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Short communication

Noninvasive fetal genotyping of human platelet antigen-1a

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We describe a reliable noninvasive fetal human platelet antigen (HPA)-1a genotyping assay on a real-time polymerase chain reaction (PCR) platform using cell-free fetal DNA isolated from maternal blood. Nonspecific amplification of maternal cell-free DNA is overcome by pre-PCR digestion of the cell-free DNA with the *Msp1* restriction enzyme. Noninvasive fetal HPA-1a genotyping offers a safe method for alloimmunised pregnant women to determine whether their fetus is at risk of fetal or neonatal alloimmune thrombocytopenia (FNAIT) and whether interventions to prevent intracranial haemorrhage are required. The availability of this test is relevant to the ongoing debate on screening pregnancies for HPA-1a-mediated FNAIT.

Keywords: Alloimmune thrombocytopenia, cell-free DNA, fetal or neonatal alloimmune thrombocytopenia, human platelet antigen-1a, maternal plasma, noninvasive prenatal diagnosis.

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Introduction

Fetal or neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies directed against paternally inherited antigens present on fetal platelets. In 80% of cases of FNAIT, antibodies are directed against the human platelet antigen (HPA)-1a.[1] FNAIT complicates about 0.1% of all pregnancies and is associated with major fetal and neonatal morbidity and mortality. The most feared complication is an intracranial haemorrhage (ICH), occurring in approximately 10–20% of cases. The majority of these bleedings occurs *in utero*. Antenatal management of FNAIT, ultimately aimed at preventing ICH, may consist of weekly administration of intravenous immunoglobulins, with or without corticosteroids, to the mother, or fetal blood sampling with platelet transfusions.[2] In most centres, elective caesarean section is the preferred mode of delivery in pregnancies at risk of FNAIT. In the absence of screening programmes, the disease is only diagnosed after the birth of a symptomatic neonate, that is, fetal or neonatal bleeding, or occasionally by chance in the case of neonatal blood tests for other reasons. Consequently, antenatal treatment is currently provided only in pregnancies subsequent to the birth of a previously affected child.[3]

In HPA-1a-alloimmunised pregnant women, knowledge of the fetal HPA-1a status determines the pregnancy management. Only if the fetus is HPA-1a positive is there a risk of FNAIT, and the pregnancy is managed as such. In about 30% of cases, there is a 50% chance that the fetus will inherit a paternal HPA-1b allele, and there is no risk of FNAIT. Currently, when the father is heterozygously HPA-1a/1b positive, the fetal HPA genotype is determined by amniocentesis. This invasive procedure carries a small risk of miscarriage and can potentially cause the boosting of antibody formation.

In 1997, Lo et al.[4] reported the presence of cell-free fetal DNA in maternal plasma and serum, and its implications for noninvasive prenatal diagnosis. Since then, polymerase chain reaction (PCR)-based noninvasive genotyping of paternally inherited fetal alleles not present in the maternal genome, for example, *RHD* genotyping in D-negative mothers and the detection of Y chromosome-specific sequences for fetal sexing, has found its way into clinical practice.[5] However, testing for the presence or absence of

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fetal alleles that only differed slightly from their maternal counterpart, for example, only one nucleotide, appeared to be more difficult and, for certain alleles, remained severely hampered by nonspecific amplification of the overwhelmingly present maternal cell-free DNA. For this reason, a reliable noninvasive fetal HPA-1a genotyping assay has not yet been described.

In this communication, we report a noninvasive fetal HPA-1a genotyping assay in which the nonspecific amplification of maternal cell-free DNA is overcome by pre-PCR digestion of the cell-free DNA with the *Msp1* restriction enzyme. This enzyme specifically recognises the maternal HPA-1b allele DNA sequence, whilst leaving the fetal HPA-1a allele DNA sequence intact (Figure S1).

Methods

Sample collection

Frozen plasma and serum samples from pregnant women with anti-HPA-1a antibodies were collected at the Leiden University Medical Centre (national referral hospital for pregnancies at risk of FNAIT) and Sanquin Diagnostic Services (national reference laboratory) under Medical Ethics Committee approval. All blood samples had been drawn from women who had undergone a previously affected pregnancy and for whom amniocentesis had been performed in the index pregnancy to determine the fetal HPA-1a status. Informed consent was obtained in all cases.

DNA extraction and *Msp1* digestion

DNA was extracted from 1 ml of plasma or serum with the QIAamp DSP Virus kit (QIAGEN Inc., Hilden, Germany) using a modified spin-column method. Briefly, 1 ml of lysis buffer was added to 100 μl of protease and 1 ml of plasma or serum and incubated at 56°C for 20 minutes. After mixing with ethanol (96%), the mixture was applied to the QIAamp column and centrifuged at 6000 × *g* for 1 minute. The columns were washed twice and incubated with an open lid at 56°C for 5 minutes. DNA was eluted with 60 μl of water. Forty-four microlitres of extracted DNA were digested with 20 units of the *Msp1* restriction enzyme (New England BioLabs Inc., Ipswich, MA, USA) at 37°C for 2 hours, during which time the HPA-1b allele was digested, followed by 20 minutes of enzyme heat inactivation at 80°C. Genomic DNA from an HPA-1b/1b donor (5 ng) and from an HPA-1a/1b donor (500 pg) was digested at the same time to serve as negative and positive controls, respectively.

