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CASE REPORT

Hee Chee Seng · Gun Suk Chyn · Rakesh Naidu
Esha Gupta · Sushela Devi Somnath
Ammu Kutty Radhakrishnan

Comparison of single nucleotide polymorphisms in the human interleukin-10 gene promoter between rheumatoid arthritis patients and normal subjects in Malaysia

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Abstract In this study, three single nucleotide polymorphisms (SNPs) located within the promoter of the human interleukin (IL)-10 gene [rs1800896 (position: –1087G > A), rs1800871 (position: –824C > T) and rs1800872 (position: –597C > A)] were investigated in 84 rheumatoid arthritis (RA) patients and 95 age- and sex-matched healthy subjects using polymerase chain reaction-restriction fragment length polymorphism method. Production of IL-10 by peripheral blood lymphocytes from the RA patients and healthy subjects cultured in the presence of Concanavalin A (Con A) was determined by using enzyme-linked immunosorbent assay. The results show that the distribution of the IL-10 genotypes did not differ significantly between RA patients and healthy subjects ($P > 0.05$). However, a significant difference was observed in allele frequencies of –824CT, –824TT, –597CA, and –597AA between the RA patients and healthy volunteers ($P = 0.04$). The –1087A/–824T/–597A (ATA) haplotype, which comprises all mutant alleles, was associated with lower IL-10 production when compared with the other haplotypes. In contrast, the RA patients who did not display the ATA haplotype produced significantly higher levels of IL-10 when compared with those carrying either one ($P = 0.012$) or two ($P = 0.005$) ATA haplotypes. Our findings suggest that there is an association between SNPs in the promoter of the human *IL-10* gene and susceptibility to RA.

Key words Interleukin-10 · Rheumatoid arthritis · Single nucleotide polymorphism

C.S. Hee (✉) · E. Gupta · S.D. Somnath · A.K. Radhakrishnan
International Medical University, No. 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia
Tel. +603-8656-7228; Fax +603-8656-7229
e-mail: cs_hee@yahoo.com

S.C. Gun
Department of Medicine, Hospital Tuanku Ja'afar, Seremban, Malaysia

R. Naidu
Department of Molecular Medicine, University of Malaya, Kuala Lumpur, Malaysia

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive arthropathy caused by autoimmune responses.[1] Clinically, RA is characterized by chronic synovitis, which often leads to destruction of cartilage and bone.[2] In RA, a large number of cytokines are produced by monocytes, macrophages, and synovial fibroblasts that are stimulated by $CD4^+$ T-lymphocytes.[1] Interleukin-10 (IL-10) is a cytokine that has been shown to exert both immunoregulatory and anti-inflammatory effects in RA by inhibiting the synthesis of pro-inflammatory cytokines.[3] The addition of IL-10 to cultures of mononuclear cells from RA patients was reported to reverse cartilage degradation.[4] In mouse and rat models of collagen-induced arthritis, administration of recombinant IL-10 was shown to exert some protective effects such as delaying the onset and reducing the severity of arthritis.[5–7] There is evidence to show that the production of human IL-10 is controlled at the transcription level, most likely through some of the regulatory sequences present in the promoter region of the *IL-10* gene.[8] A number of proximal single nucleotide polymorphisms (SNPs) have been identified in the promoter region of the human IL-10, e.g., –1087G > A (rs1800896), –824C > T (rs1800871), and –597C > A (rs1800872).[9,10] The SNPs at sites –1087G > A, –824C > T, and –597C > A are located at putative regulatory regions in the promoter of the human *IL-10* gene. The –1087G > A lies within a putative Ets transcription factor binding site, while the –824C > T is located within a putative positive regulatory region and the –597C > A lies within a putative STAT-3 binding site and a negative regulatory region.[11] Hence, polymorphisms at these sites may alter the binding sites of various transcription factors, which in turn may affect the production of IL-10. The association of SNPs in the promoter region of the human *IL-10* gene and disease susceptibility has been studied in several diseases such as myocardial infarction and coronary artery disease,[12] endometriosis,[13] Alzheimer,[14] systemic lupus erythematosus (SLE)[15] and schizophrenia.[16] Discrepancies in the association of SNPs in the promoter region of the human *IL-10*

