Association of IFI200 Gene Polymorphisms with Susceptibility to Systemic Lupus Erythematosus

To the Editor:

The *MNDA* (myeloid nuclear differentiation antigen), *IFIX* (interferon-inducible protein X), *IFI16* (interferon-inducible protein 16), and *AIM2* (absent in melanoma 2) are a group of interferon (IFN)-inducible genes whose products belong to the gene family denoted hematopoietic interferon-inducible protein with 200 amino acid repeat (IFI200). These genes map on chromosome 1q21-23, which is the major susceptibility locus of systemic lupus erythematosus (SLE). They are proposed as new candidate genes for SLE susceptibility for several reasons: (1) genetic mapping from a murine model of lupus[1,2]; (2) upregulation of all 4 genes in patients with SLE[3]; (3) the role of IFI16 as autoantigen in patients with SLE[4,5]; (4) the ability of IFI16 to bind single-strand DNA in a process of DNA repair[6]; and (5) the discovery of AIM2 as an intracellular DNA sensor leading to inflammation and apoptosis[7]. Therefore, IFI200 might be associated with abnormal inflammation and loss of tolerance to dsDNA observed in patients with SLE.

We recruited 200 SLE patients (194 women, 6 men; mean age 36.21 ± SD 10.76 yrs) from King Chulalongkorn Memorial Hospital, Bangkok, each having at least 4 of the American College of Rheumatology revised criteria for SLE[8], and 200 ethnically matched healthy volunteer blood donors from the Thai Red Cross Society (147 women, 53 men; mean age 23 ± SD 12.3 yrs)[9]. The study was approved by the ethics committee of the Faculty of Medicine, Chulalongkorn University, and all subjects gave their informed consent.

Detection of apoptosis. Within 60 minutes after blood draw, peripheral blood mononuclear cells (PBMC) from 15 healthy donors containing different IFI16 genotypes were isolated by Ficoll-Hypaque gradient (Robbins Scientific, Sunnyvale, CA, USA) and resuspended in RPMI-1640 medium (Sigma, New York, NY, USA) with 10% fetal bovine serum (Gibco, Karlsruhe, Germany) and 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) at concentration 5×10^6 cells/ml. These cells were plated into 24-well plastic tissue culture plates. PBMC were treated with doxorubicin (Pharmacia and Upjohn GmbH, Freiburg, Germany) at a concentration of 10 μg/ml and then cultured at 37°C in 5% CO_2 for 24 h. This condition was determined to be effective to induce highest p53 expression in dose (1, 10, and 100 μg/ml) and time (8 and 24 h) response experiments. p53 mRNA expression was studied using real-time reverse transcription-polymerase chain reaction (RT-PCR) with the following primers: 5'TGG CCA TCT ACA AGC AGT CAC A3' and 5'GCA AAT TTC CTT CCA CTC GGA T3', as described[10]. Finally, the percentage of apoptotic cells was measured by FACScan flow cytometry (Becton Dickinson, Mountain View, CA, USA) for annexin V-FITC and propidium iodide binding according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA). Cells positive only for annexin V were counted as apoptotic.

Quantification of IFIX and IFI16 isoforms. Expression of *IFIX* and *IFI16* isoforms was semiquantified by conventional RT-PCR using the following primers: 5'GGA ACA GAG TCA GCA TCC and 5'GTT ATT TGA TAT CCT TGT CC for *IFIX* [11]; and 5'CAT CTT CGG ACT CCT CAG and 5'GTT CAG CAC CAT CAC TTC for *IFI16*. cDNA from leukocytes of 19 SLE patients containing different genotypes was amplified in a Perkin Elmer/GeneAmp PCR 2400 system. The PCR conditions consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C, 30 s), annealing (60°C for *IFIX* gene, 62°C for *IFI16* gene, 30 s), extension (72°C, 30 s), and final extension (72°C, 7 min). PCR products were loaded in 1.5% Tris-acetate agarose gel and analyzed by electrophoresis in Tris-acetate buffer at 100 volts for 40 min followed by staining with 50 μg/ml ethidium bromide. The density of product bands was semiquantified using Gel Doc™ MZL software (Bio-Rad).

