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Research Article

Association between functional polymorphisms of *Foxp3* and *Interleukin-21* genes with the occurrence of recurrent pregnancy loss in Gaza strip-Palestine

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ABSTRACT

Background: This study was conducted in order to investigate the association between *IL-21* (rs2055979 G/T and rs13143866 A/G) and *Foxp3* (rs2232365 A/G and rs3761548 A/C) gene polymorphisms and recurrent pregnancy loss (RPL) in a group of Palestinian women residing in Gaza strip.
Methods: A retrospective case-control study was carried out during the period (January to June, 2014). A total of 200 females, 100 RPL patients and 100 controls women without previous history of RPL, aged 20–35 years were included in the study. *IL-21* rs13143866 A/G and *Foxp3* rs2232365 A/G polymorphisms were analyzed by allele-specific PCR whereas, *IL-21* rs2055979 G/T and *Foxp3* rs3761548 A/C polymorphisms were investigated by PCR-RFLP.
Results: No statistically significant difference existed between RPL cases and controls in terms of the allelic and genotypic distribution of *IL-21* rs2055979 or rs13143866. On the other hand, the two *Foxp3* gene polymorphisms showed statistically significant association with RPL: the rs2232365 A/G allele G and its homozygous genotype G/G and the rs3761548 A/C allele A and its A/A genotype were significantly higher in RPL group.
Conclusions: *Foxp3* rs2232365 A/G and rs3761548 A/C polymorphisms represent risk factors for RPL in Gaza strip women. *IL-21* polymorphisms rs2055979 G/T and rs13143866 A/G however, do not pose tangible risk for RPL in our population. Study results should be confirmed on a larger sample.

Keywords: IL-21 polymorphisms, Foxp3 polymorphisms, Abortion, Recurrent pregnancy loss

INTRODUCTION

Recurrent pregnancy loss (RPL), which is currently defined as two or more consecutive pregnancy losses before the 20th week of gestation, occurs in approximately 1% to 5% of women at reproductive age.[1] Although many known causes of RPL including anatomic (15%), infectious (1%–2%), hormonal (20%), immunological (20%), and genetic (2%–5%) have been identified, a significant number of cases (approximately 40%–50%) do not have known causes, and these cases are called unexplained recurrent pregnancy losses.[2]

In the face of unknown etiological factor(s), dysregulated immunity was proposed as a potential mechanism underlying unexplained RPL.[3] This reportedly includes autoimmune abnormalities (e.g., positive anti-phospholipid, anti-nuclear and anti-microsomal antibodies), increased cell-mediated immunity and altered T helper Th1-Th2-Th17-Treg balance.[4,5] Additionally, various cytokines have been implicated in the maintenance of pregnancy through modulating the maternal immune system.[6]

Unregulated and excessive Th1 type immunity may be harmful to the fetus.[7] This is highlighted by a predominance of anti-inflammatory Th2 cytokines in normal pregnancy, and by the fact that poor pregnancy outcome is associated with heightened expression of pro-inflammatory Th1 cytokines.[8]

Interleukin (IL)-21 gene encodes a pro-inflammatory cytokine that is produced by activated $CD4^+$ T cells and natural killer (NK) cells, and is expressed at high levels by Th17 cells.[9] IL-21 augments the differentiation of Th17 cells and NK cell activity.[9,10] Elevated levels of Th17 cells in the peripheral blood and decidua and raised levels of decidual NK cells have been observed in women suffering from RPL.[1,11]

Nucleotide variations in *IL-21* gene may affect its immune-modulating function. For instance, it has been found that the rs2055979 C/A and rs13143866 A/G polymorphisms of *IL-21* and the levels of IL-21 were significantly associated with various immune-mediated diseases.[12-14]

Foxp3 (transcription factor forkhead box p3) is a member of the forkhead winged-helix transcription-factor family.[15] *Foxp3* is expressed primarily in regulatory T-regulatory (Treg) cells: a subset of T-cells that are immunosuppressive.[16] Treg cells play a critical role in the induction of a privileged tolerant microenvironment at the fetal-maternal interface.[4]

