Development of new technological applications for post- and prenatal diagnosis of the hemoglobinopathies

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Chapter 3.2

Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis

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Abstract

Objective: The aim of this study was to develop a pyrophosphorolysis-activated polymerization (PAP) assay for non-invasive prenatal diagnosis (NIPD) of β-thalassemia major and sickle-cell disease (SCD). PAP is able to detect mutations in free fetal DNA in a highly contaminating environment of maternal plasma DNA.

Methods: Pyrophosphorolysis-activated polymerization primers were designed for 12 informative SNPs, genotyped by melting curve analysis (MCA) in both parents. The PAP assay was tested in a series of 13 plasma DNA samples collected from pregnant women. A retrospective NIPD was performed in a couple at risk for SCD.

Results: All PAP reactions were optimized and able to detect <3% target gDNA in a background of >97% wildtype gDNA. In all 13 cases, the paternal allele was detected by PAP in maternal plasma at 10 to 18 weeks of gestation. For the couple at risk, PAP showed presence of the normal paternal SNP allele in the maternal plasma, which was confirmed by results of the chorionic villus sampling analysis.

Conclusions: In contrast to other methods used for NIPD, the combined PAP and MCA analysis detecting the normal paternal allele is also applicable for couples at risk carrying the same mutation, provided that a previously born child is available for testing to determine the linkage to the paternal SNPs.

Introduction

Hemoglobinopathies are hereditary microcytic hypochromic anemias, characterized by a reduced, altered or absent synthesis of either the α- globin or β-globin chains and represent the most common autosomal recessive disorders worldwide. Approximately 7% of the world population is a healthy carrier of a hemoglobinopathy, resulting in ~350 000 severely affected newborns annually (1). Hemoglobinopathies are common in areas where malaria is or has been endemic, but the burden on health care in north Europe is increasing due to recent migration (2) and absence of effective prevention strategies.

Because of the severity of the disease and the lack of treatment options, prenatal diagnosis is offered in many countries as part of a national prevention program (3;4) by invasive tests using chorionic villus sampling (CVS) or amniocentesis (AC). However, these tests carry a risk of fetal loss of approximately 0.5-1% (5;6). Furthermore, the test is costly because it requires hospitalization and expert personnel to perform the procedure.

The discovery of cell-free DNA from the fetus in the maternal blood circulation has led to the possibility of non-invasive prenatal diagnosis (NIPD) (7). This has been applied successfully for fetal sex determination, RhD genotype and trisomy 21 detection (8-12). Free fetal DNA (ffDNA) is detectable very early during pregnancy and the average amount of cell ffDNA during the first and second trimester is ~10% of the total amount of cell-free DNA (13).

Recently, the pyrophosphorolysis-activated polymerization (PAP) technique was successfully applied in fetal sex determination (14). Because of the serial coupling of pyrophosphorolyis and polymerization, this reaction has a very high specificity (one mutated allele can be detected in 10^9 wildtype alleles) (15;16). PAP uses oligonucleotides with a blocked 3' end, which can only be removed (and thus permitting extension) when they anneal to specific sequences of target DNA. It has been shown that if a mismatch occurs within 16 nt from the 3'-terminus of the PAP-primer, amplification is still inhibited (17). In this way, it is possible to

specifically amplify DNA inherited from father by using paternally specific mutations or polymorphisms that are absent in mother.

The aim of the current study was to develop a PAP assay for NIPD of β-thalassemia major and sickle-cell disease (SCD). In the Netherlands, most requests for prenatal diagnosis concern homozygosity for the sickle-cell mutation or compound heterozygosities causing SCD, or a variety of β-thalassemia causing mutations that commonly occur in the Dutch immigrant population (18). This makes mutation-specific detection using ffDNA impossible as the paternally and maternally inherited alleles are indistinguishable. Therefore, we decided to use paternal-specific SNPs in linkage to the normal or mutant allele to determine the risk of having an affected fetus. This procedure is suitable for the >200 β-thalassemia mutations (19) without the design of separate assays for each individual molecular defect. The advantage of this approach is that it reduces theamount of reactions to be designed and optimized to the limited number of informative SNPs. Furthermore, the use of SNPs also makes it possible to perform NIPD when both parents carry the same mutation, as is often the case in populations with a high incidence of a small number of mutant alleles. Mutation-specific assays can only detect the disease-causing allele, whereas SNPs can be a target for the normal allele as well. Melting curve analysis (MCA) was used to quickly screen family members for the informative SNP markers and to determine linkage to the normal or mutant paternal allele. In this way, it is possible to pre-select the SNPs that need to be tested with the PAP technique for at risk pregnancies. An invasive procedure is unnecessary when the normal paternal allele is detected in the maternal plasma.

