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## 【参考資料】

### 1. 本研究に用いた症例調査データベースの内訳

66施設（産科系、内科系、その他）から758症例。うち、本研究の登録条件を満たす732症例を対象とした。

### 2. 本研究の特徴と限界

本研究では、全国の医療機関の協力により、700例を越える抗SS-A抗体陽性妊娠例の登録を達成した。このような大規模な抗SS-A抗体陽性妊娠の登録研究はわが国には前例がなく、本登録より新たな知見が得られることが期待される。しかしながら、抗SS-A抗体陽性妊娠は比較的少ない妊娠例であり、それからさらに少数のCHB発症をエンドポイントとした分析を行うには、700例の登録でもなお統計的検出力の不足は常につきまとう問題である。したがって、今回の研究結果で有意な関連が検出できなくても、それによって直ちにその関連が否定されるものではない。一方、未知のバイアスによって、偶然に有意な関連を検出する可能性もある。

その他の本研究の限界について列挙する。第1に、本研究では抗SS-A抗体陽性妊娠例を多施設から集積しているものの、抗SS-A抗体検査の標準化はなされていない。第2に、現時点では抗SS-A抗体検査は、一般に妊娠判明時にルーチンで行われているわけではないため、大多数の無症候性抗SS-A抗体陽性妊娠例は、本研究では把握できていない。第3に、事前に予測したCHBの発症率（約1%と予想）から割り出した予想症例数（7例程度）よりも、実際に登録された新生児心ブロックの症例数の方が多かった（約50例、妊娠前に抗体陽性であることが判明した症例に限っても約20例）ことは、CHB非発症例よりも発症例の方が登録されやすかった可能性を示唆する。第4に、抗SS-A抗体陽性妊娠例が、児のCHB発症をきっかけに判明するなど、妊娠前には判明していなかった場合、そうした症例の多くは妊娠中には症状が乏しく治療を受けていないため、その例を含めて症状や治療についての分析をすると結果を歪める可能性がある。

本研究の実施に当たっては、これらの限界点に極力留意しつつ分析を行った。例えば、第3の問題に対しては、症例対照研究の手法を用いて解析することとし、第4の問題に対しては、臨床症状や治療に関する分析では症例を妊娠前に抗SS-A抗体陽性であることが判明した症例に限ることとした。

本研究で得られた結果として、抗SS-A抗体高値がCHB発症のリスク因子で、DID法で32倍をカットオフ値とすることが有用である可能性が示されたことと、妊娠16週以前からのプレドニゾロン換算10mg/日以上の投与がCHBの発症予防につながる可能性が示されたことがある。

本研究で構築したデータベースからはCHBのリスク因子、予防因子を明らかにする以外にも多く情報が得られる可能性があるが、それらについては今後学会や論文などで呈示していきたいと考えている。いずれにしても結果の解釈に当たっては、これらの限界点に注意する必要がある。

## 3. 本研究で集積した症例データベースからの単純集計（一部抜粋）

≪注意≫以下は本研究の登録条件を満たす全症例を対象としたもので、CHB を発症してから抗 SS-A 抗体陽性が判明した症例や CHB 既往があるために予防治療を行った症例も含まれている単純集計であり、ここで示されている数値のみを取り上げて臨床での判断根拠とすることは、適切ではないことに留意されたい。

① 抗 SS-A 抗体の測定法の内訳（定性のみの施設あり）（有効対象　725 例）

| | 症例数 |
|---|---|
| DID | 292 |
| ELISA | 500 |
| DID＋ELISA | 67 |

② 抗 SS-A 抗体陽性妊娠例（732 例）の抗 SS-B 抗体と CHB 発症の有無との関係

| | 症例数 | CHB 発症あり | CHB 発症なし |
|---|---|---|---|
| 抗 SS−B 抗体陽性 | 188 | 18 | 171 |
| 抗 SS−B 抗体陰性 | 474 | 34 | 442 |
| 抗 SS−B 抗体不明 | 70 | 1 | 69 |

③ 抗 SS-A 抗体陽性妊娠例（732 例）の基礎疾患

| 基礎疾患 | 症例数 | | |
|---|---|---|---|
| なし | 157 | | |
| あり | 575 | SS | 275 |
| | | SLE | 281 |
| | | MCTD | 40 |
| | | RA | 31 |
| | | APS | 30 |
| | | その他 | 49 |

④ その他自己抗体の測定状況と陽性率（対象　732 例）

| | 抗核抗体 | 抗 DNA 抗体 | 抗 U1RNP 抗体 | 抗 Sm 抗体 | 抗リン脂質抗体 | 抗甲状腺抗体 |
|---|---|---|---|---|---|---|
| 測定数(%) | 680(92.9) | 566(77.3) | 437(59.7) | 434(59.3) | 587(80.2) | 215(29.3) |
| 陽性数(%) | 638(93.8) | 173(30.6) | 122(27.9) | 41(9.4) | 70(11.9) | 75(34.9) |

⑤　抗 SS-A 抗体価と CHB 発症の関係

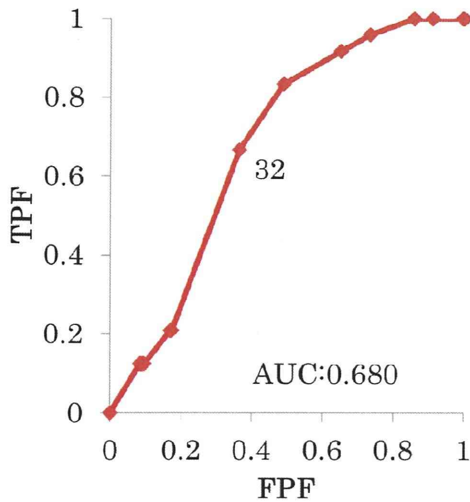



図1. 抗体価(DID法)とCHB発症における
ROC曲線での検討（n=231）

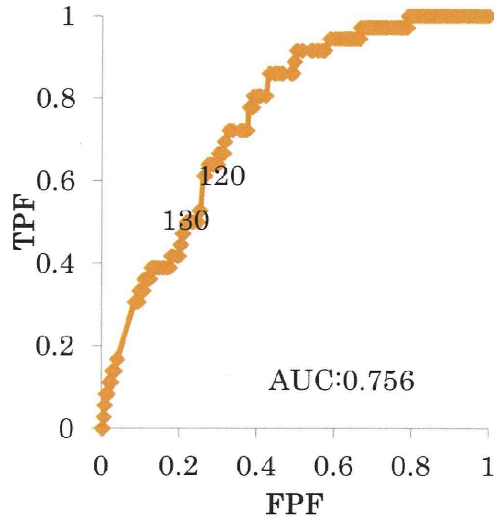
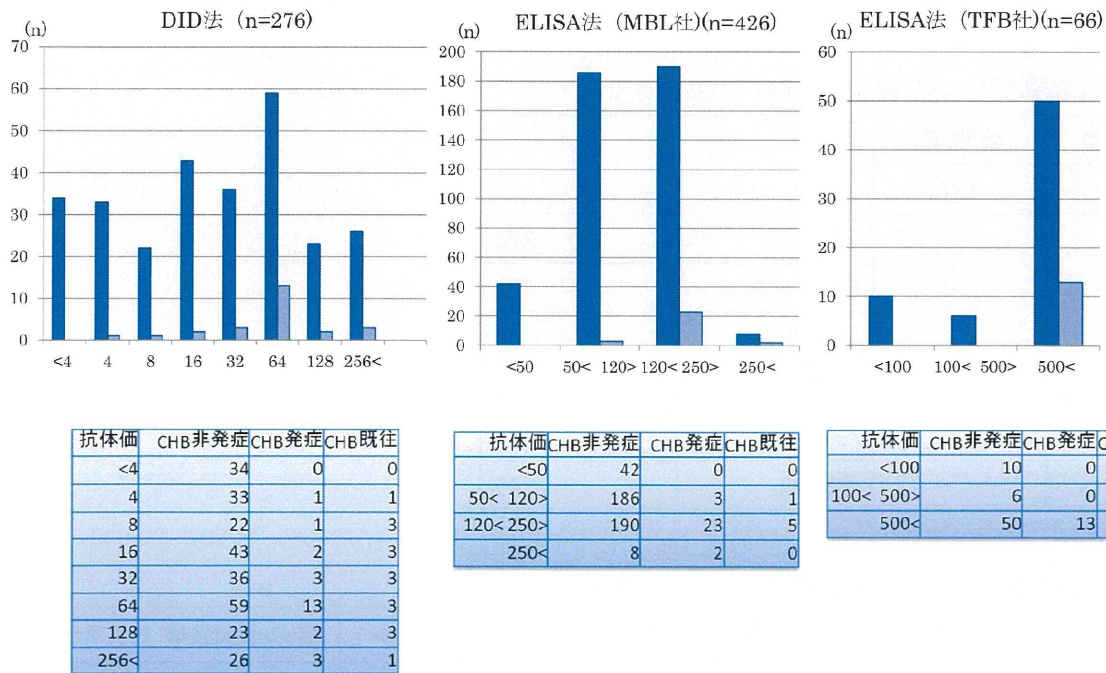



