Synergistic activation of NF-κB by TNFAIP3 (A20) reduction and UBE2L3 (UBCH7) augment that synergistically elevate lupus risk

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Abstract

Background: Systemic lupus erythematosus (SLE) is an autoimmune inflammatory rheumatic disease. SLE susceptibility is affected by multiple genetic elements, environmental factors, and their interactions. We aimed in this study to statistically and functionally characterize a gene-gene interaction (epistasis) recently documented to affect SLE risk.

Methods: Two single-nucleotide polymorphisms, rs2230926 in TNFAIP3 (A20) gene and rs131654 in UBE2L3 (UBCH7) gene, were genotyped in all 3525 Korean participants, and their SLE risk association and epistasis were statistically analyzed by calculating odds ratio (OR), 95% confidence interval (CI), and P values in genotype comparisons between 1318 SLE patients and 2207 healthy controls. Furthermore, their effects on gene functions were assessed by comparatively examining separate and combined effects of TNFAIP3 and UBE2L3 knockdowns on NF-κB transcription factor activity in human cells.

Results: SLE susceptibility is associated with TNFAIP3 rs2230926 (OR = 1.9, 95% CI 1.6–2.4, P = 8.6 × 10⁻¹¹) and UBE2L3 rs131654 (OR = 1.2, 95% CI 1.1–1.4, P = 1.1 × 10⁻⁴) in a Korean population of this study. Their risk-associated alleles synergistically elevate SLE susceptibility in both multivariate logistic regression analysis (ORinteraction = 1.6, P = 0.0028) and genotype-stratified analysis (ORinteraction = 2.4), confirming the synergistic TNFAIP3-UBE2L3 interaction in SLE risk. Additionally, the SLE-susceptible alleles confer decreased TNFAIP3 expression (P = 1.1 × 10⁻⁶, n = 610) and increased UBE2L3 expression (P = 9.5 × 10⁻¹¹, n = 475), respectively, in B cell analysis of the International HapMap Project individuals with adjustment for ethnicity. Furthermore, when compared with TNFAIP3 non-knockdown and UBE2L3 knockdown in human HeLa cells, TNFAIP3 knockdown and UBE2L3 non-knockdown synergistically increase three cytokines, CCL2, CXCL8 (IL8), and IL6, all regulated by NF-κB in the human TNFR signaling pathway.

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Background

Systemic lupus erythematosus (SLE) [MIM 152700], the most common type of lupus, is an autoimmune inflammatory rheumatic disease. SLE is a complex disease, as its susceptibility is affected by multiple genetic and non- genetic factors. Its genetic heritability is 44–66% [1, 2], but all the SLE-associated genetic elements discovered so far can explain roughly only 24% [3]. The missing genetic heritability can be partly attributed to non-linear effects arising from gene-gene interactions, among others [4].

The genetic interaction where the phenotypic effect of a gene allele depends on the presence of one or more modifier gene alleles is referred to as gene-gene interaction, or epistasis [5]. This phenotypic dependence could result from an underlying functional interaction of the genes. Some epistases have been identified for SLE, but no functional validation has been achieved to explain how the interacting genetic variants can influence SLE development.

Recently, an interaction between two NF-κB modulator genes in SLE risk has been found in Han Chinese [6] and European populations [7]. The interaction was synergistic between TNFAIP3 gene encoding TNF alpha-induced protein 3 (A20) and UBE2L3 gene encoding ubiquitin-conjugating enzyme E2 L3 (UBCH7). However, the examined single-nucleotide polymorphisms (SNPs) were different in the two case-control studies, reporting very different P values for the SLE risk interaction, $P = 3 \times 10^{-14}$ versus 0.04 [6, 7]. In this study, we reexamined their gene-gene interaction in Korean SLE patients and controls, and explored their functional consequences in human cells.

