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Association of the polymorphism 12109g>A from the *REN* gene as a risk factor for preterm birth

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Abstract

Introduction: Preterm birth is the most important cause of neonatal mortality and morbidity. It is a multifactorial disease with different etiologies, including genetic factors. Genetic variability is represented by single nucleotide polymorphisms (SNPs) in genes of proteins involved in the contractile activity. We determine the association between SNP 12109G> A in *REN* associated with preterm birth and premature rupture of membrane.

Materials and methods: A study of cases (*N*=112, 22–36 weeks of gestation; mean: 31, 95% confidence interval 30.7–32.2) and controls (*N*=66; 38–40 weeks of gestation from the last menstrual period; mean: 39.8, 95% confidence interval 38.9–39.4) was performed. Genomic DNA was isolated in all patients from peripheral blood. The SNP 12109G> A (*Mbo* I) in *REN* was typified by PCR-restriction fragment length polymorphism.

Results: A significant difference in the case group for the SNP 12109G>A was observed. The A allele was increased in women with preterm birth (81% cases *vs*. 15% control, $p<0.0000004$). There was also a significant difference between genotypes, mainly an excess of G/A heterozygotes in women with preterm birth (60% cases *vs*. 23% controls). The phenotype 12109G> A has odds ratio 6.62 (95% confidence interval 3.14–14.15), which means a high risk of preterm birth/premature rupture of membrane in presence of allele A, both in homozygotes and in heterozygotes.

Conclusion: Allelic frequency of A of SNP 12109G>A was higher in women with preterm birth than in women with normal vaginal delivery and could be considered a risk factor.

Keywords
Preterm birth, *REN* gene, SNPs, PCR-RFLP, *Mbo* I

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Introduction

Preterm birth (PTB) is considered a syndrome characterized by effacement and dilation of the cervix, or uterine irritability increased due to various factors. This varies according to the gestational age, the presence of intrauterine infection and systematic, utero-placental ischemia, excessive distention of the uterus and abnormal immune response of the fetus or the mother.[1] PTB is the most important cause of neonatal mortality and morbidity, and it is responsible for almost 70% of neonatal deaths in Mexico with an incidence of 5% to 10%.[2] PTB also a risk factor of cardiovascular diseases and diabetes of the offspring in later life.[3] The pathologies most frequently associated with PTB are premature rupture of membranes (PROM), infectious complications that cause chorioamnionitis and

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neonatal sepsis.[4] Some nutritional factors, such as maternal vitamin D deficiency and fetal[5] and zinc deficiency[6] are related to the PTB. Although scientific and technological advances in neonatal care have managed to increase survival in newborns, there has not been a positive impact on long-term morbidity.[7]

Epidemiological studies designed to recognize associated factors may explain below 40% of cases of PTB. Nevertheless, different genetic factors play an important role in its etiology.[8] There are allelic variants or single nucleotide polymorphisms (SNPs) in genes involved in the contractile activity of the uterus. SNPs may predispose events of PTB or even alter the response to drugs used to treat it.

The renin–angiotensin system (RAS) includes a set of neurohumoral factors and mechanisms involved in the regulation of blood pressure. RAS is also involved in regulating the body's sodium balance and urinary excretion of potassium.[9] Renin (REN) is a member of the aspartyl proteinase enzyme family and it is synthesized as a pre-proprotein. In humans, the gene encoding this protein is located on chromosome 1 (1q 32–1q 42) and it has sequences of steroid receptors on the 5′ promoter region. Transcription produces a 1.5 kb mRNA and the proenzyme has 340 amino acids, of which the first 43 are cleaved to produce for cAMP control and a number are an active enzyme.[10] Different proteins may arise from the same *REN* gene by differential splicing; some polymorphisms in introns altered the alternative processing mechanisms in different kinds of cells.[11]

Several studies have proposed the involvement of RAS in different pathologies, such as hypertension and heart failure.[12–15] Some of the SNPs of genes from this system have been studied in pregnancy complications; for example, preeclampsia. The attempt to determine the link between *REN* and high blood pressure (HBP) has been analyzed using affected siblings by determining haplotypes using diallelic polymorphic markers in the *REN* locus. Allelic variations were distinguishable by digestion with restriction enzymes *Taq* I, *Hinf* I, *Hind* III, *Bgl* I and *Bgl* II[16] specific for populations. For example, the SNP characterized by *Bgl* I was significantly associated with HBP in American and Caucasian populations with hypercholesterolemia. This association was found in Chinese populations, but using *Hind* III enzyme.[17,18] Some of the *REN* gene SNPs have been studied in some preterm delivery[19] or birth complications such as preeclampsia,[20] however, the polymorphism 12109G> A (*Mbo* I) has not been studied in this disease.

