

# ASSOCIATION OF INTRON-2 VARIABLE NUMBERS OF AN 86-BP TANDEM REPEAT-POLYMORPHISMS OF INTERLEUKIN-1 RECEPTOR ANTAGONIST GENE AND IDIOPATHIC RECURRENT SPONTANEOUS ABORTION

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## ABSTRACT

**Background:** It has been proposed that abnormal modulation of inflammatory response is involved in the physiopathology of idiopathic recurrent spontaneous abortion (iRSA). Factors that may participate in this process include the genetic background such as carrying specific polymorphisms of genes with functional effects. **Objective:** The objective is to study the association between iRSA and the frequency of intron-2 variable number tandem repeat-polymorphisms of interleukin-1 receptor antagonist gene (*IL1RN*). **Methods:** We conducted a case-control study including 108 women with iRSA and 103 controls. Five allelic variants of *IL1RN* were determined by polymerase chain reaction (PCR) product length analysis. **Results:** The most frequent *IL1RN* allele in this population was *IL1RN\*1*, which was present in 78% of cases and 94% of controls, and allele *IL1RN\*2*, in 45 (20.8%) cases and 12 (5.8%) controls. Allele *IL1RN\*2* was significantly associated with iRSA (odds ratio = 4.28, 95% confidence interval 2.2-8.4; p = 0.000). **Conclusion:** Carrying allele *IL1RN\*2* had a strong association with iRSA in Mexican women. This polymorphism codifies for a low-function protein, which may allow for increased activity of IL-1 pro-inflammatory axis in iRSA. (REV INVES CLIN. 2018;70:96-102)

**Key words:** Recurrent spontaneous abortion. Variable numbers of an 86-bp tandem repeat polymorphisms. Interleukin-1 receptor antagonist.

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## INTRODUCTION

Intrauterine induction of the inflammatory response is associated with a very specific and tightly regulated window during embryo implantation<sup>1</sup>. Abnormal inflammatory responses have been related to loss of gestation<sup>2,3</sup> including idiopathic recurrent spontaneous abortion (iRSA). iRSA is defined as the spontaneous loss of two or more clinical pregnancies before 22 completed weeks of gestational age in which no associated clinical factors are identified<sup>4,5</sup>.

Physiopathology of this reproductive disorder is still incompletely understood; however, different evidence points to the participation of mediators of inflammation in the mechanisms of damage. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a potent pro-inflammatory cytokine that initiates and amplifies a wide variety of effects associated with innate immunity and host responses to microbial invasion and tissue injury<sup>6</sup>. Expression of this cytokine during implantation and early placentation has been associated with successful pregnancy both in animal models and humans<sup>7,8</sup>.

On the other hand, overexpression of IL-1 $\beta$  has been related to the induction of miscarriage<sup>9,10</sup>. Functional effects of IL-1 $\beta$  are regulated, among other factors, by the presence of the IL-1 receptor antagonist (IL-1ra), a molecule that selectively blocks the actions of equimolar concentrations of IL-1 cytokine family<sup>11,12</sup>. An imbalance favoring cytokine concentration over its natural inhibitor has been proposed as an explanation to abnormal expression of IL-1 $\beta$  effects, such as in arthritis<sup>13</sup>, autoinflammatory disease<sup>14</sup> and sepsis<sup>15</sup>.

The *IL1RN* gene, located on the human chromosome 2q14 region<sup>16</sup>, encodes IL-1ra and the presence of several functional polymorphisms has been described. Among them, *IL1RN* intron 2 polymorphisms that include 2–6 variable numbers of an 86-bp tandem repeat (VNTR) sequence have been identified. The most common polymorphism is allele 1 (frequency 0.736)<sup>17</sup>. There is evidence that carrying allele 2, the second most common polymorphism, is associated to increased bioactivity of IL-1 in iRSA, as well as in other perinatal outcomes<sup>18–20</sup>. However, other publications have not found such relationship<sup>21,22</sup>.

In this paper, we evaluate the association between intron 2 polymorphisms of IL-1 receptor antagonist gene (*IL1RN*) and iRSA in a sample of Mexican women.

## MATERIAL AND METHODS

A case–control clinical trial was conducted at the Regional Hospital No. 1, Instituto Mexicano del Seguro Social (Mexican Social Security Institute), in Culiacán, Sinaloa, Mexico. The local Investigation Committee approved this project (Register R-1998-2501-01 IMSS). Samples were collected from January 2010 to September 2014.