SNPs of the two genes are associated with multiple autoimmune diseases including SLE [8], and their proteins are involved in diverse regulations of NF-κB. TNFAIP3 plays a potent suppressor of NF-κB pathways for immune homeostasis using its diverse ubiquitin-related activities. First, TNFAIP3 is an E3 ubiquitin ligase that catalytically modifies substrate proteins with a Lys-48-linked ubiquitin chain using a C-terminal domain (K48ub writer) [9, 10]. Second, TNFAIP3 is a deubiquitinating enzyme that catalytically removes a Lys-63-linked ubiquitin chain from modified proteins using the N-terminal ovarian tumor domain (K63ub eraser) [9]. Third, TNFAIP3 is a ubiquitin receptor that non-catalytically binds a Met-1-linked ubiquitin chain of partner proteins using a zinc finger domain (M1ub reader) [11–13].

In the human TNFR signaling pathway (Fig. 1), TNFAIP3 inhibits NF-κB activation in multiple ways. First, TNFAIP3 mediates degradation of RIPK1. As a K48ub writer, TNFAIP3 attaches K48ub to RIPK1 so that RIPK1 is degraded by proteasome [9]. Second, TNFAIP3 inactivates RIPK1, which regulates IκB kinase (IKK) [14]. As a K63ub eraser, TNFAIP3 detaches K63ub from RIPK1 so that RIPK1 cannot interact with other modulators [9]. Third, TNFAIP3 inhibits IKK activation. As an M1ub reader, TNFAIP3 binds M1ub of NEMO (IKKγ), which forms IKK complex with IKKα and IKKβ, and inhibits the phosphorylation of IKKβ by TAK1 [11–13]. All these activities of TNFAIP3 disable IKK activation in the cytoplasm to reduce NF-κB activity in the nucleus.

On the other hand, UBE2L3 is an E2 ubiquitin-conjugating enzyme that pairs up with an E3 ubiquitin ligase of HECT (homologous to the E6AP carboxyl terminus) type or RBR (ring between ring) family, but not of ring type, for catalytic ubiquitylation [15]. In the human TNFR pathway (Fig. 1), UBE2L3 E2 enzyme provides activated ubiquitin to LUBAC E3 enzyme (RBR family) for M1 ubiquitylation of RIPK1 and NEMO [16, 17]. All these activities of UBE2L3 enable the cytoplasmic IKK activation to enhance the nuclear NF-κB activity.

Thus, TNFAIP3 and UBE2L3 participate in the same pathway but exert opposite effects on sequential activation of IKK and NF-κB and subsequent induction of inflammatory cytokines (Fig. 1). In principle, two genes functionally participating in one pathway can interact with each other in affecting disease susceptibility even without their direct protein-protein interaction [18]. Accordingly, we aimed in this study to validate whether TNFAIP3 reduction and UBE2L3 augment influence each other’s effect on NF-κB transcription factor activation for cytokine induction.
Materials and methods

Human subjects and SNP genotyping
Korean participants were recruited at six university-affiliated hospitals in Seoul, Daejeon, and Daegu, Republic of Korea, in a study approved by the Institutional Review Board of Hanyang University Medical Center, and all provided written informed consent [19, 20]. Upon approval from the Institutional Review Board of Korea Advanced Institute of Science and Technology, peripheral blood samples from all subjects were genotyped for SNPs using the MassARRAY® system from Sequenom [20, 21].

Statistical analyses
Logistic regression analysis of the PLINK v1.07 program [22] was performed to calculate odds ratio (OR), 95% confidence interval (CI), and P values for SLE risk association and epistasis with adjustment for age and gender of participants by using the -logistic and -covar options [20, 23–25]. SNP association was corrected for multiple testing.

