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Genetic Influences on Preterm Birth in Argentina

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Abstract

Objective—To investigate genetic etiologies of preterm birth (PTB) in Argentina through evaluation of single-nucleotide polymorphisms (SNP) in candidate genes and population genetic admixture.

Study Design—Genotyping was performed in 389 families. Maternal, paternal, and fetal effects were studied separately. Mitochondrial DNA (mtDNA) was sequenced in 50 males and 50 females. Y-chromosome anthropological markers were evaluated in 50 males.

Results—Fetal association with PTB was found in the progesterone receptor (*PGR*, rs1942836; p= 0.004). Maternal association with PTB was found in small conductance calcium activated potassium channel isoform 3 (*KCNN3*, rs883319; p= 0.01). Gestational age associated with PTB

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in *PGR* rs1942836 at 32 –36 weeks (p= 0.0004). MtDNA sequencing determined 88 individuals had Amerindian consistent haplogroups. Two individuals had Amerindian Y-chromosome consistent haplotypes.

Conclusions—This study replicates single locus fetal associations with PTB in *PGR*, maternal association in *KCNN3*, and demonstrates possible effects for divergent racial admixture on PTB.

Keywords

Prematurity; genetic admixture

Introduction

Preterm birth (PTB) complicates nearly 13 million pregnancies worldwide each year[1]. Rates of PTB are currently increasing in many countries including Argentina[2]. Health care costs for infants born premature are substantial[3] and the risk of lifelong disabilities for survivors, including chronic lung disease, cognitive impairment, and cerebral palsy, are considerable[4]. While most PTB occurs spontaneously[5], the greatest single risk factor for delivering an infant prematurely is a history of a prior PTB[6].

A genetic contribution to PTB is widely accepted[7]. Twin studies and familial recurrences provide evidence that up to 40% of PTB risk is heritable[8, 9]. Racial differences in PTB rates are significant[10] and may indicate unique genetic susceptibilities in certain populations[11]. Both maternal and paternal race have been shown independently to influence PTB[12, 13, 14]. Admixture studies in Latin America have shown tremendous geographic variability in Amerindian and European contributions to ancestry[15] and in Argentina have shown extensive paternal directional mating between historic populations of European fathers and Amerindian mothers[16, 17].

The complex interplay between genetics, population admixture, and the environment makes PTB a challenging condition to study. Candidate gene studies associating single nucleotide polymorphisms (SNP) with PTB have been difficult to replicate[18]. We created a biorepository of infants born prematurely in Argentina, including samples from their parents and extended families. We hypothesized that gene studies of our population with its unique admixture would complement previous studies of PTB and allow the discovery of distinct novel gene variation.

Methods

Collection of samples began in November 2005. Two public maternity centers in Argentina, the Instituto de Maternidad y Ginecología Nuestra Señora de las Mercedes in Tucumán and Hospital Provincial de Rosario in Rosario participated in sample collection. Written informed consent was obtained from all participants and approved by both a local institutional review board and the University of Iowa (IRB200411759). Cases were identified when a singleton was delivered before 37 weeks of completed gestation. Gestational age (GA) was estimated by last menstrual period, ultrasound, or physical examination at the time of birth.

We attempted to collect samples from three generations including the preterm infant, the infant's parents, and maternal grandparents. This study design allowed separate investigation of transmission of genetic risk to a mother (maternal contribution to PTB) and the affected infant (fetal contribution to PTB). Maternal sisters with a history of PTB and premature siblings were included for study if available. Samples consisting of placental blood, cord blood, whole blood, or saliva were obtained during hospitalization. Extraction of DNA from

biosamples was completed at either the Centro de Educación Médica e Inverstigaciones Clínicas in Buenos Aires or the University of Iowa.

Sample Genotyping

SNP genotyping was performed with TaqMan assays (Applied Biosystems, Foster City, CA) as previously described[19]. Sequence Detection Systems 2.2 software (Applied Biosystems) was utilized for allele determination. Genotypes were entered into a Progeny database (Progeny Software, LLC, South Bend, IN). The overall approach mirrored previously reported studies[19, 20]. Candidate genes were selected through literature review of biological pathways implicated in PTB. Attempts were made to replicate significant results from the study of a separate population of infants born prematurely at the University of Iowa. The genes/SNPs studied are shown in Table 1.

