TNFSF15 transcripts from risk haplotype for Crohn’s disease are overexpressed in stimulated T cells

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TNFSF15 is a susceptibility gene for Crohn’s disease (CD). It remains to be elucidated how the associated single nucleotide polymorphisms (SNPs) in TNFSF15 affect the susceptibility to CD. Because there are no non-synonymous SNPs in TNFSF15, we speculated that one or more of the SNPs associated with CD may act as cis-regulatory SNPs. To reveal the effects of the SNPs on the transcriptional activity of TNFSF15, we first examined the allelic expression imbalance of TNFSF15 in peripheral blood mononuclear cells (PBMCs). When PBMCs stimulated by phytohemagglutinin (PHA) were examined, the allelic ratio of mRNA transcribed from the risk haplotype to the non-risk haplotype increased, compared with the ratio without stimulation. When peripheral blood T cells and Jurkat cells stimulated by phorbol 12-myristate 13-acetate + ionomycin were examined, an allelic expression imbalance similar to that observed in PBMCs stimulated by PHA was confirmed. The promoter assay in stimulated Jurkat cells showed that the luciferase activity of the promoter region (−979 to +35) of the risk haplotype was significantly higher than that of the non-risk haplotype, and deletion and mutagenesis analysis demonstrated that this difference resulted from the −358T/C SNP. The promoter activity of −358C (risk allele) was higher than that of −358T (non-risk allele) in stimulated T cells. This effect of −358T/C on the transcriptional activity in stimulated T cells may confer susceptibility to CD.

INTRODUCTION

Crohn’s disease (CD; MIM 266600) is a chronic condition characterized by remitting and relapsing inflammation of the small and/or large intestines. The causes of CD are still unknown, but CD is considered to be a complex, multifactorial disease partly determined by a genetic predisposition (1,2). Over the last decade, linkage and association studies using positional mapping and candidate gene approaches identified specific genetic variants associated with CD, including three genetic variants in CARD15/NOD2 (3,4) and, recently, genome-wide association studies and their meta-analyses have shown more than 30 distinct susceptibility loci for CD (5).

In 2005, Yamazaki et al. (6) performed a large-scale case–control study using single nucleotide polymorphism (SNP) markers and reported that polymorphisms in tumor necrosis factor superfamily 15 (TNFSF15) conferred susceptibility to CD in both Japanese and UK populations. These two populations have two major haplotypes (haplotypes A and B) which is formed by five SNPs (rs3810936, rs6478108, rs6478109, rs7848647 and rs7869487): haplotype A is a CD risk haplotype and haplotype B is a CD non-risk haplotype. This association has been replicated by several studies including our previous study in Japan (7) and meta-analyses of genome-wide association studies in European populations (5). In addition to these genetic studies, several studies have already shown the significance of TNFSF15 in the pathogenesis of CD. TNFSF15 is a novel member of the TNFSF (8), and its mRNA and protein expression are upregulated in macrophages and CD4+/CD8+ lymphocytes of the inflamed intestine in inflammatory bowel disease, particularly in CD (9).
TNFSF15 binds to the death-domain receptor 3 and provides costimulatory signals to activated lymphocytes (8). Through this interaction, TNFSF15 induces the secretion of IFN-gamma (10,11) and may participate in the development of T helper (Th)-1-type effector responses (9). Therefore, overexpressed TNFSF15 promotes a Th-1-mediated immunological state and initiates or exacerbates the mucosal inflammation of CD. In addition, Takedatsu et al. (12) showed that TNFSF15 enhanced not only the effector functions of Th-1 T cells but also those of Th-17 T cells in the intestinal mucosa of murine chronic colitis.

