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Original Article

Preimplantation and prenatal genetic diagnosis of aromatic L-amino acid decarboxylase deficiency with an amplification refractory mutation system-quantitative polymerase chain reaction

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Abstract

Objectives: To develop a diagnostic platform for preimplantation genetic diagnosis (PGD) and prenatal genetic diagnosis (PND) to prevent births of aromatic L-amino acid decarboxylase deficiency (AADC) patients.
Materials and Methods: Five Taiwanese families carrying AADC were enrolled. A novel technique, amplification refractory mutation system-quantitative polymerase chain reaction (ARMS-qPCR), was developed for both of PGD and PND. For PGD, blastomere biopsies of day-3 cleavage-stage embryos were subjected to ARMS-qPCR. Villi, cultured amniocytes, or both were used to confirm the PGD result; this approach could also be used as the sole method for PND after *in vivo* conception).
Results: Unaffected live births were achieved in four of the five families, except one with ongoing PGD. The ARMS-qPCR correctly classified blastomeres (from day-3 cleavage-stage embryos) as affected (homozygous mutant), carrier (heterozygous for mutant and wild-type alleles), or normal (homozygous wild-type) within 1 working day.
Conclusions: To our knowledge, this is the first report of successful PGD of AADC. The molecular technique we devised (ARMS-qPCR) was applicable for PGD as well as PND of AADC. Furthermore, it has great potential for similar applications in other monogenic disorders.

Keywords: AADC; ARMS; PGD; PND; qPCR

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Introduction

Prenatal diagnosis and genetic counseling are essential for at-risk couples who wish to avoid giving birth to children with an inherited disorder. Preimplantation genetic diagnosis (PGD), used in association with some reproductive technologies, e.g., in vitro fertilization (IVF), can determine the genetic makeup of embryos before implantation, and thus minimize the possibility of an affected pregnancy. Therefore, PGD is an alternative to prenatal diagnosis (PND) and has become a feasible option for couples at risk for any one of several familial genetic disorders [1].

The aromatic L-amino acid decarboxylase deficiency (AADC; MIM #608643) is a rare inborn error of neurotransmitter metabolism that causes severe developmental delays, an oculogyric crisis, and autonomic dysfunction. Defects in the *DDC* gene (located in 7p12.1p12.3), cause this condition [2]. A Taiwanese couple, with a history of three neonatal deaths and two induced abortions due to AADC, requested IVF and PGD to achieve an unaffected pregnancy. Based on a preliminary molecular evaluation, this couple had identical heterozygous mutations in *DDC*, IVS6 + 4A>T (a hotspot reported in more than 80% of Taiwanese patients; a founder effect has been proposed) [3]. So far, about 30 *DDC* mutants were described in no more than 50 AADC cases worldwide [4]. However, apparently no successful PGD for AADC had been reported. Therefore, we developed and applied a technique, namely amplification refractory mutation system-polymerase chain reaction (ARMS-qPCR), for PGD in carrier parents with a *DDC* mutation that causes AADC. To confirm and extend the clinical application of the ARMS-qPCR, the technique was adapted for PGD and PND, and used in five AADC families.

The technique involved two technologies. The first was ARMS-PCR, a PCR-based method that can detect single base-pair changes or small insertions or deletions, based upon sequence-specific primer sets [5]. That this method enables amplification of test DNA only when the target allele is contained within the sample, makes it especially suitable for PGD. However, such PCR-based technologies are prone to errors, e.g., contamination or allelic dropout (ADO; only one of two alleles in a heterozygous cell was amplified, resulting in misdiagnosis) [6]. Therefore, the second technology, real-time quantitative PCR (qPCR), was used to minimize errors. The qPCR is a highly sensitive technique for differential allele detection in single cells [7]; it can accurately quantify the number of copies of a particular amplicon, and determine the ratio of mutant to wild-type alleles in a sample.

