Potential role of a single nucleotide polymorphism in the gelatinase A promoter as a risk factor for premature birth

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Abstract

Background: Gelatinase A (matrix metalloproteinase-2) is an important enzyme in many biologic processes. Prevailing data reveal a functional polymorphism at bp -1575 in the human gelatinase A promoter, which is associated with diminished transcriptional response to estrogen and genetic fitness. The reason for the disequilibrium of the -1575AA genotype within the Hardy-Weinberg distribution remains unknown. We therefore screened full-term and premature newborns to investigate whether the -1575AA genotype might increase the risk of premature delivery, which is often associated with decreased survival of infants.

Methods: DNA from 959 full-term and 358 premature newborns of North German Caucasian origin was amplified from dried blood on filter paper used for standard newborn screening in Germany. Genotypes were defined by restriction digest of PCR products.

Results: No statistically significant difference in Hardy-Weinberg distribution was discovered between full-term and premature infants. However, a trend towards the expected number of homozygous mutants was seen in premature females.

Conclusions: Our results warrant the hypothesis that the disequilibrium in -1575AA mutational variant might be due to early abortions; the reduced responsiveness to estrogen stimulation in this genotype might be responsible for inadequate estrogen-stimulated gelatinase A enhancement during trophoblast invasion and uterine implantation of the embryo. Further genotyping of couples seeking help from fertility clinics might help to answer this question.

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Introduction

Premature birth represents a major cause of perinatal morbidity and mortality [1]. The short- and long-term sequelae of prematurity have serious consequences for newborn survival and health in later life. Despite major progress in obstetrics, perinatology and neonatology, an increase in the percentage of premature births has been observed over the years [1]. Many risk factors for premature birth have been identified, including lower social class, a lower level of education, single marital status, low income, younger maternal age, low body weight, ethnicity, smoking, premature rupture of membranes, infection, multiple pregnancies, fetal and uterine abnormalities and chorioamnionitis [2]. Preterm birth is also a frequent problem in women who undergo treatment for infertility [3]. Most interventions intended to prevent preterm delivery, such as prevention of preterm labor, have not have the desired effect,

although there is growing evidence that infection and neuroendocrine processes are often involved [4]. In 1991, the hypothesis of a genetic predisposition to preterm birth was established [5]. This hypothesis is strongly supported by considerable evidence in the literature describing a familial or intergenerational influence on low birth weight or preterm delivery [6, 7]. Human twin studies have also yielded clues for heritability factors of preterm delivery and gestational length [8, 9].

Genetic polymorphisms revealing an association with premature delivery or preterm premature rupture of the membranes have been detected in a number of genes, e.g. those encoding interleukins 4, 6, and 10, thyrotropin receptor, and tumor necrosis factor-alpha [10–13]. However, in these cases, correlations between genetic polymorphisms and premature birth are merely descriptive and no functional mechanism leading to premature birth has been elucidated. Despite this, the search for functionally relevant polymorphisms has continued, because their discovery would open new possibilities for intervention. Two mutations – a maternal glutathione S-transferase T1 null genotype [14], and a single nucleotide polymorphism in the matrix metalloproteinase-1 promoter [15] – have been identified as playing a role in premature births. Furthermore, it has also been discovered that the matrix metalloproteinases gelatinase A (MMP-2) and gelatinase B (MMP-9) play a crucial functional role during gestation, and at term as well as preterm labor [16, 17]. Critically ill preterm infants had significantly lower MMP-2 activity than corresponding neonates without bronchopulmonary dysplasia and intraventricular hemorrhage [16].

