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Genetic Polymorphisms of Interleukin-4 Third Intron Region in the Malaysian Patients with Systemic Lupus Erythematosus

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In this study, we analysed the genetic polymorphisms present in the third intron region of Interleukin-4 gene in Malaysian patients with Systemic Lupus Erythematosus. Overall, the RP I and II alleles were found evenly distributed in both the SLE patients and control individuals. There was no significant association observed in the distribution of allelic and genotypic frequencies between SLE patients and healthy controls. The result obtained is similar to a previous study carried out on SLE Chinese patients in Taiwan.

Key words: Interleukin-4, systemic lupus erythematosus, genetic polymorphisms, third intron, variable number of tandem repeat

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INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a well-known, chronic autoimmune disease involving multiple-organ inflammation. The brain, the heart, the skin, joints, kidneys, lungs, and blood vessels are damaged upon deposition of immune complexes (Klinman and Steinberg, 1987; Manson and Rahman, 2006; Truedsson *et al*., 2007). SLE is difficult to diagnose at the initial stages because the symptoms developed are similar and common to other diseases (Tucker, 2007). The butterfly rash is one of the distinctive characteristic features of SLE, which is always present over the nose bridge. Despite medications prescribed by medical doctors to treat various symptoms of SLE, there is still no cure for the disease (Wallace and Hahn, 2002).

Although extensive research projects were conducted worldwide to investigate the aetiology of SLE, the exact cause of the disease remains unknown until today. It was suggested that the onset of SLE is a combined effect of two factors, i.e., genetic and environment (Wakeland *et al*., 1999). The expression of multiple genes and their interaction with the environment could eventually trigger the onset of the disease. It is hard to draw a clear association of certain genes to the occurrence of SLE, due to the poorly understood pathogenesis and the complexity of genetic interactions. Environmental factors, such as ultra-violet (UV) light, drugs, external stress, and infectious agents are thought to play a role in the onset of SLE (Jonsen *et al*., 2007; Molina and Shoenfeld, 2005; Moon *et al*., 2004). These factors could serve as a trigger to excessive autoantibody production that leads to organ-end damage in genetically susceptible individuals.

Several studies provided solid and important evidence that genetics could be one of the major predisposing factors in SLE (Fairhurst *et al*., 2006; Perdriger *et al*., 2003). It was reported that about 5 to 12% of SLE cases are familial (Tsao and Grossman, 2001). Further more, the chance for a first-degree relative of a SLE patient to get the disease is twenty times higher than that of the general population (Arnett, 1997). SLE occurs up to ten times more often in females than males (Cervera *et al*., 2003). This may due to the differences between these sexes, such as life styles and hormone levels. It often strikes women at their childbearing age, especially those who have relatives affected by autoimmune diseases (Danchenko *et al*., 2006). In Malaysia, the mortality rate of SLE patients is 20.2% and active disease contributes to 19.0% of the deaths (Yeap *et al*., 2001).

Interleukin-4 (IL-4) is a T-helper type-2 cytokine with anti-inflammation properties. IL-4 is secreted by antigen-activated T-lymphocytes and plays a role in both adaptive and humoral-mediated immunity, as it promotes differentiation of both B and T cells (Funauchi *et al*., 1999). Thus, regulatory abnormalities of the gene expression could affect the disease progression. The gene encoding IL-4 has been mapped to the chromosome 5(q23-31) (Le Beau *et al*., 1989). The entire gene of 10 kbp composes of four exons and three introns (Arai *et al*., 1989). In the present study, we investigate the polymorphisms in the third intron region of the IL-4 gene. The polymorphisms consist of a variable number of tandem repeats (VNTRs) of a 70 bp repeating sequence (Fig. 1). Although the exact function of the third intron is unknown, we hypothesize that the polymorphisms present in the third intron might have regulatory

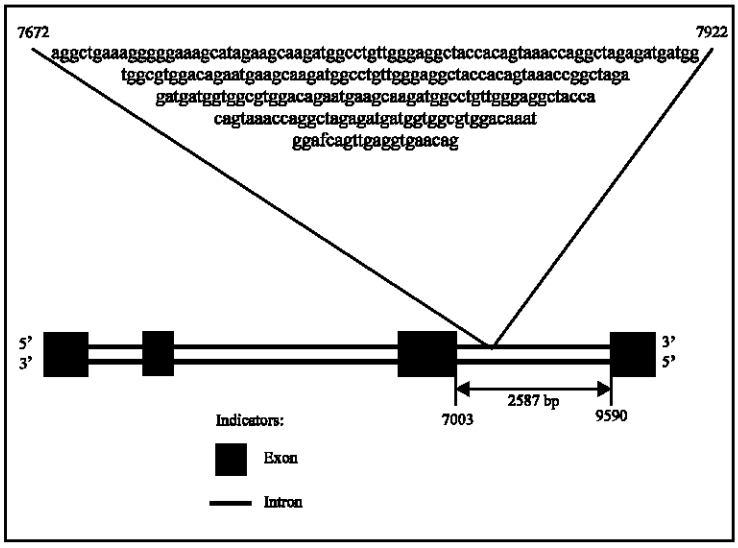

Fig. 1: Schematic representation of IL-4 gene and the VNTR polymorphisms present at the third intron region

effects on the expression of the IL-4 gene. In other words, the serum level of IL-4 might be altered depending on the presence of different variants in this polymorphic region.

