

Original article

Novel genetic marker in *TGFB2* gene associated with expression of TGF- β 2 in leukocyte and SLE susceptibility

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Background: TGF- β 2 has a role in immune regulation, and genetic variance within the gene might contribute to systemic lupus erythematosus (SLE) pathogenesis. The *TGFB2* gene is one candidate gene within the major SLE genetic susceptibility loci.

Objective: Investigate the *TGFB2* gene located on chromosome 1q41 as a SLE susceptibility gene.

Materials and methods: One hundred fifty three SLE patients and 133 healthy controls participated in this study. Four markers selected in two haplotype blocks that have a minor allele frequency greater than 5% in Thai population were genotyped and analyzed in the association study.

Results: There was no significant association between SLE susceptibility and the polymorphism in the promoter area (+67_68insACAA) and +89835 (A/G) at the intron 5 of *TGFB2* gene. Instead, minor allele of the two new genetic markers at the intron 1 (+720) (corrected p-value = 0.024, OR = 0.4141, 95%CI = 0.22-0.80) and intron 6 (+94399_94400) (corrected p-value = 0.000143, OR = 0.3367, 95%CI = 0.20-0.58) were independently associated as a protective factor to SLE. Additionally, the real time RT-PCR results showed that patients with the protective allele (minor allele) at the +94399_94400 position have higher TGF- β 2 mRNA level in leukocytes than patients with the risk allele (p=0.011).

Conclusion: Two new genetic markers at intron1 (+720) and intron 6 (+94399_94400) were independently associated with SLE. The observed results have to be confirmed in other populations with a large sample size.

Keywords: Gene polymorphism, *TGFB2*, SLE, Thai population

Positional genetic studies in both murine and human supported the relation of chromosome 1 with systemic lupus erythematosus (SLE) susceptibility especially on chromosome 1q41 as one of the major loci [1]. TGF- β 2 has a role in immune regulation, and genetic variance within the gene might contribute to systemic lupus erythematosus (SLE) pathogenesis. The *TGFB2* gene is one candidate gene within the major SLE genetic susceptibility loci.

Emerging data suggests that the TGF beta family is an important cytokine in immunoregulation. TGF- β 2, in particular, is an important factor in the generation of regulatory T cells [2, 3]. So far, there has been a report of negative association in Caucasian

using one marker at the 5'UTR of *TGFB2* gene with SLE susceptibility [4]. Another genetic approach by the transmission disequilibrium test (TDT) using microsatellite marker upstream of *TGFB2* gene also gave negative results [5]. Since *TGFB2* gene spans approximately 100 kb and consists of at least two haplotype blocks, the negative association using single marker cannot rule out the importance of this gene. Recently, polymorphisms within all exons and nearby introns within *TGFB2* gene have been reported by DNA sequencing of 32 Thai healthy controls [6]. Out of the seven polymorphisms that were detected within *TGFB2* genes in Thai population, we selected four markers that have minor allele frequency greater than 5% for our association study. The two markers (+67_68insACAA and +720(T/G) in 5'UTR and proximal intron 1 region, respectively) are located within one haplotype block. The other two markers (+89835(A/G) and +94399_94400insA in intron 5 and

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intron 6, respectively) are located within another block.

In this paper, we investigated the *TGFB2* gene located on chromosome 1q41 as a SLE susceptibility gene using 153 SLE patients and 133 healthy controls.

Materials and methods

Subjects

One hundred and fifty-three Thai SLE patients attended at King Chulalongkorn Memorial Hospital, who fulfilled at least four of the American College of Rheumatology (ACR)-revised criteria for SLE, were included in this study. One hundred and thirty-three normal controls for the population based case-control association study were recruited from unrelated healthy donors. To avoid the effect of disease activity, we recruited 22 SLE patients with inactive lupus nephritis (LN). These are defined by not having one of the following criteria: a total urinary protein level of more than 0.5 gram per day, an increment of serum creatinine levels of more than 0.5 mg/dL during one-month period of follow-up, or presence of pyuria, hematuria, or urinary cast by microscopic examination more than 10 cells. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, and the subjects gave their informed consent.

DNA extraction

DNA was isolated from buffy coat collected with ethylene diamine traacetic acid (EDTA) as an anticoagulant, using salting out method [7].

Genotyping methodology

We performed the 5' end labeling analysis to detect a 4-base pair (bp) insertion (ACAA) polymorphism of *TGFB2* at the 5' untranslated region

(5' UTR) region upstream 109 bp of the initiation codon and a 1 bp insertion (A) polymorphism in intron 6. The genomic DNA was amplified with the *TGFB2* specific primers as shown in **Table 1**.