For testing multiplicativity in gene-gene interaction, we added an interaction term in logistic regression: \( \logit\{P(\text{disease} = 1)\} | \text{SNP}_1 = A, \text{SNP}_2 = B, \text{interaction SNP}_1 \times \text{SNP}_2 = AB, \text{age} = C, \text{and gender} = D\} = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 AB + \beta_4 C + \beta_5 D. \) When OR of the risk-associated genotype in one SNP is \( OR_1 = e^{\beta_1} \), that in the other SNP is \( OR_2 = e^{\beta_2} \), and that in both SNPs is \( OR_{1\&2} = e^{\beta_3} \), a multiplicative interaction between the two genes is represented by \( OR_{\text{int}} = OR_{1\&2}/(OR_1 \times OR_2) \) [18, 26].

We used CRAN R package “epiR” to calculate an attributable proportion (AP) due to interaction, 95% confidence interval (CI), and \( P \) value in Rothman’s additive model [27]. The combined relative risk of the two SNPs (\( RR_{11} \)) was estimated from their individual relative risks (\( RR_{10} \) and \( RR_{01} \)) by \( RR_{11} = RR_{10} + RR_{01} - 1 \), as the presence of risk allele is denoted by subscript 1 and its absence by subscript 0. AP is a derivative measure of the relative excess risk caused by additive interaction: \( AP = (RR_{11} - RR_{10} + 1)/RR_{11} \) [26, 28].

Gene knockdowns
Human HeLa cells purchased from the American Type Culture Collection were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum by incubation at 37 °C in 5% CO2 atmosphere. The cells were seeded in 6-well plates at \( 1 \times 10^5 \) cells/well, and 24 h later transfected with one or two small
interfering RNAs (siRNAs) or a control of 15 nM each. At 72 h after transfection, the cells were treated with 10 ng/ml of human recombinant TNFa, 210–TA from R&D System, for 0, 2, 4, 8, and 12 h.

Three siRNAs, HS S110861 and HS S110862 from Thermo Fisher Scientific and S10086989 from Qiagen, were individually used for TNFAIP3 knockdown and are denoted here by siA20-1, siA20-2, and siA20-3, respectively. An anti-UBE2L3 siRNA SI05191242 from Qiagen, denoted here by siUBE, was used to knockdown UBE2L3 [17]. AccuTarget Control siRNA SN-1002 from Bioneer, denoted here by siCon, served as a negative control.

Protein and RNA quantifications
The whole-cell lysates were obtained using the passive lysis buffer from Promega. The protein extracts (50 μg) were subjected to western blotting using anti-TNFAIP3 antibody 59A426 from Merck Millipore, anti-UBE2L3 antibody 3848S from Cell Signaling Technology, and anti-β-actin antibody SC-1616-R from Santa Cruz Biotechnology.

Total RNA was isolated from cells using the RNAspin kit from inTRON Biotechnology and used for cDNA library construction using oligo-dT primer and ImProm-II reverse transcriptase from Promega. Quantitative polymerase chain reaction (qPCR) was performed with SYBR green fluorescent dye using CFX96™ real-time qPCR detection system from Bio-Rad Lab and gene-specific primers such as GTCTCCTCTGACCTTTACACAGCC (forward) and ACCACCTGTGTTGCTTAGCCAA (reverse) for GAPDH [29], GAAAGTCTCTGCCGCCCTT and ATGATTGTCACTGTCGAGGCC (reverse) for CCL2 [7], AGCTCTCTGGACCCCAAG and GAATTCTCAG CCTCTTCAAAAC for CXCBL8 [30], and CCCCCCGG AGAAGATTTCA and GCTGCTTTCAACATGTT ACTCTTG for IL6 [31]. Relative mRNA levels were estimated using the comparative Ct method, ΔΔCt method [32].

Results
TNFAIP3-UBE2L3 interaction in SLE risk
In this study, a total of 3525 Korean participants were all genotyped for TNFAIP3 rs22230926 and UBE2L3 rs131654 SNPs, which had shown the lowest P value among the SNPs of each respective gene locus in three previous genome-wide association studies on Asian SLE susceptibility [19, 33, 34]. For example, their P values were $1 \times 10^{-17}$ and $3 \times 10^{-16}$, respectively, in a Han Chinese population [33]. The SNP genotype distributions among the control subjects were in Hardy-Weinberg equilibria.