図2. 抗体価(ELISA法)とCHB発症における
ROC曲線での検討(n=452)

図3. 測定法別の抗SS-A抗体価とCHB発症の関係

■：CHB発症なし　■：CHB発症あり




| 抗体価 | CHB非発症 | CHB発症 | CHB既往 |
|---|---|---|---|
| <4 | 34 | 0 | 0 |
| 4 | 33 | 1 | 1 |
| 8 | 22 | 1 | 3 |
| 16 | 43 | 2 | 3 |
| 32 | 36 | 3 | 3 |
| 64 | 59 | 13 | 3 |
| 128 | 23 | 2 | 3 |
| 256< | 26 | 3 | 1 |

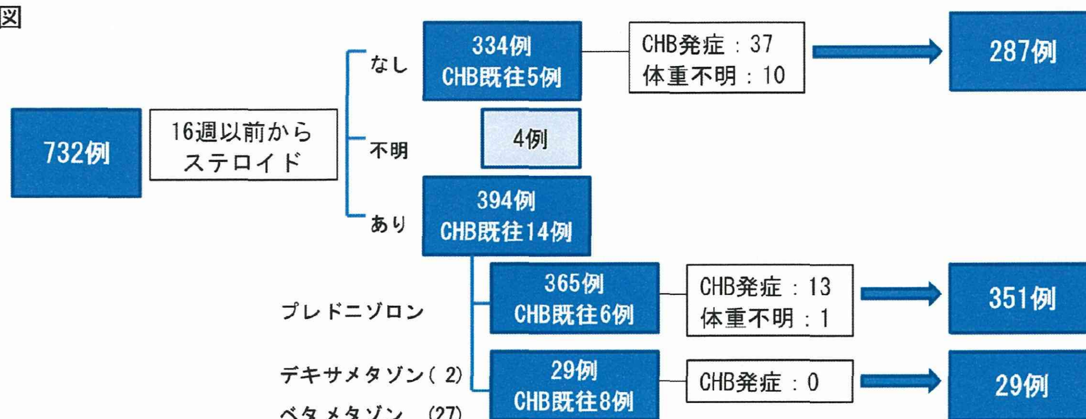
| 抗体価 | CHB非発症 | CHB発症 | CHB既往 |
|---|---|---|---|
| <50 | 42 | 0 | 0 |
| 50< 120> | 186 | 3 | 1 |
| 120< 250> | 190 | 23 | 5 |
| 250< | 8 | 2 | 0 |

| 抗体価 | CHB非発症 | CHB発症 | CHB既往 |
|---|---|---|---|
| <100 | 10 | 0 | 0 |
| 100< 500> | 6 | 0 | 0 |
| 500< | 50 | 13 | 5 |

症例データベースを用いた ROC 曲線解析により、CHB 発症を予測するためのカットオフを DID 法では 32 倍と設定した（図 1）が、4 倍、8 倍でそれぞれ 1 症例ずつ、16 倍で 2 症例と 32 倍未満でも CHB を発症している症例があることにも留意した説明が必要である。ELISA 法全体では 120-130U/ml をカットオフ値とできるのではないかとの考察がされた（図 2)。ELISA 法（MBL 社）に絞っても同様の傾向にあるが、120 未満の 3 症例（83,101,112）でも CHB 発症があった（図 3)。ELISA 法（TFB 社）では 500U/ml 以上は最終値を出していない場合が多く、本法単独でカットオフ値をどこに定められるか今後の課題である。なお、参考までにそれぞれの表に CHB 既往症例数を示した。

⑥　妊娠初期（16 週以前）からのステロイド投与の有無、種類と児の体重

図




表

| | 症例数 | 早産 | 児の子宮内発達（SD） |
|---|---|---|---|
| ステロイド投与なし | 287 | 51 （18%） | −0.53±1.18 |
| プレドニゾロン | 351 | 106 （30%） | −0.54±1.34 |
| フッ化ステロイド | 29 | 4 （14%） | −1.48±1.27 |
| 　　デキサメタゾン | 2 | | |
| 　　ベタメタゾン | 27 | | |

図：フッ化ステロイドを妊娠 16 週以前から投与された 29 例（CHB 既往の 8 例を含み、ほとんどが CHB 予防目的と考えられる）では全く CHB 発症がなかったのに対し、プレドニゾロン投与例では 365 例（CHB 既往の 6 例を含む）中、13 例に CHB が発症、ステロイド非投与例では 334 例（CHB 既往の 5 例を含む）中、37 例に CHB が発症していた。

表：母体へのステロイド投与が児の発育に及ぼす影響を見るために、それぞれの群で CHB を発症せず、かつ出生時体重の明らかな症例の在胎期間別出生時体重基準値（性別・初経別も考慮した）からの標準偏差（SD）を比較したところ、フッ化ステロイド剤投与群でやや低い傾向にあった。

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抗 SS-A 抗体陽性女性の妊娠に関する診療の手引き
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# IV.　研究成果の刊行に関する一覧表

# 研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
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# V.　研究成果の刊行物・別刷

# A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Eyrthematosus in Japanese

**Yukinori Okada[1,2,3], Kenichi Shimane[1,2], Yuta Kochi[1,2*], Tomoko Tahira[4], Akari Suzuki[1], Koichiro Higasa[3], Atsushi Takahashi[3], Tetsuya Horita[5], Tatsuya Atsumi[5], Tomonori Ishii[6], Akiko Okamoto[2], Keishi Fujio[2], Michito Hirakata[7], Hirofumi Amano[8], Yuya Kondo[9], Satoshi Ito[9], Kazuki Takada[10], Akio Mimori[11], Kazuyoshi Saito[12], Makoto Kamachi[13], Yasushi Kawaguchi[14], Katsunori Ikari[14], Osman Wael Mohammed[15], Koichi Matsuda[15], Chikashi Terao[16,17], Koichiro Ohmura[16], Keiko Myouzen[1], Naoya Hosono[18], Tatsuhiko Tsunoda[19], Norihiro Nishimoto[20], Tsuneyo Mimori[16], Fumihiko Matsuda[17], Yoshiya Tanaka[12], Takayuki Sumida[9], Hisashi Yamanaka[14], Yoshinari Takasaki[8], Takao Koike[5], Takahiko Horiuchi[21], Kenshi Hayashi[4], Michiaki Kubo[18], Naoyuki Kamatani[3], Ryo Yamada[1,17], Yusuke Nakamura[15], Kazuhiko Yamamoto[1,2]**

