

A risk haplotype of *STAT4* for systemic lupus erythematosus is over-expressed, correlates with anti-dsDNA and shows additive effects with two risk alleles of *IRF5*

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Systemic lupus erythematosus (SLE) is the prototype autoimmune disease where genes regulated by type I interferon (IFN) are over-expressed and contribute to the disease pathogenesis. Because signal transducer and activator of transcription 4 (*STAT4*) plays a key role in the type I IFN receptor signaling, we performed a candidate gene study of a comprehensive set of single nucleotide polymorphism (SNPs) in *STAT4* in Swedish patients with SLE. We found that 10 out of 53 analyzed SNPs in *STAT4* were associated with SLE, with the strongest signal of association ($P = 7.1 \times 10^{-8}$) for two perfectly linked SNPs rs10181656 and rs7582694. The risk alleles of these 10 SNPs form a common risk haplotype for SLE ($P = 1.7 \times 10^{-5}$). According to conditional logistic regression analysis the SNP rs10181656 or rs7582694 accounts for all of the observed association signal. By quantitative analysis of the allelic expression of *STAT4* we found that the risk allele of *STAT4* was over-expressed in primary human cells of mesenchymal origin, but not in B-cells, and that the risk allele of *STAT4* was over-expressed ($P = 8.4 \times 10^{-5}$) in cells carrying the risk haplotype for SLE compared with cells with a non-risk haplotype. The risk allele of the SNP rs7582694 in *STAT4* correlated to production of anti-dsDNA (double-stranded DNA) antibodies and displayed a multiplicatively increased, 1.82-fold risk of SLE with two independent risk alleles of the *IRF5* (interferon regulatory factor 5) gene.

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INTRODUCTION

Systemic lupus erythematosus (SLE, OMIM # 15200) is an autoimmune disease characterized by production of antinuclear autoantibodies, immune complex formation and inflammation in multiple organs. Patients with SLE have raised serum levels of IFN(α), which correlates to both disease activity and severity (1), and with an increased expression level of type I IFN-regulated genes peripheral blood cells (an IFN signature) (2–4). The reason for this IFN signature may be an ongoing production of type I IFN, which is caused by RNA- or DNA-containing immune complexes that trigger plasmacytoid dendritic cells (DCs) to synthesize type I IFNs via the Toll-like receptor (TLR) 7 or TLR9 (5). Because the type I IFNs cause activation and maturation of DC, stimulation of Th1 and Tc cells as well as enhanced immunoglobulin production by B-cells, this mechanism of a continuous activation of the type I IFN system could be directly involved in the etiopathogenesis of SLE (5,6).

We have previously observed that the interferon regulatory factor 5 (*IRF5*) and tyrosine kinase 2 (*TYK2*) genes, which are involved in both the production and the effects of the type I IFNs are associated with susceptibility to develop SLE (7). These observations have been replicated in multiple subsequent studies (8,9). The transcription factor *IRF5* regulates the expression of type I IFN genes, while the Janus kinase *TYK2* binds to the type I IFN receptor (IFNAR) and is required for signaling through this receptor. In addition, *IRF5* is involved in cell adhesion, apoptosis, cell cycle regulation and early immune activation (10), while *TYK2* interacts with the cellular receptors of IL(interleukin)-6, IL-10 and IL-12/23 cytokine families, in addition to IFNAR (11). Thus, both *IRF5* and *TYK2* have diverse functions in the immune system that can affect several pathways in autoimmune reactions.

In order to identify other molecules within the type I IFN system with a possible association with SLE, we studied the signal transducer and activator of transcription 4 (*STAT4*) transcription factor, which plays a key role in the IFNAR signaling by being activated and translocated to the nucleus after IFNAR ligation (12). In addition, *STAT4* transmits signals from the receptors of IL-12 and IL-23, and can therefore contribute to autoimmune responses by affecting the functions of several innate and adaptive immune cells (13). By analyzing 53 single nucleotide polymorphisms (SNPs) in *STAT4* for their association with SLE in Swedish patients and Control individuals we identified a common risk haplotype type for SLE formed by 10 SNPs in introns of *STAT4*. During the course of our study, an association of the SNP rs7574865 in *STAT4*, which tags this risk haplotype was found to be associated with SLE in three North American SLE-cohorts of European ancestry (14). Furthermore, two recently published genome-wide association studies of SLE confirmed this association (15,16). In our study we show that the risk haplotype identified in *STAT4* is over-expressed in primary human cells of mesenchymal origin, but not in transformed B-cells, and that the risk haplotype of *STAT4* is over-expressed in mesenchymal cells (osteoblasts) carrying the risk haplotype, compared with cells with the non-risks haplotype. We also show that the risk allele of *STAT4* correlates with increased frequency of immunological manifestations in the SLE

patients, including production of anti-dsDNA (double-stranded DNA) autoantibodies. Because *IRF5* mediates type I IFN production and *STAT4* affects the response to type I IFN, we also determined the joint effects of the risk alleles of *STAT4* and two recently identified independent risk alleles of *IRF5* with strong effects on SLE (17). This analysis revealed a strong multiplicatively increased risk for SLE by these three polymorphisms located in *STAT4* and *IRF5*, which are two of the most strongly associated SLE-genes and belong to the same cell signaling pathway.