Thus, it is likely that TNFSF15 is involved in the pathogenesis of CD. However, there have been limited studies regarding how the associated SNPs in TNFSF15 affect the susceptibility to CD. There are no non-synonymous SNPs in TNFSF15, and since the odds ratios of −358T/C (rs6478109) and −638A/G (rs7848647) in the 5′-flanking region were the highest in our previous association study (7) and the mRNA and protein expression of TNFSF15 were reported to be up-regulated in CD (9), we speculated that one or both of these two SNPs (−358T/C and −638A/G) may act as cis-regulatory SNPs. To reveal the effects of the two SNPs on the transcriptional activities of TNFSF15, we first examined the allelic imbalance of TNFSF15 mRNA transcribed from the risk haplotype (−638G/−358C/+15524G) and non-risk haplotype (−638A/−358T/+15524A) in peripheral blood mononuclear cells (PBMCs) in heterozygote samples using rs3810936 (+15524A/G) in exon 4 as a haplo- typic marker. Because we did not know the cell types in which these associated SNPs act as cis-regulatory SNPs, we used the allelic imbalance in gene expression as a guide to find these cell types (13). We have determined the allelic expression imbalance of TNFSF15 in peripheral blood T cells and Jurkat cells stimulated using phorbol 12-myristate 13-acetate (PMA) and ionomycin. Then, using Jurkat cells, promoter analysis was performed in vitro. We show that −358T/C SNP affects the transcriptional activity of TNFSF15 in activated T cells. These results suggest that −358T/C SNP acts as regulatory SNP in stimulated T cells and contributes to the genetic susceptibility to CD.

RESULTS

Allelic expression imbalance of TNFSF15 in stimulated T cells

To quantify the allelic ratios of TNFSF15 mRNA transcribed from the TNFSF15 risk haplotype to that from the non-risk haplotype, a calibration curve was made using standard mixtures consisting of various ratios of two plasmids, as described in Materials and Methods (Fig. 1). The intensity (peak height) ratios of the standard mixtures were plotted against the known mole ratios of standard plasmids (n = 3). A linear relationship was obtained from the expression ratio of 0.5 to that of 2.0, and the coefficient of determination (R²) was 0.99. The linear regression equation was as follows: the allelic expression ratio of TNFSF15 = 0.5α + 0.1, where α denotes the ratio of the peak height of the risk-haplotype-specific transcript to that of the non-risk-haplotype-specific transcript. The allelic ratios of the heterozygotes’ genomic DNA were 1.09 ± 0.08 (n = 13, Fig. 2), which also support the appropriateness of this assay.

PBMCs from healthy volunteers who were heterozygotes of the CD risk and non-risk haplotypes were obtained for analysis of the allelic expression imbalance of TNFSF15. In PBMCs under the lipopolysaccharide (LPS) and phytohemagglutinin (PHA) stimulation conditions, the expression level of TNFSF15 mRNA was significantly up-regulated (Fig. 2A and C). Then, the allelic expression ratio in PBMCs without stimulation was examined. The allelic expression ratios in stimulated PBMCs by LPS were not changed significantly compared with those in the non-stimulated PBMCs (Fig. 2D). In contrast, the allelic expression ratio in stimulated PBMCs by PHA (1.39 ± 0.34) was significantly higher than that of genomic DNA (1.09 ± 0.08, P = 0.007) and significantly increased compared with the ratio in the non-stimulated PBMCs (0.80 ± 0.36, P = 0.0002) (Fig. 2B). Because PHA stimulates T cells relatively specifically, these results suggested that an allelic expression imbalance may occur in stimulated T cells. Then, the allelic expression ratio was examined using isolated peripheral blood T cells. Without stimulation, the expression levels of TNFSF15 mRNA in peripheral blood T cells were too low to determine the allelic expression ratio; thus, the allelic expression ratio was examined only in stimulated peripheral blood T cells. The results are shown in Figure 2B. The allelic expression imbalance was confirmed in peripheral blood T cells stimulated using PMA + ionomycin (1.35 ± 0.08, n = 6, versus genomic DNA, P = 0.011). These results strongly suggested that some of the SNPs located in the major haplotypes of TNFSF15 act as regulatory SNPs at least in T cells and that the transcriptional activity of the risk haplotype may be higher than that of the non-risk haplotype in stimulated T cells.

