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*Chapter 6*

# Modifying Effect of Smoking on the Association between SLE and the Genetic Polymorphisms Involved in ROS Production

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## Abstract

Exposure to reactive oxygen species (ROS) via cigarette smoking is thought to contribute to the development of systemic lupus erythematosus (SLE). ROS increase immunogenicity of DNA, LDL and IgG, generating ligands for which autoantibodies show higher avidity. To evaluate modifying effect of the genetic polymorphisms in ROS production on the association of cigarette smoking with SLE risk could be important for understanding of the pathogenesis of SLE. The relationship of the genetic polymorphisms of cytochrome P450 (*CYP*) *1A1* rs4646903 and glutathione S-transferase (*GST*) *M1* deletion, N-acetyltransferase 2 (*NAT2*) and tumor necrosis factor receptor superfamily, member 1B (*TNFRSF1B)* rs1061622 to SLE risk with attention to interaction with cigarette smoking were investigated. *CYP1A1* rs4646903 and *NAT2* genotypes were significantly associated with SLE risk. The allele (genotype) presumed to increase the risk of SLE is designated as the "at-risk" allele (genotype). The multiplicative interaction

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between the four genetic polymorphisms and smoking was far from significant. As for the four genetic polymorphisms, smokers with "at-risk" allele (genotype) had a significantly higher risk of SLE than never smokers with no "at-risk" allele (genotype). The observed high ORs were attributed largely to the effect of ever-smoking, however. There were significant additive interactions between smoking and any one of the following: *CYP1A1* rs4646903, *NAT2* or *TNFRSF1B* rs1061622. Specifically, about 50 % of the excess risk for SLE in smokers with the "at-risk" genotype was due to the additive interaction. Testing replication in different populations is an important step and additional studies are warranted to confirm interaction between the genetic polymorphisms associated with ROS production and smoking suggested in Japanese samples.

# Introduction

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 all been hypothesized to be associated with the development of SLE [1-4], although the strength of the evidence implicating each of these factors varies.

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 [5, 6]. However, identification of these genetic factors has been slow. The genetic basis of SLE is very complex; it has been estimated that over 100 genes may be involved in SLE susceptibility [7], but it is difficult to predict how many genes contribute to SLE susceptibility.

SLE, like other common multifactorial diseases such as cancers, diabetes, asthma, obesity, and cardiovascular disease, results from a complex interplay of genetic and environmental risk factors. However, triggering events for SLE may include many environmental factors [8]. A previous meta-analysis of nine studies revealed a significantly increased risk for the development of SLE among current smokers compared with that among non-smokers (summary odds ratio (OR) = 1.50, 95% confidence interval (CI) = 1.09 - 2.08) [3]. it has been reported that cigarette smoke affects a wide range of immunological functions in humans [9, 10]. In our study, compared with non-smoking, current smoking was significantly associated with an increased risk of SLE (OR= 3.06, 95% CI = 1.86 - 5.03) [11]. A recent study also showed that a history of smoking is significantly though modestly associated with the development of SLE (OR = 1.65, 95% CI = 1.17–2.33) [12]. Like SLE, rheumatoid arthritis (RA) is autoimmune disease characterized by altered inflammatory and impaired immune responses causing immune-mediated destruction of tissues and organs. Smoking constituted a significant risk factor for the development of RA in a meta-analysis (OR = 1.27, 95% CI = 1.12 - 1.44) [13]. The result of the meta-analysis supports our finding that smoking contributes to an increased risk of SLE.

Metabolism of carcinogens related to cigarette smoke is regulated by a balance of a number of steps that involve production and detoxification of reactive oxygen species (ROS). ROS bind covalently to DNA that leads to somatic mutation or disruption of cell cycle. ROS is also considered to promote the autoimmune response [14]. The enzyme activities of cytochrome P450 (CYP)1A1 and glutathione S-transferase (GST) M1 are critical for the

functionalization of xenobiotics, such as genotoxins in cigarette smoke. CYP1A1 contributes to the Phase I metabolic activation and formation of ROS, whereas GSTM1 plays a critical role for Phase II detoxification of activated carcinogens or ROS [15, 16]. Extensive studies have been performed on the possible associations between polymorphisms of such genes as *CYP1A1* and *GSTM1* and cancer susceptibility [17-19]. The enzyme of N-acetyltransferase (NAT) is involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as ROS [20]. It has been suggested that N-acetylation of polycyclic aromatic hydrocarbons (PAHs) by NAT2 may be associated with ROS production [21]. ROS increase immunogenicity of DNA, LDL and IgG, generating ligands for which autoantibodies show higher avidity [14]. Tumor necrosis factor r superfamily, member 1B (TNFRSF1B) is a receptor for TNF-α and is considered to mediate various biological effects including generation of ROS and the subsequent intracellular pro-inflammatory signaling events [22]. Therefore, we selected one representative functional polymorphism for each gene, namely *CYP1A1* rs464903, *GSTM1* deletion, *NAT2* genotypes determined by *NAT2**4, *5B, *6A or *7B allele and *TNFRSF1B* rs1061622. Considering that exposure to ROS via cigarette smoking may be contribute to the development of SLE, it is important to study the association between SLE and the polymorphisms involved in metabolism of tobacco smoke and ROS production. In this paper, we summarized our recent studies on interaction between smoking and the genetic polymorphisms involved in ROS production in SLE.