We described a functional G/A polymorphism at bp -1575 in the human gelatinase A [MIM# 120360, OMIM] promoter, which affects the transcriptional response of the promoter to estrogen and is correlated with reduced genetic fitness in adults [18]. This polymorphism, adjacent to an estrogen receptor-alpha binding site, is linked with a C/T transition within an Sp1 binding site at bp -1306. Because the -1575G/A transition results in an incomplete loss of estrogen responsiveness *in vitro* and the allelic distribution of the -1575A-1306T variant showed a significant deviation from the expected Hardy-Weinberg genotype frequency, we hypothesized that these linked polymorphisms might play a functional role in obstetric complications and preterm births caused by a decreased catabolism of collagen type IV.

Methods

Patients and collection of dried blood samples

Dried blood samples were collected from 358 premature (185 female, 173 male) and 959 full-term (488 female, 471 male) newborns of North German Caucasian origin. Samples were provided by the Screening Center North (Department of Pediatrics, University Hospital Hamburg-Eppendorf, Germany) and were anonymized before analysis. The project was approved by state of Hamburg ethics committee.

Prematurity was defined as the infant having been born less than 33 weeks into gestation and having a body weight of less than 2000 g. On average, neonates included in our study were born after 30 weeks of gestation and had a body weight of 1305 g. To avoid including children more than once, only first samples were considered.

For neonatal screening, blood samples were provided on standardized filter paper cards (Whatman 903 paper, Dassel, Germany). Blood was transferred without additives onto filter paper and allowed to dry at room temperature. Dried blood spots provide a convenient tool for the determination of biochemical markers (amino acids, enzyme activities) since compounds are more stable, the material simplifies handling and shipping, and DNA (deoxyribonucleic acid) can be isolated from dried blood with relative ease. As all neonates (full-term and premature) are screened in our area, surplus material was available without requiring additional blood for this study.

Isolation of genomic DNA

The QIAamp DNA Minikit (Qiagen, Hilden, Germany) was used to isolate genomic DNA. Briefly, punched-out circles (3 mm in diameter) were incubated in ATL buffer at 85°C for 10 minutes, before adding 20 µl of proteinase K stock solution and incubating at 56°C for 1 hour. Two hundred microliters of buffer AL was added and samples were incubated at 70°C for 10 minutes. Two hundred

microliters of ethanol (100%) were then added, before applying the mixture to a QIAamp spin column and centrifuging according to manufacturers' directions. DNA was extracted from the column after several washing steps, according to manufacturer's directions. PCR (polymerase chain reaction) was performed as described below.

PCR from dried blood spots

Initially, genomic DNA was isolated and used for PCR of the required fragment as described above. To simplify the procedure and to save time and costs we developed a method for direct PCR from dried blood spots. Dried blood spots (3 mm in diameter) were put into Eppendorf caps with PCR buffer (200 mM TRIS, 500 mM KCl) and heated in a water bath at 100 °C for 10 min. This step oxidizes hemoglobin, which otherwise inhibits the PCR reaction. Samples were put on ice for 1 min and 2 µL of supernatant were used for each PCR. The PCR reaction was run with 0.75 µL $MgCl_2$ (50 mM), 2.5 µL PCR buffer, 2 µL NTPs (10 mM each), 0.75 µL primer (50ng/µL each) and 0.25 µL Taq polymerase (Invitrogen, Karlsruhe, Germany). After the initial denaturation, 38 cycles of the following steps were used: denaturation at 94 °C for 0.5 min, annealing at 58 °C for 1.5 min and elongation at 72 °C for 1.5 min. The following flanking primers were used: 5'-CACACCCACCAGACAAGCCT-3' and 5'-TGGGGAATATGGGGAATGTT-3', which generate a 349-bp fragment spanning bp -1665 to -1317 relative to the dominant transcriptional start site of the human gelatinase A gene.

Determination of genotypes by restriction enzyme digest

To determine the genotype of each individual PCR, products were digested with restriction endonuclease *Rca I* and restriction fragments were separated on an agarose gel. The -1575A polymorphism in the gelatinase A 5'-regulatory region introduces a *Rca I* cleavage site within the PCR product resulting in two fragments of 260 bp and 89 bp in size.

