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A Novel Targeted Approach for Noninvasive Detection of Paternally Inherited Mutations in Maternal Plasma

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The challenge in noninvasive prenatal diagnosis for monogenic disorders lies in the detection of low levels of fetal variants in the excess of maternal cell-free plasma DNA. Next-generation sequencing, which is the main method used for noninvasive prenatal testing and diagnosis, can overcome this challenge. However, this method may not be accessible to all genetic laboratories. Moreover, shotgun next-generation sequencing as, for instance, currently applied for noninvasive fetal trisomy screening may not be suitable for the detection of inherited mutations. We have developed a sensitive, mutation-specific, and fast alternative for next-generation sequencing—mediated noninvasive prenatal diagnosis using a PCR-based method. For this proof-of-principle study, noninvasive fetal paternally inherited mutation detection was performed using cell-free DNA from maternal plasma. Preferential amplification of the paternally inherited allele was accomplished through a personalized approach using a blocking probe against maternal sequences in a high-resolution melting curve analysis—based assay. Enhanced detection of the fetal paternally inherited mutation was obtained for both an autosomal dominant and a recessive monogenic disorder by blocking the amplification of maternal sequences in maternal plasma. *(J Mol Diagn 2015, 17: 590—596; http://dx.doi.org/10.1016/j.jmoldx.2015.05.006)*

Since the successful introduction of noninvasive prenatal testing for fetal trisomy screening, requests to expand the repertoire for noninvasive prenatal diagnostics (NIPD) have been increasing. NIPD can be performed on small fragments of cell-free fetal DNA (cffDNA) that are present in maternal plasma.[1] During a mean of approximately 7 to 9 weeks of gestation, the amount of cffDNA is sufficient to be detected noninvasively in maternal plasma.[2] Current clinical application of NIPD includes fetal sex determination, fetal rhesus D determination, and the diagnosis of several monogenic disorders.[3,4] For the latter, NIPD can be applied in both autosomal dominant and recessive cases, most efficiently when the mother does not carry the mutant allele and/or carries a different mutation compared with the father, respectively.[3,4]

One of the biggest challenges of noninvasive detection of paternally inherited sequences in the fetus is the excess of maternal cell-free DNA (cfDNA) in plasma. Here a parallel can be drawn with cancer genetics, which faces similar challenges in the need to detect mosaic or low-level somatic mutations in the presence of excess wild-type sequences.[5] Deep sequencing approaches using various next-generation sequencing (NGS)

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platforms can be used to overcome these challenges for both NIPD and cancer genetics (eg, targeted NGS approaches for both cancer detection and therapy).[6–8] Even though the application of NGS for both these purposes is expanding, implementation and proper validation of novel applications for NGS in diagnostics is currently still quite expensive, especially when this method is applied for the detection of merely one or two variants. Moreover, NGS may be less suitable for the detection of variants in certain regions of the genome, such as GC-rich regions and repeat areas, and may therefore not be the most eligible method of choice for mutation detection. Therefore, this study aimed to develop an alternative noninvasive paternal mutation detection method that does not require NGS. Such an alternative needs to be accessible for genetic diagnostic laboratories and needs to be sensitive enough to detect the low levels of fetal sequences in maternal plasma.

High-resolution melting curve analysis (HR-MCA) is a relatively simple, fast, and low-cost technique for genotyping and mutation scanning and is frequently used in routine molecular and cancer diagnostics.[5,9–11] It combines (asymmetric) PCR with a short post-PCR melting step to detect sequence variations using a saturating double-stranded DNA binding dye.[9] Although HR-MCA is a relatively sensitive technique, the detection of mosaic or low-level mutations may still be challenging and variant dependent.[12] Therefore, variations in traditional HR-MCA methods have been developed to overcome this challenge.[5,13–16] Most of these studies describe the use of either peptide nucleic acid or locked nucleic acid (LNA) probes. Addition of such probes to the PCR reaction results in clamping or blocking the specific undesired PCR products by inhibiting amplification.[5,13–15] LNA is a bicyclic high-affinity nucleic acid analog that contains a ribonucleoside link between the 2′-oxygen and the 4′-carbon atoms with a methylene unit (2′-O, 4′-C-methylene bridge).[15,17] The thermal stability, binding capacity, and affinity of LNA to complementary DNA increases substantially with each LNA base incorporated, resulting in suppressed amplification of these complementary sequences.[15,17] More importantly, in case of a mismatch, the LNA probe does not bind to the template with high affinity, enabling primer extension and preferential amplification of the allele of interest. This principle of allele-specific blocking could be of use in NIPD to obtain preferential amplification of the paternally inherited allele through targeted blocking of the maternal allele. By first determining both parental genotypes, target-specific LNA probes against maternal sequences could be designed, enabling preferential amplification and specific detection of the paternally inherited mutation in maternal plasma.