Materials and methods

Patients

During 2009–2011, five Taiwanese families with AADC (CCH001–CCH005) who came to our clinic for PGD, PND, or both, were enrolled in this study. The relevant clinical and genotypic information are summarized (Table 1) and family

Table 1
Summary of the five AADC carrier couples seeking preimplantation/prenatal genetic diagnosis (PGD/PND).

Family No.	Paternal genotype	Maternal genotype	Pregnancy cycle	PND / PGD (sample examined)	No. of embryos biopsied for PGD	No. of embryos implanted	Fetal genotype (Amniocentesis)	Result
CCH001	IVS6 + 4A > T/wt	IVS6 + 4A > T/wt	1	PND (villus)	–	–	IVS6 + 4A>T/IVS6 + 4A > T	Temination
			2	PND (villus)	–	–	IVS6 + 4A > T/IVS6 + 4A > T	Temination
			3	PGD (blastomere)	5	2 (1 IVS6 + 4A > T/wt and 1 wt/wt)	IVS6 + 4A > T/wt	Live singleton (boy)
CCH002	IVS6 + 4A > T/wt	IVS6 + 4A > T/wt	1	PND (villus)	–	–	IVS6 + 4A > T/wt	Live singleton (girl)
			2	PND (villus)	–	–	wt/wt	Live singleton (girl)
CCH003	c. 1234C > T/wt	c. 1297 dupA/wt	1	PND (villus)	–	–	wt/wt	Live singleton (boy)
CCH004	IVS6 + 4A > T/wt	IVS6 + 4A > T/wt	1	PND (villus)	–	–	IVS6 + 4A > T/IVS6 + 4A > T	Termination
			2	PND (villus)	–	–	wt/wt	Live singleton (girl)
CCH005	c. 1297 dupA/wt	IVS6 + 4A > T/wt	1	PGD (blastomere)	Ongoing	Ongoing	–	–

pedigrees and genotypes are shown (Fig. 1). PGD was performed in family CCH001 and is now ongoing in family CCH005, whereas PND was used for families CCH002–CCH004.

Mutation analysis

The DNA was extracted from single blastomeres biopsied from day-3 (8-cell cleavage-stage) embryos with good

