

The Potential Role of Interferon-regulatory Factor 7 Among Taiwanese Patients with Systemic Lupus Erythematosus

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ABSTRACT. *Objective.* Type I interferons (IFN), especially IFN- α , have been proposed to underlie the pathogenesis of systemic lupus erythematosus (SLE). Members of the IFN regulatory factor (IRF) family, which regulate IFN expression, have been implicated as risk factors for SLE. Our aims were to investigate the expression of IRF7 and its correlation with disease activity and to explore the association in Taiwanese patients between 2 genetic single-nucleotide polymorphisms (SNP) of IRF7 and SLE.

Methods. IRF7 messenger RNA (mRNA) levels were measured in peripheral blood mononuclear cells by real-time reverse transcription polymerase chain reaction in 51 adult patients with SLE and 65 age-matched and sex-matched controls. Their serum IFN- α levels were determined by ELISA and the clinical manifestations were recorded at the same time. Two IRF7 SNP, rs1061501 and rs1061502, were examined by genotyping across 92 patients with SLE and 92 age and sex-matched healthy control subjects.

Results. Compared with controls, the expression of IRF7 mRNA was significantly increased in patients with SLE and was positively correlated with both the serum level of IFN- α and lupus disease activity. The distribution of SNP rs1061501 by genotype (CC, CT, and TT) and by allele (C, T) was significantly different between the SLE and the control group ($p = 0.028$ for genotype and $p = 0.009$ for allele). There were no significant differences for SNP rs1061502.

Conclusion. The results suggest that dysregulation of IRF7 might mediate an excessive production of IFN- α , which then exerts a crucial effect on the pathogenesis of human SLE. The IRF7 SNP rs1061501 TT genotype and T allele are enriched in Taiwanese patients with SLE and thus would seem to be associated with an increased risk of developing SLE. (First Release June 1 2011; J Rheumatol 2011;38:1914–19; doi:10.3899/jrheum.101004)

Key Indexing Terms:

INTERFERON REGULATORY FACTOR 7

GENE POLYMORPHISM

SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is the prototype of various systemic autoimmune diseases with unknown etiology and is characterized clinically by multisystem involvement, mainly of joints, skin, and kidneys. Many risk factors, including genetic predisposition, the presence of environmental factors, hormone levels, gender, viral infection, and dysregulation of cytokine production, have been suggested as causative factors. Many previous serological studies of cytokine levels in patients with SLE have repeatedly demonstrated a significant

increase in type I interferons (IFN), especially IFN- α ^{1,2,3,4}. More recently, various analyses of gene expression profiles using microarrays among patients with SLE and controls have revealed enhanced expression of IFN gene signatures^{5,6,7}. The suggestion that overproduction of IFN- α might lead to the development of SLE has been further supported by studies of patients with hepatitis C who have undergone IFN- α therapy, some of whom developed antinuclear autoantibodies; eventually, in some cases, the development of full-blown SLE was even triggered^{8,9}. Together, these results strongly suggest that type I IFN plays an important role in the pathogenesis of SLE^{10,11}.

Family members of patients with SLE have been found to have increased basal IFN- α production even though they have not developed SLE¹². This indicates that the influence of genetic background on IFN- α production may be important. The investigation of single-nucleotide polymorphisms (SNP) in genes of the type I IFN pathway has identified an association between SLE and the interferon regulatory factor 5 (IRF5) rs2004640 and the tyrosine kinase 2 rs2304256 SNP in Swedish, Finnish, and Icelandic populations¹³. Some of these

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Accepted for publication April 11, 2011.

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alleles have been functionally analyzed and shown to increase the production of IRF5^{14,15,16}; this would presumably promote IFN- α production. An association between IRF5 rs2004640 SNP and SLE has also been found for Korean populations¹⁷. Additionally, another report has shown that a common IRF5 haplotype is associated with increased serum IFN- α activity in patients with SLE, and this effect is most prominent in patients showing the presence of anti-RNA binding protein or anti-dsDNA antibodies¹⁸. All this evidence suggests that IRF5 plays a central role in the pathogenesis of SLE. In addition, an IRF3 allele that reduces IRF3 expression has been shown to be protective against SLE development¹⁹. To our knowledge, the association between IRF7 expression, its SNP, and SLE has not been investigated. Therefore, we conducted the study to investigate the potential role of IRF7 in the pathogenesis of SLE.