The discovery of a genetic etiological factor that may predispose PTB and some of its causes, such as PROM, should be a major link for future research in the biology, diagnosis and therapy of this syndrome. Because of this, we evaluated the association of the SNP 12109G> A (*Mbo* I) of the *REN* gene with PTB.

Materials and methods

Study population

A retrospective, descriptive, case–control study was performed. Samples were collected from a DNA biobank from 2000 to 2004. The samples came from pregnant Mestizo Mexican women who attended in the Obstetrics and Gynecology Department of the Obstetrics and Gynecology Hospital of the Western National Medical Center of the Mexican Institute of Social Security and General Hospital No. 45 (Ayala) in the metropolitan area of Guadalajara, Mexico. The case group was integrated with a total of 112 samples belonging to women with an age range of 16–40 years diagnosed with PROM $\geqslant$ 24 h and PTB with pregnancy termination between 22 and 36 weeks of gestation from the date of the last menstrual period. General data were recorded for analysis (name, age, gynecological and obstetric history) in each case. The gestational age and the birth canal and the presence or absence of the variables were obtained from medical records, corroborating data with a personal interview with the patient. The variables considered for this study in the case group were: weeks of gestation, way of termination, history of preterm labor, PROM, infection at admission (genitourinary), diabetes mellitus, hypertensive disorders (hypertension in pregnancy or preeclampsia/eclampsia). The control group consisted of 66 women with an age range of 15–38 years, and at least one normal vaginal delivery (NVD) at term (38–40 weeks of gestation), and no history of PTB and PROM. Patients with drug treatment to prevent PTB and/or serious diseases (heart disease, cancer, HIV) and/or other obstetric complications (previous placenta, oligohydramnios, fetal death) were excluded.

Analysis of SNPs by PCR-restriction fragment length polymorphism

A venous blood sample was obtained in all patients by venipuncture. DNA was extracted from each sample using the methodology established by Miller.[20] For SNP identification from the *REN* gene, a 250-bp segment was amplified using PCR, under the following conditions: 1 cycle of 94°C, 5 min; 28 cycles of 94°C, 1 min; 65°C, 1 min; 72°C, 2 min; and 1 cycle of 72°C. The concentrations of the reaction components were 1X buffer, $MgCl_2$ 1 mM, dNTPs 0.2 mm, *Taq* polymerase 0.03 U/µl, DNA 100 ng/µl, and 6 pM of each primer for the SNP 12109G>A. The sequences of the primers were 5′-TGAGGTTCGAGTCGGCCCCCT-3′ and 5′-TGCCCCAAACATGGCCACACAT-′3 for sense and antisense, respectively.

Subsequently, the amplified products were digested with the restriction enzyme *MboI* (↓GATC↑) using 10X buffer, 1 U of enzyme, 1µl of PCR product and completed with nuclease-free water. The mixture was incubated for 2 h at 37°C. The slices made it possible to differentiate

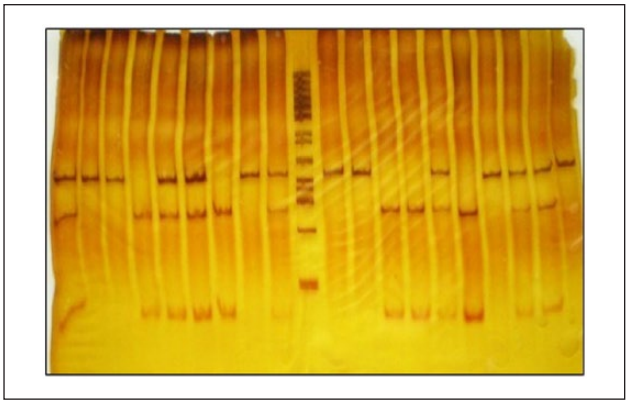

Figure 1. Characterization of polyacrylamide gel for SNP 12109G> A (Mbo I) by PCR restriction fragment length SNP. Lines are numbered from left to right. Homozygous G/G (Lanes 2, 3, 8, 11, 12, 17 and 20), homozygous A/A (lanes 4, 7, 13 and 14) and heterozygous G/A (Lanes 1, 5, 6, 15, 18 and 19) are observed. Molecular weight marker 50 bp (Lane 10).