In this proof-of-principle study, we describe a fast and sensitive alternative for NGS-mediated NIPD using a PCR-based method. We explored the use of HR-MCA in combination with target-specific blocking LNA probes to obtain allele-specific blocking of maternal sequences for the enhanced detection of the fetal paternally inherited allele in maternal plasma. We found that this novel approach for NIPD can be applied in both an autosomal dominant and recessive monogenic disorder.

Materials and Methods

Patients

Two couples who opted for prenatal diagnosis visited the Department of Clinical Genetics. Both mothers underwent an invasive procedure [chorionic villus sampling (CVS)] for prenatal diagnosis to determine fetal genotype for a familial mutation. In case 1, the father was a carrier of a pathogenic *BRCA2* mutation (c.5682C>G, p.Tyr1894*). In case 2, both parents were carriers of a different heterozygous mutation in the *HBB* gene. The mother was heterozygous for the hemoglobin (Hb) C mutation (c.19G>A, p.Glu7Lys), and the father was heterozygous for the HbS mutation (c.20A>T, p.Glu7Val). A sibling was a carrier of the HbS mutation. Maternal blood withdrawal was performed at 10^{+6} and 11^{+1} weeks$^{+\text{days}}$ of gestation for cases 1 and 2, respectively, after informed consent was obtained.

Sample Processing

Maternal ($n = 2$) and paternal ($n = 1$) plasma (input of 800 μL) was isolated, processed, and measured as previously described.[18] Isolated plasma DNA was concentrated to 20 μL using the Zymo Clean & Concentrator -5 kit (Zymo Research, Irvine, CA). As a control, the total amount of cell-free DNA (fetal and maternal) was determined by real-time PCR detection of *CCR5* as previously described.[19] Total concentrations of 112 and 350 pg/μL were obtained for the *BRCA2* and *HBB* case, respectively. Genomic DNA (gDNA) from all parents was isolated from peripheral blood cells using automated isolation (Qiagen, Venlo, the Netherlands). Fetal gDNA was isolated from CVS on the QIAcube according to manufacturer's instructions (Qiagen).

Control Samples

Several positive and negative control samples [gDNA and freshly isolated anonymized wild-type (WT) plasma DNA] were used to optimize the assay. All control samples were isolated similarly to the parental DNA samples. For *BRCA2* a total of 20 control samples were analyzed: anonymized WT plasma DNA ($n = 12$), WT gDNA ($n = 6$), and gDNA from individuals heterozygous for the *BRCA2* mutation ($n = 4$). For *HBB* a total of 23 control samples were analyzed: anonymized WT plasma DNA ($n = 12$), WT gDNA ($n = 4$), gDNA heterozygous for HbC ($n = 2$), gDNA heterozygous for HbS ($n = 2$), gDNA homozygous for HbC ($n = 1$), gDNA homozygous for HbS ($n = 1$), and gDNA from an individual compound heterozygous for HbC/HbS ($n = 1$).

Assay Design

PCR for HR-MCA was performed using target-specific primers and a mutation-specific unlabeled detection probe (mutation detection probe) with a 3′ C3-spacer (Biolegio, Nijmegen, the Netherlands) and was executed both with and

Table 1 Primer and Probe Sequences Used for PCR and High-Resolution Melting Curve Analysis

Description	Sequence
*BRCA2*_NIPD_MCA_F	5′-CAACGAGAATAAATCAAAAATTTG-3′
*BRCA2*_NIPD_MCA_R	5′-TGCGTGCTACATTCATCATTA-3′
*BRCA2*_NIPD_MCA_P_Me*	5′-CCGTCCAACAATCCTCCGTAACCT-3′
*BRCA2*_LNA (WT)	5′-T+T+G+T+TA+C+G+A+G+GC-3′
*HBB*_NIPD_MCA_F	5′-GACACAACTGTGTTCACTAGCA-3′
*HBB*_NIPD_MCA_R	5′-CCACCAACTTCATCCACGTTCA-3′
*HBB*_NIPD_MCA_P_Me*	5′-GCAGACTTCTCCACAGGAGTCAG-3′
*HBB*_LNA1 (wild type)	5′-+T+G+A+C+TC+C+T+G+A+G-3′
*HBB*_LNA2 (hemoglobin C)	5′-C+T+C+C+T+A+A+G+G+A+G-3′

Forward (F) and reverse (R) primers are depicted for both cases. Mutation detection probes (P) contain a 3′ C3-spacer (Me*). Locked nucleic acid (LNA)—modified bases in the target-blocking probes are depicted with + before the base. Target-blocking probes were designed to perfectly match maternal sequences. Position of the altered nucleotide is underlined.