### Patients

We used the definition and clinical classification of recurrent spontaneous abortion (RSA) from the American Society for Reproductive Medicine and the ESHRE Special Interest Group, Early Pregnancy, stating that RSA is defined when two or more pregnancy losses before 20 weeks of gestation occur<sup>4,5</sup>. Cases were women with a clinical history of RSA in which no associated cause explaining miscarriage was identified during clinical protocol and classified as iRSA. Clinical protocol to rule out associated conditions included general studies (hematology, clinical chemistry, urinalysis, and coagulation tests), karyotype of both members of the couple, hormonal profile (follicle-stimulating hormone, luteinizing hormone, estradiol, androstenedione, prolactin, and thyroid-stimulating hormone), autoimmune conditions (lupus anticoagulant, antiphospholipid antibodies and antinuclear antibodies), pelvic ultrasound, hysterosalpingography, and infections panel (toxoplasma, cytomegalovirus, rubella, herpes, *Mycoplasma hominis*, and *Ureaplasma urealyticum*). Confirmation of miscarriage included identification of embryo or placental tissues by histopathology.

Women with no medical history of miscarriage and at least two consecutive pregnancies delivering at term with normal spontaneous labor and healthy newborn were included as controls. Women attending postpartum follow-up and who had a normal course during pregnancy and delivery, including normal routine laboratory examinations and no evidence of other pathologies in the clinical records, were invited to participate and granted signed consent.

Table 1. Clinical characteristics of iRSA patients and controls

Clinical characteristics	Group		p value
	iRSA* (n = 108)	Controls (n = 103)	
Age in years	30.9 ± 4.6 (18-49)	29.3 ± 2.6 (23-37)	0.005
Parity	3.4 ± 1.3 (1-7)	2.8 ± 0.7 (2-3)	0.000
Deliveries	1.2 ± 1.2 (0-6)	2.8 ± 0.7 (2-3)	0.000
Abortions	2.2 ± 1.4 (2-4)	0	NA
Gestational age at abortion (weeks)	15.45 ± 2.6 (8-20)	NA	NA

\*iRSA was defined when two or more pregnancy losses before 20 weeks of gestation occurred. Mean ± SD, (range).

NA: not applicable, iRSA: idiopathic recurrent spontaneous abortion, SD: standard deviation.

## Polymorphism identification

A sample of 5 mL of blood was obtained and processed for DNA extraction and purification using DNeasy Blood and Tissue kits (Qiagen, Germantown, MD). Purified DNAs were stored at  $-80^{\circ}\text{C}$  until assayed. Polymorphism identification was done using the protocol of Genc et al.<sup>23</sup>, which briefly consists of DNA amplification with a PCR protocol of 35 cycles (60 s  $94^{\circ}\text{C}$ , 60 s  $60^{\circ}\text{C}$ , and 60 s  $72^{\circ}\text{C}$ ), using the pair of primers: Forward: 5'-CTCAGCAACACTCCTAT-3' and reverse: 5'-TCCTGGTTCGACGGTAA-3'. Amplification products were analyzed in 1.5% agarose gels. This technique allows the identification of five alleles<sup>17</sup>: allele *IL1RN\*1* was identified as a product of 410-bp band (four copies of the 86-bp repeat); allele *IL1RN\*2* was a 240-bp band (two repeats); allele *IL1RN\*3* was a 500-bp band (five repeats); allele *IL1RN\*4* was a 325-bp band (three repeats); and allele *IL1RN\*5* was a 595-bp band (six repeats).

## Statistical analysis

Hardy-Weinberg equilibrium was verified in the control group. The association between specific alleles

and genotypes with iRSA was evaluated using Chi-square test, and odds ratio (OR) and 95% confidence interval (CI) were calculated. Values of  $p < 0.05$  were considered as statistically significant.

## RESULTS

We included 108 women diagnosed with iRSA (cases) and 103 controls. The clinical characteristics of patients are shown in table 1.