# Materials and Methods

## Study Subjects

The Kyushu Sapporo SLE (KYSS) study was a case-control study to evaluate risk factors for SLE among females. SLE patients (n = 129) were recruited from outpatients of Kyushu University Hospital, Saga University Hospital and their collaborating hospitals in Kyushu from 2002-2005, while 51 SLE patients were recruited from outpatients of Sapporo Medical University Hospital and its collaborating hospital in Hokkaido from 2004 to 2005. All patients (n = 180) fulfilled the American College of Rheumatology 1982 revised criteria for SLE [23]. The mean duration (± SD) of SLE was 11.9 (8.55) years. An antinuclear antibody (ANA) test is ordered as a routine screening test if there is a reasonable suspicion of SLE from family history and/or physical findings. Therefore, we performed an ANA test for all SLE patients and almost all of the patients had a positive ANA test result. The rheumatologists in charge asked eligible SLE patients to take part in this study and obtained written informed consent from them. SLE patients with cognitive dysfunction were not included in this study.

Controls were not, individually or in larger groups, matched to cases. Controls were recruited from nursing college students and care workers in nursing homes in Kyushu (n = 325), while in Hokkaido, controls were recruited from participants at a health clinic (n = 188).

In analysis, 18 subjects (eight cases and ten controls) were excluded because of male sex. A portion of the participants agreed to donate blood samples, which were stored until use for DNA extraction and genotyping of the candidate genes of SLE. Only females who agreed to donate blood samples were included in this study (152 cases and 427 healthy controls).

All SLE patients and controls provided written informed consent for cooperation in the study. The present study was approved by the institutional review boards of Kyushu University Graduate School of Medical Sciences, Sapporo Medical University, St. Mary's College and the other institutions involved.

## Questionnaire Survey

Cases were asked to complete a self-administered questionnaire about their lifestyles before the diagnosis of SLE while controls completed the questionnaire about their current lifestyles. Subjects were considered current smokers if they smoked or had stopped smoking less than one year before either the date of diagnosis (SLE patients) or the date of completion of the questionnaire (controls). The relevant ages would be age at diagnosis (SLE patients) and age at time of questionnaire (controls). Non-smokers were defined as those who had never smoked in their lifetime. Former smokers were those who had stopped smoking one year or more before either the date of diagnosis (SLE patients) or the date of completion of the questionnaires (controls).

Similarly, subjects were considered current drinkers if they consumed alcohol before either the date of diagnosis of SLE (SLE patients) or completion of the questionnaire (controls). Details of the health examination and the self-administered questionnaire have been documented elsewhere [24, 25].

## Genetic Analysis

Genomic DNA was extracted from buffy coat stored at -80 $^{o}$C using the QIAamp blood kit (QIAGEN, Inc, Santa Clarita, CA). The genotype of *CYP1A1* rs4646903 (T3801C) polymorphism was determined by PCR-restriction fragment length polymorphism (PCR-RFLP) method as described previously [26]. The *CYP1A1* rs4646903 polymorphism was classified into three genotypes; predominant homozygous alleles (TT), heterozygous alleles (TC), and minor homozygous alleles (CC). *GSTM1* genotypes are divided into two categories in relation to enzymatic activity [27]. Lack of activity is caused by the homozygous deletion of an intact gene (the null genotype). The *GSTM1* non-null genotype is the wild type or heterozygote. As for the *NAT2* polymorphism, the most common mutations in the Japanese population at positions C481T, G590A, and G857A of *NAT2* were analyzed using *Kpn* I, *Taq* I and *Bam*H I by the PCR-RFLP method, as described elsewhere [28-30]. According to the nomenclature of *NAT2*, wild-type and three variant alleles were defined as *NAT2**4 and *5B, *6A, *7B. Subjects were classified by this genotyping into three groups: homozygous for the major allele *4/*4 (rapid acetylator), heterozygous for the major and minor alleles *4/*5B, *4/*6A, and *4/*7B (intermediate acetylator), and homozygous for the minor alleles *5B/*5B, *5B/*6A, *5B/*7B, *6A/*6A, *6A/*7B, and *7B/*7B (slow acetylator). As for *TNFRSF1B* rs1061622 (Met196Arg, 587T>G), the genotype was determined by a PCR-subsequent single-strand conformation polymorphism method described elsewhere [31]. The *TNFRSF1B* rs1061622 polymorphism was classified into three genotypes; predominant homozygous alleles (TT), heterozygous alleles (TG), and minor homozygous alleles (GG).

For genotyping quality control, we retyped randomly selected samples (10% of previously typed samples) with the same method and confirmed the complete agreement of genotyping.

### Statistical Analysis

We used $\chi^2$ statistics for homogeneity to test for case-control differences in the distribution of several covariates and the genotypes of the four polymorphisms. The distribution of the genotypes in controls was compared with that expected from Hardy-Weinberg equilibrium by the chi-square test. Unconditional logistic regression was used to compute the ORs and their 95%CIs with adjustments for several covariates (age, region of residence, smoking status, alcohol consumption). Age was treated as a continuous variable. The remaining covariates were treated as categorical variables. Region of residence fell into two categories (Kyushu and Hokkaido), as did smoking status (current and former smokers combine and non-smokers), alcohol drinking status (current and former drinkers combined and non-drinkers), and the NAT2 status (slow and intermediated acetylators combined and rapid acetylators).

The trend was assessed by assigning ordinal values for categorical variables. The interaction between the genotypes of the four polymorphisms and smoking on the risk of SLE was statistically evaluated based on the likelihood ratio test, comparing the logistic models with and without (multiplicative scale) terms reflecting the product of the genotype and smoking for interaction [32]. In a logistic regression model, interaction refers to a departure from multiplicativity. Rothman has argued that interaction estimated as departure from additivity better reflects biologic interaction [33]. Three measures for biologic interaction as departure from additivity, namely the relative excess risk due to interaction (RERI), attributable proportion (AP) and synergy index (SI) were calculated by the method described by Andersson et al. [34]. Biological interaction was absent if RERI and AP are equal to zero and SI and multiplicative interaction term are equal to one.

