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Original Article

Detection of SEA-type α-thalassemia in embryo biopsies by digital PCR

Ta-Hsien Lee [a], Ya-Chiung Hsu [b], Chia Lin Chang [b, *]

[a] Institute of Biomedical Science, Chang Gung University, No. 259, Wenhua 1st Road, Guishan Township, Taoyuan City 33302, Taiwan
[b] Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital Linkou Medical Center, Chang Gung University, 5 Fu-Shin Street, Gueishan Township, Taoyuan County 333, Taiwan

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ABSTRACT

Objective: Accurate and efficient pre-implantation genetic diagnosis (PGD) based on the analysis of single or oligo-cells is needed for timely identification of embryos that are affected by deleterious genetic traits in *in vitro* fertilization (IVF) clinics. Polymerase chain reaction (PCR) is the backbone of modern genetic diagnoses, and a spectrum of PCR-based techniques have been used to detect various thalassemia mutations in prenatal diagnosis (PND) and PGD. Among thalassemias, SEA-type α-thalassemia is the most common variety found in Asia, and can lead to Bart's hydrops fetalis and serious maternal complications.

Materials and methods: To formulate an efficient digital PCR for clinical diagnosis of SEA-type α-thalassemia in cultured embryos, we conducted a pilot study to detect the α-globin and SEA-type deletion alleles in blastomere biopsies with a highly sensitive microfluidics-based digital PCR method. Genomic DNA from embryo biopsy samples were extracted, and crude DNA extracts were first amplified by a conventional PCR procedure followed by a nested PCR reaction with primers and probes that are designed for digital PCR amplification.

Results: Analysis of microfluidics-based PCR reactions showed that robust signals for normal α-globin and SEA-type deletion alleles, together with an internal control gene, can be routinely generated using crude embryo biopsies after a 10^6-fold dilution of primary PCR products.

Conclusion: The SEA-type deletion in cultured embryos can be sensitively diagnosed with the digital PCR procedure in clinics. The adoption of this robust PGD method could prevent the implantation of IVF embryos that are destined to develop Bart's hydrops fetalis in a timely manner. The results also help inform future development of a standard digital PCR procedure for cost-effective PGD of α-thalassemia in a standard IVF clinic.

Introduction

Alpha-thalassemia (α-thalassemia) is one of the most serious inherited autosomal diseases in Asian populations, and has a high prevalence in the tropical and sub-tropical areas. The majority of α-thalassemia can be attributed to genetic aberrations on chromosome 16p13.3 [1,2]. Large segment deletions of α-globin gene clusters cause defects in hemoglobin synthesis, and beget mild to severe anemia [3]. The most common α-thalassemias in South and Southeast Asia include the South-East Asian (SEA), Philippines (PHI), and Thailand (THAI) type deletions. Among them, the SEA-type deletion has the highest prevalence, and the carrier frequency ranges from 3 to 11% in most populations.

Most SEA-type carriers are asymptomatic or display mild symptoms, yet homozygous SEA-type deletion can lead to Hb Bart's hydrops fetalis in the offspring when both parents are carriers [4–7]. In addition, the presence of Hb Bart's hydrops fetalis can lead to serious maternal complications. Although the overall chance of having a baby with Hb Bart's hydrops fetalis is low in a population with a 3% prevalence of SEA-type carriers, it represents a serious concern for many couples, especially those with infertility issues. Because there is a one in four chance of having a baby with the Hb Bart's hydrops fetalis when both parents are carriers, it is prudent to diagnose potential homozygous embryos during the prenatal stage. This has led to a wide usage of prenatal diagnosis (PND) among high risk pregnant women, and later the application of pre-

* Corresponding author. Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital Linkou Medical Center, Chang Gung University, 5 Fu-Shin Street, Guishan Township, Taoyuan, Taiwan. Fax: +886-33288252.
E-mail address: amego@cgmh.org.tw (C.L. Chang).

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implantation genetic diagnosis (PGD) in IVF patients prior to the beginning of pregnancy.

PGD, which examines the genetic makeup of pre-implantation embryos for inheritable gene defects, aims to prevent the possibility of transmitting genetic disorders among fertilized embryos in IVF clinics before embryo implantation, thereby allowing carriers of a genetically defective allele to conceive a healthy baby [8,9]. Most of the PND and PGD procedures utilize polymerase chain replication (PCR) to amplify the affected genetic component(s). These diagnoses use a wide spectrum of methods ranging from multiplex conventional PCR, real-time PCR, microsatellite PCR to droplet digital PCR [9–11]. Currently, the standard diagnosis for α-thalassemia carriers includes conventional PCR and real-time PCR. For example, studies using a multiple probe GAP-PCR approach indicated that this technique could allow 100% detection of SEA-type deletion in carriers and embryonic tissues [10]. Likewise, a recent study of multiplex fluorescent PCR demonstrated the possibility of using this technique to detect SEA-type deletion with genomic DNA from Hb Bart's hydrops fetalis and single-cell embryo samples [8,9]. However, the efficiency of obtaining acceptable amplification from single cells was only about 85%, and the method is still prone to erroneous readout, suggesting that a more reliable procedure that provides convenient and timely evaluation of select genotypes in cultured embryos is much needed.

