Association between prostaglandin E receptor 3 polymorphisms and Stevens-Johnson syndrome identified by means of a genome-wide association study

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Background: Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory vesiculobullous reactions of the skin and mucosa. They often affect the ocular surface and can result in permanent visual dysfunction.

Objectives: We sought to discover genetic markers for SJS/TEN susceptibility.

Methods: We performed a genome-wide association study with 60 patients and 300 control subjects. We applied stringent filter and visual assessments for selecting single nucleotide polymorphisms (SNPs) and a high false discovery rate threshold. We fine-mapped the region where a candidate SNP was found and confirmed the results by means of sequencing. We evaluated the function of agonist-activated prostaglandin E receptor 3 (EP3), the gene for which contained several SNPs, in regulating cytokine production in human conjunctival epithelial (CE) cells. The expression levels of EP3 in the CE cells from patients and control subjects were also compared.

Results: We identified 3 SNPs that passed the false discovery rate threshold. One (rs17131450) was close to the *EP3* gene. Therefore we analyzed the *EP3* region in detail and identified 5 other SNPs. We confirmed the association between SJS/TEN and all 6 SNPs. Activated EP3 was expressed in control CE cells, and it suppressed polyI:C-stimulated cytokine production, suggesting that EP3 might help prevent ocular surface inflammation. Concordantly, the EP3 levels were much lower in the CE cells of the patients than in those of the control subjects.

Conclusion: We demonstrated, using both genetic and functional analyses, that *EP3* could be a key player in the pathogenesis of SJS/TEN accompanied by ocular complications. (J Allergy Clin Immunol 2010;126:1218-25.)

Key words: *Prostaglandin E receptor 3, Stevens-Johnson syndrome, toxic epidermal necrolysis, genome-wide association study, single nucleotide polymorphism*

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are acute-onset mucocutaneous diseases (Fig 1, *A*) induced by infectious agents or an adverse reaction to a drug.[1-8] Although the annual incidences of SJS and TEN are very low, 0.4 to 1 and 1 to 6 cases per million persons, respectively,[8] they have a significant public health effect because the mortality rate is high (ie, 3% and 27%, respectively). Healthy children and adults can suddenly get these diseases, and any drug approved worldwide is a candidate instigator.[3,9-12] Associations between HLA type and drug-induced severe cutaneous adverse reactions, including SJS and TEN, have been reported.[13-21]

Patients with ocular involvement (50% to 68%)[8,11] exhibit severe conjunctivitis, and corneal epithelial defects often persist because of ocular surface inflammation.[4,22] Even after the skin lesions have healed, ocular surface complications, such as conjunctival invasion of the cornea, severe dryness of the eye, and, in some instances, keratinization of the ocular surface, can persist (Fig 1, *B*).[23] Representative causative drugs of SJS/TEN with ocular involvement are cold remedies, antibiotics, and nonsteroidal anti-inflammatory drugs (NSAIDs).[4,5,7,23] In this study we focused exclusively on patients with SJS/TEN with ocular involvement. Hereafter, "SJS/TEN" denotes SJS/TEN accompanied by ocular complications.

Although the pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established, the extreme rarity of the cutaneous, mucosal, and ocular surface reactions to drug therapies led us to suspect individual susceptibility. Previously, we performed a single nucleotide polymorphism (SNP) association analysis of candidate genes to investigate whether a genetic predisposition for SJS/TEN exists and to identify culpable polymorphisms. We found SJS/TEN-associated polymorphisms in the genes encoding Toll-like receptor 3 (TLR3),[5-7] IL-4 receptor,[24,25] and Fas ligand[26] in ethnic Japanese patients. We also showed that in Japanese patients HLA-A*0206 is strongly associated with the disease.[27,28] Therefore it is quite obvious that not only environmental but also genetic factors contribute to the cause of SJS/TEN.

