Microsatellite Markers within —SEA Breakpoints for Prenatal Diagnosis of HbBarts Hydrops Fetalis

Sherry Sze Yee Ho,1 Samuel S. Chong,2,4 Evelyn S.C. Koay,3,4 Yiong Huak Chan,5 Ponnusamy Sukumar,1 Lily-Lily Chiu,4 Wen Wang,2 Ashim Roy,1 Mary Rauff,1 Lin Lin Su,1 Arijit Biswas,1 and Mahesh Choolani1*

Background: We sought to develop a rapid prenatal diagnostic test for simultaneous detection of HbBarts hydrops fetalis and exclusion of maternal contamination.

Methods: We developed a multiplex quantitative fluorescent PCR (QF-PCR) test that detects the presence/absence of 2 microsatellite markers (16PTEL05/16PTEL06) located within breakpoints of the Southeast Asia (—SEA) deletion. HbBarts hydrops fetalis (—SEA/—SEA) is diagnosed by absence of both markers, and maternal contamination of fetal DNA is excluded by absence of noninherited maternal alleles. Fetal and parental DNA samples from 50 families were analyzed in a blinded clinical validation study, and QF-PCR results were compared with their respective molecular genotypes.

Results: The multiplex QF-PCR results included correct diagnoses of HbBarts hydrops fetalis in 11 of the fetuses tested, correct verification as unaffected in 20 fetuses, and correct identification as either carriers (αα/—SEA) or unaffected homozygotes in 18. Misidentification as unaffected occurred for 1 carrier. Sensitivity for diagnosis of HbBarts hydrops fetalis was 100% [lower 95% confidence interval, 76.2%], and specificity was 100% (lower 95% confidence interval, 92.6%). None of the samples tested showed any traces of noninherited maternal alleles; thus false-positives because of maternal contamination were eliminated.

Conclusions: In this QF-PCR method, detection of maternally and paternally inherited fetal alleles allowed diagnosis of the double-deletion syndrome, and the ability to differentiate between these alleles allowed simultaneous exclusion of maternal contamination of the fetal genetic material. This novel strategy using cell-free fetal DNA in maternal plasma could form the basis for noninvasive testing for HbBarts hydrops fetalis.

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α-Thalassemia (OMIM 141800 and 141850), an inherited anemia syndrome, is the most common of the inherited hemoglobin synthesis disorders, which are the most common monogenic diseases (1, 2). α-Thalassemia is characterized by decreased or complete absence of α-globin chain synthesis (3–5), caused by deletion of or mutation (nondeletional) in the α-globin genes (1, 6). Clinically, 4 variants of the syndrome are recognizable, with increasing severity of the disease manifestation depending on how many normal α-globin genes are present (3, 2, 1 or none) (7, 8). Retention of 3 normal α-globin genes results in a silent carrier state, with minimal complications. Individuals with 2 normal α-globin genes develop microcytosis (heterozygous α-thalassemia). Those with 1 normal α-globin gene often have microcytosis and hemolysis (HbH disease). Loss of all 4 α-globin genes, as can occur in the common Southeast Asian (—SEA)6 deletion, leads to HbBarts hydrops fetalis (9, 10). Affected fetuses develop severe intrauterine anemia and become hydropic, usually in the 2nd and 3rd trimesters. They die either in utero or soon after birth. Maternal complications such as hypertension, preeclampsia, polyhydramnios, and severe post-partum hemorrhage can lead to fatal consequences in late gestation and at delivery (11). Of the many mutations that have been described, deletions at the α-globin gene locus

Departments of1 Obstetrics & Gynaecology, 2 Paediatrics, and 3 Pathology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

4 Molecular Diagnosis Centre, National University Hospital, Singapore.

5 Biostatistic Unit, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

* Address correspondence to this author at: Department of Obstetrics and Gynaecology, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074. Fax 65-6779-4753; e-mail obgmac@nus.edu.sg.

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account for most α-thalassemia cases. In —SEA, the most common α-globin gene deletions encountered are the single gene deletions, α^3.7α and α^4.2α, and the double gene deletions in cis, —SEA, —FIL, and —THAI. The —MED and —(α)20.5 double-gene deletions are more prevalent in the Mediterranean region. Because these deletions are regionally specific (12), programs of screening, genetic counseling, and prenatal diagnosis of specific mutations have been developed for individual regions (13, 14).