MATERIALS AND METHODS

Patients. All patients with SLE enrolled in our study were identified at the National Cheng Kung University Hospital, a medical center located in southern Taiwan. All patients had the research explained to them in detail and fully informed consent was obtained from each patient before blood was drawn. Fifty-one patients, all of whom had fulfilled at least 4 of the criteria of the American College of Rheumatology classification for SLE, were enrolled for the IRF7 mRNA and serum IFN- α study. The mean age of the patients was 36.65 ± 13.52 years with a female to male ratio of 9:1. Patients with a current or recent infection, chronic viral hepatitis, or malignancy were excluded. Sixty-five age-matched and sex-matched healthy hospital employees were used as the control group. At the time of blood drawing for IFN- α and IRF7 determination, the clinical manifestations and disease activity in each patient were assessed according to the Safety of Estrogens in Lupus Erythematosus: National Assessment — Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) instrument, which produces a score ranging from 0 to 105; in addition, flare composite scores were also obtained²⁰. Twenty-one (41.2%) patients had lupus nephritis based on either proteinuria > 0.5 g/day or the presence of urinary cellular casts. The presence of an infection, renal stones, or other causes of these symptoms were excluded among patients diagnosed with lupus nephritis. Anti-dsDNA antibodies were detected by the standard *Crithidia luciliae* indirect immunofluorescence assay. Twenty-eight patients (54.9%) were found to be positive for anti-dsDNA antibodies. Thirty-four patients (66.6%) had low serum C3. Peripheral blood mononuclear cells (PBMC) and serum were prepared from the heparinized peripheral blood by Ficoll-hypaque separation (Histopaque-1077; Sigma, St. Louis, MO, USA).

For SNP study, a total of 184 subjects were enrolled, including 92 in the SLE group and 92 in the control group. There were only 4 men in the SLE group; they were matched by 4 in the control group. The median age was 38.0 years (Q1, Q3: 30.3, 46.0 yrs) in the control group and 36.0 years (26.0, 45.0 yrs) in the SLE group.

Serum IFN- α level. We measured the IFN- α level in the serum samples with the Human IFN Alpha Serum Sample ELISA Kit (PBL Medical Laboratories, Piscataway, NJ, USA) following the manufacturer's protocol. The detection range of IFN- α by the kit was 0.1–100 pg/ml. Samples and controls (human IFN- α standard from the kit) were serially diluted in an anti-IFN- α antibody-coated microtiter plate, reacted with anti-IFN- α antibody, reacted with horseradish peroxidase-conjugated secondary antibody, developed with tetramethyl-benzidine, and read on a microtiter plate reader at 450 nm. To calculate the concentration of IFN- α , the serum sample data were compared to a standard curve generated from the controls.

IRF7 mRNA expression by real-time polymerase chain reaction (RT-PCR). Aliquots of the blood samples were treated with Blood RNA Stabilization Reagent (Qiagen, Chatsworth, CA, USA) to stabilize the total RNA, and then

centrifuged to pellet nucleic acids. The pellet was washed, resuspended, incubated in buffered proteinase K (Qiagen, Cat. No. 19133) to digest any proteins present, and then centrifuged through a Shredder spin column (Qiagen, Cat. No. 79654) to homogenize the cell lysate while removing residual cell debris. The supernatant was mixed with chloroform in a new microfuge tube and centrifuged. The RNA sample was then mixed with ethanol and centrifuged through an RNeasy Mini spin column (Qiagen, Cat. No. 74106), which was then washed multiple times, treated with DNase I (Qiagen, Cat. No. 79254) to digest any DNA present, and finally eluted into nuclease-free water.