Further, to examine this allelic expression imbalance in T cells, Jurkat cells were selected from various T cell lines, because these cells are heterozygotes of the CD risk haplotype/non-risk haplotype and so the allelic expression imbalance can be assayed. By stimulation of Jurkat cells using PMA and ionomycin, the mRNA of TNFSF15 increased significantly (relative expression, >500; n = 3) compared with that in non-stimulated cells (relative expression, 1), and the
assay of the allelic expression imbalance was then performed. Because in non-stimulated Jurkat cells, the expression level of TNFSF15 mRNA was too low to obtain an accurate value of the allelic expression ratio, the ratio was examined only in the stimulated Jurkat cells. The allelic expression ratio in stimulated Jurkat cells was 1.93 +/− 0.32 (n = 3), indicating that stimulated Jurkat cells also showed an allelic expression imbalance of TNFSF15 mRNA, and the amount of mRNA transcribed from the risk haplotype was more than that from the non-risk haplotype. These findings were similar to the results of peripheral blood T cells stimulated by PMA and ionomycin (Fig. 2B). Therefore, further promoter analysis was performed using Jurkat cells.

Higher promoter activity of TNFSF15 CD risk haplotype

Although the results concerning the allelic expression imbalance suggested that the promoter activity of the risk haplotype was higher than that of the non-risk haplotype in stimulated T cells, these results do not indicate which SNPs influence the differences in transcriptional activity between the risk haplotype and the non-risk haplotype in stimulated T cells. As the odds ratios of −358T/C (rs6478109) and −638A/G (rs7848647) in the 5′-flanking region of TNFSF15 were the highest in our previous association study, these two SNPs were further examined in reporter assays. To estimate the effect of the promoter sequences of the risk haplotype (−358C/−638G) and the non-risk haplotype (−358T/−638A) on the transcriptional activity, the luciferase activities were compared between pGL4-989(−638G/−358C) and pGL4-989(−638A/−358T) (Fig. 3). In stimulated Jurkat cells, the luciferase activity of pGL4-989(−638G/−358C) was significantly higher than that of pGL4-989(−638A/−358T), whereas in non-stimulated Jurkat cells, there were no differences in the luciferase activities between them (Fig. 4A and B). To determine the SNP responsible for the difference in the luciferase activity between pGL4-989(−638G/−358C) and pGL4-989(−638A/−358T) in stimulated Jurkat cells, an additional two reporter plasmids, pGL4-989(−638G/−358T) and pGL4-989(−638A/−358C), were also examined (Fig. 3). Among the four reporter plasmids, the luciferase activities of pGL4-989(−638G/−358C) and pGL4-989(−638A/−358C) were significantly higher than those of pGL4-989(−638A/−358T) and pGL4-989(−638G/−358T) (Fig. 4B). These results indicated that
result that the luciferase activity of pGL4-500(−989 to +35) was significantly higher than that of the CD non-risk haplotype in stimulated Jurkat cells, but not in non-stimulated Jurkat cells and the difference in the promoter activity resulted from the SNP at position −358.

We first examined the allelic imbalance in mRNA expression in PBMCs in order to determine whether some of the SNPs located in the major haplotypes of TNFSF15 (CD risk haplotype or CD non-risk haplotype) acted as regulatory SNPs. This allelic imbalance was examined by measuring the allelic expression ratio of the CD risk haplotype-specific transcript to the CD non-risk haplotype-specific transcript. Because this method measures both haplotype-specific transcripts in the same sample, the measurement is much less impacted by the biological variation that occurs when comparing expression levels between different samples. In addition, this method does not require normalization to a house-keeping gene. Although several reports have already demonstrated that an allelic imbalance in gene expression can serve as a guide to determine cis-acting regulatory SNPs (15–17), the results of allelic imbalance in the present study offered more useful information than expected (18). These findings, indeed, provided important information for us to design promoter assays, namely, that one of the cell lines used in the promoter assay should be a T cell line for comparison between with and without stimulation. Since a previous report showed that the TNFSF15 protein is expressed mainly on macrophages in the involved intestine of CD (9), without the results of the allelic imbalance study, we would have used only cell lines of monocytes in the promoter assay and would have obtained no significant results. In Figure 2B and D, there was one sample in which the allelic expression ratio in the non-stimulated PBMCs was higher than in the others. These two samples were obtained from the same healthy subject (subject 7). The expression level of TNFSF15 in that sample was the same as that in other samples, and subject 7 was also confirmed to have haplotypes A and B using the parents’ genotype. We again obtained PBMCs from subject 7 1 month later after the first sampling and examined the allelic expression imbalance of TNFSF15, but the results were the same as in the first experiments. At present, we cannot explain the exact reason, but there is a possibility that subject 7 has unexpected rare mutation near TNFSF15.

