Genetic associations of relaxin: preterm birth and premature rupture of fetal membranes

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OBJECTIVE: Relaxin H2 (RLN2) is a systemic hormone (sRLN) that is produced by the corpus luteum, whereas decidual RLN (dRLN) acts only locally. Elevated sRLN is associated with spontaneous preterm birth (sPTB) and elevated dRLN with preterm premature rupture of membranes (PPROM). Associations were sought between single nucleotide polymorphisms (SNPs) in the *RLN2* promoter with levels of dRLN and sRLN in Filipino patients with sPTB, PPROM, or normal term delivery.

STUDY DESIGN: Stringent selection of women with sPTB (n = 20) or PPROM (n = 20) and term control subjects (n = 20) was made from >8000 samples from Filipino patients who delivered at 34-36 weeks' gestation. Twelve SNPs were genotyped on maternal blood, with 9 excluded based on the high linkage disequilibrium or being the same as in the control population. Quantitative immunocytochemistry on parietal decidual tissue was performed (n = 60); sRLN was measured by enzyme-linked immunosorbent assay in a subset of patients (n = 21).

RESULTS: SNP rs4742076 was associated significantly with PPROM ($P < .001$) and increased expression of dRLN ($P < .001$). The genotype TT had increased dRLN in PPROM ($P < .05$). SNP rs3758239 was associated significantly with both PPROM and sPTB ($P < .01$), and genotype AA had increased dRLN expression ($P < .05$). The sRLN showed a trend of higher levels in PPROM and sPTB, but was not significant.

CONCLUSION: SNP rs4742076 in the *RLN2* promoter was associated with increased dRLN expression and PPROM; SNP rs3758239 was associated with both PPROM and sPTB in these Filipino patients. Specific homozygous genotypes were identified for both SNPs and were shown to be associated with increased dRLN tissue expression.

Key words: polymorphism, preterm birth, preterm premature rupture of membranes, quantitative immunocytochemistry, relaxin

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Preterm birth (PTB) is the leading cause of neonatal morbidity and death in the United States, and its cause is associated with ethnicity.[1] Socioeconomic parameters are associated with ethnic disparity but are not the cause of a higher incidence of PTB.[2] Genetic factors appear to contribute significantly to the complex gene-environment interactions that result in prematurity, with the maternal genetic component being substantially more important than the fetal genetic component.[3,4]

Immigration to Hawaii over the past 2 hundred years has resulted in 1 of the most heterogeneous populations known. However, the Filipino subpopulation in Hawaii, while having access to similar health care, has a substantially higher rate of PTB (11.7%) than either white (7.2%) or other Asian populations (9.0%) of these islands.[5] A recent study identified ancestry informative markers, showed major variations in East-Asian Americans, and concluded that Filipino women could be distinguished genetically from other East-Asian Americans.[6]

There are 3 genes for human relaxin (*RLN1, 2,* and *3*).[7,8] Both *RLN1* and *RLN2* are expressed in human decidua and placenta. However, *RLN2* is the major form, with *RLN1* being only minimally expressed by the decidua. *RLN2* is also produced by the corpus luteum and enters the systemic circulation in pregnancy (sRLN). The action of *RLN2* from the maternal decidua (dRLN) and fetal trophoblast is purely autocrine/paracrine; it does not enter the systemic circulation. This was determined from patients with ovum donation pregnancies and no corpora lutea who were shown to have undetectable sRLN levels.[9] A different pattern of serum RLN levels during gestation is associated with PTB compared with normal control subjects. Women with spontaneous PTB (sPTB) have lower RLN levels in early pregnancy but higher levels in later gestation, compared with women who deliver at term.[10] On the other hand, increased expression of intrauterine RLN has been shown in

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patients with preterm premature rupture of membranes (PPROM) without infection.[11] RLN has been shown to cause a dose-dependent increase in the expression of specific genes and proteins and the activities of some regulatory matrix metalloproteinases that are involved in PPROM.[12,13] It can also modulate the production of the proinflammatory cytokines directly; interleukin 6 and 8 are produced by both the chorion and decidua.[14,15] However, there has been no attempt to date to integrate the data on the systemic and intrauterine RLN in either normal gestation or in sPTB.