The SNP genotypes were statistically compared between 1318 SLE patients (34.4 ± 12.4 years; 93% female) and 2207 healthy controls (40.7 ± 15.4 years old; 77% female) using logistic regression analysis (Table 1). Parts of these Korean samples previously demonstrated no genetic stratification in principal component analyses [19, 35], but the case and control groups of this study were different in age distribution and gender ratio, so the logistic regression was adjusted for age and gender of the participants. SLE susceptibility is associated with both TNFAIP3 and UBE2L3 genes in conferring SLE risk. Furthermore, this SNP pair revealed a similar interaction (AP of interaction = 0.42; 95% CI 0.01–0.81) additionally in Rothman’s additive model based on additivity of the individual relative risks [27].

This TNFAIP3-UBE2L3 interaction was further tested by using genotype-stratified analysis. According to the genotypes of the two SNPs, all study subjects are stratified into six rather than nine subgroups in Table 2, as heterozygotes in the TNFAIP3 SNP are grouped together with homozygotes of the risk-associated minor allele G, which are too few to form a subgroup alone. Thus, the TNFAIP3 SNP genotypes are classified into risk-associated allele carriage (R) and non-carriage (N), while the UBE2L3 SNP genotypes are denoted by nn, nr, and rr, comprising of the risk-associated major allele T (r) and the non-risk-associated minor allele G (n).

ORs of the R/rr, R/nn, and N/rr subgroups are 4.1-, 1.3-, and 1.3-fold greater than the N/nn subgroup, yielding OR$_{int}$ = 4.1/(1.3 × 1.3) = 2.4, a synergistic interaction. That is, the combined effect of R and rr is 2.4-fold greater than the sum of their separate effects versus N and nn. Additionally, a comparison among the ORs for R/nn, R/rr, and N/rr subgroups versus N/nn subgroup yields a 1.5-fold increased combined effect, OR$_{int}$ = 2.3/(1.3 × 1.2) = 1.5.

A similar gene-gene interaction was recently found in Han Chinese susceptibility to SLE with TNFAIP3 rs2230926 and UBE2L3 rs463426 SNPs ($P = 3.1 \times 10^{-14}$) in generalized multifactor dimensionality reduction analysis [6]. Another similar interaction was found in European susceptibility to SLE with TNFAIP3 rs80126770 and UBE2L3 rs140490 SNPs (OR$_{int}$ = 1.3), but the P value was not very low ($P = 0.039$) [7]. Although the documented SNP pairs were all different, the three independent epistasis studies appear to support for the
mRNA is associated with the risk-associated allele T of TNFAIP3 mRNA (G risk-associated allele)

bUsing logistic regression with adjustment for age and gender of the participants

aIn the genotypes of systemic lupus erythematosus, SLE

| Subgroup | N/mm | N/nr | N/nr | R/mm | R/nr | R/nr |
|----------|------|------|------|------|------|------|
| Controls | T/TG | T/TG | T/TT | (G+GG)/GG | (G+GG)/Tg | (G+GG)/TT |
| Cases    | 232  | 999  | 481  | 63   | 117  | 38   |
| P        | 0.13 | 0.0090 | 0.21 | 1.6×10⁻⁷ | 8.4×10⁻¹³ |
| OR       | 1.0  | 1.2  | 1.3  | 1.3  | 2.3  | 4.1  |
| ORₑₑ      | –    | –    | –    | –    | 1.5  | 2.4  |

SLE = systemic lupus erythematosus, OR = odds ratio, CI = confidence interval

aIn the genotypes of TNFAIP3 rs2230926/UBE2L3 rs131654, shown in bold are their risk-associated alleles, which are more frequent in patient cases than healthy controls

bUsing logistic regression with adjustment for age and gender of the participants
different sequences in TNFAIP3 mRNA (Fig. 3). The UBE2L3 knockdown is effective with siUBE, as its efficiency and specificity have been demonstrated under the same conditions as this study, i.e., in HeLa cells under TNFR activation [17]. Furthermore, siCon is an adequate negative control as it does not affect CCL2, CXCL8, or IL6 expression.

