CD72 polymorphisms associated with alternative splicing modify susceptibility to human systemic lupus erythematosus through epistatic interaction with FCGR2B

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We previously reported association of FCGR2B-Ile232Thr with systemic lupus erythematosus (SLE) in three Asian populations. Because polymorphism of CD72, another inhibitory receptor of B cells, was associated with murine SLE, we identified human CD72 polymorphisms, tested their association with SLE and examined genetic interaction with FCGR2B in the Japanese (160 SLE, 277 controls), Thais (87 SLE, 187 controls) and Caucasians (94 families containing SLE members). Four polymorphisms and six rare variations were detected. The former constituted two major haplotypes that contained one or two repeats of 13 nucleotides in intron 8 (designated as *1 and *2, respectively). Although association with susceptibility to SLE was not detected, the *1 allele was significantly associated with nephritis among the Japanese patients (P = 0.024). RT–PCR identified a novel alternatively spliced (AS) transcript that was expressed at the protein level in COS-7 transfectants. The ratio of AS/common isoforms was strikingly increased in individuals with *2/*2 genotype when compared with *1/*1 (P = 0.000038) or *1/*2 (P = 0.0085) genotypes. Using the two Asian cohorts, significant association of FCGR2B-232Thr/Thr with SLE was observed only in the presence of CD72-*1/*1 genotype (OR 4.63, 95% CI 1.47–14.6, P = 0.009 versus FCGR2B-232Ile/Ile plus CD72-*2/*2). Minigene assays demonstrated that the 13-nucleotide repeat and 4 bp deletion within the same haplotype of intron 8 could regulate alternative splicing. The AS isoform lacks exon 8, and is deduced to contain 49 amino acid changes in the membrane-distal portion of the extracellular domain, where considerable amino acid changes are known in CD72c allele associated with murine SLE. These results indicated that the presence of CD72-*2 allele decreases risk for human SLE conferred by FCGR2B-232Thr, possibly by increasing the AS isoform of CD72.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by production of an array of autoantibodies and immune complexes that leads to inflammation and damage of multiple organs. Although the precise mechanism of disease development is not clear, genetic epidemiological data indicate substantial contribution of genetic factors (1,2). Linkage studies identified multiple chromosomal regions linked with disease susceptibility.
and/or specific clinical manifestations, at least some of which are dependent on genetic or environmental background of populations (3).

In parallel with positional approach, several susceptibility genes have been identified through candidate gene approach. Candidate gene approach has higher detection power for disease associations when compared with positional approach (4), and is considered particularly advantageous in SLE where plenty of functional candidate genes are already available from studies of human SLE as well as mouse models (5).

Genes coding for molecules involved in B cell activation or regulation have been considered strong candidate susceptibility genes. By means of systematic polymorphism screening of genes such as CD22 (6), Src-homology-2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1, PTPN6) (7), BCMA (TNFRSF17) (8) and BLyS/BAFF (TNFSF13B) (9), we previously identified association of dinucleotide repeat polymorphism within 3' -untranslated region (3'-UTR) of CD19, a positive regulator of B cell receptor (BCR) signaling (10) and also a SNP coding for an amino acid substitution, Ile232Thr, in the transmembrane region of an inhibitory receptor, FCGR2B (11). The latter association was confirmed in Thai and Chinese populations (12,13), but not in Caucasians and African-Americans (14–16). Instead, a promoter haplotype of FCGR2B has recently been shown to be associated with SLE in Caucasians (17,18), thereby suggesting that FCGR2B may be a susceptibility gene of SLE shared by multiple populations.

CD72 is a 45 kDa type II transmembrane protein containing a C-type lectin domain (19–22). CD72 is expressed as a homodimer in B cells of most developmental stages except for plasma cells, and also in dendritic cells and macrophages (19–21,23). CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail, one of which recruits SHP-1. CD72 functions as a negative regulator of BCR signaling (24–28). Importantly, CD72 has been shown to downregulate signaling of BCR containing IgG as well as IgM, whereas CD22 downregulates signals from IgM-BCR but not from IgG-BCR (24). Thus, if present, CD72 polymorphisms may be more relevant to SLE, where autoantibodies of IgG class play a pathogenic role. Recently, a model has been proposed that CD100, a ligand of CD72, turns off negative regulation of CD72 and leads to B cell activation both in human and in mouse (28–30). On the other hand, several lines of evidence indicate that CD72 also possesses potentials for positive signaling (31,32), which could be at least partially accounted for by the recruitment of Grb2 to the other ITIM, leading to association with B cell linker protein (BLNK, BASH, SLP-65) (33).