Methods

Samples

Twenty-four SNPs along the β-globin gene cluster were selected on the basis of previous studies (20;21) (Table 1). DNA samples from healthy individuals of seven subsets of different populations were genotyped by MCA to test their informativity. These included 50 Dutch, 50 Czech, 20 Turks, 20 Moroccans, 20 Greek, 20 Cypriots and 30 Surinamese individuals. Thirteen pregnant couples referred to our laboratory for carrier diagnostics were randomly selected and used to optimize and test the PAP reactions. In one family, only the mother was available for testing. A retrospective NIPD was performed in a Turkish couple at risk for SCD, referred to our clinic for CVS analysis. Their previously born unaffected child was also included in the study to determine the linkage of informative SNPs with the paternal wildtype allele. Prior to the CVS, a blood sample was drawn from mother at 18 weeks of gestation, to be used for the non-invasive test.

All individuals of whom blood and/or DNA was used in this study consented to use their material in scientific research.

Isolation of DNA

Genomic DNA was isolated from leukocytes using the Autopure LS robotic workstation (Gentra Systems, Minneapolis, MN, USA). Maternal blood samples were centrifuged to separate the plasma from the leukocytes, erythrocytes and platelets within 48 h after blood drawing. Plasma samples were stored at -80 °C until they were used for testing. Extraction of free DNA from the plasma was performed with the EZ1 Advanced workstation and the included EZ1 Virus Mini Kit v2.0 protocol (Qiagen, Venlo, The Netherlands).

Melting curve analysis (MCA)

With the use of Primer3 (22) and Beacon Designer software (Bio-Rad Laboratories Inc, Hercules, CA, USA), primers were designed to amplify 12 different fragments along the β-globin gene cluster covering all 24 selected SNPs (Figure 1). In addition, 20 MCA probes were designed to cover SNPs specifically. Design of the primers and probes was based on the UCSC Genome Browser, February 2009 assembly (hg19). One of the probes covered three SNPs, and two probes covered two SNPs (Table 1). Because of the presence of highly homologous regions in the β-globin gene cluster, the PreG frame, XmnI site, F2 and F3 fragments were amplified in a first external reaction to obtain a specific polymerase chain reaction (PCR) product. This product was subsequently used as template for a second nested amplification to generate a shorter fragment, suitable to perform MCA. The other eight fragments were suitable for direct use for MCA without nested PCR. For the healthy control population, PCR products were also analyzed by direct sequencing to confirm the results and to validate the MCA technique.

The external PCR reaction was performed in a 20 μL volume containing 10x PCR buffer with $MgCl_2$ (Roche, Basel, Switzerland), 0.2 mM dNTPs (Roche), 6 μM of each primer (Biolegio, Nijmegen, The Netherlands), 1 unit GoTaq polymerase (Promega, Madison, WI, USA) and 20 ng genomic DNA. The reaction was initiated by 3 min denaturation at 94 °C, followed by 33 cycles of 30 s denaturation, 1 min annealing at optimal annealing temperature (Table 1) and 90 s extension at 72 °C. Final extension occurred for 6 min at 72 °C.

The PCR for MCA was performed asymmetrically to generate an excess of one of the strands for the probe to hybridize. The reaction was performed in 10x PCR buffer with $MgCl_2$ (Roche), 0.2 mM dNTPs (Roche), 1 μM forward primer, 10 μM reverse primer, 5 μM probe (Biolegio), 1 μL LCGreen® Plus dye (Idaho Technology Inc., Salt Lake City, UT, USA), 1 unit FastStart Taq polymerase (Roche) and 20 ng genomic DNA. In case of the nested PCR, 1:100 diluted external PCR product was used instead of genomic DNA. The PCR was performed in a 4Titude Framestar 96 wells plate (Bioké, Leiden, The Netherlands) and 15 μL mineral oil (Sigma) was added to prevent evaporation. The PCR was initiated with a 10 min hold at 95 °C. Thermal cycling was performed for 40 cycles of 20 s denaturation at 95 °C, 30 s annealing at optimal annealing temperature (Table 1) and 40 s extension at 72 °C. Finally, the reaction was elongated at 72 °C for 5 min and heteroduplexes were generated in an additional step at 95 °C for 1 min. Subsequently, samples were melted in the LightScanner HR96 (Idaho Technology Inc.) by using a melt range of 55 °C to 98 °C at a hold temperature of 50 °C. Data analysis of the melt curves was performed using the Call IT 1.5 software (Idaho Technology Inc.).