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## Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Although recent genome-wide association studies (GWAS) have contributed to discovery of SLE susceptibility genes, few studies has been performed in Asian populations. Here, we report a GWAS for SLE examining 891 SLE cases and 3,384 controls and multi-stage replication studies examining 1,387 SLE cases and 28,564 controls in Japanese subjects. Considering that expression quantitative trait loci (eQTLs) have been implicated in genetic risks for autoimmune diseases, we integrated an eQTL study into the results of the GWAS. We observed enrichments of cis-eQTL positive loci among the known SLE susceptibility loci (30.8%) compared to the genome-wide SNPs (6.9%). In addition, we identified a novel association of a variant in the AF4/FMR2 family, member 1 (*AFF1*) gene at 4q21 with SLE susceptibility (rs340630; $P = 8.3 \times 10^{-9}$, odds ratio = 1.21). The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels ($P < 0.05$). As *AFF1* transcripts were prominently expressed in $CD4^{+}$ and $CD19^{+}$ peripheral blood lymphocytes, up-regulation of *AFF1* may cause the abnormality in these lymphocytes, leading to disease onset.

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## Author Summary

Although recent genome-wide association study (GWAS) approaches have successfully contributed to disease gene discovery, many susceptibility loci are known to be still uncaptured due to strict significance threshold for multiple hypothesis testing. Therefore, prioritization of GWAS results by incorporating additional information is recommended. Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Considering that abnormalities in B cell activity play essential roles in SLE, prioritization based on an expression quantitative trait loci (eQTLs) study for B cells would be a promising approach. In this study, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS and identified *AFF1* as a novel SLE susceptibility loci. We also confirmed cis-regulatory effect of the locus on the *AFF1* transcript. Our study would be one of the initial successes for detecting novel genetic locus using the eQTL study, and it should contribute to our understanding of the genetic loci being uncaptured by standard GWAS approaches.


## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production, complement activation, and multi-organ damage [1]. Familial aggregation demonstrates that both genetic and environmental factors play a role in pathogenesis of SLE [2]. Genetic studies using candidate gene-approaches, and recently, genome-wide association studies (GWAS), have uncovered more than 25 SLE susceptibility genes, including *HLA-DRB1*, *IRF5*, *STAT4*, *ITGAM*, *BLK*, *TNFAIP3*, and others [3–18]. However, most of these studies were conducted in European populations [3–13,15,17], and few studies have been conducted in Asian populations [14,16,18]. Since the epidemiology of SLE has demonstrated that the prevalence of disease substantially differs among populations, genetic backgrounds of SLE should be also heterogeneous across populations [19,20]. Therefore, additional studies in Asians might provide novel insights. It is of note that GWAS for SLE in Chinese populations identified novel loci that had not been detected in Europeans, such as *ETS1*, *IKZF1*, and *WDFY4* [14,16].

Another issue raised by the previous GWASs for complex diseases is that many susceptibility loci still remained uncaptured, owing to its strict significance threshold for multiple hypothesis testing [21]. In SLE, for example, the 26 risk loci identified by the previous GWAS explained only an estimated 8% of the total genetic susceptibility to the disease [15]. Therefore, it is still important to examine the sub-loci of GWAS, in order to reveal the entire picture of genetic etiology. To effectively explore these uncaptured loci, prioritization of GWAS results by incorporating additional information implicated in the disease pathophysiology is recommended [22,23]. Considering that abnormalities in B cell activity play essential roles in SLE [1] and that expression quantitative trait loci (eQTL) have been implicated to comprise approximately a half of genetic risks for autoimmune diseases [24], prioritization based on an eQTL study for B cells would be a promising approach for SLE [25]. Moreover, an eQTL itself assures the presence of functional variant(s) that regulate gene expression. Thus, eQTL increases the prior probability of the presence of disease-causal variant(s) in the locus more effectively and unbiasedly, compared to other knowledge-based prioritizations such as gene pathway analysis [24].

Here, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS, which effectively enabled to detect a novel SLE susceptibility locus.

## Results

### GWAS for SLE

In the GWAS, 891 SLE cases and 3,384 controls in Japanese subjects were genotyped over 550,000 single nucleotide polymorphism (SNP) markers (Table S1, S2 and Figure 1). We applied stringent quality control (QC) criteria and evaluated associations of 430,797 autosomal SNPs, as previously described [26]. No substantial population stratification was demonstrated through principal component analysis (Figure S1) or a Quantile Quantile plot of $P$-values (inflation factor, $\lambda_{GC} = 1.088$, Figure S2), suggesting homogenous ancestries of our study population [27].

We identified significant associations in six chromosomal loci that satisfied the genome-wide significance threshold of $P<5.0\times10^{-8}$ (Table 1 and Figure 2A). These loci have been reported to be associated with SLE susceptibility (*STAT4*, *TNFAIP3*, *HIP1*, *BLK*, *ETS1*, and the HLA region) [3–18]. We also observed significant replications in 17 of the previously reported SLE susceptibility loci [3–18] ($\alpha = 0.01$; Table 2). Of these, significant replications were enriched in the loci identified through the studies in Asian populations (80%; 8 of the 10 loci), including *RASGRP3*, *IKZF1*, *HIP1*, *WDFY4*, intergenic region at 11q23, *ETS1*, *SLC15A4*, *ELF1*, and *HIC2-UBE2L3* [14,16,18], compared to those in European populations (56.3%; 9 of the 16 loci) [3–13,15,17].

### Incorporation of eQTL study into GWAS results

For the selection of SNPs incorporated in the replication studies of the potential association signals, we evaluated cis-eQTL effects of the SNPs using publically available gene expression data [28], and prioritized the results of the GWAS. After applying QC criteria, we evaluated the expression levels of 19,047 probes assayed in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals [29] using Illumina's human whole-genome expression array (WG-6 version 1) [28]. For each of the SNPs included in our GWAS, probes located within ±300 kbp regions were focused on as cis-eQTLs (average 4.93 probes per SNP). We denoted the SNPs which exhibited significant associations with expression levels of any of the corresponding cis-eQTLs as eQTL positive (false discovery rate (FDR) $Q$-values<0.2). We observed enrichments of eQTL positive loci among the SLE susceptibility loci (30.8%; 8 of the 26 evaluated loci) including a well-known eQTL gene of *BLK* [11,25] (Table 2), compared to the genome-wide SNPs (6.9%) and compared even to the SNPs in the vicinity of expressed loci (among the SNPs located within ±10 kbp of probes used for the expression analysis, 13.1% were eQTL positive; Table S3).