Statistical analysis. The association between genotypes and the expression of isoforms or apoptosis was analyzed by GraphPad Prism, version 4.0

Table 1. Putative functional single-nucleotide polymorphisms (SNP) and genotyping methods.

Genes	SNP	Location and Functional Prediction	Genotyping Method	Sequence of primers (5'→3')	Restriction Enzyme
MNDA	A2706G (rs7513873)	Intron; A: strengthens donor site and creates cryptic acceptor site/G:–	PCR-RFLP	ACT CAT CCT CAC CAA CAC TGG CGA CAG GGC GAA ACT C	Nla III
	C16432T (rs2276403)	Exon; histidine → tyrosine	PCR-RFLP	GGA TCC ATG GAT GTA GTG ATG AGG AAC CTG GTG TTC	Rsa I
IFIX	G13792T (rs856084)	Intron; T: abolishes SC35 binding site/G:–	PCR-RFLP	GCA ACG ATT GCT GAC CAC CCA GTG ATG AGA TGG GAG AA	AhdI
	C-223T (rs16841336)	Promoter	Taqman SNP genotyping assay	—	—
	A40309G (rs10908697)	Intron	Taqman SNP genotyping assay	—	—
	C43235T (rs1615480)	Intron	Taqman SNP	—	—
IFI16	C-7217T (rs4657618)	Promoter; T: AML1a or RUNX1/C:–	PCR-RFLP	GCC AGC CTG AAA TAG AAG GTA ACT CTG GCT CTT GAG	Hae III
	C6771G (rs866484)	Exon; threonine → serine	PCR-RFLP	GCC GTT CCC CAT CTC CCA AGC ATC AAG TAT CCC TGT GAA AGC	HpyCH4 III
	A23201G (rs1772414)	Intron; A: abolishes donor site/G:–	PCR-FRLP	CCC ATT TCC CCT TTG CTT ATT CTG TGA ATT GGG GCA GAA T	Dra III
AIM2	G-151T (rs16841642)	Promoter; G: AP–1/T:–	PCR-RFLP	CAC TAG CAG CCA CAG AAG GGG TGT CGT TGG TTT TGC	Dra III
	C3452T (rs2276405)*	Exon; glutamic acid → lysine	PCR-SSP	GCC TGT GGC AAT ATT AAA CTC GCC TGT GGC AAT ATT AAA CTT GGC TGA TCC CAA AGT TGT GCC	—
Internal control			PCR	TTC CCA ACC ATT CCC TTA TCA CGG ATT TCT GTT GTG TTT C	—

* Not polymorphic in the Thai population. PCR-FRLP: polymerase chain reaction-restriction fragment length polymorphism; PCR-SSP: polymerase chain reaction-sequence specific primer.

(Graph-Pad Software, San Diego, CA, USA). The Mann-Whitney U test was used in comparisons between 2 groups, while Kruskal-Walls H was applied in case of analysis of more than 2 groups. A p value < 0.05 was considered significant.

Eleven putative functional single-nucleotide polymorphisms (SNP) were selected from *MNDA*, *IFIX*, *IFI16*, and *AIM2* genes according to computational prediction and genotyped as described in Table 1. The percentage of genotyping for each SNP was 100%. There were no missing genotyping data. All SNP were in Hardy-Weinberg equilibrium when we compared the observed with the expected genotype frequencies of each SNP ($p > 0.05$). PLINK v1.05 program[12] was used to calculate p values, empirical p values (100,000 permutations), and odds ratios (95% confidence intervals) for genotype and haplotype associations. Linkage disequilibrium was determined by JLIN, a Java-based linkage disequilibrium plotter[13] (Table 2).