Digital PCR is a high-resolution detection method that has been used in the detection of short-fragment tumor DNA in the blood of patients, and for non-invasive prenatal diagnosis of inheritable disorders [12–14]. Because the readout of digital PCR is based on signals in thousands of independent PCR reaction compartments, its readout is less prone to influence by contaminants when compared to single-tube PCR reactions [12,15]. It is also less affected by the amplification efficiency when compared to other PCR procedures [12,15]. Therefore, it could be an optimal tool for quick and accurate detection of inherited genetic disorders in cultured embryos at IVF clinics. While digital PCR is ideal for the quantification of individual targets in complex samples, the power of this method is limited in cases where known targets are relatively simple. To take advantage of the power of digital PCR, in the present study we evaluated the feasibility and optimal conditions of using a nested digital PCR approach to identify embryos with the SEA-type deletion allele before embryo transfer in an IVF clinic. The results may help inform future application of digital PCR for robust and cost-effective PGD of α-thalassemias in IVF clinics and reduce the occurrence of Hb Bart's hydrops fetalis [11].

Materials and methods

Patients, sample collection, and DNA extraction

All studies were conducted with the approval of the Institutional Review Board (IRB) and Human Research Ethics Committee at Chang Gung Memorial Hospital, and with informed consent from each patient. The IRB No. is 102-0050C. This study enrolled a total of 20 cases from March 2010 to May 2014. These patients aged 29–39, and have an average age of 33. Nineteen of these cases were normal, and only one couple carried the SEA-type thalassemia in both the maternal and paternal genomes. From this couple, we obtained 9 samples of 3 pro-nuclei (PN) zygotes, 27 samples of Day 3 arrested 2 PN embryos, and 4 samples of blastomere biopsies.

Biopsied blastomere(s) from the cleavage-stage or trophectoderm cell-stage embryos were collected into 0.2 ml Eppendorf tubes with 3 μl cell lysis buffer (Universal ^{TM} extraction buffer, Yeastern biotech CO. Ltd.) [16]. DNA was released following heating at 95 °C for 5 min, and all DNA samples were kept at −20 °C before the assay. The α-thalassemia positive control DNA samples were collected from the peripheral blood of well characterized SEA-type deletion carriers, and were extracted with the QIAamp DNA mini-kit (Qiagen, Germany). The concentration of DNA was quantified with a Thermo Nanodrop 1000 spectrophotometer.

Detection with the conventional PCR procedure

To identify normal α-globin and SEA-type deletion alleles, DNA from all blood and embryo biopsy samples were amplified with a nested PCR protocol (Fig. 1, Table 1). DNA samples were first amplified with a set of flanking PCR primers. The amplification was performed with the Qiagen HotstarTaq Plus DNA polymerase kit. The reactions each contained 2.5 μl 10X PCR buffer, 1 μl $MgCl_2$ (25 mM), 0.5 μl HotstarTaq Plus DNA polymerase (5 U/μl), 5 μl Q

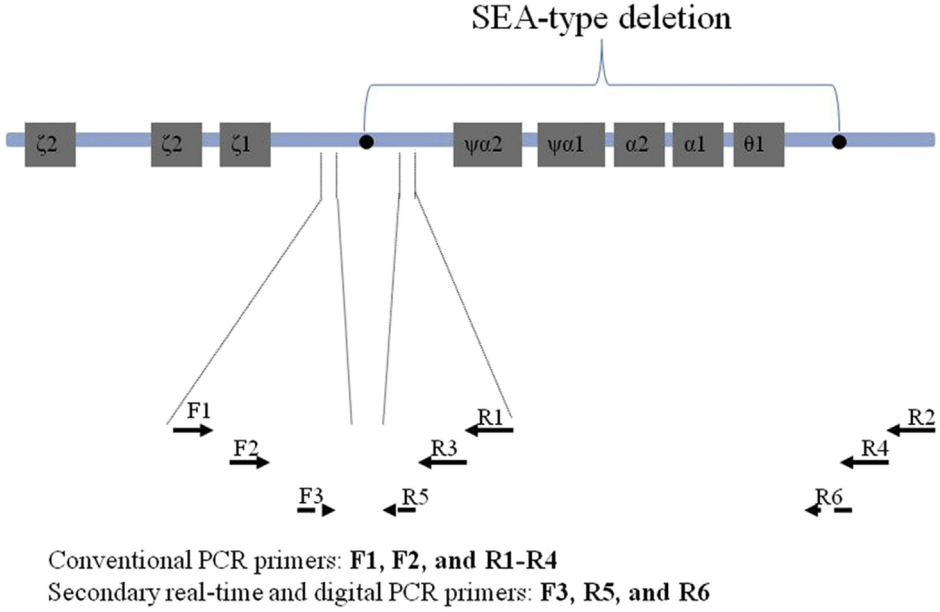

Fig. 1. Design of nested PCR primers and probes for the detection of SEA-type deletion in DNA of embryo biopsies. The positions of primary and secondary primers within the α-globin gene clusters are indicated by arrows. The position of SEA-type deletion is indicated by an arrow bracket.