PCR analysis

Real-time PCR analysis was performed with the StepOne-Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Taqman chemistry. DNA was analysed for HPA-1a using an allele-specific reverse primer. To reduce primer annealing to the (remaining) nontarget HPA-1b allele, the third nucleotide at the 3′ end of the allele-specific primer was mismatched. Primer and probe sequences were as follows: forward primer, 5′-GTAGAGA GTCGCCATAGCTCTGATT-3′; reverse primer (allele-specific), 5′-CACAGCGAGGTGAGCACA-3′; probe, 5′-FAM-CCTGTAAGACAGGAGCCCAAAGAGAAGTC-TAMRA-3′. Part of the *albumin* gene was amplified as a control for DNA isolation.[5]

The singleplex reactions were set up in a volume of 25 μl, using 12.5 μl of Taqman Universal PCR Master Mix (Applied Biosystems) and 10 μl of extracted DNA (3 μl for the *albumin* PCR). Primers and probes were used at final concentrations of 300 and 100 nmol/l. Cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of denaturation for 15 seconds at 95°C and primer annealing and elongation for 1 minute at 60°C.

DNA was analysed for HPA-1a in triplicate. Test results were considered to be valid only if amplification with the digested DNA from the HPA-1b/1b control donor was not observed. All test results were compared with the genotyping results of amniotic fluid cells.

Results

A total of 34 samples was collected (plasma: n = 28; serum: n = 6). Twenty-eight women carried an HPA-1a-positive fetus and six women carried an HPA-1a-negative fetus. The median gestational age at the time of blood sampling was 32 weeks (range, 12–39 weeks). All 28 samples from women carrying an HPA-1a-positive fetus showed positive PCR results in three of the three replicates, with a mean cycle threshold value (C_t) of 34.0 (±2.2 SD) (Figure 1). In the six samples from women carrying an HPA-1a-nega-

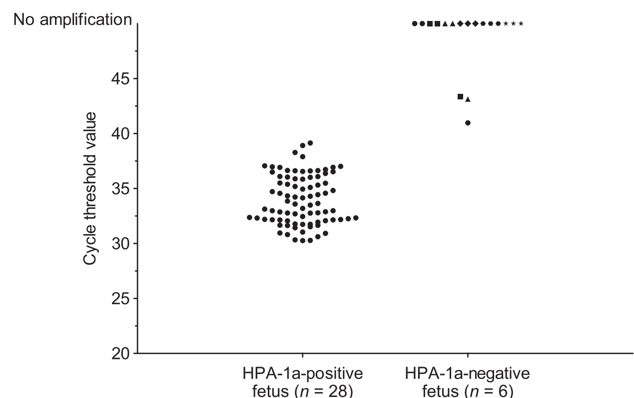

Figure 1. Cycle threshold (C_t) values for human platelet antigen (HPA)-1a-positive and HPA-1a-negative fetuses. The C_t values of all three replicates per sample are shown. The different symbols for the HPA-1a-negative fetuses represent the six different cases.

tive fetus, no amplification ($n = 3$) or amplification in one of the three replicates ($n = 3$) was observed (C_t values of 41.0, 43.1 and 43.4, respectively).

Discussion

We have developed a reliable noninvasive fetal HPA-1a genotyping assay on a real-time PCR platform. False-positive results caused by nonspecific amplification of maternal cell-free DNA were completely excluded by sequence-specific enzyme digestion. At the same time, the sensitivity of the assay was retained, as no false-negative results were observed.

The HPA-1a/b polymorphism results from a single nucleotide change (196T>C) in the *ITGB3* gene, leading to a phenotypic leucine to proline amino acid change in the glycoprotein. This subtle difference in DNA sequence between the HPA-1a and HPA-1b alleles has thus far prevented the development of a reliable fetal HPA-1a genotyping assay using cell-free fetal DNA isolated from maternal blood. As cell-free fetal DNA represents only a small fraction (3–10%) of the *total* cell-free DNA in the maternal blood, the maternally derived cell-free DNA molecules greatly outnumber the fetal derived cell-free DNA molecules. Previous attempts to develop a reliable noninvasive fetal HPA-1a genotyping assay in our laboratory were hampered by mispriming of the HPA-1a-specific primer to the overwhelmingly present maternal HPA-1b allele, resulting in false-positive or inconclusive test results (data not shown). The use of extra mismatches in the HPA-1a-specific primer, the incorporation of locked nucleic acids in the HPA-1a-specific primer and the use of a peptide nucleic acid probe (blocking the HPA-1b allele) all resulted in invalid assays.