A significant association of the minor allele frequency from 3 SNP of *IFIX* and *IFI16* genes was found between SLE patients and healthy controls (Table 3). The G13792T (rs856084) in *IFIX* and the C6771G (rs866484) and A23201G (rs1772414) in *IFI16* showed significant differences (OR 0.73, 95% CI 0.54–0.98, empirical p = 0.033; OR 1.33, 95% CI 1.00–1.75, empirical p = 0.05; and OR 1.37, 95% CI 1.03–1.80, empirical p = 0.030, respectively). The association of both SNP (rs866484 and rs1772414) in *IFI16* gene seems to be a recessive effect model. The presence of 2 G alleles (GG) conferred a significant p = 0.009 (empirical p = 0.023) for rs866484 and p = 0.013 (empirical p = 0.032) for rs1772414. The marginal significance results are probably due to the limited sample size in this study. We performed haplotype analysis of significant SNP rs866484 and rs1772414 with the strongest linkage disequilibrium (D' = 0.814 and $r^2 = 0.643$) by comparing each haplotype with another 3 haplotypes between SLE patients and controls. Our finding showed that the GG was a risk haplotype (OR 1.41 and p = 0.017, empirical p = 0.031), whereas CA was a protective haplotype (OR 0.73 and p = 0.032, empirical p = 0.041).

Regarding the selection as putative functional SNP, we tried to prove our hypothesis of 3 positive SNP as follows. SNP rs866484 (C6771G) of *IFI16* is a nonsynonymous SNP whose amino acid is altered from threonine to serine. This SNP is located on an A-type repeat containing p53 binding site. Binding of IFI16 protein to the C-terminus of p53 has been reported to stimulate the transcription of p53-responsive reporter plasmids and leads to susceptibility to apoptosis of cells[14]. We hypothesized that the G risk allele may bind p53 with higher affinity and lead to more apoptosis. We conducted our experiment by inducing the highest p53 mRNA expression with doxorubicin at a concentration of 10 μg/ml for 24 h. The association of SNP rs866484 and apoptosis rate was studied by flow cytometry analysis. We were unable to determine the association between SNP and apoptosis (Figure 1A). This negative finding may be because threonine and serine are in the same amino acid group. Nevertheless, further study should be performed using direct protein-protein interaction methods to prove the direct role of this SNP in protein structure that affects p53 binding. In addition, we tested the association of the 2 SNP within the intron of *IFIX* (rs856084) and *IFI16* (rs1772414) genes with the expression of the isoforms (α and ß for *IFIX*; A, B, and C for *IFI16)* by reverse transcription-polymerase chain reaction, as described[3]. However, we found no differences in isoforms among the genotypes (Figure 1B, 1C, 1D).

Our results suggest that these SNP are not likely to be functional SNP. We could not exclude the possibility that these SNP are in linkage disequilibrium with nearby causative SNP. Interestingly, one report in SLE in Caucasian patients also suggested an association signal in the *IFIX-IFI16* intergenic region[15]. More extensive research using dense SNP and increased sample sizes is required to clarify the role of *IFIX* and *IFI16* gene in SLE.

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Table 2. Linkage disequilibrium coefficients (D' and r^2) among single-nucleotide polymorphisms (SNP) within *MNDA, IFIX, IFI16,* and *AIM2*.

	rs7513873 (MNDA)	rs2276403 (MNDA)	rs16841336 (IFIX)	rs856084 (IFIX)	rs10908697 (IFIX)	D' rs1615480 (IFIX)	rs4657618 (IFI16)	rs866484 (IFI16)	rs1772414 (IFI16)	rs16841642 (AIM2)
r^2 rs7513873 (MNDA)	—	0.7741	0.8634	0.1401	0.2054	0.051	0.288	0.189	0.024	0.08
rs2276403 (MNDA)	0.391	—	0.7808	0.0083	0.4456	0.139	0.117	0.38	0.268	0.23
rs16841336 (IFIX)	0.678	0.3469	—	0.0752	0.4062	0.065	0.256	0.3	0.114	0.08
rs856084 (IFIX)	0.004	0.00001	0.0005	—	0.9449	0.376	0.452	0.208	0.297	0.26
rs10908697 (IFIX)	0.008	0.022	0.0321	0.4237	—	0.189	0.307	0.042	0.051	0.03
rs1615480 (IFIX)	0.0009	0.0044	0.0017	0.0972	0.0117	—	0.738	0.899	0.836	0.55
rs4657618 (IFI16)	0.016	0.0017	0.015	0.1707	0.0547	0.309	—	0.413	0.498	0.33
rs866484 (IFI16)	0.004	0.0164	0.01	0.0345	0.0015	0.466	0.159	—	0.814	0.67
rs1772414 (IFI16)	0.0009	0.008	0.0016	0.0691	0.0016	0.367	0.224	0.643	—	0.78
rs16841642 (AIM2)	0.001	0.0061	0.0007	0.0542	0.0004	0.172	0.103	0.448	0.579	—