Pyrophosphorolysis-activated polymerization (PAP)

The PAP reaction was performed to detect the paternal allele in DNA isolated from maternal plasma. Primer pairs were designed for 12 SNPs, containing a blocked dideoxynucleotide at the 3' terminus (ddC or ddA) of either the forward or reverse primer. Primers were designed in such a way that the blocked nucleotide is complementary to the position of the SNP, or maximally 1 nt downstream of the SNP. Amplicon lengths ranged from 79 to 214 bp, which is suitable to amplify the fragmented ffDNA. The blocked primers were ordered from Fidelity Systems Inc. (Gaithersburg, MD, USA), the unblocked contra-primers were ordered from Biolegio. An overview of all the primers is given in Table 2. The PAP reaction was performed in PCR buffer (250 mM Tris-HCl pH 7.8, 80 mM $(NH_4)_2SO_4$, 17.5 mM $MgCl_2$, 125 μM dNTPs (each) and 450 μM Na_4PPi), 2.5 U Klentaq

Fragment	SNP (position*)	Upstream primer (5' > 3')	Downstream primer (5' > 3')	Probe (5' > 3'-phos)	Ta (°C)
External reactions					
preGframe		TTTCTTTCCTTTCTTATTCAAC	TGACATATTGGCCACTTAAC		58
XmnI-site		GAAACTGTTGCTTTATAGGAT	GCTTGTGATAGTAGCCTTGTC		62
F2		CCAGTGACTAGTGCTGCAAGAA	AAGTGTGGAGTGTGCACATGA		63
F3		AATCTCAGGCTTTGAGGGAAGTTAAC	TAAATGAGGAGCATGCACACAC		63
MCA reactions					
F1	rs113040651 (5291564)	TCCCACTGTGGACTACTTGCT	TTGCCTTAAAGGTGGTGACA	GCCCTGTTTTTGTCAACTGTCACCACCTTT	63
preGframe SNP 1	rs2855121 (5277291)	GCCTTACACAGGATTATGAAGTCTGAAAG	AGAAACTCTGGAAATCTGGCTTATTGG	TAATTCCTATCAACCTGATAAGTTAGGG	63
preGframe SNP 2	rs2855122 (5277236)	GCCTTACACAGGATTATGAAGTCTGAAAG	GGCAGGGACTGTTTTATTGACTAATAG	CCAGAGTTTCTGACGTCATAATCTACCAA	63
preGframe SNP 3	rs112035597 (5277116)	CCAATAAGCCAGATTTCCAGAGTTTC	GGCAGGGACTGTTTTATTGACTAATAG	GAATACTTCCTGCCATGTTAAGTGG	63
XmnI-site	rs7482144 (5276169)	TGAATCGGAACAAGGCAAAGGC	CCTCACTGGATACTCTAAGACTATTGG	AAATATCTGTCTGAAACGGTTCCTGGC	65
F2 SNP 1	rs113425530 (5274720)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 2	rs2070972 (5274717)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 3	rs113047906 (5274708)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 4	rs60097179 (5274452)	TAGGCTTGATTCTGGGTGGA	GAACTGAAGACAACCATGTGTGA	GGCTTTATTCTGCAAGCAATACAAATAATA	63
F3 SNP 1	rs28379094 (5269806)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	TCTGGGTGGAAGCTGGGTGTGTAGTTATCT	63
F3 SNP 2	rs28440105 (5269799)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	TCTGGGTGGAAGCTGGGTGTGTAGTTATCT	63
F3 SNP 3	rs5789383 (5269586)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	AGATACCACTGAGCCTCTTGCCCATGATT	63
F3 SNP 4	rs3841756 (5269534)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	GGCTTTATTCTGCAAGCAATACAAATAAT	64
F4	rs10128556 (5263683)	TCACAATATGTTGGGGTAGTGA	GCACCTCCCATTGTGTCCTA	GGGGTAGTGAGTTGGCATAGCAAGTAAGA	61
F5	rs968857 (5260458)	CCTAAACTGAGGAACCTTTGG	TGATGTGAATAAATGCATGACAC	GTTTGATTAGTCAGCAAGCATGTGTCA	61
F6	rs16911905 (5249290)	CGATCACGTTGGGAAGCTAT	TAGGGGTAGGAGGGGAAAAG	TAGGAACTTGAATCAAGGAAATGATTT	61
voBRsa1 SNP 1	rs10742584 (5248770)	GCACAGAGCACATTGATTTGTATTTATTC	CAGAATATGCAAAATACTTACAGGACAGAATG	GGAGAAGATATGCTTAGAACTGAGGTAGAG	61

Table 1 Sequences of the primers and probes used for MCA.

