# Cigarette Smoking, *STAT4* and *TNFRSF1B* Polymorphisms, and Systemic Lupus Erythematosus in a Japanese Population

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***ABSTRACT.*** ***Objective.*** Recent studies have identified signal transducer and activator of transcription 4 (*STAT4*) as a susceptibility gene for systemic lupus erythematosus (SLE) in different populations. Similarly, tumor necrosis factor receptor superfamily, member 1B (*TNFRSF1B*) has been reported to be associated with SLE risk in Japanese populations. Along with environmental factors such as smoking, both polymorphisms may modulate an individual's susceptibility to SLE. We investigated these relationships in a case-control study to evaluate risk factors for SLE among Japanese women.

***Methods.*** We investigated the relationship of the *STAT4* rs7574865 and *TNFRSF1B* rs1061622 polymorphisms to SLE risk with special reference to their combination and interaction with cigarette smoking among 152 SLE cases and 427 controls.

***Results.*** The TT genotype of *STAT4* rs7574865 was significantly associated with increased risk of SLE (OR 2.21, 95% CI 1.10–4.68). Subjects with at least one G allele of *TNFRSF1B* rs1061622 had an increased risk of SLE (OR 1.56, 95% CI 0.99–2.47). The attributable proportion due to the interaction between the *TNFRSF1B* rs1061622 genotypes and smoking was estimated to be 0.49 (95% CI 0.07–0.92), indicating that 49% of the excess risk for SLE in smokers with at least one G allele was due to an additive interaction. A lack of significant associations of *STAT4* with smoking was observed. No significant gene-gene interactions were found among polymorphisms of *STAT4* and *TNFRSF1B*.

***Conclusion.*** Our findings suggest that the association between cigarette smoking and SLE could be differentiated by the *TNFRSF1B* rs1061622 T allele among female Japanese subjects. This preliminary exploratory result should be confirmed in a larger study. (First Release August 15 2009; J Rheumatol 2009;36:2195–203; doi:10.3899/jrheum.090181)

*Key Indexing Terms:*

| | | |
|---|---|---|
| *STAT4* | *TNFRSF1B* | SMOKING |
| SYSTEMIC LUPUS ERYTHEMATOSUS | INTERACTION | EPIDEMIOLOGY |

Despite intensive research, the etiology of systemic lupus erythematosus (SLE) remains unclear. Many environmental exposures including smoking, ultraviolet light, medications, infectious agents, hair dyes, and dietary factors have been hypothesized to be associated with the development of SLE[1-5], although the strength of the evidence implicating each of these factors varies. Our previous study found that current smoking conferred a 2.0-fold increase in the risk of

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SLE[6] among Japanese women. A recent metaanalysis of the 9 existing studies examining this relationship revealed a small but significantly increased risk for development of SLE among current smokers compared with nonsmokers (OR 1.50, 95% CI 1.09–2.08)[5].

Studies of twin concordance are commonly used in epidemiology to estimate the role of genetics and the influence of environmental factors on disease susceptibility. Disease concordance is much higher in monozygotic twins (24%–57%) than in dizygotic twins (2%–5%), suggesting a genetic component to SLE[7-9]. However, progress in identifying these genetic factors was initially slow. The genetic basis of SLE is very complex and it is difficult to predict how many genes contribute to SLE susceptibility; it has been estimated that over 100 genes may be involved in SLE susceptibility[10]. Advances in gene identification have been made by candidate gene association studies and, more recently, by genome-wide association studies (GWAS). GWAS combine the power and resolution of a conventional association study with the hypothesis-free methodology of a linkage scan. Two GWAS in SLE, performed in populations of European descent, have been published to date[11,12]. Even before the GWAS, there was growing evidence to implicate single-nucleotide polymorphisms (SNP) in the HLA region, interferon regulatory factor 5 and *STAT4*[13-15]. In addition to these, SNP in B lymphoid tyrosine kinase, chromosome 8 open reading frame 13, integrin alpha M, integrin alpha X, PX domain containing serine/threonine kinase, and C-terminal domain-binding SR-like protein rA9 have been established as new candidate genes for SLE by GWAS[11,12]. The advent of GWAS has had an unprecedented influence on our knowledge of the genetics of SLE, bringing to light many unexpected candidate genes and biological pathways. There is more to come, given that the current risk loci do not fully account for the genetic contribution to disease susceptibility (e.g., 32 risk loci for another autoimmune disease, Crohn's disease, explained one-fifth of the disease heritability, and many additional loci remain to be identified)[16]. The GWAS have important limitations, including their potential for false-positive results, lack of information on gene function, insensitivity to rare variants and structural variants, requirement for large sample sizes, and possible biases due to case and control selection and genotyping errors[17]. Therefore use of GWA findings in screening for disease risk at present is problematic[17].

