Peptide-nucleic acid-mediated enriched polymerase chain reaction as a key point for non-invasive prenatal diagnosis of β-thalassemia

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ABSTRACT

The presence of fetal DNA in maternal plasma can be exploited to develop new procedures for non-invasive prenatal diagnosis. Tests to detect 7 frequent β-globin gene mutations in people of Mediterranean origin were applied to the analysis of maternal plasma in couples where parents carried different mutations. A mutant enrichment amplification protocol was optimized by using peptide nucleic acids (PNAs) to clamp maternal wild-type alleles. By this approach, 41 prenatal diagnoses were performed by microelectronic microchip analysis, with total concordance of results obtained on fetal DNA extracted from chorionic villi. Among these, 27/28 were also confirmed by direct sequencing and 4 by pyrosequencing.

Key words: non-invasive prenatal diagnosis, fetal DNA in maternal plasma, PNA-clamping.

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Introduction

Since the identification of fetal DNA in maternal plasma,[1] several attempts have been made to develop alternative approaches for non-invasive prenatal diagnosis of genetic diseases. The identification of paternally inherited mutations in maternal plasma is particulary challenging mainly due to the predominant presence of maternal DNA (>95%) which shares the same alleles with the fetus and competes with the fetal DNA during the amplification process. Recently, assays to detect 4 β-globin gene mutations causing thalassemia in Southeast Asians were performed by MALDI-TOF mass spectrometry analysis.[2] Paternally inherited alleles were correctly identified in 20 of the 23 pregnancies. This approach is sufficiently specific to detect fetal mutations directly from maternal plasma, although the instrumentation is too sophisticated and expensive for routine clinical use. Subsequently, a size separation strategy was developed using electrophoresis and excision of the gel fraction containing short-sized DNA for selective enrichment of fetal sequences.[3,4] This protocol was used in combination with PCR amplification with a peptide-nucleic-acid (PNA) clamp, a strategy previously proposed by our group to selectively suppress amplification of maternal allele,[5,6] and was applied to non-invasive diagnosis of β-thalassemia in 32 pregnancies, with 100% sensitivity and 93.8% specificity in mutation detection.[3] Nevertheless, the enrichment of fetal sequences through gel elution is problematic and prone to contamination. As an alternative approach, we evaluated a microelectronic microchip technology that proved to be highly reliable in the identification of mutations in several disease genes[7-11] coupled with PNA-clamping of maternal wild-type allele to preferentially amplify fetal mutated sequences. PNAs are DNA analogs in which the ribose-phosphate backbone is replaced by a peptide backbone. PNA/DNA hybrids have much higher thermal stability than corresponding DNA-DNA hybrids, but are more destabilized by single base-pair mismatches. Thanks to their properties, specifically designed PNAs could interfere with amplification of the wild-type allele while still allowing amplification of the mutant allele. Therefore, any PCR-based mutation detection technology can be combined with allelic suppression by sequence specific-PNA clamping.[12,13] The strategy was applied to the identification of fetal pater-

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nally inherited mutations on DNA extracted from maternal plasma in couples at risk for beta-thalassemia and other hemoglobinopathies undergoing invasive prenatal diagnosis. In addition, we also evaluated the direct sequencing and pyrosequencing[14,15] methodologies in a subset of diagnoses.

Design and Methods

Patients

Plasma samples were collected prior to chorionic villus sampling (9-12 weeks of gestation) from 41 women in couples at risk of β-thalassemia where the two parents carried different mutations. Detailed written informed consent, as approved by the local Ethical Review Boards, was obtained for each woman.

Sample preparation

DNA was extracted from 500 μL of maternal plasma[16] with the QIAamp DSP Virus kit, Qiagen.

Polymerase chain reaction conditions

PCR was performed in 50 μL containing 15 μL of plasma DNA, 200 μM dNTPs, 10 mM Tris-HCl, 1.5 mM MgCl2 1.3 U of thermostable DNA polymerase (AmpliTaq Gold; Applied Biosystems), 20 pmoles of each primer and PNA. Cycling conditions were: 1 cycle at 95°C for 10 mins., 47 cycles at 95°C for 45 secs., 60°C for 45 secs., (for PNA annealing),[17] 45 secs., at the annealing temperature specific for each primer set (for primer annealing and extension), followed by 1 cycle at 72°C for 10 mins. For the Lepore-Boston mutation, primers specific for the rearranged chromosome were use[18] (Table 1).