Because this method measures both haplotype-specific transcripts in the same sample, the measurement is much less impacted by the biological variation that occurs when comparing expression levels between different samples. In addition, this method does not require normalization to a house-keeping gene. Although several reports have already demonstrated that an allelic imbalance in gene expression can serve as a guide to determine cis-acting regulatory SNPs (15–17), the results of allelic imbalance in the present study offered more useful information than expected (18). These findings, indeed, provided important information for us to design promoter assays, namely, that one of the cell lines used in the promoter assay should be a T cell line for comparison between with and without stimulation. Since a previous report showed that the TNFSF15 protein is expressed mainly on macrophages in the involved intestine of CD (9), without the results of the allelic imbalance study, we would have used only cell lines of monocytes in the promoter assay and would have obtained no significant results. In Figure 2B and D, there was one sample in which the allelic expression ratio in the non-stimulated PBMCs was higher than in the others. These two samples were obtained from the same healthy subject (subject 7). The expression level of TNFSF15 in that sample was the same as that in other samples, and subject 7 was also confirmed to have haplotypes A and B using the parents’ genotype. We again obtained PBMCs from subject 7 1 month later after the first sampling and examined the allelic expression imbalance of TNFSF15, but the results were the same as in the first experiments. At present, we cannot explain the exact reason, but there is a possibility that subject 7 has unexpected rare mutation near TNFSF15.

Although the in vitro promoter assay clearly showed that the promoter activity (−989 to +35) of the CD risk haplotype was significantly higher than that of the CD non-risk haplotype in stimulated Jurkat cells and that this difference in the promoter activity resulted from the −358T/C SNP, this study could not confirm that the −358T/C SNP fully explains the allelic imbalance detected in peripheral blood T cells stimulated using PMA + ionomycin, because not all of the SNPs located in the CD risk/non-risk haplotypes were examined in the promoter assay. Although this is a limitation of this study, it is likely that the −358T/C SNP explains the allelic

DISCUSSION

In the present study, we report that, in stimulated peripheral T and Jurkat cells, the expression of TNFSF15 mRNA transcribed from the CD risk haplotype was higher than that from the CD non-risk haplotype. In the reporter assays, the promoter activities of the CD risk haplotype (−989 to +35) were significantly higher than those of the CD non-risk haplotype in stimulated Jurkat cells, but not in non-stimulated Jurkat cells and the difference in the promoter activity resulted from the SNP at position −358.

SNP at position −358 affected the luciferase activity, and the plasmids containing the C allele showed higher luciferase activity than those containing the T allele. In addition, the result that the luciferase activity of pGL4-500(−358T/C) was significantly higher than that of pGL4-500(−358C) and pGL4-500(−358T) have the wild-type sequence. The reporter plasmids mutated (from C to T) at position −358 of pGL4-989(−638G/−358T), pGL4-500(−358C) and pGL4-500(−358T) have the wild-type sequence. The reporter plasmids mutated (from C to T) at position −358 of pGL4-989(−638G/−358T) and (from T to C) at position −358 of pGL4-989(−638A/−358T) were named pGL4-989(−638G/−358T) and pGL4-989(−638A/−358C), respectively.