Admixture Analysis

To estimate genetic admixture in our study population, we evaluated anthropologically variable regions of mitochondrial DNA (mtDNA) and the Y-chromosome. 100 individuals from the Tucumán study site (50 males and 50 females) were selected for MtDNA sequencing and Y-chromosome sequencing. Sequencing of purified DNA was performed by Functional Biosciences (Madison, WI, USA). Chromatograms were loaded to a UNIX workstation and displayed with the CONSED program (v. 4.0), as previously described[19].

MtDNA was sequenced in hypervariable region 1 (HVR1) and hypervariable region 2 (HVR2). HVR1 was analyzed from 16049 – 16503 bp; HVR2 from 066 – 420 bp. Polymorphisms in mtDNA sequence were compared to the revised Cambridge Reference Sequence[21], publicly available mtDNA sequence[22], and to previously published works[16, 23] in order to assign haplogroups.

Y-chromosome sequencing was performed for DYS19 and DYS199. DYS19 is a Y-chromosome short tandem repeat which owes it diversity to a variable number of GATA repeats[24]. Sequencing of DYS19 was preformed utilizing primers as described by Hammer and Horai[25]. DYS199 is a Y-chromosome polymorphism with greater than 90% prevalence of the `T' allele in Amerindian populations[26]. Sequencing of DYS199 was performed utilizing primers as described by Underhill *et al.*[26].

Statistical Analysis

SNP genotype data was analyzed for non-mendelian inheritance utilizing PedCheck[27]. Fetal and maternal associations with PTB were determined using the Family Based Association Test (FBAT)[28, 29] and PLINK software (version 1.07)[30]. When multiple SNPs were tested in a gene, haplotype analysis was performed using FBAT. Maternal and paternal allelic transmissions were separately analyzed using PLINK to investigate parental differences in transmission. GA and birth weight (BW) were evaluated as continuous variables for SNP associations. GA was also evaluated in 4-week windows to determine allelic frequency at specific intervals of gestation. Given that analysis was completed for four independent tests (fetal affected status, maternal affected status, GA, BW), formal Bonferroni correction for multiple testing in this study at an α level of 0.05 was $p < 0.0002$.

Results

A total of 387 families were studied. 294 prematurely born probands were available for genotyping. Sampling of three generations, including the proband infant, parents, and maternal grandparents, was complete for 96 families. An additional 103 infants had both

parents available for study. Mean GA of case infants was 33.2 ± 2.6 weeks. Mean BW was 1886 grams ± 542 grams. GA distribution of study infants is shown in Figure 1.

Population Admixture

Analysis of mtDNA sequence HVR1 and HVR2 classified 88 individuals into Amerindian haplogroups A2 (n= 13), B1 (n= 9), C1 (n= 37), or D1 (n= 29). Two individuals had evidence of African ancestry, L1b and L2a. Other haplogroups represented included X and I, with one individual each. Eight individuals were not strictly classifiable due to either lack of informative polymorphisms or poor sequence quality. Mitochondrial sequence data can be viewed at GenBank (http://www.ncbi.nlm.nih.gov/genbank) with accession numbers HM592299 though HM592389 (HVR1) and HM592390 through HM592487 (HVR2).

Y-chromosome sequencing of DYS19 was successful in 44 individuals. Six individuals genotyped with the most common Amerindian GATA repeat number, 13 GATA repeats. Twenty-five individuals had the most common European variant of 14 repeats. Seven individuals had 15 repeats, three had 16 repeats, and three had 17 repeats. DYS19 sequence data can be viewed at GenBank (http://www.ncbi.nlm.nih.gov/genbank) with accession numbers HM592488 through HM592531. Of the six individuals with 13 DYS19 GATA repeats, only two also had the T allele of the Amerindian marker DYS199. No other individuals in our study cohort had the T allele.

Fetal Effects

Genetic predisposition for PTB in the fetus was associated ($p < 0.05$) with multiple genes in our study as shown in Table 2. The T allele of rs1942836 in *PGR* showed the strongest fetal association with PTB ($p = 0.004$).

In haplotype analysis, *TRAF2* showed multiple positive fetal associations with PTB as shown in Figure 2. Notably, the combination of a T allele at rs3750512 and a G allele at rs4880166 in *TRAF2* was observed in 55% of our population ($p = 0.004$).

Maternal Effects

Maternal associations ($p < 0.05$) with PTB are shown in Table 3. The T allele of rs883319 in *KCNN3* showed the strongest maternal association with PTB ($p = 0.01$).