By prioritizing the results of the GWAS using the eQTL study, we selected 57 SNPs from 1,207 SNPs that satisfied $P<1.0\times10^{-3}$ in the GWAS. We subsequently referred the associations of the selected SNPs using the results of the concurrent genome-wide scan for SLE in an independent Japanese population (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, 447 SLE cases and 680 controls of Japanese origin were evaluated using a pooled DNA approach [30]. We selected SNPs if any association signals were observed in the neighboring SNPs of the

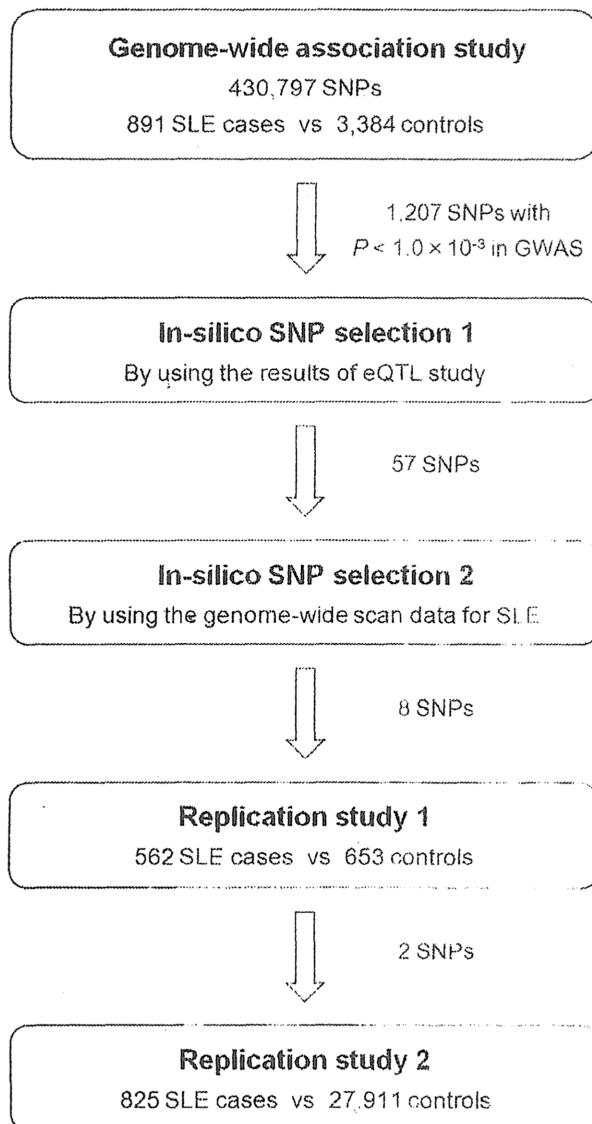


**Figure 1. Design of the GWAS and multi-stage replication studies for SLE in Japanese subjects.** A total of 2,278 SLE cases and 31,948 controls were enrolled. The clinical characteristics of the subjects are summarized in Table S1 and S2. Details of the genome-wide scan data for SLE referenced in the *in silico* SNP selection 2 are described elsewhere (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009).
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pooled analysis. As a result, 8 SNPs remained for further investigation (Table S4).

## Replication studies and identification of *AFF1*

Then, we performed two-stage replication studies using independent SLE cohorts for Japanese subjects (cohort 1 with 562 SLE cases and 653 controls, and cohort 2 with 825 SLE cases and 27,911 controls). First, we evaluated the selected 8 SNPs in the replication study 1. In the replication study 2, 2 SNPs that satisfied $P<1.0\times10^{-6}$ in the combined study of GWAS and replication study 1 were further evaluated (Figure 1). Among the evaluated SNPs, we observed significant replications in the SNP located in the genomic region of the AF4/FMR2 family, member 1 gene (*AFF1*) at 4q21 (rs340630; $P=4.6\times10^{-5}$ and $P=0.0094$ in the two individual cohorts, respectively; Table 3, Table S5, and Figure 2B). The combined study for the GWAS ($P=1.5\times10^{-4}$) and the replication studies demonstrated significant associations of rs340630 that satisfied the genome-wide significance threshold ($P=8.3\times10^{-9}$, OR = 1.21, 95% CI 1.14–2.30).

## Cis-eQTL effect of rs340630 on *AFF1* transcripts

Since the landmark SNP in the *AFF1* locus, rs340630, was prioritized through the eQTL study as an eQTL positive SNP (Table 3), we further validated its cis-eQTL effect using Epstein-Barr virus (EBV)-transfected B cell lines established from Japanese individuals (Pharma SNP Consortium (PSC) cells, $n=62$). The correlation between rs340630 genotypes and the expression levels of *AFF1* was significant in the PSC cells stimulated with phorbol myristate acetate (PMA) ($R^2=0.074$, $P=0.033$; Figure 3A). The expression levels increased with the number of SLE-risk (A) alleles. To further confirm this cis-regulatory effect, we performed allele-specific transcript quantification (ASTQ) of *AFF1*. The transcript levels of each allele were quantified by qPCR using an allele specific probe for a SNP in the 5′-untranslated region (rs340638), which was in absolute LD with rs340630 ($r^2=1.0$, $D'=1.0$). We examined PSC-cells ($n=17$) that were heterozygous for both rs340630 and rs340638. The mean ratio of each transcript (A over G allele: the A allele comprises a haplotype with the risk (A) allele of rs340630) were significantly increased to 1.07 compared to the ratio of the amount of DNA (1.00, $P=0.012$) (Figure 3B). These results suggest that rs340630, or SNP(s) in LD with it, are a regulatory variant predisposing SLE susceptibility through increased expression levels of *AFF1*.

## Expression of *AFF1* in CD4$^+$ and CD19$^+$ peripheral blood lymphocytes

*AFF1* is known to be involved in cytogenetic translocations of acute lymphoblastic leukemia (ALL) [31]. Its fusion protein with the mixed-lineage leukemia gene (*MLL*) is implicated in the regulation of transcription and the cell cycle of lymphocytes [31]. To investigate the expression pattern of *AFF1* in normal tissues, we evaluated the transcript levels of *AFF1* in a panel of various tissues. We observed prominent expression of *AFF1* in CD4$^+$ and CD19$^+$ peripheral blood lymphocytes, implying an important role for *AFF1* in helper T-cells and B-cells (Figure 3C).

## Discussion

Through a GWAS and multi-staged replication studies consisting of 2,278 SLE cases and 31,948 controls in Japanese subjects, our study identified that the *AFF1* locus was significantly associated with SLE susceptibility.

As well as the identification of the novel SLE susceptibility locus, we observed significant replications of associations in the previously reported susceptibility loci. The replications were especially enriched in the loci identified through the studies in Asian populations, compared to those in European populations. Considering the ethnical heterogeneities in the epidemiology of SLE [19,20], these observations suggest the similarities in the genetic backgrounds of SLE shared within Asian populations, and also the existence of the both common and divergent genetic backgrounds encompassed between European and Asian populations.

**Table 1.** Results of a genome-wide association study for Japanese patients with SLE.

| rsID[a] | Chr | Position (bp) | Cytoband | Gene | Allele[b] | No. subjects | | Allele 1 freq. | | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1/2 | Case | Control | Case | Control | | |
| rs10168266 | 2 | 191,644,049 | 2q32 | *STAT4* | T/C | 891 | 3,384 | 0.37 | 0.27 | 1.59 (1.42–1.78) | $2.7\times10^{-16}$ |
| rs9501626 | 6 | 32,508,322 | 6p21 | HLA region | A/C | 891 | 3,381 | 0.20 | 0.12 | 1.86 (1.62–2.13) | $1.0\times10^{-18}$ |
| rs2230926 | 6 | 138,237,759 | 6q23 | *TNFAIP3* | G/T | 891 | 3,377 | 0.11 | 0.069 | 1.75 (1.47–2.08) | $1.9\times10^{-10}$ |
| rs6964720 | 7 | 75,018,280 | 7q11 | *HIP1* | G/A | 891 | 3,384 | 0.25 | 0.19 | 1.43 (1.27–1.63) | $1.3\times10^{-8}$ |
| rs2254546 | 8 | 11,381,089 | 8p23 | *BLK* | G/A | 891 | 3,384 | 0.78 | 0.72 | 1.42 (1.61–1.25) | $4.1\times10^{-8}$ |
| rs6590330 | 11 | 127,816,269 | 11q24 | *ETS1* | A/G | 891 | 3,368 | 0.48 | 0.39 | 1.44 (1.30–1.60) | $1.3\times10^{-11}$ |

