Non-invasive first-trimester detection of paternal beta-globin gene mutations and polymorphisms as predictors of thalassemia risk at chorionic villous sampling

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Abstract

Objective: The objective was to evaluate the beta-globin gene mutations and polymorphisms in cell-free fetal DNA in the early first trimester (7–9th weeks' gestation) for the prediction of thalassemia risk at chorionic villous sampling (CVS).

Study design: Beta-globin gene mutations and polymorphisms were analyzed in 97 carrier families and 100 control couples. Using conventional PCR-DGGE we carried out cell-free fetal DNA analysis in 37 couples in whom only the father was an *IVSI-110* carrier.

Results: Beta-globin gene mutations have 80% information content in contrast to 39% of polymorphisms. By non-invasive early first-trimester identification of the paternally transmitted *IVSI-110* mutation, we reached a sensitivity and specificity of 96 and 100%, respectively. Although the detection rate of the Y chromosome in male fetuses was as high as 100%, beta-thalassemia allele drop-out cannot be excluded.

Conclusions: Even though there is high sensitivity in non-invasive paternally transmitted beta-thalassemia mutation detection, intense effort must be made to avoid misdiagnoses before the clinical application of this approach.

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1. Introduction

Beta-thalassemia is mostly caused by a spectrum of point mutations in the beta-globin gene with more than 170 different nucleotide substitutions described worldwide. The prenatal genetic diagnosis of fetuses affected by beta-thalassemia relies on invasive procedures such as chorionic villous sampling (CVS), with a small but finite risk of fetal loss and injury.

Intact fetal cells, isolated from maternal blood, have been used in a single-cell polymerase chain reaction for the non-invasive prenatal diagnosis (PND) of hemoglobinopathies [1,2]. The major limitations to applying these techniques clinically are the low number of fetal cells in maternal circulation during pregnancy (one fetal cell/ml of maternal blood) [3] and the complexity of the isolation process. In contrast, cell-free fetal DNA analysis has the advantage of being rapid and reliable. The non-invasive prenatal detection of a beta-globin gene mutation, using cell-free fetal DNA, has been reported by Chiu et al. [4]. Recently, the detection of four paternally inherited point mutations for beta-thalassemia, using size-fractionated cell-free DNA in maternal plasma, was also reported [5].

The chromosomal regions bearing the mutant alleles are in strong linkage disequilibrium with specific patterns of DNA restriction site polymorphisms [6]. These patterns, known as beta-globin gene haplotypes, are useful markers in the PND of beta-thalassemia [7]. In this study, we compare the information content of the haplotypes (RFLP) with mutations in beta-globin gene mutation carrier couples in order to assess the informativity of the available molecular markers for non-invasive prenatal detection of beta-thalassemia.

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The non-invasive prenatal detection of the paternal specific mutation or marker is used as a prognostic test to predict the diagnosis with invasive procedures such as amniocentesis or chorionic villous sampling, in order to evaluate the ability to prognose the risk of transmission in the first trimester.

2. Materials and methods

2.1. Materials

We studied a total of 97 couples (194 carriers) and their parents, carriers of beta-thalassemia mutations. Identification of beta-globin gene mutations and haplotypes was performed in these families during the weeks 7–9 of pregnancy. All the couples gave informed consent for invasive and non-invasive PND in accordance with the Helsinki declaration and the local ethics board directive. In addition, we identified the haplotypes in 100 control non-heterozygote couples, so as to determine differences in the haplotype versus mutation information content.

In 37 heterozygote couples, in which the father was the carrier of an *IVSI-110* mutation, maternal blood samples were collected in a Wasserman tube at the above-mentioned gestational age. The blood samples were processed 20 min after venipuncture and were centrifuged at 3000 rpm for 10 min. Serum was removed carefully and transferred into fresh polypropylene tubes. Then the samples were recentrifuged at 3000 rpm for 10 min and the serum samples were transferred into a fresh tube. Cell-free fetal DNA was extracted from 5-mL serum samples by the QIAamp DNA Blood Kit (Qiagen), according to the "blood and body fluid protocol," with minor modifications. The cell-free fetal DNA was used for non-invasive beta-globin gene mutation detection and screening for the paternal contribution.

2.2. Methods

2.2.1. Mutation analysis

The beta-globin gene mutations were amplified by polymerase chain reaction (PCR) and subsequently analyzed by denaturing gradient gel electrophoresis (DGGE) according to the protocol described by Losekoot et al. [8]. The reverse dot blot [9] and amplification refractory mutation system (ARMS) methods were used for the detection of the 11 most common mutations in Greece, based on a protocol previously described [10]. PCR products were separated by 2% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining.