without the addition of a target-specific blocking LNA probe (target-blocking probe) (Exiqon, Vedbaek, Denmark) that binds to the WT or mutant maternal allele. Primer/probe design was based on parental Sanger sequencing results of the region of interest (Table 1). Amplicons of 117 and 115 bp were designed for the detection of the familial *BRCA2* and *HBB* mutations, respectively, using LightScanner Primer Design (Idaho Tech/BioFire Diagnostics, Salt Lake City, UT). In both cases, mutation detection probes were designed against the forward strand. Target-blocking probes were designed against maternal templates in the same region as the mutation detection probes and were directed to the reverse strand.

PCR and HR-MCA

PCR and HR-MCA without target-blocking probe were performed as previously described.[10,11] In short, asymmetric PCR (to preferentially amplify the forward strand) was performed in 96-well nontransparent plates (Framestar, 4titude, Surray, UK) in a total reaction volume of 10 μL containing 1× LightScanner Master mix (Idaho Tech/BioFire Defense), 5 pmol of forward primer, 1 pmol of reverse primer, 5 pmol of mutation detection probe, and 2 ng of gDNA template. Primer-specific optimal annealing temperature for both primer sets was determined using a PCR gradient (58°C to 64°C). Asymmetric PCR was performed with a reverse primer, forward primer, and mutation detection probe ratio at 1:5:5, respectively. All samples were tested in duplicate. A range of 50°C to 98 was used for HR-MCA melting. Melt temperature of normalized melting peaks was determined using the unlabeled probe genotyping analysis tool of the LightScanner software with Call-IT version 2.0 (Idaho Tech/BioFire Diagnostics). Target-blocking probe was titrated into each reaction in a mutation detection probe to target-blocking probe ratio of 1:1 to 1:10 (ie, 5 to 50 pmol per reaction) and optimized for each set. Cycling protocol for testing the target-blocking probe was 95°C for 5 minutes, 50 cycles of 10 seconds at 95°C, 20 seconds at 72°C, and 30 seconds at the primer-specific annealing temperature of 58°C or 63°C for *BRCA2* and *HBB,* respectively, to obtain target-blocking probe binding before amplification (modified from Oh et al[5]).

Determining the Detection Limit of the Assay

As a control, the detection limit of the assay was determined using a mix of paternal gDNA [mutation carrier (MUT)] heterozygous for the familial mutation and maternal gDNA for each case, mimicking an artificial pregnancy (with the paternal gDNA representing the fetus). A relative serial dilution range of 33% to 1% paternal gDNA mixed into maternal gDNA was created using a total amount of approximately 425 pg of mixed gDNA (maternal and paternal) per reaction. Both parental samples were also tested separately (100% paternal or 100% maternal gDNA). All samples were tested in duplicate using the optimal ratio of mutation detection probe to target-blocking probe of 1:5 and 1:2 for *BRCA2* and *HBB,* respectively, in each PCR reaction.

Conditions for Testing Maternal Plasma Samples

Maternal plasma samples were tested together with corresponding parental gDNA, CVS gDNA, and several positive and negative controls (see *Control samples*) using the cycling protocol for target-blocking probes. When testing plasma samples, total reaction volume was increased 1.5×, enabling an input of 7.5 μL of concentrated plasma DNA template per reaction. Plasma samples were tested at least in duplicate. Total gDNA input per reaction for control samples was 2 ng. Results were confirmed in at least two independent tests.

Results

Optimization of HR-MCA

To optimize parameters for HR-MCA mutation scanning using a mutation detection probe, DNA samples from all parents and several controls with known genotypes (anonymized plasma DNA and gDNA) were used. With the optimal annealing temperature for the primers determined (ie, 58°C or 63°C for *BRCA2* and *HBB,* respectively), the target-blocking probes specific for the maternal allele(s) were tested subsequently, together with the mutation detection probe. The selected PCR conditions used for testing the target (WT)—specific blocking LNA probes enabled binding of the target-blocking probe to unwanted target sequences before primer extension (Figure 1). To determine optimal concentrations, target-blocking probe was titrated into each PCR reaction, resulting in optimal ratios of mutation detection probe to WT target blocking probe of 1:5 and 1:2 for *BRCA2* and *HBB,* respectively.