The thymine to cytosine substitution (T>C) in the HPA-1b allele creates a DNA sequence recognition site (5′-CCGG-3′) for the *Msp1* restriction enzyme (Figure S1). Therefore, pre-PCR processing of the cell-free DNA with *Msp1* will digest the maternally derived HPA-1b alleles, whilst leaving the eventually present fetal HPA-1a alleles unimpaired. Indeed, positive PCR results were obtained in all replicates of the samples from women carrying an HPA-1a-positive fetus, whereas no amplification signals were observed in 15 of the 18 replicates of the six samples from women carrying an HPA-1a-negative fetus (Figure 1). In the three other replicates, C_t values of more than 41 were observed, pointing to nonspecific amplification of the remaining HPA-1b molecules. These single replicates could clearly be distinguished from true-positive amplification (three of three replicates with C_t values of <39), and thus did not influence correct assignment.

In our study, blood samples ranging from as early as 12 weeks of gestation to the late third trimester were tested. Although we have shown that fetal HPA-1a genotyping using cell-free fetal DNA isolated from maternal blood can be performed in the first trimester, the potential risk of false-negative results caused by low fetal DNA concentrations can be avoided by performing the test later in pregnancy (e.g. early in the second trimester), as the amount of cell-free fetal DNA in the maternal circulation gradually increases during pregnancy.

Noninvasive fetal HPA-1a genotyping offers a safe and relatively easy method for HPA-1a-alloimmunised pregnant women with a heterozygous partner to determine whether or not their fetus is at risk of FNAIT and whether antenatal interventions to prevent ICH are required. It allows for the determination of the fetal HPA-1a status without exposing the mother or fetus to the risks associated with invasive procedures, which are currently performed in up to 30% of alloimmunised pregnancies because of a heterozygous father. In view of the current focus on the noninvasive antenatal management of FNAIT, in which fetal blood sampling and intrauterine platelet transfusions are minimised or eliminated completely,[2] a noninvasive test to establish the fetal risk is clearly called for. A knowledge of the fetal HPA-1a status may further contribute to the avoidance of unnecessary caesarean sections in HPA-1a-alloimmunised pregnant women with a heterozygous partner.

Noninvasive fetal HPA-1a genotyping could also play an important role in FNAIT screening programmes. FNAIT is mainly caused by HPA-1a antibodies and, in the European population, the prevalence of HPA-1a negativity is about 2%.[3] In analogy with red cell antibody screening programmes, screening pregnancies for HPA-1a-mediated FNAIT has been advocated by several groups.[3,6] Drawbacks to its introduction have been the lack of consensus on how to identify the pregnancies at risk and on the optimal antenatal management of screen-positive cases. Nonetheless, in such an FNAIT screening setting, noninvasive fetal HPA-1a genotyping would help to optimise the selection of pregnancies at risk without the need for paternal zygosity testing or invasive procedures to determine the fetal HPA-1a status.

The assay described in this communication is applicable only to pregnancies complicated by alloimmunisation to HPA-1a, involving 80% of FNAIT cases. Noninvasive assays to determine the fetal status of other clinically relevant, but rarer, HPAs, such as HPA-5b or HPA-15b, have not yet been developed.

Conclusions

We have developed a reliable noninvasive fetal HPA-1a genotyping assay that offers a safe method for HPA-1a-alloimmunised pregnant women to determine whether their fetus is at risk of FNAIT and whether antenatal treatment

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should be initiated. The availability of this test adds an important perspective to the ongoing debate on whether or not to screen pregnancies for HPA-1a-mediated FNAIT.

Disclosure of interests

We declare that we have no conflicts of interest.

Contribution to authorship

All authors were involved in the design of the study. PGS, AAS and OJHMV performed the experiments and analysed the data. DO and GCMLP-C provided patient samples and critically revised the manuscript for intellectual content. MdH and CEvdS supervised data discussion and critically revised the manuscript for intellectual content. PGS wrote the manuscript. The final version of the manuscript was approved by all authors.

Details of ethics approval

This study was approved by the Medical Ethics Committee of the Leiden University Medical Centre (P04.203).

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Supporting information

The following supplementary materials are available for this article:

Figure S1. *Msp1* digestion.

Additional Supporting Information may be found in the online version of this article.

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References

1 Knight M, Pierce M, Allen D, Kurinczuk JJ, Spark P, Roberts DJ, et al. The incidence and outcomes of fetomaternal alloimmune thrombocytopenia: a UK national study using three data sources. *Br J Haematol* 2011;152:460–8.

2 Mechoulan A, Kaplan C, Muller JY, Branger B, Philippe HJ, Oury JF, et al. Fetal alloimmune thrombocytopenia: is less invasive antenatal management safe? *J Matern Fetal Neonatal Med* 2011;24:564–7.

3 Kamphuis MM, Paridaans N, Porcelijn L, De Haas M, Van Der Schoot CE, Brand A, et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG* 2010;117:1335–43.

4 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.

5 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, Bossers B, van Erp F, de Haas M. Reliability of fetal sex determination using maternal plasma. *Obstet Gynecol* 2010;115:117–26.

6 Tiller H, Killie MK, Skogen B, Oian P, Husebekk A. Neonatal alloimmune thrombocytopenia in Norway: poor detection rate with non screening versus a general screening programme. *BJOG* 2009;116:594–8.