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

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Abstract

Objectives—Relaxin H2 (RLN2) is a systemic hormone (sRLN) produced by the corpus luteum, whereas decidual (dRLN) only acts 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 controls (n=20) was made from over 8,000 samples from Filipino patients delivered at 34–36 weeks gestation. Twelve SNPs were genotyped on maternal blood with exclusion of nine based on high linkage disequilibrium (LD) or being the same as in the control population. Quantitative immunocytochemistry on parietal decidual tissue was performed (n=60) and sRLN measured by ELISA in a subset of patients (n=21).

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Results—SNP rs4742076 was significantly associated with PPROM ($p<0.001$) and increased expression of dRLN ($p<0.001$). The genotype TT had increased dRLN in PPROM ($p<0.05$). SNP rs3758239 was significantly associated with both PPROM and sPTB ($p<0.01$), and genotype AA had increased dRLN expression ($p<0.05$). The sRLN showed a trend of higher levels in PPROM and sPTB, but was not significant.

Conclusions—SNP rs4742076 in the *RLN2* promoter was associated with increased dRLN expression and PPROM while 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.

Keywords

Relaxin; preterm birth; preterm premature rupture of membranes; polymorphisms; quantitative immunocytochemistry

INTRODUCTION

Preterm birth (PTB) is the leading cause of neonatal morbidity and mortality in the United States and its etiology 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 two hundred years has resulted in one 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 Caucasian (7.2%) or other Asian populations (9.0%) of these islands.[5] A recent study identified Ancestry Informative Markers (AIMs), and showed major variations in East-Asian Americans (EAA) concluding that Filipinos could be genetically distinguished from other EAA.[6]

There are three 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 and 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 systemic RLN levels.[9] A different pattern of serum RLN levels during gestation is associated with PTB compared to normal controls. Women with sPTB have lower RLN levels in early pregnancy, but higher levels in later gestation compared to women who delivered at term.[10] On the other hand, increased expression of intrauterine RLN has been shown in patients with PPROM without infection.[11] RLN has been shown to cause a dose-dependent increase in the expression of specific genes, proteins and activities of some regulatory matrix metalloproteinases (MMPs) involved in PPROM.[12,13] It can also directly modulate the production of the proinflammatory cytokines, IL-6 and IL-8 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 using a homogeneous Danish population showed that women 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

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delivering at preterm due to sPTB or PPROM, but this may be important as different mechanisms are likely 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 etiologies. 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 with samples available in the University of Hawaii Biospecimen Repository (HiBR), collected between 2005 and 2011. The HiBR is approved by the Western Institutional Review Board (IRB) and deemed our study proposal exempt from IRB review since all the data and samples were de-identified. There were 94 patients with Filipino ancestry up to, and including, all four grandparents (self-reported), with singleton pregnancies, spontaneous preterm delivery between 34 weeks 0 days and 36 weeks 6 days by documented estimated due date (EDD) in the absence of medical complications of pregnancy, and either PPROM (n=20) or sPTB (n=23) according to their diagnosis at admission. For the latter group, 20 patients were randomly selected. Preterm gestational age was selected based upon results of *RLN* SNP analysis in a Danish population.[16] Controls (n=20) were randomly selected from 45 patients meeting the same ethnicity and inclusion/exclusion criteria, with normal spontaneous vaginal term delivery between 39 weeks 0 days and 40 weeks 0 days. Exclusion criteria were: delivery due to medical complications during pregnancy (preeclampsia, fetal growth restriction, histological and/or clinical chorioamnionitis, renal disease, HELLP), multiple gestations, pregnancies resulting from infertility treatment, fetal anomaly, uterine anomaly, induced preterm delivery or placental conditions (placental abruption, polyhydramnios, positive urine drug screen for cocaine or amphetamines), pregnancies with EDD based on third trimester ultrasound.

We genotyped maternal blood in all patients for 12 SNPs located in the promoter region of the *RLN2* and quantitated the RLN protein in the maternal decidual cells (n=60) as well as 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 using the Autopure system (Qiagen, Valencia, CA) and 10ng 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 (PCRs) were performed on an ABI GeneAmp 9700 thermocycler and allelic determination was carried out on an ABI 7900HT Fast Real-Time PCR 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) containing de-identified demographic and clinical information for statistical analysis.

SNPs were chosen to cover the promoter of the *RLN2* from circa 2.2 kb to 6.6 kb before the 5′UTR, 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 dbSNP identification (rs) numbers and chromosome 9 base location is shown in Table 1. Allele and genotype frequencies were calculated for each SNP. Of the twelve genotyped,

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seven matched the reference allele and were therefore excluded from further analysis. The remaining five SNPs met Hardy-Weinberg equilibrium (HWE) at the 5 % alpha level.