Fragment	SNP (position*)	Upstream primer (5' > 3')	Downstream primer (5' > 3')	Probe (5' > 3'-phos)	Ta (°C)
voBRsa1 SNP 2	rs10742583 (5248641)	GCAGGAAGAGATCCATCTACATATC	CGTAATATTTGGAATCACAGCTTGG	TTTAGTGCATCAATTTCTTATTTGT	67
voBRsa1 SNP 3	rs16911894 (5248614)	GCAGGAAGAGATCCATCTACATATC	CGTAATATTTGGAATCACAGCTTGG	TAATAAGAAAATTGGGAAAACGATCTTC	65
β frame SNP 1	rs10768683 (5247791)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	GGTGAGTCTATGGGACCCTTGATGTTTTC	65
β frame SNP 2	rs7480526 (5247733)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	TAGGAAGGGGAGAAGTAACAGGGTAC	65
β frame SNP 3	rs7946748 (5247726)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	TAGGAAGGGGAGAAGTAACAGGGTAC	65
F7	rs10837631 (5246356)	CCCATTTGCTTATCCTGCAT	TTCAGGGGAAAGGTGGTATC	ATCTCTCAGCCTTGACTCCACTCAGTTCT	65
naBHpa1 SNP 1	rs4426157 (5240771)	GGCAAAGGGATCTATTCAAGAAG	GCTTGGTGCAGAGCTGAGTC	CAACGAGACAGAAAGTTAACAAGGA	65

Table 1 (continued) Sequences of the primers for the four external polymerase chain reactions, and primers and probes covering the 24 SNPs used for MCA. The optimal annealing temperature (Ta) for each reaction is indicated in the last column. MCA, melting curve analysis. *Positions are according to the UCSC Genome Browser, February 2009 (hg19).

SNP name	Position*	RS-nomen-clature	WT	mutant	Upstream primer (5' > 3')	Downstream primer (5' > 3')
preGframe SNP2	5277236	rs2855122	G	A	GTAGAGCTCTCCTCCAATAAGCCAGATTTCCAGA TCTCCTCCAATAAGCCAGATTTCCAGAGTTTCTGAC[dd**A**]	CTCTGAACTCGATCCATGACCTTGGTAGATTATGA[dd**C**] CTTTTGTTTTTTCTCTGAACTCGATCCATGACCTTGGTAG
F2 SNP2	5274717	rs2070972	T	G	GGCCAGTGACTAGTGCTGCAAGAAGAAC	AGCCTGGCCTCCAGATAACTACACACC[dd**C**]
F2 SNP4	5274452-5274453	rs60097179	-	ins T	CTTTCAAGGATAGGCTTTATTCTGCAAGCAAT[dd**C**]	GACAACCATGTGTGATCTCTTAGCAGAATAGA
F3 SNP3	5269586	rs5789383	A	del A or G	CCCTGTCCTCCAGATACCACTGAGC[dd**C**]	CAAACAGGCATGCAGAAATACACATACACACTTCCC
F3 SNP4	5269534	rs3841756	A	del A	TCAGAGCTTTCAAGGATAGGCTTTATTCTGCAAGCAAT[dd**A**]	GCAGAAATACACATACACACTTCCCTCAATATAAACCC
F5	5260458	rs968857	A	G	TTAGTATTTATAGGTCATGAGGTTCCTTCCTCTGC CCTTCCTCTGCCTCTGCTATAGTTTGATTAGTCA**A**[ddC]	CTGATGTGAATAAATGCATGACACATGCTTG[dd**C**] GGTTGAGTAACTGATAATGGTTTGTCCTTTCCTTGATGG
F6	5249290	rs16911905	G	C	GGAGGTTTAAACAAACAAAATATAAAGAGAAATAGGAACTTGAAT[dd**C**] GTCCACTAAGAATACTGCGTTTTAAAATCATTTCCTT[dd**C**]	AGGGGAAAAGGTCTTCTACTTGGCTCAGATTAT TCACGTTGGGAAGCTATAGAGAAAGAAGAGTA
voBRSal SNP2	5248641	rs10742583	G	A	GAAGATCGTTTTCCCAATTTTCTTATTACACAAATAAGAA[dd**A**] GGTAGACAAAACTCTTCCACTTTTAGTGCATCAA[dd**C**]	CGCAGGAAGAGATCCATCTACATATCC GGAATCACAGCTTGGTAAGCATATTGAAGATCGTT
β-frame SNP1	5247791	rs10768683	C	G	TGAGAACTTCAGGGTGAGTCTATGGGAC[dd**C**] CCATAGAAAAGAAGGGGAAAGAAAACATCAAG[dd**C**]	CTGTACCCTGTTACTTATCCCCTTCCTATGAC TTTGCCACACTGAGTGAGCTGCACTGT
β-frame SNP2	5247733	rs7480526	A	C	GGTGAGTCTATGGGACGCTTGATGTTTTCTTTCC GGTGAGTCTATGGGACGCTTGATGTTTTCTTTCC	CGTCTGTTTCCCATTCTAAACTGTACCCTGTTACTT[dd**A**] CGTCTGTTTCCCATTCTAAACTGTACCCTGTTACTT[dd**C**]
β-frame SNP3	5247726	rs7946748	G	A	TAAGTTCATGTCATAGGAAGGGGAGAAGTAA[dd**C**] ATTCGTCTGTTTCCCATTCTAAACTGTACCCT[dd**A**]	TAAAACGATCCTGAGACTTCCACACTGATGC CTTCCCCTTCTTTTCTATGGTTAAGTTCATGTC
F7	5246356	rs10837631	T	A	TACCCATTTGCTTATCCTGCATCTCTCAGCCTTG[dd**A**]	GCCGTAAAACATGGAAGGAACACTTCAGGGGAA