PCR was carried out using Applied Biosystems/GeneAmp PCR system 9600 (Applied Biosystems, Foster City, USA) under specific PCR condition, that consisted of an initiation denaturation at 95°C for five minutes, followed by 30 cycles of denaturation (95°C, one minute), annealing (59°C, one minute) and extension (72°C, one minute) and final extension at 72°C for seven minutes. The products were further analyzed by DNA sequencing using 6% denaturing polyacrylamide gel in tris-boric acid EDTA (TBE) buffer and visualized under the Molecular Dynamics Phosphoimager (Amersham Biosciences, Piscataway, USA).

We performed the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the single nucleotide polymorphism at positions +720 (G/T) and +89835 (A/G). The genomic DNA was amplified with the *TGFB2* gene specific primers as shown in **Table 1**. The reaction volume for the amplification reaction was 20 µL, under specific PCR condition, that consisted of an initiation denaturation at 95°C for five minutes, followed by 35 cycles of denaturation (95°C, one minute), annealing (59°C, one minute) and extension (72°C, one minute) and final extension at 72°C for five minutes. For +720 (T/G) in intron 1, the PCR product size was 281 base pairs (bp). Amplified DNA were digested with five units of specific restriction enzyme TaaI (Tsp4CI) (Fermentus, Burlington, Canada) in 1X buffer tango in a total volume of 15 µL at 65°C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 40 minutes. If G was present at this position,

Table 1. Primers used for analysis of the *TGFB2* gene polymorphism.

Polymorphic marker	Polymorphic Position	Sequence of primers (5' → 3')
rs10482719 (4bp insertion)	+67_68 5'UTR	Forward: TTTTGGAACACTGGCCTTTTC Reverse: ACTACTGTGTGCTGAGCGCT
rs1106569 (SNP)	+720 Intron 1	Forward: TTTGACTTCCATCCCTGAG Reverse: AGTTCTTAGTCGGCCACCA
rs6684205 (SNP)	+89835 Intron 5	Forward: GCGGCCTATTGGTTAGGTA Reverse: GCTCTCTGCTCCTCAGAACAA
Th1725 (1bp insertion)	+94399_94400 Intron 6	Forward: ATGTATGTGCAGCCAAGCAT Reverse: GTTCCCGTGTGGAAAATAATGA

TaaI would cut the 281 bp PCR product into two fragments: 185 bp and 96 bp. No digestion would occur if T was present. For position +89835(A/G) in intron 5, PCR product size was 311 bp fragment and the amplified DNA were digested with seven units of specific restriction enzyme FspBI (Meal) (Fermentas, Burlington, Canada) in 1X buffer tango in a total volume of 15 μ L at 37°C for 14-16 hours, followed by a 3% agarose gel electrophoresis at 100 volts for 40 minutes. If G was present at this position, FspBI would cut the 311 bp PCR product into two fragments: 277 bp and 84 bp. No digestion would occur if A was present. A 100 bp ladder (Promega, Madison, USA) was used to estimate the size of the PCR fragments. Additionally, the selected PCR products were analyzed to confirm the results of four sites of single nucleotide polymorphisms (SNP) for TGF- β 2 genotyping by DNA sequencing.

Gene expression analysis

Total RNA was isolated from leukocytes using a QIAamp RNA blood mini kit (Qiagen, Chatworth, USA) according to the manufacturer's instruction. The total RNA concentration was measured with a spectrophotometer. Complementary DNA (cDNA) was constructed using TaqMan Reverse Transcription kit (ABI, California, USA) as described previously [8, 9]. The mRNA levels of TGFB2 and housekeeping gene, 18s rRNA, were determined by LightCycler® machine (Roche Molecular Biochemicals, Indianapolis, USA). The sequence of primers and probes are shown as follow: TGFB2 sense 5'CCT GAA CAA CGG ATT GAG CTA T 3' and antisense 5'GTT ACA TCG AAG GAG AGC CAT T 3'; 18s rRNA sense 5'GCC CGA AGC GTT TAC TTT GA 3', antisense 5'TCC ATT ATT CCT AGC TGC GGT ATC 3' and probe 5'FAM AAA GCA GGC CCG AGC CGC C TAMRA3'. All primer pairs were designed to span across an intron-exon boundary to distinguish amplification of the genomic DNA. The PCR reactions were carried out in 20 μ L total volume, which composed of QuantiTect SYBR or QuantiTect probe (Qiagen, Chatworth, USA) for TGFB2 and 18s rRNA, respectively, 0.5 μ M forward primer, 0.5 μ M reverse primer, 0.2 μ M probe and 2 μ L cDNA template. TGFB2 gene expression was analyzed using a comparative C_T method [10].