Carrier frequencies of the —SEA deletion in the general population range from 4.5% in Hong Kong to 14% in northern Thailand (15). In Singapore, 5.6% of the population carries α-thalassemia mutations, and of these carriers, 27% carry deletion (18). When both parents carry the —SEA deletion, there is a 1 in 4 chance that the fetus will inherit defective alleles from both parents and be affected with HbBarts hydrops fetalis. Couples at risk should be identified and offered genetic counseling and prenatal diagnosis so that they can make an informed choice. A number of methods can be used to perform prenatal diagnosis with DNA isolated from chorionic villi, amniocytes, or fetal blood samples.

Southern blot analysis (19, 20), formerly the standard method to detect gene deletions, is time-consuming, laborious, and technically demanding; with a limited detection rate of 60%–80%, this method is not suitable for large-scale screening (21). DNA sequence analysis of each deletion breakpoint has now enabled PCR-based testing (22–26). Several techniques based on PCR amplification of normal and affected chromosomes (26–28) have been developed to more rapidly identify globin gene mutations. These techniques include single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis (29, 30), direct sequencing, amplification refractory mutation system PCR (31), reverse dot-blot analysis (32), and Gap-PCR, which is based on the multiplex amplification of junctional segments of several different breakpoints (33–36). The latter technique enables screening and diagnosis of several common deletions in a single test. The advantages of a multiplex-PCR test are reductions in cost and time. Although PCR-based techniques are highly sensitive and require only a small amount of DNA to make a diagnosis, they are also prone to false-negative results from allele dropout (37) and to false-positive results due to amplification of contaminating maternal DNA that may be present in the fetal samples. Chan et al. (1997) reported a misdiagnosis rate of 3.8% attributable to maternal DNA contamination (19). Current established PCR-based diagnostic tests require a separate test to exclude maternal contamination.

We describe a novel technique that enables the simultaneous diagnosis of HbBarts hydrops fetalis and exclusion of maternal contamination. Using multiplex quantitative fluorescent (QF)-PCR, we amplified polymorphic microsatellite markers within the —SEA deletion breakpoints. Complete absence of these markers suggests a deletion on both alleles, because these microsatellite markers are located within the breakpoints. The polymorphic nature of these microsatellite markers enables differentiation between maternal and paternal alleles, enabling the exclusion of maternal contamination by confirming the absence of noninherited maternal alleles within the fetal DNA samples. The additional cost and time required for a separate test to exclude maternal contamination are eliminated.

**Materials and Methods**

**SAMPLE COLLECTION AND DNA ISOLATION**

Controls. We used the Puregene DNA Purification Kit (Genta Systems Inc) to isolate DNA from 3 cell lines obtained from the Coriell Cell Repositories (GM10799, GM03037, GM03433). GM10799 was initiated from the B-lymphocytes obtained from an α-thalassemia carrier (αα/−SEA), and both GM03037 and GM03433 were initiated from fibroblasts obtained from patients with HbBarts hydrops fetalis (−SEA/−SEA). DNA was also isolated from EDTA-anticoagulated blood samples (3 mL each) from 2 α-thalassemia carriers (carrier-1, carrier-2) with the genotype αα/−SEA and 2 healthy volunteers (normal-1, normal-2). The buffy layer was separated from the plasma by a 10-min centrifugation at 1600 g and diluted (1:1) with 1× phosphate-buffered saline (137 mol/L NaCl, 10 mol/L phosphate, 2.7 mol/L KCl, pH 7.4). DNA was isolated from 200 μL of the diluted buffy layer with High Pure Template DNA Purification Kit (Roche GmbH) according to the manufacturer’s recommendations. The α-globin genotypes were determined by the α-thalassemia 7-deletion multiplex PCR as previously described (38).

**Paired Parental and Fetal Samples.** In the blinded clinical validation study, we analyzed 50 sets of stored DNA samples consisting of maternal, paternal, and fetal DNA obtained from the DNA Bank of the Molecular Diagnosis Centre at the National University Hospital. Use of the banked DNA samples for this study complied with the regulations set by our Institutional Review Board, including informed patient consent and anonymization of source. Sources were peripheral blood for parental DNA and amniotic fluid (AF), chorionic villi, or fetal blood for fetal DNA. Maternal contamination of the fetal DNA samples was monitored by PCR amplification of the D1S80 variable number of tandem repeat (VNTR) polymorphism (39). In all prenatal samples, parental and fetal samples were haplotyped simultaneously to exclude maternal contamination. As part of the routine diagnostic protocol, the α-globin genotypes of these samples had been previously determined according to the method of Tan et al. (38), but these results were not made known to the analysts conducting the clinical validation study for the new QF-PCR method.