RESULTS

Identification of risk alleles and one major risk haplotype of *STAT4* in SLE

The *STAT4* gene located on chromosome 2q32.3 consists of 24 exons spanning a 120 kb region (Fig. 1). We genotyped 53 SNPs, distributed across *STAT4*, in DNA samples from 485 Swedish patients with SLE and 563 Control individuals and observed 10 SNPs that yielded uncorrected *P*-values < 0.001 , with *P*-values for the association signals calculated by Fischer's exact test ranging from 4.5×10^{-4} to 7.1×10^{-8} (Fig. 1, Supplementary Material, Table S2). The strongest signal of association [odds ratio (OR) = 1.71 with 95% confidence interval (CI) 1.40–2.08] was observed for two SNPs located in intron 3 of *STAT4*, namely the SNPs rs10181656 and rs7582694, which are in perfect linkage disequilibrium (LD) ($r^2 = 1$) with each other in our genotype data. The 10 SNPs that display association signals with SLE are located in introns 3–24 of *STAT4*, and are all correlated with the most strongly associated SNPs rs10181656 and rs7582694 ($r^2 > 0.41$) (Fig. 1 and Supplementary Material, Table S3). We constructed haplotypes using the genotype data from these 10 SNPs and tested the association of the formed haplotypes with SLE (Table 1). This analysis defines a major risk haplotype for SLE that occurs with a frequency of 0.24 in the SLE-patients, compared with 0.17 in the Controls and is strongly associated with SLE [unadjusted *P*-value = 1.7×10^{-5} and OR 1.59 (95% CI 1.28–1.98)]. By conditional logistic regression analysis we found that the SNPs rs7582694 (or rs10181656) that displayed the strongest signals of association with SLE accounts for the association signals observed from all other SNPs in *STAT4* (Table 2). According to the haplotype mapping (HapMap) phase II data there are five additional SNPs in *STAT4* that are in strong LD ($r^2 > 0.9$) with the SNPs rs10181656 and rs7582694. These are: rs11889341 ($r^2 = 1.0$), rs7574865 ($r^2 = 1.0$), rs7568275 ($r^2 = 1.0$), rs8179673 ($r^2 = 0.95$), rs10174238 ($r^2 = 0.90$). One of them, rs7574865, was recently shown to be associated with SLE with high significance ($P = 1.87 \times 10^{-9}$, OR = 1.55) in SLE patients from the US with European ancestry (14), and two consequent genome-wide association studies confirm this finding (15,16).

Allelic and total expression levels of *STAT4*

Because there are no known common coding SNPs in *STAT4* that are in LD with the SLE-associated *STAT4* SNPs, we investigated if a possible functional role for these intronic SNPs that accounted for the association signal with SLE

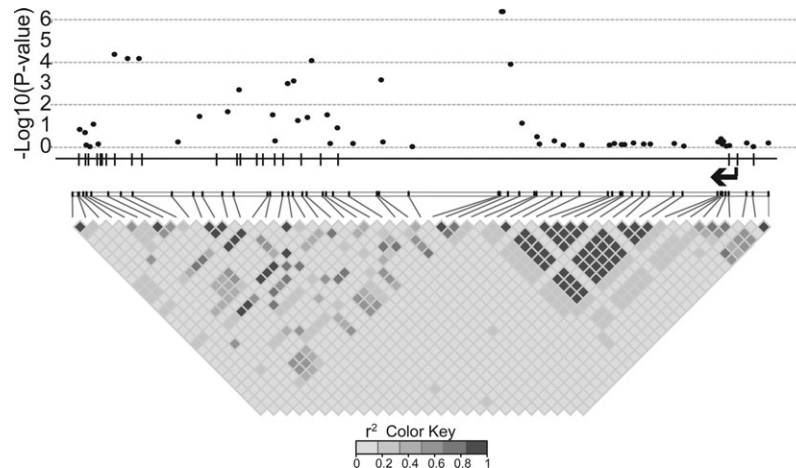


Figure 1. Schematic view of the genetic variation of *STAT4*. The positions of the exons are marked with vertical lines and the translation start site is indicated by an arrow. The negative \log_{10} of the *P*-value for the association of each SNP with SLE determined by Fisher's exact test is shown by a dot in the diagram. The pair-wise LD r^2 -values between the SNPs in *STAT4* according to the combined data from the Swedish SLE patients and Controls are shown in the lower part of the figure. The exact LD values (D' and r^2) are provided in Supplementary Material, Table S3.

