Evaluation of Fetal and Maternal Genetic Variation in the Progesterone Receptor Gene for Contributions to Preterm Birth

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ABSTRACT: Progesterone plays a critical role in the maintenance of pregnancy and has been effectively used to prevent recurrences of preterm labor. We investigated the role of genetic variation in the progesterone receptor (PGR) gene in modulating risks for preterm labor by examining both maternal and fetal effects. Cases were infants delivered prematurely at the University of Iowa. DNA was collected from the mother, infant, and father. Seventeen single nucleotide polymorphisms (SNP) and an insertion deletion variant in PGR were studied in 415 families. Results were then analyzed using transmission disequilibrium tests and log-linear-model-based analysis. DNA sequencing of the PGR gene was also carried out in 92 mothers of preterm infants. We identified significant associations between SNP in the PGR for both mother and preterm infant. No etiologic sequence variants were found in the coding sequence of the PGR gene. This study suggests that genetic variation in the PGR gene of either the mother or the fetus may trigger preterm labor. (*Pediatr Res* 62: 630–635, 2007)

Preterm labor and preterm birth are major public health problems throughout the world. In the United States, preterm birth has been increasing in birth prevalence over the last two decades and now affects approximately 500,000 infants per year. Besides its association with mortality, there are both acute and chronic morbidities associated with the preterm birth, including long-term sequelae such as cognitive and motor delays. Genetic factors play a strong role in preterm birth (1). The best predictor for preterm delivery is the birth of a previous preterm infant to that woman (2–4). In addition, a family history of preterm delivery in the mother herself (5) or the mother's sister (6) strongly supports an underlying genetic component. In twin studies, the heritability of preterm delivery has been suggested to be approximately 40% (7). Progesterone, and in particular 17- α hydroxyprogesterone caproate (17p), has been shown to reduce the risk of a subsequent preterm delivery by approximately 30% in women who have had at least

one preterm child (8–14). Further, the earlier the gestational age of the first infant, the more effective progesterone is in prolonging the gestation of a subsequent pregnancy.

While other mammals show a decrease in serum progesterone levels before parturition, no consistent evidence of this has been seen in humans. It has been suggested that changes in isoform ratio and/or the expression of the progesterone receptor may provide a “functional” progesterone withdrawal, leading to the initiation of labor (15–20).

Variation in the genes involved in progesterone synthesis or metabolism could be hypothesized as playing a role in preterm delivery as have single nucleotide polymorphisms (SNP) playing a role in inflammation, fetal membrane stability, immune response, sympathetic nerve action, angiogenesis, and clotting (21). In this report, we evaluated the role of genetic variation in the fetal and maternal progesterone receptor genes to identify women who may be at higher or lower risk of preterm delivery compared with the general population risk. Uncovering genetic variants in the progesterone receptor that are associated with a high risk of preterm birth could lead to targeted use of progesterone interventional therapies in the future. We evaluated risk using SNP in a family-based study that measured risk with either the mother or the fetus as the index case. We complemented this analysis with a DNA sequencing-based strategy to search for new variants in the progesterone receptor gene (PGR) that might play a role in prematurity using the mother as the primary risk case.

METHODS

Cases consisted of preterm babies admitted to the Neonatal Intensive Care Unit of the University of Iowa Hospitals and Clinics after being born in house, as well as transferred from referring units during the first 28 d of life. All families provided signed informed consent (IRB199911068) for inclusion in a registry of births at the University of Iowa Children's Hospital that had limited maternal data available in accordance with IRB guidelines.

Preterm delivery was considered as delivery before 37 completed weeks of gestation. Gestational age was estimated from the first day of the last menstrual period and was confirmed by ultrasound examination and pediatric assessment at birth. A total of 440 premature infants with gestational ages

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Abbreviations: PGR, progesterone receptor gene; PR-A, progesterone receptor isoform A; PROGINS, an *alu* insertion polymorphism in an intron of the progesterone receptor (PR) gene; SNP, single nucleotide polymorphisms

between 22 and 36 wk (mean, 31.2 ± 3.6 wk) and one or both parents have been enrolled in this study. To stratify for analysis, preterm infants were classified into three different groups based on the gestational age (GA): 1) early GA (between 22 and 27 wk); 2) middle GA (between 28 and 33 wk); and 3) late GA (between 34 and 36 wk).