Reverse transcription. To prepare a complementary DNA (cDNA) pool from each RNA sample, total RNA (5 μ g) was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) and the resulting samples were diluted 40 times with DNase-free water. Each cDNA pool was stored at -20°C until RT-PCR analysis. Specific oligonucleotide primer pairs were selected from the Roche Universal Probe Library for the RT-PCR assays. The specificity of each primer pair was validated by performing an RT-PCR reaction using a common reference RNA (Stratagene, La Jolla, CA, USA) as a template, and the size of the PCR product was checked using a DNA 1000 chip (Agilent, Santa Clara, CA, USA) run on a Bioanalyzer 2100 (Agilent). Primer pairs generating the predicted product size and no other side-product were chosen to conduct the following RT-PCR reaction. RT-PCR reactions were performed on the Roche LightCycler Instrument 1.5 using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Cat. 03 515 885 001, Castle Hill, Australia). Briefly, 10 μ l reactions were used that contained 2 μ l Master Mix, 2 μ l of 0.75 μ M forward primer and reversed primer, and 6 μ l cDNA sample. Each sample was run in triplicate. The RT-PCR program consisted of 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s. At the end of the program a melt curve analysis was done. At the end of each RT-PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. The LightCycler 3 Data analysis software automatically calculated the C_p value for each reaction. The fold expression or repression of the target gene relative to the internal control gene, peptidylprolyl isomerase H in each sample, was then calculated using the formula:

$$2^{-\Delta\Delta C_p} \text{ where } \Delta C_p = C_p \text{ target gene} - C_p \text{ internal control} \\ \text{and } \Delta\Delta C_p = \Delta C_p \text{ test sample} - \Delta C_p \text{ control sample}$$

IRF7 polymorphism genotyping. We investigated 2 IRF7 polymorphism sites, NCBI Reference Sequences NM_004031.2 [IRF7 mRNA 776 G>G/A(R); rs1061501] and NCBI Reference Sequences NM_004031.2 [IRF7 mRNA 984 A.A./G(R); rs1061502]. The rs1061501 polymorphism is a synonymous change, while the rs1061502 polymorphism results in a missense mutation. ABI Taqman assays (VIC-C allele; FAM-T allele) were performed for the SNP study, the reverse sequence for the rs1061501 polymorphism is GGT CGG CCG GGT CCC CCG AGT TATC [C/T] CGC AGCA TCA CGA AGC GAC GGG TGC, and ACT GCC TGG AGC AGG AGG TCC CCCT [C/T] GT CAC CAG CTG GGG CAG GGA GGG GG for the rs1061502 polymorphism.

ABI TaqMan SNP genotyping assay. TaqMan SNP Genotyping Assays (Applied Biosystems Inc., Foster City, CA, USA) were used to determine genotypes using archived genomic DNA isolated from PBMC. Assays-on-Demand primers and probes were obtained from Applied Biosystems. The TaqMan assays were performed following the manufacturer's instructions on an MX3000P instrument (Stratagene). The PCR was carried out in a total reaction volume of 25 μ l, which contained 12.5 μ l of 2 \times TaqMan Universal PCR Master Mix, 1.25 μ l of 20 \times TaqMan SNP Genotyping Assay Mix, 1 μ l containing 1–20 ng DNA sample with ddH₂O added to give a 25 μ l final volume. The PCR program was as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 92°C for 15 s, and annealing/extension 60°C for 1 min. At the end of each PCR run, the products were automatically analyzed by the system and an

amplification plot was generated for each DNA sample. Each genotyping plate contained positive and negative controls. About 10% of the samples were duplicated to assure the quality control of the genotyping.

Statistical analysis. Normally distributed continuous data were compared by independent 2-sample t-tests and categorical variables were compared by chi-squared tests. When the data were far from normality, Mann-Whitney U tests were applied. Continuous data were displayed as mean \pm SD. Categorical data were represented by number (%), and nonparametric data were presented as medians (interquartile range). Statistical assessments were 2-sided and evaluated at the 0.05 level of significant difference. Statistical analyses were performed using SPSS 15.0 statistical software (SPSS, Chicago, IL, USA).

RESULTS

There was a wide range of serum IFN- α levels in patients with SLE, from 0.80 to 7.26 pg/ml with a mean of 1.84 pg/ml. Compared with healthy controls, the patients with SLE showed a significant elevation in serum IFN- α level (mean 1.84 vs 0.39 pg/ml; $p < 0.001$). Since renal involvement is considered to be an important prognostic factor in SLE, a comparison of IFN- α level between patients with and without nephritis was performed. We failed to find a significant correlation between renal involvement and serum IFN- α level ($p = 0.13$). However, importantly, we did find that patients with SLE had a significantly higher expression of IRF7 mRNA than the healthy controls (3.69 ± 0.94 vs 1.96 ± 0.80 ; $p < 0.001$). Among patients with SLE, the level of IRF7 mRNA was positively correlated with the serum IFN- α level (Figure 1). Meanwhile, the IRF7 mRNA level was shown to be significantly and positively correlated with SLE disease activity as assessed by the physician's global assessment and SELENA-SLEDAI score (Figure 2). The levels of IRF7 mRNA also paralleled various lupus serological activities markers, such as a positive correlation with serum anti-DNA titers and a negative correlation with C3 concentration (Figure 3).

