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Genetic Polymorphisms of the TNF-α and TNF-β Genes in Malaysian SLE Patients

Chua Kek Heng[1*] • Lau Tze Pheng[1] • Foo Chui Ting[1] • Tan Si Yen[2] • Lian Lay Hoong[1]

[1] Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2] Department of Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Corresponding author: * **khchua@um.edu.my**

ABSTRACT

A total of 100 SLE patients and 100 normal healthy controls were included in this study. Blood samples were collected and genomic DNA was extracted by using the conventional phenol-chloroform method. The DNA samples were then used to examine the association between the genetic polymorphisms of TNFs and SLE in the Malaysian population. In this study, the distribution pattern of the TNF-α -308 and TNF-β +252 genetic polymorphisms were demonstrated, in addition to their association with susceptibility to SLE. With regards to the TNF-α gene polymorphisms, the frequency of the TNF1/1 homozygote was significantly higher ($\chi^2 = 9.1912$, $p < 0.05$) in healthy controls, while the frequency of TNF1/2 heterozygote was significantly higher ($\chi^2 = 9.1912$, $p < 0.05$) in SLE patients. Interestingly, the TNF2/2 homozygote was not present in either SLE patients or in the healthy control group. In terms of the TNF-β gene polymorphisms, there was no significant association between the different alleles or genotypes and SLE susceptibility.

Keywords: genetic association, PCR-RFLP, single nucleotide polymorphism, SLE susceptibility

INTRODUCTION

Autoimmunity is a state where the immune response is directed against our body's own cells, tissues and other cellular components. Generally, autoimmune diseases can be categorized into two main types, i.e., organ-specific and systemic autoimmune diseases (Goldsby *et al.* 2003). Systemic Lupus Erythematosus (SLE) is one of the prominent examples of systemic autoimmune diseases. SLE is more prevalent in females than males at a 10:1 ratio. In addition, a higher prevalence rate of SLE is observed in women of child-bearing years, i.e., between 20 to 40 years of age (Goldsby *et al.* 2003). This may be due to the hormonal changes that occur during puberty and the child-bearing years. The populations of African Americans, Afro-Caribbeans and Asians also show a greater prevalence and incidence rates than Caucasians (Rus and Hochberg 2002). Kaslow's study (Kaslow 1982), which was previously carried out in 1982, had reported that the mortality rates are three-fold higher among blacks (8.4/100,000) and two-fold higher among Asians (6.8/100,000), compared to whites (2.8/100,000).

In SLE, any part of the body can be affected during the course of the disease, particularly the skin, joints, heart, lungs, liver, kidneys, blood vessels and nervous system. There are various signs and symptoms which may appear either serially or simultaneously, these include the characteristic butterfly-shaped rash (or malar rash), discoid rash, oral ulcers, arthritis, serositis and others (Wallace and Hahn 1997). The pathogenesis of SLE is mainly due to the overproduction of disease-causing subsets of T lymphocytes, autoantibodies and immune complexes. In addition, failure to appropriately regulate the synthesis or removal of the autoantibodies, immune complexes and activated T-cells will result in their precipitation on tissues, which subsequently initiate tissue destruction in SLE patients (Hahn 2002).

Broadly, the etiology of SLE involves genetic, environmental and endocrine-metabolic factors. The genetic factor consists of both major histocompatibility complex (MHC) and non-MHC genes (Tsao 2002). Examples of non-MHC genes include those that encode for interleukins, immunoglobulins and mannose-binding lectins (MBL) (Vyse and Kotzin 1998). Owing to its polygenic nature, the susceptibility of SLE is affected by multiple genes that interact with each other (Tsao 2002).

