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IL-10 gene promoter and intron polymorphisms and changes in IL-10 secretion in women with idiopathic recurrent miscarriage

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STUDY QUESTION: Is recurrent pregnancy loss (RPL) associated with polymorphisms in the promoter and intron regions of the interleukin-10 (*IL-10*) gene?

SUMMARY ANSWER: *IL-10* rs1518111 was found to be associated with RPL but the commonly studied promoter variants rs1800872, rs1800871 and 1800896 were not.

WHAT IS KNOWN ALREADY: Reduced expression of IL-10 is implicated in RPL, due to defective maternal immune tolerance (causing early miscarriages) or placental vascular insufficiency (causing late losses). IL-10 production is in part inherited, and *IL-10* gene variants associated with reduced IL-10 expression have been analyzed for their association with RPL, often with inconclusive results.

STUDY DESIGN, SIZE, DURATION: A retrospective case–control study was performed between January 2011 and April 2012. The subjects comprised 296 RPL cases and 305 control women.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Genotyping of the *IL-10* intron (rs1878672, rs3024492, rs1554286, rs1518111, rs3024491, rs3024490) and promoter (rs1800872, rs1800871, rs1800896) variants was done by real-time PCR, with defined clusters.

MAIN RESULTS AND THE ROLE OF CHANCE: A higher minor allele frequency (MAF) of rs1518111 ($P = 0.03$) was in seen RPL cases; but the MAFs of the remaining SNPs were comparable between cases and controls. Setting the homozygous major allele genotype (1/1) as the reference, significantly higher frequencies of heterozygous rs1554286 and rs1800872, and homozygous rs1800896 genotype carriers, and a reduced frequency of homozygous rs1518111 genotype carriers, were seen in RPL cases, while the distribution of the remaining genotypes were comparable between cases and controls. Serum IL-10 levels were significantly reduced in RPL cases compared with control women ($P = 0.002$), and this correlated with rs1518111 and rs1800871 genotypes in both groups, and with the rs1800872 genotype among control women. A nine-locus (rs1878672, rs3024492, rs1554286, rs1518111, rs3024491, rs3024490, rs1800872, rs1800871 and rs1800896) haploview analysis demonstrated an increased frequency of haplotype 112112121 in RPL cases, thus conferring a disease susceptibility nature to this haplotype.

LIMITATIONS, REASONS FOR CAUTION: The main limitation of this study was that it was limited to Bahraini Arabs, thereby necessitating parallel studies of other ethnic groups. Another limitation is the study design, which prompts speculation on whether it is a cause–effect relationship.

WIDER IMPLICATIONS OF THE FINDINGS: While the lack of association of the various *IL-10* promoter variants with RPL was in agreement with reports from varied ethnic groups, this is the first study to confirm the association between *IL-10* rs151811 intronic variant and RPL.

† R.H.Q. and K.M. contributed equally to the work.

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Key words: haplotypes / idiopathic recurrent miscarriage / interleukin-10

Introduction

Recurrent pregnancy loss (RPL) is a significant obstetric complication, which affects 2–4% of healthy women. While studies have identified several modifiable and non-modifiable factors, including parental chromosomal and structural abnormalities, immunologic factors and thrombophilia, along with nutritional and environmental factors (Bogdanova and Markoff, 2010; Coulam and Acacio, 2012), most RPL cases are idiopathic (Jaslow *et al.*, 2010). Successful initiation of pregnancy is coordinated by ovarian progesterone and estrogen, whereby a surge in progesterone inhibits estrogen-dependent effects, thus facilitating endometrium-directed migration of natural killer (NK) and other immune cells, and up-regulation of the expression of adhesion molecules needed for blastocyst adherence and implantation (Cha *et al.*, 2012; Salker *et al.*, 2012). While failure in these mechanisms results in subfertility, an extended altered progesterone-estrogen imbalance is linked with early pregnancy loss (Cha *et al.*, 2012; Salker *et al.*, 2012).

Successful pregnancy also depends on the induction of maternal tolerance to foreign fetal tissues, and a distinct cytokine production profile which facilitates placental development and fetal growth (Salamonsen *et al.*, 2007; Houser, 2012). In this regard, it has been shown that decidual cells around the early conceptus inhibit maternal immunity, so as to protect the allogeneic fetus from infiltrating cytotoxic T cells (CTLs). A Th1/Th2 paradigm has been proposed to describe altered inflammatory changes linked with early pregnancy (Saito *et al.*, 2010; Nakashima *et al.*, 2012), in which a Th1-type response mediated by IL-2, IL-12 and interferon-γ is regarded to be required for early implantation, while the maintenance of pregnancy is dependent of the contribution of regulatory (Treg) or anti-inflammatory (Th2-type) cytokines, in particular Interleukin (IL)-10; this proposal later developed into the Th1/Th2/Th17/Treg paradigm (Saito *et al.*, 2010). This was made evident by the shift in the Th1:Th2 cytokine ratio in favor of enhanced Th1 and reduced Th2 expression in the maternal circulation and placental villous tissue in first trimester miscarriages (Prigoshin *et al.*, 2004; Saito *et al.*, 2010; Nakashima *et al.*, 2012).