Growing evidence suggests that women with unexplained RPL had remarkably reduced levels of Treg cells in peripheral blood as well as in deciduas.[17-19] The reduction of Treg cell in unexplained RPL patients has been linked to decreased expression of *Foxp3*.[19] Moreover, a reduced suppressive capacity of Treg cells has also been implicated in unexplained RPL.[5,20] Therefore, it is hypothesized that the reduced number and/or activity of Treg cells predisposes to miscarriage. Single nucleotide polymorphisms of *Foxp3* gene may change its functional role or quantity.[21]

Due to their important regulatory role in the immune system and their involvement in immune-related diseases, this study was designed in order to test whether *IL-21* (rs2055979 G/T, and rs13143866 A/G) and *Foxp3* (rs2232365A/G and rs3761548A/C) polymorphisms are associated with RPL in Palestinian women.

METHODS

Study Population

The study was conducted on 100 Palestinian women, 18–35 years old, who had at least two RSAs ≤20 weeks of gestation. Age and ethnicity matched 100 women with at least two live births and without a previous history of abortion or pregnancy-associated complications served as the control group.

Ethical Considerations

Informed consent was obtained from all participants.

DNA extraction and polymorphism determination

The DNA was isolated from whole blood samples using Wizard DNA extraction kit (Promega, USA) as described by the manufacturer. The isolated DNA was stored at -20C◦ until analysis of *IL-21* and *Foxp3* gene polymorphisms.

1. Analysis of IL-21 polymorphisms by AS-PCR & PCR-RFLP

IL-21(G1472T) rs2055979 genetic polymorphism was analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) protocol using the primers presented in Tables 1 and 2.[22] The PCR conditions were as described in Table 2. The PCR products were digested with 0.2 μL of NlaIII restriction enzymes (New England Biolabs, UK) at 37°C for 16 hours and then separated on a 2% agarose gel.

Table 1: Primers, restriction enzyme and PCR Protocols used for genotyping *Foxp3* and *IL-21* SNPs.

SNP	Enzyme	Primers5'-3'	Method
IL-21 rs2055979	NlaIII	**F:** GCTCTGAACCCAAACACTCTC	PCR-RFLP
		R: ACAGCCAGGAAACTCTGGAA	
IL-21 rs13143866 A Allele	-----	**F:** AACAGTTAAACAAGGTGCATGAGATGCTAGAAATA	AS-PCR
		R: AAATGTTTTCATTGTCACAGACCTTTAATTGCATAA	
IL-21 rs13143866 G Allele	-----	**F:** CAGTTAAACAAGGTGCATGAGATGCTAGAGATG	
		R: AAATGTTTTCATTGTCACAGACCTTTAATTGCATAA	
Foxp3 rs2232365 A Allele	-----	**F:** CCCAGCTCAAG AGACCCCA	AS-PCR
		R: GGGCTAGTGAGGAGGCTATTGTAAC	
Foxp3 rs2232365 G Allele	-----	**F:** CCAGCTCAAGAGACCCCG	
		R: GCTATTGTAACAGTCCTGGCAAGTG	
Foxp3 rs3761548	PstI	**F:** GCCCTTGTCTACTCCACGCCTCT	PCR-RFLP
		R: CAGCCTTCGCCAATACAGAGCC	

Table 2: The PCR mix and reaction conditions used for genotyping *Foxp3* and *IL-21* SNPs.

SNP	PCR thermocycling conditions	PCR mix condition
IL-21 rs2055979 NlaIII PCR-RFLP	94°C, 4 min 94°C, 1 min; 64°C, 30 sec; 72°C, 30 sec; 30 cycle 72°C, 7min	Total volume of 10 μL, with 5 μL Taq PCR Master mix (Promega, USA), 1μL(10pmol) of primers, 2μL Nuclease-Free Water, 1μL (20ng) of genomic DNA,
IL-21 rs13143866 AS-PCR A Allele &G Allele	94°C, 3 min 94°C, 30sec; 66°C, 30 sec; 72°C, 40 sec; 35 cycle 72°C, 10 min	Total volume of 20μL, with 10 μL Taq PCR Master mix, 2μL (10pmol) of each primer,4μL Nuclease-Free Water, 2 μL (20ng) of genomic DNA. Note: each allele was determined in a separate tube.
Foxp3 rs2232365 AS-PCR A Allele &G Allele	95°C, 3 min; 95°C, 30 sec; 66°C, 45 sec; 72°C, 50 sec; 5 cycle; 95°C, 30 sec; 61°C, 50 sec; 72°C, 50 sec; 15 cycle; 95°C, 50 sec; 61°C, 1 min; 72°C, 1.5 min; 15 cycle; 72°C, 7min.	
Foxp3 rs3761548 PstI PCR-RFLP	95°C, 3 min; 95°C,30 sec; 63°C, 30sec; 72°C, 1min; 35 cycle; 72°C, 7min.	Total volume of 20 μL, with 10 μLTaq PCR Master mix, 2 μL (10pmol) of primers, 4 μL Nuclease-Free Water, 2 μL (20ng) of genomic DNA.