Human CD72 gene is encoded on human chromosome 9p13.2, which has not previously been detected by linkage analyses. However, in a murine model of SLE, MRL/lpr, CD72 allele has been shown to be associated with systemic vasculitis phenotype in a recessive manner (34). Furthermore, the same CD72 allele has been shown to be responsible for the genetic background of murine SLE induced by immunization of a synthetic peptide representing an epitope of Sm autoantigen, through epitope spreading (35).

These observations led us to hypothesize that CD72 polymorphisms may also be associated with SLE in humans. Thus far, systematic polymorphism screening of CD72 has not been reported. In this study, we performed human CD72 polymorphism screening and association study of CD72 polymorphisms with SLE in Japanese, Thais and Caucasians. In addition, because both FcγRIIB and CD72 possess inhibitory potentials on BCR signaling, epistatic interaction between FCGR2B and CD72 polymorphisms in relation to the susceptibility to SLE was examined. Furthermore, functional relevance of CD72 polymorphisms was investigated.

RESULTS

New variations of human CD72 gene

Variation screening of all exons with ~100 bp of flanking introns and promoter region (≤1 kb) was performed on genomic DNA from 10 Japanese SLE patients and 10 Japanese controls by direct sequencing. Ten variations were detected (Fig. 1A). Four of them, c.109A>G, c.338G>A, c.701C>T and c.728G>A, were located in the coding region and resulted in non-synonymous substitutions, Ile37Val, Cys113Tyr, Pro234Leu and Ser243Asn, respectively [mutation nomenclature is based on the work of den Dunnen and Antonarakis (36)].

Other six variations were located in introns. One of them encoded a repeat polymorphism constituted of one or two repeats of 13 nucleotides, IVS8_13T (Fig. 1B and C). Thereafter, we considered IVS8VNTR as a haplotype-tagging polymorphism, and all remaining subjects were genotyped for this polymorphism. Variation screening of all exons with 100 bp of flanking introns and promoter region resulted in non-synonymous substitutions, Ile37Val, Cys113Tyr, Pro234Leu and Ser243Asn, respectively [mutation nomenclature is based on the work of den Dunnen and Antonarakis (36)].

All remaining subjects were genotyped for this polymorphism.

Association of CD72 polymorphisms with susceptibility to lupus nephritis

We next carried out case–control association analyses of the detected CD72 variations. Initially, 98 Japanese patients and 95 controls were genotyped to estimate allele frequencies and linkage disequilibrium. Among the variations, four of them, c.109A>G, c.338G>A, c.701C>T and c.728G>A, were located in the coding region and resulted in non-synonymous substitutions, Ile37Val, Cys113Tyr, Pro234Leu and Ser243Asn, respectively [mutation nomenclature is based on the work of den Dunnen and Antonarakis (36)].

Other six variations were located in introns. One of them encoded a repeat polymorphism constituted of one or two repeats of 13 nucleotides, IVS8_13T (Fig. 1B and C). Thereafter, we considered IVS8VNTR as a haplotype-tagging polymorphism, and all remaining subjects were genotyped for this polymorphism.

Tables 1 and 2 show the results of case–control association study in the Japanese and Thais. The control genotypes were not deviated from Hardy–Weinberg equilibrium. Significant association was not observed between SLE and controls. However, when the patients were divided into subgroups in accordance with the presence of lupus nephritis,
significant increase was observed in the allele and allele carrier frequencies of CD72-1/C3 in the patients with nephritis in the Japanese, and a similar tendency was observed in the Thais.

In contrast, association of CD72 polymorphisms with susceptibility to SLE or lupus nephritis was not observed in 94 Caucasian SLE families using transmission/disequilibrium test (total SLE: 1/C3 allele transmitted 35, non-transmitted 31; SLE with nephritis: 1/C3 allele transmitted 7, non-transmitted 10).