All statistical analyses were performed using the computer program STATA Version 12.1 (STATA Corporation, College Station, TX). P values were two-sided, with those less than 0.05 considered statistically significant.

## Results

There were 152 females with SLE and 427 healthy females enrolled in this study. As shown in Table 1, the age (mean, 95% CI) of patients with SLE (41.2, 39.2 - 43.3) was significantly higher than that of controls (31.9, 30.5 - 33.2) ($P < 0.0001$). From the questionnaire, the mean age (95% CI) at the time of diagnosis of SLE was 29.1 (27.3 - 31.0) (data not shown). There was also a significant difference between the age of diagnosis (SLE) and age of completion of the questionnaire (controls) ($P = 0.04$, data not shown). Compared with controls, cases were more likely to report a history of smoking ($P = 0.001$). On the other hand, controls tended to drink alcohol more frequently than SLE patients ($P <0.0001$). The frequencies of the three genotypes of the *CYP1A1* rs4646903 were marginally different

between cases and controls (P = 0.051). The distribution of the *NAT2* genotypes was significantly different between cases and controls (P = 0.001) while the genotype distributions of the *TNFRSF1B* rs1061622 and the *GSTM1* deletion polymorphism were not. The genotype distribution of *CYP1A1*rs4646903 was consistent with HWE in controls ($P_{HWE}$ = 0.05001) (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).

**Table 1. Selected characteristics of SLE cases and controls**

| Characteristics | Cases (n =152) | Controls (n = 427) | P |
|---|---|---|---|
| Age (year), mean (95% CI) | 41.2 (39.2 - 43.3) | 31.9 (30.5 - 33.2) | <0.0001 |
| Region of residence, n (%) | | | |
| Hokkaido | 51 (33.6) | 176 (36.4) | 0.096 |
| Kyushu | 101 (66.5) | 251 (63.6) | |
| Cigarette smoking status*, n (%) | | | |
| Non- smoker | 98 (64.9) | 339 (79.6) | 0.001 |
| Former smoker | 7 (4.64) | 18 (4.23) | |
| Current smoker | 46 (30.5) | 69 (16.2) | |
| Alcohol drinking status*, (%) | | | |
| Non-drinker | 68 (45.0) | 120 (28.1) | <0.0001 |
| Former drinker | 0 (0.00) | 0 (0.00) | |
| Current drinker | 83 (55.0) | 303 (71.6) | |
| *CYP1A1* rs4646903 | | | |
| TT | 61 (40.4) | 180 (42.8) | |
| TC | 66 (43.7) | 204 (48.5) | |
| CC | 24 (15.9) | 37 (8.79) | 0.051 |
| *GSTM1* deletion | | | |
| Non-null | 75 (49.7) | 227 (53.9) | |
| Null | 76 (50.3) | 194 (46.1) | 0.367 |
| *NAT2* genotype** | | | |
| Rapid acetylator genotype | 23 (15.1) | 130 (30.4) | 0.001 |
| Intermediate acetylator genotype | 89 (58.6) | 207 (48.5) | |
| Slow acetylator genotype | 40 (26.3) | 90 (21.1) | |
| *TNFRSF1B* rs1061622 | | | |
| TT | 97 (64.2) | 310 (73.1) | |
| TG | 53 (35.1) | 111 (26.2) | |
| GG | 1 (0.66) | 3 (0.71) | 0.114 |

CI, confidence interval

*Several observations with missing values.

**Rapid, *4/*4; Intermediate, *4/*5B, *4/*6A, *4/*7B; Slow, *5B/*5B, *5B/*6A, *5B/*7B, *6A/*6A*6A/*7B, *7B/*7B

Table 2 shows the association between the polymorphisms involved in ROS production and risk of SLE. When adjusted for age, region, smoking status and alcohol consumption, the CC genotype of *CYP1A1* rs4646903 was significantly associated with an increased risk of SLE (adjusted OR ($OR_{adj}$) = 2.47, 95% CI = 1.28 - 4.78) compared with at least one T allele. There was no association between the *GSTM1* deletion polymorphism and SLE risk. Based on the associations between *CYP1A1*/*GSTM1* genotypes and SLE, we designated the combined metabolic genotype presumed to increase the risk of SLE as the combined "at-risk" genotype (high risk genotype, *CYP1A1* rs4646903 CC and *GSTM1* null genotypes combined).

**Table 2. Association between the polymorphisms involved in ROS production and risk of SLE**

| Polymorphism | OR (95% CI) | |
|---|---|---|
| | Crude | Adjusted* |
| *CYP1A1* rs4646903 | | |
| TT | 1.0 (reference) | 1.0 (reference) |
| TC | 0.96 (0.64 - 1.43) | 0.92 (0.58 - 1.46) |
| CC | 1.94 (1.07 - 3.49) | 2.37 (1.18 - 4.78) |
| CC vs. TT + TC | 1.98 (1.14 - 3.43) | 2.47 (1.28 - 4.78) |
| *GSTM1* deletion | | |
| Non-null | 1.0 (reference) | 1.0 (reference) |
| Null | 1.21 (0.84 - 1.75) | 1.13 (0.74 - 1.73) |
| *CYP1A1/GSTM1* genotypes combined | | |
| High risk** vs. Low risk† | 2.07 (0.96 - 4.44) | 4.30 (1.79 - 10.4) |
| *NAT2* genotype‡ | | |
| Rapid acetylator genotype | 1.0 (reference) | 1.0 (reference) |
| Intermediate acetylator genotype | 2.43 (1.46 - 4.04) | 2.18 (1.24 - 3.85) |
| Slow acetylator genotype | 2.51 (1.41 - 4.48) | 2.34 (1.21 - 4.52) |
| Non-rapid vs. Rapid | 2.45 (1.50 - 4.00) | 2.34 (1.36 - 4.02) |
| *TNFRSF1B* rs1061622 | | |
| TT | 1.0 (reference) | 1.0 (reference) |
| TG | 1.52 (1.02 - 2.27) | 1.59 (1.01 - 2.53) |
| GG | 1.07 (0.11 - 10.4) | 0.86 (0.07 - 10.1) |
| GG + TG vs. TT | 1.51 (1.02 - 2.25) | 1.56 (0.99 - 2.47) |

CI, confidence interval; OR, odds ratio

*Adjusted for age, region, smoking status and alcohol consumption.