−358T/C polymorphism influences nuclear protein binding

Electrophoretic mobility shift assay (EMSA) was performed to assess whether the −358T/C SNP is within a binding domain for nuclear proteins. To evaluate the specificity of the shift band, competition assays with excess amounts of unlabeled double-stranded oligonucleotides were performed. Oligonucleotides that contained the T allele (biotin-labeled probe-358T) showed specific binding to nuclear protein extracted from stimulated Jurkat cells, whereas oligonucleotides that contained the C allele (biotin-labeled probe-358C) showed specific binding to nuclear protein extracted from stimulated U937 cells. The reporter assay was performed using anti-GATA1, anti-GATA2 and anti-GATA3 antibodies, but no supershift bands were observed (data not shown).
imbalance detected in stimulated peripheral blood T cells to a certain extent, as both the allelic imbalance and the differences in the promoter activity were found relatively specifically in T cells and T cell lines as well as specifically in a stimulated condition.

EMSA showed that the $-358T/C$ SNP influences nuclear protein binding, and this protein binding was observed specifically when nuclear protein extracted from Jurkat cells was used. These results also strongly suggest that the $-358$ SNP is a regulatory SNP that functions specifically in T cells. However, since we have not determined the transcription factor yet, further experiments are required to confirm that this differential protein binding causes the difference in promoter activity between the TNFSF15 CD risk haplotype and non-risk haplotype.

According to a previous report (6), UK populations have $-358T/C$ SNP (C allele frequency of UK control = 67%), the SNP of which was associated with a combined group of CD and ulcerative colitis. In addition, HapMap data show that Utah residents with ancestry from northern and western Europe (CEU) also have $-358T/C$ SNP (C allele frequency = 64%). Although it is unclear how accurately HapMap samples of CEU reflect the patterns of genetic variation in people with northern and western European ancestry, these data also suggest that some European populations have $-358T/C$ SNP. These data collectively suggest that $-358T/C$ SNP may play an important role in susceptibility to CD in European as well as in Japanese populations.

Several studies have already shown the significance of TNFSF15 in mucosal inflammation of CD and in murine
models (9,19). Prehn et al. (10) reported that INF-gamma production by lamina propria lymphocytes of patients with CD was dose-dependently augmented by TNFSF15. Bamias et al. (19) showed in a mouse model of chronic ileitis that TNFSF15 was expressed on antigen-presenting cells, acts on memory CD4\(^+\)/CD45RB\(^lo\) murine lymphocytes and enhanced the secretion of INF-gamma in the inflamed intestine. Takedatsu et al. (12) reported that, in addition to enhancing the effector functions of Th1, TNFSF15 enhanced IL-17 production from Th17 cells of inflamed intestine. All these results strongly suggest that TNFSF15 enhances the functions of the late/effector phase of Th1 and Th17 immunity. In this study, we show that the transcriptional activity of the TNFSF15 CD risk haplotype is increased in stimulated T cells, whereas we have not yet examined whether the CD risk haplotype increases their expression of TNFSF15 protein. Thus, we cannot explain precisely how the CD risk haplotype actually affects the susceptibility to CD. However, we speculate that the CD risk haplotype increases the protein expression of TNFSF15 in stimulated T cells, on
which the DR3 receptor is expressed, and that TNFSF15 enhances the functions of the late/effector phase of Th1 and Th17 immunity through autocrine/paracrine mechanisms.

In conclusion, the promoter activity of $2^{358}$C (risk allele) was higher than that of $2^{358}$T (non-risk allele) in stimulated T cells. This effect of $2^{358}$T/C on the transcriptional activity in stimulated T cells may confer susceptibility to CD. In addition, the results of the allelic imbalance study provided much information for constructing an efficient in vitro promoter assay.