2.2.2. Analysis of polymorphisms and haplotypes

The PCR technique was applied for the amplification of the seven most common beta-globin gene polymorphic restriction sites (RFLPs): AvaII/β, AvaII/ψβ, HindII/5′ψβ, HindII/3′ψβ, HindIII/Gγ, XmnI/Gγ, and HinfI/β. The PCR amplification was performed in a total volume of 25 μL consisting of 1 μL DNA, 0.08 mM dNTPs, 1.2 μM of each primer [11], 1× Taq DNA polymerase buffer, 1.5 mM MgCl$_2$, and 0.2 U of Taq DNA polymerase. The thermal cycling was as follows: denaturation at 93 °C for 1 min, 28 cycles of 93 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, with a final cycle of 93 °C for 1 min, 56 °C for 1 min, and 72 °C for 3 min. The amplified fragments, after restriction enzyme digestion, were separated by 2% agarose gel electrophoresis and visualized using ethidium bromide staining and ultraviolet light. Haplotypes were identified according to previous studies [11,12].

2.2.3. Non-invasive genetic analysis

For the amplification of the *IVSI-110* mutation in serum cell-free fetal DNA two-round PCR analysis was used. The first round of amplification was carried out using the Region B-specific sequence of the beta-globin [13]. The PCR amplification was performed in a total volume of 50 μL consisting of 10 μL serum extracted DNA, 0.2 mM dNTPs, 1 μM of each primer, 1× Ampli Taq Gold polymerase buffer, 3 mM MgCl$_2$, and 5 U of Ampli Taq Gold polymerase. In parental samples, we used 1 μL of the blood DNA and 2.5 U of Ampli Taq Gold polymerase. The thermal cycling was as follows: denaturation at 96 °C for 10 min, 40 cycles of 96 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 5 min. Following the first round, sequencing and a nested PCR reaction were carried out using 1-μL aliquot of the first PCR reaction. The conditions of this PCR and the subsequent DGGE analysis have been described before [8]. The only modification was the use of the Ampli Taq Gold polymerase. Samples were assigned according to the results in two groups, the 0% risk group and the 50% risk group.

Y-chromosome-specific sequences *DYS14* and *DYZ1* for the identification of the paternal contribution were employed. The primer sets Y1.7/Y1.8 and Y1.1/Y1.2 were used to amplify *DYS14* and *DYZ1*, respectively, according to a protocol previously described [14]. In brief, PCR amplification was performed in a total volume of 25 μL containing 5 μL of serum extracted DNA and 2.5 U of platinum Taq polymerase high fidelity.

3. Results

The percentage of each mutation was estimated from the beta-globin gene mutation analysis in the carrier group (194 individuals). *IVSI-110*, *Codon 39*, and *IVSI-1* were the most frequent mutations (33.5, 19, and 10.8%, respectively). 80% of the couples had different paternal and maternal mutations, which were informative for non-invasive screening of the paternal contribution.

The beta-globin gene haplotype analysis in the mutation carrier group and the control group, by PCR-RFLP analysis, revealed the percentage of the couples who presented at least

Table 1
Percentage of the couples with at least one informative polymorphism of the beta-globin gene

Number of informative polymorphisms	Mutation carrier couples (%)	Control couples (%)
1	28	9
2	5.5	10
3		3
4	5.5	6
5		1
Total percentage	39	29

one informative polymorphism (Table 1) that was linked to the mutation and that was different from the father and mother. Thirty-nine percent of the mutation carrier couples and 29% of the control couples had at least one informative polymorphism. HindIII/Gγ and HindII/3′ψβ were the most informative polymorphisms, in both groups, whereas AvaII/β and AvaII/ψβ presented 2–3 times higher information content in carrier couples compared with controls. The χ^2-test used to evaluate whether a significant difference existed between the patients and the control groups resulted in statistically non-significant P values. Although each mutation may be associated with one or more different haplotypes [6], one specific haplotype was found to be linked to every mutation in our series.

Mutation analysis, as expected, showed that *IVSI-110* is the most frequent mutation and probably the most informative for carrying out non-invasive PND. In 37 informative couples, in whom the paternal mutation *IVSI-110* was different from the maternal, the non-invasive detection of the mutation in maternal serum samples was performed (Fig. 1). Of the couples with this profile, we found that 25 of the 37 fetuses were carriers of the paternal mutation IVSI-110, using our non-invasive approach. At CVS we identified one more fetus carrier of the paternal *IVSI-110* mutation, resulting in sensitivity of 96% and specificity of 100% using our non-invasive approach (Table 2). The one missed paternal mutation detection was observed in 1 of 12 that were negative for the *IVSI-110* serum samples, collected in the 7th week of pregnancy, leading to a misdiagnosis rate of 8%, potentially due to the very low cell-free fetal DNA concentration. Nevertheless, the fetal gender was correctly determined in this sample by Y chromosome amplification. These results were verified in the 12th week of pregnancy by CVS and fetal DNA analysis in routine PND.