IL-21 (C1455T) rs13143866 genetic polymorphism was analyzed by allele-specific PCR (AS-PCR) using the primers described in Tables 1 and 2. The PCR conditions were as described in Table 2. The AS-PCR primers were designed using WebSNAPER software (http://pga. mgh.harvard.edu/cgi-bin/snap3/ websnaper3.cgi).

2. Analysis of Foxp3 gene polymorphisms by AS-PCR and PCR-RFLP

Genotypes of rs2232365A/G were determined by using the AS-PCR primers shown in Table 1.[23] A "touch-down" protocol was applied after an initial denaturing step for 3 min at 95°C. The PCR parameters used for amplification of this SNP were as presented in Table 2.

Genotyping of rs3761548A/C Polymorphisms were determined using PCR-RFLP (Tables 1 and 2) method. The parameters for PCR are presented in Table 2. A 10μL aliquot was digested with 1μL of PstI restriction enzyme (NEB, UK) at 37°C for 16 hours and then separated on a 2% agarose gel.

Statistical analysis

The genotype, allele frequency in RPL patients and the controls were analyzed by standard Chi-square test and odds ratio (OR) for risk of RPL at 95% confidence intervals (CI). All statistical analyses were performed using the SPSS 17.0 software package (SPSS, Chicago, I/l, USA). Hardy-Weinberg equilibrium (HWE) was tested using a freely available software: (http://www. oege. org/software/hwe-mr-calc.shtml).

RESULTS

Genotypes of IL-21

In PCR-RFLP genotyping of rs2055979 G/T, the major G allele should yield two bands (162 and 51 bp) whereas, the polymorphic T allele should give one band (213 bp). Consequently the genotypes where defined as G/G (162 and 51bp), G/T (213, 62,51bp) and T/T (213bp). AS-PCR genotypes of rs13143866 A/G where defined by the

presence of one or two different band(s): G/G (373bp), A/G (373 bp and 375 bp), and A/A (375 bp).

Genotypes of Foxp3

For AS-PCR genotyping rs2232365A/G, the A/A, A/G, and G/G genotypes should produce (442 bp), two (442 bp and 427 bp), and one (427 bp) bands, respectively. Genotypes of rs3761548A/C were defined by PCR-RFLP on basis of the presence of three different patterns of bands as A/A (487 bp), A/C (487 bp, 329 bp and 158 bp), and C/C (329 bp, 158 bp).

Genotypic and allelic distribution of IL-21 and Foxp3 polymorphisms in RPL subjects and controls.

The genotypic and allelic frequencies of the study population are shown in (Table 3). Statistical analyses revealed that no statistically significant differences existed between RPL cases and controls in terms of the *IL-21* rs2055979 G/T (p>0.05) or rs13143866 A/G (p>0.05) polymorphisms. Taking the homozygous wild-type as a reference, Chi-square and odds ratio analyses demonstrated no significant association between RPL and the homozygous or heterozygous genotypes of the polymorphic allele rs2055979 G/T [OR (95% CI)= 0.78 (0.36-1.67), 0.79 (0.41-1.52), respectively] or rs13143866 A/G [OR (95% CI)= 0.72 (0.33-1.59), 0.77 (0.42-1.43), respectively] genotypes (Table 3).

Table 3: Genotype and allele frequencies of *IL-21* and *Foxp3* polymorphisms in controls and RPL patients.