The signal transducer and activator of transcription 4 (*STAT4*) is uniquely activated by interleukin 12 (IL-12) through its receptor, which has an essential downstream role in Th1 cell differentiation and proliferation[18]. In addition, it has been reported that *STAT4* is necessary for the development of Th17 cells (IL-17-producing CD4+ T cells)[19]. Since Th1 cells and Th17 cells play an important role in chronic inflammatory disorders, and since *STAT4* is considered to be a key molecule in both the Th1 and Th17 lineages, *STAT4* may play a crucial role in the development of autoimmune diseases such as rheumatoid arthritis and SLE. The association between the *STAT4* rs7574865 and SLE has been reported in several populations[13,20-24].

Tumor necrosis factor (TNF) is a pleiotropic cytokine that plays a crucial role in a wide variety of proliferative responses, inflammatory effects, and immune responses. TNF has the ability to bind 2 distinct TNF receptors, TNF receptor superfamily, member 1A (TNFRSF1A, also known as TNFR1), and TNFRSF1B (also known as TNFR2)[25]. TNFRSF1A is known to initiate the majority of TNF's biological activities[26]. Both TNFRSF1A and TNFRSF1B are coexpressed on virtually all cells and initiate distinct signal transduction pathways by interacting with different signaling factors[27]. TNFRSF1A contains an intracellular cell-death domain, which is required for signaling of apoptosis and activation of the proinflammatory transcription factor nuclear factor-κB1 (NF-κB1)[28]. Ligand-binding to TNFRSF1A can lead to either apoptotic or antiapoptotic cascades, depending on the recruitment of different cellular factors that bind to the intracellular death domain, including TNF receptor-associated factor 2 (TRAF2), TNF receptor-associated death domain, and Fas-associated death domain[29,30]. On the other hand, TNFRSF1B usually is associated with activation of NF-κB1 and antiapoptotic cell-signaling cascades, mediated by binding to TRAF1/TRAF2 heterodimers and cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/cIAP2). Recently, a role of TNFRSF1B as a modulator of TNFRSF1A-mediated apoptotic mechanisms has been proposed[31]. Also, TNFRSF1B has been proposed to play a key role in chronic inflammatory disorders[32]. Women with mild endometriosis (stages I and II) have been shown to have deficient expression of TNFRSF1B[33]; however, little is known about the pathophysiological effects, if any, of the differential expression of TNF receptors in endometriosis. Accumulating evidence suggests the involvement of TNFRSF1B and its receptors in the pathogenesis and development of SLE. Several studies reported that the rs1061622 (Met196Arg) polymorphism of the *TNFRSF1B* gene is associated with SLE risk[34-36], but other studies have not replicated the finding[37-40].

SLE, an autoimmune disease, results from a complex interplay between genetic and environmental risk factors, as do other common diseases such as cancer, diabetes mellitus, and cardiovascular disease. Studying gene-environment interactions in relation to the risk of SLE may be valuable because positive findings would clearly implicate the disease-causing exposures, clarify SLE etiology, and point to environmental modifications for disease prevention. Cigarette smoking, a well known environmental etiological factor for SLE, has been suggested to influence STAT4[41] and TNFRSF1B[42,43] production. In SLE, risk effects of individual genes have turned out to be notably lower than the estimated total genetic risk. Therefore, it is possible that

some combinations of risk genes (epistasis or gene-gene interaction) might interact and cause unexpected joint risk effects.

To find such possible genetic interactions, it is reasonable to start evaluating combinations of already identified risk-modifying genes. A further useful consideration in searching for such epistatic effects might be any known biological interactions. *STAT4* and *TNFRSF1B* genes have been independently reported to be associated with SLE. We conducted a case-control genetic association study of SLE among Japanese women to evaluate gene-smoking and gene-gene interactions.

## MATERIALS AND METHODS

*Study subjects and data collection*. The Kyushu Sapporo SLE (KYSS) study was a case-control study to evaluate risk factors for SLE among women. One hundred and one SLE patients were recruited from outpatients of Kyushu University Hospital, Saga University Hospital, and their collaborating hospitals in Kyushu during the period 2002-2005, while 51 SLE patients were recruited from outpatients of Sapporo Medical University Hospital and its collaborating hospital in Hokkaido during the period 2004-2005. All patients (n = 152) fulfilled the American College of Rheumatology 1982 revised criteria for SLE[44]. Controls (n = 251) were recruited from nursing college students and care workers in nursing homes in Kyushu, while in Hokkaido, controls (n = 176) were recruited from participants at a health clinic in a local town.