To elucidate the pathophysiology of SJS/TEN in more detail, in the current study we performed a genome-wide association study (GWAS) and analyzed more than 300,000 SNPs. This method

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Abbreviations used
BRLMM: Bayesian Robust Linear Model with a Mahalanobis distance classifier
CE: Conjunctival epithelium
EP3: Prostaglandin E receptor 3
FDR: False discovery rate
GAPDH: Glyceradlehyde-3-phosphate dehydrogenase
GWAS: Genome-wide association study
HapMap-CHB: HapMap Han Chinese
HapMap-JPT: HapMap Japanese
LD: Linkage disequilibrium
MAF: Minor allele frequency
NSAID: Nonsteroidal anti-inflammatory drug
PGE_2: Prostaglandin E_2
PHCjE: Primary human cultivated conjunctival epithelial
QC: Quality control
SJS: Stevens-Johnson syndrome
SNP: Single nucleotide polymorphism
TEN: Toxic epidermal necrolysis
TLR3: Toll-like receptor 3

permits the identification of genetic loci and genes associated with complex human traits without bias or *a priori* knowledge of the function or involvement of any gene in the disease pathway. For example, by using this strategy, our group identified SNPs in 3 different genomic loci that have modest associations with primary open-angle glaucoma.[29] In the GWAS we found 3 SNPs that were significantly associated with SJS/TEN.

Using a fine-mapping approach, we found several SNPs in the prostaglandin E receptor 3 *(EP3)* gene that were significantly associated with SJS/TEN. Supporting the genetic association of these polymorphisms with the disease, we also found that EP3 suppressed the production of cytokines induced by polyI:C stimulation and that EP3 expression was greatly reduced compared with that seen in control subjects in the conjunctival epithelium (CE) of patients with SJS/TEN, suggesting EP3 contributes functionally to the pathogenesis of SJS/TEN.

METHODS

Patients

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki. The purpose of the research and experimental protocols was explained to all the participants, and their prior written informed consent was obtained.

The diagnoses of SJS and TEN were based on a confirmed history of acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites, including the ocular surface. In the patients with SJS/TEN receiving a diagnosis in the acute stage at our hospital, a histological diagnosis using skin biopsy was also performed (Fig 1, *A*).[30-32] The detailed information of the patients with SJS/TEN and the control subjects who were analyzed is shown in the Methods section and Table E1 of this article's Online Repository at www.jacionline.org.

GWAS and subsequent fine-mapping of SNPs to the *EP3* region

To identify SNPs associated with SJS/TEN by means of a GWAS, we used an Affymetrix GeneChip Mapping 500K Array Set (Affymetrix, Santa Clara, Calif), according to the manufacturer's instructions (see the Methods section in this article's Online Repository).[29]

Fine-mapping analysis of the *EP3* region was performed with the iSelect Custom Infinium Genotyping system (iSelect; Illumina, Inc, San Diego, Calif), according to the manufacturer's instructions (see the Methods section in this article's Online Repository).[29]

SNP confirmation by means of direct sequencing

The 6 SJS/TEN-associated SNPs that showed significant associations ($P < .01$) in the fine-mapping analysis were confirmed by means of sequencing, as described previously (see the Methods section in this article's Online Repository).[7,24-26] The primers for both PCR and sequencing are shown in Table E2 in this article's Online Repository at www.jacionline.org. Each allele was assessed as an independent variable, and separate P values were calculated for each SNP. P values of less than .05 were regarded as statistically significant. In addition, the P values were corrected according to the number of samples tested (Bonferroni correction).

Human conjunctival tissues and primary human cultivated CE cells

For RT-PCR of the human CE, we used human CE cells obtained from healthy volunteers by means of impression cytology. The primary human cultivated conjunctival epithelial (PHCjE) cells were obtained from conjunctival tissue acquired during surgical intervention to treat conjunctivochalasis.

For immunohistochemistry, human conjunctival tissues were prepared from samples obtained during surgeries to reconstruct the ocular surface as treatment for various ocular surface diseases, including SJS and pterygium. As the control, we used the nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes because of aging, resulting in a foreign body sensation on the ocular surface.

