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**SHORT COMMUNICATION**

# Lack of association between CD28 IVS3 +17T/C SNP and the susceptibility to SLE in the Malaysian population

Tze-Pheng Lau[1], Lay-Hoong Lian[1], Suat-Moi Puah[1], Ching-Hoong Chew[1], Si-Yen Tan[2] and Kek-Heng Chua[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.*

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**Abstract.** Systemic Lupus Erythematosus (SLE) is a generalized autoimmune disease which results in multiorgan damage. The pathogenesis of SLE is mainly due to the over-production of autoantibodies and immune complexes, as well as abnormal regulation of their production and clearance. One of the many factors that lead to the over-production of autoantibodies is the hyperactivity of T- and B-cells. Owing to the costimulatory function of the CD28 molecule during T-cell activation, the CD28 gene is the target of our study. The results show that the T allele of the CD28 gene and its corresponding homozygous genotype scored the highest frequency amongst the Malaysian population. However, the CD28 gene is not significantly associated with the susceptibility of SLE when comparing between diseased and normal healthy subjects.

**Keywords:** CD28, Malaysian, SLE

## INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease, which results in extensive tissue damage in various organs due to the deposition of immune complexes. Various parts of the body can be affected during the course of disease such as the kidneys, joints, skin, lungs, blood vessels, the heart and the nervous system. As a result, serious complications may occur during the late phase of disease, i.e., arthritis, myositis, vasculitis, pleuritis, pericarditis, kidney failure and occasionally, neurological disorders (Wallace and Hahn, 1997). As previously known, the genetic etiology of SLE is polygenic in nature. The human genes, which were found to be responsible as the predisposing factor for SLE, are the major histocompatibility (MHC), immunoglobulin, Fcγ receptor (FcγR), apoptosis and cytokine genes (Caroll *et al.,* 1987; Wallace and Hahn, 1997).

In this study, we focused on the susceptibility of SLE in the Malaysian population and the costimulatory molecule CD28 gene, which is located on the q arm of the human chromosome 2 at band 33-34 (June *et al.,* 1994). A T/C single nucleotide polymorphism (SNP) can be observed at the +17 position in intron 3 (CD28 IVS3 +17T/C) (Nakao *et al.,* 2000). This gene encodes for the costimulatory molecule CD28, which is expressed as a homodimeric receptor on T-cell surfaces (Thompson *et al.,* 1989). These molecules are important in T-cell proliferation, cytokine production and the prevention of T-cell anergy (Lenschow *et al.,* 1996). In this context, the CD28 molecule will interact with the B7.1 or B7.2 molecules on the surfaces of antigen-presenting cells (APCs) to costimulate the T-cell activation in T-cell mediated immune response (Greenfield *et al.,* 1998).

## MATERIALS AND METHODS

***Patient and control samples.*** A total of 100 SLE patients and 100 matched healthy control blood samples were collected from the University Malaya Medical Centre (UMMC) in Kuala Lumpur. Informed consent from the volunteers had been obtained prior to the blood sample collection (Ethics Approval No: 380.1). All of the SLE patients had fulfilled the American College of Rheumatology (ACR) criteria in confirming the diagnosis of SLE (Tan *et al.,* 1982). The blood samples were collected in EDTA vacutainer tubes and stored at -70ºC for further use. DNA is then extracted by using the conventional phenol-chloroform DNA extraction method (Sambrook and Russell, 2001). The extracted DNA is then subjected to spectrophotometric measurement

* Author for correspondence: Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel: 603-79676607; Fax: 603-79676600; E-mail: khchua@um.edu.my

at 260 and 280 nm to check for the purity levels.