Next, we determined the detection limit of this assay. For each case, paternal (MUT) gDNA was mixed into maternal gDNA, mimicking an artificial pregnancy using amounts of gDNA resembling the quantities of cfDNA found in maternal plasma early in gestation. Without the addition of a target blocking probe, a dilution effect is observed in the detection

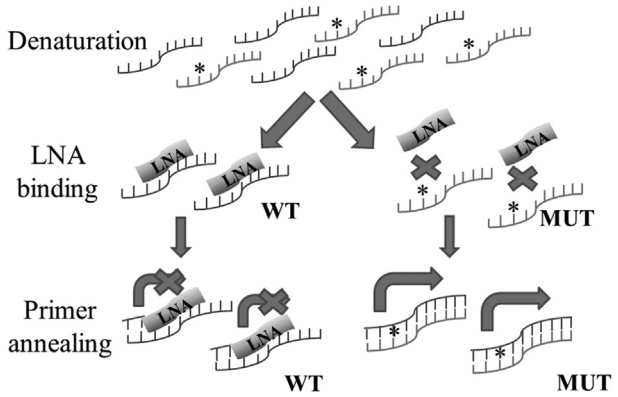

Figure 1 Principle of target-blocking locked nucleic acid (LNA) probe binding in high-resolution melting curve analysis. The **top left arrow** depicts target-blocking probes designed against maternal wild-type (WT) sequences are able to bind denatured single-stranded WT sequences. No primer extension and amplification can occur (**left arrow with cross**). **Top right arrow** depicts the situation when the DNA template harbors a mutation (**asterisk**), target-blocking probes will not bind to paternal mutant (MUT) sequences (**right crosses**), enabling primer extension and amplification.

signal of the MUT allele, whereas the detection signal of the WT-specific melting peak was increased because of the high background of WT sequences (Figure 2A). Without blocking, the mutant allele could no longer be detected in a relative paternal gDNA percentage of approximately 16% and lower [ie, approximately 10 genome equivalents (GE) based on a conversion factor of 6.6 pg of DNA per cell]. However, addition of a target blocking probe directed against maternal sequences resulted in preferential amplification and enhanced detection of the paternal mutation at approximately 1% to 2% paternal gDNA (ie, approximately 0.5 to 2 GE) in a background of maternal sequences.

Testing Maternal Plasma Samples

Paternal mutation detection was performed on total cfDNA from maternal plasma using a mutation detection probe and target blocking probe(s) for selective blocking of maternal template amplification during PCR amplification.

For case 1, results from WT plasma DNA indicate that with the use of only the *BRCA2* mutation detection probe, one WT-specific normalized melting peak is present in HR-MCA, as expected in a WT individual, with a melt temperature calling at approximately 66°C (Figure 2A). Paternal gDNA reveals two melting peaks, with a melt temperature calling at 66°C for the WT and 72°C for the *BRCA2* MUT-specific peak, respectively, as expected for an individual heterozygous for this mutation (Figure 2A). Similar results were obtained for paternal plasma (data not shown). As a control, we mixed heterozygous paternal gDNA with maternal WT gDNA (25% paternal gDNA in 100% maternal gDNA). As expected, without the use of a target blocking probe, a dilution effect of the MUT-specific melting peak was observed (Figure 2A). The detection signals were skewed toward detection of the WT-specific melting peak as also previously observed in the aforementioned serial dilution range of mixed parental gDNA. To improve paternal mutation detection, we used a WT target blocking probe together with the *BRCA2* mutation detection probe, resulting in inhibition of amplification of the WT *BRCA2* allele during PCR (Figure 2B). As a result, the *BRCA2* mutation detection probe could no longer detect a WT PCR product in HR-MCA as shown for WT maternal gDNA (Figure 2B). In CVS and paternal gDNA samples heterozygous for the *BRCA2* mutation, only the *BRCA2* MUT-specific normalized melting peak could be detected (Figure 2B). In the maternal plasma sample, the paternally inherited mutation in the fetus could only be detected when the maternal WT template was blocked, indicating that for this mutation, the addition of a single target-specific blocking LNA probe was sufficient to enhance the detection of the paternally inherited *BRCA2* mutation (Figure 2B).

For case 2, the situation is more challenging. In this case, both parents carry a different mutation in the *HBB* gene. These mutations even affect the same codon, and the position of the mutations is only 1 bp apart. As a result, the template region covered by the paternal HbS mutation detection probe also covers the adjacent maternal HbC mutation. When using only this HbS mutation detection probe (Figure 3, A and B), results from parental, CVS, and control gDNA samples reveal a specific normalized melting peak pattern in the HR-MCA assay for all three different alleles (HbC, WT, and HbS with melt temperature calling at 62°C, 66°C, and 70°C, respectively) (Figure 3, A and B). As expected, maternal gDNA reveals a melting peak for the HbC and WT allele because the mother is heterozygous for the HbC mutation (Figure 3B). Results from paternal gDNA (heterozygous for the HbS mutation) reveal

