Consultations in Molecular Diagnostics

Prothrombin Gene Variants in Non-Caucasians with Fetal Loss and Intrauterine Growth Retardation

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Thrombotic predisposition may affect pregnancy outcome, but in non-Caucasians the contributing genetic factors are poorly characterized. Two recently identified prothrombin gene mutations (20209C>T and 20221C>T) have been observed in non-Caucasian patients with thrombosis. The mutations are located near the commonly identified variant 20210G>A and have not been reported in Caucasian patients. The authors report a novel connection with pregnancy complications. The identification of sequence variants other than 20210G>A in the 3'-untranslated region of the prothrombin gene suggests that additional nucleotide substitutions may contribute to the development of thrombotic events and adverse pregnancy outcomes, especially in less well-characterized populations. (*J Mol Diagn* 2003, 5:250–253)

Thrombophilia has been associated with a wide range of clinical manifestations including deep vein thrombosis and maternal or fetal morbidity and mortality. Genetic risk factors for thrombosis such as factor V Leiden, prothrombin 20210G>A, and 677C>T in the MTHFR gene have been reported to contribute to pregnancy complications including intrauterine growth retardation (IUGR), idiopathic pregnancy loss, and late intrauterine fetal demise.^{1–4} All three genetic variants are common in Caucasians, but less prevalent in other ethnic groups. In fact, genetic risk factors for both thrombosis and obstetric complications are poorly defined in non-Caucasian populations.

Two novel prothrombin mutations have recently been identified in Middle-Eastern and African-American patients with thrombotic events.^{5,6} We present the case histories of three non-Caucasian patients who had obstetrical complications including recurrent spontaneous

abortions, IUGR, and neonatal demise. The three patients and three of four neonates were studied for the presence of factor V Leiden (1691G>A), the prothrombin gene variant 20210G>A, and the thermolabile MTHFR variant (677C>T). DNA was analyzed by real-time PCR and melting curve analysis on the Roche LightCycler (Roche, Indianapolis, IN).⁷ After atypical probe dissociation curves were noted, direct DNA sequencing demonstrated transitions in the direct vicinity of the commonly identified prothrombin variant (20210G>A), at position 20209 (C>T) and 20221 (C>T).⁸ These changes correspond to those identified in the other reports^{5,6} and point to a possible additional relation to adverse pregnancy outcomes. Although these mutations have been identified in only a few patients thus far, our report provides further support for a potential contribution to a thrombophilic phenotype. The identified changes may influence mRNA processing, stability, and/or the process of translation similar to 20210G>A. As neither mutation has been identified in Caucasians, population-based studies of non-Caucasian individuals are warranted to establish the true prevalence and clinical significance.

Cases

Case 1

Patient 1 is a 40-year-old South Asian female with a family history of thrombosis and a poor obstetric history. The patient's mother had a spontaneous deep venous leg thrombosis (DVT) and a DVT in her maternal grandmother led to a massive fatal pulmonary embolus. This DVT, however, occurred after a fracture. The patient had late pregnancy losses at 15 and 20 weeks gestational age, and a term pregnancy complicated by mild pre-eclampsia. She was delivered by cesarean section for failure to progress in labor. The fourth (index) pregnancy was complicated by first and second trimester bleeding, intrauterine growth restriction (IUGR) and absent end diastolic

Accepted for publication June 19, 2003.

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flow in the umbilical vessels at 35 weeks. Before delivery, the patient was hypertensive, but not diagnosed with pre-eclampsia. She delivered a 4 pound male (<10th percentile for gestational age) with Apgar scores of 8 and 9. The newborn was admitted to the neonatal intensive care unit for IUGR and prematurity. His neonatal course was complicated by hyperbilirubinemia, feeding difficulties, and hypospadias. He was discharged after 18 days.

