Chapter 5

A novel targeted approach for noninvasive detection of paternally inherited mutations in maternal plasma

Chapter 5: A novel targeted approach for noninvasive detection of paternally inherited mutations in maternal plasma

Jessica van den Oever

Ivonne van Minderhout

Kees Harteveld

Nicolette den Hollander

Bert Bakker

Nienke van der Stoep

Elles Boon

J Mol Diagn. 2015 Jul 7. pii: S1525-1578(15)00126-9. doi: 10.1016/j.jmoldx.2015.05.006

Abstract

The challenge in noninvasive prenatal diagnosis (NIPD) 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 (NGS), 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 NGS 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 NGS-mediated NIPD using PCR methodology. 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 (HR-MCA) 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.

Introduction

Since the successful introduction of noninvasive prenatal testing for fetal trisomy screening, there has also been a growing request to expand the repertoire for noninvasive prenatal diagnostics (NIPD). NIPD can be performed on small fragments of cell-free fetal DNA (cffDNA) that are present in maternal plasma (Lo *et al.*, 1997). On average, from about 7-9 weeks in gestation the amount of cffDNA is sufficient to be detected noninvasively in maternal plasma (Hill *et al.*, 2010). Current clinical application of NIPD include fetal sex determination, fetal Rhesus D (*RhD*) determination and the diagnosis of several monogenic disorders. 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 to the father respectively (Daley *et al.*, 2014; Bustamante-Aragones *et al.*, 2012).

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 towards 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 (Oh *et al.*, 2010). Deep sequencing approaches using various Next Generation Sequencing (NGS) platforms can be used to overcome these challenges for both NIPD and cancer genetics (e.g. targeted NGS approaches for both cancer detection and therapy) (Chang *et al.*, 2013; Hagemann *et al.*, 2013; Lo *et al.*, 2010). Even though the application of NGS for both these purposes is expanding, currently implementation and proper validation of novel applications for NGS in diagnostics is still quite expensive, especially when this method is applied for the detection of merely 1 or 2 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 is 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 (Montgomery *et al.*, 2007; Oh *et al.*, 2010; Almomani *et al.*, 2009; van der Stoep *et al.*, 2009). It combines (asymmetric) PCR with a short post-PCR melting step to detect sequence variations using a saturating double-stranded DNA binding dye (Montgomery *et al.*, 2007). Although HR-MCA is a relatively sensitive technique, the detection of mosaic or low level mutations may still be challenging and variant dependent (Out *et al.*, 2015). Therefore, variations in traditional HR-MCA methods have been developed to overcome this challenge (Chou *et al.*, 2005; Oh *et al.*, 2010; Laughlin *et al.*, 2010; Warshawsky *et al.*, 2011; Macher *et al.*, 2012). The majority of these studies describe the use of either peptide nucleic acid (PNA) or locked nucleic acid (LNA) probes. Addition of such probes to the PCR reaction results in clamping or blocking specific undesired PCR products by inhibiting amplification (Chou *et al.*, 2005; Oh *et al.*, 2010; Laughlin *et al.*, 2010; Warshawsky *et al.*, 2011). LNA is a bicyclic high affinity nucleic acid analogue that contains a ribonucleoside link between the 2'-oxygen and the 4'-carbon atoms with a methylene unit (2'-O,4'-C-methylene bridge) (Mouritzen *et al.*, 2003; Warshawsky *et al.*, 2011). 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 (Mouritzen *et al.*, 2003; Warshawsky *et al.*, 2011). 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 methodology. We have 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 show that this novel approach for NIPD can be applied in both an autosomal dominant and recessive monogenic disorder.

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 HbC mutation (c.19G>A, p.Glu7Lys) and the father was heterozygous for the HbS mutation (c.20A>T, p.Glu7Val). A previous child was also shown to be a carrier of the HbS mutation. Maternal blood withdrawal was performed at 10+6 and 11+1 weeks of gestation for case 1 and 2 respectively after informed consent was obtained.