between *REN* gene haplotypes. We observed in G/G homozygotes a 250 bp fragment (absence of the restriction site); the A/A homozygotes had two fragments of 169 and 81 bp each, and heterozygotes presented three fragments. The digested amplified products were visualized by electrophoresis in 6% polyacrylamide gel stained with silver nitrate (Figure 1).[21]

Statistical analysis

Comparisons between observed and expected genotypic proportions were performed by Hardy–Weinberg equilibrium, using a Finetti generator.[22] SPSS version 20.0 was used for data analysis and a $p \leqslant 0.05$ value was considered a statistically significant difference. Chi-square analysis was applied to contrast differences in the distribution of genotypes, alleles and phenotypes[23] and multivariate analysis and to detect the existence of any confounding variable.[24] Relative risk of the SNP was estimated and the odds ratio (OR) was calculated by Epi Info v.7.1.

Results

A total of 112 patients diagnosed with PTB were analyzed for this study; genotypic, phenotypic and allelic frequencies are shown in Table 1. A significant difference between genotypes was found, mainly an excess of GA heterozygotes in women with PTB (60% of cases *vs*. 23% of controls). The comparison of cases and controls showed a significant difference for the polymorphic marker *REN* 12109G>A, where an increase in the A allele in the PTB group was found (41% of cases with allele A *vs.* 15% in controls; $p<0.0000004$). The difference for phenotype A (genotypes AA + AG) was significant; this phenotype was increased in patients with PTB (66% of cases *vs*. 23% in controls; $p < 0.0000001$). The OR for the phenotype 12109G> A *(Mbo* I) was 6.62 (95% confidence interval (CI) 3.14–14.15). Our results indicate that the A allele was associated with a high risk of PTB in homozygotes as well as heterozygotes. No interaction among variables and no confounding variables were found in the cases group.

Discussion

The study of gene SNPs of RAS for several years has focused on hypertension due to its direct involvement in blood pressure.[25,26] However, the system has been implicated in various tissues, having various effects for different diseases, as in the pregnancy case.[26] The counterbalance changes of RAS during the normal pregnancy are rigid. There is marked activation of the RAS with increased production of renin angiotensinogen, angiotensin I and angiotensin II, whose primary effect is the release of aldosterone, which contributes to the retention of sodium and water in the proximal tubule.[27] Initially in pregnancy, there is decreased systemic vascular tone and vasodilation mediated by progesterone. In addition, sustained volume expansion associated with peripheral vasodilatation, renal vasodilation and RAS stimulation occurs. If a genetic change occurs that modifies the proteins of RAS, there is support for a pathophysiology.[28] However, the etiology of this dysfunction requires further exploration, to the level of the genes involved in the RAS. The components of the RAS have been found both in maternal decidua and in fetal placental tissues.[29] In this report we found a SNP of a RAS component that could change the normal mechanisms in pregnancy, culminating in a PTB risk.

Various factors related to PTB; one limitation of the study is that there was not stratified in the different variables for studying REN 12109G> A (*Mbo* I) SNP (gestational age, diabetes mellitus, infections, hypertensive disorders) that showed patients with PTB, since subgroups of each variable would be too small to have representative statistical power. The presence of hypertensive disorders in all women with PTB (hypertension/preeclampsia/eclampsia) was 12%; slightly greater than the prevalence among pregnant women with these abnormalities, which is considered to be 8% to 10%. It has been observed that levels of RAS components in plasma increase normally during an uncomplicated pregnancy;[30] however, the levels of angiotensinogen are diminished in pregnancy with preeclampsia or hypertension, and strongly associated with the risk of a PROM and PTB.[31] However, it is unknown whether this suppression of RAS components is linked to hypertension in pregnancy or secondary to another process.[9] In contrast, plasma renin levels increase in preeclampsia.[32] The mechanisms that contribute to decrease or increase in plasma levels of RAS components in the pathogenesis of PTB or complications in pregnancy

Table 1. Distribution of genotypes, alleles and phenotypes in the case and control groups. The presence of genotypes with alleles in the case group has a highly significant relationship with PTB events.

