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Single-nucleotide polymorphisms of transforming growth factor-β1 gene in Taiwanese patients with systemic lupus erythematosus

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Transforming growth factor-β1 (TGF-β1) is involved in the generation of CD8+ T suppressor cells, natural killer (NK) cells and regulatory T (Th3) cells for down-regulatory effects on antibody production. We studied TGF-β1 activity in patients with systemic lupus erythematosus (SLE) to try to clarify whether the dysregulation by TGF-β1 is genetically determined. Sera from 55 patients with clinically inactive SLE, who were taking minimal steroids and/or hydroxychloroquine, and 40 healthy controls, along with supernatants from concanavalin A-stimulated peripheral blood mononuclear cell (PBMC) cultures from 18 patients with SLE and 10 controls were subjected to TGF-β1 enzyme-linked immunosorbent assay. A total of 138 patients with SLE and 182 controls were genotyped for 5 single-nucleotide polymorphisms (SNPs) of TGF-β1: -988C/A, -800G/A, -509C/T, Leu^{10}/Pro^{10} and Arg^{25}/Pro^{25}. Patients with SLE had lower serum levels of TGF-β1 compared with controls (p=0.052). The unstimulated and stimulated TGF-β1 production of PBMCs in patients with SLE was higher than in controls, although these differences did not reach significance (p=0.073 and 0.074, respectively). None of the TGF-β1 SNPs was strongly associated with SLE in Taiwanese patients or had any prognostic significance in lupus nephritis. Hence these polymorphisms do not represent a genetic predisposition to SLE. The intrinsic capability of immunoregulation for spontaneous B cell hyperactivity through PBMC TGF-β1 production was presumed to be intact in clinically stable SLE in Taiwanese. Whether the lower serum TGF-β1 level that causes the defective immune regulation in SLE is primarily under genetic control or secondary to the influence of ongoing cellular interactions in the cytokine context needs to be elucidated.

Key words: Case-control studies, single nucleotide polymorphism, systemic lupus erythematosus, transforming growth factor beta

Systemic lupus erythematosus (SLE), the most common autoimmune disease in Taiwan [1],is characterized by polyclonal B cell hyperactivity, a plethora of autoantibodies, and subsequent diverse clinical manifestations. Both the persistence of autoreactive helper T cells and the lack of maturation or appearance of appropriate down-regulating T cells play a vital role in the perpetuation of pathogenic autoantibodies formation [2]. It has become increasingly clear that cytokines contribute markedly to the pathogenesis of SLE.

Transforming growth factor-β (TGF-β) is a family of proteins (TGF-β1, 2, and 3), secreted by many cell types, with pleiotropic signalling properties and multifunctional effects. In general, TGF-β has a stimulatory effect on cells of mesenchymal origin and an inhibitory effect on cells of epithelial or neuroectodermal origin. Various studies have shown that TGF-β plays an important role in a number of diseases, including osteoporosis [3], spinal osteophytosis [4], myocardial infarction [5], Alzheimer's disease [6], proliferative diabetic retinopathy [7], and breast cancer [8].

In the immune system, TGF-β1 is produced by dendritic cells, macrophages, lymphocytes, and natural killer (NK) cells. TGF-β1 can suppress T or B cell function, and serves as a costimulatory factor in the development of regulatory T cells. Therefore, the production of this cytokine by antigen-specific T cells defines a unique regulatory (Th) subset, Th3 [9]. It has been established that peripheral lymphocytes produce levels of active TGF-β that enable CD8+ cells to develop inhibitory activity [10,11], and that lymphocyte

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production in both the total and biologically active form of TGF-β is reduced in subjects with SLE [12]. Furthermore, decreased production of total TGF-β1 inversely correlates with disease activity, although that of active TGF-β1 does not have such a correlation [13]. It has been shown that the lack of interleukin (IL)-2 [11,14,15] or tumor necrosis factor-α [12,16], increased amounts of IL-10 [11,17], and decreased protease activity in subjects with SLE [18-20] contribute to decreased active TGF-β production that blocks the generation of regulatory T cells.