For ELISAs, PHCjE cells were cultured as previously described (see the Methods section in this article's Online Repository at www.jacionline.org).[33]

RT-PCR

RT-PCR was performed, as previously described.[34,35] Amplification was performed with DNA polymerase (Takara, Shiga, Japan) for 40 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute for human *EP3* (GeneAmp; Applied Biosystems, Foster City, Calif). The primers for human *EP3* and human glyceradlehyde-3-phosphate dehydrogenase *(GAPDH)* were, respectively: forward 5′- CGT GTA CCT GTC CAA GCA GCG TTG GGA GCA -3′ and reverse 5′- CCG TGT GTG TCT TGC AGT GCT CAA CTG ATG -3′; forward 5′- CCA TCA CCA TCT TCC AGG AG-3′ and (reverse) 5′- CCT GCT TCA CCA CCT TCT TG-3′.

Immunohistochemistry

The human conjunctival tissues were embedded in OCT compound (Sakura Finetek, Torrance, Calif) and flash-frozen in liquid nitrogen. Sections 6 μm thick were cut and fixed in 100% acetone at 4°C for 10 minutes. Immunohistochemistry was performed as previously described (see the Methods section in this article's Online Repository).[35]

ELISA

The amounts of CXCL11, CCL20, IL-6, and IL-8 released into the culture supernatant were determined by means of ELISA with the Human CXCL11, CCL20 DuoSet (R&D Systems, Inc, Minneapolis, Minn) or the OptEIATM IL-6 and IL-8 set (BD PharMingen, San Diego, Calif), respectively, according to the manufacturer's instructions.

Quantitative RT-PCR

Quantitative RT-PCR analyses for *CXCL11*, *CCL20*, and *IL6* mRNAs were performed on an ABI-prism 7700 (Applied Biosystems), as previously reported.[33-35] The primers and probes for human *CXCL11*, *CCL20*, *IL6*, and *GAPDH* were from Applied Biosystems.

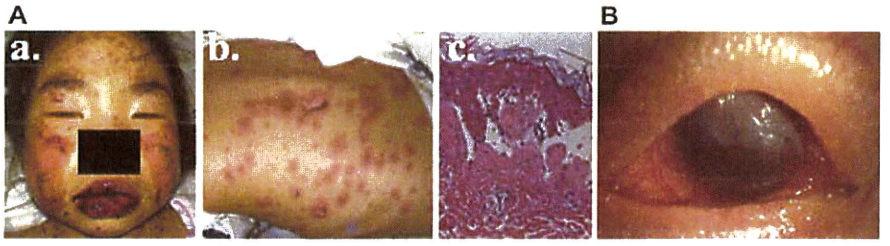

FIG 1. A, Skin eruptions accompanying the mucocutaneous illness of patients with SJS/TEN at the acute stage. *a*, Face with vesiculobullous lesions, conjunctivitis, and swollen crusted lips. *b*, Vesiculobullous lesions of the skin. *c*, Skin biopsy specimens of the erythematous macules showing necrotic keratinocytes and liquefaction degeneration. B, Ocular surface complications of patients with SJS/TEN. Conjunctival invasion results in severe vision loss.

Data analysis

To manage the genotype data and perform statistical analysis, we used a laboratory information management system, LaboServer (World Fusion, Tokyo, Japan). For the genotype frequency comparisons of SNPs between cases and control subjects, we used Hardy-Weinberg equilibrium analysis and the χ^2 test.

For the ELISA and quantitative RT-PCR analysis, data were expressed as mean ± SEM and were evaluated by using the Student *t* test.