**MATERIALS AND METHODS**

**Cell isolation and culture**

A total of 13 healthy volunteers who were heterozygotes of the CD risk and non-risk haplotypes were selected for analysis of the allelic expression imbalance of TNFSF15 in PBMCs; one healthy volunteer who was a homozygote of the CD risk haplotype and one healthy volunteer who was a homozygote of the non-risk haplotype were selected for the polymerase chain reaction (PCR) template for constructing the reporter plasmid among the healthy volunteers who had been genotyped in the previous study. The haplotypic phases of the healthy volunteers were determined using their parents’ genotypes. The healthy volunteers gave written and informed consent to participate in this study. The study protocols were approved by the Ethics Committee of Tohoku University School of Medicine. PBMCs were isolated using Lymphoprep Tube (AXIS-SHIELD) from the 13 healthy volunteers. Untouched T cells (>94% purity) were prepared from PBMCs of five healthy volunteers using Pan T Cell Isolation kit (Miltenyi Biotec). PBMCs and peripheral blood T cells were then suspended in RPMI 1640 containing 10% fetal calf serum. After 24 h in culture, PBMCs were incubated at a concentration of $5 \times 10^6$ cells/6 cm dish with or without 1 $\mu$g ml$^{-1}$ LPS (Sigma-Aldrich Corp.) or 10 $\mu$g ml$^{-1}$ PHA (Invitrogen) for 24 h, and peripheral blood T cells were incubated with or without 40 ng ml$^{-1}$ PMA (BIOMOL International L.P.) and 1.5 $\mu$M ionomycin (BIOMOL Research Laboratories Inc.) for 24 h. The Jurkat human leukemia T cell line (TKG 0209), which had already been confirmed as heterozygous for CD risk and non-risk haplotypes, HeLa cells (TKG 0331) and U937 cells (TKG 0279) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. The Jurkat and U937 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and HeLa cells in MEM supplemented with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. Jurkat cells were stimulated with 40 ng ml$^{-1}$ PMA and 1.5 $\mu$M ionomycin for 24 h.

**Extraction of RNA and cDNA synthesis**

Total RNA was extracted using an illustra RNAspin Mini RNA Isolation Kit (GE Healthcare), and the quality of the RNA was verified by running the samples on a 1% agarose gel. The RNA was quantified by measuring the ultraviolet emission at 260 and 280 nm. Ten micrograms of RNA was treated with DNase I to remove genomic DNA. Five micrograms of RNA was reversely transcribed to single-stranded cDNAs with SuperScript III First-Strand Synthesis SuperMix (Invitrogen) using random hexamer primer according to the manufacturer’s protocol.

**SNaPshot analysis**

SNaPshot was used to quantify the allelic ratio of TNFSF15 mRNA transcribed from the risk haplotype to that from the non-risk haplotype. SNaPshot is a primer extension method that relies on the addition of a single dye-labeled dideoxynucleotide to primers localized adjacent to an SNP. The accuracy of quantification using SNaPshot has been shown in several studies (20,21). In this study, rs3810936 (+15524A/G) in exon 4 was used as a haplotypic marker (Fig. 7). The position of the SNP in TNFSF15 was determined according to the reference sequence GenBank NM_005118.2., and the first nucleotide of the exon 1 start site was defined as position 1. The fragment including the SNP (rs3810936) in exon 4 was amplified from cDNA by PCR using a pair of primers (primer 1: 5’-ggagaggcctgtgcagtt-3’/primer 2: 5’-taggaactcggtggcagagg-3’), in which the primers span introns of the genomic sequence so that the PCR product (>15 000 bp) from genomic contamination can be distinguished from the
product (619 bp) generated from the cDNA. In addition to cDNA, the fragment including the SNP (rs3810936) in exon 4 was amplified from genomic DNA by PCR using a pair of primers (primers 2 and 3: 5'-ccgccacacgagctc-3'). An aliquot of the PCR product was subjected to SNaPshot analysis, according to the manufacturer’s protocol (ABI Prism SNaPshot™ Multiplex Kit; Applied Biosystems Inc.). The primer sequences used for SNaPshot analysis were 5'-gtcagtcggatcagctgtggcgggaaagtcgt-3' (primer 4). The product of the primer extension reaction was analyzed on an ABI 310 sequencer (Applied Biosystems). To determine the allelic ratios of the mRNA transcribed from the risk haplotype to that from the non-risk haplotype, a calibration curve (linear regression line) was drawn using the standard samples described below. The standard samples were made using pGEM3Z (Promega). Fragments of the TNFSF15 risk allele and non-risk allele of the SNP (rs3810936) in exon 4 were amplified from the genomic DNA by PCR using a pair of primers (primer 5: 5'-gggtgtaggacgtctgcaacctgaacagca-3'/primer 6: 5'-actccccggagggctgctgctgcaaggg-3'); underlined sequences are recognition sequences for KpnI or Smal, respectively). After digestion of the PCR fragment using KpnI (Takara Bio, Inc.) and Smal (Takara Bio Inc.), the fragment was subcloned in sense orientation into the KpnI/Smal site of pGEM3Z for propagation in bacteria. After propagation, the plasmids were isolated from the bacteria using HiSpeed™ Plasmid Midi kit (Qiagen). The sequence of each plasmid (CD risk allele and CD non-risk allele) was confirmed by direct sequencing. Standard samples (0.01 pmol µl⁻¹) consisting of various ratios of these two plasmids were made for making the calibration curve. To check the appropriateness of this assay, allelic ratios of the heterozygotes’ genomic DNA, where the two alleles are present in an equimolar ratio, were examined.