Identification of a novel splicing isofrm of CD72

Because polymorphisms encoding 13-nucleotide repeat and four-nucleotide deletion were found in intron 8, we investigated a possibility that these polymorphisms were associated with...
alternative splicing. RT–PCR of RNA derived from peripheral blood mononuclear cells (PBMCs) using primers placed within exon 6 and exon 9 revealed two amplification products (Fig. 2A). By sequencing, the longer product was found to be the normal CD72 mRNA (common form), whereas the shorter band encoded alternative splicing isoform caused by exon 8 skipping (AS form). In the common form, termination codon appears within exon 8, and exon 9 constitutes 3' UTR. In the AS form, exon 9 sequence was deduced to be translated into polypeptide until the new termination codon appears. As a result, C-terminal 42 amino acids in the membrane-distal portion of extracellular region of normal CD72 protein are thought to be replaced with new 49 amino acids in the AS form. In the common form, termination codon appears within exon 8, and exon 9 constitutes 3' UTR. In the AS form, exon 9 sequence was deduced to be translated into polypeptide until the new termination codon appears. As a result, C-terminal 42 amino acids in the membrane-distal portion of extracellular region of normal CD72 protein are thought to be replaced with new 49 amino acids in the AS form. Thus, alternative form of CD72 with exon 8 skipping paradoxically codes for a longer polypeptide of 366 amino acids, rather than 359 amino acids of the common isoform (Fig. 2B).

Full-length cDNAs coding for the common and AS CD72 isoforms were both readily amplified from PBMC cDNA, thereby confirming the presence of the transcripts of the isoforms. Sequence analysis of multiple cDNAs revealed no other variations in the AS mRNA. Because an anti-human CD72 antibody that recognizes the extracellular domain of the AS isoform is not currently available, we confirmed the expression of the CD72 isoforms by western blotting with anti-human CD72 antibody directed against the cytoplasmic domain (H-96).

Association of CD72 genotypes with relative quantity of alternatively spliced isoform

We next investigated the expression of common and AS mRNA isoforms in PBMCs from 32 healthy individuals with different CD72 genotypes (13 with *1/*1, 11 with *1/*2, 8 with *2/*2), using semi-quantitative RT–PCR. To standardize the difference in the numbers of CD72 positive cells among individuals, the quantity of AS isoform was normalized by that of the common form in each subject. As shown in Figure 3, the ratio of AS/common isoforms was strikingly increased in individuals with *2/*2 genotype (n = 8) when compared with *1/*1 (n = 13, P = 0.000038) or *1/*2 (n = 11, P = 0.0085). The ratio of AS isoform in heterozygotes was significantly higher than in *1/*1 homozygotes (P = 0.0049), indicating that the expression of AS isoform is dependent on the dosage of *2 allele.

Contribution of intron 8 polymorphisms to differential expression of alternative splicing products

To directly test whether the intron 8 polymorphisms influence the difference in the expression of splicing isoforms, minigene
assays were conducted. Minigene constructs containing genomic DNA segments derived from exon 7 through exon 9 of CD72 were prepared from individuals carrying *1/*1 and *2/*2 genotypes, representing one or two repeat alleles of IVS8VNTR, respectively. Because of the linkage disequilibrium, only the former minigene construct contained CTCA deletion of intron 8. This construct was designated as *1/del. On the other hand, the latter did not contain the four-nucleotide deletion, and was designated as *2/+.

In addition, to gain further insight into the individual contribution of these two polymorphic sites, chimeric constructs containing each of the VNTR and deletion were prepared (*1/+ and *2/del) (Fig. 4A). These four minigene constructs were introduced into COS-7 cells. COS-7 cells were used because these cells do not express endogenous CD72, and also because previous studies have successfully employed COS-7 cells for minigene assays (37). RT–PCR was performed using primers placed within exon 7 and exon 9.

As shown in Figure 4B, two mRNA isoforms with and without exon 8 were detected in the minigene-introduced COS-7 cells. Unlike in PBMCs, the exon 8-excluded isoform was more abundant in all COS-7 transfectants. Such a difference was probably related to the cellular environment and structure of the introduced genes. Nonetheless, clear difference was observed among the constructs. Exon 8 exclusion from mRNA was strongly increased in *2/+ construct when compared with *1/del, thereby confirming that intron 8 polymorphisms can regulate alternative splicing.