IL-10 is a pleiotropic cytokine produced by activated Th2 cells, B-cells, monocytes and macrophages, which play a central role in the maintenance of maternal–fetal tolerance (Denney *et al.*, 2011; Wang *et al.*, 2011). IL-10 acts by down-regulating the production of Th1 cells- and macrophages-derived chemokines and cytokines (Sabat *et al.*, 2010), by interfering with antigen presentation (Sabat *et al.*, 2010), and by inhibiting CTL or NK cell responses (Zhai *et al.*, 1998; Sabat *et al.*, 2010). A role for IL-10 in regulating pregnancy outcomes has been reported. The expression of IL-10 by cytotrophoblasts and decidual T cells, and predominance of the Th2 program during early pregnancy and the peri-implantation period has been shown to facilitate successful pregnancy (Fan *et al.*, 2011; Piao *et al.*, 2012). Reduced IL-10 levels have been seen in RPL (Bates *et al.*, 2002) and have been associated with defective maternal immunotolerance in early pregnancy or with placental vascular insufficiency in late pregnancy failure (Hanna *et al.*, 2000; Cochery-Nouvellon *et al.*, 2009).

Since cytokine expression is partly constitutional, several studies have examined the association of cytokine gene polymorphisms (as surrogates of cytokine expression) with altered levels of cytokine production and key obstetric complications, often with inconclusive findings (Karhukorpi *et al.*, 2001; Zammiti *et al.*, 2006; Cochery-Nouvellon *et al.*, 2009; Kaur and Kaur 2011). The *IL-10* gene is located on chromosome 1 (1q31–q32), and there are several functional single-nucleotide polymorphisms (SNPs) which control IL-10 secretion (D'Alfonso *et al.*, 2000; Mormann *et al.*, 2004), in particular the promoter SNPs −1082A>G (rs1800896), −819T>C (rs1800871) and −592A>C (rs1800872) (D'Alfonso *et al.*, 2000; Mormann *et al.*, 2004). The association of *IL-10* promoter SNPs with recurrent miscarriage has been reported by several studies but with inconclusive findings (Kamali-Sarvestani *et al.*, 2005; Zammiti *et al.*, 2008; Cochery-Nouvellon *et al.*, 2009; Parveen *et al.*, 2013). Here we analyzed the association of the common *IL-10* promoter SNPs (−1082A>G, −819T>C and −592A>C), and novel intronic SNPs, with RPL in Bahraini women.

Methods

Subjects

This retrospective case–control study was performed at outpatient OB/GYN clinics in Manama and Rifaa (Bahrain), between January 2011 and April 2012. Study subjects comprised 296 consecutively recruited women with confirmed RPL (mean age 31.6 ± 5.4 years). RPL assessment was based on Royal College of Obstetricians and Gynecologists (RCOG) guidelines (http://www.rcog.org.uk/guidelines), which included screening of antiphospholipid antibodies (lupus anticoagulant or anticardiolipin antibodies), karyotyping of both partners, pelvic ultrasound scan for evaluating uterine anatomy (hysteroscopy or sonohysteroscopy), and inherited thrombophilias screening (factor V Leiden, factor II G20210A). In all cases, these procedures were performed. The inclusion criteria were three or more miscarriages of unknown etiology and with the same partner, which occurred during the first trimester of gestation. Exclusion criteria included older age (>40 years) at first pregnancy, Rh blood group incompatibility, previous history of pre-eclampsia defined as elevated systolic and diastolic blood pressure (BP) > 145/95 mmHg, or a rise in systolic/diastolic BP > 30/15 mmHg on at least two occasions, and preclinical miscarriages and/or biochemical pregnancy. Patients were also excluded if they reported systemic autoimmune disease, diabetes mellitus, thyroid dysfunction, anatomical disorders, infections (toxoplasmosis, HCMV, rubella, HIV, Group B streptococci, Chlamydia trachomatis, hepatitis B and C and bacterial vaginosis) or liver function abnormalities.

The control group comprised 305 multiparous women with 2 or more successful pregnancies, and no miscarriages (spontaneous or induced), and a negative family history of miscarriage. The control women were university and hospital employees, or volunteers, who were recruited following a routine checkup after an uncomplicated pregnancy, and were matched to cases according to age and self-declared ethnic origin.

Blood samples were taken from all participants in EDTA-containing tube for total genomic DNA extraction and in plain tubes (no preservatives) for

serum preparation. All women were required to sign a consent form before inclusion in the study. The Research and Ethics Committee of the Arabian Gulf University approved the study protocol.

IL-10 genotyping

We selected polymorphisms in the *IL-10* gene with a minor allele frequency (MAF) of >5% in Caucasians, using the SNPbrowser software (version 4.0, Applied Biosystems, Foster City, CA, USA). *IL-10* genotyping was performed using the allelic (VIC- and FAM-labelled) discrimination method. TaqMan assays, as assay-on-demand, were ordered from Applied Biosystems: C_12084302_20 (rs1878672), C_22274509_10 (rs3024492), C_8828812_10 (rs1554286), C_8828803_1_ (rs1518111), C_15983669_10 (rs3024491), C_15983670_10 (rs3024490), C_1747363_10 (rs1800872; −592 C>A), C_1747362_10 (rs1800871; −819C>T) and C_1747360_10 (rs1800896; −1082A>G). The reactions were performed in 6 μl volumes on a StepOne real-time PCR system, according to the manufacturer's instructions (Applied Biosystems). Replicate blinded quality control samples were included to assess reproducibility of the genotyping procedure; concordance was >99%.

Serum IL-10 Measurements

Serum was prepared by centrifugation of coagulated blood tubes at 2000*g* for 10 min at room temperature, and was stored in 200 μl aliquots at or below −30°C. Samples were tested for IL-10 using a human IL-10 sandwich enzyme-linked immunosorbent assay (catalogue number D1000B; R&D Systems, Minneapolis, MN, USA). Assay sensitivity was 3.9 pg/ml, and inter-assay and intra-assay precision (CV%) ranged from 5.9–7.5% and 1.7–5.0%, respectively.