In our study, 2 IRF7 SNP, rs1061501 and rs1061502, were evaluated for distribution among SLE and control subjects.

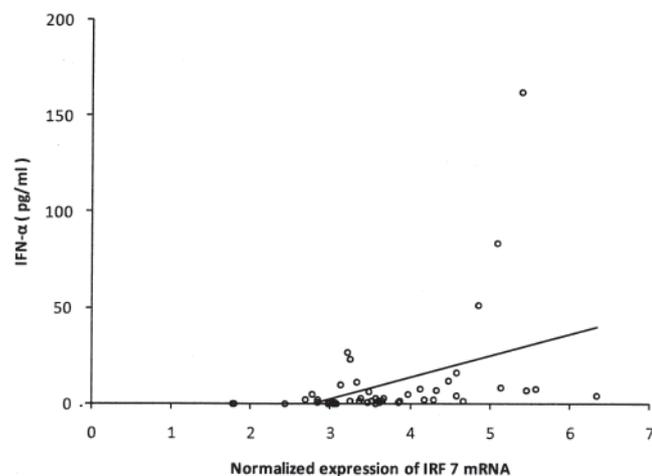


Figure 1. The expression levels of interferon (IFN) regulatory factor (IRF7) messenger RNA (mRNA) in peripheral blood mononuclear cells were significantly correlated with serum IFN- α levels in patients with systemic lupus erythematosus ($p < 0.001$).

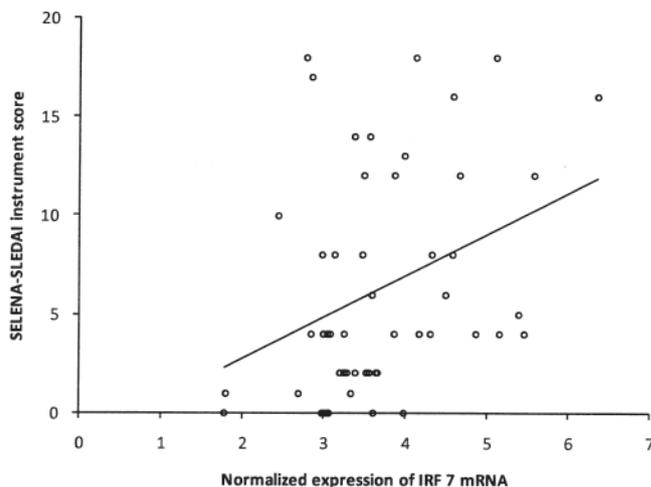


Figure 2. The expression levels of interferon regulatory factor (IRF7) messenger RNA (mRNA) in peripheral blood mononuclear cells were positively correlated with the Safety of Estrogens in Lupus Erythematosus: National Assessment - Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) instrument scores of patients with SLE ($p = 0.01$).

Both SNP were found to be in Hardy-Weinberg equilibrium (p value from Hardy-Weinberg equilibrium test was 1.000 for both). The distributions of the SNP rs1061501 and rs1061502 between the SLE and control groups are shown in Table 1. The distribution of rs1061501 with respect to genotype (CC, CT, and TT) and allele (C vs T) differed significantly between the SLE and control groups ($p = 0.028$ for genotype and $p = 0.009$ for allele). However, there were no significant differences for the SNP rs1061502 between the SLE and control groups. The distribution of the SNP rs1061501 and rs1061502 according to lupus nephritis status among the 92 SLE subjects was also compared and no significant differences between patients with and without nephritis ($p = 0.805$ for rs1061501 and $p = 1.000$ for rs1061502) were found.