When the difference between *1/del and *1/+ was compared with that between *1/del and *2/del, it was suggested that although VNTR seems to play a greater role, each of the VNTR and the deletion possesses an independent effect on splicing, and both act in an additive manner to achieve full activity on exon 8 exclusion in the haplotype 2 (*2/+). This was also supported from the comparison of *2/+, *1/+ and *2/del.

Epistatic interaction between CD72 and FCGR2B polymorphisms in conferring susceptibility to SLE

Finally, we examined whether genetic interaction between CD72 and FCGR2B polymorphisms plays a role in conferring susceptibility to SLE, because both molecules are involved in negative regulation of BCR signaling. The analysis was done in the Japanese and Thai cohorts, in which significant association of FCGR2B-232Thr/Thr genotype had been already demonstrated (11,12). As shown in Tables 3 and 4, the risk
of CD72-232Thr/Thr genotype was observed only in the presence of CD72-*1/*1 genotype in both cohorts. In the Caucasian samples, frequency of FCGR2B-232Thr was too low to be analyzed for epistatic interaction (14).

When the data from these two cohorts were combined by meta-analysis, significant association with the onset of SLE was observed only in FCGR2B-232Thr/Thr plus CD72-*1/*1 genotypes when compared with FCGR2B-232ile/ile plus CD72-*2/*2 genotypes (Table 5). In all comparisons, no heterogeneity was detected in meta-analysis. Taken together, these observations indicated that CD72-*2 allele, associated with preferential production of AS isoform, decreases risk of development of SLE conferred by FCGR2B-232Thr allele product in an epistatic manner.

### DISCUSSION

In the present study, we demonstrated that human CD72 polymorphisms are associated with the relative quantity of a novel alternative splicing product, and also with the presence of nephritis among the patients with SLE. Of particular interest, when the genetic interaction between CD72 and FCGR2B was examined, the previously detected SLE susceptibility conferred by FCGR2B-232Thr/Thr genotype (11–13) substantially decreased in the presence of CD72 allele associated with increased quantity of the alternative splicing product. This alternative mRNA form was confirmed to be expressed at the protein level. These observations suggest that CD72 and FcγRIIB may directly or indirectly interact at the protein level to regulate B cell activation, and their inhibitory potentials are influenced by their polymorphisms.

Because CD72 is a type II membrane protein, C-terminal region constitutes the membrane-distal portion of extracellular region (20). In the deduced human AS isoform, a stretch of 42 amino acids out of 243 amino acids in the extracellular region is replaced with a totally different sequence of 49 amino acids. Nevertheless, this alteration is predicted to preserve C-type lectin structure (38), thereby suggesting that the AS isoform represents a functional polymorphism possessing differential properties. In mice, four alleles of CD72 with amino acid substitutions are known, some of which are caused by differences in the mode of splicing (39–42). This implies that regulation of CD72 function by splicing may be conserved in evolution. Among murine alleles, CD72* has been shown to be involved in the susceptibility to vasculitis in MRL/lpr mice (34), and also to SLE induced by an immunization of a synthetic peptide representing Sm B/B’ sequence (35). Of particular interest, this allele contains multiple amino acid substitutions as well as a deletion of seven amino acid stretch in the membrane-distal portion of the extracellular domain that is altered in human CD72 AS isoform. Functional difference in mouse CD72 alleles has not been investigated, although

### Table 3. Epistatic interaction between FCGR2B and CD72 for the susceptibility to SLE in the Japanese population

<table>
<thead>
<tr>
<th>FCGR2B</th>
<th>CD72-*1/*1</th>
<th>SLE (n = 51)</th>
<th>ctr (n = 98)</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr/Thr</td>
<td>6 (11.8)</td>
<td>3 (3.1)</td>
<td>3.20 (0.76–13.5)</td>
<td>9 (11.0)</td>
</tr>
<tr>
<td>Ile/Thr</td>
<td>18 (35.3)</td>
<td>38 (38.8)</td>
<td>0.76 (0.34–1.67)</td>
<td>29 (35.4)</td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>27 (52.9)</td>
<td>57 (58.2)</td>
<td>0.76 (0.37–1.56)</td>
<td>44 (53.7)</td>
</tr>
</tbody>
</table>

See Table 4 for definitions.