PNA-mediated enriched polymerase chain reaction

PNAs (Applied Biosystems) were designed complementary to the wild-type sequence of 6 beta-globin gene mutations: IVSI-1(G>A) IVSI-6(T>C), IVSI-110(G>A), Cd39(C>T), IVSII-745(C>G) and HbS(A>T) (Table 1). Mutations IVSI-1 and IVSI.6 share the same PNA. For the Lepore-Boston mutation, PNA clamping is not necessary since the rearranged chromosome is

Table 1. Sequences of primers, reporters, stabilizers, oligonucleotides and peptide-nucleic acids.

Mutation	*Primer sequence Tm (°C)*	*PCR fragment (bp)*	*Reporter and stabilizer sequences*	*Thermal stringency Tm (°C)*	*PNA sequences Tm (°C)*	*Optimal range of PNA concentration (pmoles)*
IVSI-110 (G>A)	F 5-'gactctct**ctgcctat-3'** Rb-5'gcagctcactcagtgt-3' (48)	223	WT probe 5'-Cy3-cctattggtctatt-3' MUT probe 5'-Cy5- gcctattagtctatt-3' Stab 5'-agactcttgggtttctgataggcactgact-3'	31-32	5'-ctgcctattggtctat-3' (62)	0.15/0.25/ 0.35/0.45/0.55
IVSI-1 (G>A)	Fb 5'-atctactcccaggag-3' R 5'-ctgtcttgt**aaccttga-3** (48)	239	WT probe 5'-Cy3- ccaacctgcc-3' MUT probe 5'-Cy5- ccaatctgccc-3' Stab 5'-agggcctcaccaccaacttcatccacgttcacc-3'	37-8	5'-aaccttgataccaacc-3' (62)	0.005/0.010/ 0.015/0.020/0.025
IVSI-6 (T>C)	Fb 5'-atctactcccaggag-3' R 5'-ctgtcttgt**aaccttga-3'** (48)	239	WT probe 5'-Cy3-ccttgataccaa-3' MUT probe 5'-Cy5-cttgatgccaa-3' Stab 5'-agggcctcaccaccaacttcatccacgttcacc-3'	24-25	5'-aaccttgataccaacc-3' (62)	0.005/0.010/ 0.015/0.020/0.025
HbS (A>T)	Fb 5'-ctgtggagccacac-3' R 5'-taacgg**cagacttctc-3'** (48)	198	WT probe 5'-agacttctcctcactgagtccgaacattgag-3'* MUT probe 5'-agacttctccacagcagtatatcgcttgaca-3'* Stab 5'-ggagtcaggtgcaccatggtgtctgtttgaggt-3'	33-34	5'-cagacttctcctcag-3' (61)	0.95/1.05/ 1.15/1.25/1.35
Cd39 (C>T)	Fb 5'-tcttgggtttctgata-3' R 5'-gcagctcactcagt-3' (44)	245	WT probe 5'-tggacccagaggtctgagtccgaacattgag-3' * MUT probe 5' -ggacctagaggtgcagtatatcgcttgaca-3' * Stab 5'-ctttgagtcctttggggatctgtccactcctg-3'	24-25	5'-cttggacccagaggt-3' (63)	0.065/0.075/0.085/ 0.095/0.105
IVSII-745 (C>G)	F 5'-taatagcagct**acaatc-3'** Rb 5'-tctgataggcagcct-3' (48)	216	WT probe 5'-Cy3-ctacaatccagc-3' MUT probe 5'-Cy5-ctacaatccagg-3' Stab 5'-taccattctgcttttattttatggttgggataaggc-3'	37-38	5'-acaatccagctaccat-3' (62)	0.020/0.040/ 0.060/0.080/0.10
Lepore-Boston	F 5'-gttttcctaccctcagatt-3' Rb 5'-aatcattcgtctgtttccca-3' (57)	356	WT probe 5'-gccacactgagctgagtccgaacattgag-3'* MUT probe 5'-tctcagctgagtgcagtatatcgcttgaca-3'* Stab 1 5'-gagctgcactgtgacaagctgc-3' Stab 2 5'-ctggacaacctcaagggcacct-3'	32-33	–	–

**WT-Universal probe - CTC AAT GTT CGG ACT CAG-Alexa532 **MUT- Universal probe - TGT CAA GCG ATA TAC TGC-Alexa647

*Forward oligonucleotide primer sequences were used for direct sequencing of all fragments except for those containing the IVSII-745 and IVSI-110. For each primer, the sequence overlapping with the wild-type PNA is indicated in bold. b: 5' biotinylated primer. stab: stabilizer. *Universal reporter formats which hybridize to the universal probes (**). In PNA sequences, the position of the wild-type nucleotide involved in each mutation of interest is underlined.*

not present in maternal DNA. PNAs were designed to have a melting temperature approximately 15°C higher than that of the PCR primer sets.