Statistical analysis

Statistical levels of significance for deviations from expected Hardy-Weinberg distributions and allele distribution in the different groups were determined by χ^2 analysis.

Results

To evaluate the distribution of the -1575G/A polymorphism in the human gelatinase A 5'-regulatory region, DNA from dried blood spots of premature and full-term born infants was amplified using flanking primers spanning a region of 349 bp between bp -1665 to -1317. Initially, genomic DNA was isolated from dried blood using a Qiagen kit. This method was time-consuming and expensive, so we developed a protocol allowing direct amplification of DNA from dried blood spots. PCR products were digested with the restriction endonuclease *Rca I*, which cleaves the mutant -1575A variant but not the wild type -1575G (Fig. 1).

Figure 1. Double-stranded DNA sequence between bp -1585 and -1565 of the human gelatinase A 5'-regulatory region showing the allelic variant -1575G/A.

The variant -1575A can be digested with the restriction endonuclease *Rca I,* while the wildtype allele -1575G cannot be cleaved.

Digest of DNA from a homozygous wildtype yields one 349 bp band, while heterozygous infants reveal three bands (349 bp, 260 bp and 89 bp), and homozygous mutants only show bands at 260 and 89 bp (Fig. 2).
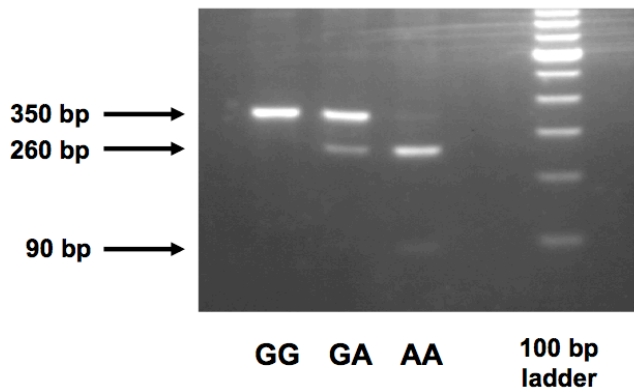
Figure 2. DNA fragments of the different genotypes after restriction digest with *Rca I*. Genotype GG (homozygous wildtype): no allele is cleaved, one band at 349 bp.

Genotype GA (heterozygous variant): one allele is not cleaved, one band at 349 bp. The other allele is cleaved into two fragments, bands at 260 bp and 89 bp (very faint). Genotype AA (homozygous mutant): no band at 349 bp; both alleles are cleaved into fragments of 260 bp and 89 bp.

To investigate whether there was a difference in the allelic distribution of the -1575G/A polymorphism in premature and full-term born infants, DNA from 959 (488 female, 471 male) full-term and 358 (185 female, 173 male) premature newborns were studied, all of Caucasian origin in Northern Germany (Table 1).

Table 1. Genotyping of premature and full-term-born children for the -1575G/A polymorphism in the gelatinase A promoter

Genotype	GG	GA	AA	Total
Female				
Premature	77 (42%)	96 (52%)	12 (6%)	185
Full-term	284 (58%)	193 (40%)	11 (2%)	488
Male				
Premature	78 (45%)	88 (51%)	7 (4%)	173
Full-term	254 (54%)	204 (43%)	13 (3%)	471

The values predicted by the assumption of Hardy-Weinberg equilibrium for the polymorphism at bp -1575 were GG:GA:AA = 58.9:35.6:5.5, respectively, whereas the actual enumerated values for full-term borns were GG:GA:AA = 56.1:41.4:2.5, respectively

($p<0.05$ by χ^2 analysis). The distribution for prematurely born infants was also in Hardy-Weinberg disequilibrium, and there was no statistically significant difference between males and females, whether for full-term born or premature born infants. However, a slight trend towards the expected number of homozygous mutants is seen in prematurely born females.