All SLE patients and controls provided written informed consent for cooperation in the study. Cases were asked to complete a self-administered questionnaire about their lifestyles before the diagnosis of SLE. Details of the health examination and the self-administered questionnaire have been documented[6]. A portion of the participants also agreed to donate blood samples, which were stored until use for DNA extraction and genotyping of the candidate genes of SLE. Only women who agreed to donate blood samples were included in this study, while 175 cases and 517 controls were enrolled in the previous study[6].

The present study was approved by the institutional review boards of Kyushu University Graduate School of Medical Sciences, Sapporo Medical University, and each of the other institutions involved.

*Genetic analysis*. Genomic DNA was extracted from buffy-coat stored at –80°C using the QIAamp blood kit (Qiagen Inc., Santa Clarita, CA, USA). Genotyping of *STAT4* rs7574865 (G→T) was performed using the polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP) method. PCR amplification was carried out using primers 5'-GGT GTG GAT GGA GGT AAG GA-3' and 5'-ATC CCC TGA AAT TCC ACT GA-3'. The 182 PCR product was digested with *Mse* I. While the T (ancestral) allele yielded 3 fragments of 91 bp, the G (nonancestral) allele yielded 2 fragments of 130 bp and 52 bp. Details of the genotyping method for *TNFRSF1B* rs1061622 have been described[34]. For genotyping quality control, we retyped randomly selected samples (10% of previously typed samples) with the same method and confirmed the results.

*Statistical analysis*. We used chi-square statistics for homogeneity to test for case-control differences in the distribution of genotypes or alleles of the STAT4 rs7574865 and *TNFRSF1B* rs1061622. The distribution of the *STAT4* rs7574865 and *TNFRSF1B* rs1061622 genotypes in controls was compared with that expected from Hardy-Weinberg equilibrium (HWE) by the chi-square test. Unconditional logistic regression was used to compute the odds ratios (OR) and 95% confidence intervals (95% CI), with adjustments for several covariates (age, smoking status, alcohol intake, study region). Age was treated as a continuous variable. The relevant ages were age at diagnosis (SLE patients) and age at the time of the questionnaire (controls). Pack-years of smoking (years of smoking multiplied by packs of 20 cigarettes) were categorized in tertiles based on the distribution among controls (0 to < 1, 1 to ≤ 5, and > 5 pack-years). Alternatively, subjects were considered current smokers if they had smoked or had stopped smoking < 1 year before either the date of diagnosis (SLE patients) or the date of completion of the questionnaire (controls). Nonsmokers were defined as those who had never smoked in their lifetime. Former smokers were those who had stopped smoking ≥ 1 year before either the date of diagnosis (SLE patients) or the date of completion of the questionnaire (controls). Similarly, subjects were considered current drinkers if they had consumed alcohol before either the date of diagnosis of SLE (SLE patients) or the completion of the questionnaire (controls). Nondrinkers were defined as those who had never consumed alcohol in their lifetime. Since very few subjects were former drinkers, they were included in the current-drinkers category. Region was divided into 2 groups, namely Kyushu and Hokkaido. Trend of cumulative exposure was assessed by ordinal scores: 0 (never-smoker), 1 (0 to < 1 pack-years), 2 (1 to ≤ 5 pack-years), and 3 (> 5 pack-years). Trend of genotype influence was assessed by a score test for each genotype as follows: 0, homozygous for major allele; 1, heterozygote; and 2, homozygous minor allele.

To test for biological interactions between the *STAT4* genotypes and smoking status, we entered interaction terms (statistical interaction) reflecting the product of genotype and smoking status into the logistic models. In a logistic regression model, interaction refers to a departure from multiplicativity. Statistical interaction is used to refer to departure from the underlying form of a statistical model (additive or multiplicative). Rothman has argued that interaction estimated as departure from additivity better reflects biologic interaction on the basis of the sufficient component-cause model[45-47]. This is because information concerning an additive interaction between 2 factors is more relevant to disease prevention and intervention. Three measures for biologic interaction as departure from additivity, namely the relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP), and synergy index (S), were calculated as described by Andersson, *et al*[48]. The RERI is the excess risk due to interaction relative to the risk without exposure. AP refers to the attributable proportion of disease that is due to interaction among individuals with both exposures. S is the excess risk from exposure (to both factors) when there is interaction relative to the risk from exposure (to both factors) without interaction. Biological interaction was absent if RERI and AP are equal to 0 and S and the multiplicative interaction term are equal to 1.