Microchip analysis

We used the NMW 1000 NanoChip™ Molecular Biology Workstation by Nanogen (San Diego, CA, USA). In this system, the amplified DNA fragment is directed electrophoretically into the chip, and then hybridized with fluorescently labeled DNA probes specific for the mutant and wild type sequences, and the presence or absence of the mutation is detected by the fluorescence signal. Conditions for stabilizer and reporter design, microchip addressing, hybridization, thermal stringency and fluorescence detection have been previously described.[7-11]

Direct sequencing

Direct sequencing was performed using ABI Prism 3100 Genetic Analyzer (PE Biosystems, Foster City, CA, USA).

Pyrosequencing

Pyrosequencing (Pyrosequencing AB, Uppsala, Sweden) is a real-time sequencing method that relies on the sequential incorporation of nucleotides in a primer-directed polymerase extension reaction.[14,15]

Conditions were developed for the analysis of IVSI-110, Cd39, IVSI-1 and IVSI-6 mutations on amplicons amplified with the same primers used for the microchip analysis (Table 1). For the pyrosequencing reaction, primer 5'-CACTGACTCTCTCTGCCT-3' was used for IVSI-110, 5'-GGTCTACCCTTGGACC-3' for Cd39 and 5'-TTGGTGGTGAGGCCC-3' for IVSI-1 and IVSI-6 mutations.

Results and Discussion

In an attempt to identify efficient and accessible methodologies for non-invasive prenatal diagnosis of genetic diseases we evaluated a microelectronic chip and two sequencing-based techniques. They were all coupled with a PNA-mediated mutant enrichment amplification strategy because these methodologies are not sufficiently sensitive to directly identify fetal mutated alleles in maternal plasma. One of the advantages over other microarrays is that the microelectronic microchip is highly reliable in the detection of sequence variations and can be easily customized by the end-user.[10] We also evaluated other highly accurate and user-friendly methodologies. Among accessible technologies for genotyping, nucleic acid sequencing is fast becoming a diagnostic tool in routine use in clinical laboratories. In our study, the sequencing approach proved to be reliable and could be used to confirm

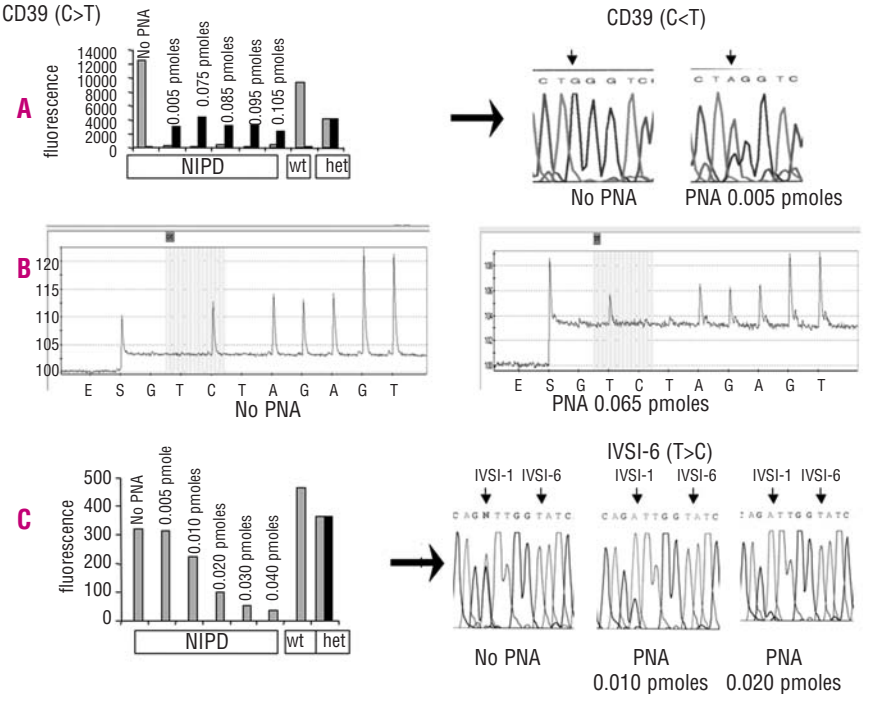

Figure 1. Non-invasive prenatal diagnosis on maternal plasma performed by the microchip system (A and C: left panels), direct sequencing (A and C: right panels) and pyrosequencing (panels B) for the Cd39 (A and B), and IVSI-6 (C) mutations in pregnant women. For the microchip analysis, maternal plasma was analyzed in the absence of PNA and in the presence of increasing amounts of PNA (pmoles in 50 µL final volume of the PCR reaction), in parallel with wild-type (wt) and heterozygous (het) genomic DNA control samples (25 ng). Fluorescence hybridization signals for wild-type (grey) and mutant (black) alleles are shown. In couples analyzed in panels A and B the fetus inherited the paternal mutated allele, in panel C the fetus inherited the paternal wild-type allele.