Sample processing

Maternal (n=2) and paternal (n=1) plasma (input 800 µL) was isolated, processed and measured as previously described (van den Oever *et al.*, 2012). Isolated plasma DNA was concentrated to 20 µL using the Zymo Clean & Concentrator™ -5 kit (Zymo Research, Irvine, USA). As a control, the total amount of cell-free DNA (fetal + maternal) was determined by Real Time PCR detection of *CCR5* as previously described (Boon *et al.*, 2007). A total concentration of 112 pg/µL and 350 pg/µL was 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, Venlo, the Netherlands).

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 n= 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 n=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 (from now on referred to as "mutation detection probe") with a 3' C3-spacer (Biolegio, Nijmegen, the Netherlands) and was executed both with and without the addition of a target specific blocking LNA probe (from now on referred to as "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 bp 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, USA). 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 (van der Stoep *et al.*, 2009; Almomani *et al.*, 2009). In short, asymmetric PCR (to preferentially amplify the forward strand) was performed in 96-well non-transparent plates (Framestar, 4titude, Surray, United Kingdom) in a total reaction volume of 10 µL containing 1x LightScanner Master mix (Idaho Tech/ BioFire Defense), 5 pmol forward primer, 1 pmol reverse primer, 5 pmol mutation detection probe and 2 ng gDNA template. Primer specific optimal annealing temperature (Ta) for both primer sets was determined using a PCR gradient (58-64°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 from 50 to 98°C was used for HR-MCA melting. Melt temperature (Tm) of normalized melting peaks was determined using the unlabeled probe genotyping analysis tool of the LightScanner software (Idaho Tech/ BioFire Diagnostics, Salt Lake City, USA). Target blocking probe was titrated into each reaction in a mutation detection probe to target blocking probe ratio from 1:1 to 1:10 (i.e. 5-50 pmol/reaction) and optimized for each set. Cycling protocol for testing the target blocking probe was 95°C for 5 min, 50 cycles of 10 s at 95°C, 20 s at 72°C and 30 s at the primer specific Ta of 58°C or 63°C for *BRCA2* and *HBB* respectively to obtain target blocking probe binding prior to amplification (modified from Oh *et al.*) (Oh *et al.*, 2010).

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 from 33% to 1% paternal gDNA mixed into maternal gDNA was created using a total amount of ~425 pg 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 1.5x 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 2 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 utilized. With the optimal Ta for the primers determined (i.e. 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 prior to primer extension (Fig. 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.

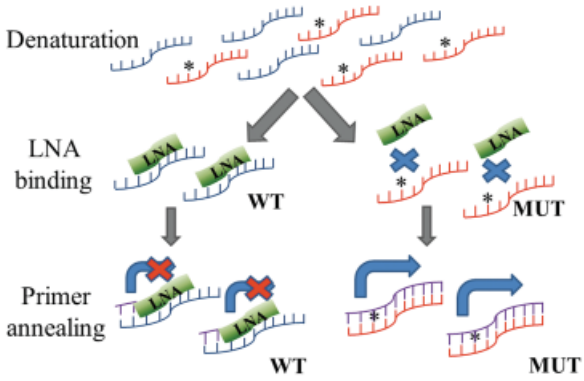

Figure 1: Principle of target blocking LNA probe binding in HR-MCA.
Target blocking probes designed against maternal wild type (WT) sequences are able to bind denatured single stranded WT sequences. No primer extension and amplification can occur (blue arrow, red cross). In case of a mutation (*) target blocking probes will not bind to paternal mutant (MUT) sequences (blue cross), enabling primer extension and amplification.

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 signal

of the MUT allele, while the detection signal of the WT specific melting peak was increased because of the high background of WT sequences (Fig. 2A). Without blocking, the mutant allele could no longer be detected in a relative paternal gDNA percentage of ~16% and lower (i.e. ~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 ~1% - 2% paternal gDNA (i.e. ~0.5-2 GE) in a background of maternal sequences.