Table I Demographics and clinical characteristics of cases and controls.

	Cases[a]	Controls[b]	*P*[b]
Age at inclusion in study[c]	31.6 ± 5.4	31.6 ± 4.9	0.88
Body mass index (kg/m^2)[c]	**26.3 ± 5.4**	**25.2 ± 4.3**	**0.006**
Obesity [*n* (%)][d]	**58 (19.6)**	**37 (12.1)**	**0.02**
Smokers [*n* (%)][d]	30 (10.1)	32 (10.8)	0.69
Systolic blood pressure (mmHg)[c]	**114.1 ± 11.9**	**120.2 ± 17.0**	**<0.001**
Diastolic blood pressure (mmHg)[c]	**72.0 ± 8.4**	**75.8 ± 9.1**	**<0.001**
Glucose (mmol/l)[c]	5.1 ± 0.9	5.2 ± 0.7	0.55
Menarche (years)[c]	**12.2 ± 1.1**	**12.8 ± 1.0**	**<0.001**
Number of pregnancies[c]	4.2 ± 1.5	4.0 ± 1.1	0.11
Number of children[c]	**0.8 ± 1.1**	**4.0 ± 1.1**	**<0.001**
Miscarriages[c]	**3.6 ± 1.0**	**0.0 ± 0.1**	**<0.001**
Serum IL-10 (pg/ml)[c]	**5.3 ± 1.8**	**6.1 ± 1.2**	**0.002**

Boldface indicates significant differences.
[a]A total of 296 RPL cases and 305 control women were included.
[b]Student's *t*-test (continuous variables), Pearson's χ^2 test (categorical variables).
[c]Mean ± SD.
[d]Percent of total within each group/subgroup.

Table II *IL-10* SNPs analyzed.

SNP	Location[a]	Alleles	HWE	Cases[b]	Controls[b]	χ^2	*P*[c]	OR (95% CI)	Power (%)
rs1878672	206943713	C:A	0.36	205 (0.36)	201 (0.33)	0.92	0.34	1.12 (0.88–1.43)	71
rs3024492	206944112	T:A	0.07	90 (0.16)	85 (0.14)	0.71	0.40	1.15 (0.83–1.58)	43
rs1554286	206944233	C:T	0.06	153 (0.27)	141 (0.23)	2.17	0.14	1.22 (0.94–1.59)	69
rs1518111	**206944645**	**G:T**	**0.44**	**141 (0.24)**	**181 (0.29)**	**4.78**	**0.03**	**0.75 (0.58–0.97)**	**89**
rs3024491	206945046	G:T	0.22	192 (0.33)	183 (0.30)	1.51	0.22	1.17 (0.91–1.49)	73
rs3024490	206945311	G:T	0.07	201 (0.35)	192 (0.31)	1.56	0.21	1.17 (0.92–1.49)	54
rs1800872	206946407	C:A	0.80	194 (0.33)	182 (0.30)	1.37	0.24	1.16 (0.91–1.48)	92
rs1800871	206946634	C:T	0.05	202 (0.34)	181 (0.29)	3.00	0.08	1.24 (0.97–1.58)	74
rs1800896	206946897	A:G	0.26	230 (0.39)	216 (0.35)	1.74	0.19	1.17 (0.93–1.48)	84

MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; Boldface indicates significant differences.
[a]Location on chromosome based on dbSNP build 125.
[b]Minor allele defined based on frequency in controls.
[c]Adjusted *P*-value, adjusted for BMI, menarche, and systolic and diastolic blood pressure.

Statistical analysis

Statistical analysis was performed on SPSS v. 20.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as percentages of total (categorical variables) or as mean ± SD (continuous variables). Student's *t*-test was used to determine differences in means, and Pearson χ^2 or Fisher's exact test was used to assess inter-group significance. Allele frequencies were calculated by the gene-counting method, and genotypes were tested for departures from Hardy–Weinberg equilibrium (HWE) in the control population using Haploview version 4.2 (http://www.broad.mit.edu/mpg/haploview). Bonferroni multiple-comparisons correction method was employed in calculating the corrected *P*-value, as per: $Pc = 1 - (1 - P)^n$, where n = number of comparisons.

All analyses were conducted under additive genetic effect, as it is the conservative model, using the SNPStats software (bioinfo.iconcologia.net/snpstats/). We used CaTS Power Calculator (www.sph.umich.edu/csg/abecasis/cats) to calculate the power to detect an association between *IL-10* variants and RPL in our cohort. The parameters used were: 296 RPL cases and 305 control women, genotypic relative risk for heterozygotes (1/2) and minor allele homozygotes (2/2), and the MAF for RPL cases and controls for the 9 tested SNPs, and assumed a 2.5% population prevalence of RPL (unpublished Bahrain Ministry of Health statistics). Assuming these parameters, we calculated the overall power (72.1%) as the average power of the nine tested SNPs. Linkage disequilibrium analysis was performed using Haploview 4.2, and haplotype reconstruction (linear arrangements of alleles on the same chromosome inherited as a unit) was performed by the expectation maximization method (Haploview 4.2) using the default method of Gabriel *et al.* (2002). Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence intervals (95% CI) associated with the RPL risk, taking the control as the reference group. Statistical significance was set at $P < 0.05$, with statistically significant differences being designated as boldface in the tables.