DISCUSSION

In concordance with previous reports for other ethnic groups of patients with SLE, our study showed a significant increase in serum IFN- α levels among Taiwanese patients with SLE, confirming again the relevance of IFN- α to human SLE. IFN- α is normally produced in response to viral or bacterial infections through activation of IRF family transcription factors²¹, and is a crucial cytokine that is needed to defeat invading bacteria and viruses. The role of microorganism infection in the overactivation of the IFN system, as occurs in patients with SLE, remains to be clarified. In contrast to this possible role of microorganisms in SLE, another theory suggests that the stimulation may occur through DNA, RNA, and nuclear proteins that are derived from patients themselves, instead of foreign microorganisms^{10,11}. Although IFN have been implicated in lupus nephritis^{22,23}, we found no statistically significant relationship between serum IFN- α levels and lupus nephritis. We interpret this to mean that the relationship

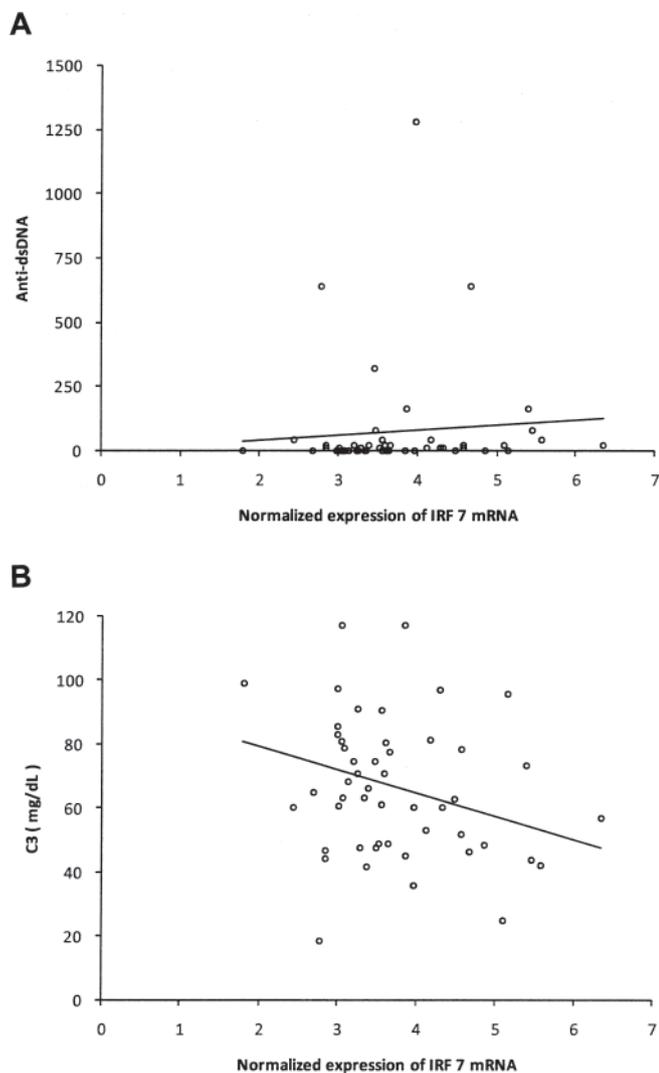


Figure 3. A. The expression levels of interferon regulatory factor (IRF7) messenger RNA (mRNA) in peripheral blood mononuclear cells were positively correlated with anti-dsDNA titers ($p = 0.017$) in patients with systemic lupus erythematosus (SLE). B. The levels were negatively correlated with serum C3 level ($p = 0.032$) in patients with SLE.

between the IFN pathway and lupus nephritis is at the local cellular level. Perhaps plasmacytoid dendritic cells accumulating in the kidneys may be more active and create more local IFN stimulation in lupus nephritis. The number of IFN produced locally in the kidneys and not the IFN- α level in the serum, which is what we measured, may be what is important to lupus nephritis. Of course, other possible reasons may explain this conflicting result, such as patient variation and patient ethnicity. SLE is well known to be a very heterogeneous disease that is characterized by tremendously diverse clinical features, especially across different racial groups.

The main novel findings of our study were that the expression of IRF7 mRNA in patients with SLE increased significantly and was correlated positively with IFN- α serum levels and SELENA-SLEDAI status. In addition, the level of IFN7

Table 1. Frequency of the interferon regulatory factor 7 rs1061501 and rs1061502 single-nucleotide polymorphisms (SNP) compared between patients with systemic lupus erythematosus (SLE) and healthy controls. Data are shown as n (%). Percentages may not add up to 100% because of rounding up or down.