gene and RA susceptibility have been reported by several researchers.[17–22] Crawley et al.[17] reported that IL-10 SNP rs 1800896/1800871/1800872 ATA haplotypes were associated with juvenile RA children, but no association was detected in British Caucasians.[18] The rs1800896 G/G was reported to be associated with joint destruction in female patients in Netherlands,[19] but no such association was observed in the rs1800896 G/A alleles in French,[20] Finnish,[21] or British[22] RA patients. Variations were also observed between IL-10 SNPs and the production of IL-10. IL-10 production was reported to be significantly lower in those with the ATA/ATA genotype in LPS-stimulated cultures by some researchers.[23,24] Keijsers et al.[24] reported that the rs1800896 A allele was associated with higher IL-10 production when compared with the rs1800896 G allele.[24] In this study, our aim was to investigate whether there is an association between SNPs in the promoter region of the human *IL-10* gene and susceptibility to RA. Our research hypothesis for this study was that SNPs in the promoter region of the human *IL-10* gene is associated with lower IL-10 protein and that this might influence the susceptibility to RA.

Methods

Recruitment of patients and volunteers

All the RA patients were recruited from Hospital Tuanku Ja'afar, (formerly known as Hospital Seremban) located in Seremban, Malaysia. The healthy subjects were recruited from both Hospital Tuanku Ja'afar and the International Medical University. A total of 84 RA patients and 95 sex ($\chi^2 = 0.340$, $P = 0.560$)- and age ($\chi^2 = 7.373$, $P = 0.117$)-matched healthy subjects were recruited. The patient and control cohorts comprised the three major ethnic groups in Malaysia, i.e., Malay, Chinese, and Indian. Within the RA patient group, there were 23 Malay, 27 Chinese, and 34 Indian patients recruited, and the healthy subjects' group consisted of 29 Malay, 17 Chinese, and 49 Indian volunteers. The recruitment of RA patients was based on the 1987 revised criteria of the American Rheumatism Association.[25] The control subjects were defined as having no rheumatologic or other significant medical illnesses. Informed consent was obtained from all the subjects recruited for this study. This study was approved by the Research and Ethics Committees of the International Medical University.

Genotyping of IL-10 polymorphisms

About 5 ml of blood was drawn from the RA patients and healthy subjects into heparinized tubes (Meus, Piove di Sacco, Italy). Genomic DNA was extracted from peripheral blood leucocytes using a commercially available DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The promoter region of the human *IL-10* gene containing the three SNPs, i.e., –1087G > A, –824C > T, and –597C > A[9,10] was amplified by polymerase chain reaction (PCR) using published forward and reverse primers[26] (see Table 1). PCR was performed using commercially available PCR premix (AccuPower PCR PreMix, BIONEER, Daejeon, Korea) according to the manufacturer's recommended protocol. Into a 0.2 ml PCR tube containing the AccuPower PCR PreMix, 1 µl template DNA (about 50 ng/µl), 3 µl of each primer (1 pmol/µl) and 13 µl DNase-free water were added. The total volume for the PCR was 20 µl. PCR was performed using a PTC-100 Peltier Thermal Cycler (MJ Research) using the following cycling conditions: 30 cycles at 95°C for 30 s, 45°C for 30 s and 72°C for 1 min. The PCR products were purified using a commercial kit (GENOMED, Bad Oyenhausen, Germany). The purified PCR product was then digested with appropriate restriction enzymes (see Table 1) as described earlier.[26]

DNA sequencing

The DNA sequencing was performed on one-third of the samples to validate the restriction fragment length polymorphism (RFLP) data. PCR for DNA sequencing was performed using a forward primer tagged with the M13 primer and a reverse primer as described earlier (see Table 1). The purified PCR products and primers were couriered to Macrogen (Seoul, Korea), a company that provides DNA sequencing services. The DNA sequencing was performed in Macrogen using an ABI PRISM 3730XL Sequencer (Applied Biosystems) and the results from the DNA sequencing were obtained via e-mail.