Hardy-Weinberg equilibrium was demonstrated in the control group ( $p = 0.242$ ). The most frequent genotype was homozygous *IL1RN\*1/1*, which was present in 69 cases (64.4%) and 92 controls (89.3%). Genotype *IL1RN\*1/2* and *IL1RN\*2/2* were present in 31 (28.9%) and 7 (6.5%) cases and in 10 (9.7%) and 1 (0.9%) controls, respectively. No *IL1RN\*3* and 4 were found in these patients. Only one case of 1/5 polymorphism was found in the whole iRSA group. Carrying genotype *IL1RN\*1/2* or *IL1RN\*2/2*, compared with *IL1RN\*1/1* was significantly associated with iRSA (OR = 4.61, 95% CI 2.2-9.6;  $p = 0.001$ ) (Table 2).

Table 2. Association by genotype

Group	Genotype			Statistical analysis*		
	1/1	1/2	2/2	p value	OR	CI 95%
Control (n = 103)	92 (89.3%)	10 (9.7%)	1 (0.9%)			
iRSA (n = 107)	69 (64.4%)	31 (28.9%)	7 (6.5%)	0.001	4.61	2.2-9.6

\*Comparing genotype 1/1 against any genotype containing allele 2, using control group as contrast. One case with genotype 1/5 was excluded from analysis.

OR: odds ratio, CI: confidence interval, iRSA: idiopathic recurrent spontaneous abortion.

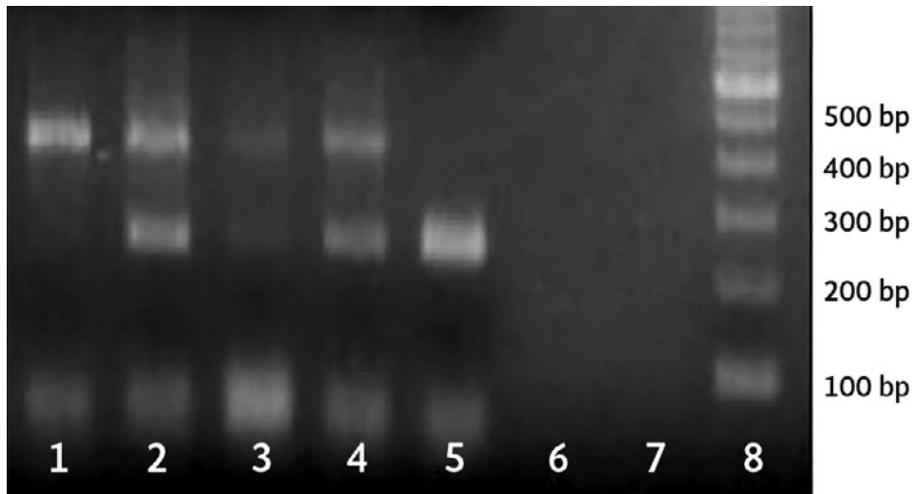
Table 3. Association by allele

Group	Allele		Statistical analysis*		
	1	2	Sig.	OR	95% CI
Control (n = 206)	194 (94.1%)	12 (5.8%)	0.000	4.28	2.2-8.4
iRSA (n = 214)	170 (78.7%)	45 (20.8%)			

\*Comparing allele 1 versus Allele 2, using control group as contrast. One case with allele 5 was excluded from analysis.

OR: odds ratio, CI: confidence interval, iRSA: idiopathic recurrent spontaneous abortion.

Figure 1. Polymerase chain reaction (PCR) products evaluation. Purified DNAs from five patients were subjected to PCR amplification of *IL1RN* intron-2 using the protocol described in Material and Methods and analyzed by gel electrophoresis. Allele *IL1RN\*1* appears as a product of 412 bp and Allele *IL1RN\*2* appears as a band of 240 bp. Lanes 1 and 3 show *IL1RN\*1* homozygosity, Lanes 2 and 4 show 1/2 heterozygosity, and Lane 5 shows *IL1RN\*2* homozygosity. Lane 8 shows 100-bp ladder molecular weight marker.



As expected, the most frequent allele in this population was allele 1, present in 170 (78.7%) cases and 194 (94.1%) controls. Allele 2 was present in 45

(20.8%) cases and 12 (5.8%) controls. Carrying allele *IL1RN\*2* compared to allele *IL1RN\*1* was significantly associated with iRSA (p = 0.000) (Table 3). Figure 1

shows typical results of the electrophoresis of PCR products, including alleles 1 and 2.

Logistic regression analysis revealed that carrying the allele *IL1RN\*2* best fits the dominant model.