Tumor necrosis factors (TNFs) are a group of low molecular weight proteins that regulate the inflammatory process in our body. The term "tumor necrosis factor" originates from its function as a mediator in tumor necrosis in animal serum treated with lipopolysaccharides (Abbas *et al.* 1994). The members of the TNF family consists of tumor necrosis factor alpha (TNF-α) and beta (TNF-β), which are also known as cachectin and lymphotoxin, respectively (Ruddle and Waksman 1968; Beutler *et al.* 1985). Both of these molecules are encoded by genes located at the short arm of human chromosome 6 (Spies *et al.* 1989). TNF-α is a cytokine produced by monocytes and/or macrophages, while TNF-β is a lymphokine secreted by lymphoid cells (Vilček and Lee 1991). Aside from having different cells of origin, both of these molecules play different roles in the immune system. TNF-α is responsible for the pathogenesis of certain autoimmune diseases, while TNF-β is vital in the monitoring of the normal immune system (Pujol-Borrell *et al.* 1987; Bettinotti *et al.* 1993).

In 2004, Parks and his collaborators revealed that TNFs are one of the determinants responsible for the initiation and progression of SLE. The synthesis of TNF was found to be altered in SLE patients and associated with the disease activity (Aringer *et al.* 2002). Polymorphisms of the TNF-α and TNF-β genes are associated with the susceptibility of SLE to a great extent. Preceding studies had demonstrated the association between TNF-α-308 G/A and TNF-β+252 A/G polymorphisms with SLE (Bettinotti *et al.* 1993; Kim *et al.* 1996; Zhang *et al.* 1997; Parks *et al.* 2004). As a result, our current study aimed to investigate the association between both TNF-α and TNF-β genetic polymorphisms with SLE in the Malaysian population.

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Original Research Paper

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 100 SLE patients, who had attended the University Malaya Medical Centre (UMMC) in Kuala Lumpur, Malaysia, were randomly selected for this study. These patients had fulfilled the revised American College of Rheumatology (ACR) criteria for SLE (Tan *et al.* 1982). An additional 100 healthy individuals were also included as normal controls. Blood samples were collected from these volunteers with written informed consent (Ethics Approval No: 380.1). Subsequently, genomic DNA was extracted by using the conventional phenol-chloroform method and stored at -70°C until further use.

Analysis of the TNF-α gene polymorphisms

The TNF-α gene polymorphisms were analyzed by polymerase chain reaction (PCR) followed by agarose gel electrophoresis (AGE). The forward and reverse primers used in this analysis were previously described by Zúñiga *et al*. (2001), with some modifications in the PCR conditions. The PCR conditions were as follows: denaturation step at 95°C for 1 min, annealing step at 65°C for 1 min and an extension step at 72°C for 1 min. This PCR cycle was repeated for 30 rounds. The PCR products obtained were separated and identified via 2.0% (w/v) AGE, with the 50 bp DNA ladder (Fermentas, USA) as the indicator for the size of DNA fragments produced. The gel was stained with ethidium bromide and visualized under ultraviolet (UV) light.

Analysis of the TNF-β gene polymorphisms

The analysis of TNF-β gene polymorphisms involved both PCR and restriction enzyme (RE) digestion. Initially, PCR was carried out by using primers that were previously described in Bettinotti *et al*. (1993) and the PCR conditions were modified, as follows: denaturation step at 95°C for 1 min, annealing step at 67°C for 1 min and extension step at 72°C for 1 min. The PCR cycle was repeated for 30 rounds. After PCR, a 740 bp PCR product was obtained and analyzed on a 1.5% (w/v) agarose gel. Subsequently, RE digestion was carried out on the amplified products by using the *Nco*I enzyme (Fermentas, USA). The fragments yielded were analyzed and visualized by using a 2.0% (w/v) ethidium bromide-stained agarose gel, where a 100 bp DNA ladder (Fermentas, USA) was used as the marker for the size of DNA fragments.

Statistical analysis

The data obtained from the analysis of TNF-α and -β gene polymorphisms were statistically analyzed by using the allelic and genotypic frequencies, Chi-square (χ^2) test, probability (p) value, odds ratio (OR) and 95% confidence interval (CI) values (Joseph *et al.* 1994).