Case 2

Patient 2 is a 26-year-old African-American female with no family history of thrombosis. The patient's mother, however, had two pregnancy losses at 16 and 20 weeks. The patient had one first trimester miscarriage followed by the conception of spontaneous dichorionic, diamniotic twins. At 14 weeks gestation, growth discordance was first noted and growth restriction of baby A was confirmed by ultrasound evaluation at 19 weeks. At 23 weeks the growth of baby A was <10th percentile for gestational age, whereas baby B was at the 30th percentile. The patient declined amniocentesis. Fetal echocardiograms were normal. By 25 weeks, both fetuses demonstrated growth restriction (A <10th percentile, B <12th percentile). The patient underwent cesarean section at 27 weeks for severe fetal heart rate decelerations in baby A. At delivery, baby A weighed 421 g (15 ounces) with Apgar scores of 1 and 5, and baby B weighed 582 g (1 pound, 4 ounces) with Apgars of 2 and 8. Both birth weights were less than the 10th percentile for gestational age. Placental pathology revealed small necrotic lesions comprising less than 10% of the placental volume for twin A. The placenta of baby B was normal on pathological examination. The weight of the combined placentas was 525 g.

The hospital course of baby A was complicated by a grade I intraventricular hemorrhage (IVH), sepsis, spontaneous bowel perforation, diffuse intravascular coagulation (DIC), and pulmonary hemorrhage leading to cardiac decompensation requiring CPR. Due to these complications, support was withdrawn and the infant expired at approximately 1 month of age. Baby B's hospital course was complicated by grade IV IVH, respiratory distress syndrome, coagulopathy, necrotizing enterocolitis, anemia, and thrombocytopenia requiring blood transfusions and platelet transfusions. Due to the poor neurological prognosis and severe cardiopulmonary compromise secondary to pneumonia, support was withdrawn at 2 months of age.

Case 3

Patient 3 is a 22-year-old African-American G7P1 female, who was 39 weeks pregnant at the time of the study. The pregnancy was uneventful and a healthy infant was delivered at 41 weeks gestational age. During her first term pregnancy, the patient was hospitalized for several weeks due to intractable emesis in the first trimester. She delivered a healthy male infant at a gestational age of 42 weeks. This patient's medical history is remarkable for five spontaneous first trimester abortions of unknown

cause, confirmed by ultrasound and dilatation and curettage. The patient's mother (G4P2) had two spontaneous abortions at 3 to 4 months gestation, whereas the maternal grandmother had one spontaneous abortion in the first trimester, and four live births. There is no family history of venous thromboembolism.

Discussion

Nucleotide change 20210G>A in the prothrombin gene was first described by Poort et al.,⁹ who identified this genotype in 18% of patients with a documented family history of venous thrombosis, but in only 1% of healthy controls. The vast majority of heterozygous carriers have prothrombin levels in the highest quartile. Only two additional point mutations have been described in the 3'-UTR of this gene. The first is a C > T transition at position 20221 in three family members of Lebanese/Syrian extraction. The 9-year-old proband experienced acute vascular rejection of an allogeneic renal transplant with segmental intrarenal arterial thrombosis.⁵ The second point mutation is a recently reported C>T transition at position 20209, identified in four African-American individuals. Three had a history of venous thrombosis or stroke, whereas the fourth had hepatic cirrhosis.⁶

We identified atypical prothrombin probe dissociation curves in three non-Caucasian patients (Figure 1). To identify the exact nucleotide change, we designed PCR and sequencing primers (5'-ACTCATATTCTGGGCTC-CTGC-3' and 5'-CTGGCTCTTCCTGAGCC-3' for the forward and reverse direction, respectively) and characterized the nucleotide composition of the area in question. Patient 1 was heterozygous for sequence variant 20221C>T (Figure 2a), 11 nucleotides downstream of the common prothrombin mutation. This sequence variant was also present in DNA from her newborn. In patient 2, the wild-type C was replaced by a T at nucleotide position 20209 (Figure 2b) (GenBank Accession No. NM000506). The same sequence change was identified in baby B, whereas baby A had inherited the wild-type allele. Patient 3 carried the same sequence change as patient 2. All sequences were confirmed by direct DNA sequencing in the opposite direction. Coagulation studies demonstrated high levels of factor II in all patients (Table 1). Other coagulation studies were unremarkable except for protein S levels in both patients 1 and 3. Reduced levels of protein S, however, are common during pregnancy.