Association studies were performed using Fisher's exact test (permutation test) and logistic regression with the controls as the reference group and neonatal gender as the covariate. The data were tested for LD by calculating R^2. Three SNPs (rs13293410, rs3758239, rs7029400) were in high LD (R^2>0.9). As this indicates redundancy, two 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 $p< 0.05$. The inheritance model with the smallest p-value was considered to be the best-fitting model for the respective SNP and was used to calculate odds ratios (ORs). Due to small sample size, interaction between RLN genotype and dRLN expression was evaluated by bootstrap analysis with tissue RLN-genotype interaction being considered significant at $p< 0.05$. As this was an exploratory study, no adjustment to the alpha level was made for multiple comparisons, and all comparisons are reported. SAS (SAS Institute, Cary, NC) and R (www.r-project.org) were used for the statistical analysis.

Quantitative Immunocytochemistry

Slides of formalin fixed fetal membrane rolls (n=60) were treated in sodium citrate buffer (10mM, pH 6.0) for 30 minutes for antigen retrieval, followed by 0.3% hydrogen peroxide then 2.5% horse serum. They were incubated for one hour with rabbit polyclonal antibody (IgG) to human RLN (8μg/ml) (Calbiochem/EMD Biosciences, San Diego, CA). Negative controls were non-immune IgG (DAKO North American Inc, Carpenteria, CA). Color was developed using the ImmPRESS reagent (Vector Labs, Burlingame, CA) for 30 minutes and DAB (3, 3-diaminobenzidine) for 5 minutes.

A series of brightfield images were acquired between 420 – 700 nm at 20 nm intervals using a multispectral imaging system comprised of an Olympus BX51 microscope and a CRI Nuance spectral analyzer (Caliper Life Sciences, Hopkinton, MA). The images were stacked to create a three-dimensional image cube. Quantitative image analysis of the unmixed data was performed with Inform software (PerkinElmer, Waltham, MA version 1.4.0). The average signal intensity per pixel from five different fields of decidua from each patient was collected and results expressed as mean ± SD. Immunocytochemical data were analyzed using non-parametric analysis (GraphPad Software Inc., San Diego, CA), and the Kruskal-Wallis ANOVA used to compare differences in protein expression.

sRLN was measured in a subset of patients (n=21) by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. ELISA results were analyzed as above.

RESULTS

The demographics and clinical characteristics of the study population are shown in Table 2, and none of the covariates were distributed unevenly among strata.

Our results show that one SNP (rs4742076) was associated only with PPROM, whereas SNP rs3758239 was associated with both PPROM and sPTB. There was no significant association ($p>0.05$) of rs10115467 in any patient group (Table 3). The TT genotype of SNP rs4742076 had a significant association ($p<0.001$) with PPROM with an odds ratio of 11.0 (CI 2–60.5) when compared to both the controls and sPTB (Table 3A and 3B). Genotype AA of SNP rs3758239 showed a significant association ($p<0.001$) between PPROM [odds

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ratio of 12.5 (CI 2.7–50)] and the term controls (Table 3A). It also had a significant association ($p<0.01$) with sPTB [odds ration of 7.1 (CI 1.7–25)] when compared to the term controls (Table 3C).

Examples of decidual cell staining for RLN are shown in Figure 1(A–D) showing darker cytoplasmic stain in PPROM (Figure 1A) compared to sPTB (Figure 1B) or at term delivery (Figure 1C). The control non-immune IgG on a section from a PPROM patient showed very low background immunostaining (Figure 1D). The methodology for quantitation of this staining using the Inform software program does not require the negative controls. Instead, it uses pattern recognition of stained cells by spectra in the stained cells as shown in Figure 1E, where the nuclear membranes are outlined in green, the extracellular matrix (ECM) pink and the individual decidual cells delineated in different colors. Thus, the program excludes the nuclei and the ECM and quantifies only the relevant cytoplasmic RLN. The quantitative results for all patients showed significantly increased cytoplasmic RLN expressed in the PPROM group compared to both the sPTB ($p<0.001$) and term controls ($p<0.001$) Figure 1F.

Only a subset of samples was available for RLN measurement by ELISA, therefore only a subpopulation of each group could be studied; 5 for PPROM, 7 for sPTB and 9 for term controls. The results showed a trend for higher levels of RLN in PPROM and sPTB compared to term controls, but this did not achieve statistical significance (Figure 2).