### Table 4. Epistatic interaction between FCGR2B and CD72 for the susceptibility to SLE in the Thai population

<table>
<thead>
<tr>
<th>FCGR2B</th>
<th>CD72-*1/*1</th>
<th>SLE (n = 25)</th>
<th>ctr (n = 47)</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr/Thr</td>
<td>6 (24.0)</td>
<td>2 (4.3)</td>
<td>7.88 (1.51–41.1)</td>
<td>4 (10.3)</td>
</tr>
<tr>
<td>Ile/Thr</td>
<td>7 (28.0)</td>
<td>20 (42.6)</td>
<td>0.93 (0.28–3.00)</td>
<td>17 (43.6)</td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>12 (48.0)</td>
<td>25 (53.2)</td>
<td>1.26 (0.43–3.66)</td>
<td>18 (46.2)</td>
</tr>
</tbody>
</table>

Proportions of the patients or controls with particular FCGR2B genotypes among the total patients or controls with each CD72 genotype are shown in parentheses. Odds ratio (OR) and 95% confidence interval (CI) are calculated against CD72-*2/*2 plus FCGR2B-232ile/ile. In the Thais, eight SLE and 22 control samples could not be genotyped for FCGR2B due to DNA conditions, and were excluded from this analysis.

### Table 5. Meta-analysis of the combinatorial effect of CD72 and FCGR2B polymorphisms for the susceptibility to SLE in the Japanese and Thai cohorts

<table>
<thead>
<tr>
<th>FCGR2B</th>
<th>CD72</th>
<th>*1/*1</th>
<th>*1/*2</th>
<th>*2/*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr/Thr</td>
<td>4.63 (1.47–14.6)</td>
<td>1.60 (0.67–3.86)</td>
<td>1.58 (0.32–7.90)</td>
<td></td>
</tr>
<tr>
<td>Ile/Thr</td>
<td>0.80 (0.41–1.55)</td>
<td>1.33 (0.73–2.42)</td>
<td>0.77 (0.26–2.34)</td>
<td></td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>0.89 (0.49–1.61)</td>
<td>0.91 (0.52–1.58)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

The data shown in Tables 3 and 4 were combined by meta-analysis under the random effects model. Odds ratio and 95% confidence interval of each genotype combination against FCGR2B-232ile/ile plus CD72-*2/*2 are shown. Although the FCGR2B-232Thr genotype had been shown to be associated with SLE in both populations (11,12), significant association was observed only in the presence of CD72-*1/*1 genotype (P = 0.009).
considerable structural difference has been suggested for \textit{CD72} from the difference in the reactivity with monoclonal antibodies (43).

The binding sites on \textit{CD72} with putative ligands such as membrane Ig (24) or CD100 (28) have not yet been mapped. The protective effect of \textit{CD72}*-2 allele might be explained if the AS product has stronger inhibitory potential on BCR signaling, for example stronger affinity to BCR or weaker affinity to CD100. \textit{FCGR2B}-Ile232Thr polymorphism has been shown to be associated with changes in the inhibitory potential, although the effect seems to be inconsistent in different experimental systems (15,44). The inhibitory potential of FcγRIIb is dependent on phosphorylation of ITIM through positive BCR signaling (45); thus, it is likely that differences in \textit{CD72} function may influence the potential for inhibitory signals mediated by FcγRIIb. Alternatively, it is also possible that positive signals from \textit{CD72} may be decreased in the AS isoform product, thereby rendering carriers of *2 allele protective for the development of SLE or nephritis.

The expression of AS isoform in PBMC is considered to be stable at least at the mRNA level, because substantial quantity of AS isoform was detected in virtually all PBMCs from 32 individuals (Fig. 3), and the full-length cDNA of the AS isoform was readily amplified from PBMCs. Because antibodies that can specifically detect the AS protein product are not currently available, we cannot examine whether the AS isoform is differentially distributed among subsets of B cells or dendritic cells. Such studies as well as functional consequences of \textit{CD72} AS expression should be addressed in future studies, some of which are underway in our laboratory.