From the total of 440 preterm babies, 312 were born as single births, the remainder resulting from multiple gestation pregnancies (103 twin babies and 25 triplets/quads). Triplets and quadruplets were excluded from analysis leaving 415 infants included in this analysis. Single-birth pregnancies were analyzed separately from the multiple (twin) pregnancies. One randomly selected infant was chosen from twin pairs for inclusion in any genetic analysis that used twins so that only a single infant contributed genotype data. DNA was extracted from cord blood for the infants born at the University of Iowa Children's Hospital or from discarded blood or buccal swabs for infants transferred to the hospital. Venous blood or buccal swabs were used to collect samples for DNA from the parents and all DNA extracted using standard protocols.

Genotyping for SNP markers was performed using the TaqMan chemistry as designed by Applied Biosystems (Foster City, CA). SNP Genotyping Assays were ordered from Applied Biosystems using either the Assay-on-Demand or the Assay-by-Design service. The genotyping assay mixes included primers for amplification of the region containing the SNP of interest and two TaqMan Minor Groove Binder probes specific to the polymorphic variants at the site labeled with different fluorescent reporter dyes, FAM and VIC. Reactions were carried out using standard conditions supplied by the company. Fluorescence levels of the FAM and VIC dyes were read following thermocycling. A total of 18 tagging SNP were selected to encompass the entire haplotype block structure of PGR (22). Locations of the SNP tested (and an *alu* insertion, PROGINS) as well as the structure of the PGR gene and surrounding region is shown in Figure 1.

The *alu* insertion, PROGINS, was genotyped using a gel-based assay. Primer sequences and protocols were modified from those published by Kurz *et al.* (23). SNP were chosen to cover the large haplotype block surrounding the PGR gene and are listed in Table 1 with their position on chromosome 11 based on the July 2003 University of California Santa Cruz (UCSC) assembly. The known functional SNP V660L [reference sequence (rs)1042838] was not evaluated directly but is in complete linkage disequilibrium with rs1042839 and PROGINS, both of which are included in this study and serve as surrogates for the V660L (24).

All 8 exons of PGR were sequenced in both directions. Primer sequences and PCR conditions are available upon request. Cycle sequencing was performed in a 10- μ L reaction using 0.25 μ L of Applied Biosystems Big Dye Terminator (version 1.1) sequencing reagent, 0.5 μ L of 5 μ M sequencing primer, 0.5 μ L of dimethyl sulfoxide (DMSO), 1 μ L of 5 $\times$ buffer, and 1 μ L of DNA template, and 6.75 μ L of ddH₂O. Following a denaturation step at 96°C for 30 s, reactions were cycle sequenced at 96°C for 10 s, 53°C for 5 s, and 60°C for 4 min for 40 cycles. Magnetic bead cleanup was performed using standard protocols. Samples were resuspended in 60 μ L of ddH₂O and 15 μ L were then injected on an Applied Biosystems 3730 sequencer. The Applied Biosystems sequence software was used for lane tracking and first-pass base calling. Chromatograms were transferred to a UNIX workstation, base called with PHRED (version 0.961028), assembled with PHRAP (version 0.960731), scanned by POLYPHRED (version 0.970312), and the results viewed with CONSED (version 4.0).

Statistical analysis. Each SNP was assessed using the program PedCheck (25) for any departures from Mendelian inheritance patterns. Maternal and fetal genetic effects were then evaluated.

Maternal genetic effects. We used a log-linear (26) model-based approach to study the effects of mother's genotypes on her delivering a premature baby. In the log-linear model, the unit of analysis is the "triad," consisting of an

affected offspring and the two parents. Tests of maternally mediated genetic effects are based on the symmetry assumption of allele counts between the mothers and the fathers in the source population, as defined by Schaid (27). The log-linear approach provides likelihood ratio tests (LRT) of the genetic effects as well as maximum-likelihood estimators of the genetic relative risks for maternally mediated genetic effects. This approach places no assumptions on the underlying disease-inheritance model. The expectation-maximization (EM) algorithm was applied to fully use the families with missing parental genotypes (28). We tested maternal-mediated effects using a 2-degree of freedom test based on all singleton birth families.