Because increased dRLN expression was highly significant in PPROM, we linked this with the genotype analysis using bootstrap analysis for pairwise comparisons. Thus, by comparing CC with TT for SNP rs4742076 within all subjects (n=60), the presence of the genotype TT showed significantly increased ($p<0.05$) dRLN tissue expression with a 95% confidence interval between (0.000 – 0.007). For the SNP rs3758239 the genotype AA when compared to AG also demonstrated significantly higher dRLN expression ($p<0.05$) within all subjects with a CI (0.00012 to 0.00619).

DISCUSSION

We have demonstrated some significant differences in the frequency of specific SNPs in the *RLN2* promoter in both PPROM and sPTB, in a Filipino population with a high risk of sPTB. In addition, we have successfully associated two genotypes with increased phenotypic tissue expression of dRLN with PPROM and sPTB.

Our study population was selected from a biorepository and ethnicity based upon self-reported information Our patient numbers were relatively small due to our stringent selection of patients; rigorous inclusion/exclusion criteria were used including only late PTB (34 weeks to 36 weeks, 6 days). Multiple studies have shown a high correlation between early gestational age sPTB and infection, suggesting that genetic influences in sPTB and PPROM may be more significant in late sPTB.[17,18] A study which sought associations between SNPs in the *RLN2* promoter and sPTB, stratified the patients by early and late sPTB showing significant associations only in patients with late sPTB.[16]

There have been remarkably few genetic studies on RLN and sPTB, considering that altered levels in serum have long been associated with sPTB.[19,20]. It is clear that ovarian RLN enters the systemic circulation, whereas decidual/placental RLN only has autocrine/paracrine actions within the uterus. Thus, sRLN could target the decidua via its blood supply and influence both the structural properties of the fetal membranes, as well as its proinflammatory milieu.[11] Although these actions have been shown for intrauterine RLN, there has been no plausible explanation to date why alterations in sRLN are associated with sPTB.

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Our study shows a significant association between SNP rs4742076 and PPROM. In a homogeneous Danish population, the homozygous allele of this SNP also had a significant association with patients having late PTB, in which PPROM was included but not distinguished.[16] However, there were no tissue or serum RLN analyses performed. We have confirmed this same association in a genetically different population and extended it to show that the patients with the genotype TT of this SNP had an increased risk of PPROM. However, we found no association between rs4742076 and sPTB. This may be due to the small size of our patient population or the inclusion of PPROM together with sPTB in the Danish study, which caused them to identify this SNP with PTB in general, rather than specifically with PPROM.[16] In addition, we show that dRLN was increased in these same PPROM patients, compared to the sPTB or the controls. Indeed, patients with the homozygous T allele showed a significantly increased dRLN, suggesting a potential genetic effect on decidual cell phenotype. We also attempted to link sRLN levels with the genetic analyses in a subset of patients, based upon the previous finding of mid-pregnancy increase in serum RLN in patients destined to have sPTB.[10] We showed that patients with both PPROM and sPTB tended to have higher systemic RLN levels than patients with normal term deliveries, but in neither case was this significant.

We showed that polymorphisms in SNP rs3758239 were associated with both PPROM and sPTB outcomes in Filipinos. This SNP was studied in a Danish population and no associations with sPTB were found.[16] On the other hand, these authors showed a significant association between SNP rs10115467 and sPTB, whereas we failed to find this. These differences may be due to the very different populations or the relatively low numbers of patients, both here and in the Danish study.[16] Certainly, there is a need for further studies using highly selected but larger patient cohorts.

The association between carriers of genotype AA of SNP rs3758239 and both PPROM and sPTB may indicate sharing of molecular pathways, such as activation of proinflammatory cytokines.[21] However, an increase in MMPs and apoptotic pathways predominate in PPROM and are either absent or minimally presented in sPTB, suggesting that specific pathways prior to the common terminal pathway are also involved.[22] Thus, different RLN SNPs may be associated with PPROM and sPTB. The local action of RLN upon the extracellular matrix of the fetal membranes caused by increased dRLN may be of greater consequence to PPROM than to preterm labor. This agrees with a recent study showing that alterations in genes involved in the integrity of the maternal extracellular matrix are of special importance to PPROM.[23]

In conclusion, complimentary positive results have been found by SNP analysis and immunocytochemical phenotypic expression of RLN in decidual cells. Thus, we have shown that patients with the genotype TT of rs4742076 in the *RLN2* promoter have an increased risk of PPROM. In addition, patients that were carriers of genotype AA of rs3758239 had an increased risk of PPROM and sPTB. Therefore the presence of these polymorphisms may allow the identification of pregnancies at high risk for PPROM and sPTB, which could lead to proper risk stratification and ideally improved outcomes for these patients.