***Genotyping of CD28 IVS3 +17T/C polymorphism.*** The genotyping of the CD28 IVS3 +17T/C polymorphism is performed by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. In the PCR method, the forward and reverse primers used are as previously described by Nakao and his colleagues (2000). The PCR conditions however, are modified, with the denaturation step at 94ºC for 30 sec, annealing step at 60ºC for 30 sec and extension step at 72ºC for 30 sec. The PCR cycle is repeated for 30 rounds, and the region encompassing the intron 3 of CD28 gene is amplified. The resulting PCR product is analyzed via 2 % (w/v) agarose gel electrophoresis (AGE) with the help of 50 bp DNA ladders (Fermentas, USA). Subsequently, the amplified products are subjected to restriction enzyme (RE) digestion using Eco47III (Nakao *et al.,* 2000). The presence or absence of the restriction site within the intron 3 region is determined by the T/C SNP. The restriction fragments produced are analyzed via 3 % (w/v) agarose ethidium bromide-stained gel, and the 50 bp DNA ladder (Fermentas, USA) is again used to estimate the RE fragments between 20 and 130 bp.

In addition, the allele-specific PCR (ASPCR) analysis is carried out in order to verify the accuracy of the PCR-RFLP. Briefly, the ASPCR is performed by using two specific primers for T and C alleles, along with one common primer, as previously described by Ahmed and his collaborators (2001).

***Statistical analysis.*** The data obtained in this study were statistically interpretated, i.e., determination of allelic and genotypic frequencies (n), Chi-square ($\chi^2$), p, odds ratio (OR) and 95 % confidence interval (CI) values (Dawson and Trapp, 2004).

## RESULTS

Post PCR, a 148 bp amplified product was produced, which contained the region encompassing the intron 3 sequence of CD28 gene (Figure 1). After RE digestion, two types of RE fragments were produced corresponding to the type of alleles present. If a T allele is present, the Eco47III restriction will generate two fragments of 126 and 22 bp, as shown in Figure 2. However, the 22 bp fragment is unable to be visualized by the 3 % (w/v) agarose gel. As a result, the presence of 126 bp band on the agarose gel indicates the presence of the T allele (Figure 2). On the other hand, the amplified product will remain intact if a C allele is present, i.e., by the observation of the presence of the 148 bp band on the 3 % (w/v) agarose gel in Figure 2. The T/C heterozygote however, exhibits both the 148 and 126 bp bands (Figure 2). Generally, a 212 bp amplicon could be observed when the integrity of the PCR-RFLP was confirmed using ASPCR analysis.

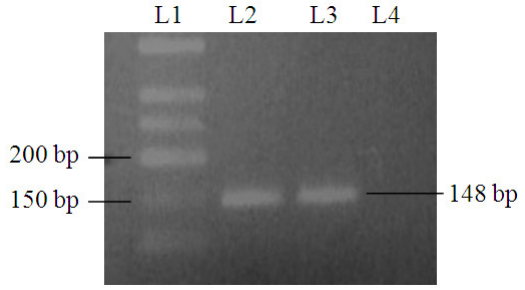


**Figure 1.** Two percent agarose gel showing the PCR product before *Eco*47III digestion. L1, 50 bp DNA ladder; L2, PCR product for SLE group; L3, PCR product for healthy control group; L4, Negative control.

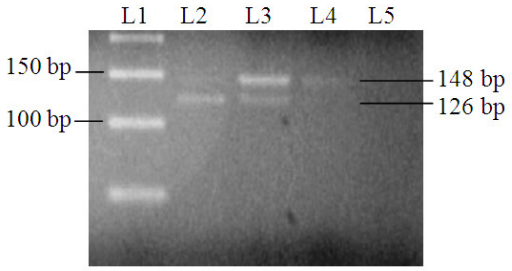


**Figure 2.** Three percent agarose gel showing the RE fragments after *Eco*47III digestion. L1, 50 bp DNA ladder; L2, Genotype - Homozygous T; L3, Genotype - Heterozygous T/C; L4, Genotype - Homozygous C; L5, Negative control.

## DISCUSSION

In the analysis of the CD28 gene SNP, the presence or absence of the Eco47III restriction site within the intron 3 region of CD28 gene is determined by the type of CD28 allele. As a result, the type of RE fragments produced after PCR-RFLP directly indicates the type of CD28 allele/genotype present in an individual.