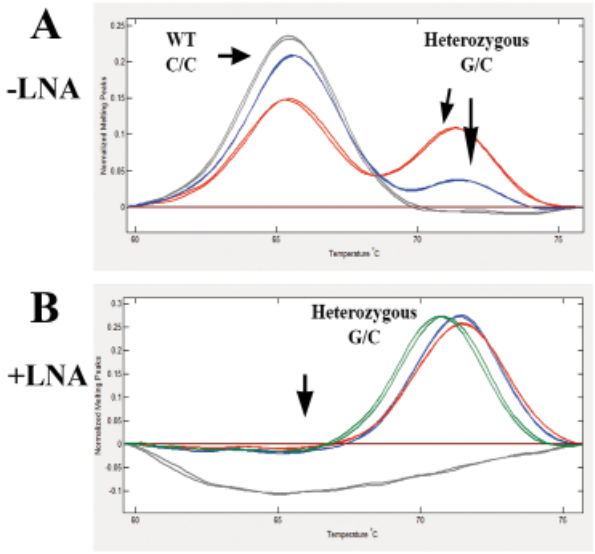

Figure 2: HR-MCA results using the *BRCA2* mutation detection probe.
Panel A: Without target blocking LNA probe: Gray: Wild type (WT) plasma DNA; Red: heterozygous paternal gDNA; Blue: control paternal gDNA (25%) diluted in WT maternal gDNA. Panel B: With target blocking LNA probe: WT signal is blocked (arrow). Gray: maternal WT gDNA; Red: heterozygous CVS gDNA; Blue: heterozygous paternal gDNA; Green: maternal plasma.

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 show 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 Tm calling at around 66°C (Fig. 2A, gray line). Paternal gDNA shows two melting peaks, with a Tm calling at 66°C for the WT and 72° for the *BRCA2* MUT specific peak respectively, as expected for an individual heterozygous for this mutation (Fig. 2A, red line). 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 (Fig. 2A, blue line). The detection signals were skewed towards 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 de-

tection probe, resulting in inhibition of amplification of the WT *BRCA2* allele during PCR (Fig. 2B, arrow). As a result, the *BRCA2* mutation detection probe can no longer detect a WT PCR product in HR-MCA as shown for WT maternal gDNA (Fig. 2B, gray line). In CVS (red line) and paternal (blue line) gDNA samples heterozygous for the *BRCA2* mutation, only the *BRCA2* MUT specific normalized melting peak could be detected (Fig. 2B). In the maternal plasma sample, the paternally inherited mutation in the fetus could only be detected when the maternal WT template was blocked, showing that for this mutation, the addition of a single target specific blocking LNA probe is sufficient to enhance the detection of the paternally inherited *BRCA2* mutation (Fig.2B, green line).