[a]SNPs that satisfied the threshold of $P<5.0\times10^{-8}$ were indicated.
[b]Based on forward strand of NCBI Build 36.3.
SLE, systemic lupus erythematosus; OR, odds ratio.
doi:10.1371/journal.pgen.1002455.t001

To effectively detect the novel SLE susceptibility locus, we integrated cis-eQTL effects of the SNPs and prioritized the results of the GWAS. In addition to identifying a novel locus for SLE-susceptibility, our study demonstrated approximately 30% of confirmed SLE-susceptibility loci were comprised of cis-eQTLs. We also confirmed cis-regulatory effect of the landmark SNP in the *AFF1* locus, rs340630, on *AFF1* transcripts, which had been prioritized through the eQTL study. These results would suggest that accumulation of quantitative changes in gene expression would accelerate the disease onset of SLE. It would also demonstrate the validity of applying eQTL study in the search of the susceptible genes for SLE or other autoimmune diseases, as previously suggested in the study for celiac disease [24]. To our knowledge, this is one of the initial studies to successfully discover a new locus by prioritizing GWAS results using eQTLs, and should contribute to the approaches assessing genetic loci still being uncaptured by recent large-scaled GWASs due to stringent significance threshold for multiple hypothesis testing [21].

We observed prominent expression levels of *AFF1* in CD4$^+$ and CD19$^+$ peripheral blood lymphocytes, which would imply an important role for *AFF1* in helper-T-cells and B-cells. In fact, *AFF1* is essential for normal lymphocyte development, as demonstrated in mice deficient for *AFF1*; severe reduction were observed in the thymic double positive CD4/CD8 population and the bone marrow pre-B and mature B-cell numbers [32]. The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels. As the *AFF1* locus was also demonstrated as an eQTL in primary liver cells [33], the cis-regulatory effect may hold in primary cells as well as lymphoblastoid cells used in the present study. However, because the mechanism of transcriptional regulation is substantially different among cell types [34], cell-type specific analyses including those for primary T-cells and B-cells are needed for understanding the precise role of *AFF1* variant in primary lymphocytes. Although further functional investigation is necessary, our observation suggested that *AFF1* is involved in the etiology of SLE through the regulation of development and activity of lymphocytes. It is of note that *AFF3*, which also belongs to the AF4/FMR2 family, is associated with susceptibility to autoimmune diseases [35].

One of our study's limitations is the selection of SNPs for the replication study using the results of the pooled DNA approach [30], which used a different genotyping platform from that of the present GWAS. Moreover, the association signals based on Silhouette scores in pooled analysis would be less reliable compared to those based on individual genotyping. Since direct comparisons of the association signals of the same single SNPs between the studies would be difficult due to these issues, we adopted the complementary approach that referred the association signals of the multiple SNPs in the pooled analysis for each of the single SNPs in the GWAS, taking account of LD and physical distances between the SNPs. However, there would exist a possibility that the variant(s) truly associated with SLE was left not to be examined in the replication study. It should be noted that only 1 SNP among the 8 selected SNPs yielded the significant association with SLE, although further enrichments of the significant associations might be anticipated. To elucidate effectiveness and limitation of our approach, further assessments of the studies on the remaining loci would be desirable. It should also be noted that the control-case ratio of the subjects were relatively high in the replication study 2 (=33.8), and this disproportionate ratio could have induced potential bias on the results of the association analysis of the SNPs. However, considering the homogeneous ancestries of the Japanese population [27] and that principal component analysis did not demonstrate significant population stratification in the control subjects of the replication study 2 (data not shown), the bias owing to population stratification might not be substantial.

In summary, through a GWAS and multi-staged replication studies in a Japanese population integrating eQTL study, our study identified *AFF1* as a novel susceptibility locus for SLE.

## Materials and Methods

### Subjects

We enrolled 2,278 systemic lupus erythematosus (SLE) cases and 31,948 controls. SLE cases enrolled in the genome-wide association study (GWAS) ($n=891$) or part of the 2nd replication study ($n=83$) were collected from 12 medical institutes in Japan under the support of the autoimmune disease study group of Research in Intractable Diseases, Japanese Ministry of Health, Labor and Welfare: Hokkaido University Graduate School of Medicine, Tohoku University Graduate School of Medicine, the University of Tokyo, Keio University School of Medicine, Juntendo University School of Medicine, University of Occupational and Environmental Health, University of Tsukuba, Tokyo Medical and Dental University, National Center for Global Health and Medicine, Nagasaki University, Wakayama Medical University, and Jichi Medical University. SLE cases ($n=562$) and controls ($n=653$) enrolled in the 1st replication study were collected from Kyushu University. Some of the SLE cases ($n=742$) and controls ($n=27,911$) enrolled in the 2nd replication study were collected from Kyoto University, Tokyo Women's