SNPs	Genotype or allele	RPL %	Controls %	X2 value	P value	OR (95% CI)
IL-21 rs2055979	G/G	32	27	0.604	0.739	1
	G/T	45	48	0.494	0.482	0.79 (0.41-1.52)
	T/T	23	25	0.423	0.515	0.78 (0.36-1.67)
	G/T+ T/T	68	73	0.601	0.438	0.79 (0.43-1.45)
	Normal G	109	102	0.491	0.483	0.87 (0.59-1.29)
	Mutant T	91	98			
IL-21 rs13143866	G/G	52	45	1.006	0.605	1
	A/G	33	37	0.680	0.410	0.77 (0.42-1.43)
	A/A	15	18	0.655	0.418	0.72 (0.33-1.59)
	A/G+ A/A	48	55	0.981	0.322	0.76 (0.43-1.32)
	Normal G	137	127	1.114	0.291	0.80 (0.53-1.21)
	Mutant A	63	73			
Foxp3 rs2232365	A/A	20	25	10.701	0.005*	1
	A/G	49	63	0.006	0.937	0.97 (0.48-1.95)
	G/G	31	12	6.898	0.009*	3.23 (1.33-7.85)
	A/G+ G/G	80	75	0.717	0.40	1.33 (0.68-2.60)
	Normal A	89	113	5.761	0.016*	1.62 (1.09-2.40)
	Mutant G	111	87			
Foxp3 rs3761548	C/C	33	44	10.646	0.005*	1
	C/A	37	42	0.249	0.617	1.18 (0.63-2.21)
	A/A	30	14	7.195	0.007*	2.86 (1.31-6.22)
	C/A+ A/A	67	56	3.38	0.066	1.72 (0.96-3.06)
	Normal C	103	130	7.494	0.006*	1.75 (1.17-2.61)
	Mutant A	97	70			

*P value ≤ 0.05 is considered statistically significant.

On the other hand, the genotypic and allelic distributions of *Foxp3* rs2232365 A/G and rs3761548 A/C were statistically different between the RPL and the controls. Regarding rs2232365 G allelic distribution and as shown in Table 3, the risk of RPL in women with the minor G allele (and its homozygous genotype G/G) was significantly higher than that in women with A allele (OR =1.62, 95% CI= 1.09-2.40).

Similarly, the distribution of genotypes and alleles at the rs3761548 SNP were significantly different between the RPL and controls. The AA genotype and the A allele frequency were significantly higher in RPL women with OR = 2.86, 95% CI= (1.31-6.22) and OR = 1.75, 95% CI= (1.17-2.61)), respectively.

Hardy-Weinberg equilibrium

All the four SNPs were tested for HWE. The *IL-21* rs2055979 and *Foxp3* rs2232365 genotypic distributions were in HWE. In the contrary, the genotypes of *IL-21* rs13143866 and *Foxp3* rs3761548 polymorphisms deviated from HWE.

Combined genotypes of the investigated IL-21 and Foxp3 polymorphisms in RPL subjects and controls.

Statistical analysis revealed that the various combinations of the two *IL-21* genotypes are not significantly different between the two study groups. On the other hand, the *Foxp3* genotypic combination GG (of rs2232365 A/G) and AA (of rs3761548 A/C) are significantly more prevalent in the RPL group (Table 4).

Table 4: Combined *Foxp3* genotypes and risk of RPL.

Combined genotypes	RPL	Control	$\chi 2$ value	*P* value	OR (95% CI)
AA+CC	53	69	25.707	0.001	1
AA+AC	57	67	0.159	0.69	1.11 (0.67-1.83)
AA+AA	50	39	3.341	0.068	1.67 (0.96-2.90)
AG+CC	82	107	0.000	0.992	0.998 (0.63-1.58)
AG+AC	86	105	0.076	0.783	1.1 (0.68-1.69)
AG+AA	79	77	1.423	0.233	1.34 (0.83-2.15)
GG+CC	64	56	2.37	0.124	1.49 (0.90-2.47)
GG+AC	68	54	3.689	0.055	1.64 (0.99-2.72)
GG+AA	61	26	14.572	0.000	3.1 (1.71-5.47)

DISCUSSION

RPL is a frequent multifactorial complication of pregnancy, that has been studied tremendously but the causes and treatment for this ailment are not fully resolved yet. Dysregulated immunity was proposed as a potential mechanism underlying RPL[3,24] and that inter-individual variation in the anti-inflammatory and pro-inflammatory cytokine levels may modify the risk of unexplained RPL.[7,25]