We cannot exclude that the combination of high prothrombin levels, a mutation in the 3' end of prothrombin, and pathology such as the intrarenal thrombosis in the proband from Lebanon,⁵ the vascular disease in the African-American patients,⁶ and the obstetric complications of the patients in our study, have occurred by chance. A recent study of polymorphisms MTHFR 677C>T, factor V Leiden 1691G>A, and prothrombin 20210G>A revealed no support of a causal link between the common maternal or fetal thrombophilia risk factors and an increased risk of IUGR.^{10,11} Previously reported associations between pregnancy complications and

Figure 1. LightCycler analysis. **a:** The melting profile of a heterozygous control (20210G>A, shown in **blue**) reveals one peak at the typical melting temperature for the wild-type sequence (58°C), and one that corresponds to presence of the mutation, which decreases the melting temperature to 49°C. The atypical melting curve of the sample from patient 1 (shown in **green**) is superimposed onto this curve and demonstrates one wild-type melting temperature (58°C) and one unknown allele at 53°C. **b:** The melting curve of the heterozygous control (shown in **red**) demonstrates peaks at 58°C and 49°C, as expected for the 20210G and 20210A alleles, respectively. The melting curve of patient 2 (superimposed in **pink**), however, exhibits probe-melting from the wild-type allele at 58°C, and another, unknown allele, which results in a melting temperature of 53°C. The melting pattern of patient 3 is identical to that of patient 2.

these polymorphic sites may be explained by lower statistical power of earlier studies, but may also be due to different inclusion criteria. The 44 newborns investigated by Kupfermanc et al¹² had a mean birth weight of 1387 g and the majority of these infants (64%) were delivered before 36 weeks of gestation. The mean birth weight reported by Infante-Rivard et al,¹⁰ on the other hand, was higher (2393.5 g) and 83% of these infants were born between 36 and 40 weeks of gestation.¹³ These differences may have affected final interpretations. Only a

Figure 2. Direct DNA sequencing. **a:** This segment of the reverse prothrombin gene sequence from patient 1 includes nucleotide position 20221, at which both the wild-type G as well as a second peak are present. The additional peak corresponds to an A on the other allele, and demonstrates G/A heterozygosity (**large arrow**). The **small arrow** identifies position 20210, at which only the anti-sense consensus sequence is seen. **b:** Sequence analysis of patient 2 in the forward direction shows two closely overlapping peaks at prothrombin nucleotide position 20209 (**large arrow**). The ABI software, unable to interpret the true nature of the sequence, indicates this with an N. The position adjacent to the new mutation, however, is a G, which reflects homozygosity for the wild-type sequence at position 20210 (**small arrow**).

large, prospective study with uniform inclusion criteria at all participating institutions will definitively solve this quandary for the commonly studied thrombophilia risk factors.

In favor of a pathogenic role of the two sequence variants in our patients is the fact that they occur in the direct vicinity of prothrombin variant 20210G>A, which is located at the last nucleotide of the 3'-UTR. Given its location in a non-coding region, the pathological basis remained unclear until the identification of a new genetic mechanism of increased mRNA 3' end formation efficiency that results in a gain-of-function with elevated prothrombin plasma concentrations.¹⁴ Nucleotide changes other than 20210G>A within the same gene segment could influence mRNA processing and stability in ways similar to variant 20210G>A.

The atypical prothrombin melting curves have been observed in only three of approximately 900 prothrombin studies on the LightCycler performed in this laboratory to date. Ethnic background is often not provided, but most of our patients are Caucasian and the two new sequence

Table 1. Coagulation Studies of Three Patients with Prothrombin Variants

	Patient 1	Patient 2	Patient 3	Reference range
Factor II	134%	144%	130%	72–136%
Lupus anticoagulant	negative	negative	np	negative
Anticardiolipin antibody				
IgG	3	7	9	<23
IgM	4	6	7	<11
Antithrombin III	81%	93%	np	82–124%
Protein S	25%	83%	48%	61–140%
Protein C	112%	157%	111%	78–168%

np, not performed.

variants may be much more prevalent in other ethnic groups. Larger and population-specific studies of non-Caucasian individuals will be necessary to establish the true prevalence of the 20209C>T and 20221C>T mutations, and their obstetric consequences.

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