The human gene encoding TGF-β1 is located on chromosome 19q13. Several single nucleotide polymorphisms within the TGF-β1 genes have been identified: 3 in the promoter region at positions -988 (C→A), -800 (G→A), -509 (C→T) and 2 in the signal sequence at positions +869 (T→C) and +915 (G→C) [6,21]. The polymorphism at position +869 changes codon 10 (Leu→Pro), that at position +915 changes codon 25 (Arg→Pro), and both were shown to correlate with interindividual variation in levels of TGF-β1 production [21]. Associations of these genetic polymorphisms with immune diseases such as allergies/asthma (-509T) [22], post-transplant allograft fibrosis (Arg^{25}) [21], and multiple sclerosis [23], have been reported.

TGF-β1 seems to be of utmost importance in the regulation of autoimmunity. The production of TGF-β1 might be under genetic control, which in turn influences the development of autoimmunity. In this study, we investigated the association of TGF-β1 polymorphisms with susceptibility to SLE in Taiwanese patients and attempted to identify their functional correlations and association with lupus nephritis.

Materials and Methods

Patients and subjects

A total of 138 unrelated patients (128 females, 10 males, average age 29.2 years) fulfilling the classification criteria of the 1982 American Rheumatism Association for SLE [24] were followed up for at least 5 years at the rheumatology clinic of Veterans General Hospital-Kaohsiung, Taiwan. Sixty one patients (44.2%) had biopsy-documented renal involvement, 5 (3.6%) had central nervous system disease, 12 (8.7%) had severe hematologic involvement, and 60 (43.5%) had mild disease mainly affecting the skin and joints. 182 unrelated healthy blood donors were selected as controls. All patients with SLE and controls underwent TGF-β1 genotyping.

Serum collection

Serum samples were collected from 55 of the 138 patients with SLE, who were clinically stable and taking minimal doses of prednisolone (less than 10 mg per day) with or without hydroxychloroquine or cytotoxic drugs, and 40 controls, who were free of any medical diseases or minor infections, and kept frozen at -70°C.

Cell culture

Peripheral blood mononuclear cell (PBMC) culture was performed in 18 of the 55 patients with inactive SLE and 10 controls. PBMCs were isolated from heparinized venous blood of these 28 individuals by centrifugation over a Ficoll-Hypaque density gradient (Pharmacia Fine Chemicals, Portage, Michigan, USA) at 400 × g for 30 minutes. The cells were resuspended twice in RPMI 1640 supplemented with fetal calf serum (10%), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) [Gibco, Grand Islands, New York, USA]. The cells, at a concentration of 2×10^5 cells/mL, were activated by concanavalin A (Con-A, 10 μg/mL; Sigma, St Louis, Missouri, USA) and incubated in 5% CO_2 at 37°C for 48 hours. The supernatants were collected and stored immediately at -70°C before TGF-β1 cytokine assay. Control tubes without Con-A added were processed the same way.

Quantitation of TGF-β1 cytokine titer

Using acid activation and neutralization, the latent TGF-β1 contained in the serum and cell culture supernatants was activated to the immunoreactive form and subjected to "sandwich" enzyme immunoassay in duplicate (Quantikine; Cat no. DB100, R & D Systems, Minneapolis, USA). The diluted active TGF-β1 binds to TGF-β1 soluble receptor type II coated on the microtiter plate. After washing, an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells to "sandwich" the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TGF-β1 bound in the initial step. The color development was stopped and the intensity of the color was measured by spectrophotometer at 450 nm. The results were calculated by reference to the standard curve and expressed as picograms per milliliter (pg/mL).

Genotyping

Genomic DNA from whole blood was extracted and purified using the phenol-chloroform method

recommended in the 11th International HLA Workshop [25]. Based on the published sequences of the TGF-β1 gene [21,26], 2 fragments were amplified by polymerase chain reaction (PCR) with 2 sets of primers (5'-GCTGCCAGCTTGCAGGCTATGGAT-3' and 5'-GGAGAAGGAGGGTCTGTCAACATG-3' for fragment 1 and 5'-ACTGCGCCCTTCTCCCTG-3' and 5'-GTTGTGGGTTTCCACCATTAG-3' for fragment 2). Reactions were performed in a total volume of 50 μL containing 0.5 μg of genomic DNA, 10 pmol of each primer, 0.2 mM each of the dNTPs, 1 unit of AmpliTaq DNA polymerase (Perkin Elmer), 50 mM KCl, 2 mM $MgCl_2$, 10% dimethylsulfoxide (fragment 2 only), and 10 mM Tris-HCl (pH 8.3). The cycling reaction for fragment 1 consisted of initial denaturation at 95°C for 1 minute; 40 cycles of denaturation at 94°C for 15 seconds; annealing at 61°C for 30 seconds; extension at 72°C for 40 seconds; and a final extension at 72°C for 5 minutes. The thermocycling procedure for fragment 2 consisted of initial denaturation at 95°C for 5 minutes; 5 cycles of 1 minute at 94°C, 60°C and 72°C; then 40 cycles of 30 seconds at 94°C, 56°C, and 72°C; and a final extension at 72°C for 1 minute. The PCR products were purified and subsequently sequenced by an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) and the 5 polymorphic sites were identified.

