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GENETICS

The mutation-free embryo for in vitro fertilization selected by MALBAC-PGD resulted in a healthy live birth from a family carrying PKD 1 mutation

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Abstract
Background Autosomal dominant polycystic kidney disease (ADPKD, autosomal dominant PKD or adult-onset PKD) is the most prevalent and potentially lethal kidney disease that is hereditary and lacks effective treatment. Preimplantation genetic diagnosis (PGD) of embryos in assistant reproductive technology (ART) helps to select mutation-free embryos for blocking ADPKD inheritance from the parents to their offspring. However, there are multiple pseudogenes in the PKD1 coding region, which make blocking ADPKD inheritance by PGD complicated and difficult. Therefore, this technique has not been recommended and used routinely to ADPKD family plan.
Methods and Results Here, we report a new strategy of performing PGD in screening (target-) mutation-free embryos. We firstly used a long-range PCR amplification and next generation sequencing to identify the potential PKD1 mutant(s). After pathogenic variants were detected, multiple annealing and looping-based amplification cycles (MALBAC), a recently developed whole genome amplification method, was used to screen embryo cells. We successfully distinguished the mutated allele among pseudogenes and obtained mutation-free embryos for implantation. The first embryo transfer attempt resulted in a healthy live birth free of ADPKD condition and chromosomal anomalies which was confirmed by aminocentesis at week 18 of gestation, and by performing live birth genetic screening.
Conclusions The first MALBAC-PGD attempt in ADPKD patient resulted in a healthy live birth free of ADPKD and chromosomal anomalies. MALBAC-PGD also enables selecting embryos without aneuploidy together and target gene mutation, thereby increasing implantation and live birth rates.

Keywords Polycystic kidney · Autosomal dominant · Preimplantation diagnosis · MALBAC

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited condition characterized by irreversible pathological development of cysts throughout the kidneys and decline in renal function [1]. It affects about 1/400 to 1/1000 live births [2] and accounts for 7–10% of all the patients with end-stage renal disease worldwide [3].

ADPKD has always been one of the major topics in nephrology and genetics research communities. Increasing basic researches contribute to illustrate the pathogenesis of ADPKD. It is now clear that ADPKD is caused by mutations in *PKD1* gene encoding polycystin-1 (85% cases) [4–7] or *PKD2* encoding polycystin-2 (15% cases) [8, 9]. The mutation in either of these two genes may result in the phenotype of ADPKD. Compared to the PKD2 mutation, PKD1 mutation usually indicates more severe disease, earlier onset, and poorer prognosis. Therefore, blocking PKD1 mutation is more beneficial for the patient. While new knowledge about how mutant polycystin-1/2 result in extensive cysts growth is still needed, emerging clinical

investigations focus on potential interventions minimize renal failure in ADPKD patients. While there was insufficient evidence that these treatments could improve patient outcome [10], the most promising breakthrough in this field is the proved efficiency of tolvaptan by a randomized controlled trial. Tolvaptan treatment resulted in a 50% reduction in the annual rate of kidney growth and a 33% reduction in the rate of decline in kidney function per year compared with placebo [11].

Due to the nature of ADPKD as a single-gene inherited disease, blocking the *PKD1* mutant heredity with preimplantation genetic diagnosis (PGD) is the most effective way, theoretically, of reducing the incidence of ADPKD. However, technical difficulties in single-cell whole-genome amplification and sequencing of *PKD1* hinder the generalization of PGD in this disease. With the development of a new whole-genome amplification method, MALBAC [12], sensitivity of detecting *PKD1* mutation with single embryo cell was greatly improved. After rigorous technical tests and ethical check, we performed MALBAC-PGD for one ADPKD couple. MALBAC-PGD enables simultaneous prevention of the mutated allele, as well as chromosomal abnormalities, thereby increasing success rates of clinical pregnancy and live birth. Furthermore, the female ADPKD patient gave birth to a healthy baby on August 3, 2016 as a result of this treatment. To our knowledge, this is the first application of MALBAC-PGD in ADPKD and chromosomal disease prevention.

Methods

Ovarian stimulation, in vitro fertilization, and embryo transfer processes were performed according to the standard protocol. If more than one mutation-free embryo is retrieved, only one embryo will be transferred and others will be frozen. And after PGD process, if *PKD1* mutation is still found in amniocentesis at week 18 of gestation, the female patient would decide whether to terminate the pregnancy.