Quantitative real-time PCR

For measurement of the total amount of the TNFSF15 mRNA transcripts, real-time monitoring of the PCR was performed using LightCycler 1.5 (Roche Diagnostics) and SYBR ExScript RT-PCR Kit (Takara Bio Inc.) according to the manufacturer’s protocol. The expression of β-actin was used as the normalization control. The expression of non-stimulated cells in sample 1 was set at 1, and data were expressed as the relative expression. Melting-point analysis of the RT–PCR product was used to confirm that a single product was generated. The primer sequences were as follows: TNFSF15 primer 7, 5'-gaggggtgtcttggtccatccta-3', TNFSF15 primer 8, 5'-cctagtctcctcagcagtc-3'; β-actin primer 9, 5'-tgacctctttgctgtggcagaaatg-3', β-actin primer 10, 5'-taagctgtctgctcctgcaagaa-3'.

Plasmid construction of luciferase reporter genes

A promoter fragment of the 5' flanking region in the TNFSF15 risk haplotype (−638G/−358C) or non-risk haplotype (−638A/−358T), from −989 to +35 relative to the transcription start site, was amplified from human genomic DNA by PCR using a pair of primers (primer 11: 5'-cgggttgtagccttatggtaaagtgta-3'/primer 12: 5'-cttcgggtgtctgtcagtaa-3'); underlined sequences indicate recognition sequences for KpnI or EcoRV, respectively) (Fig. 2). After digestion of the PCR fragment using KpnI and EcoRV (Takara Bio Inc.), each fragment was subcloned in sense orientation into the KpnI/EcoRV sites of pGL4.11 firefly luciferase vector (Promega) for propagation in bacteria. After propagation, the plasmids were isolated from bacteria using a HiSpeed™ Plasmid Midi kit (Qiagen). Each plasmid was named pGL4-989(−638G/−358C) or pGL4-989(−638A/−358T), respectively.

The 5'-deleted promoter fragments from −500 to +36 were amplified from pGL4-989(−638G/−358C) and pGL4-989(−638A/−358T) by PCR using primers 12 and 13 (5'-ggtgtgtagcagcagtcgctcactac-3'), underlined sequence indicates recognition sequence for KpnI). These fragments were also subcloned into pGL4.11 vectors, and the plasmids were isolated from bacteria. These constructs were named pGL4-500(−358C) or pGL4-500(−358T), respectively.

To exchange the −358C nucleotide of pGL4-989 (−638G/−358C) for the −358T nucleotide, a fragment from −495 to −211 of TNFSF15 was amplified from pGL4-989(−638A/−358T) using a primer pair (primer 14, 5'-ggggtgtagcagcagtcgctcactac-3'/primer 15, 5'-gaaggtgtagcagcagtcgctcactac-3'), underlined sequences indicate recognition sequences for AvrII and EcoRI). After digestion of the PCR fragment and pGL4-989(−638G/−358C) using AvrII (Takara Bio Inc.) and EcoRI (Cosmo Bio Co., Ltd, Tokyo, Japan), the fragment was subcloned in sense orientation into the digested pGL4-989(−638G/−358C). This construct was named pGL4-989(−638G/−358C). To exchange the −358T nucleotide of pGL4-989(−638A/−358T) for the −358C nucleotide, a fragment from −497 to −213 of TNFSF15 was amplified from pGL4-989(−638G/−358C) and was subcloned into the digested pGL4-989(−638A/−358T). This construct was named pGL4-989(−638A/−358C). All the constructs were sequenced to ensure fidelity.