Fetal genetic effects. Fetal genetic effects were assessed by the transmission disequilibrium test (TDT), a family-based method introduced by Spielman *et al.* (29). Alleles at each marker were tested for association with the preterm delivery, using the Family Based Association Test (FBAT) (30–32). Additionally, haplotype FBAT (HBT) was performed for sliding windows of 3, 4, and 5 SNP across the PGR gene.

Multiple testing. In this study, the following groupings of the families were all analyzed for fetal and maternal genetic effects: all singletons, all singletons and one twin from each twin family, and for each of the three gestational age groupings (early, middle, late) in addition to all gestational ages together. Given the multiple testing, for statistical significance at an α level of 0.05, the conservative Bonferroni correction can be applied; (*i.e.* p values ≤ 0.0003 would be considered significant evidence of association under the most conservative model).

RESULTS

Table 1 shows the individual genetic variants used in this study. No deviations from Hardy-Weinberg equilibria were seen in any of the SNP. SNP showing significant p values of <0.05 or <0.01 are shown in Figure 1 for both fetal and maternal effects.

Fetal genetic effects. Figure 2 shows the p values for testing child genetic effects using the entire set of singleton birth data as well as subsets defined by gestational age. Figure 3 shows the analogous p values when singletons and one of the twins are analyzed. No SNP was significantly associated under the Bonferroni corrected threshold of $p < 0.0003$, however, several SNP had notable results. In the single pregnancy group, two variants (rs1942836 and rs1893505) had p values <0.01 (75 and 61 informative trios, respectively, where informative trios refer to trios with nonzero contribution to the test statistic) when middle gestational pregnancies were considered. Only rs1942836

Table 1. List of SNP in the PGR gene

SNP	Base change	Location (UCSC Genome Browser March 2006 build)	Minor allele frequency
1 rs1893505	C/T	100,573,168	0.41
2 rs10501973	A/G	100,569,036	0.27
3 rs954723	C/T	100,568,391	0.49
4 rs1942836	C/T	100,554,807	0.2
5 rs471811	A/G	100,549,663	0.3
6 rs568157	A/G	100,529,742	0.45
7 rs474320	A/T	100,520,009	0.15
8 rs4754732	C/T	100,513,962	0.3
9 rs3740753	C/G	100,504,231	0.15
10 rs582691	A/G	100,500,326	0.09
11 rs493957	A/G	100,494,908	0.33
12 rs503362	C/G	100,467,287	0.25
13 rs653752	C/G	100,453,570	0.39
14 rs666553	C/T	100,444,128	0.15
15 rs578029	A/T	100,427,864	0.26
16 rs1042839	A/G	100,427,662	0.14
17 Progin		Intron G (7&8)	0.14
18 rs500760	A/G	100,415,451	0.26
19 rs471767	A/G	100,410,757	0.31

Figure 1. Genomic structure of the PGR gene with location of each SNP tested (numbers in), exon and intron boundaries and significant SNP indicated. Regions of homology with greater than 80% human-mouse nucleotide identity over at least 100 bp are also shown. All SNP reside in a single large haplotype block.

Figure 2. The p values for the entire singleton pregnancies data set, as well as subsets defined by gestational age.

Figure 3. The p values when all singleton and one twin are analyzed.

was suggestive ($p < 0.01$) when all gestational ages together were analyzed ($p = 0.002$, 35 informative trios). In the singletons plus one twin birth group, rs1942836 had suggestive association when the middle gestational group was analyzed. In the sliding windows analysis, two regions of significance were consistently observed for windows of 2, 3, 4, or 5 SNP in all births/all gestations or all births/middle gestations as well as in single births alone (all gestations and middle gestations only). The results from 4-SNP-haplotype sliding windows (which showed the most significant results of the 2, 3, and 4 SNP windows tested) are shown in Figure 4, with one cluster in PGR yielding p values as low as 0.0002.

Figure 4. Haplotype association results for SNP within the PGR gene, calculated for the total data (singletons and one twin, from all gestational groups).