** At least one T allele of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype, at least one T allele of *CYP1A1* rs4646903 and the *GSTM1* null genotype or the CC genotype of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype

†The CC genotype of *CYP1A1* rs4646903 and the *GSTM1* null genotype

‡ Rapid, *4/*4; Intermediate, *4/*5B, *4/*6A, *4/*7B; Slow, *5B/*5B, *5B/*6A, *5B/*7B, *6A/*6A*6A/*7B, *7B/*7B

The rest of the combined genotypes of *CYP1A1* rs4646903 and *GSTM1* deletion polymorphisms were defined as a low risk genotype (at least one T allele of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype, at least one T allele of *CYP1A1* rs4646903 and

the *GSTM1* null genotype or the CC genotype of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype). The high risk genotype of *CYP1A1* rs4646903 and the *GSTM1* polymorphisms combined was significantly associated with an increased risk of SLE ($OR_{adj}$ = 4.30, 95% CI = 1.79 - 10.4) compared with the low risk genotype. As for the *NAT2* acetylator genotypes, the slow acetylator and intermediate acetylator genotypes were significantly associated with an increased risk of SLE ($OR_{adj}$ = 2.18, 95% CI = 1.24 - 3.85 and $OR_{adj}$ = 2.34, 95% CI = 1.21 - 4.52, respectively). The $OR_{adj}$ of the intermediate acetylator and slow acetylator genotypes combined was 2.34 (95% CI = 1.36 - 4.02). The possession of *TNFRSF1B* rs1061622 G allele was marginally associated with an increased risk of SLE ($OR_{adj}$ = 1.56, 95% CI = 0.99 - 2.47).

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 (ever-smokers). As the patients presented with the first clinical symptom 0.42 years (median) before the diagnosis [35], there shouldn't be a problem of estimating the SLE risk for current and former smokers combined. Ever-smoking was associated with an increased risk of SLE ($OR_{adj}$ = 2.86; 95% CI = 1.78 - 4.60) (data not shown). To achieve adequate statistical power, the genotypes were also categorized into two groups. As significantly higher CYP1A1 enzyme induction was observed in subjects with the CC genotype compared with the TT or TC genotype (there was no significant difference in enzymatic activity between the TT genotype and TC genotype) by exposure to smoking [36], the T allele may appear to act in a dominant fashion. The *NAT2* genotypes were classified as rapid acetylator genotype and non-rapid acetylator genotype. As for *TNFRSF1B* rs1061622, the minor G allele carriers were combined due to low prevalence of the minor allele (the GG and TG genotypes combined and TT genotype).

**Table 3. Interaction between smoking and *CYP1A1* rs4646903 genotypes**

| *CYP1A1* rs4646903 genotype + Smoking status | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | P | Adjusted* | P |
| T allele carrier genotype + Non-smoking | 1.0 (reference) | | 1.0 (reference) | |
| T allele carrier genotype + Ever-smoking | 2.01 (1.29 - 3.13) | 0.002 | 2.73 (1.64 - 4.54) | <0.0001 |
| CC genotype + Non-smoking | 1.77 (0.91 - 3.44) | 0.090 | 2.13 (0.98 - 4.63) | 0.055 |
| CC genotype + Ever-smoking | 5.49 (1.90 - 15.9) | 0.002 | 9.72 (2.73 - 34. 6) | <0.0001 |
| Multiplicative interaction measure | 1.54 (0.43 - 5.54) | 0.510 | 1.68 (0.37 - 7.44) | 0.503 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 2.71 (-3.13 - 8.55) | 0.363 | 5.85 (-6.33 - 18.0) | 0.347 |
| Attributable proportion due to interaction | 0.49 (-0.09 - 1.07) | 0.098 | 0.60 (0.08 - 1.13) | 0.025 |
| Synergy index | 2.52 (0.57 - 11.0) | 0.222 | 3.04 (0.67 - 13.9) | 0.151 |

CI, confidence interval; OR, odds ratio.

*Adjusted for age, region and alcohol consumption.

Table 3 shows the modifying effect of the *CYP1A1* rs4646903 genotypes on the association of smoking with SLE risk. Subjects with the CC genotype ($OR_{adj}$ = 9.72, 95% CI = 2.73 - 34.6) presented a higher risk of SLE than those with at least one T allele ($OR_{adj}$ = 2.73, 95% CI = 1.64 - 4.54 in smokers relative to non-smokers with at least one T allele. The multiplicative interaction between the *CYP1A1* rs4646903 genotypes and smoking was not significant. For assessment of additive interaction, adjusted measures (95% CI) of RERI and SI were 5.85 (-6.33 - 18.0) and 3.04 (0.67 - 13.9), respectively. These values suggested no significant biologic (additive) interactions. Meanwhile, the adjusted AP due to interaction between the *CYP1A1* rs4646903 genotypes and smoking was estimated to be 0.60 (95% CI = 0.08 - 1.13; $P$ = 0.025), indicating that 60% of the excess risk for SLE in smokers with the CC genotype was due to additive interaction.