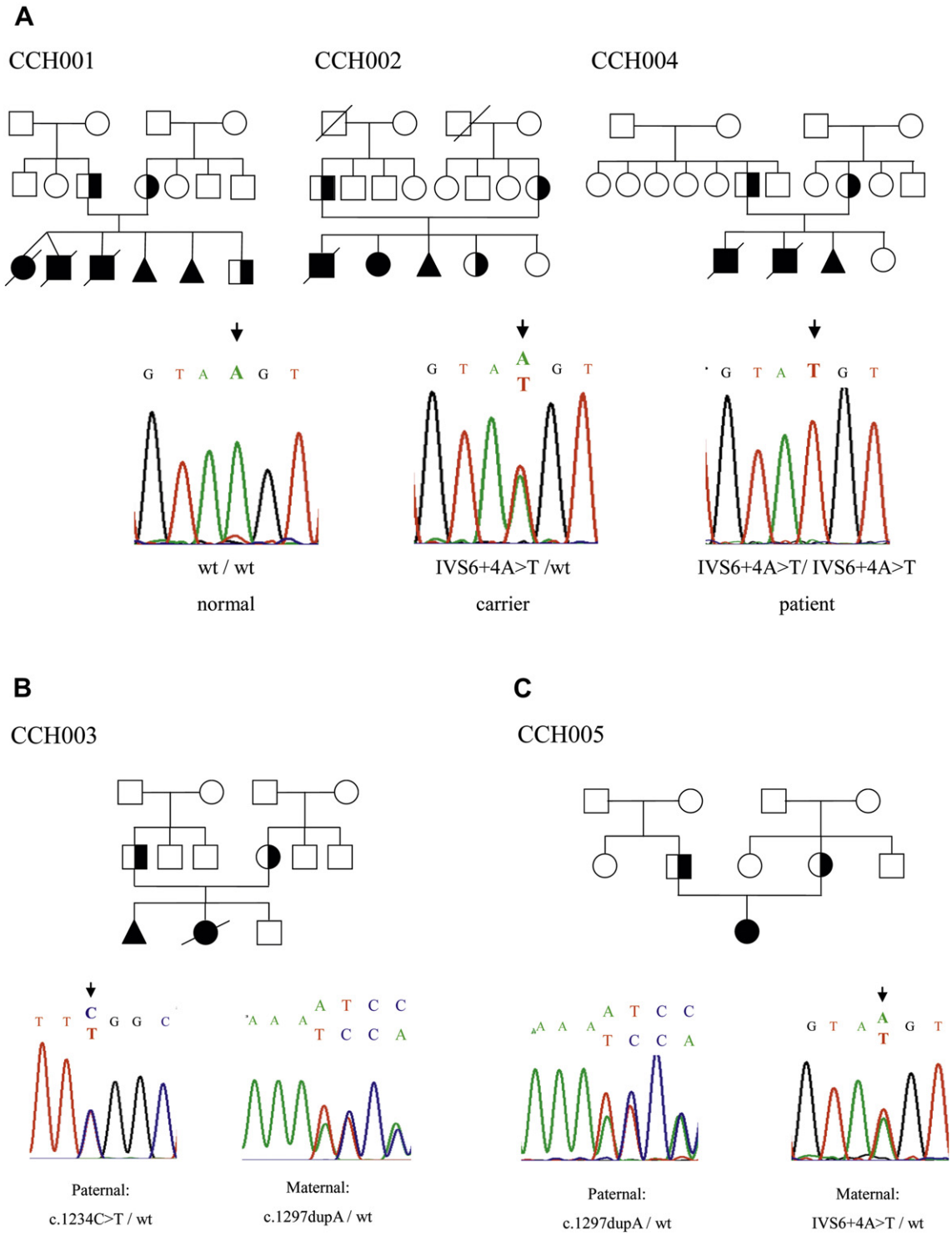

Fig. 1. Pedigrees with *DDC* mutations in five families with aromatic L-amino acid decarboxylase deficiency (AADC). (A) Parents from three families (CCH001, CCH003 and CCH004) carried the same mutation (IVS6 + 4A>T). (B) Parents of CCH002 carried two heterozygous mutations (paternal: c.1234C>T; maternal: c.1297dupA). (C) Parents of CCH005 also carried two heterozygous mutations (paternal: c.1297dupA; maternal: IVS6 + 4A>T). Black arrows indicate the mutation position.

morphology (for PGD) and from fetal cells by chorionic villus sampling at 11–14 weeks of pregnancy (for PND), using the Genomic DNA Mini Kit (Geneaid, Tao-Yuan, Taiwan). Furthermore, DNA from peripheral lymphocytes of reference individuals, including parents, was also extracted and examined.

All 14 exons of the *DDC* gene were amplified by polymerase chain reaction (PCR), with the corresponding intronic primers designed from the available genomic DNA sequence (NCBI Reference Sequence: NG_008742.1) using Oligo 6.71 (Molecular Biology Insights, Cascade, CO, USA). Amplified fragments were subjected to bidirectional sequencing with the Big-Dye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