PCR amplification with peripheral blood sample and library preparation

Genomic DNA was extracted from peripheral blood lymphocytes using a DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN, Inc., Valencia, CA, USA). The entire coding region, most of 5′ and 3′ untranslated regions, and the exon-intron boundaries of *PKD1* and *PKD2* were amplified in a total of six (four reactions for *PKD1* gene exons 1–34 and two reactions for *PKD1* gene exons 34–46 and *PKD2*) distinct PCR reactions using primers anchored either in the rare mismatched region with the human homologs (Fig. 1). The long-range PCR (LR-PCR) primers for *PKD1* gene exons 1–34 were shown in Table 1, as published previously [13–16] and the multiplex PCR primers for *PKD1* gene exons 34–46 and *PKD2* were designed using software Ion AmpliSeq™ Designer. LR-PCR amplification reaction system and amplification conditions for the various LR-PCR fragments were described in Table 2 and Table 3, respectively, which were optimized based on published article previously [13]. Ampliseq multiple PCR amplification reaction system and amplification conditions were set as manufacturer's instructions. The LR-PCR products (except for *PKD1* exon 1 with an extremely high content of GC) of *PKD1* gene exons 2–34 were purified with Agencourt AMPure XP Beads, then, quantified, fragmented by NEBNext Fast DNA Fragmentation Kit (New England Biolabs, Ipswich, MA, USA). Sequencing library of LR-PCR products and other Ampliseq multiplex PCR products were constructed by Ion Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) and then sequenced with Ion PGM™ 200 Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). *PKD1* exon 1 was amplified in another PCR reaction using specific nested primers and sequenced by Sanger method through 3730xl DNA analyzer.

Bioinformatics analysis and mutation identification and classification

All sequencing reads were mapped to the *PKD1*/2 reference genome with the Torrent Server TMAP software, and then we could get bam files. The reference sequences were NM_001009944.2 for *PKD1* and NM_000297.2 for *PKD2*. Then, bam files were analyzed using the Ion Reporter Software (version 4.4).

We adjusted ACMG (2015) classification principle [17] with PKDB classification and the actual needs of our PGD then classified variants into four different classes (Table 4). The first three classes were confirmed by long PCR and then

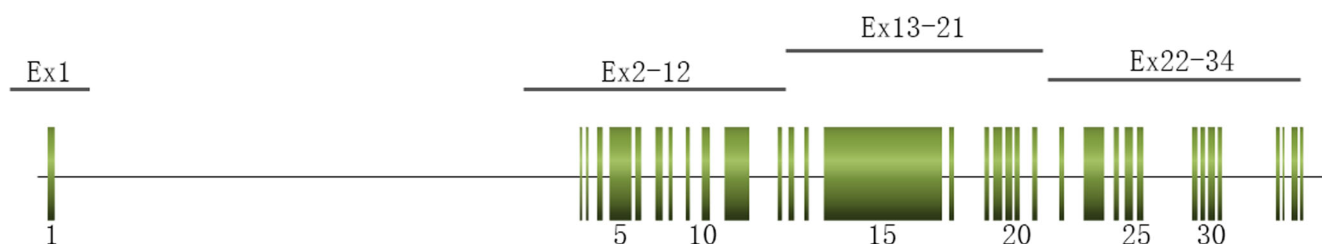

Fig. 1 Structural gene map and positional primer locations for *PKD1*

Table 1 PKD1 Gene Exons 1–34 Primers for LR-PCR

Fragment	Primers	Sequence (5′–3′)	Size(Kb)	Genomic location
*PKD1*_Ex1 [13–15]	*PKD1* _1F	CGCAGCCTTACCATCCACCT	2.3	chr16:2185030-2187307
	PKD1 _1R	TCATCGCCCCTTCCTAAGCA		
*PKD1*_Ex2-12 [13]	*PKD*1 _2-12F	CCAGCTCTCTGTCTACTCACCTCCGCATC	8.7	chr16:2163080-2171636
	*PKD1*_2-12R	CCACGGTTACGTTGTAGTTCACGGTGACG		
*PKD1*_Ex13-21 [13]	*PKD1*_13-21F	TGGAGGGAGGGACGCCAATC	7.9	chr16:2155145-2163036
	*PKD1*_13-21R	ACACAGGACAGAACGGCTGAGGCTA		
*PKD1*_Ex22-34 [16]	*PKD1*_22-34F	CCGTGTAGAGAGGAGGGCGTGTGCAAGGA	7.5	chr16:2147212-2154714
	*PKD1*_22-34R	TCGGCAAGGACCTGCTGGATCAGGTCTTC		

Sanger sequencing to prevent the false positivity due to high-through put sequencing error detection or mismatch in long segment amplification.