Table 4 shows the modifying effect of the *GSTM1* genotypes on the association of smoking with SLE risk. Smokers with the *GSTM1* non-null genotype ($OR_{adj}$ = 2.32, 95% CI = 1.19 - 4.52) and smokers with the *GSTM1* null genotype ($OR_{adj}$ = 3.35, 95% CI = 1.76 - 6.39) had a significantly increased risk of SLE. All interaction measures (multiplicative and additive) between the *GSTM1* genotypes and smoking were far from significant, however.

As both phase I and phase II enzymes are involved in ROS production, combined *CYP1A1*/*GSTM1* genotypes in combination with cigarette smoking may be contribute to the risk of SLE. The interaction between the *CYP1A1*/*GSTM1* combined genotypes and smoking is shown in Table 5. Smokers with the high risk genotype ($OR_{adj}$ = 17.5, 95% CI = 3.20 - 95.9) had a higher risk of SLE than those with the low risk genotype ($OR_{adj}$ = 2.80, 95% CI = 1.70 - 4.60), relative to non-smokers with the low risk genotype (reference). The adjusted AP due to interaction between the combined metabolic genotypes and smoking was estimated to be 0.68 (95% CI = 0.011 - 1.25), indicating that 68% of the excess risk for SLE in smokers with the combined "at-risk" genotype was due to additive interaction. However, no multiplicative interaction of smoking and the combined metabolic polymorphism with SLE was observed. The RERI and SI additive interaction measures between the combined metabolic genotypes and smoking did not also reach statistical significance.

**Table 4. Interaction between smoking and *GSTM1* genotypes**

| *GSTM1* genotype + Smoking status | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | P | Adjusted* | P |
| Non-null genotype + Non-smoking | 1.0 (reference) | | 1.0 (reference) | |
| Non-null genotype + Ever-smoking | 1.80 (1.01 - 3.22) | 0.047 | 2.32 (1.19 - 4.52) | 0.013 |
| Null genotype + Non-smoking | 1.07 (0.68 - 1.68) | 0.768 | 0.98 (0.59 - 1.65) | 0.947 |
| Null genotype + Ever-smoking | 2.62 (1.48 - 4.65) | 0.001 | 3.35 (1.76 - 6.39) | <0.0001 |
| Multiplicative interaction measure | 1.36 (0.60 - 3.10) | 0.463 | 1.47 (0.58 - 3.69) | 0.415 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 0.75 (-0.83 - 2.33) | 0.352 | 1.04 (-1.17 - 3.25) | 0.356 |
| Attributable proportion due to interaction | 0.29 (-0.23 - 0.80) | 0.270 | 0.31 (-0.24 - 0.86) | 0.269 |
| Synergy index | 1.86 (0.44 - 7.84) | 0.398 | 1.79 (0.48 - 6.69) | 0.386 |

CI, confidence interval; OR, odds ratio
*Adjusted for age, region and alcohol consumption.

**Table 5. Interaction between smoking and combined CYP1A1/GSTM1 metabolic genotypes**

| Combined metabolic genotype + Smoking status | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | P | Adjusted* | P |
| Low risk genotype**+ Non-smoking | 1.0 (reference) | | 1.0 (reference) | |
| Risk genotype*** + Non-smoking | 2.04 (1.33 - 3.12) | 0.001 | 2.80 (1.70 - 4.60) | <0.0001 |
| Low risk genotype + Ever-smoking | 1.76 (0.69 - 4.50) | 0.235 | 3.77 (1.36 - 10.4) | 0.011 |
| Risk genotype + Ever-smoking | 5.88 (1.37 - 25.1) | 0.017 | 17.5 (3.20 - 95.9) | 0.001 |
| Multiplicative interaction measure | 1.63 (0.28 - 9.38) | 0.582 | 1.66 (0.23 - 11.2) | 0.615 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 3.08 (-5.54 - 11.7) | 0.483 | 12.0 (-17.7 - 41.6) | 0.430 |
| Attributable proportion due to interaction | 0.52 (-0.22 - 1.27) | 0.171 | 0.68 (0.11 - 1.25) | 0.019 |
| Synergy index | 2.71 (0.37 - 19.8) | 0.326 | 3.62 (0.52 - 25.4) | 0.195 |

CI, confidence interval; OR, odds ratio.
*Adjusted for age, region and alcohol consumption.
** At least one T allele of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype, at least one T allele of *CYP1A1* rs4646903 and *GSTM1* null genotype or the CC genotype of *CYP1A1* rs4646903 and the *GSTM1* non-null genotype
*** The CC genotype of *CYP1A1* rs4646903 and the *GSTM1* null genotype

**Table 6. Interaction between smoking and *NAT2* genotypes**

| NAT2 genotype + Smoking status | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | P | Adjusted* | P |
| Rapid acetylator genotype + Non-smoking | 1.0 (reference) | | 1.0 (reference) | |
| Rapid acetylator genotype + Ever smoking | 1.90 (0.73 - 4.95) | 0.186 | 2.16 (0.76 - 6.15) | 0.151 |
| Non rapid acetylator genotype + Non-smoking | 2.32 (1.28 - 4.23) | 0.006 | 2.07 (1.07 - 4.00) | 0.030 |
| Non-rapid acetylator genotype + Ever-smoking | 5.17 (2.65 - 10.09) | <0.0001 | 6.44 (3.07 - 13.52) | <0.0001 |
| Multiplicative interaction measure | 1.17 (0.40 - 3.37) | 0.774 | 1.44 (0.45 - 4.62) | 0.536 |
| Additive interaction measure | | | | |
| Relative excess due to interaction | 1.91 (-0.62 - 4.43) | 0.138 | 3.29 (-0.47 - 7.05) | 0.086 |
| Attributable proportion due to interaction | 0.38 (-0.02 - 0.79) | 0.066 | 0.50 (0.12 - 0.88) | 0.010 |
| Synergy index | 1.92 (0.73 - 5.03) | 0.185 | 2.40 (0.83 - 7.00) | 0.108 |

CI, confidence interval; OR, odds ratio
*Adjusted for age, region and alcohol consumption.