Parental Specific Transmission and PTB

Parental specific allele transmission effects on PTB are shown in Table 4. Although, none of the SNP transmissions achieved statistical significance, a trend towards significance was shown in *DEFA6*, *PGR*, and *PTGIS*.

Birth Weight/Gestational Age Effects

The T allele of *PGR* rs1942836 was associated with higher BW in infants born prematurely when studied as a continuous variable ($p = 0.007$). The T allele of *PGR* rs1942836 remained significant when evaluating the association of higher GA (studied as continuous outcome) and genotype ($p = 0.03$). The A allele of *HPGD* rs8752 was also associated with higher GA as a continuous outcome ($p = 0.03$).

In evaluation of GA effects on PTB utilizing 4 week windows, the *PGR* rs1942836 T allele had association with PTB at GA 33 to 36 weeks ($p = 0.0004$). In addition, the T allele was associated with longer gestation and higher BW when the effects of GA and BW on PTB were evaluated as continuous variables. Both *TRAF2* rs4880166 and rs2811761 had associations with PTB at GA 29 to 32 weeks ($p = 0.002$ for both SNPs). A 4 week window

haplotype analysis of *APOE* showed association between the rs405509 A allele, rs429358 T allele, and PTB at 32 to 35 weeks (p= 0.003). Additionally, a 4 week window haplotype analysis of *PTGIS* showed association between the rs6095545 G allele, rs493694 G allele, and PTB at 33 to 36 weeks (p= 0.001).

Discussion

Global reduction in PTB rates could have a substantial impact on worldwide infant morbidity and mortality. Discovering genetic susceptibilities to PTB could allow for novel treatments and interventions that might lessen the impact PTB has on families and society. In this study, we attempted to replicate previous candidate gene investigations that found associations with PTB and to uncover associations unique to our Argentine cohort.

When evaluating fetal associations with PTB, we were able to replicate significance in a SNP previously studied in the *PGR* gene. This SNP, rs1942836, is located in a possible regulatory region of *PGR*. Fetal association with PTB in rs1942836 was first reported by Ehn *et al.* with p= 0.002 (23). SNP rs1942836 had a similar association in our population with p= 0.004. We speculate that this region of *PGR* may have a role in altering expression of progesterone receptor isoforms. Progesterone has an important role in pregnancy maintenance particularly in later gestation[31, 32]. Supplementation of progesterone in high-risk pregnancies has been effective in reducing the incidence of repeat PTB[33]. Progesterone has also been shown to protect fetal membranes from TNF-induced apoptosis[34]. Progesterone receptors are found in many pregnancy associated tissues including fetal amniotic and chorionic membranes[35, 36]. Our finding that polymorphisms in rs1942836 are associated with PTB in the latter GA window of 32 to 36 weeks (p= 0.0004) is consistent with the important role of progesterone in maintaining uterine quiescence late in pregnancy.

Fetal association with PTB was also found in haplotype analysis of *TRAF2*. The haplotype block containing rs3750512 (T allele) and rs4880166 (G allele) in *TRAF2* had association with PTB at p= 0.004. Evaluating polymorphisms in *TRAF2* for gestational age effects revealed that rs4880166 was most strongly associated with PTB at GA 29 to 32 weeks (p= 0.002). *TRAF2*, which encodes TNF receptor-associated factor 2, is involved in signal transduction in the TNF cascade and is an activator of the transcription factor NF-kappa B[37, 38]. NF-kappa B works in conjunction with *TRAF1, TRAF2,* to suppress caspase-8 induced apoptosis of cell membranes[39]. Elevations in amniotic fluids levels of TNF have been demonstrated in both preterm labor and premature rupture of membrane[40, 41]. Previous studies have also linked genetic polymorphisms of TNF and TNF Fas-mediated pathways to premature membrane rupture[42, 43]. Our findings suggest that alterations of TNF pathway proteins may lead to PTB.

The strongest maternal association with PTB was seen in *KCNN3* rs883319 with p= 0.01. KCNN3 (also known as SK3) channels are expressed in tissues responsible for maintaining gestation and inducing parturition including the uterine myometrium[44] and are subject to regulation by estrogen[45]. Overexpression of *KCNN*3 has been linked to compromised parturition through reduced uterine contractility in mouse models[46], and overexpression of this channel precludes the ability of mice to give birth prematurely[47]. We recently reported both association and sequence data suggesting a role for both rare and common variants in KCNN3 in European populations further supporting the observations made in this South American population[48]. *KCNN3* rs883319 was specifically associated with early PTB in that study.