Table 1. Technical data for the genotyping of single nucleotide polymorphisms in the human interleukin (IL-10) gene promoter

Genotyping method	Primer sequences	Restriction enzyme	References
RFLP (–1087G > A)	5′-AAG ACA ACA CTA CTA AGG CTT CCT T-3′ 5′-TAA ATA TCC TCA AAG TTC C-3′	*Eco*NI	Padyukov et al.[26]
RFLP (–824C > T)	5′-AAG ACA ACA CTA CTA AGG CTT CCT T-3′ 5′-TAA ATA TCC TCA AAG TTC C-3′	*Mae*III	Padyukov et al.[26]
RFLP (–597C > A)	5′-AAG ACA ACA CTA CTA AGG CTT CCT T-3′ 5′ TAA ATA TCC TCA AAG TTC C 3′	*Rsa*I	Padyukov et al.[26]
DNA sequencing	5′-TGT AAA ACG ACG GCC AGT ACA CAC AAA TCC AAG ACA AC-3′ 5′-AGG AAC ACG CGA ATG AGA AC-3′	–	CGAP, National Cancer Institute

RFLP, restriction fragment length polymorphism; CGAP, cancer genome anatomy project

Detection of IL-10 level

About 4 ml of blood from RA patients and healthy volunteers was collected into heparin tubes (Meus). The blood was transferred into 15-ml tubes Falcon), to which 8 ml of G-Dex II RBC lysis buffer (iNtRONS, Seongnam, Korea) was added. The mixture was vortexed and then centrifuged (2000 g at 4°C for 10 min) to recover the peripheral blood leucocytes. The pellet containing peripheral blood leucocytes was reconstituted with 5 ml of complete RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with L-glutamine (Gibco, Invitrogen), 10% foetal bovine serum (Gibco, Invitrogen), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Gibco, Invitrogen). Haemocytometer (Neubauer, La Fontaine, Forst-Bruchsal/Dynatech, Denkendorf, Germany) was used to perform cell count. The cell count was adjusted to 2.5×10^5 cells/ml with complete RPMI medium and 100 µg/ml Concanavalin A (Con A) (Sigma-Aldrich, St. Louis, MO, USA) was added to the final cell suspension; 200 µl of this cell suspension was plated out in two sterile 96-well flat-bottom tissue culture plates (Falcon, Becton Dickinson, USA). The culture plates and their lids were sealed with surgical tape (3M) and incubated at 37°C in a humidified 5% CO_2 incubator (SHEL LAB, Cornelius, OR, USA). The plates were removed after 24 and 48 h and stored at –80°C until further analysis. The amount of IL-10 produced by the peripheral blood leucocytes was measured by commercially available enzyme-linked immunosorbent assay (ELISA) human IL-10 kit, according to the manufacturer's recommendation (eBioscience, San Diego, CA, USA).

Statistical analysis

The data were first tabulated in Excel (Microsoft, Redmond, WA, USA) and then analyzed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). The Hardy-Weinberg Equilibrium Test was performed to analyze the genotype distribution. Where appropriate, the differences between patient and control genotype and allele frequencies were measured using χ^2 test with Yates' correction. The haplotype frequencies and the differences between the haplotype frequencies (χ^2 test) were calculated using an Estimating Haplotype (EH) program.[27] Normality tests, i.e., the Kolmogorov–Smirnov test with a Lilliefors significance level and Shapiro–Wilk statistic were performed on the amount of IL-10 produced by the mitogen-stimulated peripheral blood leucocytes. The Mann–Whitney test and Kruskal Wallis test were used to make inferences about the differences between the amounts of IL-10 produced.

Results

The IL-10 promoter polymorphisms

The RFLP patterns obtained following the PCR-RFLP analysis are shown in Fig. 1. As shown in Table 2, there was no significant deviation of the genotype distribution from the Hardy–Weinberg Equilibrium observed in either RA patients or healthy subjects. The SNPs at positions –824 and –597 in the promoter region of the human *IL-10* gene were shown to be in complete linkage disequilibrium and therefore the genotype distributions for these two SNPs were found to be identical (see Table 2a). The genotype distribution at positions –1087, –824, and –597 in RA patients did not differ significantly from that observed for the healthy subjects group ($P > 0.05$). However, allele frequencies of –824C > T and –597C > A differed significantly ($P = 0.04$) between RA patients and healthy subjects (see Table 2b). The odds ratio for IL-10 –824T, and –597A carriers in the RA patients versus IL-10 –824C, and –597C carriers in the control groups was 1.55 (95% CI 1.01–2.36). Four haplotypes were identified including a rare haplotype –1087G/–824T/–597A (GTA) (see Table 2c). No significant difference was observed between these haplotype frequencies between RA patients and control subjects. The relationship