Table 6 shows the modifying effect of *NAT2* genotypes on the association of smoking with SLE risk. Individuals with the non-rapid acetylator genotype ($OR_{adj}$ = 6.44, 95% CI = 3.07 - 13.52) presented a higher risk of SLE than those with the rapid acetylator genotype

($OR_{adj}$ = 2.16, 95% CI = 0.76 - 6.15) in smokers, relative to non-smokers with the rapid acetylator genotype. The multiplicative interaction between the *NAT2* genotypes and smoking was far from significant. For assessment of additive interaction, adjusted measures (95% CI) of RERI and SI were 3.29 (-0.47 - 7.05) and 2.40 (0.83 - 7.00), respectively. These values suggested no significant biologic (additive) interactions. Meanwhile, the adjusted AP due to interaction between the *NAT2* genotypes and smoking was estimated to be 0.50 (95% CI = 0.12 - 0.88), indicating that 50% of the excess risk for SLE in smokers with the non-rapid acetylator genotype was due to additive interaction.

Table 7 shows the modifying effect of the *TNFRSF1B* rs1061622 genotypes on the association of smoking with SLE risk. Subjects with at least one G allele ($OR_{adj}$ = 5.42, 95% CI = 2.48 - 11.84) showed a higher risk of SLE than those with the TT genotype ($OR_{adj}$ = 2.39, 95% CI = 1.36 - 4.23) in smokers relative to non-smokers 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 (95% CI) of RERI and SI were 2.65 (-1.35 - 6.65) and 2.54 (0.80 - 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 - 0.92), indicating that 49% of the excess risk for SLE in smokers with at least one G allele was due to additive interaction.

**Table 7. Interaction between smoking and *TNFREF1B* rs1061622 genotypes**

| TNFREF1B rs1061622 genotype + Smoking status | OR (95% CI) | | | |
|---|---|---|---|---|
| | Crude | P | Adjusted* | P |
| TT genotype + Non-smoking | 1.0 (reference) | | 1.0 (reference) | |
| TT genotype + Ever-smoking | 1.92 (1.16 – 3.17) | 0.011 | 2.39 (1.36 – 4.23) | 0.003 |
| G allele carrier +Non-smoking | 1.38 (0.85 – 2.23) | 0.186 | 1.33 (0.77 – 2.32) | 0.307 |
| G allele carrier + Ever-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 – 3.44) | 0.345 | 2.65 (-1.35 – 6.65) | 0.194 |
| Attributable proportion due to interaction | 0.33 (-0.18 – 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 |

CI, confidence interval; OR, odds ratio

*Adjusted for age, region and alcohol consumption.

# Discussion

*CYP1A1* rs4646903 and *NAT2* genotypes were significantly associated with SLE risk. The high risk genotype of *CYP1A1* rs4646903 and *GSTM1* combined was also significantly associated with an increased risk of SLE. The carriage of *TNFRSF1B* rs1061622 G allele was

marginally associated with an increased risk of SLE. There was no association between the *GSTM1* deletion polymorphism and SLE risk.

The associations between *CYP1A1* polymorphisms and chronic inflammatory diseases such as RA [37] and SLE [38] have been reported. The *CYP1A1* rs4646903 polymorphism located in the 3' untranslated region of the *CYP1A1* gene might be involved in the augmented expression of *CYP1A1* mRNA [39]. Moreover, the CC genotype of this polymorphism was associated with increased CYP1A1 inducibility [36]. It is possible that *CYP1A1* rs4646903 is involved in formation of ROS, thereby culminating in inflammation as well as modification of antigens that increases their antigenicity [14]. Four studies [37, 40-42] reported no associations while one Japanese study reported that the *CYP1A1* rs4646903 polymorphism was significantly associated with SLE risk (OR = 1.98, 95% CI = 1.14 - 3.43) [38].

As enzymatic deficiency in the GSTM1 isoform is correlated with increased risk of certain diseases associated with oxidative damage, then it is also possible that there is an association between the *GSTM1* polymorphisms and SLE risk. Glutathione detoxifies ROS, reduces peroxides and detoxifies multiple compounds through GST conjugation [43, 44]. As homozygous gene deletions of both the *GSTM1* gene (the *GSTM1* null genotype) result in complete lack of enzyme activity, the *GSTM1* null genotype would be associated with higher ROS. The *GSTM1* null genotype was significantly associated with an increased risk of SLE in a Chinese population (OR = 1.66, 95% CI = 1.19 - 2.32) [41] while the recent large GWAS performed in a Chinese population did not detected a significant association [45]. In an Indian population, the *GSTM1* null genotype was marginally associated with a decreased risk of SLE [42]. Other studies did not find significant associations [38, 46-49].