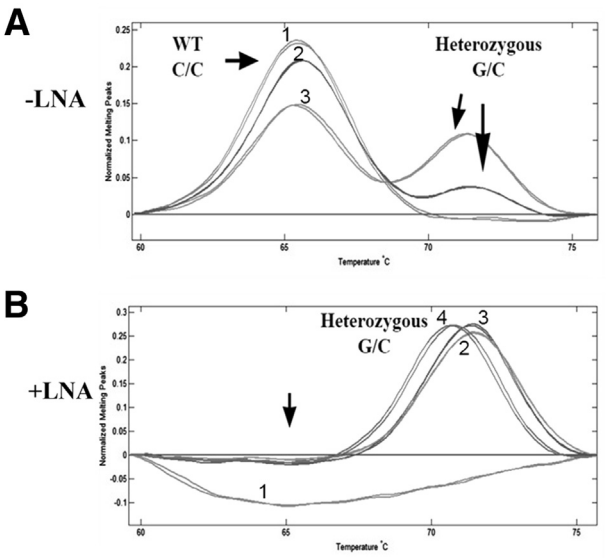

Figure 2 High-resolution melting curve analysis results using the *BRCA2* mutation detection probe. **A:** Without target-blocking locked nucleic acid (LNA) probe. Curve 1: wild-type (WT) plasma DNA; curve 2: control paternal gDNA (25%) diluted in WT maternal gDNA showing the dillution effect (**elongated arrow**); curve 3: heterozygous paternal genomic DNA (gDNA; **short arrow**). **B:** With target-blocking LNA probe: WT signal is blocked (**arrow**). Curve 1: maternal WT gDNA; curve 2: heterozygous chorionic villus sampling gDNA; curve 3: heterozygous paternal gDNA; curve 4: maternal plasma.

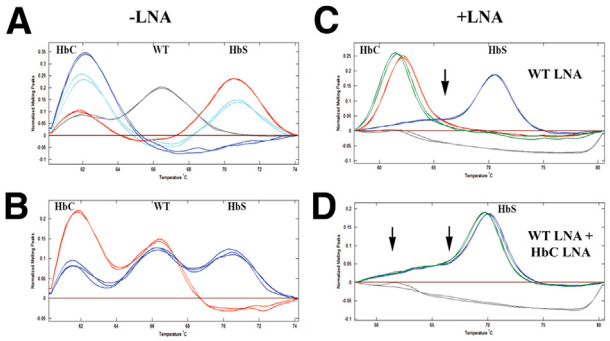

Figure 3 Representation of the high-resolution melting curve analysis (HR-MCA) melting peak patterns for *HBB* from controls, parents, and fetus using the hemoglobin (Hb) S mutation detection probe. **A:** Selection of the positive and negative controls scanned for optimization of settings for HR-MCA tested without a target-blocking locked nucleic acid (LNA) probe: wild-type (WT) (gray), control homozygous for HbS (red), control homozygous for HbC (dark blue), and control compound heterozygous for HbS/HbC (light blue). **B:** Melting peak patterns of maternal (red; heterozygous for HbC), paternal, and chorionic villus sampling gDNA (blue; both heterozygous for HbS), without addition of a target-blocking LNA probe to the PCR reaction. **C:** Samples tested with WT target-blocking LNA probe directed to block only the maternal WT sequences (**arrow**): WT genomic DNA (gDNA) (gray), paternal gDNA heterozygous for HbS (blue), maternal gDNA heterozygous for HbC (red), and maternal plasma (green). **D:** Additional blocking with an HbC target blocking LNA probe directed to the maternal HbC allele together with a WT-blocking LNA probe (**arrows**): maternal gDNA heterozygous for HbC (gray), paternal gDNA heterozygous for HbS (blue), and maternal plasma (green).

two peaks for both the WT and HbS alleles, respectively (Figure 3B). CVS gDNA displays a pattern similar to the father (Figure 3B). The addition of a WT target (*HBB*) blocking probe to the PCR reaction completely blocked amplification of the WT *HBB* allele. As expected, no PCR product can be detected by the HbS mutation detection probe in WT control plasma DNA (data not shown) and WT gDNA (Figure 3C), whereas in heterozygous maternal and paternal gDNA only the HbC and HbS MUT peaks are visible (Figure 3C). More importantly, results from maternal plasma indicate that blockage of only the maternal WT *HBB* allele is not sufficient to detect the fetal paternally inherited HbS mutation (Figure 3C). In maternal plasma, only the maternal HbC-specific melting peak is visible because the excess of HbC allele is not blocked by the WT *HBB* target blocking probe (Figure 1). Hence, an HbC target blocking probe was designed and additionally titrated into the PCR reactions together with both the HbS mutation detection probe and the WT (*HBB*) target blocking probe. The optimal ratio of the HbS mutation detection probe, WT target blocking probe, and HbC target blocking probe per reaction was 1:2:2. As expected, no signal is detected in maternal gDNA (Figure 3D) when simultaneously blocking WT and HbC templates (Figure 3D). In paternal gDNA, only the HbS peak was visible (Figure 3D). Subsequently, in maternal plasma the paternally inherited HbS mutation in the fetal cfDNA could be detected after simultaneously blocking amplification of both the maternal WT *HBB* and HbC alleles (Figure 3D).