RESULTS

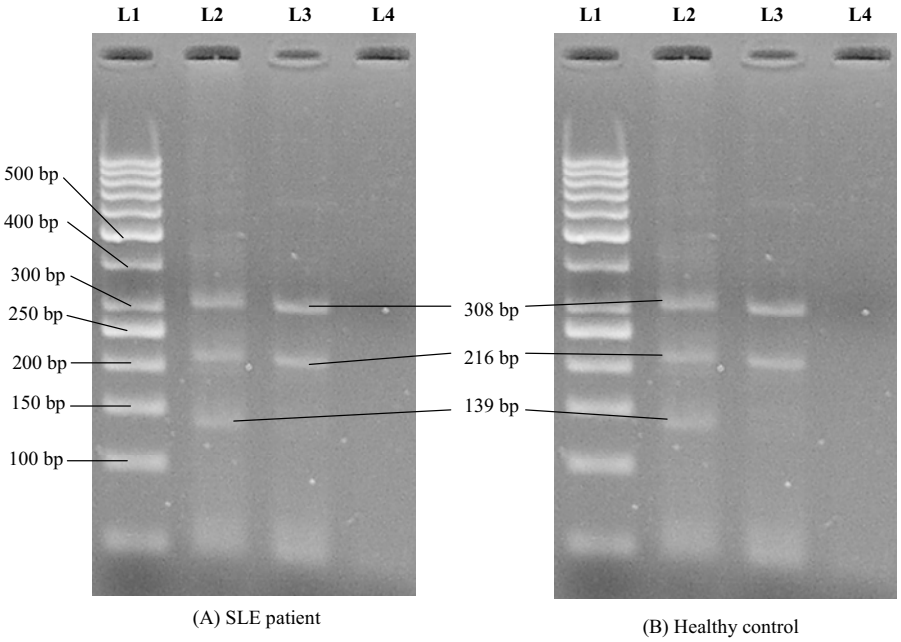
Analysis of the TNF-α gene polymorphisms

Post-PCR amplification, different lengths of PCR fragments were obtained. The TNF1 homozygote produced two fragments of 308 and 216 bp, while the TNF2 homozygote was represented by 308 and 139 bp fragments. As for the heterozygote, three fragments of 308, 216 and 139 bp were observed. **Figs. 1A** and **1B** reveal the banding patterns that represent different genotypes on 2% (w/v) agarose gels observed in both SLE patients and healthy control groups, respectively. Both the TNF1 and TNF2 alleles were observed in the SLE patients and normal control samples. Analysis of the allelic frequency of the TNF-α gene showed that the TNF1 allele was more frequent than the TNF-2 allele, and was significantly associated ($\chi^2 = 7.44$, $p < 0.05$) with healthy controls. In contrast, the TNF2 allele was significantly associated ($\chi^2 = 7.44$, $p < 0.05$) with SLE (**Table 1**). In addition, the TNF1 allele showed a lower disease penetration to SLE (OR = 0.4650), whilst the TNF2 allele showed a higher penetration to this disease (OR = 2.1507) (**Table 1**).

In this study, the most common TNF-α genotype observed in the Malaysian population was the TNF1 homozygote, followed by the heterozygote (**Table 2**). The analysis of genotypic frequency showed that the TNF1/1 homozygote was significantly higher ($\chi^2 = 9.1912$, $p < 0.05$) in healthy controls, thus indicating that this genotype was inversely associated with SLE and might be protective against this disease. On the other hand, the frequency of the TNF1/2 heterozygote was significantly higher ($\chi^2 = 9.1912$, $p < 0.05$) in the SLE patients and could be directly associated with the susceptibility of SLE (**Table 2**). In addition, the TNF1 homozygote showed a lower penetration to SLE (OR = 0.3895), compared to the heterozygote. Interestingly, there were no TNF2/2 homozygotes observed in either the SLE patient or healthy control groups (**Table 2**).