Acknowledgments

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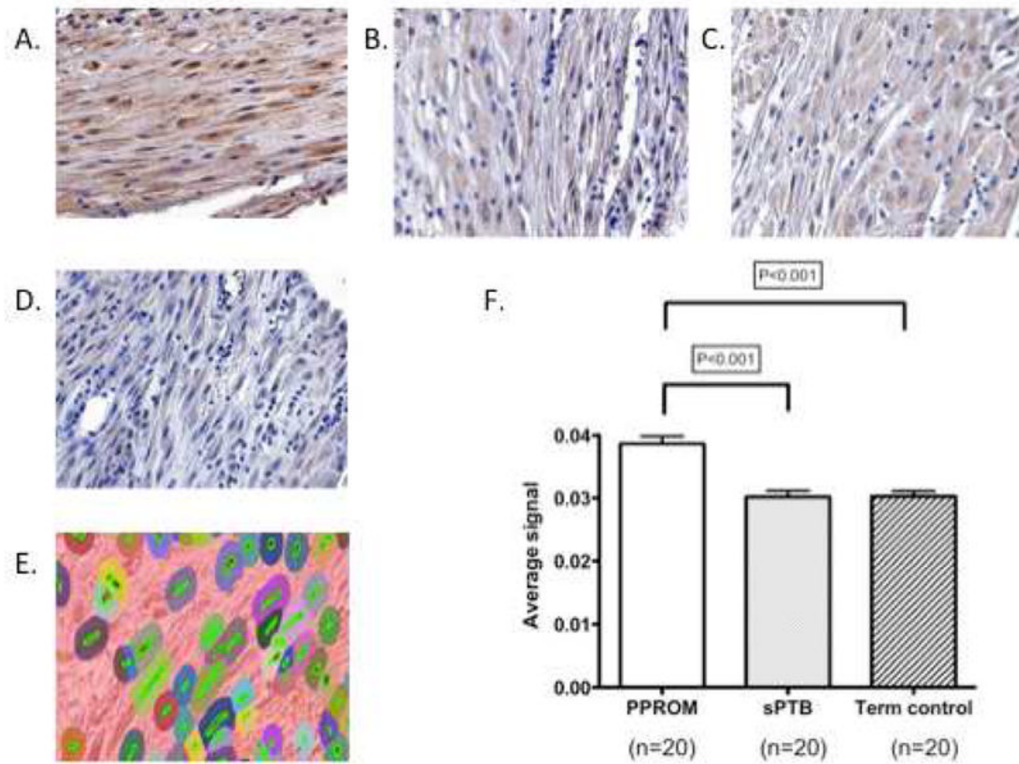

Figure 1.

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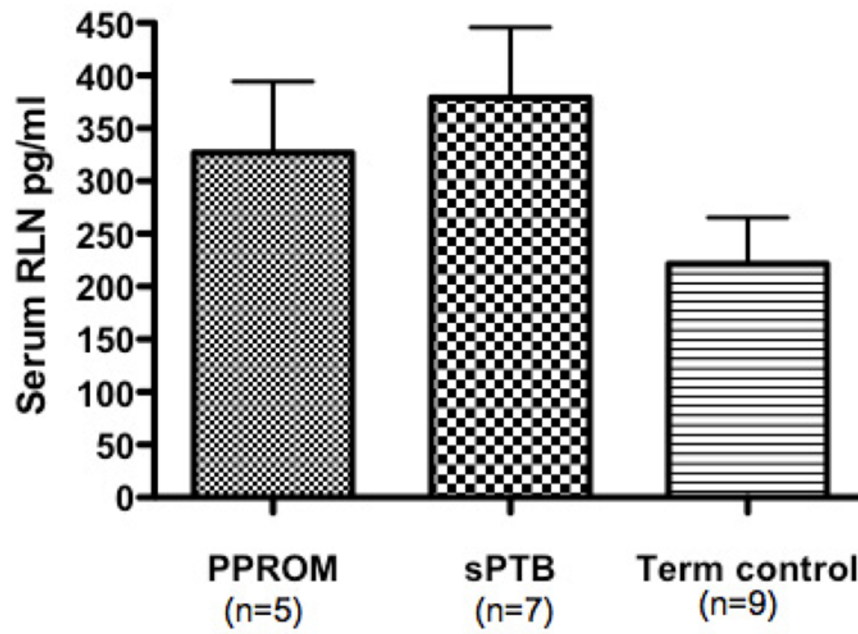

Figure 2.