Synergistic activation of NF-κB

From the four cell lines constructed using different combinations of siA20-1, siUBE, and siCon, the three cytokine mRNAs were quantified in a time-course manner at 0, 4, 8, and 12 h after TNFα treatment (Fig. 4). Either TNFAIP3 reduction or UBE2L3 augment alone increases all three cytokine mRNAs. The combined effect of TNFAIP3 reduction and UBE2L3 augment on each cytokine (fold change of mRNA) is greater than the sum of their individual effects at any of the three time points, indicating their consistent synergistic effects.

When the synergy (\( s \)) is calculated as \( s = \text{combined effect} \div (\text{TNFAIP3 reduction effect} \times \text{UBE2L3 augment effect}) \), the synergy on CCL2 or CXCL8 expression is higher at 12 h (1.3- or 1.7-fold, respectively) than at 4 or 8 h after TNFα treatment. By contrast, the synergy on IL6 expression is maximal at the earliest time point, 4 h (1.4-fold). Thus, the synergy peaks with IL6 expression sooner than CCL2 or CXCL8 expression.

This set of experiments was repeated using two other anti-TNFAIP3 siRNAs, siA20-2 and siA20-3, separately in place of siA20-1. Although the interaction extents vary with the siRNAs, their effects on CCL2, CXCL8, and IL6 expressions are reproduced (Fig. 5), excluding possible off-target effects of anti-TNFAIP3 siRNAs, and confirming the synergistic effects of TNFAIP3 reduction and UBE2L3 augment on their activation of NF-κB transcription factor and subsequent elevation of inflammatory cytokines.

Discussion

Here, we report the first functional validation of gene-gene interaction in conferring susceptibility to SLE, an autoimmune inflammatory rheumatic disease. First, a synergistic interaction between TNFAIP3 and UBE2L3 in SLE risk is evident with their respective SNPs, rs2230926 and rs131654 (Table 2). Second, their risk-associated alleles...
are associated with decreased TNFAIP3 mRNA and increased UBE2L3 mRNA, respectively (Fig. 2). Third, while TNFAIP3 reduction and UBE2L3 augment separately elevate NF-κB transcription factor activity, they together additionally show a synergism in NF-κB activation for cytokine induction (Figs. 4 and 5).

The synergistic activation of NF-κB in the nucleus can be ascribed to a mechanistic connection between TNFAIP3 and UBE2L3 in the cytoplasm. In the human TNFR pathway (Fig. 1), an extracellular activation signal is transduced to latent transcription factor NF-κB through a series of sequential events in the cytoplasm: activation of IKK, inactivation of IκB, and activation and nuclear import of NF-κB [11–13]. There is another positive route for NF-κB activation: UBE2L3 E2 enzyme supplies activated ubiquitin to LUBAC E3 enzyme for linear ubiquitylation of NEMO and subsequent activation of IKK [17, 47]. Then, activated NF-κB induces TNFAIP3 expression to exert a negative feedback on NF-κB activation, among others. Accordingly, with higher UBE2L3, NF-κB could be less inhibited by TNFAIP3.

The synergistic effect on NF-κB is evident when UBE2L3 stimulation overrides TNFAIP3 inhibition (Figs. 4 and 5). UBE2L3 augment increases the three cytokines 1.1–2.7-fold at 4, 8, and 12 h time points in comparisons of the two cell lines without TNFAIP3 knockdown. The same cytokines increase 1.3–3.7-fold in comparisons of the two cell lines with TNFAIP3 knockdown. Thus, the UBE2L3 augment effect tends to be greater with low TNFAIP3 than with high TNFAIP3, although the ranges overlap.