Analysis of the TNF-β gene polymorphisms

After *Nco*I digestion of the PCR product, two fragments of 555 and 185 bp were produced by the TNFB*1 homozygote, while the PCR product remained intact for the TNFB*2 homozygote. As for the heterozygote, three fragments of



(A) SLE patient

(B) Healthy control

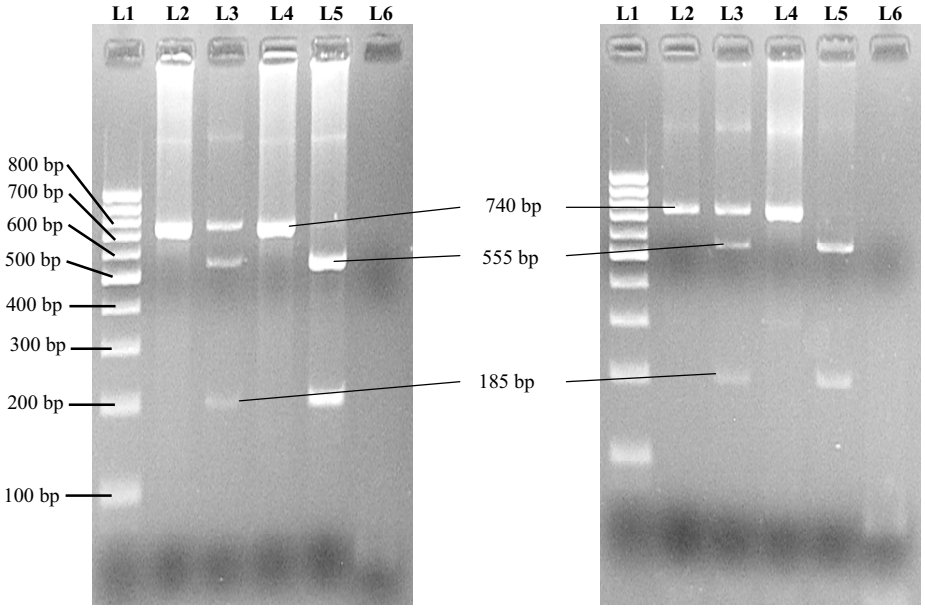
Fig. 1 Amplified products of the TNF-α gene polymorphisms for (A) SLE patients and (B) healthy controls on 2% (w/v) agarose gels. L1: 50 bp DNA ladder; L2: Heterozygote; L3: TNF1 homozygote; L4: DNA blank.

Table 1 The allelic frequency (n), χ^2 (*p*) and OR (95% CI) values of the TNF-α gene polymorphisms in Malaysian SLE patients and healthy controls.

Allele	SLE patient (n)	Healthy control (n)	χ^2 value (*p* value)	OR value (95% CI)
TNF1	158	178	7.44 (0.0064)	0.4650 (0.2660 – 0.8129)
TNF2	42	22		2.1507 (1.2303 – 3.7597)
Total	200	200		

Table 2 The genotypic frequency (n), χ^2 (*p*) and OR (95% CI) values of the TNF-α gene polymorphisms in Malaysian SLE patients and healthy controls.

Genotype	SLE patient (n)	Healthy control (n)	χ^2 value (*p* value)	OR value (95% CI)
TNF1/1	58	78	9.1912 (0.0024)	0.3895 (0.2100 – 0.7224)
TNF2/2	0	0		-
TNF1/2	42	22		2.5674 (1.3843 – 4.7617)
Total	100	100		



(A) SLE patient (B) Healthy control

Fig. 2 Amplified and digested products of the TNF-β gene polymorphisms for (A) SLE patients and (B) healthy controls on 2.0% (w/v) agarose gels. L1: 100 bp DNA ladder; L2: PCR product, undigested; L3: Heterozygote, digested; L4: TNFB*2 homozygote, digested; L5: TNFB*1 homozygote, digested; L6: DNA blank.