Panel B: maternal plasma DNA analysis performed by pyrosequencing in a couple where the father carried the Cd39, in the absence (left) and in the presence (right) of PNAs. **Panel C:** non-invasive diagnosis in a couple where the mother carries the IVSI-1 mutation (G>A) and the father carries the IVSI-6 mutation. The sequencing profiles show that in the presence of increasing amounts of the PNA (which clamps the wild-type sequence for both mutations), the maternal wild-type allele for the IVSI-1 mutation (G) is proportionally inhibited.

Table 2. Non-invasive prenatal diagnoses of β-thalassemia on maternal plasma.

N. samples analyzed for each mutation	*Paternal mutated allele*	*Paternally inherited allele*	
		mutated	*wild-type*
14	IVSI-110	5 (1 p, 1 ds)	9 (6 ds)
8	Cd39	5 (2 p, 4 ds)	3 (3 ds)
7	IVSI-6	1	6 (1 p, 4 ds)
4	Lepore Boston	3 (3 ds)	1
3	IVSII-745	-	3 (2 ds)
3	IVSI-1	-	3 (3 ds)
2	HbS	1 (ds*)	1 (1 ds)

(p): analysis also performed by pyrosequencing, paternal allele correctly identified; (ds) analysis also performed by direct sequencing, paternal allele correctly identified; (ds): analysis also performed by direct sequencing, where the paternally inherited fetal mutation was not detected.*

results obtained by other methods. Pyrosequencing is also a good approach because it is flexible and easy to use. In the present study, it proved to be highly effective in detecting minority mutated alleles. A total of 41 prenatal diagnoses on maternal plasma were performed by the microchip analysis, with complete concordance with results obtained on fetal DNA extracted from chorionic villi and analyzed with standard procedures. Among these, 27/28 were also confirmed by direct sequencing and 4/4 by pyrosequencing (Table 2 and Figure 1). The results of two independent analyses perfomed by the microchip system did not agree only in one couple tested for the paternal Cd39. The first analysis indicated the presence of a fetal mutated allele in 3 out of 5 PNA-clamped amplified samples. The second analysis showed the absence of a mutated fetal allele in all the 5 PNA-clamped samples. Sequencing confirmed the absence of the mutated allele at all PNA concentrations, and the fetus was diagnosed as having inherited the wild-type paternal allele, in accordance with results on chorionic villi. This discrepant result could be ascribed to a contamination problem since in the same PCR session 25 ng of genomic DNA samples either heterozygous or homozygous for the Cd39 mutation had been amplified as controls. This amount of genomic DNA is much more abundant than plasma DNA and its amplification for 47 cycles may give rise to contamination. To overcome this problem, in all subsequent analyses only plasma positive control samples (father carrying the mutation under investigation) were included in the PCR session. No further contamination problems were observed. The correct identification of the fetal wild-type paternal allele in a couple where a previous fetus had been correctly identified as a carrier of the paternal mutated Lepore-Boston allele further confirms the complete clearance of fetal DNA from maternal plasma after delivery thus preventing diagnostic errors due to persistence of fetal DNA from previous pregnancies.[19] A key point of our strategy is the enrichment of minority mutated fetal alleles by PNA-clamping. The major drawback of this approach is the assessment of the optimal PNA concentration which needs to be established for each mutation and may be difficult to be applied for routine analysis and large scale clinical applications despite the potential clinical usefulness. For non-invasive prenatal diagnosis, each maternal plasma sample was tested for the optimal range of PNA concentrations specific for each mutation of interest (over the range shown in Table 1). Due to the variability of total and fetal DNA content in each maternal plasma sample, slight refinements of this scale were sometimes necessary. For each method (microelectronic microchips, direct sequencing and pyrosequencing), independent PCR reactions in the presence of the optimal PNA concentration specific for each mutation of interest (Table 1) were performed on DNA extracted from the same maternal plasma samples. The strategy we propose could provide the basis for developing additional assays to identify paternally inherited β-globin gene polymorphisms to also extend non-invasive diagnosis to couples where both parents carry the same mutation. Our data suggest that a wide range of easy accessible approaches could be used in combination with PNA clamping for non-invasive prenatal testing of a variety of genetic diseases, with a view to future application in clinical diagnostic settings. Non-invasive approaches could avoid the risk of fetal loss and the costs associated with invasive procedures such as chorionic villus sampling and amniocentesis in the 50% of couples for whom the fetus did not inherit the paternal mutated allele. The use of non-invasive approaches early in pregnancy would still allow the remaining couples time to undergo subsequent invasive procedures.