PGD procedure

Whole-genome amplification of each embryo biopsy samples was performed by MALBAC WGA kit (Yikon Genomics, Shanghai, China) following the instructions of the manufacturer. Sixty SNP markers linked to mutation alleles were selected for linkage analysis. The mutation site, as well as the SNPs, were amplified by using specific primer pairs. Then, the mixed amplification products were pooled with the MALBAC WGA products and sequenced. The chromosomal copy number as well as the mutation site and SNPs, were analyzed as published previously [18].

Results

Patient and genetic background

One couple (male, 35 years old, seemingly healthy; female, 31 years old, ADPKD patient, G0, P0) attended our reproductive center for artificial insemination with the husband's semen following preimplantation genetic diagnosis. They were seen by a genetic counselor and signed a consent form approved by ethical board of our hospital before further treatment. Basic characters and routine tests results of this couple were shown in Table 5. There were no contraindications for PGD. Long-range PCR and next-generation sequencing for the couple peripheral blood sample revealed a previously reported pathogenic mutation (*PKD1*, IVS21-2_1del AG) in the female patient (Fig. 2). Since the female patient has clear family history of ADPKD, peripheral blood sample of her parents was collected for linkage analysis. Her mother was revealed to be the parent who transmits her *PKD1* mutation to the daughter (Figs. 2 and 3).

In vitro fertilization

Ovarian stimulation was performed using protocol with GnRHa, recombinant FSH, and hCG for twice, and 17 oocytes were collected. After intracytoplasmic sperm injection procedure, totally six embryos were cultured in GIII series culture media. Embryo biopsies were carried out by mechanical method on days 5–7 for PGD.

Table 2 LR-PCR amplification reaction system

Components	Exons 2–12 and Exons 22–34 volume (μL)	Exons 1 and Exons 13–21 Volume (μL)
Template DNA	≥ 100 ng (XμL)	≥ 100 ng (XμL)
Forward primer	2	2
Reverse primer	2	2
TAKARA LA Taq	0.5	0.5
2*GC Buffer I	25	0
2*GC Buffer II	0	25
dNTP mixture	8	8
dd Water	Up to 50	Up to 50

Table 3 LR-PCR amplification conditions

PKD1 fragments	PCR conditions
*PKD1*_Ex1	94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, with a final extension step of 72 °C for 10 min
*PKD1*_Ex2-12 *PKD1*_Ex13-21 *PKD1*_Ex22-34	Touch-down protocol comprised of initial step of 94 °C for 2 min; followed by 14 cycles of 94 °C for 15 s, 66 °C for 30 s with decreasing 0.5 °C per cycle, and 68 °C for 6 min; followed by 25 cycles of 94 °C for 15 s, 59 °C for 30 s, and 68 °C for 6 min, with a final extension step of 72 °C for 10 min

Table 4 Variants' classification

Definitely pathogenic variants	Variants which had been reported definitely pathogenic variants
	Novel severe damage variants which include nonsense variants, frameshift variants caused by small inserts or deletions, splice site variants, and large inserts and deletions, and which were co-separation with symptoms and consistent with Mendel's law of inheritance
High suspicious pathogenic variants	Variants had been reported high suspicious variants
	Codon variants caused by inserts or deletions with three bases
	Novel severe damage variants while the pedigree could not be verified or not consistent with Mendel's law of inheritance
Likely pathogenic variants	Variants had been reported likely pathogenic variants
	Novel synonymous variants or missense variants or intron variants, of which functional structure prediction were serious damage, and which were consistent with Mendel's law of inheritance.
Other variants	Variants had been defined uncertain variants, benign variants or likely benign variants by ACMG.