\textit{CD72} polymorphisms per se were not associated with SLE, but were associated with the presence of nephritis in SLE. SLE is a heterogeneous disease, and difference in the genetic background with respect to clinical manifestations has frequently been reported (1,3). Notably, \textit{FCGR2B}-232Thr was also preferentially associated with lupus nephritis (11,13). Furthermore, association with \textit{FCGR2B}-232Thr is observed in the Asians, but not in the Caucasians (11–16), where allele frequency of \textit{FCGR2B}-232Thr is low. Thus far, association of \textit{CD72} polymorphisms with SLE has also been observed in the Asians, but not in the Caucasians. The frequency of \textit{CD72}*-2/1/1 appears to be slightly lower in the Caucasians. These observations raise the possibility that the effect of \textit{FCGR2B}-232Thr and \textit{CD72} polymorphisms on SLE susceptibility is dependent on each other. All these hypotheses should be addressed by further association studies on multiple populations and by functional analysis at the protein level. Specifically, it would be important to test the genetic interaction between \textit{CD72} and \textit{FCGR2B} in African-Americans, where the allele frequency of \textit{FCGR2B}-232The is rather high (15). It would also be interesting to examine the interaction of \textit{CD72} with \textit{FCGR2B} promoter polymorphism, which was recently shown to be associated with SLE in the Caucasians (17,18).

The epistatic interaction between \textit{FCGR2B} and \textit{CD72} was slightly different from that between the Japanese and Thai cohorts. In the Japanese, the difference between \textit{CD72}*-1*/1 and *1/2 groups appeared to rest more in the controls than in the patients. In the patients, striking difference in the \textit{FCGR2B} genotype distribution was observed between carriers (*1/*1 + *1/*2) and non-carriers (*2/*2) of \textit{CD72}*-1 allele. On the other hand, in the Thai population, striking difference in the \textit{FCGR2B} genotype distribution was observed between \textit{CD72}*-2 absent (*1/*1) and present (*1/*2 + *2/*2) groups. Such a difference could perhaps be ascribed to rather small sample size of each population. By combining the data of the two cohorts using meta-analysis, statistically significant effect of \textit{FCGR2B}-232Thr/Thr was observed only in the \textit{CD72}*-1/*1 genotype (Table 5).

Although the gender ratio was substantially different between cases and controls in the Asian cohorts, adjustment for gender ratio did not alter the results, because the genotype distributions were not different between male and female controls. The patient populations were older than the controls, but because of the low prevalence of SLE, difference in age distribution should not affect the results significantly, and even if it did, it should have made the analyses more conservative. It is possible that some of the patients classified as ‘without nephritis’ may later develop nephritis; however, in most of the patients, disease duration was >1 year at the time of the study, and because the proportion of the patients with nephritis (59% in both populations) was similar to general prevalence of nephritis in SLE, misclassification with respect to the presence of nephritis is not considered to pose a substantial problem.

Recently, a number of observations indicated physiological or pathological significance of alternative splicing and its role in the genetic background of diseases (46). In the immune system, alternative splicing products are known for important genes such as CD28 (47), CTLA-4 (CD152) (48,49) and CD40 (50). Notably, it was recently reported that genomic polymorphism of human CTLA-4 is significantly associated with expression of alternatively spliced variants and with susceptibility to Grave’s disease, autoimmune hypothyroidism and type 1 diabetes (48). Our present observations further emphasize the role of genomic polymorphism in the regulation of alternative splicing and its relevance in complex diseases. Our knowledge of the mechanism of alternative splicing is still limited (45,51). In the case of \textit{CD72}, our present observations suggested that both VNTR and four-nucleotide deletion act in combination as cis-acting intronic splicing enhancer or silencer.

It is estimated that at least half of the human genes are alternatively spliced (52,53), and this proportion is especially higher in genes coding for molecules in the immune system (52). Search for functionally relevant alternative splicing variants in a genome-wide manner (53) and their relationship with genomic polymorphisms as well as mechanisms of regulation of alternative splicing will provide important clues for the understanding of genetic background of complex diseases and new therapeutic approaches.

