing on each couple. Our study also emphasized the limitation of the direct diagnosis based on the PCR in PGT for DM1. For the "noninformative" couples, an alternative diagnostic method, such as haplotype analysis using SNP array, needs to be evaluated the accuracy and usefulness.

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DISCLOSURES

Conflict of interest: Hiroshi Senba, Kou Sueoka, Suguru Sato, Nobuhiko Higuchi, Yuki Mizuguchi, Kenji Sato, Mamoru Tanaka declare that they have no conflict of interest. *Human rights statements and informed consent*: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all couples for being included in the study. *Animal studies*: The protocol and procedure of PGT were approved by the ethics committee both of our institution and the Japan Society of Obstetrics and Gynecology (JSOG) in every couple.

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REFERENCES

1. Nakagawa M, Nakahara K, Yoshidome H, et al. Epidemiology of progressive muscular dystrophy in Okinawa, Japan. *Neuroepidemiology*. 1991;10:185-191.
2. Norwood F, Harling C, Chinnery P, Eagle M, Bushby K, Straub V. Prevalence of genetic muscle disease in Northern England: in-depth analysis of a muscle clinic population. *Brain*. 2009;132:3175-3186.
3. Udd B, Krahe R. The myotonic dystrophies: molecular, clinical, and therapeutic challenges. *Lancet Neurol*. 2012;11:891-905.
4. Rudnik-Schöneborn S, Zerres K. Outcome in pregnancies complicated by myotonic dystrophy: a study of 31 patients and review of literature. *Eur J Obstet Gynecol Reprod Biol*. 2004;114:44-53.
5. De Rademaeker M, Verpoest W, De Rycke M, et al. Preimplantation genetic diagnosis for myotonic dystrophy type 1: upon request to child. *Eur J Human Genet*. 2009;17:1403-1410.
6. Frey UH, Bachmann HS, Peters J, Siffert W. PCR-amplification of GC-rich regions: 'slowdown PCR'. *Nat Protoc*. 2008;3:1312-1317.
7. Piyamongkol W, Bermúdez MG, Harper JC, Wells D. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. *Mol Hum Reprod*. 2003;9(7):411-420.
8. Wilton L, Thornhill A, Traeger-Synodinos J, Sermon KD, Harper JC. The cause of misdiagnosis and adverse outcomes in PGD. *Hum Reprod*. 2009;1(1):1-8.
9. Kakourou G, Dhanjal S, Mamas T, Serhal P, Delhanty JD, SenGupta SB. Modification of the triplet repeat primed polymerase chain reaction method for detection of the CTG repeat expansion in myotonic dystrophy type 1: application in preimplantation genetic diagnosis. *Fertil Steril*. 2010;94:1674-1679.
10. Davies J, Yamagata H, Shelbourne P, et al. Comparison of the myotonic dystrophy associated CTG repeat in European and Japanese populations. *J Med Genet*. 1992;29:766-769.
11. Ambrose KK, Ishak T, Lian L-H, et al. Analysis of CTG repeat length variation in the DMPK gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia. *BMJ Open*. 2017;7:1-9.
12. Deka R, Majumder PP, Shriver MD, et al. Distribution and evolution of CTG repeats at the myotonin protein kinase gene in human populations. *Genome Res*. 1996;6:142-154.
13. Dreesen J, Drüsedau M, Smeets H, et al. Validation of preimplantation genetic diagnosis by PCR analysis: genotype comparison of the blastomere and corresponding embryo, implications for clinical practice. *Mol Hum Reprod*. 2008;14(10):573-579.
14. Harton GL, De Rycke M, Fiorentino F, et al. ESHRE PGD consortium best practice guidelines for amplification-based PGD. *Hum Reprod*. 2011;26(1):33-40.
15. Mulley JC, Gedeon AK, White SJ, Haan EA, Richards RI. Predictive diagnosis of myotonic dystrophy with flanking microsatellite markers. *J Med Genet*. 1991;28:448-452.
16. Piyamongkol W, Harper JC, Sherlock JK, et al. A successful strategy for preimplantation genetic diagnosis of myotonic dystrophy using multiplex fluorescent PCR. *Prenat Diagn*. 2001;21:223-232.
17. Kakourou G, Dhanjal S, Daphnis D, et al. Preimplantation genetic diagnosis for myotonic dystrophy type 1: detection of crossover between the gene and the linked marker APOC2. *Prenat Diagn*. 2007;27:111-116.
18. Natesan SA, Bladon AJ, Coskun S, et al. Genome-wide karyomapping accurately identifies the inheritance of single-gene defects in human preimplantation embryos in vitro. *Genet Med*. 2014;16:838-845.

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