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Protease-activated-receptor 1 polymorphisms correlate with risk for unexplained recurrent pregnancy loss: a pilot study querying an association beyond coagulation

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ABSTRACT

Background: Recurrent pregnancy loss (RPL) has been associated with impaired maternal–fetal communication. Protease-activated-receptor 1 (PAR1) is critical for trophoblast invasion and establishment unrelated to its role in vascular biology.
Objectives: To analyze whether polymorphisms of PAR1 [-1426C/T], [-506I/D], and/or IVS[-14A/T] are associated with unexplained RPL.
Patients/methods: A case-control pilot study conducted in 39 healthy women with history of unexplained RPL and 98 women with a full-term, uncomplicated deliveries and no history of RPL.
Results: Women with RPL were significantly more likely to be heterozygous for [-1426C/T] (12.8% *versus* 3.2%; $p = 0.049$); the heterozygous state for IVS[-14A/T] was also more common (15.4% *versus* 4.4%; $p = 0.064$). There was no difference between groups for [-506I/D] genotypes. The functional consequence for [-1426C/T] and IVS[-14A/T] polymorphisms is underscored by the markedly low PAR1 mRNA levels in those women. Bioinformatics indicate generation of a new consensus motif for repressor Kruppel-like factor 3 (KLF3) in [-1426T]. Moreover, chromatin immunoprecipitation (ChIP) analysis confirmed a physical association between KLF3 protein and the hPar1 DNA obtained from women with the [-1426C/T] polymorphism.
Conclusions: We hypothesize that the significantly low PAR1 levels impact placenta establishment and consequently pregnancy outcome, thereby profiling a novel risk factor for unexplained RPL.

Introduction

Early pregnancy loss is an unresolved but critical issue in obstetrical care [1]. It has been proposed that women experiencing recurrent pregnancy loss (RPL) may have blood clotting disorders and thrombophilias [2]. Microthrombi are common in placental vasculature of women with RPL in association with certain thrombophilic defects [3]. Nevertheless, adverse pregnancy outcomes can occur also in women in the absence of placental thrombosis [4], suggesting pathophysiological pathways other than coagulation disorders. Moreover, it has been shown in a genetically engineered thrombomodulin gene knock-out mouse model that in the non-viable embryos that were generated, there was no evidence of thrombi among the placental defects [5]. Hence, a putative role for thrombomodulin in the maintenance of pregnancy is hypothesized, discrete from its function in coagulation. In addition, the formation of the placental matrix plate involves deposition of fibrin into decidual veins at the sites of trophoblast invasion [4,6]. Indeed, the proteolytic system of urokinase-plasminogen-activator (uPA) and its receptor (uPAR) with the plasminogen activator inhibitor 1 (PAI-1) play a role in proper placenta implantation process [7]. A PAI-1polymorphism 4G/5G has been associated with reduced fibrinolytic activity and RPL [8,9]. This is attributed to impaired uPA-R-PAI-1 function in proteolysis/remodeling of maternal tissue during placenta formation [10]. Protease-activated-receptor 1 (PAR1) plays a role in hemostasis, vascular biology, and epithelia malignancies [11,12].

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During the physiological invasion process PAR1 acts early in throphoblast invasion mediating anchoring of placenta villi into the uterine wall and remodeling of the extracellular microenvironment [13,14]. While initially PAR1 was identified as the cell sensor for thrombin, the main effector protease of the coagulation cascade, a wide spectrum of proteases has recently been defined as PAR1 activators. Among these are plasmin and the zinc-dependent matrix metalloprotease 1 (MMP-1), also known as collagenase. Infact, MMP-1 activation of PAR1 provides a direct mechanistic link connecting PAR1 to the tumor-microenvironment [15].

In the present pilot study, we chose to examine known polymorphisms described in human PAR1 (hPar1) and their association with unexplained RPL. Three genetic polymorphisms have been identified in the PAR1 gene: two in the 5′ regulatory region; [-1426C/T] and [-506 I/D], and the third, IVS [-14 A/T], occurs at the intervening sequence upstream of the exon II coding region. These polymorphisms have been linked with decreased platelet PAR1 receptor expression [16,17]. In order to provide a mechanistic clarification, we searched for the presence of consensus motifs for transcription suppressors. Bioinformatics analyses (MatInspector Release professional 8.02) showed that the [-1426C/T] polymorphism in hPar1 promoter generates a consensus motif for the transcription repressor Kruppel-like factor 3 (KLF3). The optimal values found for KLF3 family motifs are 0.952 and 0.961. We aimed to demonstrate a physical association between KLF3 protein and hPar1 chromatin using chromatin immunoprecipitation (ChIP) analysis in samples taken from individuals carrying [-1426C/T] polymorphism that is not seen in samples from controls without that polymorphism.

Methods

Study design and patients

The study was approved by the Institutional Ethics Committee and the Ministry of Health Pharmaceutical Department's Clinical Trials Unit (no. 18/07).