Statistical analysis

Comparison among the groups was performed by chi-squared test with Yate's correction and Fisher's exact test. Bonferroni's correction was applied to adjust for multiple comparisons. Comparisons of cytokine quantitations among the groups were analyzed by the Student's *t* test for the independent samples. All calculations were performed using SPSS software.

Results

Serum level and PBMC production of TGF-β1

To minimize the influence of disease activity and medication on serum and PBMC production of TGF-β1, the patients with SLE included in this study were all clinically stable and taking low doses of steroids and/or hydroxychloroquine or cytotoxic drugs. As shown in Fig. 1, the serum level of TGF-β1 was lower in patients with SLE compared with controls (818.0 ± 257.81 vs 1042.75 ± 102.53 pg/mL; p=0.052). In addition, the serum level of TGF-β1 was slightly lower in patients with SLE with nephritis compared to patients with SLE without nephritis (760.71 ± 187.32 vs 889.61 ± 325.20 pg/mL); however, this difference did not reach significance. Thereafter, we compared PBMCs from control donors and patients with SLE for their ability to produce TGF-β1 either constitutively or after stimulation

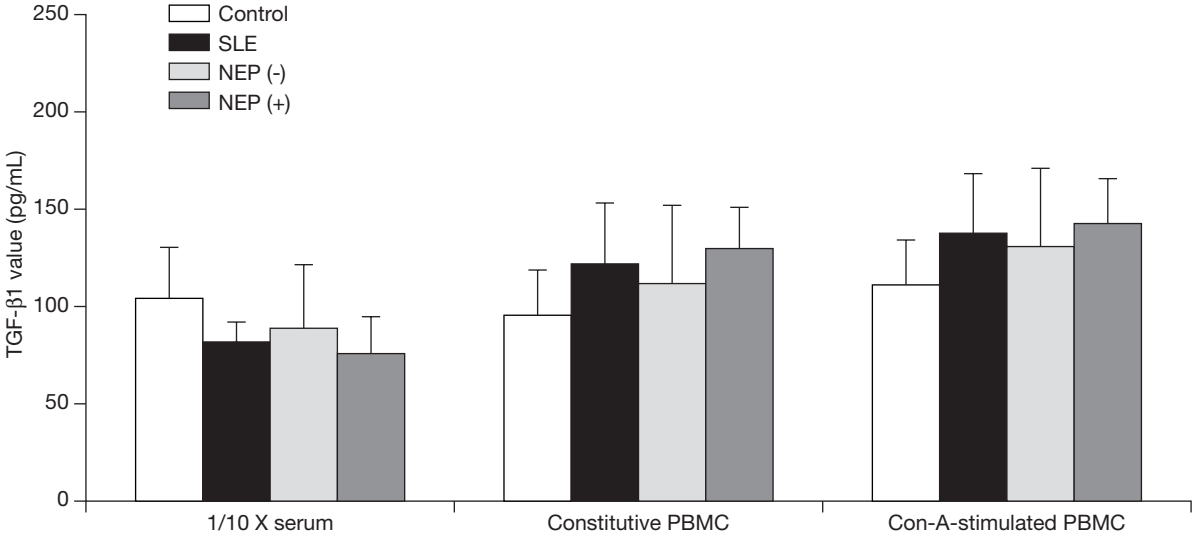

Fig. 1. Mean and standard deviation of serum transforming growth factor (TGF)-β1 levels and peripheral blood mononuclear cell (PBMC) TGF-β1 production in patients with systemic lupus erythematosus (SLE) and controls and patients with SLE with and without nephritis (NEP). Data for serum TGF-β1 level was multiplied by 0.1 to fit in the figure. The concanavalin A (Con-A) stimulated PBMCs were treated with Con-A for 48 hours, while the constitutive PBMCs were not stimulated with Con-A. The TGF-β1 levels were determined by a commercial enzyme-linked immunosorbent assay kit.