A recent study with an homogeneous Danish population showed that women who are homozygous for specific single nucleotide polymorphisms (SNPs) in the promoter region of *RLN2* have a genetic susceptibility for PTB.[16] No distinction was made in this study between women who deliver at preterm because of sPTB or PPROM, but this may be important because different mechanisms likely are involved. Thus, the aims in this exploratory study were to confirm these genetic results in a different ethnic population with a known high incidence of sPTB and to investigate whether DNA polymorphisms in *RLN2* are associated with sPTB of different causes. In addition, we examined whether such a genetic change might alter either maternal dRLN and/or ovarian RLN in the systemic circulation (sRLN).

Materials and Methods

Study population

The study population was selected from 8000 patients for whom samples were available in the University of Hawaii Biospecimen Repository that had been collected between 2005 and 2011. The University of Hawaii Biospecimen Repository is approved by the Western Institutional Review Board and deemed our study proposal exempt from institutional review board review because all the data and samples were deidentified.

There were 94 patients with Filipino ancestry up to and including all 4 grandparents (self-reported), with singleton pregnancies, sPTB between 34 weeks 0 days' and 36 weeks 6 days' gestation by documented estimated due date in the absence of medical complications of pregnancy and either PPROM (n = 20) or sPTB (n = 23) according to the diagnosis at admission. For the latter group, 20 patients were selected randomly. Preterm gestational age was selected based on results of *RLN* SNP analysis in a Danish population.[16] Control subjects (n = 20) were selected randomly from 45 patients who met the same ethnicity and inclusion/exclusion criteria, with normal spontaneous vaginal term delivery between 39 weeks 0 days and 40 weeks 0 days gestation.

Exclusion criteria were delivery because of medical complications during pregnancy (preeclampsia, fetal growth restriction, histologic and/or clinical chorioamnionitis, renal disease, HELLP [hemolysis, elevated liver enzymes, and low platelet count syndrome]), multiple gestations, pregnancies from infertility treatment, fetal anomaly, uterine anomaly, induced preterm delivery or placental conditions (placental abruption, polyhydramnios, positive urine drug screen for cocaine or amphetamines), and pregnancies with an estimated due date that was based on third-trimester ultrasound scanning.

We genotyped maternal blood in all patients for 12 SNPs that were located in the promoter region of the *RLN2* and quantitated the RLN protein in the maternal decidual cells (n = 60) and measured the maternal sRLN in a subset of the same patients (n = 21). All samples were blinded, with the disclosure of clinical characteristics/outcomes only after the analyses were completed.

Genotyping and analysis

DNA was extracted from maternal blood with the Autopure system (Qiagen, Valencia, CA); 10 ng of each sample was quality checked and used for genotyping with custom and predesigned TaqMan SNP Genotyping Assay (Life Technologies, Carlsbad, CA). Polymerase chain reactions were performed on an ABI GeneAmp 9700 thermocycler; allelic determination was carried out on an ABI 7900HT fast real-time polymerase chain reaction system (version 2.4; Applied Biosystems, Carlsbad, CA). In-house quality controls (in duplicate) were used as references to identify genotype cluster locations. Genotype data were uploaded into a database (Progeny Software, South Bend, IN) that contained deidentified demographic and clinical information for statistical analysis.

SNPs were chosen to cover the promoter of the *RLN2* from circa 2.2-6.6 kb before the 5 prime untranslated region, avoiding the region with highest homology to *RLN1*. Selection was biased toward either the functionality or the conservation of the particular SNP. A list of these SNPs, with their SNP database identification (rs) numbers and chromosome 9 base location, is shown in Table 1. Allele and genotype frequencies were calculated for each SNP. Of the 12 that were genotyped, 7 matched the reference allele and were therefore excluded from further analysis. The remaining 5 SNPs met Hardy-Weinberg equilibrium at the 5% alpha level.

Association studies were performed with the use of the Fisher exact test (permutation test) and logistic regression with the control subjects as the reference group and neonatal sex as the covariate. The data were tested for linkage disequilibrium by calculating R^2. Three SNPs (rs13293410, rs3758239, rs7029400) were in high linkage disequilibrium ($R^2 > 0.9$). Because this indicates redundancy, 2 SNPs were excluded, and only rs3758239 along with rs4742076 and rs10116567 remained for individual SNP association analyses.

Additive, codominant, dominant, and recessive inheritance models were considered. The outcome group-genotype interaction was considered significant at a probability value of $< .05$. The inheritance model with the smallest probability value was considered to be the best-fitting model for the respective SNP and was used to calculate odds ratios. Because of small sample size, interaction between RLN genotype and dRLN expression was evaluated by bootstrap analysis, with tissue RLN-genotype interaction being considered significant at a probability value of $< .05$. Because this was an exploratory study,