740, 555 and 185 bp were observed. **Figs. 2A** and **2B** demonstrate the band patterns representing different genotypes that are observed in the SLE patient and healthy control groups.

Both the TNFB*1 and TNFB*2 alleles were detected in the SLE patient and healthy control groups, but the TNFB*2 allele was more frequent. The analysis of the allelic frequency of the TNF-β gene showed that the TNFB*1 allele was not statistically significantly associated (χ^2 = 0.3672, $p > 0.05$) with either the SLE or control groups. Similarly, there was also no significant association (χ^2 = 0.3672, $p > 0.05$) between the TNFB*2 allele with the same cohort of samples (**Table 3**). In addition, the TNFB*1 allele showed a lower disease penetration to SLE with an OR value of 0.8848. On the other hand, the TNFB*2 allele showed a higher penetration to this disease (OR = 1.1303) (**Table 3**).

The analysis of TNF-β genotypic frequency revealed that the most common genotype observed among the Malaysian population was the TNFB*1/TNFB*2 heterozygote, followed by the TNFB*2 homozygote and TNFB*1 homozygote (**Table 4**). In this study, there was no significant difference (χ^2 = 4.0010, $p > 0.05$) in the distribution of TNFB*1/TNFB*1 homozygotes between the SLE patients and normal controls. Likewise, there was also no significant association between the TNFB*2/TNFB*2 homozygotes (χ^2 = 4.0010, $p > 0.05$) and the TNFB*1/TNFB*2 heterozygotes (χ^2 = 4.0010, $p > 0.05$) with SLE (**Table 4**). Furthermore, the heterozygotes showed the lowest penetration to SLE (OR = 0.5670) compared to the TNFB*1 homozygote (OR = 1.2849) and TNFB*2 homozygote (OR = 1.5653) (**Table 4**).

Table 3 The allelic frequency (n), χ^2 (*p*) and OR (95% CI) values of the TNF-β gene polymorphisms in Malaysian SLE patients and healthy controls.

Allele	SLE patient (n)	Healthy control (n)	χ^2 value (*p* value)	OR value (95% CI)
TNFB*1	83	89	0.3672 (0.5446)	0.8848 (0.5954 – 1.3148)
TNFB*2	117	111		1.1303 (0.7606 – 1.6796)
Total	200	200		

Table 4 The genotypic frequency (n), χ^2 (*p*) and OR (95% CI) values of the TNF-β gene polymorphisms in Malaysian SLE patients and healthy controls.

Genotype	SLE patient (n)	Healthy control (n)	χ^2 value (*p* value)	OR value (95% CI)
TNFB*1/TNFB*1	22	18	4.0010 (0.1353)	1.2849 (0.6407 – 2.5766)
TNFB*2/TNFB*2	39	29		1.5653 (0.8677 – 2.8237)
TNFB*1/TNFB*2	39	53		0.5670 (0.3233 – 0.9945)
Total	100	100		

DISCUSSION

Analysis of the TNF-α gene polymorphisms

Polymorphism of the TNF-α gene, which we investigated in this study, is located at the promoter region at position -308. There are two alleles indicated by the presence of a G or A base at position -308, namely the TNF1 allele and TNF2 allele, respectively (Wilson *et al.* 1997). Both of the alleles were positively scored in the Malaysian population, with the TNF1 allele occurring more commonly than the TNF2 allele. This distribution pattern is similar to that previously reported in Zúñiga *et al*. (2001) study of a Mexican population (**Table 5**). However, while the distribution pattern was similar, our study contradicts theirs, by demonstrating that the TNF-α -308 polymorphisms are significantly associated with SLE.