Maternal genetic effects. Figure 4 shows the results of tests for maternally mediated effects using all singleton pregnancies. We observed $p < 0.05$ (without correction for multiple testing) for association between prematurity and 3 SNP, rs653752 ($p = 0.007$), rs503362 ($p = 0.008$), rs4754732 ($p = 0.03$), and the *alu* insertion, PROGINs ($p = 0.04$), using all the singleton pregnancies. Three markers, PROGINs ($p = 0.03$), rs653752 ($p = 0.04$), and rs503362 ($p = 0.03$) remained significant in the middle gestational age group. SNP rs1942836 showed a p value of 0.04 in the late gestational age group. We also estimated the genetic relative risks for maternally mediated effects, *i.e.* the relative risk of disease for mothers carrying 1 or 2 copies of the variant alleles *versus* the risk for mothers carrying no copies (Table 2, Fig. 5).

Sequencing. We sequenced all 8 exons and adjacent introns of PGR on 92 mothers of premature infants to search for potential etiologic sequence variants. Previously reported SNP were detected but no missense, nonsense, or frame-shift mutations were identified.

DISCUSSION

Identifying mechanisms to prolong the length of gestation, particularly in women at risk for preterm labor and delivery, will improve both maternal and fetal outcomes. Once labor has been initiated, tocolysis as currently practiced has been only poorly effective in prolonging gestations and even when effective, may only extend gestation for a few days. While tocolysis does provide sufficient time, in some cases, for the administration of antenatal steroids to enhance fetal lung development and improve neonatal outcomes, a longer prolongation of pregnancy would be required to substantially diminish other associated morbidities and the mortality associated with preterm delivery. 17- α -hydroxyprogesterone has been shown to be effective in prolonging gestations in women who have had a previous preterm delivery, and in particular is most effective in those women who had the earliest prior delivery (8–14). Nonetheless, progesterone is only able to extend pregnancy in a proportion of cases and there has not been a recognized effect of this treatment in population-based samples of women who are unselected for prior pregnancy history.

Table 2. Relative risks for maternally mediated effects

Marker	All singletons			Early <i>p</i> Value‡	Middle <i>p</i> Value	Late <i>p</i> Value
	<i>p</i> Value	RR1*	RR2†			
Progens	0.018	0.10	2.65	0.629	0.039	0.164
rs4754732	0.025	2.41	0.87	0.274	0.034	0.082
rs503362	0.008	1.51	0.57	0.601	0.137	0.060
rs653752	0.007	0.96	0.55	0.784	0.035	0.056

* RR1 is the genetic relative risk of mother carrying one copy of the variant allele versus that of carrying no copies.

† RR2 is the genetic relative risk of mother carrying two copies of the variant alleles versus that of carrying no copies.

‡ Only *p* values of the LRT tests are shown for analyses based on early, middle, and late pregnancy subsets separately.

Figure 5. Results of tests for maternally mediated effects using all singleton pregnancies.

Pharmacogenetic variation in genes involved in either progesterone biosynthesis, absorption, metabolism, or function could be hypothesized as genetic candidates for identifying subpopulations who might be more susceptible to progesterone therapy, who might require larger or smaller dosages of progesterone for the therapy to be effective, or who may be untreatable by progesterone and in whom other mechanisms for the onset of preterm labor should be searched.

Humans do not demonstrate a decrease in serum progesterone levels before labor, as some mammals do. A functional progesterone withdrawal caused by changes in the progesterone receptor or expression of the receptor may be important in the initiation of labor (15–20). We hypothesized that variation in the progesterone receptor might underlie some of the risk for preterm delivery and evaluated a group of polymorphic variants within PGR for these effects.

We selected SNP to capture the majority of the genetic information contained within the PGR gene. PGR is located on chromosome 11 and the gene itself is contained entirely within a large, approximately 200-kb long, haplotype block in which there is strong correlation between most of the SNP within this block. This enables a comprehensive search for genetic effects of common variants that might be present within the haplotype structure (22). The few functional variants reported for PGR (24) were either tested for in this report (PROGINS Alu insertion) or were in complete linkage disequilibrium with SNP that were tested [the V660L variant (rs1042838) is in complete disequilibrium with rs1042839