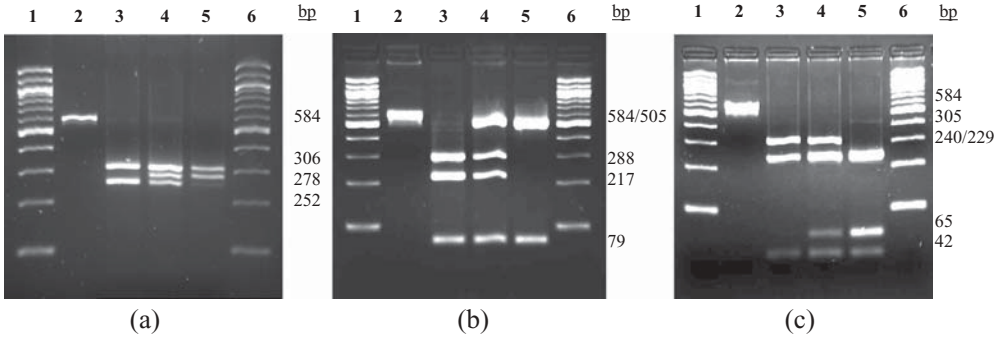

Fig. 1. Photographs of agarose gels representing typical restriction fragment length polymorphism (RFLP) patterns obtained following digestion with the restriction enzymes. Genomic DNA was amplified by polymerase chain reaction (PCR) using the primers shown in Table 1. The PCR products were purified and digested with three different restriction enzymes, i.e., (**a**) *Eco*NI, (**b**) *Mae*III, and (**c**) *Rsa*I. The restriction enzyme (**a**) *Eco*NI recognizes the SNP at site –1087G > A; (**b**) *Mae*III recognizes the SNP at site –824C > T and (**c**) *Rsa*I recognizes the SNP at position –597C > A. In all the three gels (**a**, **b**, and **c**), *lanes 1* and *6* represent the 100 bp DNA ladder; *lane 2* represents undigested PCR products, and *lane 3* represents the typical RFLP patterns obtained when only the respective homozygous wild-type alleles are present. *Lane 4* shows the typical RFLP patterns seen when the respective heterozygous (wild-type and mutant) alleles are present whereas *lane 5* represents the typical RFLP patterns seen when only the respective homozygous mutant alleles are present

Table 2. Genotype, allele, and haplotype frequency of single nucleotide polymorphisms in the promoter region of the human IL-10 gene in the rheumatoid arthritis (RA) patient and control groups

Genotype	RA patients, $n = 84$	Control subjects, $n = 95$	χ^2 (P value)	d.f.	Odds ratio	95% CI
(a) *Genotype frequency*						
–1087						
GG	3 (3.6%)	4 (4.2%)	0.975	2	–	–
GA	14 (16.7%)	16 (16.8%)				
AA	67 (79.8%)	75 (78.9%)				
–824						
CC	13 (15.5%)	21 (22.1%)	0.119	2	–	–
CT	35 (41.7%)	47 (49.5%)				
TT	36 (42.9%)	27 (28.4%)				
–597						
CC	13 (15.5%)	21 (22.1%)	0.119	2	–	–
CA	35 (41.7%)	47 (49.5%)				
AA	36 (42.9%)	27 (28.4%)				
(b) *Allele frequency*						
–1087						
G	20 (11.9%)	24 (12.6%)	0.834	1	–	–
A	148 (88.1%)	166 (87.4%)				
–824						
C	61 (36.3%)	89 (46.8%)	0.044	1	1.55	1.01–2.36
T	107 (63.7%)	101 (53.2%)				
–597						
C	61 (36.3%)	89 (46.8%)	0.044	1	1.55	1.01–2.36
A	107 (63.7%)	101 (53.2%)				
(c) *Haplotype frequency (–1087/–824/–597)*						
GCC	11%	13%	0.478	7	–	–
GTA	1%	4×10^{-6}%				
ACC	26%	34%				
ATA	62%	53%				

d.f., degrees of freedom; CI, confidence interval

between the SNPs in the human IL-10 promoter and the demographic data as well as the clinical findings of RA such as disease status, association with Sjögren's syndrome, rheumatoid factor, presence of deformities and bony erosion were also analyzed. However, we could not detect any significant association between these parameters and the SNPs in the human IL-10 promoter (data not shown).