ARMS-qPCR

For PGD, ARMS-qPCR was first evaluated in 20 lymphocytes isolated from a fully affected patient and the carrier couple, respectively, and from 15 single blastomeres obtained from a cell bank (as normal controls). Subsequently, five single blastomeres biopsied from five embryos with good morphology at day 3 (8-cell cleavage-stage) from the carrier couple were examined. Each blastomere was independently collected in a sterile PCR tube, lysed with proteinase K (125 μg/mL in 17 μM SDS; Sigma-Aldrich, St. Louis, MO, USA) at 50 °C, 60 minutes, and inactivated at 99 °C, 4 minutes. Duplex-nested PCR was then used to amplify the intron 5-6 region of *DDC*, which includes the targeted mutation. The two primer sets, designed based on the reverse strand's *DDC* gene sequence, were OF: 5′-CCTCCTGGCTG GTTTCTTTC-3′ and OR: 5′-CAGGCACTGTTTCAGGAG CTA-3′ for the first PCR (1,164 bp), and IF: 5′-AGGGTCTC GCCACTTATCCT-3′ and IR: 5′-TAAGGCAGGGATTCAA ATGG-3′ for the secondary PCR (1,005 bp; Fig. 2A). The first PCR was carried out in a 40 μL reaction, consisting of 1 × PCR buffer, 1.25 mmol/L MgCl2, 0.35 mmol/L dNTP, 0.5 μmol/L of each primer (OF + OR), 1 × GC-RICH solution, and 1U Faststart Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The cycling conditions were 95 °C, 5 minutes, followed by 25 cycles of 95 °C, 30 seconds, 55 °C, 30 seconds and 71 °C, 1 minute, and a final extension at 71 °C, 2 minutes. The PCR products were directly subjected to a second round of PCR by adding 10 μL PCR supplement with a similar PCR mixture to that used in the first PCR, except for the primer set (IF + IR). Cycling conditions were similar to the first PCR, but the number of cycles in the second step was increased from 25 to 40. To ensure accuracy of the PCR, amplified fragments were confirmed by direct sequencing. For ARMS-qPCR, two sequence-specific forward primers, modified with a mismatch at the penultimate nucleotide position of the mutation site to increase the specificity of the reaction [5], were designed: (MUF: 5′-GGCCTGATTCC TTTCTTTGTGT-3′ for the mutant allele and WTF: 5′-GGCCTGATTCCTTTCTTTGTGA-3′ for the wild-type allele). The two forward primers were respectively paired with the same reverse primer 3R: 5′-CTGCCTCAGCTT CCCGAGTA-3′ (and produced an equivalent 148 bp). The ARMS-qPCR, performed on the 7700 ABI Prism Sequence Detector (Applied Biosystems) in a 20 μL reaction, consisted of 0.5 ng of the duplex-nested PCR product, 0.5 μmol/L of each primer, 1× ROX, and 1× SYBR Green PCR Master Mix (Finnzymes, Espoo, Finland). Cycling conditions were: 95 °C, 15 minutes, followed by 45 cycles at 95 °C, 10 seconds, 60 °C, 20 seconds, and 71 °C, 10 seconds (Fig. 2B). The condition and primer sets of ARMS-qPCR used for PND were similar to those used in PGD.

Results

AADC families

Four of the five families (CCH001-CCH004) enrolled in our study had at least one successful live birth of an unaffected child (Table 1). Three families received PND, either by chorionic villus sampling or amniocentesis after natural conception (CCH002–CCH004). One couple, which had two consecutive induced abortions due to affected pregnancies ascertained by PND after natural conception, requested PGD (CCH001) and eventually had an unaffected live birth (Table 1 and Fig. 1).

ARMS-qPCR and PGD in family CCH001

The PCR amplification rate of the 60 single lymphocytes and the 15 single blastomeres was 100% (60/60) and 93% (14/15), respectively. Direct sequencing of the PCR products confirmed that the accuracy of the ARMS-qPCR was 100% (74/74). In the 40 heterozygous lymphocytes (from the couple), there was no evidence of ADO in the ARMS-qPCR (the heterozygous curves from the ARMS-qPCR almost overlapped), suggesting that the amplification efficiencies for the mutant and the wide-type alleles were similar (Fig. 2C). Of the five single blastomeres requested for clinical PGD, two were confirmed unaffected: one was homozygous wide-type, whereas the other was a heterozygous carrier. The two embryos corresponding to the two unaffected blastomeres were selected for implantation (informed consent) at day 5. A successful singleton pregnancy was eventually achieved. After 38 weeks of uneventful gestation, the woman gave birth to a healthy male baby (birth body weight 3180 g, birth body length 50 cm, and AS 8′-> 9′), whose postnatal genotyping confirmed the results of PGD and amniocentesis.