**Unpaired AF Samples.** To evaluate the polymorphism of the microsatellite markers within the Singapore population, 100 AF samples obtained from patients undergoing
routine prenatal diagnostic screening were used. Two milliliters of AF were washed and resuspended in 2 mL of 1 × PBS before DNA isolation with the QIAamp DNA Mini Kit (Qiagen GmbH) according to the manufacturer’s recommendations. QF-PCR was performed with the primer sequences for the microsatellite markers (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue2).

**Primer Design and Analysis of Microsatellite Markers**

The breakpoints of —SEA were described by Kutlar et al. (GenBank Accession AY207443) (40). These breakpoint sequences correspond to nucleotide (nt) 155395-174700 of AE006462. Two microsatellite markers (16PTEL05 and 16PTEL06) within this deleted region were found using the Marshfield Genetic Map Database (http://research.marshfieldclinic.org/genetics/home/index.asp); 16PTEL05 is located within nt 160725–160915 and 16PTEL06 is located within nt 171931–172078 (with reference to AE006462.1); 16PTEL05 contains 2 short tandem repeats (STR): an (AATA)n tetranucleotide repeat and a (CA)n dinucleotide repeat, and 16PTEL06 consists of only (CA)n dinucleotide repeats. Previous experiments had shown that the polymorphic nature of the 16PTEL05 microsatellite marker is derived from (CA)n repeats and not (AATA)n repeats (data not shown). Therefore, the primer pair 16PTEL05-F/R was designed to flank only the (CA)n repeats. The other primer pair, 16PTEL06-F/R, was designed to flank the (CA)n repeats of the 16PTEL06 microsatellite marker. All primers were designed by use of Primer Express Software v2.0 (Applied Biosystems) with the downloaded DNA sequence from GenBank (Accession AE006462). As a control, polymorphic microsatellite marker D16S539 was used. D16S539 is located on 16q24-qter, which lies outside the α-globin gene cluster. Therefore, deletions and mutations of the α-globin gene cluster will not affect the integrity of D16S539. The D16S539 primer sequences (D16S539-F/R) were obtained from the STRBase (http://www.nist.gov/div831/strbase/) website. During capillary electrophoresis all forward (F) primers were fluorescent-tagged for the detection of the amplified PCR products (see Table 1 in the online Data Supplement).

**Singleplex vs Multiplex QF-PCR.** To calculate the heterozygosities of all 3 microsatellite markers for the determination of polymorphism, we performed singleplex QF-PCR for each of the primer pairs. For the rest of the samples, D16S539-F/R, 16PTEL05-F/R, and 16PTEL06-F/R, primers were used together within a PCR reaction (single-tube multiplex-QF-PCR). PCR was performed with 3 μL of extracted DNA in a volume of 25 μL, with 1 × PCR buffer, 2.0 mmol/L MgCl₂, 0.8 mmol/L each of deoxynucleotide triphosphates (PE Biosystems), and 1 Unit of AmpliTaq Gold polymerase (Roche). We used 0.6 μmol/L of each specific primer (Proligo Primers and Probes Pty) for singleplex QF-PCR. For multiplex QF-PCR, we used 0.2 μmol/L D16S539-F/R, 0.5 μmol/L 16PTEL05-F/R, and 1.5 μmol/L 16PTEL06-F/R. Thermal cycling was performed in a PTC-200 Thermal Cycler (MJ Research Inc.) with an initial 5-min denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 s, 66 °C for 30 s, and 60 °C for 30 s, with a final extension of 60 °C for 5 min, followed by a 1-h incubation at 60 °C. Two microliters of the amplified PCR products were mixed with 9.5 μL of deionized formamide and 0.5 μL of GS-500 ROX standard (Applied Biosystems). The mix was heated at 90 °C for 2 min, followed by 4 °C for 5 min before resolving on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The resulting peaks were analyzed with GeneScan analysis software (Applied Biosystems).

**Sequencing of Microsatellite Markers.** The isolated DNA from the 2 healthy samples (healthy-1, healthy-2) were amplified with the same primer sequences (see Table 1 in the online Data Supplement), except that the forward primers are nonfluorescent labeled. The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s recommendations. Nucleotide-nucleotide BLAST (blastn, http://www.ncbi.nlm.nih.gov/BLAST/) was used to ensure that all the amplified sequences correspond to nucleotide (nt) 155395 -174700 of GenBank Accession AY207443. These breakpoint products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s recommendations. Nucleotide-nucleotide BLAST (blastn, http://www.ncbi.nlm.nih.gov/BLAST/) was used to ensure that all the target sequences of the primers were correctly amplified.