Polymorphisms in the IL-10 promoter and production of IL-10

The amount of IL-10 produced by Con A-stimulated peripheral blood leucocytes was quantified by ELISA. A standard curve was obtained using the standard pure IL-10 protein provided with the human IL-10 ELISA kit (eBioscience). The production of IL-10 by Con A-stimulated peripheral blood leucocytes reached a plateau after 24 h of culture (data not shown); therefore, the amount of IL-10 produced following 24 h of leukocyte culture was used for the statistical analysis. The difference in the amount of IL-10 produced between RA patients and healthy subjects was compared using the non-parametric Mann–Whitney test. The analysis showed that the amount of IL-10 produced by the Con A-stimulated peripheral blood leucocytes was significantly lower ($P = 0.002$) in RA patients when compared with the healthy subjects. The amount of IL-10 produced by healthy subjects carrying the homozygous mutant alleles (–1087AA) at position –1087 was found to be significantly higher when compared with the RA patients carrying the same alleles (see Table 3a). Significant differences were also detected between the level of IL-10 produced by control and RA patient subjects carrying heterozygous ($P < 0.02$) and homozygous mutant ($P < 0.015$) alleles at sites –824 and –597 (see Table 3a). Genotype frequencies consisting of SNPs (positions: –1087, –824, and –597) in the promoter region of the human *IL-10* gene were also compared using the Mann–Whitney test. Healthy subjects who carried the AA/CT/CA and AA/TT/AA genotypes were observed to produce significantly higher levels of IL-10 when compared with the RA patients carrying the same alleles (see Table 3b). The –1087A/–824T/–597A (ATA) haplotype was associated with lower production of IL-10. Study subjects were grouped according to the frequency of the ATA haplotypes, i.e., (i) did not carry the ATA haplotype, (ii) carried at least one ATA haplotype and (iii) carried two ATA haplotypes. The IL-10 production was then compared with RA patients and healthy subjects and within each group. The Mann–Whitney test showed that the difference observed in the IL-10 production between RA patients who did not carry the ATA haplotype and RA patients who carried either one ATA haplotype ($P = 0.012$) or two ATA haplotypes

Table 3. The relationship between the single nucleotide polymorphisms in the human *IL-10* gene promoters and production of IL-10

	IL-10 Production (pg/ml)[a]		Mann–Whitney's test, *P* value
	RA patients (*n* = 84)	Control subjects (*n* = 95)	
(a) *Genotype*			
–1087G > A			
GG	679.83 ± 61.08	613.75 ± 313.83	0.724
GA	415.22 ± 204.61	469.50 ± 179.49	0.533
AA	355.18 ± 178.91	470.73 ± 210.13	0.001
–824C > T			
CC	526.42 ± 188.85	507.92 ± 242.94	0.804
CT	354.52 ± 203.70	462.96 ± 189.98	0.02
TT	344.39 ± 151.82	475.48 ± 219.00	0.015
–597C > A			
CC	526.42 ± 188.86	507.92 ± 242.94	0.804
CA	354.52 ± 203.70	462.96 ± 189.98	0.02
AA	344.39 ± 151.82	475.48 ± 219.00	0.015
(b) Genotype (–1087/–824/–597)			
GG/CC/CC	679.83 ± 61.08	613.75 ± 313.83	0.742
GA/CC/CC	499.90 ± 231.6	531.8 ± 199.89	0.754
GA/CT/CA	357.29 ± 212.05	441.18 ± 171.81	0.298
GA/TT/AA	406.28 ± 19.48	0	NA[a]
AA/CC/CC	460.90 ± 166.13	462.69 ± 243.62	0.958
AA/CT/CA	353.83 ± 205.56	469.81 ± 197.19	0.034
AA/TT/AA	340.75 ± 155.53	475.48 ± 219	0.012