**MATERIALS AND METHODS**

**Subjects**

In the Japanese cohort, 160 patients with SLE (male 10, female 150, mean age ± SD 41.1 ± 13.9) and 277 controls (male 150, female 127, mean age ± SD 35.0 ± 10.0) were recruited at Juntendo University and The University of...
Tokyo (Tokyo, Japan). In the Thais, 87 patients with SLE (male 11, female 76, mean age ± SD 30.0 ± 10.5) and 187 controls (male 103, female 84, mean age ± SD 37.4 ± 9.6) were recruited at the Siriraj Hospital, Mahidol University (Bangkok, Thailand). All individuals were unrelated Japanese living in Tokyo, or unrelated present-day Thais.

Association was also examined using genomic DNA from 94 Caucasian families recruited at University of California at Los Angeles, in which one or more members had SLE (14). In all families, both parents of the SLE patients were available for study. SLE was classified according to the American College of Rheumatology (ACR) criteria (54).

Possion of nephritis was defined in accordance with the ACR criteria (persistent proteinurin >0.5 g/day or 3+ or cellular cast). Patients with nephritis at the time of the study or with previous history of nephritis were classified into ‘with nephritis’ group. In most patients, disease duration at the time of study entry was >1 year.

The study was approved by the Research Ethics Committees of the Graduate School of Medicine, The University of Tokyo, Juntendo University and Mahidol University, and by the Human Subject Protection Committee of the University of California at Los Angeles.

Genomic DNA

Genomic DNA from patients and healthy individuals was purified from peripheral blood using the QIAamp blood Kit (Qiagen, Hilden, Germany).

Variation screening of human CD72

Variations of human CD72 gene were screened using direct sequencing. Genomic DNA samples from 10 healthy individuals and 10 SLE patients were used for screening. All exons with ~100 bp of flanking introns and ~1 kb of promoter region of human CD72 gene were amplified using specific primer sets designed based on the nucleotide sequences NM_001782 and NT_008413 (LocusLink, http://www.ncbi.nlm.nih.gov/locuslink/). PCR was carried out using GeneAmp reagents and AmpliTaq Gold DNA Polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The amplification conditions consisted of initial denaturation at 96°C for 10 min, followed by 35 cycles of 96°C for 30 s, annealing temperature for 30 s and 72°C for 30 s in GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The direct sequencing analysis was performed using ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

CD72 genotyping

Genotyping of −918G>A, −223C>T, −155T>G, c.109A>G, c.338G>A, IVS4+14T>A, c.701C>T, c.728G>A and IVS8(−23)(−(20)delCTCA was done using PCR–single strand conformation polymorphism (SSCP). PCR was carried out as described earlier, followed by SSCP analysis using polyacrylamide gel (acylamide/bis, 49: 1) and constant temperature control system (AB1600 and AE6370, Atto, Tokyo, Japan). After electrophoresis, the separated fragments were visualized with silver staining (Daichi Pure Chemicals, Tokyo, Japan). The primer sequences, annealing temperatures and SSCP conditions are listed in Supplementary Material, Table S2.

Genotyping of IVS8VNTR was done using PCR–single sequence length polymorphism (SSLP). The primer sets and annealing temperatures are listed in Supplementary Material, Table S2. PCR–SSLP analysis was performed using 10% polyacrylamide gel (acylamide/bis, 29: 1). After electrophoresis, separated fragments were visualized with SYBER Gold (Molecular Probes, Eugene, OR).

FCGR2B-Ile232Thr genotyping

FCGR2B-Ile232Thr genotyping was done as previously described (11–13).

Semi-quantitative RT–PCR

The ratio of common and AS CD72 isoforms was estimated using semi-quantitative RT–PCR. PBMCs were isolated from 20 ml of venous blood samples of 13 healthy individuals with CD72*1/*1, 11 with *1/*2 and 8 with *2/*2 genotypes by density-gradient centrifugation. Total RNA was extracted from PBMCs using the RNeasy kit (Qiagen). First-strand cDNA was synthesized using Oligo(dT) primer (Promega, Madison, WI, USA) and ReverTra Ace (Toyobo, Osaka, Japan).