The functional consequences of this gene-gene interaction in NF-κB signaling need to be replicated in human immune cells instead of cervical HeLa cells used in this study. As a key modulator of diverse immune and inflammatory responses [48], NF-κB induces inflammatory cytokines and chemokines in innate immune cells, modulates inflammatory T cell functions [49, 50], and regulates inflammasome activation [51], among others. Thus, deregulated NF-κB activation is associated with multiple chronic inflammatory diseases including SLE. For example, NF-κB promotes proliferation and survival of B cells [52], and is constitutively activated in peripheral B cells from SLE patients [53]. Elevation of GLK expression in SLE patients activates NF-κB signaling under T cell receptor, and is correlated with disease.

![Fig. 4 Functional assays for synergistic induction of NF-κB-mediated cytokine expressions.](image-url)
FcyRIIB-deficient mice with SLE-like symptoms have elevated NF-κB function and immuno-}

genicity in their splenic dendritic cells [55]. While NF-κB is ubiquitously involved in diverse im-

mune cell types, both TNFAIP3 and UBE2L3 participate in B cell differentiation, and SLE risk-associated alleles of their genes affect NF-κB activation in B cells. B cell-specific ablation of Tnfaip3 in mice exaggerates NF-κB re-

sponse to CD40-induced signals, leading to SLE-like features, including elevation of germinal center B cells and plasma cells, production of autoreactive immunoglobulins, and immune complex deposits [56–58]. UBE2L3 augment increases basal and CD40-stimulated NF-κB activation in CD19⁺ B cells. An SLE risk-associated allele of UBE2L3 is associated with increased plasmablasts and plasma cells in SLE patients [7]. Accordingly, B cells could be one of the immune cells where the said synergism is connected with SLE phenotypes.

TNFAIP3 rs2230926 SNP affects TNFAIP3 gene expression (Fig. 2, left). This SNP is perfectly correlated \((r^2 = 1.0)\) with another SLE risk-associated dinucleotide polymorphism, rs148314165 and rs200820567 \((TT>A)\) located in a TNFAIP3-downstream enhancer, according to the Asian population data of the 1000 Genomes Project. The risk-associated minor allele A renders the enhancer to lack NF-κB binding and reduce TNFAIP3 expression in EBV-transformed B cells and HEK293T cells [59–61].
UBE2L3 rs131654 SNP also affects UBE2L3 gene expression (Fig. 2, right). This SNP is highly correlated with two other SLE risk-associated SNPs, rs140490 in the promoter ($r^2 = 0.76$) and rs7444 in the 3’ untranslated region ($r^2 = 0.74$) according to the Asian data of the 1000 Genomes Project. Their risk-associated alleles have been associated with increased UBE2L3 mRNA or protein in EBV-transformed B cells, CD19+ B cells, CD14+ monocytes [7, 62].

Conclusions

TNFAIP3 reduction and UBE2L3 augment together confer synergistic activation of NF-κB transcription factor and subsequent synergistic induction of inflammatory cytokines. This functional demonstration supports for the TNFAIP3-UBE2L3 gene-gene interaction that is statistically observed in synergistic elevation of SLE risk. Accordingly, inflammation in SLE could be synergistically reduced by concurrent upregulation of TNFAIP3 and downregulation of UBE2L3, and an inclusion of this synergistic genetic effect in weighted genetic risk score would improve SLE risk prediction.

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Authors’ contributions

C.K. and T.K. conceived and designed the study. T.K. and S.C.B. acquire the data. C.K. supervised the biology part, and S.C.B. supervised the clinical part. All analyzed and interpreted the data, were involved in drafting the article, and approved the final version to be published.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Review Boards of Hanyang University Medical Center and Korea Advanced Institute of Science and Technology. Every participant provided written informed consent. This study was approved by the Institutional Review Boards of Hanyang University Medical Center and Korea Advanced Institute of Science and Technology, and Prof. Gang Min Hur at Chungnam National University for their discussions and advice.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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