Statistical analyses were performed using Stata V. 8.2 (Stata Corp., College Station, TX, USA). All p values were 2-sided, with those < 0.05 considered statistically significant.

## RESULTS

One hundred fifty-two women with SLE and 427 healthy female controls were enrolled in the study. The age (mean ± SD) of patients with SLE, 41.2 ± 12.9 years, was significantly higher than that of controls, 31.9 ± 14.2 years ($p < 0.0001$). From the questionnaire, age at the time of diagnosis of SLE was 29.15 ± 11.7 years (data not shown). As shown in Table 1, there was a positive association between cigarette smoking and SLE risk, regardless of adjustment for age, region, and alcohol intake. Former smokers had a significantly increased risk of SLE compared to never-smokers (OR 3.32, 95% CI 2.00–5.53, $p < 0.0001$), while current smokers did not (Table 1). When the association was examined in terms of pack-years, the OR increased with increasing levels of cumulative exposure (p for trend < 0.0001).

As shown in Table 2, the minor allele frequencies of the *STAT4* rs7574865 and *TNFRSF1B* rs1061622 were 0.375 and 0.182, respectively, in cases and 0.290 and 0.138 in controls. The genotype distribution of the *STAT4* rs7574865 was

*Table 1.* Association between smoking and risk of SLE.

| | No. of Subjects (%) | | OR (95% CI) | | | |
|---|---|---|---|---|---|---|
| | Cases | Controls | Crude | p | Adjusted* | p |
| Smoking status** | | | | | | |
| Never smoker | 98 (64.9) | 338 (79.7) | 1.0 | | 1.0 | |
| Former smoker | 46 (30.5) | 68 (16.0) | 2.33 (1.51–3.61) | < 0.0001 | 3.32 (2.00–5.53) | < 0.0001 |
| Current smoker | 7 (4.6) | 18 (4.3) | 1.34 (0.54–3.30) | 0.523 | 1.46 (0.54–3.90) | 0.455 |
| Ever smoker | 53 (35.1) | 86 (20.3) | 2.12 (1.41–3.20) | < 0.0001 | 2.86 (1.78–4.60) | < 0.0001 |
| Cumulative exposure† | | | | | | |
| Never smokers | 98 (67.1) | 338 (80.7) | 1.0 | | 1.0 | |
| < 1 pack-year | 13 (8.9) | 32 (7.6) | 1.40 (0.71–2.77) | 0.333 | 2.19 (1.05–4.57) | 0.037 |
| 1 to ≤ 5 pack-years | 18 (12.3) | 26 (6.2) | 2.39 (1.26–4.54) | 0.008 | 3.09 (1.49–6.43) | 0.003 |
| > 5 pack-years | 17 (11.6) | 23 (5.5) | 2.54 (1.31–4.96) | 0.006 | 3.22 (1.45–7.14) | 0.004 |
| | | | p for trend < 0.0001 | | p for trend < 0.0001 | |

* Adjusted for age, region, and alcohol intake. ** Four women (1 case and 3 controls) were missing smoking status data. † Fourteen women (8 cases and 6 controls) were missing cumulative exposure data.

*Table 2.* Association between *STAT4* and *TNFRSF1B* genotypes and risk of SLE.

| | Cases/Controls | | OR (95% CI) | | | |
|---|---|---|---|---|---|---|
| | Number | Minor Allele Frequency | Crude | p | Adjusted* | p |
| *STAT4* rs7574865 | | | | | | |
| GG | 61/217 | | 1.0 | | 1.0 | |
| TG | 68/172 | | 1.41 (0.94–2.10) | 0.094 | 1.31 (0.83–2.08) | 0.242 |
| TT (ancestral) | 23/38 | | 2.15 (1.19–3.89) | 0.011 | 2.21 (1.10–4.68) | 0.027 |
| | | | p for trend = 0.008 | | p for trend = 0.029 | |
| GT + TT | 91/210 | 0.375/0.290 | 1.54 (1.06–2.24) | 0.024 | 1.46 (0.95–2.25) | 0.087 |
| *TNFRSF1B* rs1061622** | | | | | | |
| TT (ancestral) | 97/310 | | 1.0 | | 1.0 | |
| TG | 53/111 | | 1.53 (1.02–2.28) | 0.038 | 1.59 (1.00–2.52) | 0.049 |
| GG | 1/3 | | 1.07 (0.11–10.36) | 0.957 | 0.86 (0.07–10.14) | 0.904 |
| | | | p for trend = 0.050 | | p for trend = 0.075 | |
| TG + GG | 54/114 | 0.1827/0.138 | 1.51 (1.02–2.25) | 0.040 | 1.56 (0.99–2.47) | 0.056 |

