RESEARCH ARTICLE

Genetic variations in the interleukin-21 gene and the risk of recurrent idiopathic spontaneous miscarriage

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ABSTRACT. ***Objectives.*** **Insofar as recurrent spontaneous miscarriage (RSM) is linked with dysregulated immunity and inflammatory changes, and given the pro-inflammatory role of interleukin-21 (IL-21), we examined the association between *IL-21* polymorphisms and RSM.** ***Methods and results. IL-21*** **rs2055979, rs13143866, rs9992580, and rs4833837 were genotyped in 235 cases of RSM and 235 controls. Regression analysis was employed in assessing the contribution of *IL-21* variants to the overall RSM risk. Higher minor allele and genotype frequencies of rs2055979 and rs13143866, but not rs9992580 or rs4833837, were seen in RSM patients than in the controls. *IL-21* haplotype [rs9992580/rs4833837/rs2055979/rs13143866] analysis revealed a lower frequency of the TGCG haplotype, and a higher frequency of the GGCG and GAAA haplotypes in patients, thus conferring protection from or a susceptibility to RSM by these haplotypes respectively. Regression analysis confirmed the association of TGCG [OR (95%CI)=0.09 (0.05-0.16)], and GGCG [OR (95%CI)=2.52 (1.34-4.54)] and GAAA [OR (95%CI)=4.02 (2.20-7.70)] haplotypes, after adjusting for age and BMI.** ***Conclusions.*** **Our findings indicate that *IL-21* is a novel susceptibility gene for RSM.**

Key words: interleukin-21, polymorphisms, spontaneous miscarriage

Interleukin (IL)-21 is a pro-inflammatory cytokine produced by activated $CD4^+$ T cells and NK cells [1], and is expressed at high levels by T follicular helper cells and T helper (Th)17 cells [2]. The IL-21 receptor is expressed on diverse cell types, including B cells, T cells, and NK cells [3, 4]. IL-21 augments the differentiation of Th17 cell [2, 4, 5], which also secrete IL-21, indicating that IL-21 auto-regulates its own production. IL-21 regulates T cell and B cell differentiation and proliferation. It stimulates plasma cell differentiation and immunoglobulin production, and augments $CD8^+$ T cell and NK cell activity; a Th1-Th2 shift in favor of a predominantly Th2 pathway has been suggested [6]. IL-21 was shown to contribute to the development of autoimmune disorders, including experimental arthritis [7, 8], autoimmune encephalomyelitis [5], colitis [9], and SLE [3].

Recurrent spontaneous miscarriage (RSM), defined as three or more pregnancy losses before the 20^{th} week of gestation with the same partner [10], is a major reproductive problem [10]. While inherited and non-inherited factors explain the causes of some cases of RSM [11, 12], most cases remain idiopathic, and a role for dyregulated immunity and a state of low-grade inflammation in the pathogenesis of RSM have been suggested [13]. This is highlighted by a predominance of anti-inflammatory T-helper (Th)2 cytokines in normal pregnancy, and by the fact that poor pregnancy outcome is associated with heightened expression of pro-inflammatory Th1 cytokines [13]. However, the Th1/Th2 hypothesis appears to be over-simplistic, since increased Th1 cytokine (IFN-γ) and lower Th2 cytokine (IL-4, IL-10) production was seen in controls when compared to RSM patients [14], thus prompting the need to search for additional candidate loci.

IL-21 rs907715 and rs2221903 polymorphisms have been previously associated with SLE in two independent lupus cohorts [15]. A more recent report documented the association of the *IL-21* rs13143866 SNP with juvenile idiopathic arthritis [16], indicating that *IL-21* is a susceptibility locus for immune disorders. Given the multitude of effects of IL-21 on lymphocyte activity [1], and the dysregulated immunity in RSM, we assessed the association of *IL-21* rs9992580, rs4833837, rs2055979, and rs13143866 variants with RSM in Tunisian cases and controls.