Table 5 Clinical background of the couple

	Male	Female
Age (year)	35	31
BMI (kg/m^2)	25	23
Smoking history	Non-smoker	Non-smoker
Genetic diseases, inborn	None	ADPKD *(PKD1)*
Total kidney volume (cm^3)	NA	408
Family history of ADPKD	Negative	Positive
History of past illness	None	None
Childbearing history	NA	G0, P0
WBC ($\times 10^9$)	5.6	7.1
Hb (g/L)	175	140
Alb (g/L)	50	46
TBIL (μmol/L)	13	13
ALT (U/L)	13	13
SCr (μmol/L)	90	59
eGFR (CKD-EPI) (mL/min × 1.73m^2)	95	117
Karyotype analysis	No obvious anomaly	14p12h
Semen examination	Asthenozoospermia and oligospermia	NA
Sexually transmitted diseases	None	None
Blood type (ABO(Rh))	O+	B+
Anti-sperm antibody	NA	Negative
Antral follicle count (right/left)	NA	4/2
TORCH-related antibodies	NA	HSV1 IgG+; HSV2 IgG+; others -
E2 (pg/mL)	NA	17.17
P (ng/mL)	NA	0.17
T (ng/dL)	NA	11.60
PRL (ng/dL)	NA	10.88
LH (mIU/mL)	NA	2.47
FSH (mIU/mL)	NA	7.15
FSH/LH	NA	2.89

NA not applicable

PGD

Six biopsy samples from six embryos respectively were amplified by multiple annealing and looping-based amplification cycles [12]. Amplifications were failed in two samples. *PKD1*, IVS21-2_1del AG was detected in three samples by PCR and next-generation sequencing. Linkage analysis was performed to confirm the mutation detected in embryo samples by NGS. One embryo sample was found free of this mutation and then transferred on day 6. Luteal phase supports were given routinely. Serum β-hCG levels were measured at 14 days after ET. The presence of a gestational sac and fetal heartbeat by ultrasound at 5 weeks after ET evidenced clinical pregnancy. Long-range PCR and next-generation sequencing were repeated with the amniotic fluid sample collected by amniocentesis at 18 weeks of gestation. On August 3, 2016, the female patient gave birth to a child. The genotype of this child was confirmed again by long-range PCR and next-generation sequencing. No mutation of *PKD1* was found.

Discussion

For the nature of ADPKD as a single-gene inherited disease, PGD should have been an ideal option for patients who have fertility desire. However, a few technical difficulties hinder the application of PGD in ADPKD. The genomic structure of the *PKD1* gene included 46 exons with a ~2.5-kb polypyrimidine tract in intron 21. Approximately, 70% of the gene is duplicated in highly homologous loci on chromosome 16. And exon 1 is a high GC content area [19]. PCR amplification of long fragment is necessary to overcome these difficulties [20–22].

Amplification by long PCR of the entire coding region and the exon-intron boundaries of *PKD1* and *PKD2* is a tough task. Former researchers used eight primers and five thermal cycling conditions to PCR amplify exons 1–34 of *PKD1*, and all the five reactions took more than 5 h [23]. Besides time -

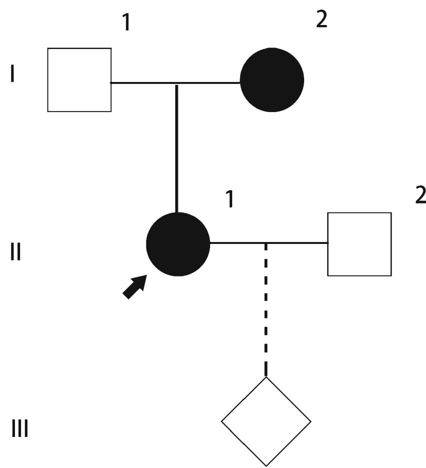

Fig. 2 Pedigree of the family

consuming, the reported method requires inputting a large amount of DNase in the PCR template that limited the usage of the method in PGD. Although the researchers had kept optimizing the reaction conditions, there had been no significant improvement so far. The update protocol proposed by Tan required 10 PCR reaction systems to cover the entire coding region, most 5′ and 3′ untranslated regions and the exon-intron boundaries of *PKD1* and *PKD2*, with five different reaction conditions [13]. To improve the efficiency of the experiment without increasing the cost significantly, we used Adrian Y. Tan's four long PCR primers in the *PKD1* polyclonal region. We amplified the *PKD1* gene 34–46 exons and the exon-intron boundaries of *PKD2* with multiplex PCR method (Ampliseq, thermofisher). Therefore, only two PCR reactions are needed in our setup. The sequencing library was then constructed with routing method of mechanical DNA fragmentation. In total, six reactions with three reaction conditions accomplished all of the amplification needed.