* Adjusted for age, region, smoking status, and alcohol intake. ** Four women (1 case and 3 controls) were missing genotype data.

consistent with HWE among controls (p = 0.640, data not shown). As for the *TNFRSF1B* rs1061622, a slight deviation from HWE in the control group was observed (p = 0.038, data not shown). The TT genotype of the *STAT4* rs7574865 genotype was significantly associated with an increased risk of SLE (adjusted OR 2.21, 95% CI 1.10–4.68, p for trend = 0.029). As the GG genotype of the *TNFRSF1B* rs1061622 was not separated due to a low prevalence of the rare G allele, we combined the TG genotype with GG genotype. The TG genotype of the *TNFRSF1B* rs1061622 was significantly associated with an increased risk of SLE (adjusted OR 1.59, 95% CI 1.00–2.52, p = 0.049), while the combination of the TG and GG genotypes was marginally associated with an increased risk of SLE (adjusted OR 1.56, 95% CI 0.99–2.47, p = 0.056).

Based on our results, we designated the genotype that is presumed to increase the risk of SLE as the risk genotype. Table 3 shows the modifying effect of the *STAT4* rs7574865 genotypes on the association of smoking with SLE risk. As shown in Table 1, the categorization "smoking status" instead of "cumulative exposure" did not materially change the results. To achieve adequate statistical power and to clearly determine the effect of a history of smoking on SLE risk, current and former smokers were combined (smokers) and minor allele carriers were combined. As the patients presented with the first clinical symptom 0.42 years (median) before the diagnosis[49], it should not be difficult to estimate the SLE risk for current and former smokers combined. Subjects with at least one T allele (adjusted OR 4.37, 95% CI 2.22–8.62, p < 0.0001) presented a higher risk of SLE than those with the GG genotype (adjusted OR 2.65, 95% CI 1.35–5.21, p = 0.005) in smokers relative to nonsmokers with the GG genotype. The multiplicative interaction between the *STAT4* rs7574865 genotypes and smoking was not significant. For assessment of additive interaction, adjusted measures of RERI, AP, and S were 1.37 (95% CI –1.46 to 4.20), 0.31 (95% CI –0.20 to 0.82), and 1.66 (95% CI 0.59 to 4.65), respectively. These values suggested no significant biologic (additive) interactions.

Table 4 shows the modifying effect of the *TNFRSF1B*

*Table 3.* Interaction between smoking and *STAT4* genotypes.

| | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | p | Adjusted* | p |
| GG genotype + nonsmoking | 1.0 (reference) | | 1.0 (reference) | |
| GG genotype + smoking | 2.09 (1.13–3.85) | 0.017 | 2.65 (1.35–5.21) | 0.005 |
| T allele carrier + nonsmoking | 1.52 (0.96–2.41) | 0.072 | 1.36 (0.81–2.30) | 0.248 |
| T allele carrier + smoking | 3.45 (1.90–6.24) | < 0.0001 | 4.37 (2.22–8.62) | < 0.0001 |
| Multiplicative interaction measure | 1.08 (0.47–2.47) | 0.854 | 1.21 (0.48–3.3) | 0.681 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 0.83 (–1.15 to 2.82) | 0.412 | 1.37 (–1.46 to 4.20) | 0.343 |
| Attributable proportion due to interaction | 0.24 (–0.26 to 0.74) | 0.347 | 0.31 (–0.20 to 0.82) | 0.234 |
| Synergy index | 1.53 (0.55 to 4.24) | 0.414 | 1.66 (0.59 to 4.65) | 0.336 |

* Adjusted for age, region, and alcohol intake.