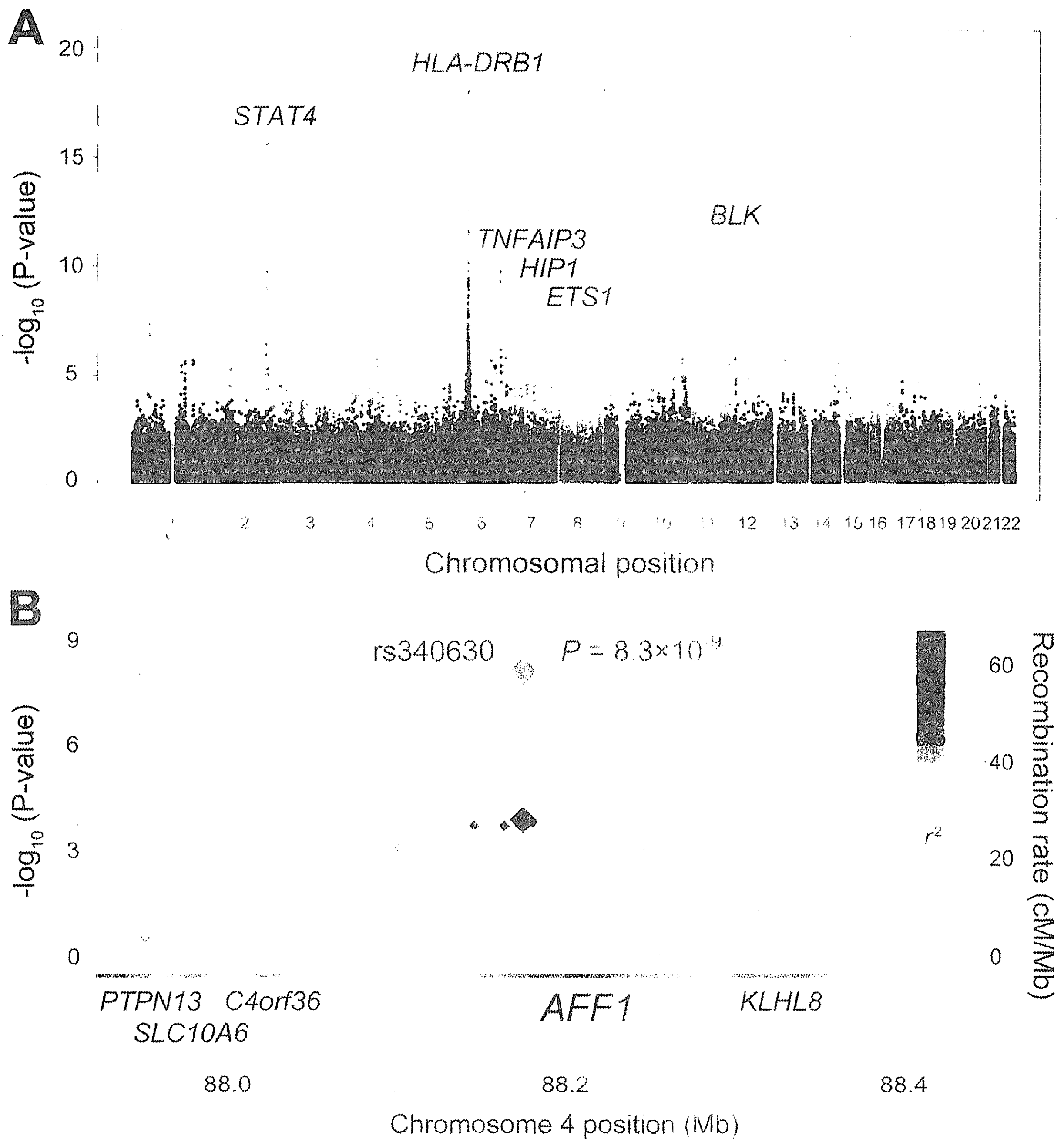


**Figure 2. Associations of the *AFF1* locus with SLE.** (A) A chromosomal plot of *P*-values in GWAS for SLE. (B) A regional plot in the *AFF1* locus. Diamond-shaped data points represent $-\log_{10}$ (*P*-values) of the SNPs. Large-sized points indicate the *P*-values of the landmark SNP, rs340630 (green for the combined study and red for the GWAS). Density of red color represents $r^2$ values with rs340630. Blue line represents recombination rates. Lower part indicates RefSeq genes. Gray dashed horizontal lines represent the threshold of $P = 5.0 \times 10^{-8}$. The plots were drawn using SNAP, version 2.1 [47].
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Medical University, the University of Tokyo, and the BioBank Japan Project [36]. All subjects were of Japanese origin and provided written informed consent. SLE cases met the revised American College of Rheumatology (ACR) criteria for SLE [37]. Control subjects were confirmed to be free of autoimmune disease. Some of the SLE cases were included in our previous studies [38–40]. Details of the subjects are summarized in Table S1 and S2. This research project was approved by the ethical committees of the University of Tokyo, RIKEN, and affiliated medical institutes.

**Table 2.** Associations among previously reported SLE-related loci.

| rsID | Chr | Position (bp) | Cytoband | Gene | Allele[a] | Allele 1 freq. | | OR (95%CI) | *P* | eQTL[b] | Identified by the studies in[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1/2 | Case | Control | | | | Caucasians | Asians |
| rs2205960 | 1 | 171,458,098 | 1q25 | *TNFSF4* | T/G | 0.23 | 0.18 | 1.35 (1.19–1.54) | $3.0\times10^{-6}$ | | + | |
| **rs3024505** | 1 | 205,006,527 | 1q32 | *IL10* | A/G | 0.019 | 0.014 | 1.34 (0.90–2.00) | 0.15 | | + | |
| rs13385731 | 2 | 33,555,394 | 2p22 | *RASGRP3* | C/T | 0.90 | 0.87 | 1.37 (1.15–1.64) | $6.0\times10^{-4}$ | + | | + |
| **rs10168266** | 2 | 191,644,049 | 2q32 | *STAT4* | T/C | 0.37 | 0.27 | 1.59 (1.42–1.78) | $2.7\times10^{-16}$ | | + | |
| rs6445975 | 3 | 58,345,217 | 3p14 | *PXK* | G/T | 0.25 | 0.23 | 1.09 (0.96–1.23) | 0.18 | + | + | |
| **rs10516487** | 4 | 102,970,099 | 4q24 | *BANK1* | G/A | 0.91 | 0.89 | 1.28 (1.07–1.53) | 0.0070 | | + | |
| rs10036748 | 5 | 150,438,339 | 5q33 | *TNIP1* | T/C | 0.75 | 0.72 | 1.16 (1.03–1.31) | 0.014 | | | + |
| **rs9501626** | 6 | 32,508,322 | 6p21 | *HLA-DRB1* | A/C | 0.20 | 0.12 | 1.86 (1.62–2.13) | $1.0\times10^{-18}$ | | + | |
| rs548234 | 6 | 106,674,727 | 6q21 | *PRDM1* | C/T | 0.40 | 0.34 | 1.30 (1.16–1.44) | $2.3\times10^{-6}$ | + | + | |
| **rs2230926** | 6 | 138,237,759 | 6q23 | *TNFAIP3* | G/T | 0.11 | 0.069 | 1.75 (1.47–2.08) | $1.9\times10^{-10}$ | + | + | |
| rs849142 | 7 | 28,152,416 | 7p15 | *JAZF1* | C/T | 0.999 | 0.999 | 2.72 (0.25–29.8) | 0.41 | | + | |
| **rs4917014** | 7 | 50,276,409 | 7p12 | *IKZF1* | T/G | 0.58 | 0.53 | 1.24 (1.11–1.38) | $8.1\times10^{-5}$ | | | + |
| rs6964720 | 7 | 75,018,280 | 7q11 | *HIP1* | G/A | 0.25 | 0.19 | 1.43 (1.27–1.62) | $1.3\times10^{-8}$ | | | + |
| **rs4728142** | 7 | 128,361,203 | 7q32 | *IRF5* | A/G | 0.16 | 0.11 | 1.48 (1.28–1.72) | $2.4\times10^{-7}$ | + | + | |
| rs2254546 | 8 | 11,381,089 | 8p23 | *BLK* | G/A | 0.78 | 0.72 | 1.42 (1.25–1.61) | $4.1\times10^{-8}$ | + | + | |
| **rs1913517** | 10 | 49,789,060 | 10q11 | *WDFY4* | A/G | 0.32 | 0.28 | 1.20 (1.07–1.35) | 0.0013 | | | + |
| rs4963128 | 11 | 579,564 | 11p15 | *KIAA1542* | T/C | 0.98 | 0.97 | 1.58 (1.03–2.44) | 0.038 | + | + | |
| **rs2732552** | 11 | 35,041,168 | 11p13 | *PDHX, CD44* | T/C | 0.75 | 0.73 | 1.13 (1.00–1.27) | 0.056 | | + | |
| rs4639966 | 11 | 118,078,729 | 11q23 | Intergenic | T/C | 0.32 | 0.28 | 1.22 (1.09–1.36) | $7.3\times10^{-4}$ | | | + |
| **rs6590330** | 11 | 127,816,269 | 11q24 | *ETS1* | A/G | 0.48 | 0.39 | 1.44 (1.30–1.60) | $1.3\times10^{-11}$ | | | + |
| rs1385374 | 12 | 127,866,647 | 12q24 | *SLC15A4* | T/C | 0.19 | 0.16 | 1.21 (1.06–1.38) | 0.0057 | | | + |
| **rs7329174** | 13 | 40,456,110 | 13q14 | *ELF1* | G/A | 0.30 | 0.25 | 1.32 (1.18–1.49) | $2.2\times10^{-6}$ | | | + |
| rs7197475 | 16 | 30,550,368 | 16p11 | Intergenic | T/C | 0.12 | 0.10 | 1.20 (1.02–0.41) | 0.031 | | | + |
| **rs11150610** | 16 | 31,241,737 | 16p11 | *ITGAM* | C/A | 0.20 | 0.19 | 1.07 (0.94–1.22) | 0.32 | + | + | |
| rs12949531 | 17 | 13,674,531 | 17p12 | Intergenic | T/C | 0.28 | 0.27 | 1.02 (0.91–1.15) | 0.73 | | + | |
| **rs463426** | 22 | 20,139,185 | 22q11 | *HIC2,UBE2L3* | T/C | 0.52 | 0.48 | 1.20 (1.08–1.33) | $6.1\times10^{-4}$ | | + | |