Transient transfection/reporter assay

For transfection, Jurkat cells, U937 cells and HeLa cells were plated on 24-well plates in 1 ml of medium at 2 × 10⁵ ml⁻¹. The cells were transiently transfected for 24 h with 0.5 µg of each reporter construct and 20 ng of pGL4.74 Renilla luciferase vector (Promega) using transfection reagent FuGene 6 (Roche Diagnostics), according to the manufacturer’s protocol. After the transfection, Jurkat cells were incubated with or without 40 ng ml⁻¹ PMA and 1.5 µM ionomycin, and U937 cells were incubated with or without 0.1 µg ml⁻¹ LPS for an additional 24 h and lysed with the passive lysis buffer (Promega). Lysates were analyzed using a Dual-Luciferase Reporter Assay System kit (Promega). The firefly and Renilla luciferase were measured on Centro LB960 (Berthold Technologies GmbH & Co. KG), and the relative luciferase activity was calculated.

Electrophoretic mobility shift assay

Jurkat, HeLa and K562 cells were seeded at 3.2 × 10⁷ per 150 cm² flask and incubated for 24 h. In addition, Jurkat cells were incubated with 40 ng ml⁻¹ PMA and 1.5 µM ionomycin for 24 h. Nuclear extracts were prepared using
a CellLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich Corp.), according to the manufacturer’s protocol. Nuclear extracts were quickly frozen at −70°C. Protein concentrations were determined using a Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Inc.), with bovine serum albumin as the standard.

The sequences of the oligonucleotides containing the SNP (C/T) at −358 in TNFSF15 were 5’-ccgtttcacaatctggaaac-3’ (probe-358C) and 5’-ccgtttcacaatctgaaacc-3’ (probe-358T). Both were prepared with or without 5’ biotin-labeling and hybridized with a complimentary oligonucleotide and used in the double-stranded form.

EMSA was performed using EMSA ‘Gel-Shift’ Kits (Panomics, Inc.), according to the manufacturer’s protocol. Briefly, a total of 5 μg nuclear extracts were incubated with biotin-labeled oligonucleotides for 30 min at 17°C. For competition assays using unlabeled probes, nuclear extracts were pre-incubated with excessive cold probes (150-fold) for 30 min at 17°C before the addition of the biotin-labeled oligonucleotides. Anti-GATA1 (sc-266; Santa Cruz Biotechnology, Inc.), anti-GATA-2 (sc-267; Santa Cruz Biotechnology, Inc.) and anti-GATA3 mAb (sc-268, sc-269; Santa Cruz Biotechnology, Inc.) were used for supershift assay. Nuclear extracts were pre-incubated with 1 μg of each antibody for 30 min at 17°C before addition of the biotin-labeled oligonucleotides. After incubation, samples were loaded onto 6.0% polyacrylamide gels and electrophoresed at 120 V. After electrophoresis, the gel was transferred to a Bio-dyne membrane (Pall Gelman Laboratory) for 45 min at 300 mA. Then, the membrane was baked for 1 h at 80°C in a dry oven. To visualize the DNA bands, the membrane was prepared with streptavidin–horseradish peroxidase conjugate and chemiluminescent substrate and exposed to X-ray film (Hyperfilm™ ECL, GE Healthcare UK Ltd).

**Statistical analysis**

Data were expressed as mean ± standard deviation. In the experiments of allelic imbalance in PBMCs, the allelic ratio of mRNA from the risk haplotype to that from the non-risk haplotype was compared with that of the genomic DNA and also compared between non-stimulated and stimulated PBMCs using paired t-test. When the allelic expression ratio in peripheral blood T cells was examined, the allelic ratio of mRNA was compared with that of genomic DNA using paired t-test. In the reporter assay, the statistical significance of differences in each assay was assessed by Student’s t-test. P-values less than 0.05 were considered significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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