Results

Study subjects

Table I summarizes the demographics and clinical characteristics of study participants. The mean age at inclusion in study ($P = 0.88$), serum glucose ($P = 0.55$), gravida ($P = 0.11$) and prevalence of smoking ($P = 0.69$) were comparable between RPL cases and control women. Significant differences were noted with respect to mean BMI ($P = 0.006$), menarche ($P < 0.001$), and systolic and diastolic blood pressure readings ($P < 0.001$) between RPL cases and control women. Although they did not constitute strong risk factors of RPL, they were nevertheless selected as the covariates that were controlled for in subsequent analyses.

Association studies

Genotype distributions of rs1878672 ($P = 0.36$), rs3024492 ($P = 0.07$), rs1554286 ($P = 0.06$), rs1518111 ($P = 0.44$), rs3024491 ($P = 0.22$), rs3024490 ($P = 0.07$), rs1800872 ($P = 0.80$), rs1800871 ($P = 0.05$) and rs1800896 ($P = 0.26$) were in HWE among study subjects. Table II summarizes the association between *IL-10* SNPs and RPL in cases and control subjects. Of the *IL-10* SNPs analyzed, a higher MAF of only rs1518111 ($P = 0.03$; $\chi^2 = 4.78$) was seen in RPL cases compared with control women; MAFs of the remaining SNPs were not significantly different between cases and controls.

The distribution of *IL-10* genotypes between RPL cases and control women is summarized in Table III. Setting the homozygous major allele

Table III *IL-10* genotype frequencies.

SNP	1/1[a]				1/2[a]				2/2[a]			
	Cases	Controls	*P*[b]	OR (95% CI)	Cases	Controls	*P*[b]	OR (95% CI)	Cases	Controls	*P*[b]	OR (95% CI)
rs1878672	128 (0.46)	134 (0.45)[c]	0.18	1.00 (Ref.)	103 (0.37)	126 (0.42)	0.10	0.84 (0.58–1.20)	46 (0.17)	37 (0.13)	0.23	1.35 (0.82–2.23)
rs3024492	207 (0.74)	227 (0.76)	0.79	1.00 (Ref.)	59 (0.21)	61 (0.20)	1.00	1.04 (0.69–1.56)	13 (0.05)	11 (0.04)	0.62	1.33 (0.58–3.05)
rs1554286	157 (0.55)	192 (0.63)	0.07	1.00 (Ref.)	**107 (0.37)**	**89 (0.29)**	**0.04**	**1.52 (1.06–2.18)**	23 (0.08)	26 (0.08)	0.74	1.04 (0.56–1.93)
rs1518111	**168 (0.57)**	**161 (0.52)**	**0.009**	**1.00 (Ref.)**	113 (0.38)	111 (0.36)	0.65	0.96 (0.68–1.37)	**14 (0.05)**	**35 (0.11)**	**0.003**	**0.37 (0.19–0.72)**
rs3024491	134 (0.46)	155 (0.51)	0.65	1.00 (Ref.)	118 (0.41)	119 (0.39)	0.82	1.09 (0.76–1.54)	34 (0.12)	31 (0.10)	0.89	1.29 (0.75–2.22)
rs3024490	130 (0.45)	160 (0.52)	0.16	1.00 (Ref.)	117 (0.40)	100 (0.33)	0.09	1.42 (0.99–2.05)	42 (0.15)	46 (0.15)	0.75	1.10 (0.68–1.80)
rs1800872	135 (0.46)	162 (0.53)	0.14	1.00 (Ref.)	**128 (0.43)**	**108 (0.35)**	**0.05**	**1.43 (1.00–2.04)**	33 (0.11)	37 (0.12)	0.71	1.10 (0.64–1.87)
rs1800871	133 (0.45)	160 (0.52)	0.28	1.00 (Ref.)	124 (0.42)	113 (0.37)	0.22	1.28 (0.90–1.83)	39 (0.13)	34 (0.11)	0.45	1.37 (0.81–2.32)
rs1800896	**124 (0.42)**	**124 (0.40)**	**0.001**	**1.00 (Ref.)**	124 (0.42)	150 (0.49)	0.12	0.68 (0.47–0.97)	**58 (0.20)**	**33 (0.11)**	**0.001**	**1.69 (1.01–2.83)**

Boldface indicates significant differences.
[a]Genotypes were coded as per '1' = major allele, '2' = minor allele.
[b]2-way ANOVA.
[c]Number of subjects (frequency).

Table IV *IL-10* serum levels.

SNP	1/1[a]		1/2		2/2		P^b
	Cases	Controls	Cases	Controls	Cases	Controls	
rs1878672	5.6 ± 2.3[c]	6.1 ± 1.4	5.1 ± 1.1	6.3 ± 1.0	5.3 ± 1.3	6.0 ± 1.2	0.822
rs3024492	5.1 ± 1.4	6.2 ± 1.2	6.5 ± 2.0	5.7 ± 1.0	5.4 ± 1.5	6.6 ± 1.0	0.433
rs1554286	5.8 ± 1.5	6.4 ± 1.1	4.9 ± 1.3	5.6 ± 1.2	5.3 ± 1.1	5.2 ± 0.9	0.119
rs1518111	**5.7 ± 2.0**	**6.5 ± 1.1**	**5.2 ± 1.5**	**5.8 ± 0.8**	**4.3 ± 1.4**	**4.2 ± 1.5**	**0.018**
rs3024491	4.9 ± 1.6	6.0 ± 1.3	5.4 ± 1.2	6.4 ± 1.1	7.2 ± 3.4	6.0 ± 1.2	0.163
rs3024490	5.7 ± 1.6	6.6 ± 1.0	5.1 ± 1.2	5.0 ± 1.1	5.0 ± 1.4	5.9 ± 1.2	0.451
rs1800872	**5.6 ± 1.3**	**6.4 ± 1.0**	**5.1 ± 1.6**	**6.1 ± 1.3**	**5.2 ± 1.8**	**4.2 ± 0.8**	**0.019**
rs1800871	**5.9 ± 2.1**	**6.5 ± 1.0**	**4.9 ± 1.3**	**6.1 ± 1.2**	**4.3 ± 1.4**	**4.3 ± 0.7**	**0.005**
rs1800896	5.0 ± 1.7	5.9 ± 1.4	5.6 ± 1.3	6.4 ± 1.0	5.5 ± 0.8	6.1 ± 0.9	0.097