Table 1. Allele frequencies in patients with SLE and controls

Locus	Allele	Controls n = 364 (%)	No. (%) of patients		
			Total n = 276	Nephritis (+) n = 120	Nephritis (-) n = 156
-988	C	364 (100)	276 (100)	120 (100)	156 (100)
	A	0	0	0	0
-800	G	364 (100)	276 (100)	120 (100)	156 (100)
	A	0	0	0	0
-509	C	173 (47.6)	122 (44.2)	57 (47.5)	65 (41.7)
	T	191 (52.4)	154 (55.8)	63 (52.5)	91 (58.3)
Codon 10	Leu	176 (48.3)	127 (46.0)	58 (48.3)	69 (44.2)
	Pro	188 (51.7)	149 (54.0)	62 (51.7)	87 (55.8)
Codon 25	Arg	364 (100)	274 (99.3)	119 (99.2)	155 (99.4)
	Pro	0	2 (0.7)	1 (0.8)	1 (0.6)

Abbreviations: SLE = systemic lupus erythematosus; Leu = leucine; Pro = proline; Arg = arginine

with Con-A, which has been shown to effectively induce suppressor cell activity [27,28]. The unstimulated production of TGF-β1 by lupus PBMCs was somewhat higher than that of control PBMCs (121.69 ± 30.92 vs 95.67 ± 23.02 pg/mL; p=0.073). The increment of TGF-β1 production in response to Con-A stimulation was insignificant in the first 48-hour culture period. Nevertheless, the stimulated PBMCs of patients with SLE produced consistently larger amounts of TGF-β1 than those of controls (137.09 ± 30.83 vs 111.25 ± 22.67 pg/mL; p=0.074). In addition, the differences of unstimulated and stimulated TGF-β1 production in PBMC cultures were not significant between patients with SLE with and without nephritis (129.42 ± 21.10 vs 112.04 ± 39.47 pg/mL and 142.30 ± 22.95 vs 130.59 ± 39.29 pg/mL, respectively; p=not significant [NS] for both comparisons).

Genotype distribution and frequency

Five polymorphisms in the TGF-β1 gene between position -1321 and +996 were analyzed: 3 substitutions in the promoter region of the gene at positions -988 (C→A), -800 (G→A), -509 (C→T), and 2 in the gene coding region for the precursor part of the TGF-β1 protein (exon 1): +869 (T→C) for codon 10 (Leu^{10}→Pro^{10}) and +915 (G→C) for codon 25 (Arg^{25}→Pro^{25}), respectively. The distributions of allele and genotype frequencies are shown in Tables 1 and 2. All patients and controls (total n=320) were -988C and -800G genotype and none had -988A and -800A genotype. Meanwhile, Arg^{25} was the

Table 2. Genotype frequencies in patients with SLE and controls

Locus	Genotype	Controls n = 182 (%)	No. (%) of patients		
			Total n = 138	Nephritis (+) n = 60	Nephritis (-) n = 78
-988	C/C	182 (100)	138 (100)	60 (100)	78 (100)
	C/A	0	0	0	0
	A/A	0	0	0 0	
-800	G/G	182 (100)	138 (100)	60 (100)	78 (100)
	G/A	0	0	0	0
	A/A	0	0	0	0
-509	C/C	39 (21.4)	28 (20.3)	15 (25.0)	13 (16.7)
	C/T	95 (52.2)	66 (47.8)	27 (45.0)	39 (50.0)
	T/T	48 (26.4)	44 (31.9)	18 (30.0)	26 (33.3)
Codon 10	Leu/Leu	39 (21.4)	27 (19.6)	15 (25.0)	12 (15.4)
	Leu/Pro	98 (53.9)	73 (52.9)	28 (46.7)	45 (57.7)
	Pro/Pro	45 (24.7)	38 (27.5)	17 (28.3)	21 (26.9)
Codon 25	Arg/Arg	182 (100)	136 (98.6)	59 (98.3)	77 (98.7)
	Arg/Pro	0	2 (1.4)	1 (1.7)	1 (1.3)
	Pro/Pro	0	0	0	0

Abbreviations: SLE = systemic lupus erythematosus; Leu = leucine; Pro = proline; Arg = arginine