The TNF1/1 and TNF1/2 genotypes were reported in both SLE patient and healthy control groups, but there was a notable absence of the TNF2/2 genotype. The significant increase in the TNF1/1 genotype in the healthy control group might imply an inverse association between the TNF1 allele and SLE. This genotype probably exerts a protective effect against the occurrence of SLE. In this context, it is assumed that the TNF1 allele might affect the production of the TNF-α protein. Hence, the synthesis of cytotoxic T cells and those that down-regulate the production of antibodies would decrease. This would then help to maintain and regulate our body's own immune system, and subsequently reduce the risk of developing SLE (Vassilli 1992).

As for the TNF2 allele, the TNF1/2 genotype was significantly higher among the SLE patients. Therefore, there was a positive association between the TNF2 allele and SLE. It is suggested that the increase in TNF2 allele transcription would result in a drastic elevation in circulating TNF levels, which might trigger SLE (Savill 1995). In addition, the TNF2 allele may also stimulate the signaling of TNF-α via type 1 receptor to accelerate apoptotic cell death in target cells, or via type 2 receptor to induce the release of proinflammatory cytokines. This proinflammatory signal would then responsible for the accumulation of immune-derived effector cells, as well with the apoptotic cells expressing the autoantigens, further contributing to the pathogenesis of SLE (Dorner *et al.* 1995). Additionally, the TNF2 allele may play some role in activating TNF-α to reduce the apoptosis of thymotic cells, as well as to enlarge the clones of autoreactive T cells (Handwerger *et al.* 1994). Zhou *et al.* (1992) proposed that the enhanced apoptosis of suppressor T cells might abolish the cells' activities and contribute to the continuation and propagation of an autoimmune response. There was also evidence linking the biological activities of the TNF and IL-1, in which the TNF2 allele has been associated with the increased synthesis of IL-1α, which is a proinflammatory cytokine. This will then contribute to the susceptibility of SLE (Danis *et al.* 1995). In addition, the TNF2 allele may also stimulate the increased levels of soluble TNF-receptors (TNF-Rs) type I and II. The binding of TNF-α to these soluble TNF-Rs prevents the membrane-bound receptor binding and subsequently, reduces the transport of proapoptotic signal to target cells. These circulating TNF-Rs will also inhibit the proteolytic cleavage of TNF-α and prolong the serum half-life of TNF-α leading to the development of a circulating pool of cytokines. As a result, the microenvironmental TNF-α concentration will increase and stimulate subsequent pathological tissue damage (Aderka *et al.* 1992). Previous studies had examined the association between the TNF-α gene and the susceptibility of SLE, based on different populations with different ethnic backgrounds. In 1996, Rudwaleit and his group had demonstrated that the TNF2 allele was significantly increased in Caucasian SLE patients when compared to healthy controls. Later in 2004, Parks and his collaborators reported an association between the TNF allele and the risk of SLE, with the presence of IL-1α -889 C/C genotype in Caucasian SLE patients in the southeastern United States.

Analysis of the TNF-β gene polymorphisms

The site of interest in the TNF-β gene is located at position +252 in the first intron of the gene, where a G or A base is present (Abraham *et al.* 1991). The two alleles, i.e., TNFB*1 and TNFB*2 can be differentiated by the presence or absence of the *Nco*I polymorphic restriction site (Abraham *et al.* 1991; Messer *et al.* 1991). In this context, the restriction site is present in the TNFB*1 allele but absent in the TNFB*2 allele (Bettinotti *et al.* 1993).

Table 5 Distribution of the TNF-α -308 allelic and genotypic frequencies in SLE patients and healthy controls in both Mexican and Malaysian populations.

Allele/Genotype	Mexican[a]		Malaysian[b]	
	SLE patient n (%)	Healthy control n (%)	SLE patient n (%)	Healthy control n (%)
Allele				
TNF1	94 (92.2)	107 (97.3)	158 (79.0)	178 (89.0)
TNF2	8 (7.8)	3 (2.7)	42 (21.0)	22 (11.0)
Genotype				
TNF1/1	44 (86.3)	52 (94.5)	58 (58.0)	78 (78.0)
TNF1/2	6 (11.7)	3 (5.5)	42 (42.0)	22 (22.0)
TNF2/2	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)

a: Zúñiga *et al.* 2001
b: This study

Table 6 Comparison of TNF-β genotypic and allelic frequencies among Korean, German, and Malaysian populations in SLE patients and healthy controls.