Boldface indicates significant differences.
[a]Genotypes were coded as per '1' = major allele, '2' = minor allele.
[b]2-way ANOVA.
[c]IL-10 serum levels ± SD (pg/ml).

genotype (1/1) as the reference, and after controlling for BMI, menarche, and systolic and diastolic blood pressure values (OR = 1.00), significantly higher frequencies of heterozygous (1/2) 1554286 (0.37 versus 0.29) and rs1800872 (0.43 versus 0.35) and homozygous (2/2) rs1800896 (0.20 versus 0.11) genotype carriers, and reduced frequency of homozygous rs1518111 (0.05 versus 0.11) genotype carriers were seen in RPL cases compared with control women. The distribution of the remaining genotypes was comparable between RPL cases and control women.

IL-10 serum levels

Mean serum IL-10 levels were lower in RPL cases than in control women ($P = 0.002$) (Table I). Table IV summarizes the IL-10 levels in the two study groups according to specific *IL-10* genotypes. Among multiparous control women, there was a progressive reduction in serum IL-10 levels in rs1518111, rs1800872 and rs1800871 minor allele-containing genotypes (Table IV). Among RPL cases, there was a parallel decline in serum IL-10 levels in rs1518111 and rs1800871 minor allele-carrying genotypes, which was seen in heterozygous (1/2), and more so in homozygous mutant (2/2) genotype carriers, when compared with homozygous major allele (1/1) genotype carriers. Compared with control women, there was an overall reduction in serum IL-10 levels in RPL cases in most of the tested genotypes (Table IV).

Haplotype analysis

Next, we evaluated the interaction between the nine tested SNPs and their mode of inheritance by analyzing the distribution of 9-locus haplotypes in PL cases and control women. The *IL-10* SNPs were aligned according to their chromosomal locations (http://www.ncbi.nlm.nih.gov/snp), and the interaction between all possible pairs of SNPs was visualized by Haploview (Fig. 1); *IL-10* haplotypes containing rs1878672, rs1554286, rs1518111, rs3024491, rs1800872, rs1800871 and rs1800896 SNPs were constructed based on the prevalence of individual SNPs and linkage disequilibrium (LD) between them (Fig. 1). Haploview analysis demonstrated heterogeneous LD among the *IL10* SNPs studied (Fig. 1). We defined the 'common haplotype' as those with frequencies >2% of the total haplotypes. Accordingly, only eight haplotypes were found to be common, capturing 68.2% of the total possible haplotypes (Table V). The remainder of the haplotypes (31.8%) had very low frequencies (<2%), and thus was deemed uncommon. An increased frequency of haplotype 112112121 ($P < 0.001$) was seen in RPL cases compared with control women after adjusting for BMI, menarche, and systolic and diastolic blood pressure, thereby conferring a disease susceptibility nature to this haplotype (Table V). This association remained significant after correcting for multiple comparisons (Pc < 0.001).

Discussion

Successful pregnancy depends on the cytokine milieu present, which can either be protective or harmful to the conceptus. The traditional paradigm proposed that maternal immunological tolerance of the allogenic fetus in pregnancy is linked with a TH1/Th2 shift to a predominant (anti-inflammatory) Th2 phenotype, with fetal loss being viewed as up-regulation of Th-1 cytokine and attenuation of Th2 cytokine activities (Raghupathy, 1997). More recent evidence indicates a significant contribution of Th17T cell subset to adverse pregnancy outcome, evidenced by the finding that the number of Th17 cells is markedly elevated in recurrent miscarriage, when compared with normal pregnancy, suggesting that increased Th1 and Th17 cell activity, and consequently reduced Th2 cell activity, is operational in women with recurrent miscarriage. IL-10 plays a dual immunosuppressive/anti-inflammatory role during pregnancy; it establishes a Th2 cytokine environment while reducing Th1 cytokine expression (Bates *et al.*, 2002; Hanlon *et al.*, 2002; Denney *et al.*, 2011; Brogin Moreli *et al.*, 2012), which has been highlighted by the findings that increased maternal IL-10 production is associated with successful pregnancy, whereas low levels are linked with recurrent fetal loss (Piccinni *et al.*, 1998; Brogin Moreli *et al.*, 2012). However contradictory findings on the involvement of IL-10 in RPL have also been reported. For example, it was reported that heightened IL-10 production was seen in RPL cases compared with fertile women (Wu *et al.*, 2001; Bates *et al.*, 2002; Denney *et al.*, 2011). While variability