*Table 4.* Interaction between smoking and *TNFRSF1B* rs1061622 genotypes.

| | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | p | Adjusted* | p |
| TT genotype + nonsmoking | 1.0 (reference) | | 1.0 (reference) | |
| TT genotype + smoking | 1.92 (1.16–3.17) | 0.011 | 2.39 (1.36–4.23) | 0.003 |
| G allele carrier + nonsmoking | 1.38 (0.85–2.23) | 0.186 | 1.33 (0.77–2.32) | 0.307 |
| G allele carrier + smoking | 3.42 (1.73–6.74) | < 0.0001 | 5.42 (2.48–11.84) | < 0.0001 |
| Multiplicative interaction measure | 1.29 (0.53–3.14) | 0.576 | 1.70 (0.62–4.61) | 0.300 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 1.12 (–1.205 to 3.44) | 0.345 | 2.65 (–1.35 to 6.65) | 0.194 |
| Attributable proportion due to interaction | 0.33 (–0.18 to 0.84) | 0.205 | 0.49 (0.07–0.92) | 0.023 |
| Synergy index | 1.87 (0.57–6.21) | 0.304 | 2.54 (0.80–8.09) | 0.114 |

* Adjusted for age, region, and alcohol intake.

rs1061622 genotypes on the association of smoking with SLE risk. Subjects with at least one G allele (adjusted OR 5.42, 95% CI 2.48–11.84, p < 0.0001) showed a higher risk of SLE than those with the TT genotype (adjusted OR 2.39, 95% CI 1.36–4.23, p = 0.003) in smokers relative to nonsmokers with the TT genotype. The multiplicative interaction between the *TNFRSF1B* rs1061622 genotypes and smoking was far from significant. For assessment of additive interaction, adjusted measures of RERI and S were 2.65 (95% CI –1.35 to 6.65) and 2.54 (95% CI 0.80 to 8.09), respectively. These values suggested no significant biologic (additive) interactions. Meanwhile, the adjusted AP due to interaction between the *TNFRSF1B* rs1061622 genotypes and smoking was estimated to be 0.49 (95% CI 0.07 to 0.92, p = 0.023), indicating that 49% of the excess risk for SLE in smokers with at least one G allele was due to additive interaction.

The interaction between the *STAT4* rs7574865 and the *TNFRSF1B* rs1061622 is shown in Table 5. The combination of the T allele carrier of the *STAT4* rs7574865 and the G allele carrier of the *TNFRSF1B* rs1061622 was significantly associated with an increased risk of SLE (adjusted OR 2.14, 95% CI 1.13 to 4.05, p = 0.020). Four interaction measures (multiplicative interaction, RERI, AP, and S) were far from statistically significant.

## DISCUSSION

Several studies have investigated whether smoking was associated with an increased risk of SLE[5]. In our study, former smokers, but not current smokers, were at increased risk for SLE. The finding may be due to chance, to higher levels of pack-years among former smokers than current smokers, or to both. In fact, pack-years of smoking were significantly higher among former smokers (2.03, 95% CI 1.87–2.18) than current smokers (1.63, 95% CI 1.29–1.99) (p = 0.039, data not shown). As the prevalence of current smokers is substantially low among Japanese women (< 5%), we could not perform a detailed analysis of SLE risk associated with pack-years of smoking. A history of smoking was significantly associated with an increased risk of SLE (adjusted OR 2.86, 95% CI 1.78–4.60, p < 0.0001). Cigarette smoking has consistently been associated with the development of rheumatoid arthritis and other autoimmune diseases. Thus, it is important to define the role of cigarette smoking in susceptibility for the development of SLE. Cigarette smoking has been proposed to be a trigger for the development of SLE, and the association has been examined in several studies, with conflicting results. Although the biologic pathway through which cigarette smoking acts to increase the instantaneous risk of SLE is not known, many potential mechanisms exist. It is possible that cigarette smoke acts via exac-

*Table 5*. Interaction between *STAT4* and *TNFRSF1B* genotypes.

| *STAT4* rs7574865+ *TNFRSF1B* rs 1061622 | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | p | Adjusted* | p |
| rs7574865 GG + rs1061622 TT | 1.0 (reference) | | 1.0 (reference) | |
| rs7574865 GG + rs1061622 G carrier | 1.73 (0.93–3.20) | 0.081 | 1.89 (0.94–3.81) | 0.076 |
| rs7574865 T carrier + rs1061622 TT | 1.67 (1.05–2.65) | 0.030 | 1.61 (0.95–2.74) | 0.076 |
| rs7574865 T carrier + rs1061622 G carrier | 2.20 (1.27–3.81) | 0.005 | 2.14 (1.13–4.05) | 0.020 |
| Multiplicative interaction measure | 0.76 (0.34–1.71) | 0.508 | 0.70 (0.27–1.77) | 0.453 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | –0.19 (–1.62 to 1.24) | 0.794 | –0.35 (–2.06 to 1.36) | 0.688 |
| Attributable proportion due to interaction | –0.09 (–0.76 to 0.58) | 0.492 | –0.16 (–0.99 to 0.67) | 0.706 |
| Synergy index | 0.86 (0.29 to 2.56) | 0.786 | 0.77 (0.22 to 2.70) | 0.682 |