Table 2 Sequences of all primers used for PAP. The position of the SNP complementary to the target strand is indicated in bold. We were able to design pyrophosphorolysis-activated polymerization (PAP) assays for 12 of the 17 SNPs that appeared to be informative in the healthy control populations. For seven SNPs, two PAP primer pairs were designed to detect both the wildtype (WT) and the mutant. *Positions are according to the UCSC Genome Browser, February 2009 (hg19).

	F1 rs113040651	preGframe SNP1 rs2855121	preGframe SNP2 rs2855122	XmnI-site rs7482144	F2 SNP2 rs2070972	F2 SNP4 rs60097179	F3 SNP1 rs28379094	F3 SNP3 rs5789383	F3 SNP4 rs3841756	F4 rs10128556	F5 rs968857	F6 rs16911905	voBRsa1 SNP2 rs10742583	β frame SNP1 rs10768683	β frame SNP2 rs7480526	β frame SNP3 rs7946748	F7 rs10837631
Turks (n=40)	57.5	70.0	42.1	75.0	42.5	70.0	70.0	27.5	12.5	26.5	55.0	5.0	12.5	12.5	40.0	10.0	27.5
Moroccans (n=40)	50.0	80.0	58.3	77.5	50.0	0.0	71.1	18.4	10.5	20.0	45.0	17.5	12.5	15.0	40.0	7.5	17.5
Czechs (n=100)	54.2	68.0	44.7	68.0	44.0	66.0	67.0	23.0	14.0	33.0	54.0	4.0	17.7	17.0	57.0	15.0	37.0
Surinamese (n=60)	33.3	86.7	73.3	86.7	63.8	83.3	73.3	10.0	21.7	13.3	38.3	13.0	40.0	36.7	63.3	18.3	14.3
Cypriots (n=40)	40.0	77.5	ND	78.9	62.5	ND	77.8	13.2	23.7	22.5	32.4	2.5	10.0	10.0	40.0	15.0	25.0
Greek (n=40)	47.5	69.4	ND	70.0	52.5	ND	71.1	21.1	18.4	27.5	47.4	10.5	18.4	11.1	62.5	20.0	47.4
Dutch (n=100)	59.2	57.1	43.6	58.2	37.0	59.0	45.9	15.0	11.2	42.7	58.2	10.2	18.4	18.4	63.3	12.2	32.3
Total (n=420)	50.2	70.5	50.9	71.2	48.1	59.6	64.9	18.1	15.3	29.0	49.5	8.5	19.4	18.4	55.0	14.1	29.8

Table 3 Overview of the frequencies of the informative SNPs in each of the control populations. Out of the 24 selected SNPs, 17 appeared informative (frequency >5% and <95%) and were used to design the pyrophosphorolysis-activated polymerization (PAP) assay. The numbers indicate the percentage of alleles in the population containing the SNP. ND, not determined, n = number of alleles tested.

(ScienTech Corp., Chesterfield, MO, USA) and 10 μL DNA isolated from maternal plasma. Thermal cycling consisted of 45 cycles of 15 s denaturation at 94 °C, a stepwise annealing for 2 min (information on the optimal conditions for each primer pair is available upon request) and extension for 40 s at 72 °C. Amplified products were visualized by electrophoresis on a 3.5% Tris/Borate/EDTA (TBE) agarose gel.

Results

Screening of healthy control populations

Along the β-globin gene cluster, 24 SNPs previously described were selected for which MCA primers were designed (20;21). Seventeen SNPs occurred with a frequency between 5% and 95% in the seven different populations and were therefore considered informative (Table 3).