[a]Based on forward strand of NCBI Build 36.3.
[b]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
[c]Based on the previously reported studies for SLE susceptibility loci [3–18].
SLE, systemic lupus erythematosus; OR, odds ratio; eQTL, expression quantitative trait locus; GWAS, genome-wide association study.
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## Genotyping and quality control

In GWAS, 946 SLE cases and 3,477 controls were genotyped using Illumina HumanHap610-Quad and Illumina Human-Hap550v3 Genotyping BeadChips (Illumina, CA, USA), respectively. After the exclusion of 47 SLE cases and 92 controls with call rates <0.98, SNPs with call rates <0.99 in SLE cases or controls,

**Table 3.** Results of combined study for Japanese patients with SLE.

| rsID | Chr | Position (bp) | Cytoband | Gene | Allele | Stage | No. subjects | | Allele 1 freq. | | OR (95%CI) | *P* | eQTL[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1/2 | | Case | Control | Case | Control | | | |
| rs340630 | 4 | 88,177,419 | 4q21 | *AFF1* | A/G | GWAS | 891 | 3,383 | 0.56 | 0.51 | 1.22 (1.10–1.36) | $1.5\times10^{-4}$ | + |
| | | | | | | Replication study 1 | 550 | 646 | 0.57 | 0.49 | 1.40 (1.19–1.64) | $4.6\times10^{-5}$ | |
| | | | | | | Replication study 2 | 820 | 27,911 | 0.56 | 0.53 | 1.14 (1.03–1.26) | 0.0094 | |
| | | | | | | Combined study | 2,261 | 31,940 | 0.56 | 0.52 | 1.21 (1.14–1.30) | $8.3\times10^{-9}$ | |

[a]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
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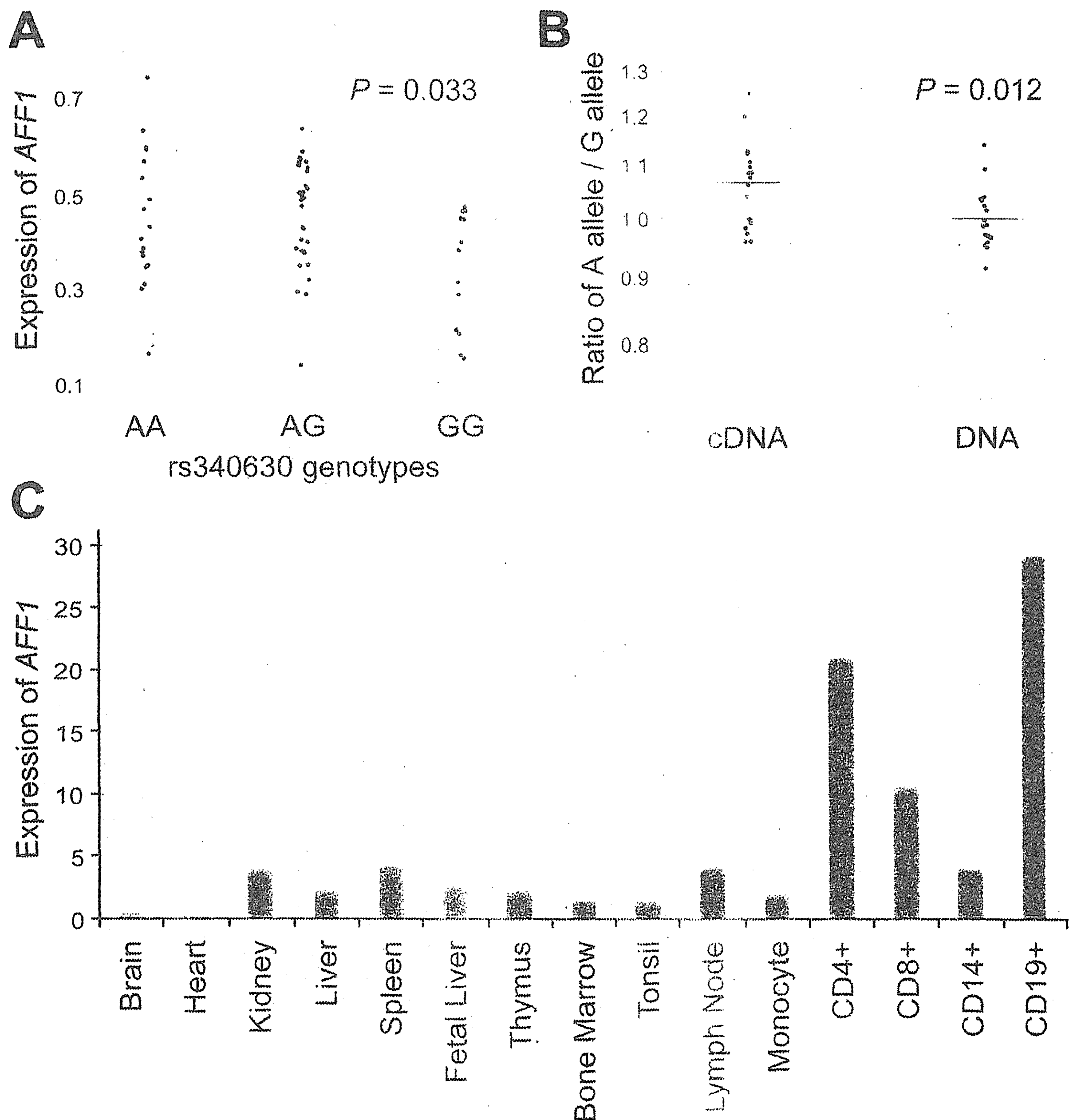


**Figure 3. Association of rs340630 with *AFF1* expression.** (A) Correlation between rs340630 genotypes and transcript levels of *AFF1* (NM_001166693) in EBV-transfected cell lines ($n = 62$) stimulated with PMA. (B) Allele-specific quantification (ASTQ) of *AFF1* transcripts. Allele specific-probes for rs340638 were used for quantification by qPCR. The ratios of A allele over G allele for the amounts of both cDNAs and DNAs were plotted in log scale for each cell line. (C) *AFF1* expression in various tissues. Transcripts levels of *AFF1* were quantified by qPCR and were normalized by *GAPDH* levels.
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non-autosomal SNPs, and SNPs not shared between SLE cases and controls, were excluded. We excluded 7 closely related SLE cases in a 1st or 2nd degree of kinship based on identity-by-descent estimated using PLINK version 1.06 [41]. We then excluded 1 SLE cases and 1 controls whose ancestries were estimated to be distinct from East-Asian populations using PCA performed along with the genotype data of Phase II HapMap populations (release 24) [29] using EIGENSTRAT version 2.0 [42]. Subsequently, SNPs with minor allele frequencies <0.01 in SLE cases or controls, SNPs with exact $P$-values of Hardy-Weinberg equilibrium test $<1.0\times10^{-7}$ in controls, or SNPs with ambiguous cluster plots were excluded. Finally, 430,797 SNPs for 891 SLE cases and 3,384 controls were obtained. Genotyping of SNPs in replication studies was performed using TaqMan Assay or Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, CA, USA).