For cases 1 and 2, successful detection of the fetal paternally inherited mutation in maternal plasma was achieved using this LNA-mediated targeted blocking approach in HR-MCA for NIPD. In case 1, this meant that the fetus would be affected, and in case 2, the fetus may either be a carrier or affected with the disease. All results were concordant to Sanger sequencing results from CVS-derived gDNA obtained after invasive procedures (Figure 4).

Discussion

The use of cffDNA isolated from maternal plasma for prenatal molecular testing or diagnostics has increased rapidly. Noninvasive prenatal testing (NIPT) for fetal trisomy screening has been introduced successfully in the past few years. Maternal plasma is easily obtainable, and very early in pregnancy sufficient amounts of cffDNA are present. All this, together with the low risk for the fetus and continuous improvements of detection methods, has provided many advances for the use of NIPD in favor of invasive testing procedures early in gestation.[3]

Advantage and Application of the HR-MCA Approach in NIPD

In this proof-of-principle study, we demonstrate the use of LNA target-specific blocking probes in HR-MCA. These target blocking LNA probes are directed against maternal background sequences to enhance the detection of fetal paternally inherited mutations in maternal plasma DNA. We chose to explore this approach because this method is sensitive, is mutation specific, and has a short turnaround time. Moreover, HR-MCA is easy to implement in diagnostics, and also equipment that is required to perform HR-MCA is relatively inexpensive. This makes this method more manageable for genetic laboratories rather than, for example, an NGS-mediated approach.

High-throughput whole genome shotgun sequencing as currently performed for NIPT is not efficient for the detection of a single paternally inherited mutation because this method will require a much higher vertical coverage of the data than currently is obtained. Targeted sequencing may be a good alternative NGS method to use for mutation detection because good vertical coverage can be obtained. Pooling of multiple

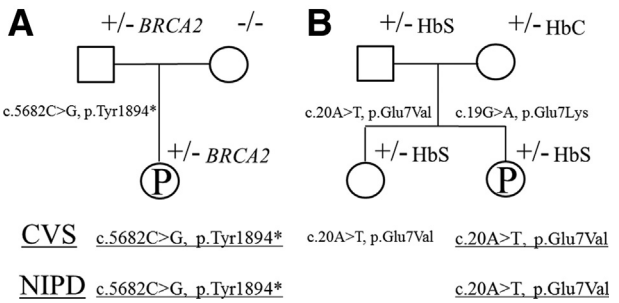

Figure 4 Family pedigrees from participating couples. **A:** Case 1: Both father and fetus are heterozygous for *BRCA2* mutation c.5682C>G, p.Tyr1894*. **B:** Case 2: Both parents are heterozygous for a different mutation in the *HBB* gene. Mother is heterozygous for c.19G>A, p.Glu7Lys [hemoglobin (Hb) C], whereas father, daughter, and the fetus are heterozygous for c.20A>T, p.Glu7Val (HbS). CVS, chorionic villus sampling; NIPD, noninvasive prenatal diagnostics.

samples is required to obtain cost reduction. However, in case of prenatal testing, a short turnaround time is demanded. Therefore, batching of samples might not always be feasible because of insufficient sample number. The advantage of HR-MCA is that it can always be performed within a short turnaround time regardless of the sample number.

When performing paternally inherited mutation detection using this novel HR-MCA–based approach in NIPD, for autosomal dominant disorders it is restricted to cases where the mother does not carry the mutation, whereas for autosomal recessive disorders, the mother and father should carry different mutations.[3] In this proof-of-principle study, we have pursued a personalized approach and used these differences in parental genotype to design target-blocking LNA probes for use in HR-MCA, which are specifically directed against the maternal sequences. This way, amplification of maternal cfDNA in plasma, including the maternally inherited fetal allele, will be blocked, providing enhanced sensitivity and specific detection of paternally inherited mutations by mutation-specific detection probes. Such an approach could be a first step toward expanding the current repertoire for NIPD toward a more general application by detecting recurrent pathogenic mutations or genotypes linked to a pathogenic haplotype.