There are several strengths in our sequencing process. First, all sequenced reads were mapped to the *PKD1/2* reference genome by the Torrent Server TMAP software to reduce false negative rate caused by true gene region alignment to pseudogene region. Second, the pathogenic mutations were confirmed by long PCR and then Sanger sequencing. In summary, we established a more effective protocol than ever to perform ADPKD-PGD in a practical manner.

Although there is only one case in this study, it is a validation of this combined PGD technology in ADPKD. To confirm efficacy and safety of this strategy, a multicenter nonrandomized controlled trial (NCT02948179) using this technology is ongoing. Although in this report, ADPKD in this case was not in rapid progression, she and her family have strong desire in PGD. When PGD is generalized in clinical practice for ADPKD couples, the indication should be specified. Patients with rapid progressive ADPKD would be preferred choice. Indicators include established TKV growth rate > 5% per year, annual eGFR decline > 5 mL/min/1.73 m^2, truncating *PKD1* mutations and elevated plasma copeptin level [24]. However, according to a recently published survey, a proportion of patients believe that PGD should be made available to prospective parents with this disease regardless of the progression of disease [25]. To the patients with less progressive ADPKD but strong desire of performing PGD, whether the PGD should be made available is open to discussion.

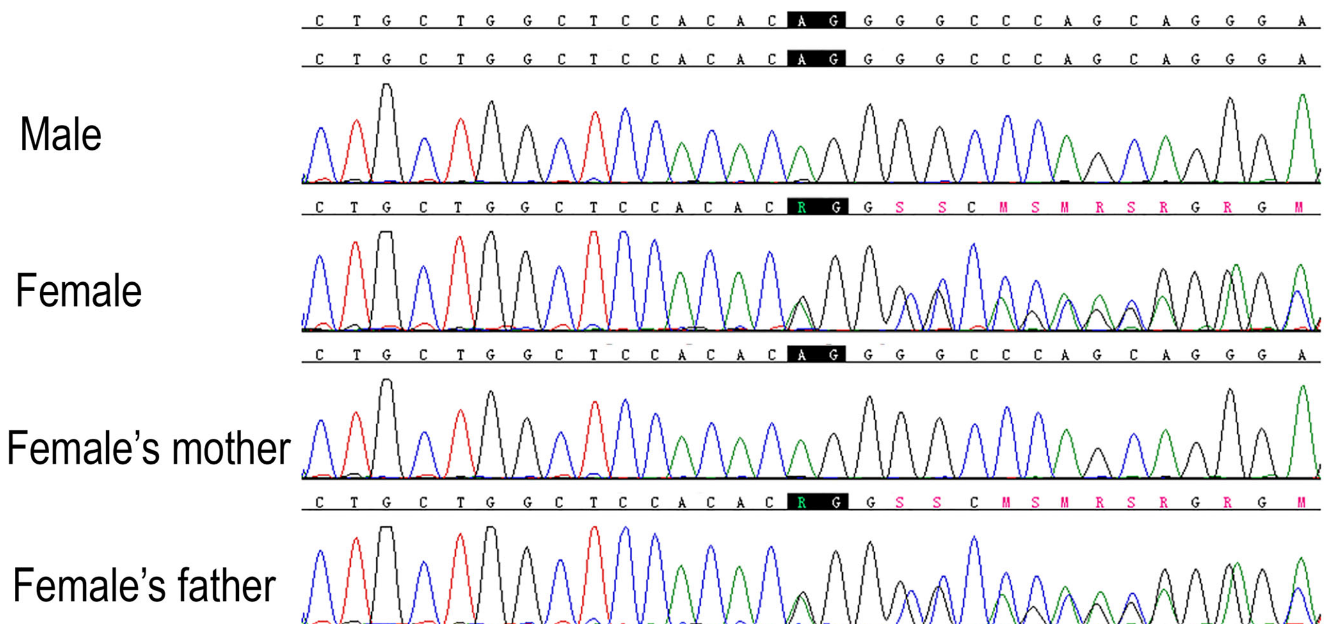

Fig. 3 Chromatogram of the peripheral blood sample from female's family

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate The study protocol and all subjects who participated in this study were approved by the Institutional Review Board of our institute in which informed consent was obtained from all families prior to participation in accordance with institutional and national guidelines.

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