Discussion

AADC

There were only four mutant genotypes in the five Taiwanese families (10 heterozygous carrier individuals) enrolled in our study, including IVS6 + 4A>T ($n = 7$), c.1234C>T ($n = 1$), and c.1297dupA ($n = 2$). The prevalent IVS6 + 4A>T

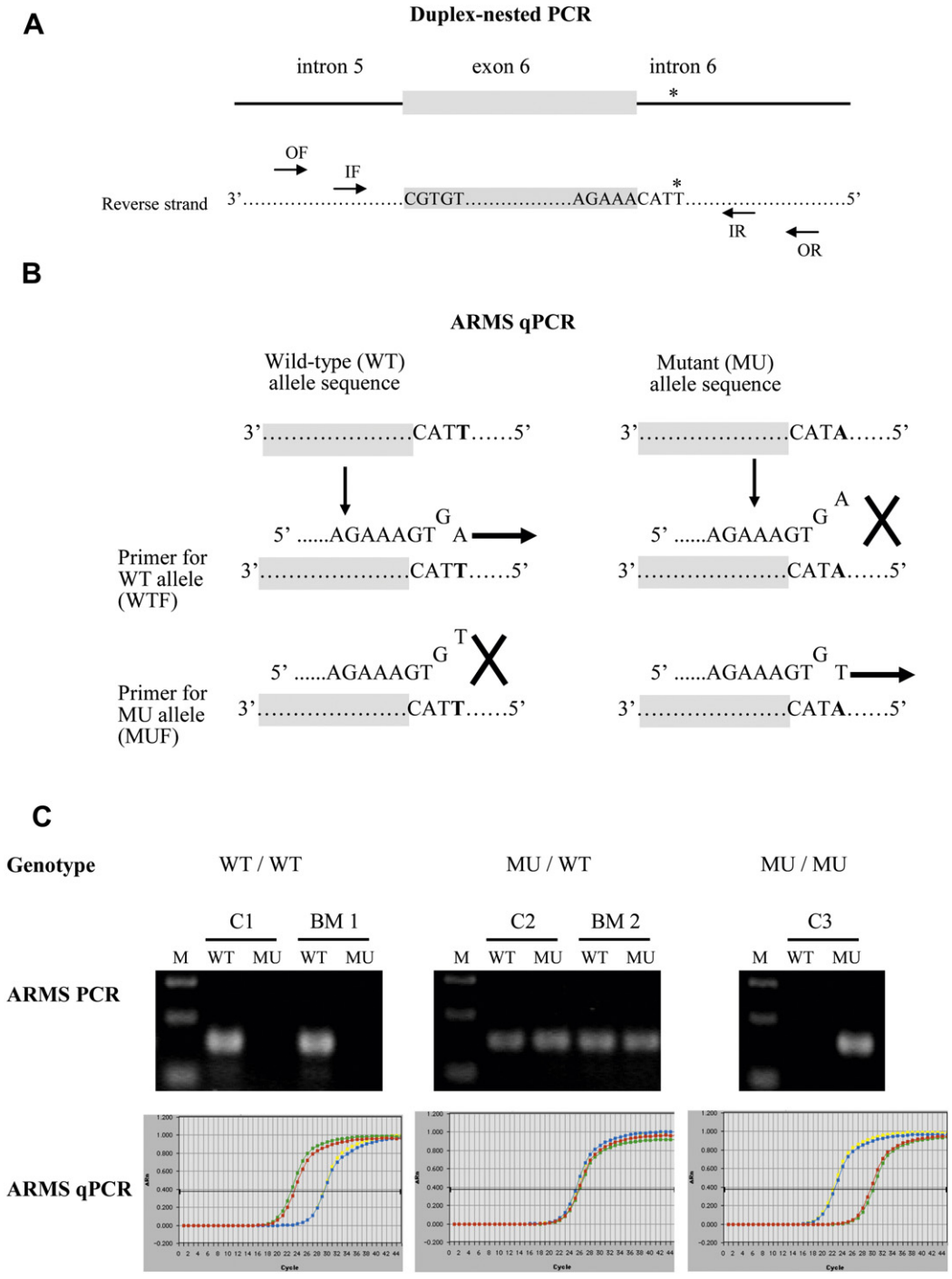

Fig. 2. Schematic diagram of a duplex-nested amplification refractory mutation system quantitative polymerase chain reaction (ARMS-qPCR) for PGD of the IVS6 + 4A>T point mutation in the *DDC* gene. (A) Duplex-nested PCR was used to amplify the region from DDC intron 5 to intron 6, including the position of the IVS6 + 4A>T mutation (*). The primers were designed using the reverse-strand sequence. OF and OR, outer primer set; IF and IR, inner primer set. (B) ARMS-qPCR to separate mutant and wild-type alleles. WTF and MUF, two primers specific for amplification of wild-type (WT) and mutant (MU) alleles, respectively. (C) A representative ARMS-PCR experiment for the following genotypes: WT/WT (including a normal control [C1] and an examined blastomere [BM1]), WT/MU (including one of the carrier parents [C2] and another examined blastomere [BM2]), and MU/MU (an affected individual lymphocyte control [C3]). M = marker. The result of the DN-ARMS-qPCR is shown at the bottom. PCR tests with WT allele-specific primers are indicated by red and green lines (duplication), whereas PCR tests with MU allele-specific primers are indicated by blue and yellow lines (duplication).

mutant allele may be the consequence of a founder effect [3]. The fact that currently available medical treatments for this condition have poor efficacy [3] justifies the use of PND and PGD in this population.

ARMS-qPCR for PGD

To our knowledge, the ARMS method has so far been used for PGD in only a few conditions, e.g., single allele-specific

ARMS in spinal muscular atrophy and double allele-specific ARMS in adenomatous polyposis [8,9]. In contrast to a single-ARMS test, where only one mutation-specific primer is used, our ARMS was based on a double allele-specific approach, thereby allowing discrimination of wild-type and mutated alleles. Well-known approaches were adapted to minimize misdiagnosis. Most PCR-based technologies used for PGD, e.g., restriction fragment length polymorphism (RFLP) [10] and primer extension mini-sequencing [11], rely on "endpoint" qualitative analysis. These methods can only assess the presence or absence of mutant and wild-type alleles, but cannot determine whether contamination or ADO occurred. In contrast, using real-time qPCR and monitoring PCR amplification, (especially reaction kinetics in the early phases of qPCR), facilitated correctly classifying the three genotypic combinations (wild-type/wild-type, wild-type/mutant, and mutant/mutant, [Fig. 2C]). In addition, contamination or ADO could be detected by assessing quantitative curves throughout the PCR process.

Recently, some real-time PCR technologies based on the principle of fluorescent resonance energy transfer (FRET) have been developed for single-cell genotyping with fluorescence-labeled FRET probes, and deemed useful as PGD methods [12]. However, designing the FRET probes increases costs. In that regard, ARMS-qPCR uses SYBR Green, a fluorescent dye that binds to the minor groove of double-stranded DNA, rather than fluorescent dye-labeled primers, making future PGD experiments easier and cheaper. Although the total time required for each experiment depends on the number of blastomeres sent for analysis, an optimized protocol should require less than 1 working day to accomplish the PGD. The ARMS-qPCR has many advantages for application in PGD, but it was suggested that cases requested for such customized design should be tested with linkage analysis to decrease the risk of misdiagnosis caused by extreme preferential amplification of one allele or ADO [13,14].

In conclusion, we report the first successful PGD of AADC using ARMS-qPCR. The initial step of duplex-nested PCR enabled the creation of specific replicates from a single blastomere in a single tube, reaching the threshold of the amount of DNA required for further amplification, followed by a novel ARMS-qPCR, to allow differential amplification of mutant and wild-type alleles. Given its high amplification rate and accuracy, ARMS-qPCR is a feasible method for PGD in couples with known genotypes. We propose that the molecular technique reported in this study will substantially contribute to PGA as well as PND of AADC.

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