Table 3. Haplotype frequencies in patients with SLE and controls

Haplotype	-509	Codon 10	Controls n = 364 (%)	No. (%) of patients		
				Total n = 276	Nephritis (+) n = 120	Nephritis (-) n = 156
1	C	Leu	121 (33.2)	87 (31.5)	43 (35.8)	44 (28.2)
2	C	Pro	52 (14.3)	35 (12.7)	14 (11.7)	21 (13.5)
3	T	Leu	55 (15.1)	40 (14.5)	15 (12.5)	25 (16.0)
4	T	Pro	120 (37.4)	114 (41.3)	48 (40.0)	66 (42.3)

Abbreviations: SLE = systemic lupus erythematosus; Leu = leucine; Pro = proline

major allele, and only 2 subjects (2/320, 0.6%) expressed Pro^{25} allele. The distributions of -509C/T and Leu^{10}/Pro^{10} allele frequencies were rather even between the SLE and control groups, and between patients with SLE with or without nephritis. There were no significant differences in -509C/T and Leu^{10}/Pro^{10} allele and genotype frequencies between these groups and subgroups. The homozygosities did not have a dose effect on disease association.The distribution and frequencies of the 4 TGF-β1 haplotypes linking -509C/T and Leu^{10}/Pro^{10} are presented in Table 3. No significant differences were found in these haplotype frequencies between the SLE and control groups, and between patients with SLE with or without nephritis.

TGF-β1 genetic polymorphisms and TGF-β1 production

As shown in Table 4, individuals with -509C homozygous genotype had a mean serum TGF-β1 concentration of 774.83 ± 220.73 pg/mL, whereas those with the -509T homozygous genotype had a mean concentration of 955.12 ± 260.80 pg/mL (*p*=NS). Individuals with Leu^{10} homozygous genotype had lower mean serum TGF-β1 concentration (779.40 ± 199.42 pg/mL), and those with Pro^{10} homozygous genotype had a higher TGF-β1 concentration (989.53 ± 277.18 pg/mL; *p*=0.064). No differences were detected in the TGF-β1 production of unstimulated and stimulated PBMC cultures among subjects with -509C, -509T, and Leu^{10}, Pro^{10} genotype.

Discussion

TGF-β consists of a multifunctional family of cytokines important in tissue repair, inflammation, and immunoregulation. NK cells, lymphocytes, and monocytes produce the TGF-β1 isoforms. TGF-β1 is known to have both beneficial and deleterious effects on lupus. SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation were observed in TGF-β knockout mice [29]. TGF-β enhances CD8 expression, and costimulates CD8+ T cells to down-regulate [30]. TGF-β inhibits T and B cell proliferation, induces apoptosis in both B cells and in the fully differentiated plasma cells, NK cell cytotoxic activity, and the generation of T cell cytotoxicity [31-33]. In our study, patients with SLE had lower serum concentrations of TGF-β1 compared with normal controls, and the serum concentration of TGF-β1 in patients with SLE and nephritis was decreased in comparison to patients with SLE without nephritis. This finding may support the idea that a deficiency in the TGF-β1 regulatory feedback loop limits expansion of the activated populations resulting in B cell hyperactivity and overproduction of autoantibodies.

Ohtsuka et al [13] reported that lymphocyte production of the total and active forms of TGF-β1 was decreased and the decrement did not correlate with disease activity or severity. They also indicated that a brief treatment of PBMCs from patients with

Table 4. TGF-β1 levels in different TGF-β1 allelic types

Allele	Serum level (pg/mL)	Constitutive TGF-β1 production (pg/mL)	Con-A stimulated TGF-β1 production (pg/mL)
-509C	774.83 ± 220.73	121.33 ± 25.37	134.90 ± 26.39
-509T	955.12 ± 260.80	116.76 ± 38.03	133.36 ± 37.58
p	NS	NS	NS
Leu^{10}	779.40 ± 199.42	117.65 ± 29.03	133.94 ± 31.36
Pro^{10}	989.53 ± 277.18	120.24 ± 37.50	134.18 ± 35.13
p	0.064	NS	NS

Abbreviations: TGF = transforming growth factor; Con-A = concanavalin A; Leu = leucine; Pro = proline; NS = not significant

SLE with IL-2 and picomolar concentrations of TGF-β can result in marked inhibition of spontaneous polyclonal immunoglobulin G and autoantibody production [14]; the deficiency of TGF-β1 in the local environment at a critical time could possibly account for the ineffectiveness of T cell regulatory function in controlling B lymphocyte activity in patients with SLE. Nevertheless, in our study, the constitutive or stimulated PBMC production of TGF-β1 of patients with SLE was higher compared with the control group, although these differences did not reach significance (p=0.073 and p=0.074, respectively). The intrinsic capability of immunoregulation for spontaneous B cell hyperactivity through TGF-β1 production was presumed to be intact in clinically stable Taiwanese patients with SLE.