Statistical analysis

The goodness of fit to the Hardy-Weinberg

equilibrium was used to calculate the expected frequencies of each genotype and comparing them with the observed values. The polymorphic markers were analyzed for an association with the disease by means of comparison of the minor allele frequency in patients and controls (basic allelic test), as well as other tests, using PLINK (genotype test of 3x2 contingency tables, test of dominant and recessive models) [11]. P-values were corrected by multiplying the number of polymorphic markers tested (Bonferroni correction). A corrected p-value (Pc) of <0.05 was considered as statistically significant. Test of independent contribution of a SNP controlling for the effect of other SNPs in the same gene was done using the logistic regression.

Statistical analysis was performed using the SPSS version 11.5 (SPSS, Chicago, USA). The expression level of common _/_ genotype was used as a calibrator. The TGF- β 2 gene expression levels were tested for normal distribution with Shapiro-Wilk test. The mRNA levels deviated significantly from normal distribution. The non-parametric test (Mann-Whitney U test) was used to compare data between the groups. All statistic results were presented with median and inter-quartile range. A p-value of less than 0.05 was considered statistically significant.

Results

The association results of TGFB2 gene polymorphisms with SLE

The two markers located within one haplotype block are +67_68insACAA at the 5'UTR and +720 (T/G) at the proximal intron1 region. The two other markers (+89835(A/G) and +94399_94400insA) locating in intron 5 and intron 6, respectively are located within another block. Each of the two markers belonging to the same hap-block was not in tight-linkage disequilibrium with each other as shown in Fig 1. We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium in the control sample, using Fisher's exact test ($p <0.05$). There were no significant deviations from Hardy-Weinberg equilibrium in all the SNPs in this study.

Of the four markers genotyped, two out of four markers showed significant association with SLE (Table 2). The +94399_94400 minor allele (A insertion) at the intron6 of TGFB2 gene was significantly decreased in SLE patients compared to healthy controls ($P_c = 0.000143$, $OR = 0.3367$, $95\%CI = 0.20-0.58$). The effect of +94400_944001 minor