In this regard, the success of pregnancy depends on maintaining a fine balance between Th1 and Th2 immunity.[26] This was reportedly characterized by a shift to Th2 (humoral) immunity, which involves augmentation in the production of Th2 cytokines accompanied by attenuation of Th1 cytokines synthesis.[3,8,26] Recently, it has been shown that T cell-derived IL-21 cytokine can overcome Treg-mediated immunosuppression and increase the level of Th17 and NK cells (favoring Th1 type immunity) in the decidua and peripheral blood in RPL patients.[27] Foxp3 is a key regulator for development and function of the immunosuppressive Treg cells and it has been proposed that Foxp3 plays a critical role in induction of fetal-maternal tolerance.[16]

Variations in the DNA sequence - SNPs in particular - may influence production and/or activity of cytokines and proteins involved in immune response, and this can modulate an individual's susceptibility to inflammatory and autoimmune diseases. Indeed, studies have shown that *IL-21* and *Foxp3* polymorphisms are associated with various inflammatory and autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and SLE, that may also predispose to RPL.[3,7,8,28-30] However, very limited studies have investigated the direct relation between *IL-21* and *Foxp3* polymorphisms and RPL.

Results of the present work showed that no statistically significant differences were evident between RPL cases and controls in terms of the allelic and genotypic distributions of *IL-21* intronic rs2055979 and rs13143866 polymorphisms. Therefore, it is concluded that these two polymorphisms do not contribute to the risk of RPL in our population. This contradicts the results of a Tunisian study where both *IL-21* rs2055979 and rs13134866 SNPs were found to be independently associated with an increased risk of RPL.[31]

On the other hand, the two *Foxp3* polymorphisms (rs2232365 and rs3761548) proved to be significantly associated with RPL. This result consolidates the findings of Chen et al., (2013) and Wu et al., (2011) who reported significant associations between pre-eclampsia and rs3761548 in Indian women and between the two polymorphisms and unexplained RPL in Chinese women.[32] In the contrary, Abbasirad et al., (2013) found no significant relation between rs3761548 and RPL in Iranian

women. The association between those *Foxp3* gene polymorphisms and adverse pregnancy outcomes could be due to abnormal transcription of *Foxp3* and indeed, Shen et al., (2010) in their study on psoriasis patients have shown that *Foxp3* rs3761548 A/A genotype causes loss of bindings to the E47 and c-Myb transcription factors, leading to defective transcription of *Foxp3* gene.

Discrepancies between results of genetic association studies like those encountered here could be due to many reasons including population genetic variation (background) unrelated to the investigated alleles, presence of nucleotide polymorphism somewhere else in the examined gene e.g., in the coding/non-coding regions, epigenetic alterations and linkage disequilibrium to other sequence variants in the vicinity of the studied loci.

Interestingly, the *IL-21* rs13143866 and *Foxp3* rs3761548 genotypes deviated significantly from HWE. In both occasions the observed homozygous genotypes were more frequent and the heterozygotes were less frequent than expected. Similar results were reported for *IL-21* rs13143866 in the Tunisian study and for *Foxp3* rs3761548 in the Chinese study.[23,31] Deviation from HWE can be caused by many factors. Assuming that there is no genotyping errors, the observed deviation could be due to the frequent non-random mating (high rate of consanguineous marriage), which is prevalent in our population, resulting in increased homozygosity. Another explanation could be the small sample size, which results in violating the HWE.

Stemming from their immunosuppressive function, Treg cells offer an attractive target for treatment of diseases associated with immune tolerance disturbances and hold great promise for reproductive application. Pharmaco-therapeutic agents that influence functioning of Treg cells already exist, and although these potential therapies are still at the experimental stage, they could offer therapeutic options for pregnant women with autoimmune diseases (e.g., SLE) in the future.[29,33]

In conclusion, results of the present study revealed that among the four investigated polymorphisms the two *Foxp3* functional polymorphisms (rs2232365 and rs3761548) proved to be significantly associated with RPL and could represent a potential risk factor for the occurrence of RPL in Palestinian women. Future work should be directed towards confirming this result in a larger RPL sample and correlating the level of Foxp3 protein with the genotypes of those polymorphisms.

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