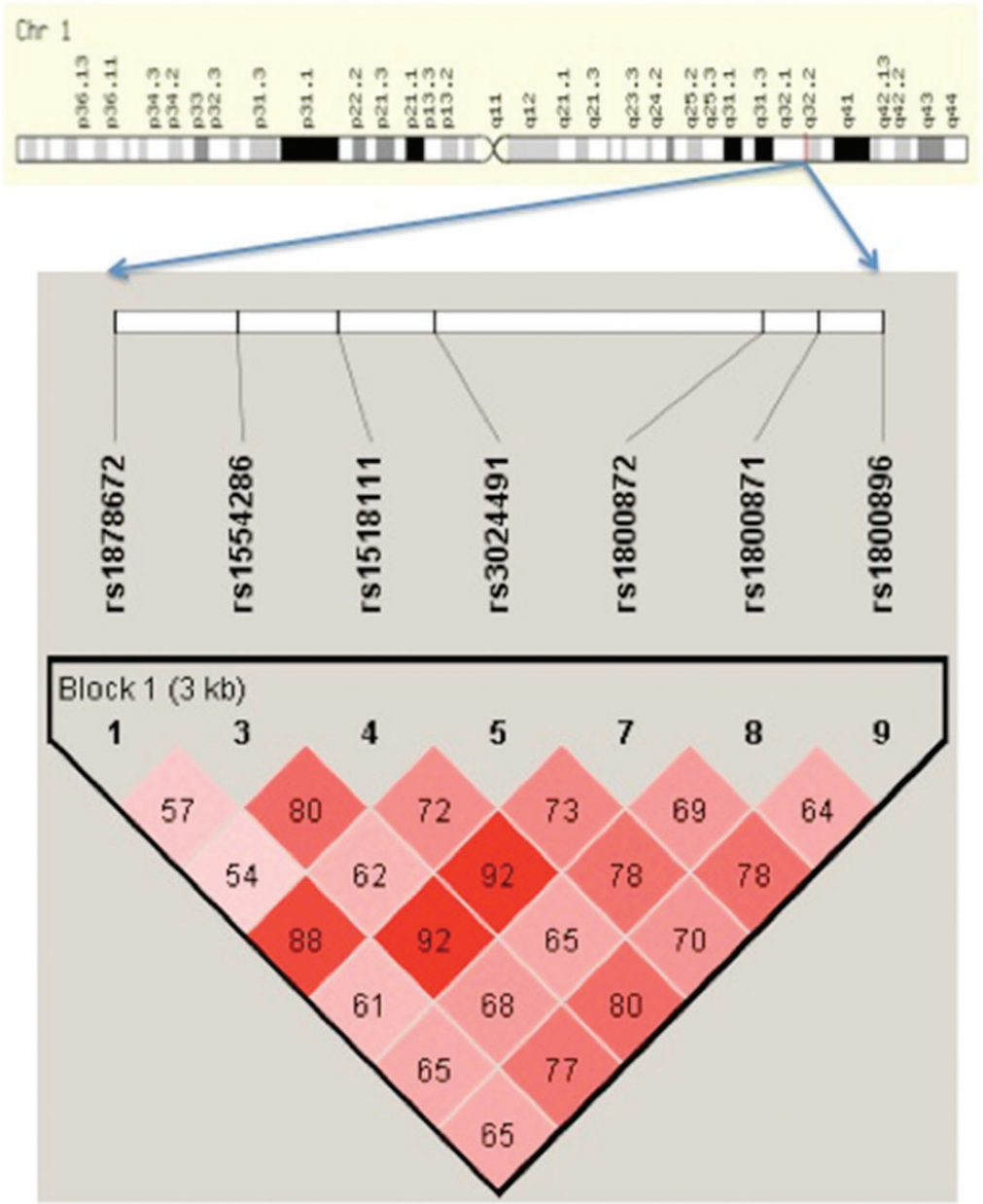

Figure 1 Linkage disequilibrium (LD) map of the *IL-10* SNPs genotyped using Haploview. (Upper panel) Chromosomal location of human *IL-10* gene (chromosome 1; 1q31–q32). (Lower panel) Haploview graph of *IL-10* SNPs analyzed. The positions of the 9 SNPs used (Build 37.3) are indicated along with the basic gene structure, and displayed above the Haploview output. The relative LD between specific pair of *IL-10* SNPs is indicated by the color scheme, which represents the LD relationships. This is based on D′ values (normalized linkage disequilibrium measure or D) multiplied by 100; D′ is calculated as D divided by the theoretical maximum for the observed allele frequencies. Values approaching zero indicate absence of LD, and those approaching 100 indicate complete LD. The red squares represent varying degrees of LD $<$ 1 and LOD (logarithm of odds) $>$ 2 scores; darker shades indicating stronger LD.

within the *IL-10* gene was shown to influence IL-10 secretion, the role of *IL-10* polymorphisms in RPL pathogenesis remains controversial.

We investigated the association of *IL-10* promoter and intron SNPs among Bahraini women with idiopathic RPL, including the commonly studied rs1800872, rs1800871 and rs1800896 promoter variants. MAF for the *IL-10* variants examined in healthy Bahraini was of interest when examined with other ethics groups, such as Asians, European Caucasians, Sub-Saharan Africans, African Americans and (Guajarati) Indians (http://www.ncbi.nlm.nih.gov/SNP/snp). While the overall power was estimated at 72.1%, variability in the power of individual SNPs was noted, which ranged from 43% (rs3024492) to 92% (rs1800872). This heterogeneity is attributed to several factors, including MAF,

genotype relative risk, effect size and extent of LD, since the power to detect association is directly related to LD (Evans and Purcell, 2012). Among Bahraini women, the MAFs of rs1554286, rs1518111, rs3024490, rs1800872 and rs1800871 were comparable to Europeans, while the MAFs of rs1878672, rs3024491 and rs1800896 in Bahraini were more comparable to Africans (Table VI). A reversal in the minor allele usage of rs1554286, rs1518111, and rs3024490, rs1800872, and rs1800871 was noted between Asians and other populations (Table VI).