Optimization of the PAP reactions

Pyrophosphorolysis-activated polymerization reactions were initially tested in genomic DNA samples with known genotype to test the sensitivity of the PAP and to determine the optimal conditions for each primer pair. Optimization of the PAP reactions included testing at different annealing temperatures and annealing times. Most reactions worked properly after trying three to four different conditions. For three SNPs, we designed new primers to obtain better results. After optimization, we tested the PAP reactions in dilution series of genomic DNA, in which we mixed a wildtype sample with a homozygous SNP genotype. For all 12 SNPs, we were able to detect <1% target gDNA mixed in > 99% wildtype gDNA isolated from whole blood. The next step included testing in cell-free DNA isolated from plasma samples from healthy control individuals. This was also performed in a dilution series by mixing a wildtype and a homozygous SNP plasma DNA sample. In all cases, ~3% of the SNP plasma DNA sample was detectable in ~97% wildtype DNA, indicating that our method was suitable to use for DNA isolated from blood plasma.

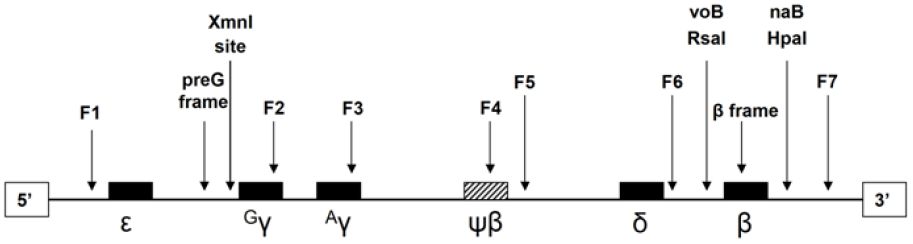

Figure 1 Overview of the positions of the 12 different fragments along the β-globin gene cluster, which are amplified by melting curve analysis (MCA). Each arrow represents one fragment covering one, two or three SNPs. Eight fragments were of suitable length (up to 400 bp) to perform MCA directly. The PreG frame, XmnI site, F2 and F3 fragments were first amplified in an external polymerase chain reaction (PCR) to obtain a specific PCR product, which was then used as template for a second nested MCA reaction.

Test cases: 13 pregnant couples

Thirteen pregnant couples were referred to our laboratory for carrier diagnostics. In all cases, only one of the parents was a carrier of a hemoglobinopathy, so the couples were not at risk of having an affected child and prenatal diagnosis was not indicated. PAP reactions were tested in plasma DNA from these pregnant women at 10 to 18 weeks of gestation to test the performance of the PAP assay in maternal plasma. The MCA technique was used to genotype the SNPs prior to the PAP test to determine which SNPs were absent in the mother but present in the father and, when detected by PAP in maternal plasma, should be of fetal origin. In all 13 couples, at least one SNP, and in eight families (~60% of the cases), two or more were informative (Table 4). Obviously, we could not determine the linkage of the SNP to the paternal mutations in these cases, as no other family members were available. However, in all cases, we were able to detect a paternal allele in maternal plasma. In ten families, genomic DNA of the baby (after birth) was available for testing, and PAP results were confirmed by direct sequencing analysis. All PAP reactions performed in this group of test cases showed the correct results. These results indicated that our method is suitable to perform NIPD for β-thalassemia major and SCD, provided that a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs.

Proof of principle: retrospective NIPD

A Turkish couple at risk for SCD was referred to our clinic for prenatal diagnosis for their second pregnancy. Their first child was born unaffectedly. We performed MCA for both parents and the first child to determine the informative SNPs, as well as the linkage to the paternal alleles. One of the SNPs in the β-globin gene, rs7480526 (wildtype A, variant C), was informative in this family. The mother had a wildtype genotype (A/A), and both the father and the child were heterozygous (A/C). From this data, it could be concluded that the paternal A allele is linked to the hemoglobin S (HbS) mutation and C is linked to the paternal wildtype allele, because the first child inherited the C allele but not the HbS mutation from the father (Figure 2).

As a proof of principle, PAP was performed for the informative SNP on free plasma DNA isolated from maternal blood. Results showed the presence of the A and C allele in the reaction with the oligonucleotide specific for the A and C nucleotide, respectively. As the mother does not carry the C allele, it can be deduced that the fetus inherited the C allele from the father, which is linked to the normal allele (Figure 2). The fetus did not inherit the mutated allele from the father and will be either a carrier of HbS or normal and thus not affected with SCD. The result was confirmed by performing MCA for this SNP on the fetal material obtained by CVS, showing heterozygosity for the SNP (A/C). In addition, results from the CVS showed that the fetus was a carrier of HbS, indicating inheritance of the maternal mutation.