Authorship and Discosures

LC contributed to the design, conduction and analysis of the study and wrote the paper; SG contributed to the design, performed the research and collected data; GR, MT, CC, LS, FP and BF contributed to acquisition, analysis and interpretation of data; MF, MS, and AF critically reviewed the manuscript. The authors reported no potential conflicts of interest.

References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997; 350:485-7.
2. Ding C, Chiu RW, Lau TK, Leung TN, Chan LC, Chan AY, et al. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. Proc Natl Acad Sci USA 2004;101:10762-7.
3. Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA 2005;293:843-9.
4. Li Y, Page-Christianes GC, Gille J, Holzgreve W, Hahn S. Non-invasive prenatal detection of achondroplasia in size-fractionated cell-free DNA by MALDI-TOF MS assay. Prenat Diagn 2007;27:11-7.
5. Cremonesi L, Galbiati S, Foglieni B, Smid M, Gambini D, Ferrari A, et al. Feasibility study for a microchip-based approach for noninvasive prenatal diagnosis of genetic diseases. Ann N Y Acad Sci 2004;1022:105-12.
6. Galbiati S, Restagno G, Foglieni B, Bonalumi S, Travi M, Piga A, et al. Different approaches for noninvasive prenatal diagnosis of genetic diseases based on PNA-mediated enriched PCR. Ann N Y Acad Sci 2006;1075:137-43.
7. Foglieni B, Cremonesi L, Travi M, Ravani A, Giambona A, Rosatelli MC, et al. β-thalassemia microelectronic chip: a fast and accurate method for mutation detection. Clin Chem. 2004;50:73-9.
8. Ferrari F, Foglieni B, Arosio P, Camaschella C, Daraio F, Levi S, et al. Microelectronic DNA chip for hereditary hyperferritinemia cataract syndrome, a model for large-scale analysis of disorders of iron metabolism.Hum Mutat 2006; 27:201-8.
9. Santacroce R, Ratti A, Caroli F, Foglieni B, Ferrarsi A, Cremonesi L, et al. Analysis of clinically relevant single-nucleotide polymorphisms by use of microelectronic array technology. Clin Chem 2002;48:2124-30.
10. Cremonesi L, Ferrari M, Giordano PC, Harteveld CL, Kleanthous M, Papasavva T, et al. An overview of current microarray-based human globin gene mutation detection methods. Hemoglobin 2007;31:289-311.
11. Stenirri S, Restagno G, Ferrero GB, Alaimo G, Sbaiz L, Mari C, Genitori L, Ferrari M, Cremonesi L. Integrated strategy for fast and automated molecular characterization of genes involved in craniosynostosis. Clin Chem. 2007 Aug 10; [Epub ahead of print]
12. Nielsen PE, Egholm M, Buchardt O. Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone. Bioconjug Chem 1994;5:3-7.
13. Orum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley C. Single base pair mutation analysis by PNA directed PCR clamping. Nucleic Acids Res 1993; 21:5332-6.
14. Fakhrai-Rad H, Pourmand N, Ronaghi M. Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. Hum Mutat 2002;19:479-85.
15. Ahmadian A, Ehn M, Hober S. Pyrosequencing: history, biochemistry and future. Clin Chim Acta 2006;363:83-94.
16. Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM, Lo YM. Effects of blood processing protocols on fetal and total DNA quantification in maternal plasma. Clin Chem 2001; 47:6107-13.
17. Hancock DK, Schwarz FP, Song F, Wong LJ, Levin BC. Design and use of a peptide nucleic acid for detection of the heteroplasmic low-frequency mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) mutation in human mitochondrial DNA. Clin Chem 2002;482:2155-63.
18. Camaschella C, Alfarano A, Gottardi E, Travi M, Primignani P, Caligaris Cappio F, et al. Prenatal diagnosis of fetal hemoglobin Lepore-Boston disease on maternal peripheral blood. Blood 1990;75:2102-6.
19. Smid M, Galbiati S, Vassallo A, Gambini D, Ferrari A, Viora E, et al. No evidence of fetal DNA persistence in maternal plasma after pregnancy. Hum Genet 2003;112:617-8.