In contrast to promoter and coding mutations, non-coding intronic variants mutations have been less extensively studied. The presence of most of the intronic mutations within or close to conserved splice sites creates altered splice donor or acceptor sites, resulting in frameshifts and premature termination codons. Evidence from next-generation sequencing documented the association of deep intronic variants with disease states, thus assigning a pathogenic nature to non-coding mutations (Flanagan *et al.*, 2013). This study is the first to investigate the association between RPL and intronic (rs1878672, rs3024492, rs1554286, rs1518111, rs3024491 and rs3024490) polymorphisms. Among the studied *IL-10* variants, rs1518111 was independently associated with RPL risk. This was the first study to link this variant with RPL risk. Previous studies that have examined the relationship between *IL-10* polymorphisms and RPL have focused on the promoter variants rs1800871 (−592C>A), rs1800872 (−819C>T) and rs1800896 (−1082A>G), given their association with altered IL-10 secretion, as evidenced by the lower transcriptional activity linked with the ATA haplotype (Ouma *et al.*, 2008; Parveen *et al.*, 2013).

Conflicting results have been reported on the association of *IL-10* promoter variants with RPL. For rs1800896 (−1082A>G), no significant association with RPL was found in this study. This was in agreement with Argentinian (Prigoshin *et al.*, 2004), Brazilian (Daher *et al.*, 2003), Cypriot (Costeas *et al.*, 2004), Finnish (Karhukorpi *et al.*, 2001), Iranian (Kamali-Sarvestani *et al.*, 2005), Tunisian (Zammiti *et al.*, 2006) and UK (Babbage *et al.*, 2001) studies, but in apparent disagreement with a study on 200 RPL and 300 control North Indian women, which documented strong association of rs1800896 ($P = 0.0004$) with RPL (Parveen *et al.*, 2013). In addition, three earlier meta-analyses yielded conflicting results regarding the association of rs1800896 with RPL. The first, involving six studies including 635 RPL cases, documented no

Table V Haplotype frequencies across 9 *IL-10* SNPs.[a]

Haplotype[b]	Frequency	Case:control frequencies	χ^2	*P*[c]	Pc[d]
111111111	0.235	0.226, 0.255	1.37	0.241	0.89
211121112	0.156	0.165, 0.156	0.17	0.678	1.00
112212121	0.128	0.130, 0.133	0.03	0.864	1.00
221121112	0.05	0.061, 0.043	2.07	0.150	0.73
111111112	0.041	0.041, 0.044	0.10	0.750	1.00
111212221	0.028	0.019, 0.037	3.41	0.065	0.42
112112121	**0.023**	**0.049, 0.000**	**29.91**	**4.5×10^{-8}**	**3.6×10^{-7}**
211121111	0.021	0.019, 0.024	0.37	0.545	1.00

Boldface indicates significant difference.
[a]*IL-10* SNP positions within haplotypes were: rs1878672, rs3024492, rs1554286, rs1518111, rs3024491, rs3024490, rs1800872, rs1800871 and rs1800896.
[b]Alleles were coded as '1' (*major allele*) and '2' (*minor allele*).
[c]Adjusted *P*-value, adjusted for BMI, menarche, and systolic and diastolic blood pressure.
[d]Pc = corrected *P* as per the Bonferroni method.

Table VI MAF of *IL-10* variants studied.[a]

SNP	Bahraini	Asians	Europeans	Sub-Saharan Afr.	African Amer.	Indians
rs1878672	0.33 (C)[b]	0.06 (C)	0.46 (C)	0.26 (C)	0.36 (C)	0.28 (C)
rs3024492	0.14 (A)		0.22 (A)		0.04 (A)	
rs1554286	0.23 (T)	0.29 (C)	0.19 (T)	0.48 (T)	0.44 (T)	0.36 (T)
rs1518111	0.29 (A)	0.29 (G)	0.24 (A)	0.48 (A)	0.48 (A)	0.38 (A)
rs3024491	0.30 (T)	0.01 (T)	0.44 (T)	0.25 (T)	0.36 (T)	0.27 (T)
rs3024490	0.31 (T)	0.31 (G)	0.26 (T)	0.48 (T)	0.50 (T/G)	0.38 (T)
rs1800872	0.30 (A)	0.31 (C)	0.23 (A)	0.47 (A)	50.0% (A)	0.38 (A)
rs1800871	0.29 (T)	0.28 (C)	0.23 (T)	0.47 (T)	0.42 (T)	0.31 (T)
rs1800896	0.35 (G)	0.06 (G)	0.46 (G)	0.27 (G)	0.37 (G)	0.28 (G)

MAF, minor allele frequency.
[a]Obtained through: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi.
[b]MAF prevalence among healthy individuals (minor allele).

significant association between rs1800896 and RPL (Bombell and McGuire, 2008), while the second, which included the six studies included in the earlier analysis of Bombell and McGuire, demonstrated an association between rs1800896 and RPL, but only under the dominant genetic model (Medica *et al.*, 2009). A more recent meta-analysis which included the studies included in the earlier meta-analysis (Bombell and McGuire, 2008; Medica *et al.*, 2009), along with their own results on North Indian women (Parveen *et al.*, 2013), documented an RPL-protective nature of the rs1800896 variant. These apparent discrepancies can likely be attributed to ethnic differences, and hence disease association, as well as to differences in interpretation of the association of rs1800896 with RPL risk.