SNP [†]	SLE, n = 92	Controls, n = 92	p**
rs1061501			
Genotype			
CC	7 (7.6)	12 (13.0)	0.028*
CT	30 (32.6)	43 (46.7)	
TT	55 (59.8)	37 (40.2)	
Allele ^{††}			
C	44 (23.9)	67 (36.4)	0.009*
T	140 (76.1)	117 (63.6)	
SNP [†]	SLE, n = 92	Controls, n = 92	p**
rs1061502			
Genotype			
CC	0 (0)	0 (0)	0.720
CT	3 (3.3)	5 (5.4)	
TT	89 (96.7)	87 (94.6)	
Allele ^{††}			
C	3 (1.6%)	5 (2.7%)	0.724
T	181 (98.4%)	179 (97.3%)	

* $p < 0.05$. [†] The SNP rs1061501 and rs1061502 genotypes were assessed for Hardy-Weinberg equilibrium (p of both = 1.000). ** p values were calculated by Fisher's exact test between groups. ^{††} Each SNP has 2 alleles and hence the total of both groups is $92 \times 2 = 184$.

mRNA expression was also correlated positively with serological disease activity markers, anti-DNA titer, and hypocomplementemia. These results suggest that IRF7 plays a role in the pathogenesis of SLE, possibly by increasing the production of IFN- α . Although the expression of IRF7 mRNA was significantly correlated with SELENA-SLEDAI status, the correlation was not very strong. Obviously, many other factors, in addition to IRF7, play a role in SLE clinical disease activity.

We showed a significant difference between the SLE and control groups for the SNP rs1061501, with enrichment of the TT genotype and T allele in patients with SLE. This result for SNP rs1061501 may indicate an increased risk of SLE in the Taiwanese population. To our knowledge, the association of IRF7 polymorphisms with SLE has not been reported in a Taiwanese population before. A study in a Japanese population showed significant distributional differences for SNP of IRF3 and IRF7 between SLE and control groups. However, the significance for the IRF7 SNP was lost after correction for multiple tests¹⁹. A recent study of IRF7 polymorphisms in SLE that used a large and genetically diverse group found associations for several SNP with the presence of dsDNA antibodies and an increased serum IFN- α level²⁴. Specifically, they found that the IRF7 SNP polymorphisms rs702966, rs4963128, and rs702966, in combination with SLE-associated autoantibodies, resulted in higher serum levels of IFN- α ;

this provides *in vivo* biological evidence for a role of this locus at a protein level in human SLE. IRF7 modulates the expression of a large number of genes associated with the IFN pathway. This includes upregulation of the genes encoding transcription factors and those involved in signal transduction and apoptosis^{21,25}. Polymorphisms affecting IRF7 have also been associated with IFN-induced hematological adverse effects in patients with chronic hepatitis C²⁶. In addition, a genomic profiling study showed an association between SNP rs1061501 and esophageal tumors, a finding that supports the idea that this SNP, along with other SNP identified in that study, might be involved in esophageal cancer susceptibility and/or development²⁷. Thus, it appears that functional genetic variants of IRF7 are involved in a variety of disease processes. In a mouse model, knockout of the DNase II gene results in arthritis, anemia, and the constitutive production of IFN- α and IFN- β ; the IFN- β production and anemia are eliminated when IRF3 and IRF7 are knocked out simultaneously with the DNase II gene²⁸. Murine plasmacytoid dendritic cells that have been stimulated with sera or IgG from patients with SLE produce IFN- α ; this has been found to be dependent on IRF7 and also to be partially dependent on IRF5²⁹. These accumulating pieces of evidence suggest that expression of IRF family members, particularly IRF3, IRF5, and IRF7, plays a pathogenetic role in SLE through the production of IFN- α and/or IFN- β .

Our results demonstrated the association of one IRF7 SNP with SLE among Taiwanese patients. Whether this SNP is a functional polymorphism remains to be elucidated. There are several potential limitations of our study; a major one is the limited sample size. Nonetheless, the sample size was large enough to show statistical significance with respect to differences in the distribution of rs1061501 by genotype and allele between the SLE and control groups. Further studies using larger sample sizes will be necessary to confirm our present findings. In addition, the levels of IRF mRNA expression detected by RT-PCR may not be correlated with IRF activity, and this also needs further investigation. Our results indicate a potential role for IRF7 in the pathogenesis of SLE and support the hypothesis that polymorphism of the IRF7 gene might be associated with an increased risk of developing SLE among Taiwanese.

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