Detection of Paternally Inherited Mutations in Maternal Plasma DNA Using HR-MCA

In this study, we describe the application of this approach for two different cases: one autosomal dominant (*BRCA2*) and one autosomal recessive monogenic disorder (*HBB*). Whereas for case 1 (*BRCA2*) maternal sequences could be blocked with the use of only a single blocking LNA probe, for case 2 (*HBB*) the situation was more challenging. Both parents were heterozygous for a different mutation in the *HBB* gene, and these mutations involved the same codon/amino acid by affecting a base pair substitute 1 bp apart. Therefore, the template region covered by the HbS-specific detection probe and the WT-specific blocking LNA probe also covered the adjacent maternal HbC mutation. Consequently, this implicated that the WT-specific blocking LNA probe would have a mismatch on the other maternal (HbC) allele, and amplification of this HbC allele could therefore still occur. Blocking only the maternal WT allele in this case appeared insufficient for selective detection of the paternally inherited mutation because of the excess of amplified HbC-specific template in maternal plasma after PCR. Both the maternal WT and HbC alleles needed to be blocked simultaneously to provide enough background reduction of maternal cfDNA to detect the paternally inherited mutation in the fetus. Considering the recessive inheritance of the disease, additional confirmation of the actual fetal genotype through an invasive procedure was still required for this case to determine whether the fetus would be affected or a carrier of the disease. Nevertheless, in cases where the paternally inherited mutation is excluded, an invasive procedure could be avoided using this approach (in approximately 50% of the cases).

Applying HR-MCA Method in NIPD

As found in this study, this method can be used successfully for NIPD. However, additional controls to confirm the presence of cffDNA in plasma are essential in NIPD to exclude false-negative results, especially when no paternally inherited mutation was detected.[19,20] Because of the fragmented nature of circulating cfDNA, there is a restriction for designing primers and probes. Fetal cfDNA is approximately 143 to 146 bp in size, which limits amplicon size for PCR.[8]

HR-MCA has previously been proposed as a useful method for NIPD.[16,21,22] In these studies, no blocking LNA probe was used. The use of a blocking LNA probe could, however, be essential for the detection of fetal mutations in case of low fetal fraction or for the detection of more challenging mutations. In the study of Yenilmez et al,[21] HR-MCA without a blocking LNA probe was performed and was not successful in case of early gestation. Levels of cffDNA may differ extensively between individuals and have been described to increase as gestation progresses.[23,24] Early in gestation, fetal paternally inherited variants may not be distinguished from the maternal background because the levels of cffDNA are too low to detect. We have previously found that the lowest detectable fraction of a variant or mosaic by a conventional HR-MCA approach (without a blocking probe) is very variant dependent and can be limited to only 25%.[12] Therefore, it will be particularly challenging for some variants to be detected at low levels of template DNA, not only in gDNA but especially in plasma DNA. For future NIPD, the use of target blocking probes to block the amplification of undesired PCR products may therefore be extremely useful for mutation detection early in gestation, if not essential.

In summary, in this proof-of-principle study we have successfully found a PCR-based target-specific detection HR-MCA approach that is suitable for the detection of paternally inherited mutations in cffDNA from maternal plasma by making use of a target-specific LNA blocking probe. We have used a personalized approach by designing primers, paternal allele-specific mutation detection probes, and maternal allele-specific target blocking probes based on parental sequences. The application of this method was used for NIPD in both an autosomal dominant and recessive monogenic disorders and can be used as a sensitive and fast alternative for NGS-based approaches.

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References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS: Presence of fetal DNA in maternal plasma and serum. Lancet 1997, 350:485–487
2. Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, Daniels G, Chitty L: Non-invasive prenatal determination of fetal sex: translating research into clinical practice. Clin Genet 2011, 80:68–75