Table 3. Genotype and allele frequencies for *MNDA, IFIX, IFI16,* and *AIM2* polymorphisms in SLE patients and healthy controls. Significant results (p value < 0.05) indicated in bold type.

Gene	SNP	Minor a	Major A	SLE, n = 200					Control, n = 200					Model of Inheritance (Recessive)		Allelic Test*	
				Genotype, n (%)			Allele, n (%)		Genotype, n (%)			Allele, n (%)					
				aa	Aa	AA	a	A	aa	Aa	AA	a	A	OR (95% CI)	Emp p	OR (95% CI)	Emp p
MNDA	rs7513873	A	G	1 (0.5)	50 (25)	149 (75)	52 (13)	348 (87)	1 (0.5)	45 (22.5)	154 (77)	47 (11.8)	353 (88.3)	NA	NA	1.12 (0.74–1.71)	0.571
	rs2276403	T	C	1 (0.5)	37 (19)	162 (81)	39 (9.75)	361 (90.25)	0 (0)	32 (16)	168 (84)	32 (8)	368 (92)	NA	NA	1.24 (0.76–2.03)	0.372
IFIX	rs16841336	C	T	4 (2)	53 (27)	143 (72)	61 (15.3)	339 (84.75)	3 (1.5)	55 (27.5)	142 (71)	61 (15.3)	339 (84.8)	NA	NA	1 (0.68–1.47)	1
	rs856084	G	T	**15 (7.5)**	**88 (44)**	**97 (49)**	**118 (29.5)**	**282 (70.5)**	**27 (14)**	**92 (46)**	**81 (40.5)**	**146 (36.5)**	**254 (63.5)**	**0.52 (0.25–1.06)**	**0.079**	**0.73 (0.54–0.98)**	**0.033**
	rs10908697	G	A	47 (24)	103 (52)	50 (25)	197 (49.3)	203 (50.75)	46 (23)	101 (50.5)	53 (26.5)	193 (48.3)	207 (51.8)	1.03 (0.63–1.68)	0.928	1.04 (0.79–1.37)	0.773
	rs1615480	C	T	25 (13)	82 (41)	93 (47)	132 (33)	268 (67)	19 (9.5)	81 (40.5)	100 (50)	119 (29.8)	281 (70.3)	1.36 (0.69–2.68)	0.551	1.16 (0.86–1.57)	0.335
IFI16	rs4657618	T	C	32 (16)	101 (51)	67 (34)	165 (41.3)	235 (58.75)	34 (17)	98 (49)	68 (34)	166 (41.5)	234 (58.5)	0.93 (0.53–1.63)	0.956	0.99 (0.75–1.31)	0.946
	rs866484	G	C	**57 (29)**	**87 (44)**	**56 (28)**	**201 (50.3)**	**199 (49.75)**	**35 (18)**	**103 (51.5)**	**62 (31)**	**173 (43.3)**	**227 (56.8)**	**1.88 (1.14–3.11)**†	**0.023**	**1.33 (1.00–1.75)**	**0.050**
	rs1772414	G	A	**57 (29)**	**93 (47)**	**50 (25)**	**207 (51.8)**	**193 (48.25)**	**36 (18)**	**104 (52)**	**60 (30)**	**176 (44)**	**224 (56)**	**1.82 (1.10–3.00)**††	**0.032**	**1.37 (1.03–1.80)**	**0.030**
AIM2	rs16841642	T	G	41 (21)	94 (47)	65 (33)	176 (44)	224 (56)	35 (18)	102 (51)	63 (31.5)	172 (43)	228 (57)	1.22 (0.72–2.07)	0.694	1.04 (0.79–1.38)	0.779
	rs2276405	C	T	200 (100)	0 (0)	0 (0)	400 (100)	0 (0)	200 (100)	0 (0)	0 (0)	400 (100)	0 (0)	NA	NA	NA	NA