Table 1. Haplotype analysis of 10 SNPs in *STAT4* associated with SLE

	Haplotypes ^a	Haplotype frequencies ^b		<i>P</i> -values ^c	OR (95% CI) ^d
		Cases	Controls		
H1	GGCCGCTCGC	0.50	0.59	5.8E-05	0.70 (0.59-0.84)
H2	AATAATCGCG	0.24	0.17	1.7E-05	1.59 (1.28-1.98)
H3	GGTAACCCGC	0.06	0.07	0.59	0.91 (0.64-1.29)
H4	GGCAACCCGG	0.04	0.06	0.028	0.62 (0.40-0.95)
H5	GGCAACCGCG	0.05	0.04	0.058	1.51 (0.98-2.31)
H6	GGCCGCCCGG	0.02	0.02	0.90	1.04 (0.55-1.95)
H7	GGTAACCGCG	0.02	0.01	0.29	1.44 (0.73-2.85)

^aThe 10 SNPs included in the analysis are in order from left to right: rs3024886, rs3821236, rs3024877, rs1517352, rs13017460, rs10168266, rs16833249, rs10181656, rs7582694 and rs16833260.

^bHaplotype frequencies estimated using Haploview v. 4.0.

^cUnadjusted *P*-values by χ^2 test using Haploview v 4.0.

^dOdds ratio (OR) and 95% confidence interval (CI) calculated using the formula $(a \times d)/(b \times c)$ where *a* and *b* are the haplotype counts in Cases and *c* and *d* are the haplotype counts in Controls.

could be to alter the expression of the *STAT4* gene. *STAT4* has two well-characterized isoforms, *STAT4* α and β , which could be relevant for SLE, because they have been shown to have distinct functions in mediating the response to IL-12 in Th1-cells from mice (18). In the *STAT4* β isoform, expression of an additional exon introduces a stop codon in the *STAT4* mRNA, which results in a truncated protein lacking a putative transcriptional activation domain in its C-terminus. We tested whether the disease-associated *STAT4* SNPs would alter expression of the alternatively spliced *STAT4* β isoform by utilizing expression data from Affymetrix Exon 1.0 arrays in two population-based panels of cells of both lymphoid (lymphoblast) and mesenchymal (osteoblast) origin (19). We did not find evidence for association with the expression level of the *STAT4* β isoform for any of the SLE-associated SNPs (data not shown).

Next we determined the allelic expression levels of *STAT4* (20) in pooled primary human osteoblast and pooled human

lymphoblast cell lines. The SNP rs8179673, which is in almost perfect pair-wise LD ($r^2 = 0.95$) with our two strongest SLE-associated SNPs rs7582694 and rs10181656 was used as indicator of allelic imbalance in pre-mRNA extracted from the cells. This analysis showed that the SLE-risk allele of the SNP rs8179673 was significantly over-expressed in the pooled osteoblasts ($P = 0.038$), but not in the lymphoblasts (Fig. 2A and B). Consequently, we determined the allelic expression levels of the SLE-risk haplotype of *STAT4* in 10 informative individual osteoblasts for which the SLE-risk haplotype could be unequivocally assigned and which were heterozygous for at least two of the SNPs rs1517352, rs3821236 and rs7574865 used as indicators of allelic imbalance. The SNP rs1517352 and rs3821236 were genotyped in our SLE samples (Table 2, Supplementary Material, Table S3), and the SNP rs7574865 is in perfect LD ($r^2 = 1.0$) with our two strongest SLE-associated SNPs. We observed significant over-expression of the SLE-risk haplotype in each of the osteoblasts carrying the SLE-risk haplotype, compared with its expression in osteoblasts with a non-risk haplotype ($P = 8.4 \times 10^{-5}$) (Fig. 2C). We also determined the total expression level of *STAT4* in 40 individual osteoblasts with known *STAT4* haplotype status using data from Illumina Human Ref-8 Bead Arrays and found that *STAT4* mRNA was over-expressed in osteoblasts carrying the risk haplotype for SLE ($P = 0.0085$) compared with osteoblasts with non-risk haplotypes (Fig. 2D).

Correlation between risk allele of *STAT4* and disease phenotype

Information concerning disease manifestations and genotype data for the *STAT4* SNP rs7582694 were available for 475 SLE patients included in the association analysis described earlier, and from an additional cohort of 220 Swedish SLE patients. We correlated the presence of at least one risk allele with age at diagnosis of SLE, individual American College of Rheumatology (ACR) criteria, total number of ACR criteria and the presence of antibodies against dsDNA. We found that patients with at least one risk allele of *STAT4*

Table 2. Conditional logistic regression analysis of SNPs in the *STAT4* gene in SLE