As NAT2 is an important xenobiotic-metabolizing enzyme and theoretically the non-acetylated xenobiotics may induce an autoimmune mechanism, the genetic polymorphism of *NAT2* may play a role in susceptibility to SLE. The first study reported a predominance of individuals with slow acetylation activity (slow acetylators) among patients with hydralazine-induced lupus [50]. Furthermore, procainamide-induced lupus appeared to be more common and to develop more rapidly after a smaller cumulative dose in slow acetylators than in rapid acetylators [51]. The observation that xenobiotics can cause a drug-induced SLE especially in slow acetylators suggests that non-acetylated xenobiotics may accumulate and convert into reactive metabolites. N-acetylation is generally accepted as a detoxifying reaction because acetylation indirectly blocks the oxidation of arylamines [52]. Hydralazine and procainamide are arylamine drugs. Tobacco smoke as a source of the carcinogenic arylamines is already well known. Toxic intermediate metabolites of smoking-related arylamines are detoxified by NAT2. Therefore, the slow acetylator status is associated with a diminished N-acetylation ability to detoxify toxic compounds, thereby increasing SLE risk. It is hypothesized that these toxic compounds might alter self-proteins presented to the immune system and thus stimulate T-cells which induce pathological and clinical signs of autoimmunity by different effector mechanisms. Furthermore, ROS can be often detoxified by phase II drug metabolizing enzymes, such as NAT2 [53]. It is plausible that the NAT2 slow acetylator status is associated with an increased risk of SLE. The genetic polymorphism of hepatic NAT2 enzyme causes inter-individual variation in the response to a variety of amine drugs and potential carcinogens [54, 55]. Different haplotypes are encoded by at least 7 single nucleotide polymorphisms (G191A, C282T, T341C, C481T, G590A, A803G, G857A) within the single 870-bp exon of *NAT2* [56]. The most common mutations in the Japanese population are at positions C481T, G590A, and G857A of *NAT2* [28-30]. The major alleles that led to a

reduction in NAT2 activity are *6A and *7B, which contain the G590A and G857A substitutions, respectively. *NAT2**5B contains the T341C, C481T, and A803G substitutions [56]. Identification of mutations at positions 481, 590, 803, and 857 will be sufficient to determine mutated alleles as the remaining mutations [56]. Our previous study found that the *NAT2* slow acetylator status may be a determinant in susceptibility to SLE [57]. The role of the acetylator phenotype (or genotype) in the determination of susceptibility to idiopathic SLE is controversial in subsequent studies. Some studies indicated an increased frequency of the slow acetylator phenotype (or genotype) in SLE patients [40, 58-62], while other studies found no association [63-70]. As ethnic differences in the *NAT2* allele frequencies are striking [55], it has been suggested that the role of the *NAT2* polymorphism on SLE may differ with ethnic group. The frequencies of T341C and G857A explain the ethnic difference in acetylator phenotypes between Caucasians and Asians [71, 72].

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 two distinct TNF receptors, TNF receptor superfamily, member 1A (TNFRSF1A, also known as TNFR1) and TNFRSF1B (also known as TNFR2) [73]. TNFRSF1A is known to initiate the majority of TNF's biological activities [74]. Both TNFRSF1A and TNFRSF1B are coexpressed on virtually all cells and initiate distinct signal transduction pathways by interacting with different signaling factors [75]. TNFRSF1A contains an intracellular cell death domain, which is required for signaling of apoptosis and activation of the proinflammatory transcription factor nuclear factor κB1 (NFκB1) [76]. 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 [77, 78]. On the other hand, TNFRSF1B usually is associated with activation of NFκB1 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 modulator of TNFRSF1A-mediated apoptotic mechanisms has been proposed [79]. Also, TNFRSF1B has been proposed to play a key role in chronic inflammatory disorders [80]. Women with mild endometriosis (stages I and II) have been shown to have deficient expression of *TNFRSF1B* [81]; however, little is known about the pathophysiological effects, if any, of the differential expression of TNF receptors in endometriosis. In the present study, the genotypic distribution of the *TNFRSF1B* rs1061622 was not consistent with HWE among controls. Departure from HWE can imply the presence of selection bias (lack of representation of the general population) in this population because this study was free from the possibility of genotyping error (e.g., systematic misgenotyping of heterozygotes as homozygotes or vice versa, or non-randomness of missing data), assay non-specificity or possible population admixture/stratification [82, 83]. The Japanese population sample could be expected to have a relatively low risk of population stratification effects [84, 85] in comparison to Caucasian populations that have a geographically broader-based inheritance. However, it is interesting to note 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 the genotyping blind to the SLE status of our study population; samples were randomly stored in DNA sample containers and randomly selected samples were retyped. In the present 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 three controls had the GG genotype of *TNFRSF1B* rs1061622 in the present study. If four controls had possessed the GG genotype, there was no longer a deviation from HWE in controls ($P = 0.08$). The G allele of *TNFRSF1B* rs1061622 induced significantly increased level of interleukin 6 compared with the T allele of *TNFRSF1B* rs1061622 after treatment with TNF-α in *in vitro* study [86]. In addition, the cytotoxic activity induced by the G allele of *TNFRSF1B* rs1061622 was more pronounced compared with that of the T allele of *TNFRSF1B* rs1061622 [86]. It is thus considered that *TNFRSF1B* rs1061622 is a functional polymorphism and the G allele of *TNFRSF1B* rs1061622transmits TNF-α-mediated signals more effectively than does the T allele. Our finding is line with the findings in a recent meta-analysis that he G allele of *TNFRSF1B* rs1061622 is associated with an increased risk of SLE, especially in Asian population [31]. Several studies reported that *TNFRSF1B* rs1061622 is associated with SLE risk [86-88] but other studies have not replicated the finding [89-92]. Lack of reproducibility of genetic associations has been frequently observed and has been varyingly attributed to population stratification, phenotype differences, selection biases, genotyping errors, and other factors [93, 94]. At present, the best way of resolving these inconsistencies appears to be additional replication studies with larger sample sizes, although this may not be feasible for rare conditions or for associations identified in unique populations [93].

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. Furthermore, a concern with respect to genetic association studies has been lack of replication studies, especially contradictory findings across studies. Replication of findings is very important 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.