## Association analysis of the SNPs

Association of SNPs in GWAS and replication studies were tested with Cochran-Armitage's trend test. Combined analysis was performed with Mantel-Haenzel method. Associations of previously reported SLE susceptibility loci [3–18] were evaluated using the results of the GWAS. Genotype imputation was performed for non-genotyped SNPs using MACH version 1.0 [43] with Phase II HapMap East-Asian individuals as references [29], as previously described [44]. All imputed SNPs demonstrated imputation scores, *Rsq*, >0.70.

## eQTL study

We analyzed gene expression data previously measured in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals using Illumina's human whole-genome expression array (WG-6 version 1) (accession number; GSE6536) [28]. Expression data were normalized across the individuals. We used BLAST to map 47,294 Illumina array probes onto human autosomal reference genome sequences (Build 36). We discarded probes mapped with expectation values smaller than 0.01 to multiple loci, or for which there was polymorphic HapMap SNP(s) inside the probe. Then, 19,047 probes with exact matches to a unique locus with 100% identity and with a mean signal intensity greater than background were obtained. Genotype data of HapMap individuals were obtained for SNPs included in the GWAS. Associations of SNP genotypes (coded as 0, 1, and 2) with expression levels of each of the cis-eQTL probes (located within ±300 kbp regions of the SNPs) were evaluated using linear regression assuming additive effects of the genotypes on the expression levels. Considering the significant overlap between eQTL and genetic loci responsible for autoimmune diseases [24], we applied relatively less stringent multiple testing threshold of FDR *Q*-values<0.2 for the definition of eQTL. SNPs that exhibited this threshold with any of the corresponding cis-eQTL probes were denoted as eQTL positive.

## Selection of SNPs enrolled in the replication studies

In order to select SNPs for further replication studies, we firstly integrated the results of GWAS and eQTL study. SNPs that satisfied $P<1.0\times10^{-4}$ in GWAS, or the SNPs that satisfied $1.0\times10^{-4}\leq P<1.0\times10^{-3}$ in GWAS and denoted as eQTL positive, were selected. Among these, SNPs most significantly associated in each of the genomic loci and not included in the previously reported SLE susceptibility loci [3–18] were further evaluated.

Then, the results of the concurrently proceeding genome-wide scan for SLE in the Japanese subjects using a pooled DNA approach were referred (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, DNA collected from 447 SLE cases and 680 controls of Japanese origin were pooled respectively, and genotyped using GeneChip Human Mapping 500K Array Set (Affymetrix, CA, USA). SNPs were ranked according to the Silhouette scores estimated based on relative allele scores (RAS) between SLE cases and controls, and rank-based *P*-values were assigned [30]. By referring to association signals in multiple neighboring SNPs in the pooled analysis, we selected SNPs for replication study 1. Namely, if the SNP of interest was in LD ($r^2>0.5$) or was located within ±100 kbp of SNPs showing association signals in the pooled analysis (rank-based $P<0.01$), it would be selected. SNPs that satisfied $P<1.0\times10^{-4}$ in the combined study of GWAS and replication study 1 were further evaluated in replication study 2 (Figure 1).

## Quantification of *AFF1* expression

EBV-transformed lymphoblastoid cell lines ($n=62$) were established by Pharma SNP Consortium (Tokyo, Japan) using peripheral blood lymphocytes of Japanese healthy individuals. Cells were incubated for 2 h in medium alone (RPMI 1640 medium containing 10% FBS, 1% penicillin, and 1% streptomycin) or with 100 ng/ml PMA. Conditions for cell stimulation were optimized before the experiment as previously described [45]. Cells were then harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen) with DNase treatment. Total RNA (1 µg) was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems). Real-time quantitative PCR was performed in triplicate using an ABI PRISM 7900 and TaqMan gene expression assays (Applied Biosystems). Specific probes (Hs01089428_m1) for transcript of *AFF1* (NM_001166693) were used. Expression of *AFF1* in various tissues was also quantified using Premium Total RNA (Clontech). The data were normalized to *GAPDH* levels. *GUS* levels were also evaluated for internal control, and similar results were obtained. Correlation coefficient, $R^2$, between rs340630 genotypes and transcript levels of *AFF1* was evaluated.

## Allele-specific transcript quantification (ASTQ)

ASTQ of *AFF1* in PSC cells was performed as previously described [46]. DNAs were extracted by using a DNeasy Kit (QIAGEN). RNA extraction and cDNA preparation were performed as described above. For PSC cells ($n=17$) that were heterozygous for both rs340630 (the landmark SNP of GWAS) and rs340638 (located in the 5′-untranslated region of *AFF1* and in absolute LD with rs340630), expression levels of *AFF1* were quantified by qPCR on an ABI Prism 7900 using a custom-made TaqMan MGB-probe set for rs340638. Primer sequences were 5′-CTAACTGTGGCCCGCGTTG-3′ and 5′-CCCGGCGCA-GTTTCTGAG-3′. The probe sequences were 5′-VIC-CGAA-GACCGCCAGCGCCCAAC-TAMRA-3′ and 5′-FAM-CGAA-GACCGCCGGCGCCCAA-TAMRA-3′. Ct values of VIC and FAM were obtained for genomic DNA and cDNA samples after 40 cycles of real-time PCR. We also prepared genomic DNA of samples homozygous for each allele and mixed them at different ratios (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2) to create a standard curve by plotting Ct values of VIC/FAM against the allelic ratio of VIC/FAM for each mixture. Using the standard curve, we calculated the allelic ratios for each genomic DNA and cDNA samples. We measured each sample in quadruplicate in one assay; tests were independently repeated twice.

## Web resources

The URLs for data presented herein are as follows.

NCBI GEO, http://www.ncbi.nlm.nih.gov/geo

BioBank Japan Project, http://biobankjp.org

PLINK software, http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml

International HapMap Project, http://www.hapmap.org

EIGENSTRAT software, http://genepath.med.harvard.edu/~reich/Software.htm

MACH and mach2qtl software, http://www.sph.umich.edu/csg/abecasis/MACH/index.html

SNAP, http://www.broadinstitute.org/mpg/snap/index.php

## Supporting Information

**Figure S1** Principal component analysis (PCA) plot of the subjects. PCA plot of subjects enrolled in the GWAS for SLE. SLE cases and the controls enrolled in the GWAS are plotted based on

eigenvectors 1 and 2 obtained from the PCA using EIGENSTRAT version 2.0 [42], along with European (CEU), African (YRI), Japanese (JPT), and Chinese (CHB) individuals obtained from the Phase II HapMap database (release 22) [29]. Subjects who were estimated to be outliers in terms of ancestry from East-Asian (JPT+CHB) clusters and excluded from the study are indicated by black arrows.
(TIF)

**Figure S2** Quantile-Quantile plot (QQ-plot) of *P*-values in the GWAS for SLE. The horizontal axis indicates the expected $-\log_{10}$ (*P*-values). The vertical axis indicates the observed $-\log_{10}$ (*P*-values). The QQ-plot for the *P*-values of all SNPs that passed the quality control criteria is indicated in blue. The QQ-plot for the *P*-values after the removal of SNPs included in the previously reported SLE susceptibility loci is indicated in black. The gray line represents $y = x$. The SNPs for which the *P*-value was smaller than $1.0 \times 10^{-15}$ are indicated at the upper limit of the plot.
(TIF)

**Table S1** Basal characteristics of cohorts.
(DOC)

**Table S2** Frequency of clinical characteristics of SLE in this GWAS.
(DOC)

**Table S3** Distributions of eQTL positivity rates of the SNPs.
(DOC)

**Table S4** Results of replication study 1 for Japanese patients with SLE.
(DOC)

**Table S5** Results of replication studies 1 and 2 for Japanese patients with SLE.
(DOC)

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