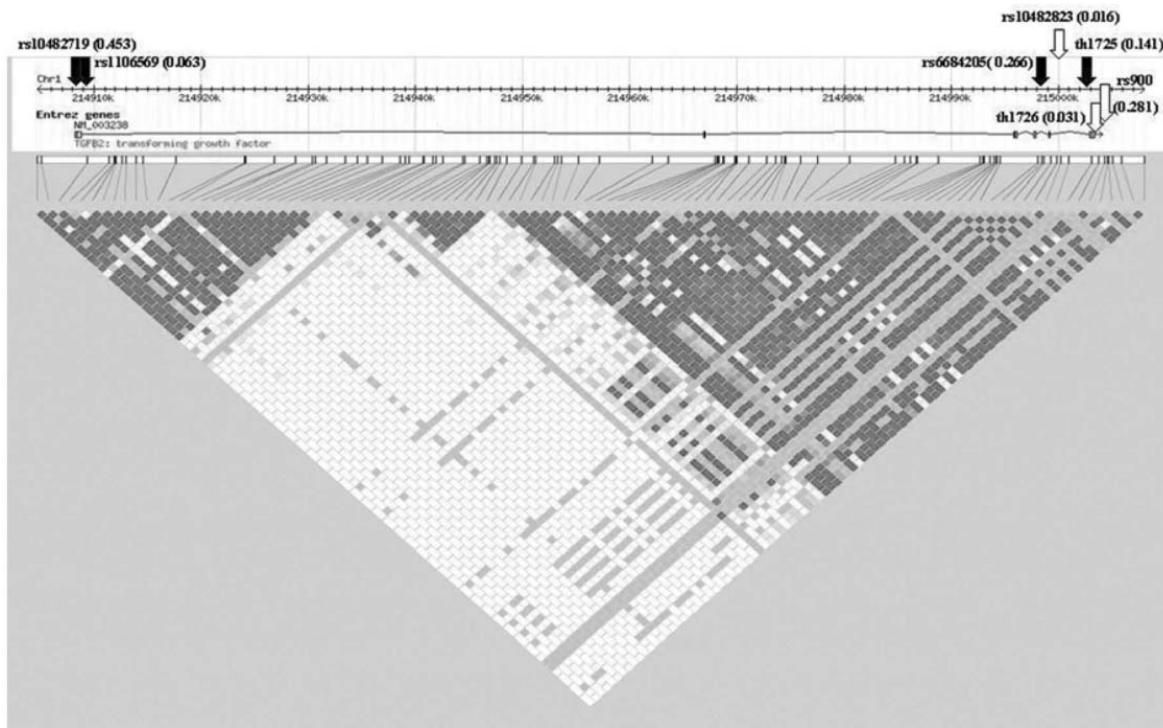


Fig. 1 Seven polymorphic markers of *TGFB2* gene and minor allele frequency in parentheses are shown. Linkage disequilibrium structure (D') was generated in Haploview based on data from Chinese and Japanese populations. The darker red color stands for higher LD. Dark arrows indicate markers used in this study.

Table 2. Polymorphic markers in *TGFB2* gene associated with SLE in Thai.

Polymorphic marker	polymorphic Position	Minor alleles	Control MAF (number,%)	SLE MAF (number,%)	OR	P-value	Pc-value
rs10482719 (4bp insertion)	+67_68 5'UTR	ACAA insertion	117/262, 44.6%	109/306, 35.6%	0.6857 (0.48, 0.96)	0.0282	0.113
rs1106569 (SNP)	+720 Intron 1	G	29/262, 11.1%	15/306, 4.9%	0.4141 (0.22, 0.80)	0.0061	0.0245
rs6684205 (SNP)	+89835 Intron 5	A	94/262, 35.9%	125/306, 40.9%	1.234 (0.88, 1.74)	0.225	0.900
Th1725 (1bp insertion)	+94399_944 00 Intron 6	A insertion	49/262, 18.7%	22/306, 7.2%	0.3367 (0.20, 0.58)	3.58×10^{-5}	0.00014

allele at the intron6 of *TGFB2* gene was similar to the autosomal dominant mode of inheritance. The presence of _/A or A/A genotype conferred with the significant OR of 0.30 (Pc = 0.00016, OR = 0.30, 95%CI = 0.16-0.57). The +720 minor allele (G allele) at the intron1 of *TGFB2* gene was found to be significantly decreased in SLE patients compared to healthy controls (Pc = 0.024, OR = 0.4141, 95%CI = 0.22-0.80). The effect of +720 G allele at the intron1 of TGF- β 2 gene was similar to the autosomal dominant mode of inheritance. The presence of T/G or G/G

genotype conferred with the significant OR of 0.38 (Pc = 0.016, OR = 0.38, 95%CI = 0.18-0.79). Association of the polymorphic markers with disease risk was also corrected with logistic regression using age and sex as covariates and the association found in this study remained significant after all corrections. Logistic regression analysis also indicated that +94399_94400insA remain significant when control the effect of +720(T/G) indicating that two makers were independent of each other.

TGFB2 mRNA expression levels and genotype

Figure 2 shows mRNA expression level with straight lines representing the median value. Despite limited sample size, the levels of TGF- β 2 gene expression were significantly higher in patients with $_{_}A$ or A/A genotype at position +94399_94400insA

($p=0.011$). Gene expression level of TGFB2 genotype at position +67_68insACAA were not significantly different between $_{_}ACAA$ or ACAA and $_{_}$ ($p=0.98$). The mRNA expression of patients with intron 1 (+720G/T) genotype were not significantly different between G/T and T/T genotypes ($p=0.36$).