SNP ID	Unadjusted <i>P</i> -values Single marker ^a	Conditional ^b on rs7582694
rs3024908	0.16	0.22
rs3024904	0.17	0.24
rs3024936	0.81	0.91
rs3024935	0.83	0.92
rs3024896	0.04	0.94
rs925847	0.53	0.23
rs3024886	3.0E-05	0.83
rs3821236	4.8E-05	0.93
rs3024877	5.9E-05	0.46
rs12999858	0.76	0.51
rs1400654	0.04	0.14
rs3771327	0.02	0.09
rs3024866	1.7E-03	0.65
rs3024851	0.03	0.12
rs932169	0.53	0.77
rs1517352	4.4E-04	0.63
rs13017460	5.1E-04	0.68
rs13389408	0.04	0.44
rs1400656	0.04	0.15
rs10168266	5.1E-05	0.80
rs7594501	0.04	0.21
rs2459611	0.81	0.58
rs7601754	0.11	0.79
rs3024921	0.69	0.72
rs16833249	2.8E-04	0.80
rs12998748	0.48	0.76
rs6434435	0.85	0.26
rs10181656	8.8E-08	NA
rs7582694	8.8E-08	NA
rs16833260	3.5E-05	0.55
rs6752770	0.05	0.56
rs7599504	0.27	0.60
rs4853543	0.61	0.37
rs4073699	0.44	0.46
rs17769077	0.85	0.92
rs4341967	0.73	0.28
rs10931483	0.70	0.29
rs4583497	0.58	0.38
rs4553370	0.69	0.32
rs10804037	0.69	0.31
rs17769459	0.65	0.91
rs6712821	0.63	0.36
rs1551443	0.62	0.37
rs7566274	0.61	0.39
rs2356350	0.98	0.23
rs10189819	0.57	0.62
rs7596818	0.44	0.81
rs11685878	0.58	0.99
rs4853546	0.43	0.79
rs1031509	0.75	0.93
rs10497711	0.74	0.73
rs7572482	0.78	0.77
rs897200	0.93	0.95

^aUnadjusted *P*-value for the association with SLE based on additive logistic regression.

^b*P*-value for the association using additive logistic regression conditional on the indicated marker. NA indicates that the marker cannot be distinguished from the conditional model.

had a higher frequency of immunological disorder (unadjusted *P*-value = 1.6×10^{-5}) and presence of antibodies against dsDNA (unadjusted *P*-value = 5.3×10^{-7}) than patients with no risk alleles (Table 3).

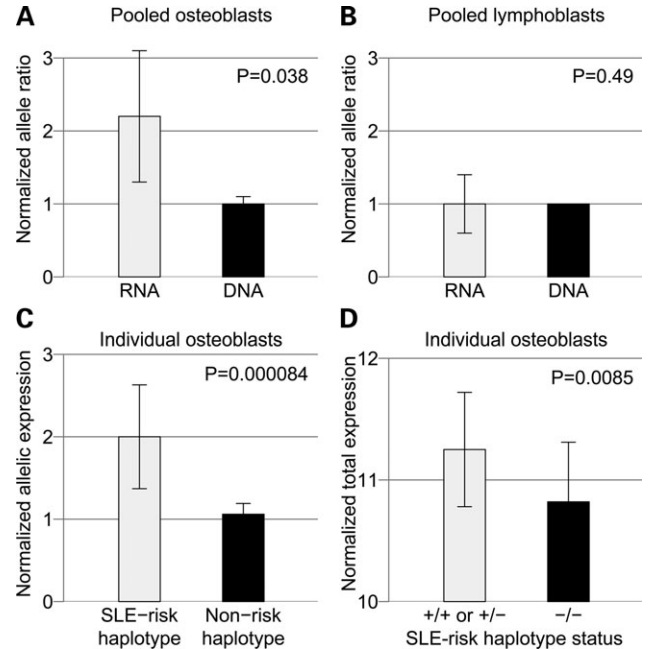


Figure 2. Expression analysis of *STAT4*. Allelic expression analysis of *STAT4* in (A) pooled primary human osteoblasts ($n = 55$) derived from unrelated Swedish individuals and (B) pooled human lymphoblastoid cell lines ($n = 55$) derived from unrelated individuals of European descent (the HapMap CEU individuals). The mean values of ratios (with SD in error bars) between the alleles (C/T) of the indicator SNP rs 8179673 in triplicate RNA samples compared with normalized heterozygote ratios in triplicate DNA samples from the corresponding cells are shown. (C) Allelic expression analysis of the SLE-risk haplotype of *STAT4* in informative individual primary osteoblasts ($n = 10$) for which the *STAT4* SLE-risk haplotype status is known and that are heterozygous for at least two of the indicator SNPs rs1517352 (A/C), rs 3821236 (A/G) and rs7574865 (G/T). The normalized mean (with SD in error bar) allelic expression levels of the SLE-risk haplotype of *STAT4* in each cell sample are shown for osteoblasts with the SLE-risk and non-risk haplotype, respectively. (D) Total expression analysis of individual primary osteoblasts with known *STAT4* haplotype status using Illumina Human Ref-8 Bead Arrays. Mean normalized total expression levels (\log_2 value on y-axis and SD of the mean in error bars) for homozygous (+/+), heterozygous (+/-, $n = 16$) carriers of the SLE-risk haplotype of *STAT4* (total $n = 17$) and non-carriers (-/-) of the risk haplotype ($n = 23$) are shown.