Picogram quantities of TGF-β are needed for suppressor cell induction [11], and nanogram amounts are essential for suppressive effects [33-35]. Strikingly, in a revealing study by Snapper et al [36], the addition of an anti-TGF-β blocking antibody to lipopolysaccharide-activated cultures led to a significant decrease in the secretion of immunoglobulins, suggesting that low levels of autocrine TGF-β may serve to enhance production and secretion of immunoglobulins under certain conditions. Therefore, the net biologic effect of TGF-β is determined by the local cytokine concentration, the cell types affected, and by the relative state of differentiation of the responsive cell. Despite the finding that intrinsic TGF-β1 production by PBMCs was not affected in our patients, exposure of T cells to insufficient picomolar concentrations of TGF-β1 at the tissue and at the time they are activated could result in either impaired down-regulation or increased up-regulation of immunoglobulin production.

The decreased serum levels of TGF-β1 activity most likely reflected an overall decreased production from all cell types. Hence, we questioned whether the decreased TGF-β1 activity could be under genetic control, as with other cytokines. The following 5 single-nucleotide polymorphic loci were studied: -988 (C→A), -800 (G→A), -509 (C→T) in the promoter region; and Leu^{10}→Pro^{10}, Arg^{25}→Pro^{25} in the signal sequence. We noted inter-ethnic differences among these polymorphic sites (Table 5). -800G, -509C, Leu^{10}, and Arg^{25} were the more common alleles in healthy Caucasian controls. Distinctively, there were no -988A or -800A and rare Pro^{25} (0.6%) alleles in this Taiwanese population, which could be excluded as potential genetic markers. The frequencies of -509C/T and Leu^{10}/Pro^{10} were similar in Taiwanese healthy controls and the frequency distributions were also similar to patients with SLE, with or without nephritis. The frequency distributions of haplotype linking -509 and codon 10 in patients with SLE were not unusual compared with the control group. Although polymorphism at codon 25 has been shown to be significantly associated with TGF-β1 production in vitro [21], there were no functional correlations with TGF-β1 production by PBMCs and -509 and codon 10 alleles in our population.

Table 5. Inter-ethnic differences of frequencies of alleles in TGF-β1 polymorphisms between patients with SLE and controls

Locus	Allele	SLE (%)	Percentage of controls				
		TW[a]	TW[a]	Japan[b]	France[c]	UK[d]	USA[e]
-988	C	100	100				
	A	0	0				
-800	G	100	100		91.2	89	94
	A	0	0		8.8	11	6
-509	C	44.2	47.6		65.7	76	66
	T	55.8	52.4		34.3	24	34
Codon 10	Leu	46.0	48.3	56	58.4	65	62
	Pro	54.0	51.7	44	41.6	35	38
Codon 25	Arg	99.3	100		91.8	90	94
	Pro	0.7	0		8.2	10	6

Abbreviations: TGF = transforming growth factor; SLE = systemic lupus erythematosus; TW = Taiwan; UK = United Kingdom; USA = United States of America; Leu = leucine; Pro = proline; Arg = arginine

[a]TW: data from this study.

[b]Japan: Yamada et al, 2000 [4].

[c]France: Cambien et al, 1996 [5].

[d]UK: Awad et al, 1998 [21].

[e]USA: Green et al, 2001 [37].

Our data suggest that these TGF-β1 polymorphisms are not associated with SLE in Taiwanese patients and have no prognostic significance in lupus nephritis; thus, their presence alone would not be a genetic risk factor for predisposition to SLE. It is well-established that patients with SLE have decreased TGF-β1 regulatory function for control of B cell hyperactivity. Whether the defect of regulation is primarily under genetic control or secondary to the influence of ongoing cellular interactions or the cytokine context needs to be elucidated.

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