Study group enrolment and data collection were performed in the Reproductive Endocrinology Division of the Clinic for Recurrent Pregnancy Loss in a large tertiary medical center in Jerusalem, Israel. The women were enrolled in a consecutive manner according to the study inclusion criteria. Control group enrolment and data collection were performed via the Admission Services of the Division for Maternal & Fetal Medicine in the same institution. Demographic data including maternal characteristics, past reproductive history, and information about previous complications during pregnancy, delivery, and neonatal period were collected. The laboratory staff was blinded as to the clinical status of the samples.

Unexplained RPL was defined as $\geq$3 consecutive spontaneous pregnancy losses at $<$20 weeks (but $>$6 weeks) of gestation with the same partner [18]. All women with unexplained RPL were [a] proven negative for common thrombophilic conditions such as prothrombin G20210A mutation, factor V (Leiden) G169A/R506Q mutation, MTHFR C677T and A1298C mutations, anti-phospholipid/co-factor syndrome, Protein S deficiency (defined as less than 65% activity), Protein C deficiency (defined as less than 70% activity), and anti-thrombin III deficiency (defined as less than 80% activity), [b] had normal uterine shape, and [c] had a negative infectious disease screen. All couples in the study group had normal karyotypes.

PAR1 polymorphism analysis

The nomenclature for the PAR1 gene is F2R22. DNA was extracted from peripheral blood cells and PCR amplification identified functional polymorphisms in the 5′ regulatory region of PAR1 gene by standard methods [19]. Polymorphism analysis was performed using primers and restriction enzymes as previously described [20]. The primers are in Table 1. For each polymorphism, five samples were sequenced and verified for comparison. All results showed 100% matching between sequence and RLFP.

Real time RT-PCR amplification

PCRs were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystem, USA) system, and the SYBR Green PCR Core Reagents Kit (Applera). The oligonucleotide primers used are in Table 1.

Quantitative values were obtained from the threshold cycle number (*Ct* value) at which the increase in fluorescent signal associated with exponential growth of PCR products begins to be detected by the laser detector. Transcripts of GAPDH as an endogenous RNA control were used. The relative gene expression level was also normalized to an arbitrarily selected reference sample. Final results, expressed as *n*-fold difference in target gene expression relative to GAPDH gene and the reference sample [17]. Each data point was determined in duplicates.

Genomatrix search

The sequence of 2000 bps upstream to the ATG of the Human Thrombin Receptor (PAR1) was scanned with MatInspector (MatInspector Release professional 8.02, February 2010). Selected groups: Human (core/matrix sim) General Core Promoter Elements (0.75/Optimized), Miscellaneous (0.75/Optimized) Vertebrates (0.75/Optimized) at the Genomatrix web site (http://www.genomatrix.de). The optimal values found for KLF3 family are 0.952 and 0.961.

ChIP analysis

To confirm that the association between KLF3 protein and [-1426C/T] hPar1 chromatin occurs *in vivo*, PCR was performed on chromatin DNA fragments [21]. Lymphocytes isolated from women carrying [-1426C/T] hPar1 and from non-carriers were compared. Immunoprecipitation was performed with an anti-KLF3 purified goat polyclonal antibody, 200 μg/0.1 ml (sc-30380X, Santa Cruz Biotechnology, Inc). DNA from the immunoprecipitated complexes were isolated and amplified by PCR using a set of primers synthesized to cover the region including the [-1426C/T] site in the hPar1 promoter are in Table 1.

Statistical analyses

PAR1 gene polymorphic incidence in the population is unknown; therefore, a convention of difference in proportions of

Table 1
Primers for PAR1 polymorphism analysis (1–3), for Real time RT-PCR amplification (4–5) and ChIP analysis (6–7).

[-1426(C/T)] sense orientation: 5′-GCTGGGTTAAGAGGAGAAGG-3′; antisense: 5′-TGCTAAGGGCCCCCAGGGGCGTCGCGGCTGGGGGTGGCC-3′
IVS[-14(A/T)] sense 5-TTTGATTCTGAAAAATAAAATTAAAAAAATTATA-3′ antisense 5′-TGAGGGGCAGAGTTTAGGAA-3′
[-506(I/D)] sense 5′-TCCTGGCCGGGGCGTCCACT-3′ antisense 5′-CCGGCGTGCAGTGAG AGTCTRCTG-3′
PAR1 sense 5′GCC GCC TGC TTC AGT CTG T-3′; antisense 5′-TGT TGC TTT TGA TTC TGG CCT G-3′
GAPDH sense 5′-ACC ACA GTC CAT GCC ATC AC-3′; antisense 5′-TCC ACC ACC CTG TTG CTG-3′
Sense: 5′CTA TTTGGG GAC CCA TTT CC-3′
Anti sense: 5′ CCTAAAGTG CGG GGA TTACA-3′