* SNP were analyzed for association with disease by comparison of minor allele frequency in SLE patients and controls. † GG compared with CG+CC genotypes. †† GG compared with AG+AA genotypes. NA: not applicable; SNP: single-nucleotide polymorphism; EMP p: empirical p value (100,000 permutations).

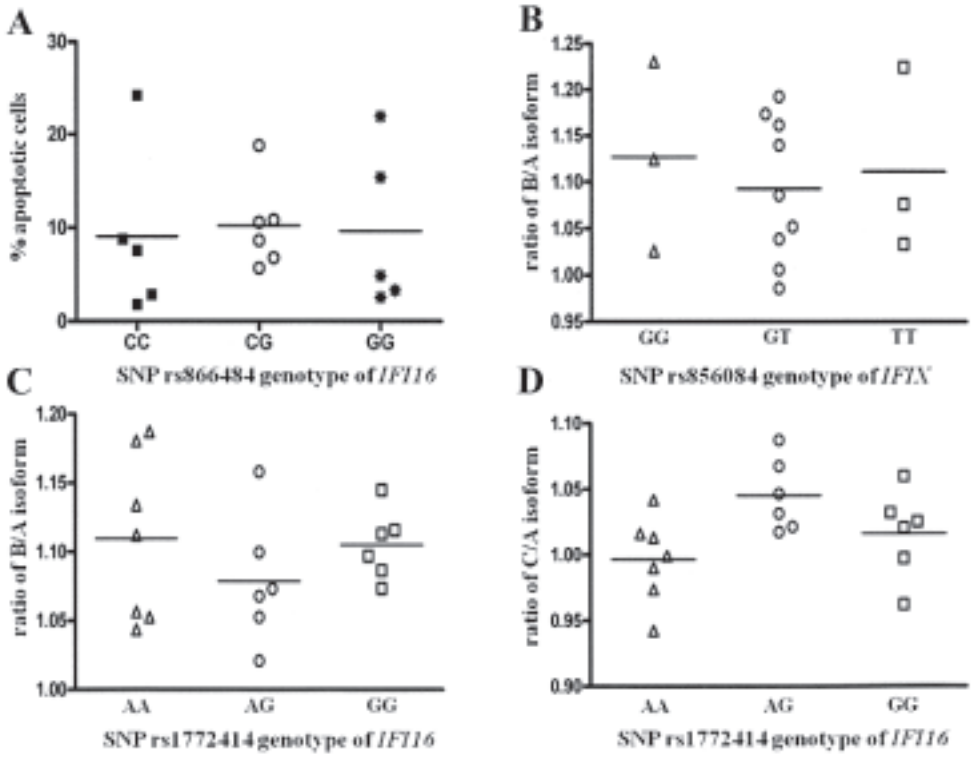

Figure 1. A. The effect of SNP rs866484 genotypes (CC, CG, and GG; n = 5, 6, and 5, respectively) on apoptotic response of healthy PBMC treated with 10 μg/ml doxorubicin for 24 h. B, C, D. The ratio of short product/full-length product grouped by SNP rs856084 genotypes of *IFIX* (GG, GT, and TT; n = 3, 9, and 3) and SNP rs1772414 genotypes of *IFI16* (AA, AG, and GG; n = 7, 6, and 6). Each data symbol represents an individual sample; horizontal lines show median values.

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