**Statistical Analysis**

SPSS 14.0 (SPSS Inc.) was used for the statistical analysis.

**Results**

**Controls**

DNA sequences from the 2 healthy volunteers (normal-1, normal-2) were obtained and aligned with the reference sequence, AE006462, by use of the nucleotide-nucleotide BLAST (blastn, http://www.ncbi.nlm.nih.gov/BLAST/). BLAST results confirmed that all the amplified sequences were specific to their respective target primers (see Table 1 in the online Data Supplement).

In DNA samples isolated from carriers with the α-thalassemia-1 trait (αα/—SEA) (carrier-1, carrier-2, GM10799), D16S539 alleles were amplified but only 1 allele was amplified for each of 16PTEL05 and 16PTEL06 (Fig. 1A). Both alleles of D16S539 in the HbBarts hydrops fetalis cell lines (GM03037, GM03433) were amplified and detected, whereas none were detected for 16PTEL05 or 16PTEL06 (Fig. 1B). The absence of both 16PTEL05 and 16PTEL06 in samples GM03037 and GM03433 suggested HbBarts hydrops fetalis (—SEA/—SEA).

**Blinded Study**

Samples were identified as HbBarts hydrops fetalis (—SEA/—SEA) when D16S539 was amplified in the absence
of both 16PTEL05 and 16PTEL06. The presence of only 1 allele in each of 16PTEL05 and 16PTEL06 suggested 2 possible genotypes: α-thalassemia-1 carriers (αα/−SEA), in which 1 of the alleles had been deleted; or normal homozygotes (αα/αα), in which both alleles were the same size and therefore could not be resolved as 2 different alleles. Normal samples (αα/αα) were identified when both alleles of either 16PTEL05 or 16PTEL06 were amplified and detected. Using this analysis protocol, we identified 11 fetal samples as HbBarts hydrops fetalis (−SEA/−SEA), 18 fetal samples as either αα/−SEA or unaffected homozygotes, and the remaining 21 fetal samples as unaffected (see Table 2 in the online Data Supplement).

In electropherogram results of Family-4 (Fig. 1C), which included α-thalassemia-1 parents (Father-4, Mother-4) with a fetus (Fetus-4) afflicted with HbBarts hydrops fetalis (−SEA/−SEA), neither of the microsatellite markers, 16PTEL05 or 16PTEL06, could be detected in Fetus-4. We compared these results with their respective known genotypes documented at the Molecular Diagnosis Centre only after all the diagnostic calls had been made in a prospective, blinded manner by the authors (see Table 2 in the online Data Supplement). From the known genotypes, 11 fetal samples were identified as HbBarts hydrops fetalis, 18 as αα/−SEA, and 21 as unaffected. Our results differentiating between fetuses with and without HbBarts hydrops fetalis were concordant in all cases (sensitivity 100%, lower 95% confidence interval, 76.2%; specificity 100%, lower 95% confidence interval, 92.6%). One fetal sample with the known carrier genotype αα/−SEA was misdiagnosed by our multiplex QF-PCR as unaffected, with the presence of 2 alleles in 16PTEL05 and 16PTEL06. Maternal contamination was excluded by the absence of 1 maternal allele of D16S539.

**Assessment of Maternal DNA Contamination**

Our multiplex QF-PCR was designed to amplify both targets (16PTEL05 and 16PTEL06) and control (D16S539) in a single tube for each sample. Therefore, simultaneously with diagnosis, maternal contamination can be excluded with the absence of D16S539 noninherited maternal alleles in fetal DNA samples. Results showed that none of the fetal DNA samples was contaminated with maternal DNA, and this was verified with VNTR analysis at the DISS80 locus.

**Heterozygosity and Polymorphism Information Content**

The number of alleles and size range of fragments obtained for each microsatellite marker obtained from the 100 AF samples are shown in Table 1. Previous experiments had shown that the polymorphism of 16PTEL05 derives from the (CA)n dinucleotide repeats and not the (AATA)n tetranucleotide repeats (data not shown). Therefore, to calculate heterozygosities of 16PTEL05, we used a primer pair of 16PTEL05 (16PTEL05-F/R) that targets only the (CA)n repeats. Allele frequencies, polymorphism
information content (PIC), and heterozygosities shown in Table 1 were calculated with PowerStats v12 freeware (http://www.promega.com/geneticidtools/powerstats). We found that the PIC values of all 3 markers ranged from 0.76 to 0.86. According to Botstein et al., a marker can be considered highly informative in a mapping population if it has an expected PIC >0.5 (41). Therefore, all 3 microsatellite markers (D16S539, 16PTEL05, and 16PTEL06) were highly informative.