Through the analysis of the allelic frequencies of the CD28 gene, both of the CD28 alleles were observed among the Malaysian population, with the T alleles scoring a higher frequency compared to the C allele (Table 1). Three types of genotype were reported, i.e., the homozygous C, homozygous T and heterozygous T/C. The homozygous T is the most common genotype observed in the Malaysian population, followed by heterozygous and homozygous C, in both of the SLE patients and healthy control groups (Table 2). In this study, both of the CD28 alleles were not statistically significant ($p > 0.05$) associated to either the SLE or healthy control groups. Similarly, none of the genotypes were statistically significant ($p > 0.05$) associated to SLE. As a result, we can conclude that none of the CD28 alleles/genotypes shows an association to the susceptibility of SLE.

**Table 1.** The allelic frequencies of the CD28 gene SNP in Malaysian SLE patients and normal healthy controls.

| Allele | SLE patient n (%) | Normal control n (%) | $\chi^2$ value (p value) | OR value (95 % CI) |
|---|---|---|---|---|
| Allele C | 46 (23) | 41 (20.5) | 0.367 (0.5446) | 1.1584 (0.7199 – 1.8641) |
| Allele T | 154 (77) | 159 (79.5) | 0.367 (0.5446) | 0.8633 (0.5365 – 1.3892) |
| Total | 200 (100) | 200 (100) | | |

**Table 2.** The genotypic frequencies of the CD28 gene SNP in Malaysian SLE patients and normal healthy controls.

| Genotype | SLE patient n (%) | Normal control n (%) | $\chi^2$ value (p value) | OR value (95 % CI) |
|---|---|---|---|---|
| Homozygous C | 8 (8) | 5 (5) | 0.7400 (0.3897) | 1.6522 (0.5213 – 5.2366) |
| Homozygous T | 62 (62) | 64 (64) | 0.0860 (0.7693) | 0.9178 (0.5168 – 1.6299) |
| Heterozygous CT | 30 (30) | 31 (31) | 0.0240 (0.8769) | 0.9539 (0.5224 – 1.7418) |
| Total | 100 (100) | 100 (100) | | |

In fact, there are not many previous studies which examined the association between the CD28 gene SNP and SLE. Many previous research carried out were to study the association of the CD28 gene polymorphisms with atopic asthma (Nakao *et al.,* 2000) and insulin-dependent diabetes mellitus (IDDM) (Ihara *et al.,* 2001). In 2004, Hebbar and his colleagues had reported the association between the soluble CD28 concentrations, rather than the CD28 gene, with the disease severity of systemic autoimmune diseases, i.e., SLE, primary Sjogen's syndrome and systemic sclerosis. It was found that there is an increase in the soluble CD28 concentrations in these systemic autoimmune diseases (Hebbar *et al.,* 2004).

Interestingly, Ahmed and his workgroup had reported the association of the CTLA-4, instead of the CD28 gene polymorphisms with SLE in the Japanese population (Ahmed *et al.,* 2001). CTLA-4 is important in regulating and controlling the in vivo physiological role of CD28. Theoretically, this observation may be applicable since the activity of CTLA-4 is closely-dependent on the activation of CD28/B7 costimulatory pathway (Greenfield *et al.,* 1998). It is assumed that any changes in the CD28 activity will result in an equivalent change in the activity of CTLA-4. Thus, the effect due to the changes in the CD28 activity is likely to be counter-balanced by CTLA-4. It is then suggested that, any defect in the CTLA-4 function rather than the CD28, is more likely to alter the activation of T-cells. Subsequently, the gene polymorphism of CTLA-4 is believed to be more prone in affecting the susceptibility to SLE, instead of the CD28 gene SNP.

Since the CD28 gene is found to be not associated with the susceptibility of SLE, further studies is suggested to investigate on the possible association between this gene and the disease severity or manifestation of SLE. Owing to the recent finding that the CTLA-4, instead of the CD28 gene, might be associated to the susceptibility of SLE, further studies can be carried out on this gene in the local population. In depth analysis regarding CD28 gene expression at mRNA and proteomic level can also be carried out in order to demonstrate the level of these gene products in influencing the disease susceptibility, severity and prognosis.

## CONCLUSION

Both of the CD28 T and C alleles are scored in the local samples, but none of them or their genotypes is reported to be significantly associated to SLE. Thus, the CD28 gene may not be one of the major susceptibility genes for SLE, with respect to the Malaysian population.

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