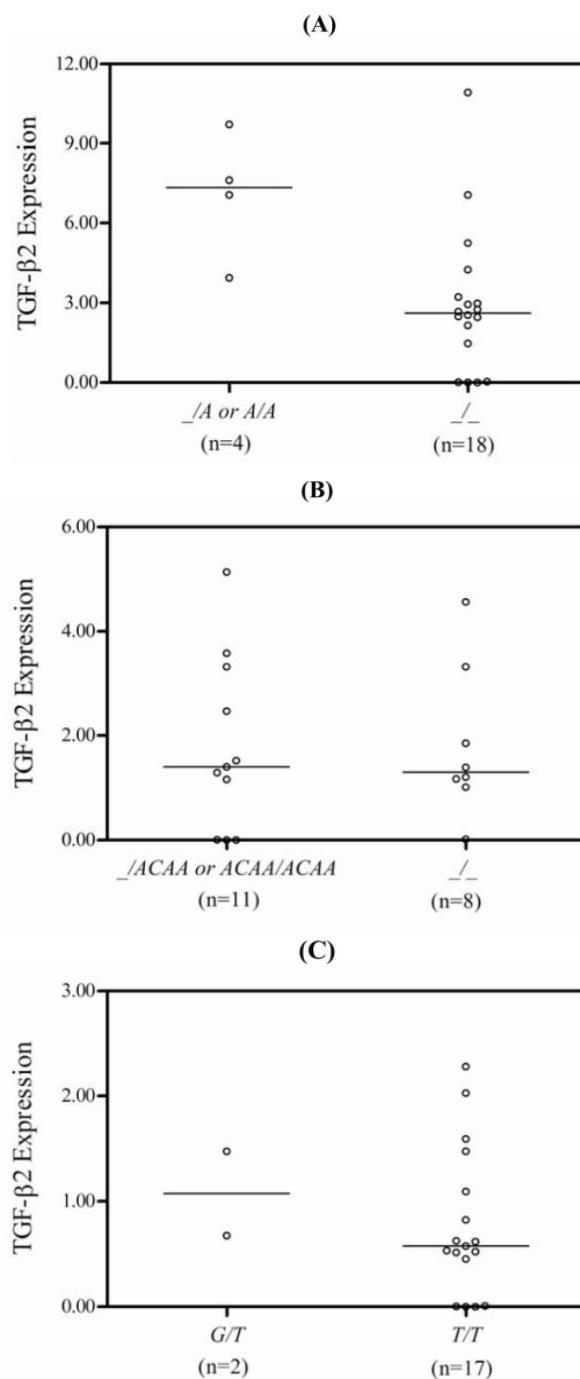


Fig. 2 Comparison of TGF- β 2 expression and genotype in lupus nephritis patients. The scattered plots show mRNA expression level and straight lines represent the median value. (A) The levels of TGF- β 2 gene expression were significantly higher in patient with $_{_}A$ or A/A genotype at position +94399_94400insA ($p=0.011$). (B) Gene expression levels of TGFB2 genotype at position +67_68insACAA were not significantly different between $_{_}ACAA$ or ACAA and $_{_}$ ($p=0.98$). (C) The mRNA expression of patients with intron1 (+720G/T) genotype were not significantly different between G/T and T/T genotypes ($p=0.36$).

Discussion

Similar to a previous study in Caucasian SLE [4], we could not find any significant association between SLE susceptibility to the polymorphism in the promoter area (+67_68insACAA) that have been suggested earlier to affect TGF- β 2 expression by bio-informatic prediction [12]. Instead, we discovered that two new genetic markers at intron1 (+720) and intron 6 (+94399_94400) were independently associated with SLE. We are particularly interested in a marker at intron 6 because of its highly significant value. Since TGF- β 2 consists of various splicing forms, these genetic markers might affect the splicing position which subsequently causes the dysregulation of the immune system. However, our attempt to analyze the splice forms of TGF- β 2 in SLE patients mainly detected only the short isoform (769 bp) (data not shown). Then, we hypothesize that the polymorphism at this position might be associated with mRNA stability and expression of this gene product, and analyzed mRNA expression using real-time RT-PCR from leukocytes of SLE patients with different genotypes. Our results showed that patients with protective allele (minor allele) at +94399_94400 position have higher TGF- β 2 mRNA level in leukocytes than patients with risk allele. However, no significant differences of TGF- β 2 expression among patients with different genotype at promoter or intron 1 position were found.

It has been shown that genetic polymorphism at 3'UTR can affect gene expression, but we did not prove in this study whether 1bp insertion polymorphism at intron 6 affects the expression of TGF- β 2 directly or indirectly. The association between genotype and expression level might be caused by causative SNP or mutation elsewhere in the intron or 3'UTR that is closely linked to our genetic marker. Since our study contains a small sample size, independent studies to confirm this result would be needed.

While TGF-B1 is an important inhibitory cytokine release from regulatory T-cells, TGF- β 2 has been shown to be an important factor that induces regulatory T-cells [2, 3]. There are also supporting evidence that regulatory T-cells are defective quantitatively and qualitatively in SLE patients [13-17]. It would be of clinical interest to analyze the association of TGF- β 2 expression in the leukocytes with regulatory T-cells in SLE patients.

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