Additive effects of the risk alleles of *STAT4* and *IRF5* in SLE

We have recently analyzed a comprehensive set of polymorphisms in *IRF5* located on chromosome 7q32 for their association with SLE in the same SLE patients and Controls that were here analyzed for SNPs in *STAT4* (17). We demonstrated that two independently associated polymorphisms in *IRF5* account for the association signals with SLE, namely a previously unknown 5 bp (CGGGG) indel (insertion–deletion) in the promoter region of *IRF5* (OR = 1.69, 95% CI 1.42–2.02) and the SNP rs10488631 or one of multiple perfectly correlated SNPs located 3' of *IRF5* (OR = 2.07, 95% CI 1.63–2.62). In the present study we investigated the joint effect on SLE of the risk allele of the SNP rs7582694 in *STAT4* and the two polymorphisms in *IRF5*. Figure 3A shows the distribution of risk alleles for SLE in *STAT4* and *IRF5* in patients and Controls. The overall significance for the difference in risk allele counts between patients and Controls

Table 3. Correlation between the *STAT4*-risk allele of the SNP rs7582694 and disease phenotype

Clinical criterion	Frequency for all patients (n = 695)	Frequency for patients with 0 risk allele (n = 300)	Frequency for patients with 1 or 2 risk alleles (n = 395)	P-value ^a
Malar rash (ACR1) ^b	0.59	0.60	0.59	0.70
Discoid rash (ACR2)	0.25	0.28	0.24	0.19
Photosensitivity (ACR3)	0.69	0.71	0.67	0.36
Oronasal ulcers (ACR4)	0.25	0.23	0.25	0.59
Arthritis (ACR5)	0.77	0.79	0.76	0.32
Serositis (ACR6)	0.45	0.42	0.47	0.17
Nephritis (ACR7)	0.32	0.27	0.35	0.040
CNS (ACR8)	0.12	0.13	0.11	0.72
Hematological (ACR9)	0.60	0.56	0.63	0.073
Immunological (ACR10) ^c	0.66	0.57	0.73	1.6×10^{-5}
ANA (ACR11)	0.98	0.98	0.98	0.79
Anti-dsDNA Ab ever ^c	0.54	0.43	0.62	5.3×10^{-7}
Number of ACR criteria ^d	5.6 ± 1.4	5.5 ± 1.3	5.8 ± 1.4	0.022
Age at diagnosis ^d	35.5 ± 14.9	36.7 ± 15.4	34.5 ± 14.5	0.11

^aFrequency of patients with 0 risk alleles compared with patients with 1 or 2 risk alleles fulfilling each clinical criterion, unadjusted *P*-value calculated by Fisher's exact test.

^bAmerican College of Rheumatology (ACR) classification criteria (39).

^cAntibodies to double-stranded DNA (dsDNA) is part of the immunological criterion.

^dMean value with 1 SD.

^eUnadjusted *P*-value calculated with the Mann-Whitney *U* test.

in all groups is high, with a one-sided *P*-value of 1.7×10^{-16} , using an additive logistic regression model. No evidence for dominance or interactions was observed between the three risk alleles using multiple logistic regression analysis. Figure 3B shows the OR for SLE in the group of patients with two or more risk alleles, using the individuals with zero or one risk allele as reference. As can be seen, the risk of SLE increases as a function of the number of risk alleles in a multiplicative manner with a 1.82-fold increase in OR for each additional risk allele. The OR for SLE is 2.08 (CI 1.53–2.83) for carriers of two risk alleles and 13.0 (3.78–44.3) for carriers of five or six risk alleles when compared with those with zero or one risk allele. No convincing correlation was found when the risk alleles in *STAT4* and *IRF5* were combined and correlated to disease phenotype in the patients with SLE (data not shown).

DISCUSSION

In the present investigation we analyzed a panel of SNPs that capture the majority of the common variation of *STAT4* for their association with SLE, and identified 10 SNPs with