Prior investigations of genetic admixture in Argentina have revealed evidence of selective mating between European males and Amerindian females[16, 17]. As a result, Amerindian

mtDNA haplogroups are found throughout Argentina in sizeable proportions but Amerindian Y-chromosome inheritance is more limited[17]. The sequencing of males and females in our study population for mtDNA haplogroup and Y-chromosome haplotype markers estimates that our population has similar historical divergent admixture with evidence of Amerindian ancestry in mtDNA but lacking in the Y-chromosome.

The contribution of genetic admixture to PTB is not known but significant racial differences exist in PTB. The risk ratio of PTB for an Amerindian in the US is 1.32 compared to Caucasians[49]. Studies of PTB in populations relatively lacking in admixture have shown that maternal genetics may play a more substantial role in PTB than fetal (and thereby paternal) contributions[50, 51] . The high prevalence of Amerindian mtDNA haplogroups in our study cohort may therefore place this population at increased risk for PTB.

Hypothesis generating candidate gene studies of association for complex diseases such as PTB have intrinsic limitations and are often difficult to replicate. A potential confounder of this study is the underlying heterogeneity of causes of PTB. Attempts were not made to stratify this population by indication for PTB. Uniformity in gestational age estimation was also limited in this study because of greatly variable levels of prenatal care in this population.

Findings in this study did not reach formal statistical significance when correcting for multiple comparisons. However, Bonferroni correction may be overly conservative when applied to studies such as PTB where intricate interactions between multiple genetic and environmental risk factors are likely contributory to disease burden[52].

In summary, this study has replicated a polymorphism in *PGR* with a fetal association and in *KCNN3* with a maternal association with PTB in a novel population. *PGR* and *TRAF2* have shown association with PTB during specific time periods in gestation in this population. Asymmetric racial ancestry exists in mtDNA and Y-chromosome markers in our cohort, highlighting potential consequences for divergent admixture and role for further investigation of parental specific effects on PTB.

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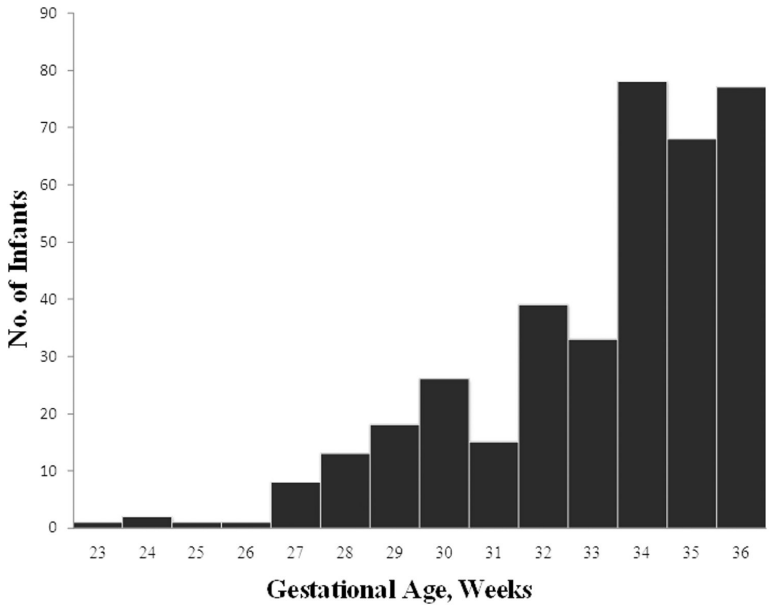