## DISCUSSION

Mechanisms of iRSA are still unknown and must be complex. Involvement of an uncontrolled inflammatory response as part of these mechanisms is plausible, and a key participation of IL-1 $\beta$  has been suggested by several authors. IL-1 $\beta$  modulates at least three different early events of implantation: Regulation of trophoblast motility<sup>24</sup>, regulation of the interaction of the trophoblast with endometrium<sup>25</sup>, and direct inhibition of decidualization<sup>26,27</sup>.

These effects appear to be mediated by regulating cyclooxygenase-2 expression and using prostaglandins as secondary signals<sup>28</sup>. Any condition that alters the physiological balance between IL-1, and its natural inhibitor IL-1ra may result in detrimental effects of the cytokine.

Studies in animal models have shown that implantation may be blocked by adding an excess of IL-1ra<sup>29</sup>. Further characterization of the extent and type of inflammatory response accompanying iRSA is extremely difficult but can be partially addressed using a serial sampling of plasma or cervicovaginal swabs in a cohort of early pregnant women already identified with iRSA.

This may be used in the future for the development of new iRSA biomarkers or for the identification of potential targets for anti-inflammatory therapeutics.

Genetic background may contribute to the final balance of IL-1 since the presence of alternative alleles of *IL1RN* results in the generation of protein products with different bioactivity. Carriage of the *IL1RN\*2* variant has been linked to the decreased bioactivity of the IL-1ra manifested as the decreased concentration of the protein<sup>30</sup> or decreased affinity to the IL-1 receptor.

Decreased mRNA expression of IL-1ra has been demonstrated in cell lines carrying allele-2<sup>31</sup>. In addition,

some authors have demonstrated increased secretion of IL-1 $\beta$  in association to the presence of *IL1RN\*2* allele<sup>32</sup> and increased production of IL-1 and decreased IL-1ra on *in vitro* stimulation<sup>33</sup>, pointing to a cross-regulation between the receptor antagonist and cytokine expression<sup>34</sup>.

A more general correlation between *IL1RN\*2* allele and presence of autoinflammatory disease strongly supports a role in the control of the inflammatory response, and iRSA may be included within this group of diseases<sup>15,35</sup>. New evidence from murine models has shown prevention of miscarriage by blocking IL-1 $\beta$ <sup>36</sup>.

We found a frequency of *IL1RN* genotypes and alleles in Mexican women that is similar to frequencies described in other Hispanic populations but different to Caucasian, Chinese, and Afro-American women<sup>37,38</sup>.

We found an association between carrying *IL1RN\*2* allele and development of iRSA, in agreement to previous findings in diverse populations<sup>19,39</sup> and in agreement with a recent meta-analysis<sup>40</sup>. Our data support the dominant transmission of this allele, but the sample size for genotype 2/2 was too small to state a final conclusion.

In this paper, we provide additional evidence of an association between iRSA and carrying an *IL1RN* allele that has been linked with decreased function of the specific inhibitor of the IL-1 family, which may result in increased bioactivity of the cytokines.

The relevance of *IL1RN* polymorphism identification is beyond the exclusive statistical association with iRSA, since we have enough information on the functional relevance of carrying *IL1RN\*2* variant that has been linked to the increased bioactivity of IL-1<sup>18,20,34,41</sup>. Other studies did not confirm an association between *IL1RN* alleles and RSA<sup>21,22,42,43</sup>.

These contradictory results may be explained by several reasons. First, the prevalence of polymorphisms of interest is variable among different populations<sup>37</sup>, and hence, statistical association may change. Second, the clinical definition of RSA is still under debate in the international literature<sup>43,44</sup> and a non-unified criteria selection for patients may introduce a bias that may explain these contradictory findings.

The strong association we found in this report between carrying the *IL1RA\*2* allele and *i*RSA opens an opportunity to develop new biomarkers for the evaluation of future risk in women presenting spontaneous abortion of unknown origin. The use of these and other genomic tools will only carry significative benefits in the arena of widely used and well-structured clinical protocols for adequate stratification of patients suffering from spontaneous abortion, something that is still in the development for *i*RSA.

It is important to emphasize that a single gene variant such as *IL1RN\*2* cannot be the direct cause of *i*RSA, but a strong evidence that innate immune mechanisms plays a role in the pathogenesis of this disease of human reproduction.

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