Discussion

The discovery of circulating ffDNA in maternal plasma during pregnancy has provided the possibility to perform non-invasive DNA tests on the fetus. In this study, we used a strategy which combined the use of the MCA and PAP technique for retrospective NIPD of β-thalassemia major and SCD. The PAP method is sensitive enough to detect the small amount of ffDNA in cell-free DNA isolated from maternal plasma. In this study we tested the PAP assay in 13 test couples and in 1 couple at risk. Our results show that this approach is suitable to detect the paternal allele in

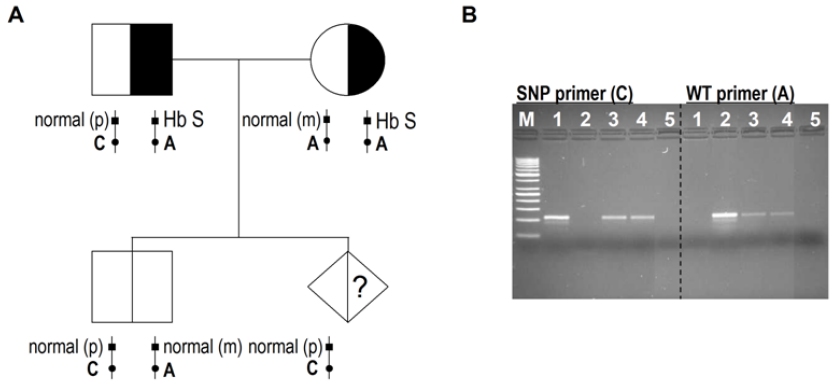

Figure 2 (A) Pedigree of the test family. The first child inherited the C allele from the father but not the HbS mutation. Therefore, it can be concluded that the C is linked to the normal allele [normal (p)]. The C allele was detected in the maternal plasma, indicating that the second child also inherited the normal paternal allele and will thus not be affected with sickle-cell disease. **(B)** Results of gel electrophoresis of the pyrophosphorolysis-activated polymerization products. The left panel shows the results from the reaction of the primer pair to detect the variant (C), the right panel depicts the results from the wildtype (A) primer pair. The A allele is obviously present, as the mother carries this allele. However, the C allele is also detected, which must be of fetal origin. (M: Gene Ruler 100 bp marker, lane 1: control sample C/C, lane 2: control sample A/A, lanes 3 and 4: DNA sample isolated from maternal plasma, lane 5: blanc)

plasma from pregnant women as we were able to detect the paternal allele in all cases. The major advantage of this kind of prenatal testing is its early and non-invasive approach. There is no increased risk for fetal loss due to the invasive procedure and this will make prenatal diagnosis more safe for pregnant women. Admission to the hospital is not necessary, which reduces the costs compared with invasive testing. Furthermore, ffDNA can be detected in maternal blood from the 6th week of gestation, which enables early prenatal diagnosis (23).

Melting curve analysis is a helpful screening method to identify which of the SNPs are informative in the family. Analysis of both parents and selection of the informative SNPs can be performed within 1 day, which is a major advantage compared to any sequence-based technology. It is highly robust, costs are relatively low and results are obtained with high sensitivity and specificity. Furthermore, SNPs that do not create or eliminate restriction sites can also be screened for with this method. The MCA method is high throughput, 96 reactions can be performed and analyzed simultaneously. MCA can be used as a quick screening method to select the informative markers, and the sensitivity of the PAP assay is necessary for detection of the ffDNA. The PAP assay can be performed in 1 day, so the complete NIPD can be performed within 2 days.

A limitation of using linkage for NIPD is the requirement of a previously born child or other family member to determine the phase of the paternal mutation in combination with the SNP. However, the number of requests for prenatal diagnosis is increasing due to the implementation of hemoglobinopathy in the newborn screening in the Netherlands as of January 2007 (24). When the first-born in a family appears to be carrier of or affected with a hemoglobinopathy, parents will be tested. If it concerns a couple at risk, prenatal diagnosis will be offered for any following pregnancies.

It is well known that the human β-globin gene cluster contains a hotspot for meiotic recombination between the δ-globin and β-globin genes (20). In previous studies, the rate of recombination in the β-globin gene cluster is estimated to be 3 to 30 times (25) and 30 to 50 times (26) greater than the genome-wide average. The presence of a recombinant β-globin haplotype in a family has significant implications when linkage is used for prenatal diagnosis (27). Therefore, it is important to perform a complete haplotype analysis and, whenever possible, use SNPs located at both the 3' and 5' end of the recombination hotspot. In the couple at risk tested in the current study, only one informative SNP was present. However, this particular SNP is located in the β-globin gene itself, which eliminates the risk of having a false positive or negative result due to meiotic recombination. If none of the SNPs within the β-globin gene are informative, it is required to test at least two SNPs, one at each side of the recombination hotspot, to obtain a reliable diagnosis.

It might occur that none of the 12 SNPs is informative in some families. To be able to perform NIPD by PAP, it is required that the father has a heterozygote genotype for one of the SNPs whereas the mother is wildtype or homozygous. Therefore, we will continue developing and optimizing PAP assays for the other five informative SNPs which will enable us to test for both the wildtype and the mutant alleles in all 17 informative SNPs. This will increase the chances of finding an informative SNP within a family and will make the test applicable to more couples at risk.