3. Daley R, Hill M, Chitty LS: Non-invasive prenatal diagnosis: progress and potential. Arch Dis Child Fetal Neonatal Ed 2014, 99:F426–F430
4. Bustamante-Aragones A, Rodriguez de AM, Perlado S, Trujillo-Tiebas MJ, Arranz JP, Diaz-Recasens J, Troyano-Luque J, Ramos C: Non-invasive prenatal diagnosis of single-gene disorders from maternal blood. Gene 2012, 504:144–149
5. Oh JE, Lim HS, An CH, Jeong EG, Han JY, Lee SH, Yoo NJ: Detection of low-level KRAS mutations using PNA-mediated asymmetric PCR clamping and melting curve analysis with unlabeled probes. J Mol Diagn 2010, 12:418–424
6. Chang F, Li MM: Clinical application of amplicon-based next-generation sequencing in cancer. Cancer Genet 2013, 206:413–419
7. Hagemann IS, Cottrell CE, Lockwood CM: Design of targeted, capture-based, next generation sequencing tests for precision cancer therapy. Cancer Genet 2013, 206:420–431
8. Lo YM, Chan KC, Sun H, Chen EZ, Jiang P, Lun FM, Zheng YW, Leung TY, Lau TK, Cantor CR, Chiu RW: Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med 2010, 2:61ra91
9. Montgomery J, Wittwer CT, Palais R, Zhou L: Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. Nat Protoc 2007, 2:59–66
10. Almomani R, van der Stoep N, Bakker E, den Dunnen JT, Breuning MH, Ginjaar IB: Rapid and cost effective detection of small mutations in the DMD gene by high resolution melting curve analysis. Neuromuscul Disord 2009, 19:383–390
11. van der Stoep N, van Paridon CD, Janssens T, Krenkova P, Stambergova A, Macek M, Matthijs G, Bakker E: Diagnostic guidelines for high-resolution melting curve (HRM) analysis: an interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner. Hum Mutat 2009, 30:899–909
12. Out AA, van Minderhout IJ, van der Stoep N, van Bommel LS, Kluijt I, Aalfs C, Voorendt M, Vossen RH, Nielsen M, Vasen HF, Morreau H, Devilee P, Tops CM, Hes FJ: High-resolution melting (HRM) re-analysis of a polyposis patients cohort reveals previously undetected heterozygous and mosaic APC gene mutations. Fam Cancer 2015, 14:247–257
13. Chou LS, Meadows C, Wittwer CT, Lyon E: Unlabeled oligonucleotide probes modified with locked nucleic acids for improved mismatch discrimination in genotyping by melting analysis. Biotechniques 2005, 39:644. 646, 648
14. Laughlin TS, Moliterno AR, Stein BL, Rothberg PG: Detection of exon 12 Mutations in the JAK2 gene: enhanced analytical sensitivity using clamped PCR and nucleotide sequencing. J Mol Diagn 2010, 12:278–282
15. Warshawsky I, Mularo F: Locked nucleic acid probes for enhanced detection of FLT3 D835/I836, JAK2 V617F and NPM1 mutations. J Clin Pathol 2011, 64:905–910
16. Macher HC, Martinez-Broca MA, Rubio-Calvo A, Leon-Garcia C, Conde-Sanchez M, Costa A, Navarro E, Guerrero JM: Non-invasive prenatal diagnosis of multiple endocrine neoplasia type 2A using COLD-PCR combined with HRM genotyping analysis from maternal serum. PLoS One 2012, 7:e51024
17. Mouritzen P, Nielsen AT, Pfundheller HM, Choleva Y, Kongsbak L, Moller S: Single nucleotide polymorphism genotyping using locked nucleic acid (LNA). Expert Rev Mol Diagn 2003, 3:27–38
18. van den Oever JM, Balkassmi S, Verweij EJ, van Iterson M, Adama van Scheltema PN, Oepkes D, van Lith JM, Hoffer MJ, den Dunnen JT, Bakker E, Boon EM: Single molecule sequencing of free DNA from maternal plasma for noninvasive trisomy 21 detection. Clin Chem 2012, 58:699–706
19. Boon EM, Schlecht HB, Martin P, Daniels G, Vossen RH, den Dunnen JT, Bakker B, Elles R: Y chromosome detection by Real Time PCR and pyrophosphorolysis-activated polymerisation using free fetal DNA isolated from maternal plasma. Prenat Diagn 2007, 27:932–937
20. van den Oever JM, Balkassmi S, Segboer T, Verweij EJ, van der Velden PA, Oepkes D, Bakker E, Boon EM: Mrassf1a-pap, a novel methylation-based assay for the detection of cell-free fetal DNA in maternal plasma. PLoS One 2013, 8:e84051
21. Yenilmez ED, Tuli A, Evruke IC: Noninvasive prenatal diagnosis experience in the Cukurova Region of Southern Turkey: detecting paternal mutations of sickle cell anemia and beta-thalassemia in cell-free fetal DNA using high-resolution melting analysis. Prenat Diagn 2013, 33:1054–1062
22. Phylipsen M, Yamsri S, Treffers EE, Jansen DT, Kanhai WA, Boon EM, Giordano PC, Fucharoen S, Bakker E, Harteveld CL: Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis. Prenat Diagn 2012, 32:578–587
23. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM, Hjelm NM: Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998, 62:768–775
24. Lun FM, Chiu RW, Allen Chan KC, Yeung LT, Kin LT, Dennis Lo YM: Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008, 54:1664–1672