RT–PCR to detect exon 8 skipping was performed using primers designed within exon 6 and exon 9 (5’-GGCAGGAGGCCAAAAACAAA-3‘ and 5’-ACCCCATTTCTACCAGGGA-3‘). One microliter of template cDNA solution was placed into 25 µl of reaction solution, and amplification was done using AmpliTaq Gold DNA polymerase (Perkin-Elmer). To achieve linear amplification, 28 cycles were found to be optimal in preliminary experiments. The amplification conditions consisted of initial denaturation at 96°C for 10 min, followed by 28 cycles of 96°C for 30 s, 70°C for 30 s and 72°C for 1 min in GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). Quantitation of AS/common isoforms ratio was performed using Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, CA, USA). These experiments were repeated three times with essentially identical results.

Expression of the CD72 isoforms in COS-7 cells

Full-length cDNA encoding common and AS forms of CD72 were amplified from first-strand cDNA derived from PBMCs with GeneAmp reagents and TaKaRa LA Taq (TaKaRa, Otsu, Shiga, Japan) and specific primer set (5’-GCAGAGCCTGCTCAGGACCAT-3‘ and 5’-ACCCCATTTCTACCAGGGA-3‘) under conditions of initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s, 64°C for 30 s and 72°C for 1.5 min in GeneAmp PCR system 9700. These cDNAs were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen-Life Technologies, Carlsbad, CA, USA) and subjected to sequence analysis. The common or AS isoform without misincorporation of nucleotides was transfected into COS-7 cells by lipofection using Lipofectamine 2000 (Invitrogen-Life Technologies). After 36 h, the cells were lysed with boiling 2% SDS sample buffer and the lysates were subjected to SDS–PAGE followed by western
blotting with a rabbit polyclonal antibody against cytoplasmic region of human CD72 (H-96, Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase, and the signals were detected using ECL chemiluminescence system (Amersham Biosciences, Uppsala, Sweden).

Minigene assay

CD72 genomic DNA segments containing 109 bp of intron 6, whole exon 7, intron 7, exon 8, intron 8 and 238 bp of exon 9 were amplified by PCR from genomic DNA derived from two donors, one with CD72 exon 9 were amplified by PCR from genomic DNA derived whole exon 7, intron 7, exon 8, intron 8 and 238 bp of CD72 genomic DNA segments containing 109 bp of intron 6, Minigene assay (Sweden).

Chemiluminescence system (Amersham Biosciences, Uppsala, Sweden) was performed using a goat polyclonal antibody against cytoplasmic vector (Fig. 4A, were recloned into VNTR were cut out between two Nco I sites, and were exchanged between EcoRI and EcoRV sites of pcDNA3.1-Zeo(+) vector (Invitrogen-Life Technologies). Therefore, the final minigene constructs were composed of 68 bp of exon 7, whole intron 7, exon 8, intron 8 and 238 bp of exon 9 (Fig. 4A, *1/del and *2/+).

To differentially examine the effects of IVS8VNTR and CTCA deletion on splicing, the other two combinations, *1/+ and *2/del, were prepared. The minigenes, *1/del and *2/+, were first subcloned into pBlueScript SK(+) (Invitrogen-Life Technologies) lacking internal NeoI site. Subsequently, the 3' portions encompassing the CTCA deletion but not the VNTR were cut out between two NcoI sites, and were exchanged between *1/del and *2/+ . These new combinations were recloned into EcoRI and NotI sites of pcDNA3.1-Zeo (+) vector (Fig. 4A, *1/+ and *2/del).

After the sequences were confirmed, these four minigene constructs were introduced into COS-7 cells by lipofection using Lipofectamine 2000 (Invitrogen-Life Technologies). After 24 h of transfection, total RNA was extracted as described earlier.

Semi-quantitative RT–PCR was performed with specific primer sets placed within exon 7 and exon 9 (5'-CATATT GGACTGGCCTCAGC-3' and 5'-ACCCCCATTCTACCATGGG GA-3') under conditions of initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s, 63°C for 30 s and 72°C for 2 min in GeneAmp PCR system 9700. PCR products were blunt-ended with T4 DNA polymerase, and the signals were detected using ECL system (Amersham). Bound antibody was detected with a goat polyclonal antibody against cytoplasmic vector (Fig. 4A, were recloned into pcDNA3.1-Zeo(+) vector (Invitrogen-Life Technologies).

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