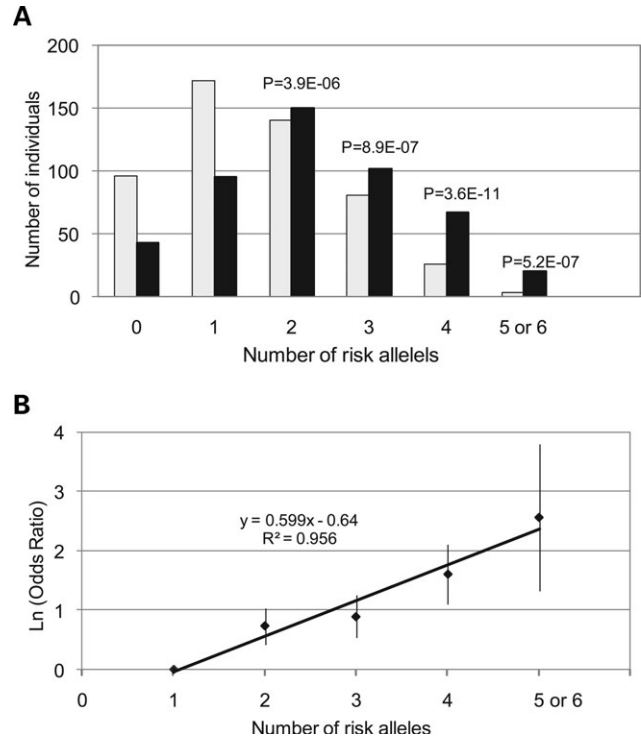


Figure 3. Joint effects of risk alleles of *STAT4* and *IRF5*. (A) Distribution of the number of patients and Controls according to number of risk alleles of SLE. The risk alleles are the C-allele of the SNP rs7582694 in *STAT4* and the longer allele (4× CGGGG) of the 5 bp indel polymorphisms and the C-allele of the SNP rs10488631 in *IRF5*. The black bars indicate patients and the light gray bars indicate Controls. The *P*-values are for the difference in allele counts between patients and Controls in the groups with two or more risk alleles, as compared with the groups with zero or one risk allele. (B) Linear regression analysis showing a multiplicative effect of the risk alleles of the SNP rs7582694 in *STAT4* and of the longer allele of the 5 bp indel polymorphisms and the C-allele of the SNP rs10488631 in *IRF5* on SLE. The OR with 95% CI are shown as a function of number of risk alleles of SLE. The slope of the line corresponds to a 1.82-fold increase in OR for each additional risk allele.

strong association signals. This result confirms a role of *STAT4* in SLE and corroborates previously reported observations of an association between individual SNPs in *STAT4* and risk to develop SLE (14–16). In our study the risk alleles of two highly correlated SNPs account for all association signals observed and define a single common risk haplotype with a strong association with SLE. However, we cannot exclude rare SNP alleles or other types of polymorphisms than SNPs as risk alleles for SLE. Identification of possible additional risk alleles must await complete re-sequencing of *STAT4* in SLE patients and Controls.

When investigating the consequences of the risk allele on the expression of *STAT4*, we observed an increased expression of the risk allele of *STAT4* in primary cells of mesenchymal origin (human osteoblasts), but not in transformed B-cells, which provides a functional hypothesis of the disease association in SLE. We recently demonstrated in comparative transcriptome analysis that human osteoblasts are closely related to human fibroblasts and chondrocytes (21). Interestingly *STAT4* was one of the most significantly over-expressed genes in the osteoblasts as compared with the two other

closely related mesenchymal cell types. The SNPs in *STAT4* that are associated with SLE are located in introns and could regulate the expression level or splicing of *STAT4*. Indeed, we show evidence supporting the former alternative, because the common risk haplotype alters expression of *STAT4* only in one of the cell types analyzed. Thus, our results indicate the potential presence of a tissue-specific intragenic enhancer, the function of which could be perturbed by common variants within the SLE-risk haplotype. Obviously, different gene variants may have different effects on the expression of *STAT4* between cell types, which could at least partially explain the diverse effects by *STAT4* in many different cells of the immune system (13).

In our study we also observed a correlation between the risk allele of *STAT4* and a higher frequency of antibodies against dsDNA among patients with the risk allele, which suggests that polymorphisms in *STAT4* contribute to the underlying autoimmune process in SLE. This effect could be due to the many different effects of STAT4 in the immune system. Besides its role in type I IFN signaling, STAT4 also transmits signals from, e.g. IL-12 and IL-23, and is thus responsible for the IL-12-dependent activation of natural killer (NK) cells and production of IFN- γ , as well as for polarization of naive CD4+ T-cells to IFN- γ producing Th1 effector cells (11). Recent studies also indicate that STAT4 regulates the IL-23-dependent expansion of TH17 cells, which are key actors in the proinflammatory immune response (22). As DC mature functionally, they express more STAT4, which is required for efficient presentation of antigen (23). Activation of STAT4 is also responsible for the IL-12-induced production of IL-10 in murine NK cells (24) and Th1 cells (25). In the presence of type I IFN, IL-10 becomes mainly proinflammatory instead of anti-inflammatory (26). Thus, STAT4 has pleiotropic and mostly stimulatory effects in the immune system, and has been described as a critical regulator of inflammation *in vivo*, including the development of autoimmune diseases (13). A risk haplotype for SLE, which is associated with increased expression of STAT4, as suggested by our data from osteoblasts, is therefore of great interest. The complex role of STAT4 *in vivo* is illustrated by the observation that *STAT4*-deficient lupus mice have aggravated nephritis and increased mortality, despite the fact that they have decreased autoantibody levels and complement depositions in the kidneys (27).

We have argued for a pivotal role of the type I IFN system in autoimmune diseases, including SLE (5). It has been proposed that type I IFNs stimulate an early autoimmune response, but may also later inhibit an ongoing autoimmune response (28). Possible inhibition of the autoimmune response by type I IFNs could occur via increased concentration and activation of STAT1 mediated by the type I IFNs, which would decrease the function of STAT4, at least in part due to competition between STAT1 and STAT4 for binding to the IFNAR (29,30). Therefore, it is possible that altered regulation of the expression of *STAT* in SLE may allow continued type I IFN stimulation of the *STAT4*-dependent autoimmune response.

Several transcription factors have been found to interact with the promoter of the *STAT4* gene, increasing its expression, including AP-1 and the NF- κ B (31). At least in DC, the proinflammatory cytokines TNF- α and IL-1 are potent stimulators

of *STAT4* expression via activation of NF- κ B (31). Interestingly, IRF5 is major mediator of TLR-triggered expression of other proinflammatory cytokines than type I IFN, such as TNF- α (32), and in this way IRF5 could promote expression of the *STAT4* gene. Thus *STAT4* and *IRF5*, which both contain polymorphisms that confer increased risk for SLE, are also functionally linked.