Allele/Genotype	Korean[a]		German[b]		Malaysian[c]	
	SLE n (%)	Control n (%)	SLE n (%)	Control n (%)	SLE n (%)	Control n (%)
Allele						
TNFB*1	67 (34.5)	260 (46.3)	136 (39.3)	111 (29.1)	83 (41.5)	89 (44.5)
TNFB*2	127 (65.5)	302 (53.7)	210 (60.7)	271 (70.9)	117 (58.5)	111 (55.5)
Genotype						
TNFB*1/TNFB*1	12 (12.4)	59 (21.0)	26 (15.0)	21 (11.0)	22 (22.0)	18 (18.0)
TNFB*1/TNFB*2	43 (44.3)	142 (50.5)	84 (48.6)	69 (36.1)	39 (39.0)	53 (53.0)
TNFB*2/TNFB*2	42 (43.3)	80 (28.5)	63 (36.4)	101 (52.9)	39 (39.0)	29 (29.0)

a: Kim *et al.* 1996
b: Bettinotti *et al.* 1993
c: This study

In this study, none of the alleles or genotypes of the TNF-β gene were significantly associated with either the SLE patients or healthy controls. Thus, the TNF-β gene is not associated to the susceptibility of SLE in the Malaysian population. However, some previous studies reported an association between the TNF-β gene and SLE. In 1993, Bettinotti *et al*. reported that the TNFB*1 allele was significantly more common among German SLE patients compared to healthy controls, while the TNFB*2 allele was not significantly different between both groups. In contrast, the findings of Kim *et al*. in 1996 demonstrated that the TNFB*1 allele was significantly higher among healthy controls while the TNFB*2 allele was significantly higher in SLE patients in a Korean population study (**Table 6**). It was also suggested that the TNFB alleles were in linkage disequilibrium with the HLA-DRB1 alleles instead (Kim *et al.* 1996). It was surprising that although the Malaysian and Korean populations shared a similar genetic background, i.e., Asian lineage, data collected from these populations were not consistent (**Table 6**). This phenomenon might be explained by the role of having different geographical backgrounds in influencing the distribution patterns of TNF-β gene polymorphisms. Consequently, this might explain the difference in the disease prevalence and susceptibility to SLE in different ethnicities. Previous studies showed that TNF-β gene polymorphisms might influence the production of TNF-α proteins. It was found that the TNFB*2 homozygote had the highest secretion of TNF-α, while the lowest secretion of TNF-α was seen in the TNFB*1 homozygote, with an intermediate level of production observed in the heterozygote (Pociot *et al.* 1993). Thus, it was proposed that the TNFB*2 allele might stimulate the production of TNF-α, while the TNFB*1 allele might inhibit the synthesis of TNF-α. Since there was no significant association between the TNF-β alleles or genotypes with SLE observed in this study, it was believed that the effects of TNFB*1 and TNFB*2 alleles had a counter-balancing effect on the production of TNF-α.

CONCLUSION

In conclusion, all of the TNF-α and TNF-β alleles were scored among Malaysians. The TNF1 and TNFB*2 alleles were the most common alleles for the TNF-α and TNF-β genes, respectively. In this study, we found that the TNF1 allele is significantly higher in the healthy controls and this might imply a potential protective effect against SLE. On the other hand, the TNF2 allele is significantly higher in the SLE patients and thus, we can hypothesize that it could be potentially associated with the susceptibility of SLE. As for the TNF-β gene, none of the alleles or genotypes were significantly associated to the risk of developing SLE.

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