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SUBJECTS AND METHODS

Study subjects

This was a retrospective, case-control study, performed at Hôpital Farhat Hached (Sousse, Tunisia). Cases comprised 235 women, who had experienced three or more pregnancy losses with the same partner, and which had occurred from between the beginning of pregnancy to the 20th week of gestation; gestational age was calculated as the time between the first day of the last normal menstrual period and the first signs of pregnancy loss. Exclusion criteria included chromosomal aberrations, Rh incompatibility, anatomical abnormalities, preclinical miscarriages, endocrine disorders (including diabetes), autoimmune disease, liver function abnormalities, abnormal thyroid function, thyroid antibodies, hyperprolactinemia prior to luteal phase defects, and fetomaternal alloimmune thrombocytopenia. Transvaginal ultrasound was performed to confirm spontaneous miscarriage (no heartbeat detected).

Control subjects comprised 235 healthy women examined at a routine check-up following uncomplicated pregnancy, and were matched with patients according to number of risk factors (smoking, alcohol consumption, previous oral contraceptive use); age distribution was comparable between cases and controls (mean age 28.9±5.9 years *vs* 28.4±3.8 years). All subjects were required to sign an informed consent form before entering the study, which was conducted after all institutional ethical requirements had been met.

IL-21 genotyping

The selection of *IL-21* SNPs was made on SNPbrowser 4.0 software (Applied Biosystems, Foster City, CA, USA); 2 intron SNPs (rs2055979, and rs13143866), and exonic (rs9992580, and rs4833837) with minor allele frequency (MAF) >10% were used. Genotyping was performed using the allelic (VIC- and FAM-labelled) discrimination method. TaqMan assay-on-demand primers were obtained from Applied Biosystems: C_1597488_10 (rs9992580), C_1597496_10 (rs2055979), C_25473096_10 (rs4833837), and C_1597498_20 (rs13143866). The reaction was performed in a 10 µl volume on a StepOne real-time PCR system (Applied Biosystems). Replicate blinded samples were genotyped to assess genotyping reproducibility; concordance was >99%.

Statistical analysis.

Statistical analysis was performed using SPSS v. 17.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as percentages of total, or frequencies; Pearson's χ^2 or Fisher's exact tests were used to determine inter-group significance. For technical reasons stemming either from insufficient samples or reagents, data were available for 232 controls and 235 cases for rs9992580, 230 controls and 233 cases for rs4833837, 225 controls and 188 cases for rs2055979, and 223 controls and 235 cases for rs13143866. Gene variants were tested for Hardy-Weinberg equilibrium and allelic frequency (gene-counting method), using HPlus 2.5 software (http://qge.fhcrc.org/hplus). *IL-21* haplotype estimation was performed using the expectation maximization method (HPlus 2.5), and haplotype assignment probability estimate was used in determining the individual contribution to that haplotype, where haplotype assignment was uncertain. The Boneferroni multiple-comparison correction method was employed for calculating the corrected *P* value derived from the number of SNPs or haplotypes tested, as per: $Pc=1-(1-P)^n$, (n=number of comparisons).

RESULTS

Genotype analysis

The distribution of *IL-21* polymorphisms is shown in *table 1*. The genotype distributions of rs9992580 (χ^2=0.040, p=0.84), and rs2055979 (χ^2=0.620, p=0.43), but not rs4833837 (χ^2=6.908, p=0.01) or rs13143866 (χ^2=14.81, p=0.001) polymorphisms were in Hardy-Weinberg equilibrium among the controls. Higher rs2055979 (p<0.001; OR: 1.94; 95% CI: 1.43-2.63) and rs13143866 (p<0.001; OR: 1.78; 95% CI: 1.34-2.35) MAF, but not rs9992580 (p=0.413) or rs4833837 (*P* = 0.378), were seen in patients. Significant differences in the rs2055979 (p<0.001) and rs13143866 (p=0.002), but not the rs9992580 (p=0.094) or rs4833837 (p=0.151) genotype distribution, were seen in RSM cases compared to controls. Taking the homozygous wild-type as reference, regression analysis demonstrated an higher risk of RSM associated with the homozygous mutant than the heterozygous rs2055979 [OR (95% CI)=1.61 (1.02-2.56) *vs* 5.13 (2.35-11.20)] and rs13143866 [OR (95% CI)=1.90 (1.18-3.06) *vs* 3.59 (1.96-6.60)] genotypes *(table 1)*.