NA, not applicable
[a] Mean ± standard deviation

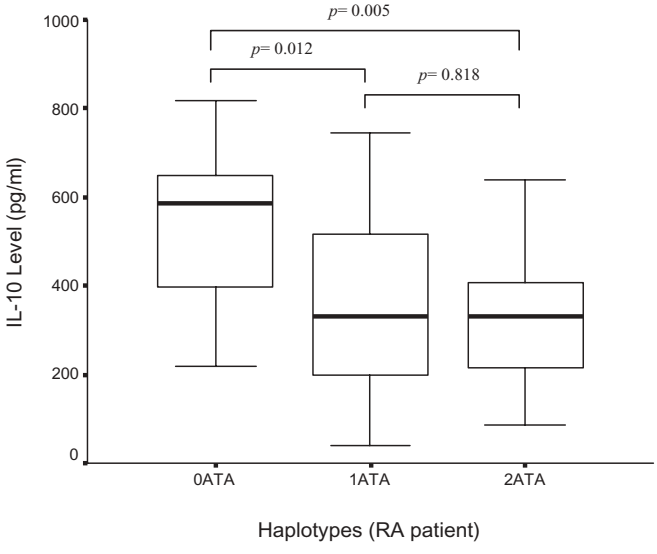

Fig. 2. Association between the amounts of interleukin-10 produced by rheumatoid arthritis (RA) patients and ATA haplotype frequencies. The *bars* show the medians

(*P* = 0.005) were statistically significant (see Fig. 2). However, no differences were observed within ATA haplotypes of the healthy subjects' group.

Discussion

The relationship between SNPs in the promoter region of the human *IL-10* gene and IL-10 production is of clinical interest because of the pivotal immunoregulatory role played by IL-10 in inflammatory and immune responses. We observed significant variation in the allele frequencies between RA patients and control subjects at positions –824 and –597. These findings have not been reported earlier in RA patients. The SNP at position –1087 in the human IL-10 promoter was reported to be associated with RA in women.[28] Interestingly, we observed significant differences in IL-10 production between RA patients and control subjects carrying the –1087AA allele (see Table 3a). The differences in the production of IL-10 amongst these SNPs have been reported earlier.[9] A significantly lower IL-10 production was observed in RA patients carrying the –824CT, –824TT, –597CA, and –597AA alleles when compared with the control subjects. These findings suggest that SNPs in the IL-10 promoter region can affect the production of IL-10. The haplotype analysis further supported the idea that mutant alleles are linked to lower IL-10 production in RA patients (see Fig. 2). In contrast, low IL-10 production was not observed in control subjects with the mutant allele. This discrepancy could be explained by the theory proposing that SNPs in different environments display different properties. For instance, studies on Icelandic SLE patients and their first-degree relatives and spouses have shown that genetic and environmental factors have some effect in determining the production of IL-10.[29] In addition, Westendorp et al., (1997) had demonstrated that environmental factors could account for up to 25% of the IL-10 production while genetic factors could account for as much as 75% of inter-individual differences in IL-10 production in monozygotic twins and first-degree relatives of meningococcal patients.[30] Environmental factors comprise endogenous and exogenous factors, which stimulate macrophages, which are the major producers of IL-10 through endotoxin (via Toll-

like receptor 4, NF-κB dependent), TNF-α (via TNF receptor p55, NF-κB-dependent) and catecholamines.[31] The SNPs in the promoter region of the human *IL-10* gene at positions –824 and –597 were in strong linkage disequilibrium and together with the SNP at site –1087, form four possible haplotypes: GCC, GTA, ACC, and ATA. The GTA haplotype was found only in some populations such as Dutch Caucasians[32] and Chinese[15,16] but not in Caucasians in the UK.[9] A significant difference was observed between the genotype/haplotype frequencies from this study and the Caucasian cohorts.[9,17] However, we observed a high degree of similarity between the SNP frequencies detected in our study and with those reported for other Asian populations.[16] The presence of genetic heterogeneity between ethnic groups in the highly polymorphic promoter region of the human *IL-10* gene has raised the possibility that in different populations, different alleles/haplotypes may be important in regulating the expression of IL-10.[33] Comparisons of SNP frequencies based on ethnicity, i.e., Malay, Chinese, and Indian were also performed between the patient and control groups, but we could not detect any significant differences. These results were most likely not convincing because the sample number in some of the groups that were compared was too small (i.e., less than 5). In conclusion, we have demonstrated that the IL-10 promoter SNPs at –824 and –597 are significantly associated with RA patients and the IL-10 production was significantly lower in –1087A/–824T/–597A associated alleles.

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