It is widely accepted that SLE development requires environmental factors acting on a genetically predisposed individual. Studying gene-environment interactions in relation to SLE risk may be valuable, as positive findings would clearly implicate the substrates with which the gene interacts as disease-causing exposures, clarifying SLE etiology and pointing to environmental modifications for disease prevention. Case-control genetic association study can be useful in investigating gene-environment interactions. A history of smoking was significantly associated with 2.86-fold increased risk of SLE. Substrates for and inducers of CYP1A1 include PAHs such as benzo(a)pyrene in tobacco smoke while GSTM1 detoxifies ROS generated during the metabolism of PAHs. Heterocyclic amines are present in cigarette

smoke and are potential substrates for NAT2 activation. Cigarette smoking has been suggested to influence TNFRSF1B production [95, 96]. Several studies have investigated that smoking was associated with an increased risk of SLE [3]. 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, several potential mechanisms exist. Exposure to ROS via cigarette smoking may be associated with increased risk of SLE. A common method for quantifying interactions is based on the calculation of the two risk factors' product term in a logistic-regression model (multiplicative). A gene-environment interaction was suggested, with "at-risk" genotype and smoking conferring significantly higher risk, compared with no "at-risk" genotype and non-smoking in the present study (Tables 3 - 7). For example, smokers with the *CYP1A1*/*GSTM1* combined "at-risk" genotype was strongly associated with increased risk of SLE ($OR_{adj}$ = 17.52, 95% CI = 3.20 - 95.9). The observed high ORs were attributed largely to the effect of ever-smoking, however. The multiplicative interaction between the genetic polymorphisms involved in ROS production investigated in the present study and smoking was far from significant, however. 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 two risk factors added to each other [33, 34]. There were significant additive interactions between smoking and any one of the following: *CYP1A1* rs4646903, *CYP1A1*/*GSTM1* combined metabolic genotypes, *NAT2* genotypes or *TNFRSF1B* rs1061622. Specifically, about 50 % of the excess risk for SLE in smokers with the "at-risk" genotype was due to the additive interaction. Thus, the results suggest evidence for additive but not multiplicative interaction. To the best of our knowledge, no studies have reported the studies on risk modification by the genetic polymorphisms involved in ROS production, such as *CYP1A1* rs464903, *GSTM1* deletion, *NAT2* genotypes determined by *NAT2**4, *5B, *6A or *7B allele and *TNFRSF1B* rs1061622, in the association of cigarette smoking and SLE. Despite the growing awareness of the relevance of gene-environment interactions in human disease, true progress in the identification of common genetic alterations that by themselves may not substantially impact 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 other genetic profile of the individual and environmental exposures; while other population-specific effects may be generated under differential gene-gene interactions in different populations, differential gene-environment interactions, or both [97]. Sample sizes for adequate power to detect interactions are prohibitively large when the frequencies of interacting variants and exposures are small [97]. In addition, assessment of gene-environment interaction also depends upon the proper statistical evaluation of interaction on the multiplicative and additive models.

Several limitations of this study warrant mention. Our study may have included a bias due to the self-reporting of smoking habits (misclassification bias). However, discrepancies between self-reported smoking habits and biochemical verification are minimal among the general population [98, 99]. Similary, the validity of consumption of coffee and tea using a self-administered questionnaire is relatively high [100, 101]. Recall bias, which occurs when cases and controls recall exposures differently, is also a well-recognized potential problem in

case-control studies. SLE patients may be more likely to report their prior exposures than healthy controls because they think they might be related to their disease. The purported link between smoking and SLE is not common knowledge, however. The possibility of recall bias in reporting smoking habit may be minimized, because SLE patients are unlikely to be aware that smoking habit may be associated with SLE risk. Inaccuracies in recall and reporting were possible and, as they were likely nondifferential, could cause dilution of a true association. Population-based case-control studies may have underestimated slightly the true association due to recall bias [102]. Case-control studies tend to be susceptible to selection bias, particularly in the control group. Selection bias may occur if the decision to participate is affected by smoking habit. In many cases, selection bias is not extreme enough to have an impact on inference and conclusions [103]. As the possibility of recall and selection biases could not be completely excluded in case-control studies, our findings should be interpreted with caution. A fundamental conceptual issue selection of controls is whether the controls should be similar to the cases in all respects other than status of the disease in question. As controls were not selected to match SLE patients on confounding factors, there were significant differences between them, such as age. Although matching is one approach to control for confounding bias in the design of the study, the confounding bias can be also controlled for by using statistical modeling approach in the analysis, as was carried out in our study.

In the present study, we carried out candidate gene association study (hypothesis-driven approach) on the genetic polymorphisms involved in ROS production (*CYP1A1* rs4646903, *GSTM1* deletion polymorphism, *NAT2* genotypes determined by *NAT2**4, *5B, *6A or *7B allele and *TNFRSF1B* rs1061622) to SLE risk with special reference to interaction with cigarette smoking among 152 SLE cases and 427 controls in female Japanese subjects. *CYP1A1* rs4646903 and *NAT2* genotypes were significantly associated with SLE risk. There were significant additive interactions between smoking and any one of the following: *CYP1A1* rs4646903, *NAT2* or *TNFRSF1B* rs1061622. Findings from gene-environment interaction analyses must be interpreted with caution due to reduced numbers of observations in the subgroups. Replication of findings is very important before any causal inference can be drawn. Testing replication in different populations is an important step. Future studies involving larger control and case populations, precisely and uniformly defined clinical classification of SLE and better exposure histories will undoubtedly lead to a more thorough understanding of the role of the genetic polymorphisms involved in ROS production in SLE development.

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