When we determined the joint effect of the risk alleles of *STAT4* and two recently identified independent risk alleles in *IRF5*, we observed a multiplicative increase in OR by a factor of 1.82 for each additional risk allele and a considerably increased risk of SLE (OR = 5.83) for those 18% of the SLE patients who carried four or more risk alleles. The importance of this finding is accentuated by the results of one of the recently published genome-wide SNP association studies, which defines *STAT4* and *IRF5*, along with HLA-DQA1 as the loci that display the strongest association with SLE among all human genes (16). These observations, as well as the findings that polymorphisms in the *STAT4* are also associated with rheumatoid arthritis (RA) (14,33), and that polymorphisms in *IRF5* are associated with RA (34), inflammatory bowel diseases (IBD) (35) and multiple sclerosis (MS) (36), suggest that *STAT4* and *IRF5* can contribute to a general loss of tolerance, whereas other genes might contribute to the disease-specific phenotypes. In this respect *IRF5* may be unique because the CGGGG indel polymorphisms in *IRF5* is associated with SLE (17), IBDs (35) and MS (36), and thus seems to promote the autoimmune process. In contrast, the SNP rs10488631 and several proxies of this SNP downstream of *IRF5* are associated only with SLE and thus, appear to promote the disease-specific phenotype.

Our study demonstrates a strongly increased risk of SLE in individuals carrying multiple risk alleles of two genes from the type I IFN signaling pathway. When more susceptibility genes of SLE, or even a complete set of susceptibility genes have been identified, a signature of multiple risk alleles could be used to improve disease prediction (37). The observed connection between the risk allele of *STAT4* and production of SLE-specific autoantibody also indicates that a genetic signature of risk alleles could be used in the future for improved classification of patients with SLE compared with the current ACR criteria. Combined genotype data from genes in the same or related molecular pathways may also be helpful when studying interactions between genes and the environment, for instance the susceptibility to disease flare during viral infections that is apparent in a subset of SLE patients (38).

MATERIALS AND METHODS

Patients and Controls

Our study included 485 Swedish SLE patients fulfilling at least four of the classification criteria for SLE as defined by the ACR (39) and 563 matched healthy Control individuals from the same geographic areas as the patients. The patients were from the rheumatology clinics at the Lund ($n = 144$), Uppsala ($n = 136$) and Karolinska (Solna) ($n = 205$) University Hospitals. The SLE patients had a mean age at disease diagnosis of 33.8 ± 14.2 years (range 9–75). Disease manifestations were scored in accordance with the ACR criteria (39) and the mean

number of criteria fulfilled was 5.7 ± 1.4 (range 4–10) out of the 11 ACR criteria. The Control samples from Lund ($n = 195$) consisted of healthy blood donors from the same region as the patients. The Control samples from Uppsala ($n = 155$) were from healthy blood donors that were matched with the patients for age, sex and region of living. The Control samples from Stockholm ($n = 213$) were population-based Control individuals matched for age and sex with the patients. DNA was extracted from blood samples of the patients and Controls using standard procedures. For correlation of *STAT4* genotype with disease phenotype we also included 220 SLE patients from the rheumatology clinic of Umeå University Hospital. These patients had a mean age of diagnosis of 39.1 ± 15.7 years and a mean number of fulfilled ACR criteria of 5.4 ± 1.4 . For studying the expression of the *STAT4* gene we used Epstein-Barr virus (EBV)-transformed human B-cells (lymphoblastoid cells) from the Human Hap Map Caucasian panel, which is derived from individuals of northern European descent (40) and human primary cells of mesenchymal origin (osteoblasts) derived from unrelated Swedish donors undergoing elective hip replacement surgery at Uppsala University Hospital. The human osteoblasts were cultured as described earlier (41). The study was approved by the regional ethical boards and all study subjects gave their informed consent to participate.

Genotyping

For genotyping we selected 64 SNPs from the *STAT4* gene, from 2 kb upstream of the first exon to 2 kb downstream of the last exon, thus including all exons and introns (Supplementary Material, Table S1). The goal of the SNP selection was to cover the *STAT4* gene region with approximately one SNP per 2 kb. The selection was guided by the Illumina Golden Gate Assay design scores and by the HapMap phase II data (20 January 2006 release, NCBI assembly B35, dbSNP b125). An Illumina score of 0.6 was used as the lower limit for inclusion in the panel. Tag-SNP information was obtained from the HapMap website, and for each group of SNPs in high LD ($r^2 > 0.95$, minor allele frequency > 0.05) at least one SNP was selected. The selected panel of 64 SNPs was genotyped in DNA samples from the Swedish patients with SLE and Controls from Lund, Uppsala and Stockholm using the Golden Gate Assay (Illumina Inc., San Diego, CA, USA). Of the 64 SNPs genotyped, seven were non-polymorphic, two failed due to poor genotype clustering and the genotypes of two SNPs deviated from Hardy–Weinberg equilibrium in the Controls ($P < 0.01$). These SNPs were excluded from further analysis. For the 53 SNPs that fulfilled the quality control criteria, the sample success rate was 96.7% and the average genotype call rate in these samples was 99.7%. Four parent–offspring trios were included in the genotyping for inheritance check, and no Mendelian inheritance errors were observed. Thirty-two samples were genotyped in duplicates with the Golden Gate Assay with 100% consistency between genotype calls. The 53 successfully genotyped SNPs capture 91% of the HapMap SNPs (MAF > 0.05) with $r^2 > 0.8$. The *IRF5* SNP rs10488631 was genotyped by the Illumina Golden Gate Assay and the 5 bp (CGGGG) indel polymorphism of *IRF5* was genotyped by polymerase chain reaction (PCR) amplification followed by