* Adjusted for age, region, and alcohol intake.

erbation of tissue hypoxia or toxin-mediated cellular necrosis to release intracellular antigens, thereby stressing the clearance functions of the immune system and precipitating clinical SLE in genetically predisposed individuals. Cigarette smoke by-products could augment numbers of autoreactive B cells in the native repertoire, predisposing to development of the disease. Prolactin and estradiol have been found to have such actions[50,51], and smoking is known to alter the metabolism of estradiol and adrenal hormones such as androstenedione and dehydroepiandrosterone[52-55]. Alternatively, smoking has known immunomodulatory effects in humans[56-58]. Thus, it is biologically plausible that cigarette smoking causes SLE.

The *STAT4* rs7574865 and *TNFRSF1B* rs1061622 genotypes were determined by PCR-RFLP in 152 cases of SLE and 427 controls. The minor allele frequencies of the *STAT4* rs7574865 and *TNFRSF1B* rs1061622 were 29.0% and 13.8% in controls, respectively. According to the HapMap SNP database[59], the frequency of the *STAT4* rs7574865 T allele is most common among Han Chinese (33.3%) and least common among Caucasians (20.0%), with Japanese (28.9%) intermediate between these groups. Therefore, the frequency of the T allele in our study was comparable with the International HapMap project data. The *TNFRSFS1B* rs1061622 G allele is most common among Caucasians (24.6%) and least common among Japanese (17.8%), with Han Chinese (20.0%) intermediate between these groups in the HapMap SNP database[59]. The frequency of the G allele in our study was somewhat lower than in the HapMap SNP database, but higher than another Japanese population (11.8%)[39]. The genotype distribution of *STAT4* rs7574865 was consistent with HWE among controls, while the genotypes of the *TNFRSF1B* rs1061622 deviated from HWE.

Departure from HWE can imply the presence of selection bias (lack of representation of the general population) in this population, because our study was free from the possibility of genotyping error (e.g., systematic misgenotyping of heterozygotes as homozygotes or vice versa, or nonrandomness of missing data), assay nonspecificity, or possible population admixture/stratification[60,61]. The Japanese population sample could be expected to have a relatively low risk of population stratification effects[62,63] in comparison to Caucasian populations that have a geographically broader inheritance. However, it is interesting that, although there was not a significant association with SLE risk, the genotype frequencies in the control population exhibited significant deviation from HWE. Alleles that are likely to be associated with disease etiology tend to exhibit deviation from expected allele or genotype frequencies. Disease-causing alleles would be expected to be significantly overrepresented among cases with the disease, but underrepresented among disease-free control subjects, and this can be manifested by deviation from HWE. Several precautious were in place to avoid detecting a spurious departure from HWE, which is usually attributed to genotyping error. We conducted this study blind to the SLE status of our study population, samples were randomly stored in DNA sample containers, and randomly selected samples were retyped. In our study, deviation from HWE in the control group was not likely because of genotyping error, but could suggest that the G allele of *TNFRSF1B* rs1061622 may be a risk allele that we could not adequately detect, or perhaps we need an alternative method to test for association. The deviation from HWE is most likely due to chance. Only 3 controls had the GG genotype of *TNFRSF1B* rs1061622. If 4 controls had possessed the GG genotype, there was no longer a deviation from HWE in controls ($p = 0.08$). As our study population was not very large, additional studies with larger sample sizes will be required.