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Table 1

Single Nucleotide Polymorphisms selected from the promoter region of *RLN2.*

SNP	Public location+ on Chr. 9	Alleles	Function or previously published association (s)
rs10115467*	5311171	A/G	Prematurity[16]
rs113390429	5310338	C/G	
rs13293410	5308584	A/G	
rs183312557	5311086	G/T	
rs3758239*	5306824	A/G	
rs4742076*	5309831	C/T	Prematurity[16]
rs62557688	5308318	A/G	
rs7029400	5309793	G/T	
rs7856237	5309087	A/G	
rs7875735	5309322	A/T	
rs79324864	5307823	A/G	
rs150032453	5311191	C/G	

*SNPs included in the genotyping analysis

+Genome Reference Consortium Human genome build 37

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Table 2

Demographics and clinical characteristics of the study population

Characteristic	PPROM (n=20)	sPTB (n=20)	Term (n=20)
Gestational age (mean)	35.8 (34.6–36.5)	36.12 (34.5–36.6)	39.76 (39.5– 40)
Neonatal gender (female/male)	[9/11]	[10/10]	[10/10]
Maternal age	28 (28–37)	27.85 (19–36)	30.25 (20–40)
Nulliparity	[8/20]	[7/20]	[4/20]
Prior preterm delivery	[3/20]	[7/20]	[1/20]
Length of ROM (hours)	13.7(+/−15.1)	2.4(+/−3.1)	5.2(+/−6.0)
Birth weight (grams)	2623(+/−316)	2522(+/−344)	3205.55(+/−345)
Baby's length (centimeters)	48.06(+/−1.74)	47.42(+/−1.80)	51.04(+/−2.58)

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Table 3

Comparisons of genotype frequencies in the three SNPs selected for analyses

A. Comparison between PPROM and Term controls

SNP (Genotypes)	PPROM/Term	OR (95%CI)	Best fitted model	OR (95% CI)	p-value
rs10115467					
GG	13/15	Reference	Dom	1.62(0.41–6.34)	0.48
AG	6/5	1.38 (0.34–5.62)			
AA	1/0	NA			
rs3758239					
AA	17/6	Reference	Dom	12.5(2.7–50)	**0.0002**
AG	3/10	0.11(0.02–0.52)			
GG	0/4	NA			
rs4742076					
CC	4/6	Reference			
CT	5/12	0.63(0.12–3.22)			
TT	11/2	8.25(1.15–59.00)	Rec	11.0(2.0–60.5)	**0.001**

B. Comparison between PPROM and sPTB

SNP (Genotypes)	PPROM/sPTB	OR (95%CI)	Best fitted model	OR (95% CI)	p-value
rs10115467					
GG	13/10	Reference	Dom	0.6(0.17–2.17)	0.43
AG	6/8	0.58 (0.15–2.21)			
AA	1/1	0.77 (0.04–13.87)			
Und	0/1				
rs3758239					
AA	17/15	Reference	Dom	0.53(0.11–2.6)	0.42
AG	3/4	0.66(0.13–3.45)			
GG	0/1	NA			
rs4742076					
CC	4/2	Reference			

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B. Comparison between PPROM and sPTB

SNP (Genotypes)	PPROM/sPTB	OR (95%CI)	Best fitted model	OR (95% CI)	p-value
CT	5/16	0.16(0.02–1.12)			
TT	11/2	2.75(0.28–26.61)	Rec	11.0(2.0–60.5)	**0.001**

C. Comparison between sPTB and Term controls

SNP (Genotypes)	sPTB/Term	OR (95%CI)	Best fitted model	OR (95% CI)	p-value
rs10115467					
GG	10/15	Reference	Dom	0.6(0.17–2.17)	0.43
AG	8/5	2.4 (0.61–9.49)			
AA	1/0	NA			
Und	1/0				
rs3758239					
AA	15/6	Reference	Dom	7.14(1.7–25)	**0.003**
AG	4/10	0.16(0.04–0.71)			
GG	1/4	0.1(0.01–1.09)			
rs4742076					
CC	2/6	Reference	Dom	3.86(0.67–22.1)	0.1
CT	16/12	4.0(0.68–23.41)			
TT	2/2	3.0(0.24–37.67)			

Und= Unable to be determined

NA= Not applicable