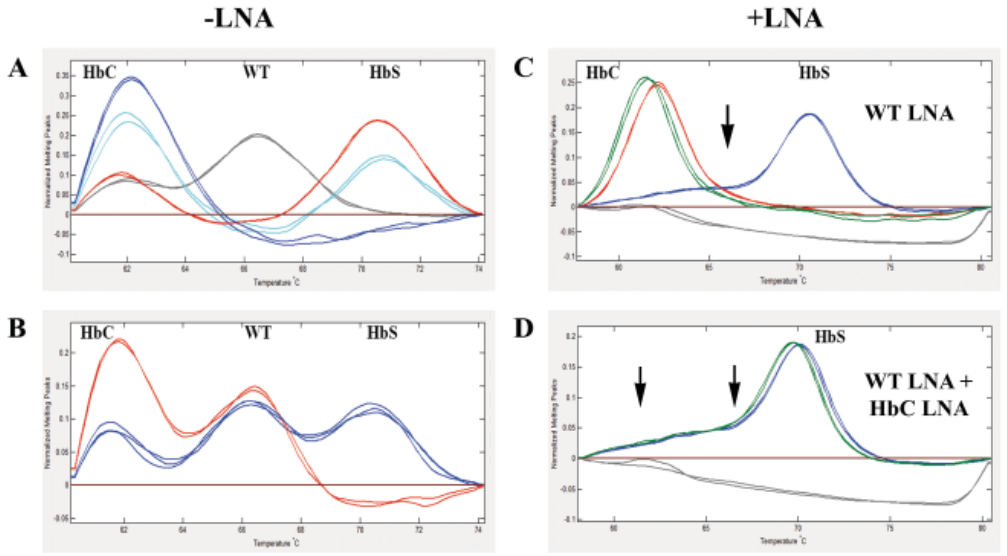

Figure 3: Representation of the HR-MCA melting peak patterns for *HBB* from controls, parents and fetus using the HbS mutation detection probe.

Panel A: Selection of the positive and negative controls scanned for optimization of settings for HR-MCA tested without a target blocking LNA probe. Gray: Wild type (WT); Dark blue: control homozygous for HbS; Red: control homozygous for HbC; Light blue: control compound heterozygous for HbS/HbC. Panel B: Melting peak patterns of maternal (red; heterozygous for HbC), paternal and CVS gDNA (blue; both heterozygous for HbS), without addition of a target blocking LNA probe to the PCR reaction. Panel C: Samples tested with WT target blocking LNA probe directed to block only the maternal WT sequences (arrow). Gray: WT gDNA; Blue: paternal gDNA heterozygous for HbS; Red: maternal gDNA heterozygous for HbC; Green: maternal plasma. Panel D: Additional blocking with an HbC target blocking LNA probe directed to the maternal HbC allele together with a WT blocking LNA probe (arrows). Gray: maternal gDNA heterozygous for HbC; Blue: paternal gDNA heterozygous for HbS; Green: maternal plasma.

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 (Fig. 3A and 3B, without LNA), results from parental, CVS and control gDNA samples show a specific normalized melting peak pattern in the HR-MCA assay for all 3 different alleles (HbC, WT and HbS with Tm calling at 62, 66 and 70°C respectively

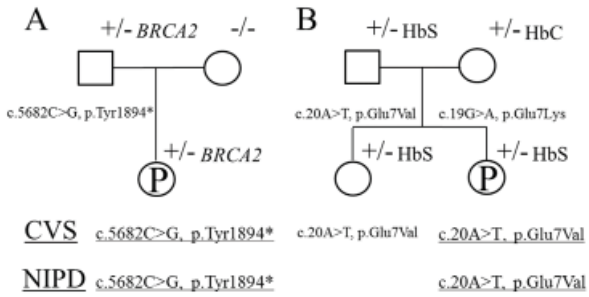

Figure 4: Family pedigrees from participating couples.
Panel A: Case 1: Both father and fetus are heterozygous for BRCA2 mutation c.5682C>G, p. Tyr1894*. Panel B: Case 2: Both parents are heterozygous for a different mutation in the HBB gene. Mother is heterozygous for c.19G>A, p.Glu-7Lys (HbC), while father, daughter and the fetus are heterozygous for c.20A>T, p.Glu7Val (HbS).

(Fig. 3A and 3B). As expected, maternal gDNA shows a melting peak for the HbC and WT allele, since mother is heterozygous for HbC mutation (Fig. 3B, red line). Results from paternal gDNA (heterozygous for the HbS mutation) show two peaks for both the WT and HbS allele respectively (Fig. 3B, blue line). CVS gDNA displays a pattern similar to father (Fig. 3B, blue line).

Table 1: Primer and probe sequences.

Description	*Sequences 5'- 3*
*BRCA2*_NIPD_MCA_F	5'-CAA CGA GAA TAA ATC AAA AAT TTG-3'
*BRCA2*_NIPD_MCA_R	5'-TGC GTG CTA CAT TCA TCA TTA-3'
*BRCA2*_NIPD_MCA_P_Me*	5'-CCG TCC AAC AAT CCT CCG TAA CCT-3'
*BRCA2*_LNA (WT)	5'-T+T+G+T+TA+C+G+A+G+GC-3'
*HBB*_NIPD_MCA_F	5'-GAC ACA ACT GTG TTC ACT AGC A-3'
*HBB*_NIPD_MCA_R	5'-CCA CCA ACT TCA TCC ACG TTC A-3'
*HBB*_NIPD_MCA_P_Me*	5'-GCA GAC TTC TCC ACA GGA GTC AG-3'
*HBB*_LNA1 (WT)	5'-+T+G+A+C+TC+C+T+G+A+G-3'
*HBB*_LNA2 (HbC)	5'-C+T+C+C+T+A+A+G+G+A+G-3'