Discussion

Preterm birth is an inhomogeneous entity and several observations suggest that it may be connected with a genetic predisposition. Earlier studies from our laboratory identified linked common polymorphisms in the gelatinase A promoter, which were associated with diminished transcriptional response to estrogen and genetic fitness in adults [18]. Our data indicate that full-term born infants reveal the same disequilibrium for the Hardy-Weinberg genotype frequency values, suggesting a prenatal cause for this disequilibrium found in full-term infants and adults. Hence, the genomic distribution of the -1575G/A polymorphism in the gelantinase A promoter, adjacent to a half-palindromic estrogen response element (ERE) and linked with the -1306C/T polymorphism within an Sp1 binding site, was studied in 358 premature and 959 full-term newborns of North German Caucasian origin. Our data indicate that no difference exists in the genetic distribution of these polymorphisms between premature and full-term children. This finding is consistent with data from other investigators, demonstrating no significant difference in gelatinase A activity in complicated and uncomplicated pregnancies [19], or in the expression of gelatinase A in human placenta and fetal membranes in relation to preterm and term labor [20].

However, we observed a slight but not significant trend towards the expected number of homozygous mutations (-1575AA) in premature females. This observation led to the hypothesis that the -1575AA genotype might be associated with reduced placental implantation resulting in miscarriage and early abortion. Gelatinase A was found to be the key regulator of trophoblast invasion in early human pregnancy [21, 22]. It is secreted by the cytotrophoblast, and is mainly localized in the chorionic villi, providing further evidence of

gelatinase A involvement in the process of trophoblast invasion [23–25]. Furthermore, studies in early baboon pregnancy revealed a correlation between estrogen levels and trophoblast implantation [26], while other studies stress the importance of estrogen receptor-alpha in modulating the adhesiveness and attachment of trophoblast cells [27]. Cytotrophoblastic cell fusion and the functional differentiation of villous trophoblasts are specifically stimulated by estradiol, and the feto-placental unit produces increasing levels of estrogen [28]. In normal placentae, nuclear estrogen receptor-alpha expression was confined to villous cytotrophoblast cells, while in abnormal placentae no cytotrophoblast cells expressing estrogen receptor-alpha were detected [29]. Breast cancer studies demonstrate that gelatinase A expression by tumor cells can serve as an indicator of strong angiogenic induction potential [30], and the gelatinase -1306TT genotype (which is genetically linked to the -1575AA genotype adjacent to the estrogen-responsive element) is associated with smaller tumors and markedly different patient survival compared to the other genotypes in women with breast cancer [31].

The strongest support to our hypothesis is a study demonstrating significantly higher mid-luteal concentrations of active gelatinase A in women who conceived, compared to women who did not conceive three days after embryo transfer [32]. Our original transcription data [18], and a study in retinal pigment epithelium, show that estrogen increases estrogen receptor-alpha expression and gelatinase A activity [33]. Conception of a gelatinase A -1575AA genotype might therefore render successful uterine implantation more difficult since similar levels of estrogen will not have the same effect of enhancing gelatinase A transcription in -1575AA individuals, in contrast to -1575GG individuals, which would eventually lead to a distorted Hardy-Weinberg distribution. Hence, -1575AA individuals would be at risk for early unnoticed abortions. In addition, the -1575AA genotype might also be a factor for unwanted childlessness. Hypothetically, couples with the heterozygous -1575GA and the homozygous -1575AA genotype would run a higher risk of unwanted childlessness than couples with the -1575GG and -1575GA genotype, which would confirm our hypothesis.

Conclusions

No association was found between the -1575AA promoter mutation in the gelatinase A gene with preterm delivery. However, certain factors lead to the hypothesis that the observed deviation in the Hardy-Weinberg distribution of the -1575AA genotype might be due to early abortions caused by reduced gelatinase A activity necessary for successful implantation. Further studies of genotyping couples seeking help for *in vitro* fertilization might reveal whether the -1575AA genotype occurs more frequently in those couples, and in problems with embryo transfer of -1575AA positive individuals.

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