tested here as well as with the PROGINS insertion with 660L in cis with the PROGINS Alu insertion]. Of the four SNP showing $p < 0.05$ in either fetal or maternal testing done here, two, showing significance in the mother (rs653752 and rs503362), are in complete disequilibrium with the V660L and the PROGINS insertion, suggesting that these variants might be providing the etiologic mechanism underlying the observation seen here. Because 660L and PROGINS Alu insertion allele is less responsive to progesterin than the 660V/no insertion allele (24), the association described may have a basis in this functional difference. We also identified strong evidence of linkage disequilibrium and presumed association using the singleton fetus/infant as a risk case with the strongest pairwise signals identified in SNPs approximately 15 and 25 kb 5' of the PGR promoter. These other two SNP with $p < 0.05$ (rs1942836 and rs4754732) are in incomplete disequilibrium with the PROGINS variants so their role is less certain. However, these two SNP are located in possible regulatory regions suggesting that a second mechanism could be acting as well, and there may be different mechanisms in the mother than in the fetus.

Our results were less statistically significant when data from twin pregnancies were included. Because twin pregnancies are more likely to deliver before 37 wk gestation, their inclusion in the analysis creates challenges, including at what gestational age they should be considered physiologically premature. At the same time, dose response effects modulated by the fetus might be more striking in twins (with twice the dose of gene products of the twins) and these effects might be evaluated in future analyses. Our use of twins was considered exploratory in this project and we anticipate that additional analytic tools will be required to properly and comprehensively make use of twins in genetic analyses of preterm labor.

We also undertook a comprehensive sequencing study of the PGR gene in mothers and focused on coding exons, splice sites, and a few highly conserved regions that might function as regulatory elements. No high-risk variants were identified in this search, although several rare variants, of yet unknown etiology, create opportunities for investigation. We assumed that if the effect of genetic variants was fetally mediated, these would be present at one half their expected frequency in the mothers of preterm infants and thus chose for reasons of efficiency to sequence only the mothers.

The PGR gene is expressed as two isoforms that are generated *via* alternate promoter elements 5' of the coding sequence of the gene (33,34). PGR is regulated *in utero* in the fetal membranes with isoform expression for the two common forms progesterone receptor isoform A (PR-A) and progesterone receptor isoform B (PR-B) having reciprocally higher expression before and after labor (35). The PR-A form seems to behave as a repressor of PR-B in the amnion as well (36). Similarly, in the myometrium, PR-A may initiate or modulate progesterone withdrawal to stimulate the onset of labor (37). Additional investigations can now focus on identifying polymorphic variants within specific conserved elements to determine whether they may play a role in altered expression of the PGR receptor and to carry out additional investigations on the

functional elements at V660L and PROGINS for the associations described.

Weaknesses of this study include the confounding effects of using a population-based collection of preterm labor cases where there is likely underlying causal heterogeneity, using the conservative Bonferroni method to correct for multiple comparisons and only indirect functional correlates with the associated genotype. Since these were registry-based samples, there was little data available on maternal indications for preterm delivery so that we were unable to stratify on this variable. The heterogeneity, however, should only predispose to false-negative results, not false positives and the characterization of functional correlates will await further study. Using the conservative Bonferroni correction, *p* values of formal significance were not seen, but as an initial exploratory investigation, several regions in the PGR gene are now identified for further investigation using larger numbers of samples and more defined phenotypes. In addition, Bonferroni is highly conservative and does not consider the linkage disequilibrium relationships across the PGR locus, which could serve to modulate the effects seen. Thus, the haplotype association may well be indicative of genuine associations.

Strengths of this study are the large sample size used, the focus on the fetus and mother as risk cases, and the extensive genotyping of the PGR gene. We also incorporated a DNA sequencing approach, which allows for detection of rare variants if the common disease/common variant hypothesis is not satisfied, as must be the case when an association-based approach is undertaken. Because many genetic disorders show substantial allelic heterogeneity, using both association and sequencing provides a comprehensive survey for variants that might be disease contributing (38). Genome-wide approaches using linkage and disequilibrium will also allow for finding those variants not suspected by our current understandings of the biology of parturition (39,40). The genetic effects identified here hold promise for use in future clinical studies to determine whether mothers or their fetuses with particular PGR genetic backgrounds might be more effectively treated with progesterone. Alternatively, there might be a subpopulation genetically resistant to progesterone and in whom alternative therapies should be tried first. A better characterization of the biologic underpinnings of these observations will provide opportunities to generate new therapeutic and preventative options for preterm labor.

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