Study group	Genotype, *n* (%)						Alleles, *n* (%)				Phenotypes, *n* (%)			
	G/G		G/A*		A/A		G		A**		G		A***	
Control, NVD=66	51	(77)	15	(23)	0	(0)	117	(87)	15	(11)	51	(77)	15	(23)
Case, PTB=112	38	(34)	67	(60)	7	(6)	143	(59)	81	(41)	38	(34)	74	(66)

NVD: normal vaginal delivery; PTB: preterm birth; *$p<0.0001$; **$p<0.0001$; ***$p<0.0001$.

are not yet defined. The action of angiotensin II is mediated by binding two receptors (AT1 and AT2). AT1 receptors are especially involved in controlling blood pressure and AT2 are primarily expressed in the fetal period,[33] causing apoptosis and fetal growth restriction.[34] These factors have a high association with PROM. The genes coding for proteins of the RAS have been studied as angiotensin-converting enzyme (ACE). The presence of the D allele of the ACE gene was related to increased risk of preeclampsia. Other RAS gene polymorphisms, such as AT1 A1166C, AGT Met235Thr, AGT Thr174Met and 83A/G-REN, seem related to preeclampsia (reviewed in Yang et al.[35]). Similarly, some *REN* polymorphisms were studied (rs5705, rs1464818, and rs3795575), the finding being that there is no associated risk of preeclampsia with these polymorphisms.[29]

It has been established that SNPs of the *REN* gene that are characterized by the digesting of *Bgl* I and *Mbo* I enzymes are associated with essential hypertension in United States' populations. Both SNPs of *Bgl* I and *Mbo* I are in the first and ninth intron of the *REN* gene, respectively. Also, while the introns are not translated into protein, changes in structure can modulate gene expression. In this genetic influence, which seems to show a recessive mode of inheritance, both increased systolic and diastolic pressure could be involved.[36] Regarding the SNP determined by *Mbo* I, the most frequent genotype was GG and the most common allele was G (Table 2). The observed distribution of G and A alleles for this study was 73.3 and 26.7%, respectively. The frequency of allele A is observed in 24% of a Caucasian population from the USA,[36] similar to that observed in this study (26.7%). This SNP has been determined in different races, including Quechua, which showed a high frequency of G allele.[37] The frequency of the G allele in Mestizo populations is consistent with that found in American Caucasians and in Mayan populations (Table 2). It has been considered that genetic changes, including those located in an intron, may influence the enzymatic activity of renin.[34] The SNP of *Mbo* I in the *REN* gene was significantly associated with a family history of hypertension in a population of Gulf Arabs from the United Arab Emirates.[37] However, none of the SNPs have been linked directly with PTB.

The association of PTB and infection (genitourinary) was reported in 38% of our study group, similar to the 30%

Table 2. Frequency of genotypes and alleles of SNP Mbo I in the REN gene in different populations. The genotype and allele frequencies determined in this study (*) are within the mean, compared with other populations and races.

Population	Genotype, %			Allele, %	
SNP *Mbo* I	AA	AG	GG	A	G
Mexico (Mestizos)*	**5.5**	**42.5**	**52.0**	**26.7**	**73.3**
US Caucasians	7.1	34	58.9	24	76
Quechua	2.1	31.6	66.3	18	82
Mayan	3.9	41.2	54.9	25	75
United Arab Emirates	14.0	44.9	41.0	36.5	63.5

SNP: single nucleotide polymorphism.

reported in the literature.[38,39] Decreased renal blood flow increases the production of REN/AT2, which in turn gives rise to fetoplacental vasoconstriction through the AT1 receptor. This causes decreased placental and renal blood flow and also fetal growth retardation, oliguria and oligohydramnios. Similarly, but through the AT2 receptor, there is vasodilation which causes PTB; in this case, metalloproteinases act by degrading membranes prematurely.[40–43] They have been described as risk factors for vascular disease and diabetes mellitus[44-47] as well as for PTB.[48] The frequency of diabetes mellitus in our study group was 3%. There are no reports that have studied the *Mbo* I SNP of *REN* gene with diabetes mellitus.

In the marker 12109G> A (*Mbo* I) evaluated in this work between study groups, allele A was more prevalent in women with PTB compared with those with NVD. Although it is well known that both gestational diabetes and preeclampsia increase the risk of PTB, the A allele was present in women who did not have any of these risk factors, according to their medical history. PTB presents as a result of a complex interaction between individual genetic background and environmental factors.[8]

This study represents the first report that determines the relationship between the SNP 12109G> A of the *REN* gene in PTB. Based on our results, the allele, genotype and phenotype frequencies for this SNP were obtained for both cases and controls. The A allele frequency was higher in women with PTB than in those with NVD. Thus it can be suggested that the SNP 12109G> A (*Mbo* I) is a possible risk factor for PTB. However, future studies are necessary

to consider other known risk factors for PTB such as socioeconomic status, gynecological clinical history and habits to obtain a complete a multiple regression model to confirm this association. Thus it can be suggested that the SNP 12109G> A (*Mbo* I) is a possible risk factor for PTB.

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