IL-21 haplotype distribution

Of the possible 16 *IL-21* haplotypes [rs9992580/ rs4833837/ rs2055979/rs13143866], nine were found to be common. A significantly lower frequency of GACG (p=0.033), and **TGCG** (p<0.001) haplotypes, and higher frequency of G**G**CG (*P* = 0.004), GA**AA** (p<0.001), and **T**ACG (*P* = 0.020) haplotypes were seen in cases, thus conferring disease protection and susceptibility to these haplotypes, respectively *(table 2)*. With the application of the Bonferroni correction, significant differences were seen for the **TG**CG haplotype (Pc<0.001), which was lower, and G**G**CG (Pc=0.035) and GA**AA** (Pc<0.001) haplotypes, which were higher among the cases of RSM *(table 2)*. Regression analysis confirmed the independent association of **TG**CG [OR (95% CI)=0.09 (0.05-0.16)], and G**G**CG [OR (95% CI)=2.52 (1.34-4.54)] and GA**AA** [OR (95% CI)=4.02 (2.20-7.70)] haplotypes, after adjusting for age and BMI.

DISCUSSION

We have previously demonstrated the association of *TNF*α [17] and *IL-10* [18] haplotypes with RSM, indicating that an altered Th1-Th2 cytokine balance is central to the pathogenesis of RSM. Here, we investigated the possible associations between *IL-21* polymorphisms and RSM, based on the central role of IL-21 in immune responses [3, 5, 7-9], and its contribution to the pathogenesis of immune diseases, including multiple sclerosis and inflammatory bowl disease [5, 9].

Table 1
IL-21 Genotype distribution.

SNP		Controls (235)	Patients (235)	P[1]	OR (95% CI)
rs9992580	**MAF**[2]	106 (22.6)	96 (20.4)	0.413	0.87 (0.64-1.18)
	GG	138 (58.7)	156 (66.4)	0.270	1.00 (Reference)
	GT	82 (34.9)	62 (26.4)	0.166	0.68 (0.39-1.18)
	TT	12 (5.1)	17 (7.2)	0.658	1.23 (0.49-3.07)
rs4833837	**MAF**	116 (24.7)	105 (22.3)	0.378	0.86 (0.64-1.17)
	AA	136 (57.9)	154 (65.5)	0.256	1.00 (Reference)
	AG	72 (30.6)	53 (22.6)	0.982	1.01 (0.56-1.81)
	GG	22 (9.4)	26 (11.1)	0.056	2.17 (0.98-4.80)
rs2055979	**MAF**	99 (21.1)	133 (28.3)	<0.001	1.94 (1.43-2.63)
	CC	138 (58.7)	87 (37.0)	<0.001	1.00 (Reference)
	CA	75 (31.9)	69 (29.4)	0.042	1.61 (1.02-2.56)
	AA	12 (5.1)	32 (13.6)	<0.001	5.13 (2.35-11.20)
rs13143866	**MAF**	114 (24.3)	178 (37.9)	<0.001	1.78 (1.34-2.35)
	GG	134 (57.0)	109 (46.4)	<0.001	1.00 (Reference)
	GA	64 (27.2)	74 (31.5)	0.008	1.90 (1.18-3.06)
	AA	25 (10.6)	52 (22.1)	<0.001	1.96-6.60)

[1] Pearson chi-square test.
[2] MAF: minor allele frequency.
[3] Number (percentage of total).

RSM is a heterogeneous disorder, and both inherited and non-inherited factors that can contribute to an altered risk of RSM have been described. The definition of RSM consists of three or more pregnancy losses in the absence of thromboembolic events, thyroid dysfunction, or hyperglycemia [12]. While obesity was linked to a heightened risk of RSM [11], this did not appear to be the situation here, since comparable frequencies of *IL-21* genotypes and haplotypes were seen between normal-weight (BMI<25 kg/m^2) and overweight/obese (BMI>25 kg/m^2) cases of RSM and controls *(data not shown)*.