MATERIALS AND METHODS

Subjects: The study included 100 SLE patients, fulfilling the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE and 100 healthy control individuals, from the University Malaya Medical Center (UMMC) located in Kuala Lumpur (Ethics Approval No. 380.1) (Tan *et al*., 1982). Blood samples were collected in EDTA tubes to prevent coagulation. This study has been conducted in 2007. Genomic DNA extraction was performed via the conventional phenol-chloroform method as described by Sambrook (2001).

PCR amplification: The PCR primers and cycling parameters were set according to the desired conditions, based on study done by Cantagrel *et al.* (1999). These conditions were optimized and used to screen all the patients as well as healthy control samples for genetic polymorphisms present in the third intron of IL-4 gene. 5'-AGGCTGAAAGGGGGAAAGC- 3' and 5'- CTGTTCACCTCAACTGCTCC- 3' are the primers used in this PCR.

Generally, PCR was performed with 10 ng genomic DNA in a final volume of 20 μL containing 10 pmol of each primers (1st Base Pte. Ltd., Singapore), 1 U *Taq* DNA polymerase (Fermentas, USA), 0.1 mM dNTP mix (Promega, USA), 2.0 μL *Taq* buffer with KCl and 1.0 mM $MgCl_2$. PCR was carried out in a thermal cycler (Mastercycler gradient, Eppendorf, Germany). The cycling program was set at 95.0°C, 5 min for initial denaturation, followed by 30 cycles at 95.0°C for 0.5 min, 60.4°C for 0.7 min, 72.0°C for 0.7 min and lastly one cycle of final extension step at 72.0°C for 5 min. The PCR products were then analysed via a 2% (w/v) Agarose Gel Electrophoresis (AGE).

Statistical analysis: Statistical analysis was carried out in order to evaluate the association between the genetic polymorphisms to the onset of SLE. Genotypic distribution and allelic frequencies of the polymorphism in all samples were generated. Chi-square (χ^2) and Odd Ratios (OR) tests with 95% Confidence Interval (CI) were also performed (Dawson and Trapp, 2004). A p-value that is less than 0.05 was considered statistically significant.

RESULTS

Allelic and genotypic frequencies: Figure 2 shows the band patterns observed for the PCR products scored in our study via 2% (w/v) agarose gel. There were three genotypic combinations observed in the study, i.e., homozygous RP I, heterozygous RP I/RP II and homozygous RP II. The RP I allele was represented by a 183 bp fragment, whereas the RP II allele was represented by a 253 bp fragment. The genotypic distribution and allelic frequencies for this study are shown in Table 1.

There was no significant difference in the distribution of both genotypic and allelic frequencies between SLE patients and the normal healthy control group in the

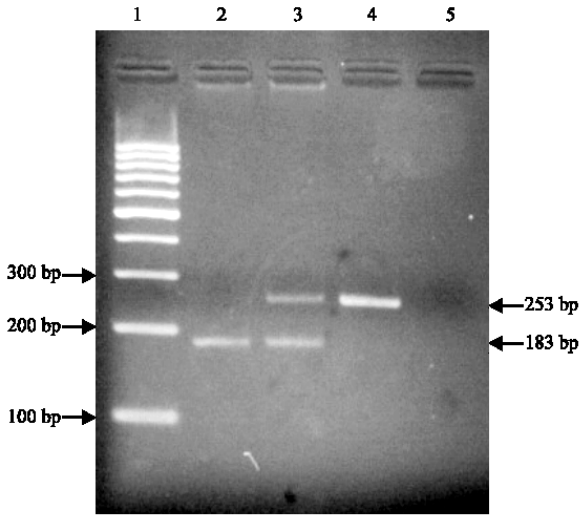

Fig. 2: IL-4 third intron polymorphism PCR products. Lane 1: 100 bp DNA marker (Fermentas Life Sciences, USA); Lane 2: Homozygous RP I (183 bp); Lane 3: Heterozygous RP I/ RP II (183 bp and 253 bp); Lane 4: Homozygous RP II (253 bp); Lane 5: DNA blank

Table 1: Genotypic distribution and allelic frequencies of the IL-4 third intron polymorphism in SLE patients and control group

	Frequency			
IL-4 Third intron	SLE Patient (%) n = 100	Control (%) n = 100	χ^2 (p-value)*	OR (95% CI)
Genotype				
RP I/RP I	53 (53.0)	48 (48)	0.5001 (0.4795)	1.2216 (0.7012-2.1282)
RP I/RP II	40 (40.0)	49 (49)	1.6398 (0.2003)	0.6939 (0.3963-1.215)
RP II/RP II	7 (7.0)	3 (3)	1.6842 (0.1944)	2.4337 (0.611-9.6942)
Allele				
RPI	146 (73.0)	145 (73)	0.0126 (0.9106)	1.0255 (0.6603-1.5927)
RPII	54 (27.0)	55 (27)		0.9751 (0.6279-1.5144)

*All p-values represent χ^2 test results

Malaysian population ($p>0.05$). The homozygous RP II genotype had the highest OR value of 2.4337. The homozygous RP I genotype was the most frequent genotype scored in the Malaysian SLE patients with 53% of the total genotypes, whereas homozygous RP I and heterozygous RP I/RP II genotypes were found almost evenly distributed in the healthy control groups (48 and 49%, respectively). However, the homozygous RP II genotype is the least common in both the patients (7%) and control group (3%).