The promoter variants rs1800872 (−592C>A) and rs1800871 (−819C>T), previously implicated in altered IL-10 secretion, were not associated with RPL among Bahraini women. This was in agreement with an earlier meta-analysis (Bombell and McGuire, 2008) and two case–control studies (Costeas *et al.*, 2004; Prigoshin *et al.*, 2004), and in partial agreement with Iranian (Kamali-Sarvestani *et al.*, 2005) and Indian (Kaur and Kaur, 2011, Parveen *et al.*, 2013) studies. According to the former, rs1800872 but not rs1800871 was associated with RPL (Kamali-Sarvestani *et al.*, 2005), while in the latter study rs1800871 more so than the rs1800872 SNP (Parveen *et al.*, 2013) was linked with RPL. Our results were in sharp disagreement with French (Cochery-Nouvellon *et al.*, 2009) and Tunisian (Zammiti *et al.*, 2006) studies, which demonstrated an association of these SNPs with exclusively early RPL (before 10 weeks of amenorrhea). This may be reconciled by differences in ethnicities (Kamali-Sarvestani *et al.*, 2005; Zammiti *et al.*, 2006; Cochery-Nouvellon *et al.*, 2009; Parveen *et al.*, 2013), selection of cases (Zammiti *et al.*, 2006; Cochery-Nouvellon *et al.*, 2009) and the failure to test for other *IL-10* promoter variants (Kamali-Sarvestani *et al.*, 2005; Kaur and Kaur, 2011; Parveen *et al.*, 2013).

To the best of our knowledge, this is the first study to test the possible association of *IL-10* intronic variants and RPL. Results obtained clearly showed the association of rs1518111, but not of rs1878672, rs3024492, rs1554286, rs3024491 or rs3024490 variants, with the presence of RPL. The rs1518111 has been shown to be associated with altered IL-10 secretion (Remmers *et al.*, 2010), and with hypertension in ischemic stroke (Park *et al.*, 2013), and Behçet's Disease (Remmers *et al.*, 2010; Xavier *et al.*, 2012), presumably by influencing inflammatory and coagulation events. Additional population-based case–control studies are needed to validate the contribution of this variant with altered RPL risk, specifically if the rs1518111 intron variant, found here to be associated with RPL, might prove to be useful in predicting miscarriage in future pregnancy.

Serum IL-10 levels were markedly lower in RPL cases than in control women, in agreement with previous findings documenting reduced IL-10 levels in patients with confirmed repeated miscarriage (Bates *et al.*, 2002). This indicates that optimal IL-10 levels are critical for successful pregnancy (Rolle *et al.*, 2013), and that altered IL-10 levels distinguish between successful pregnancies and miscarriages, as was suggested (Rolle *et al.*, 2013). However, this is an oversimplification, given the range of IL-10 serum levels obtained here (3.44–13.99 pg/ml) and elsewhere (Wilson *et al.*, 2004), and the sequential expression of cytokines at distinct stages in pregnancy in uterine, decidual and placental tissues (Chaouat *et al.*, 2004). While the role IL-10 plays in embryo–maternal interactions, in particular in early implantation (Chaouat *et al.*, 2004; Rolle *et al.*, 2013), remains to be seen, results in this study support the notion that implantation failure and miscarriage may be attributed, at least in part, to constitutional reduction in IL-10 expression and secretion.

Since the interaction of specific variants localized within a haplotype, defined as linear arrangements of alleles on the same chromosome and inherited as a unit, is more informative when compared with the analysis of the association of single variants in determining disease susceptibility, including RPL, we analyzed the linkage disequilibrium pattern (non-random association of alleles at $\geq$2 loci originating from a single chromosome) between the tested SNPs. The nine tested *IL-10* polymorphisms were in linkage disequilibrium, thus allowing for construction of 9-locus (rs1878672, rs3024492, rs1554286, rs1518111, rs3024491, rs3024490, rs1800872, rs1800871 and rs1800896) haplotypes. However, Haploview analysis revealed marked heterogeneity in the haplotypes obtained, with most of the diversity (68.2%) captured by only 8 haplotypes, the frequencies of which exceeding the threshold of >2% set for assigning 'common' nature. The 112112121 haplotype was identified as positively associated with RPL ($Pc = 3.6 \times 10^{-7}$), after correcting for multiple comparisons, and adjusting for the covariates BMI, menarche, and systolic and diastolic blood pressures. It is tempting to speculate that information obtained from haplotypic analysis could be used clinically for the diagnosis/prognosis of future miscarriages in high-risk women.

In conclusion, our study demonstrated that the *IL-10* rs1518111 SNP, but not the commonly studied promoter variants rs1800872, rs1800871 and rs1800896, is associated with RPL. Our study has strengths, namely that it was sufficiently powered, that cases and controls were ethnically matched, hence minimizing the problems of differences in genetic background and that potential covariates were controlled for in single SNP and haplotype analysis. However, our study has some limitations. It was limited to Bahraini Arabs, thereby necessitating parallel studies on different ethnic groups. Another limitation relates to the study design (retrospective case–control study), thus prompting the speculation on a cause–effect relationship. Follow-up studies on additional *IL-10* variants, and populations of related and distant ethnic origins are needed to confirm (or alternatively rule out) the association of *IL-10* variants with altered IL-10 secretion and risk of RPL.

Authors' roles

R.H.Q. was responsible for sample processing, genotyping and drafting of the manuscript. K.M. was responsible for drafting of the manuscript. F.L.S. was responsible for sample processing and genotyping. N.M. and F.E.M. were responsible for patient screening and referral. T.M. was responsible for data analysis and funding for the project. W.Y.A. was the project leader and was responsible for statistical analysis and finalizing the manuscript.

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Conflict of interest

None declared.

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