size separation by electrophoresis using 4% agarose gels, or using an ABI 3770 capillary sequencer (Applied Biosystems, Foster City, CA, USA) as previously described (17). The SNP rs7582694 in *STAT4* was genotyped in the SLE patients from Umeå using the SNPstream system (Beckman-Coulter Inc., Fullerton, CA, USA). The genotyping success rate was 90.6% and the reproducibility of genotyping was 100% according to 6% duplicated genotyping assays.

Expression analysis of *STAT4*

The *STAT4* β isoform-specific expression was determined with Affymetrix Exon 1.0 arrays (Affymetrix, Santa Clara, CA, USA) using isoform-specific probe sets 2592363 at chr2:191604056–191604086 and 2592364 chr2:191604199–191604358 according to the procedures recommended by Affymetrix. The complete data set for the EBV-transformed human B-cells ($n = 57$) has been described previously (19), and the complete data set for the human primary osteoblasts ($n = 58$) generated by the same procedures will be published separately. Allelic expression levels of *STAT4* were assessed in pooled lymphoblasts ($n = 55$) and osteoblasts ($n = 55$). The allelic ratios were determined by quantitative sequencing of RT(reverse transcriptase)–PCR products from pre-mRNA and PCR product from genomic DNA in the pooled samples (42). Allelic expression ratios in the RNA samples were calculated using normalized heterozygote ratios from genomic DNA from the same cells as reference (42). The SNP rs8179673 was used as indicator of allelic imbalance in the expression of *STAT4* in the pooled samples. The sensitivity of pooled RNA analysis in the EBV-transformed human B-cells was assessed using known loci ($n = 13$) with allelic differences in expression in individual cell lines from previous studies by us (20) and others (43,44). Of these 13 loci, 12 (92%) showed statistically significant over-expression of the expected allele in the pooled analysis (D.J. Verlaan, E. Grundberg, B. Ge, R. Hoberman, K. Lam, V. Koka, J. Dias, S. Gurd, N. Martin, Ö. Ljunggren, O. Nilsson, A. Kindmark, H. Brandström, E. Harmsen and T. Pastinen, manuscript in preparation). The individual osteoblasts have been genotyped for about half a million SNPs using Illumina 550K SNP Bead Arrays (Illumina Inc.). This data was used for assigning *STAT4* haplotypes to the individual osteoblasts using an algorithm in Phase 2.1 as described previously (45). Osteoblasts ($n = 10$) for which the haplotype could be accurately assigned using this method, and which were heterozygous for at least two out of the three SNPs rs1517352, rs3821236 and rs7574865 used as indicators of the SLE-risk haplotype, were included in the allelic expression analysis of *STAT4* (42). The mean values of triplicate measurement of the allelic expression levels determined using two or three heterozygous SNPs were used. The total expression level of *STAT4* was determined in osteoblasts with known *STAT4* haplotype ($N = 40$) in two biological replicates with Human Ref-8 Bead Arrays from Illumina Inc. using the probe ILMN_8937. A variance-stabilizing transformation, which takes advantage of a large number of technical replicates available from the Illumina BeadArrays was coupled with a robust spline normalization to obtain normalized mean expression values (46).

Statistical analysis

Fisher's exact test was used to test for Hardy–Weinberg equilibrium in the Control samples and for comparing the allele counts in the Cases and Controls. Association tests, calculation of OR and conditional logistic regression analysis were performed using the software PLINK. LD was calculated using Haploview v 4.0. The expression level of the *STAT4 β* isoform was correlated with the genotypes of the SLE-associated SNPs in *STAT4* using a linear regression model. The expression level of the SLE-risk haplotype and the total expression level of *STAT4* were correlated with the SLE-risk haplotype using a two-tailed *t*-test. A logistic regression model was used to estimate gene–gene interaction between the *STAT4* and *IRF5* SNPs and for the additive effects of the three SNPs. Fisher's exact test was used to test for the difference in *STAT4* and *IRF5*-risk allele counts between Cases and Controls. These analyses were performed using R-software and environment. Clinical criteria were analyzed using StatView software. Fisher's exact test was used for comparing the frequencies of ACR criteria between Cases and Controls and continuous variables were compared using Mann–Whitney *U* test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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