**Discussion**

The basis of PCR diagnosis of a fetus affected with homozygous α-thalassemia-1 is the absence of all 4 α-globin genes. Because of its high sensitivity, PCR requires a very small amount of DNA for diagnosis but will also detect the few copies of contaminating α-globin genes that may be present in the sample. Therefore, low levels of maternal DNA contamination may result in misdiagnosis. Current established PCR-based methods require a separate test to exclude maternal contamination. D1S80 testing, commonly used to exclude maternal contamination, is a VNTR consisting of a 16 bp repeat with at least 29 alleles ranging in size from 369 to 801 bp corresponding to 14 and 41 repeats, respectively (42,43). PCR efficiency is reduced with the amplification of large repeats such as D1S80. Differential amplification may also result in drop-out of the larger allelic product and misclassification of a heterozygous individual as homozygous for the overamplified smaller allele. Therefore, in cases in which results of D1S80 analysis are inconclusive, further tests with STR analysis are required. We developed a novel prenatal diagnostic test that uses QF-PCR to detect HbBarts hydrops fetalis in a single assay. We investigated specifically the α-thalassemia-1 type deletion because it is the most common double-gene deletion in −SEA. Our results support the hypothesis that HbBarts hydrops fetalis (−SEA/−SEA) can be diagnosed by analyzing microsatellite markers within the breakpoints. These microsatellite markers (16PTEL05 and 16PTEL06) are highly informative, with PIC of 0.80 and 0.86, respectively, allowing differentiation between paternally and maternally inherited alleles. The locations of these markers and the breakpoints of various types of other α-thalassemia double deletions, such as −MED, −FIL and −THAI, are shown in Fig. 2, which shows that the markers (16PTEL05 and 16PTEL06) also lie within the breakpoints of −FIL and −THAI. A fetus with these double gene deletions, −MED and −SEA, can survive to later gestation and present with HbBarts hydrops fetalis (10). Because of the lack of α-globin chain synthesis, fetuses that inherit the homozygous −FIL and −THAI do not survive beyond 8 gestational weeks.

PCR amplifications of these polymorphic microsatellite markers are efficient because they consist of short repeating units of 2–4 bp. Multiplex QF-PCR amplifications of both targets (16PTEL05 and 16PTEL06) and control (D16S539) enable HbBarts hydrops fetalis to be diagnosed simultaneously with the exclusion of maternal contamination. The absence of both 16PTEL05 and 16PTEL06 suggests HbBarts hydrops fetalis (−SEA/−SEA). The presence of 1 allele for each of 16PTEL05 and 16PTEL06 suggests either α-thalassemia-1 (aa/−SEA) or the normal homozygote (aa/aa). Because D16S539 is located outside the breakpoint region, it is used as a control to confirm the presence of DNA and to exclude maternal contamination. QF-PCRs of all primer pairs yield consistent results in DNA isolated from the cell lines. No amplification of 16PTEL05 and 16PTEL06 was detected in the cell lines of HbBarts hydrops fetalis (GM03037 and GM03433). In the blinded study, HbBarts hydrops fetalis was diagnosed when the microsatellite markers (16PTEL05, 16PTEL06) were not detected, in the presence of D16S539. Maternal contamination was excluded in all cases, as evidenced by the absence of noninherited maternal alleles in fetal DNA samples.

In conclusion, our findings showed that, by amplifying target microsatellite markers found within the breakpoint region of −SEA deletion, HbBarts hydrops fetalis can be
accurately identified. The target microsatellite markers are highly polymorphic, as demonstrated by the heterozygosity and PIC calculations of 100 AF samples. Because these microsatellite markers are highly polymorphic, maternally and paternally inherited alleles can be differentiated and identified in fetal DNA samples. The ability of our novel QF-PCR method to differentiate between maternally and paternally inherited allele will also be useful in the analysis of fetal DNA in maternal plasma. The detection and identification of the paternally inherited fetal alleles in the maternal plasma may be useful to exclude HbBarts hydrops fetalis; we are currently exploring this strategy for noninvasive prenatal testing (44).

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References