Figure 1.
Frequency Distribution of Study Infants by Gestational Age

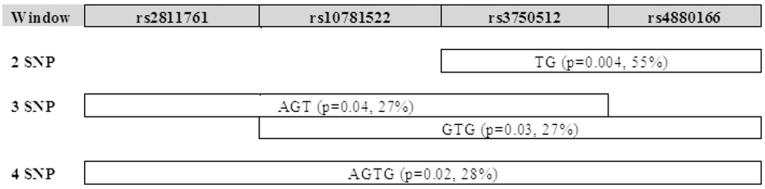

Figure 2.
TRAF2 Haplotype Blocks

Table 1

Candidate Genes

Gene	Chromosome	SNP marker(s)
LRP8	1p32.3	rs3737983
PCSK9	1p32.3	rs11591147
KCNN3	1q21.3	rs883319
CFHR1	1q31.1	rs800292
EPHX1	1q42.12	rs1009668
APOB	2p24.1	rs7575840
AGTR1	3q24	rs275649
HPGD	4q34.1	rs8752
HMGCR	5q13.3	rs3931914, rs2303152, rs12654264
IL5	5q31.1	rs743562
NR3C1	5q31.3	rs4128753
ADRB2	5q33.1	rs1042718
C2	6p21	rs7746553
IGFBP3	7p13	rs3793345
DEFA6	8p23.1	rs4458901
NAT2	8p22	rs1799930
ABCA1	9q31.1	rs2066716, rs4149313, rs2230806, rs3890182
PTGES	9q34.11	rs3844048
TRAF2	9q34.3	rs2811761, rs10781522, rs3750512, rs4880166
MBL2	10q21.1	rs5030737, rs7096206
DHCR7	11q13.4	rs3763856, rs1630498, rs2002064
SERPINH1	11q13.5	rs667531, rs681390
PGR	11q22.1	rs1042839, rs653752, rs1942836, rs1893505
MMP1	11q22.2	rs470215, rs7125062, rs996999
MMP3	11q22.2	rs650108, rs679620
ALDH2	12q24.12	rs2238151
PTGER2	14q22.1	rs17197
LIPC	15q22.1	rs1800588, rs1968685, rs1973028, rs6083
CYP11A1	15q24.1	rs2073475
CRHR1	17q21.31	rs110402
ACE	17q23.3	rs4351
SMARCA4	19p13.2	rs1529729
APOE	19q13.32	rs405509, rs429358, rs7412
APOC2	19q13.32	2288911
BP1	20q11.23	rs1341023
MMP9	20q13.12	rs17576, rs3918256
PTGIS	20q13.13	rs6095545, rs6090996, rs493694

Abbreviation: SNP, single-nucleotide polymorphism.

Table 2

Fetal Significant Effects

Gene	SNP	Allele (frequency)	Informative Families	p-value
DEFA6	rs4458901	C (77%)	71	p= 0.02
NAT2	rs1799930	G (86%)	49	p= 0.04
TRAF2	rs3750512	T (55%)	101	p= 0.03
TRAF2	rs4880166	G (64%)	97	p= 0.04
PGR	rs1942836	T (66%)	96	p= 0.004
APOC2	rs2288911	C (76%)	67	p= 0.04
MMP2	rs17576	A (79%)	94	p= 0.02

DEFA6: defensin alpha 6; *NAT2:* N-acetyltransferase 2; *TRAF2:* TNF receptor-associated factor 2; *PGR:* progesterone receptor; *APOC2:* apolipoprotein C2; *MMP2:* matrix metallopeptidase 2

Table 3

Maternal Significant Effects

Gene	SNP	Allele frequency)	Informative families	p-value
KCNN3	rs883319	T (18%)	51	p= 0.01
HMGCR	rs2303152	C (57%)	15	p= 0.05
SERPINH1	rs667531	G (60%)	56	p= 0.04
PTGER2	rs17197	T (73%)	50	p= 0.04
LIPC	rs1973028	G (53%)	53	p= 0.03

KCNN3: potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3; *HMGCR:* 3-hydroxy-3-methylglutaryl-CoA reductase; *SERPINH1:* serpin peptidase inhibitor, clade H, member 1; *PTGER2:* prostaglandin E2 receptor; *LIPC:* hepatic lipase

Table 4

Parent-of-origin specific effects for PTB infants

Gene	SNP	Allele	Paternal transmission	Paternal OR[a]	Maternal transmission	Maternal OR[a]	P-value[b]
DEFA6	rs4458901	C	*P*=0.005	2.5	*P*=0.65	1.15	0.09
PGR	rs1042839	C	*P*=0.47	1.3	*P*=0.10	1.9	0.10
PTGIS	rs493694	G	*P*=0.18	1.8	*P*=0.03	0.90	0.07

Abbreviations: PTB, preterm birth; SNP, single-nucleotide polymorphism.

[a]OR is odds ratio of having the associated allele versus the other allele.

[b]Null hypothesis: paternal odds ratio=maternal odds ratio.