There was also no significant association of allelic frequencies with the onset of SLE as the distribution of both alleles, RP I and II, was observed commonly in the SLE patients and the control group ($p>0.05$). Interestingly, the RP I allele presents three times higher in both SLE patients and control group compared to the RP II allele in this study.

DISCUSSION

Scientists proposed that the over-production of autoantibodies in SLE patients is one of the pathogenesis, which leads to the destruction of the patients' organs (Klinman and Steinberg, 1987). Thus, cytokines such as IL-4 with polymorphic gene sequences, can be potential markers of disease susceptibility and severity, as the gene expression may play a role in the disease pathogenesis. In the present study, we did not observe any significant association of the IL-4 third intron polymorphisms with the onset of SLE. Both genotypic distribution and allelic frequencies did not show significant association with an increased risk of developing SLE in Malaysian population. ORs for both genotypic distribution and allelic frequencies revealed that the presence of any genotypic or allelic combinations, posses nearly as equal chance to the onset of SLE. In other words, all genotypic combinations and alleles were not observed to significantly affect the onset of SLE in Malaysian population. Therefore, the polymorphisms can be ruled out from being one of the risk factors that influences the disease development.

Present results are similar to Wu *et al.* (2003) study in 2003 on Chinese patients with SLE in Taiwan. They revealed that the IL-4 third intron polymorphisms were not associated to the onset of SLE when compared to the normal healthy controls. However, further studies in analysing the presence of the clinical manifestation demonstrated the polymorphisms were significantly associated to the presence of certain clinical manifestations in the Taiwan Chinese SLE patients. It seemed that the homozygous RP I genotype was significantly associated to the formation of discoid rash in SLE patients (Wu *et al.*, 2003). Thus, it is suggested that the IL-4 third intron polymorphisms may not affect the onset of SLE but it may have effect on the progression and clinical manifestations of SLE in patients in Malaysia too.

Rheumatoid arthritis (RA) and SLE share several identical characteristics. RA is an autoimmune disease that involves the inflammation of joints. As in SLE, RA is suggested to be a multi-factorial disease where both genetic and environmental factors play a role in the predisposition of its onset (Cantagrel *et al.*, 1999). Autoantibodies are involved in both SLE and RA pathogenesis. The IL-4 third intron polymorphisms were significantly reported in French RA patients (Cantagrel *et al.*, 1999). Catangrel and his colleagues reported that the RP I allele was found to be significantly higher in the RA patients as compared to the healthy controls. Based on these data, we propose that the IL-4 third intron polymorphisms may play a role in the presence of certain clinical manifestations in the SLE patients, i.e., inflammation of joints. In contrast, another study completed by Buchs *et al.* (2000) demonstrated that the RP I allele is responsible for protection against further joints inflammation in another cohort of French RA patients.

Interestingly, there is the presence of the third allele, RP III that was reported in a study carried out by Vandenbroeck *et al.* (1997). The RP III allele, which is characterized by four tandem repeats, is 323 bp in length, and was observed in Caucasian and Sardinian multiple sclerosis (MS) patients (Vandenbroeck *et al.*, 1997). This allele however, is very rare compared to the other two alleles and was excluded from having any statistical disease-promoting effect (Vandenbroeck *et al.*, 1997). The third allele was not found in this study.

In the study of the effect of IL-4 in murine model, it was proposed that IL-4 is important but not necessary for autoantibody production in SLE (Deocharan *et al.*, 2003). Over-expression of the IL-4 gene can lead to kidney disease by excessive production of IL-4 and autoantibodies (Deocharan *et al.*, 2003). It is then believed that inhibiting the IL-4 production may be therapeutically effective in SLE patients. In another study on a different murine model, it showed that the presence of IL-4 is protective against lupus-like glomerulonephritis due to marked changes in serum levels of IgG subclasses. IL-4 downregulates Th-mediated IgG mechanisms that results in absence of IgG3, decreased IgG2a level, and increased IgG1 levels in the murine model (Santiago *et al.*, 1997). Thus, it is suggested that the presence of IL-4 may be able to prevent and protect against kidney problems in SLE patients. Kidney failure is one of the major causes of death in SLE patients.

Overall, our preliminary study did not show a significant association between the IL-4 third intron polymorphisms with the onset of SLE in Malaysian population, however it is suggested that the expression of the gene can affect the severity of disease as shown in other study (Wu *et al*., 2003).

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