Our hypothesis is that an altered IL-21 production, precipitated by specific *IL-21* polymorphisms, induces pregnancy loss by promoting a local inflammatory state in which IL-21-producing Th17 cells play a significant role [19]. Whereas earlier theories have suggested that pregnancy was a Th2 phenomenon, recent evidence linking Th17 cells and their cytokines (IL-17, IL-21, IL-23) with RSM, indicates that pregnancy loss is not an exclusively Th1 phenomenon, pointing to the contribution of other processes to RSM. Recent findings have demonstrated that Th17 cells play a major role in rejecting-conceptus antigens, and thus jeopardise pregnancy [19]. An increased proportion of Th17 cells was also seen in the peripheral blood and decidua of RSM women, which was paralleled by decreased frequency of Treg cells, suggesting that a Th17-Treg cell balance is critical to pregnancy outcomes [19, 20]. As such, the original Th1/Th2 paradigm has been revised to include Th17 and Treg populations.

We focused on rs9992580 and rs4833837 (coding region), and rs2055979 and rs13134866 (intronic) IL-21 polymorphisms. Both rs4833837 and rs13143866 were not

Table 2
IL-21 haplotypes in RSM.

Haplotype[1]	Controls	Patients	P[2]	Pc[3]	OR (95% CI)
G A C G	0.339±0.025	0.249±0.044	0.033	0.261	0.65 (0.49-0.86)
G A C **A**	0.183±0.024	0.158±0.017	0.340	0.976	0.83 (0.59-1.17)
T G C G	0.181±0.020	0.029±0.022	<0.001	<0.001	0.09 (0.05-0.16)
G A **A** G	0.179±0.024	0.131±0.023	0.055	0.400	0.70 (0.49-1.00)
G **G** C G	0.032±0.011	0.077±0.021	0.004	0.035	2.52 (1.34-4.54)
G A **A** **A**	0.031±0.028	0.117±0.041	<0.001	<0.001	4.02 (2.20-7.00)
T A **A** **A**	0.022±0.026	0.037±0.024	0.241	0.916	1.73 (0.78-3.68)
T A C **A**	0.011±0.008	0.029±0.024	0.064	0.449	2.86 (1.00-7.23)
T A C G	0.010±0.012	0.031±0.023	0.020	0.166	3.84 (1.23-10.16)

[1] rs9992580/rs4833837/rs2055979/rs13143866 haplotypes.
[2] Fisher's exact test.
[3] Pc = corrected P, as per the Bonferroni method [Pc=1-(1-P)^n]; n = number of comparisons.

in Hardy-Weinberg equilibrium (HWE), which, while suggesting the possibility of errors in genotyping or population stratification [21, 22], is probably attributable to the high degree of consanguinity commonly found in Islamic countries [22]. It is also due to the genetic make-up of present-day Tunisians, which is an admixture of ancient Lebanese (Phoenicians) with Carthaginians (ancestors of present-day Tunisians), Berbers (North African nomads), and Europeans, hence making tracing the genealogical origin of every participant virtually impossible. Similar conclusions were also reached by Hoskins, who also suggested that deviations from HWE are seen in polymorphisms whose minor allele frequencies exceed 0.05 [21].

Both rs2055979 and rs13134866 were independently associated with an increased risk of RSM. Homozygosity for rs2055979 and rs13134866 was associated with a progressively higher risk of RSM, compared to heterozygotes, thus establishing a "dose-effect" relationship. This was supported by haplotype analyses, in which a negative association of TGCG, and a positive association of GGCG and GAAA haplotypes were seen for RSM. This is the first report linking *IL-21* polymorphisms with RSM, and was in accordance with the reported role of IL-21-producing Th17 cells in poor pregnancy outcomes.

We propose that altered IL-21 expression, precipitated by specific *IL-21* variants or haplotypes, induces a local inflammatory state, in which pro-inflammatory Th17 cells and their cytokines act locally at the implantation site (possibly systemically) by amplifying the responses at the feto-maternal junction. The strengths of this study lie in the number of study subjects involved (235 cases of RSM and 235 controls), in the homogeneous racial make-up of the study participants, and in use of haplotype and regression analysis in studying RSM associations. However, our study has some shortcomings, namely that were not able to measure IL-21 levels, and did not examine the T cell phenotype in cases or controls. Further investigations are required to clarify the importance of IL-21, including the role of an altered Th17-Treg balance in RSM.

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