The TT genotype of the *STAT4* rs7574865 genotype was associated with a 2.2-fold increase in the risk of SLE in this study. Although rs7574865 is located in the third intron of the *STAT4* gene, high levels of STAT4 expression correlated with rs7574865 as well as the other intronic SNP rs3821236 and rs302486664. As all 3 SNP have been associated with an increased risk of SLE[64], these SNP may be responsible for splice variation or regulatory effects of *STAT4*. A recent study reported that the STAT4 risk allele was associated

with overexpression of STAT4 in osteoblasts but not in B cells[65]. To address the significance of such findings, it will be necessary to examine the effect of the risk genotype on the expression levels and splicing isoforms in T and B cells. At this time the correlation of the STAT4 function and intronic SNP should be interpreted cautiously. Comprehensive functional studies of *STAT4* will be required to confirm our finding. On the other hand, the TG genotype of the *TNFRSF1B* rs1061622 was associated with a 1.6-fold increase in the risk of SLE. The *TNFRSF1B* G allele transmits a stronger TNF-α signal than the *TNFRSF1B* T allele[36], which might be involved in the increased risk of SLE in the G allele carriers. Thus, it is biologically plausible that the *STAT4* rs7574865 T and *TNFRSF1B* rs1061622 G alleles are associated with an increased risk of SLE.

It is widely accepted that development of SLE requires environmental factors acting on a genetically predisposed individual. As cigarette smoking is a well known environmental etiological factor for SLE[5,6] and has been suggested to influence STAT4[41] and TNFRSF1B[42,43] production, we investigated the gene-environment interaction between the polymorphisms and smoking (Tables 3 and 4). A gene-environment interaction was suggested, with combination of the *TNFRSF1B* rs1061622 G allele carrier and history of smoking conferring a significantly higher risk (OR 5.42, 95% CI 2.48–11.84, $p < 0.0001$) compared with the TT genotype and no history of smoking. Studies of interaction among risk factors in the epidemiological literature have classically been performed using a departure from the additivity model originally described by Rothman, where a term is used to quantify the contribution of interaction to a disease risk, as compared with the contribution of each of the 2 risk factors added to each other[47,48]. An alternative common method for quantifying interactions is based on the calculation of the 2 risk factors' product term in a logistic-regression model (multiplicative). The AP due to interaction between the *TNFRSF1B* rs1061622 genotypes and smoking was estimated to be 0.49 (95% CI 0.7–0.92, $p = 0.023$). This measure was not equal to zero, suggesting the existence of a biological (additive) interaction. Meanwhile, the multiplicative interaction measure was not significant. Thus, the results suggest evidence for additive but not multiplicative interaction. To our knowledge, no studies on the interactions between the *TNFRSF1B* genotypes and SLE have been reported.

Understanding the genetic basis of complex diseases has been increasingly emphasized as a means of achieving insight into disease pathogenesis, with the ultimate goal of improving preventive strategies, diagnostic tools, and therapies. Case-control genetic association studies such as ours aim to detect association between genetic polymorphisms and disease. Although case-control genetic association studies can measure statistical associations, they cannot test causality. Determining genetic causation of disease is a process of inference, which requires supportive results from multiple association studies and basic science experiments combined. Further, a concern with respect to genetic association studies has been lack of replication studies, especially contradictory findings across studies. Replication of findings is required before any causal inference can be drawn. Testing replication in different populations is an important step. Additional studies are warranted to replicate our and others' findings from case-control genetic association studies. Case-control genetic association study can be useful in investigating gene-gene or gene-environment interactions, however. Despite the growing awareness of the relevance of gene-environment interactions in human disease, progress in the identification of common genetic alterations that by themselves may not substantially influence risk, but in concert with environmental exposures may lead to disease development, has been limited. Some genetic variants may exert population-specific effects that are independent of the remaining genetic profile of the individual and environmental exposures; while other population-specific effects may be generated under differential gene-environment interactions in different populations[66]. Sample sizes for adequate power to detect interactions are prohibitively large when the frequencies of interacting variants and exposures are small[66].

Gene-gene interactions have received much attention recently because most human traits may be under the control of several genetic factors, as well as environmental factors, and these factors likely interact among each other to influence these traits. Assessment of gene-gene interaction also depends upon the proper statistical evaluation of interaction on the multiplicative and additive models. The method used in the test for gene-environment interaction can also be used to quantify gene-gene interactions for unlinked loci. Gene-gene interaction was not significant between the 2 polymorphisms in this study. Additional studies are required to corroborate the association among Japanese women suggested in our investigation.

Our findings suggest that the *STAT4* rs7574865 and *TNFRSF1B* rs1061622 might be genetic factors for SLE. Our study suggested a relationship between SLE and the *TNFRSF1B* rs1061622 genotype, but our sample size may prevent any conclusive inference on the interaction of smoking and *TNFRSF1B* rs1061622. Future studies involving larger control and case populations, precisely and uniformly defined clinical classification of SLE, and better exposure histories will undoubtedly lead to more thorough understanding of the role of various genes in development of SLE.

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## APPENDIX

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