The purpose of this study was to design PAP assays for the 17 SNPs that appeared to be informative from the screening of the healthy control populations by MCA. We were able to design PAP primers for 12 of the SNPs, and two primer sets were designed for seven of these SNPs, one to detect the wildtype and one for the mutant allele. Because of the stringencies for the PAP primer design (e.g. GC content, T_m, location of the SNP), assays for the other five SNPs are not optimized yet. However, the 12 SNPs provided sufficient information to perform the current study. In the near future, we will perform the NIPD by PAP in parallel to the standard CVS and AC procedures. After a period of validation, the PAP technique will be introduced as a standard diagnostic method for prenatal diagnosis.

Other studies describing NIPD for paternally inherited β-thalassemia mutations include detection of the codon 41/42 (-CTTT) mutation by using real-time PCR (28) and hemoglobin E detection by restriction enzyme analysis of PCR products (29). However, these approaches are suitable for a single mutation and are not applicable when both parents carry the same mutation. The allele-specific arrayed primer extension (AS-APEX) method combines detection of mutations and genotyping of SNPs (30), similar to the current study. Although this approach showed to be very useful for the southern Chinese population, only four mutations can be detected by this AS-APEX assay, which would not be sufficient to apply in north European countries. Another possibility is to detect the paternal allele by the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (31). In addition, this method, in combination with multiplex PCR of single-copy DNA (M1-PCR), enables direct haplotyping without the use of pedigree data. This would make the NIPD method described in this study applicable to more pregnant couples at risk (32). These mass spectrometry methods showed high specificity and sensitivity, but the use of sophisticated and expensive equipment limits the applicability in diagnostic laboratories. PAP in combination with MCA is relatively cheap and quick to perform. In principle, this approach is suitable to perform NIPD for all β-thalassemia causing mutations and the sickle-cell mutation, even when both parents carry the same mutation.

		F2 SNP2		F2 SNP4		F5		F6		β frame SNP1		β frame SNP2		F7	
		rs2070972		rs60097179		rs968857		rs16911905		rs10768683		rs7480526		rs10837631	
Family	Member	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
1	Mother					G	G					C	C		
	Father					G	A					C	A		
2	Mother					G	G					C	C		
	Father					G	A					C	A		
3	Mother					G	G	C	C					T	T
	Father					G	A	C	G					A	A
	Child					G	A	C	G					T	A
4	Mother	T	T			A	A								
	Father	T	G			G	A								
	Child	T	G			G	A								
5	Mother			-	-										
	Father			ins T	ins T										
	Child			-	ins T										
6	Mother											A	A		
	Father											C	A		
	Child											C	A		
7	Mother	T	T			A	A					C	C		
	Father	T	G			G	A					C	A		
8	Mother											C	C		
	Child											C	A		

Table 4 Overview of the informative SNPs in each of the families.

		F2 SNP2		F2 SNP4		F5		F6		β frame SNP1		β frame SNP2		F7	
		rs2070972		rs60097179		rs968857		rs16911905		rs10768683		rs7480526		rs10837631	
Family	Member	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
9	Mother							C	C	C	C	A	A		
	Father							C	G	C	G	C	A		
	Child							C	G	C	G	C	A		
10	Mother							C	del	C	del	C	del		
	Father							C	G	C	G	C	A		
	Child							del	G	del	G	del	A		
11	Mother					G	G	C	C			C	C		
	Father					A	A	C	G			C	A		
	Child					G	A	C	G			C	A		
12	Mother	T	T												
	Father	G	G												
	Child	T	G												
13	Mother											C	C		
	Father											A	A		
	Child											C	A		
testcase	Mother											A	A		
	Father											A	C		
	Fetus (CVS)											A	C		

Table 4 (continued) Overview of the informative SNPs in each of the families. All parents were screened by melting curve analysis for the 12 SNPs for which pyrophosphorolysis-activated polymerization primers were designed. DNA of the babies (if available) was analyzed by direct sequencing to confirm the results. Only the SNPs that were informative in each family are shown in the table. CVS = material obtained from chorionic villus sampling.

In conclusion, this study has shown that the PAP assay, in combination with the MCA method, can be used for paternal allele detection in maternal plasma. This enables NIPD of β-thalassemia major and SCD, provided that a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs. If the SNP linked to the paternal mutation is detected, an invasive procedure will still be necessary to check whether the fetus also inherited the maternal mutation. However, NIPD methods will make half of the invasive procedures redundant when informative SNPs are present. The use of SNPs instead of mutations makes it possible to perform NIPD in couples carrying the same mutation. In addition, this approach might also be applicable for other genetic disorders as linkage to informative SNPs can be used for most disease genes.

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