Among the 37 maternal serum samples examined, 18 were found to be positive for the Y chromosome. These
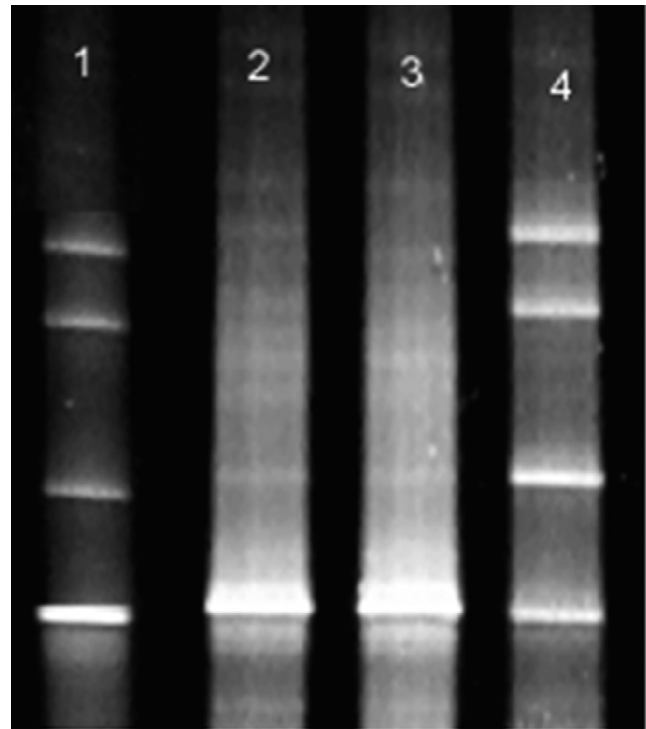

Fig. 1. Non-invasive detection of the *IVSI-110* beta-globin gene mutation (Fragment B)—sample 1: serum DNA sample (embryo *IVSI-110/N*; mother *IVSII-745/N*); sample 2: maternal DNA sample (*IVSII-745/N*); sample 3: normal DNA sample (*N/N*); sample 4: paternal DNA sample (*IVSI-110/N*).

results were also verified in the 12th week of pregnancy by CVS. Consequently, the detection rate of the paternal contribution to the Y chromosome was 100%.

4. Discussion

The majority of the couples presented with informative mutations, namely different paternal and maternal nucleotide substitutions. The information content of the mutations is double that found for the polymorphisms. Consequently, *IVSI-110* is a better marker for the non-invasive prenatal detection of the paternal contribution than linked polymorphisms and haplotypes.

As described before, the *IVSI-110* mutation represents one-third of the mutations found. The analysis of all informative couples with paternal *IVSI-110* mutation only provided very high sensitivity and specificity in beta-thalassemia prediction as early as the 7–9th weeks' gestation.

Table 2
Comparison of invasive (chorionic villous sampling—CVS) versus non-invasive (maternal serum) procedures of prenatal diagnosis

	Number of fetuses	Fetal carriers of the IVSI-110 mutation (50% risk group)	Fetal non-carriers of the IVSI-110 mutation (0% risk group)	
Non-invasive procedures (maternal serum analysis)	37	25	12	Sensitivity 96% specificity 100%
Invasive procedures (CVS analysis)	37	26	11	Allele drop-out 8%

Unfortunately, by this approach, misdiagnosis cannot be excluded in the negative *IVSI-110* tested cases, although all fetuses that were found to be male were verified in the 12th week of pregnancy at CVS. In addition, the lack of informative polymorphisms linked to the *IVSI-110* mutation in our population seems to diminish the prospect of avoiding invasive prenatal diagnosis at the present time.

Recently, the detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma has been reported [5]. The gestational ages ranged from 10 to 12 weeks, with a detection rate of 81–100%. Our serum samples were collected in the 7–9th weeks of gestation, without size fractionation, due to the very low fetal DNA concentration in the 7–9th weeks [15]. Even though in early pregnancy only 0.4% (range 0–5.2%) of the total fetal DNA has a size of 0.5–1 kb [16], our protocol permitted the amplification of a 637-bp fetal DNA by performing re-amplification. These conditions may have affected the sensitivity and raised the possibility of misdiagnosis in one of the fetuses that was positive for the mutation.

If the parents are carriers of different mutations or polymorphisms linked to mutations and the paternal mutation is present in the maternal serum, then the probability of the fetus having thalassemia is 50%. Therefore, it is necessary to use invasive procedures, like CVS and amniocentesis, for the fetal genotype analysis. Consequently, the absence of the paternal mutation may reduce this probability to 0%, making invasive techniques unnecessary. Although this is theoretically correct, in practice, according to our results and to those of others, the low quantity of DNA, which is also prone to allele drop-out, might affect the sensitivity of the PCR reactions, rendering this approach inapplicable for the time being.

In conclusion, our preliminary results show that mutations constitute a better marker for the non-invasive prenatal detection of beta-thalassemia, compared with linked polymorphic markers and haplotypes. The applicability of our approach, as early as the 7th week of pregnancy, using conventional PCR for the detection of *IVSI-110*, is limited to at-risk couples with different genotypes in the male and the female partners. Nevertheless, additional studies focusing on increasing sensitivity and eliminating allele drop-out of mutations should be done before any attempt at replacing invasive approaches. This accomplishment would be potentially useful for the future clinical application of a combined non-invasive and invasive approach to beta-thalassemia.

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