Table 1: Primer and probe sequences used for PCR and HR-MCA.
Forward (F) and reverse (R) primers are depicted for both cases. Mutation detection probes (P) contain a 3' C3-spacer (Me*). LNA (locked nucleic acid) modified bases in the target blocking probes are depicted with + prior to the base. Target blocking probes were designed to perfectly match maternal sequences. Position of the altered nucleotide is underlined.

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 (Fig. 3C, gray line and arrow), while in heterozygous maternal and paternal gDNA only the HbC and HbS MUT peaks are visible (Fig. 3C, red line (HbC) and blue line (HbS) respectively). More importantly, results from maternal plasma show that blockage of only the maternal WT *HBB* allele is not sufficient to detect the fetal paternally inherited HbS mutation (Fig. 3C, green line). In maternal plasma only the maternal HbC specific melting peak is visible, since the excess of HbC allele is not blocked by the WT *HBB* target blocking probe (Fig. 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 between HbS mutation detection probe, WT target blocking probe and HbC target blocking probe per reaction was shown to be 1:2:2 respectively. As expected, no signal is detected in maternal gDNA (Fig. 3D, gray line) when simultaneously blocking WT and HbC templates (Fig. 3D, arrows). In paternal gDNA only the HbS peak is visible (Fig. 3D, blue line). Subsequently, in maternal plasma the paternally inherited HbS mutation in the fetal cfDNA can now be detected after simultaneously blocking amplification of both maternal WT *HBB* and HbC allele (Fig. 3D, green line).

For both case 1 and case 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 would 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. (Fig. 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, have provided many advances for the use of NIPD in favor of invasive testing procedures early in gestation (Daley *et al.*, 2014).

Advantage and application of the HR-MCA approach in NIPT

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 in order to enhance the detection of fetal paternally inherited mutations in maternal plasma DNA. We choose to explore this approach since this methodology is sensitive, 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 since 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 since good vertical coverage can be obtained. Pooling of multiple 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, while for autosomal recessive disorders mother and

father should carry different mutations (Daley *et al.*, 2014). In this proof of principle study, we have pursued a personalized approach and we have 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 towards expanding the current repertoire for NIPD towards 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 2 different cases; one autosomal dominant (*BRCA2*) and one autosomal recessive monogenic disorder (*HBB*). While 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 bp 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 ~ 50% of the cases).

Applying HR-MCA method in NIPD.

As shown in this study, this method can be used successfully for NIPD. Do note that 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 (Boon *et al.*, 2007; van den Oever *et al.*, 2013). Due to the fragmented nature of circulating cfDNA, there is a restriction for designing primers and probes. Fetal cfDNA is on average around 143-146 bp in size, which limits amplicon size for PCR (Lo *et al.*, 2010).

HR-MCA has previously been proposed as a useful method for NIPD (Yenilmez *et al.*, 2013; Macher *et al.*, 2012; Phylipsen *et al.*, 2012). 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 and colleagues HR-MCA without a blocking LNA probe was performed and was not successful in case of early gestation (Yenilmez *et al.*, 2013). Levels of cffDNA may differ extensively between individuals and have been described to increase as gestation progresses (Lo *et al.*, 1998; Lun *et al.*, 2008a). Early in gestation, fetal paternally inherited variants may not be distinguished from the maternal background, since the levels of cffDNA

are too low to detect. We have previously shown 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% (Out *et al.*, 2015) 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 demonstrated 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 shown 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.

Acknowledgements

The authors would like to thank Dave van Heusden for technical assistance.