RESULTS

GWAS

After genotyping 500,568 SNPs from 60 patients with SJS/TEN (cases) and 300 control subjects, we selected 313,924 SNPs using the stringent criteria chosen for our quality control (QC) filter (see the Methods section in this article's Online Repository). To identify SNPs associated with SJS/TEN, we compared the genotype frequency of each SNP between cases and control subjects. Twenty-five SNPs passed the threshold for the false discovery rate (FDR; 0.05; see Fig E1 in this article's Online Repository at www.jacionline.org). We then visually checked the 2-dimensional cluster plots of these SNPs (see the Methods section in this article's Online Repository at www.jacionline.org), and 3 of them passed our QC test (Table I). In subsequent experiments we focused on an SNP (rs17131450) that mapped close to the *EP3* gene, which is located in the 1p31 region of the human genome (Fig 2, *A*, and Table I) because the other 2 SNPs were from the "gene desert" region (see Figs E2-E5 in this article's Online Repository at www.jacionline.org).

Fine-mapping analysis of the *EP3* region

Based on the GWAS result, we performed a fine-mapping analysis of the *EP3* region using 75 cases and 448 control subjects (see Fig E6 in this article's Online Repository). We generated a custom DNA array (see the Methods section in this article's Online Repository) to analyze the SNPs in and near *EP3* through the 2 major linkage disequilibrium (LD) blocks of the HapMap Japanese (HapMap-JPT) plus HapMap Han Chinese (HapMap-CHB) populations residing within the region (Fig 2, *A*, *green bar*). We compared the genotype frequencies of 86 SNPs selected by our stringent QC filter between the cases and control subjects (see the Methods section in this article's Online Repository). The SNP (rs17131450) that showed a significant association with SJS/TEN in the GWAS also showed a significant association ($P < .01$) in the fine-mapping analysis. We also identified 5 other significantly associated ($P < .01$) SNPs in *EP3* (rs5702, rs1325949, rs7543182, rs7555874, and rs4147114; Fig 2, *A* and *C*). All of the SNPs, except rs4147114, were in Hardy-Weinberg equilibrium ($P > .05$) in the control samples. We rechecked the 2-dimensional cluster plot for rs4147114 precisely and confirmed that the distribution of the cluster was normal. One of the SNPs in *EP3* was in an exon as a silent SNP, and the other 4 were in introns (Fig 2, *C*).

TABLE I. SJS/TEN-associated SNPs obtained from the initial GWAS

SNP ID	Chromosome	SNP type	MAF	HWE in control*	Call rate†	*P* value (−log P)‡
rs1325975	6	Intergenic	0.11	0.12	0.99	5.83
rs17131450	1	Intergenic	0.09	0.11	1.00	5.77
rs11238074	11	Intergenic	0.12	0.04	0.99	5.62

*P value for the deviation from Hardy-Weinberg equilibrium.
†Call rate per SNP in cases plus control subjects.
‡P value for genotype frequency comparison between cases and control subjects.

Sequencing analysis of the SJS/TEN-associated SNPs

Finally, we assessed the association of the 6 SNPs obtained from the fine-mapping analysis by sequencing samples from 100 cases and 160 control subjects. A summary of the case-control analysis based on the sequence data is shown in Table II. The association of all 6 SNPs was statistically significant, even with the Bonferroni correction ($P < .0083$). in the dominant model (Table II and Fig 2, *B*). All were in Hardy-Weinberg equilibrium ($P > .001$) in both the case and control samples. Four of the 5 SNPs in *EP3* (rs5702, rs1325949, rs7543182, and rs7555874) showed a strong LD with each other (average $D' > 0.9$, $r^2 > 0.7$; Fig 2, *B*). We identified 2 major haplotypes (types 1 and 2) of these 4 SNPs (Table III), and we also observed a significant association with SJS/TEN in various combinations of haplotypes. Consequently, from the results of the initial GWAS to those of direct sequencing, we successfully identified 6 SNPs associated with SJS/TEN, 5 of which were located within the *EP3* gene.

EP3 mRNA and protein expression in human ocular surface epithelium

We previously reported that EP3 is constitutively expressed in murine CE.[35] Given the association between SNPs in *EP3* and SJS/TEN and the murine expression pattern, we examined the

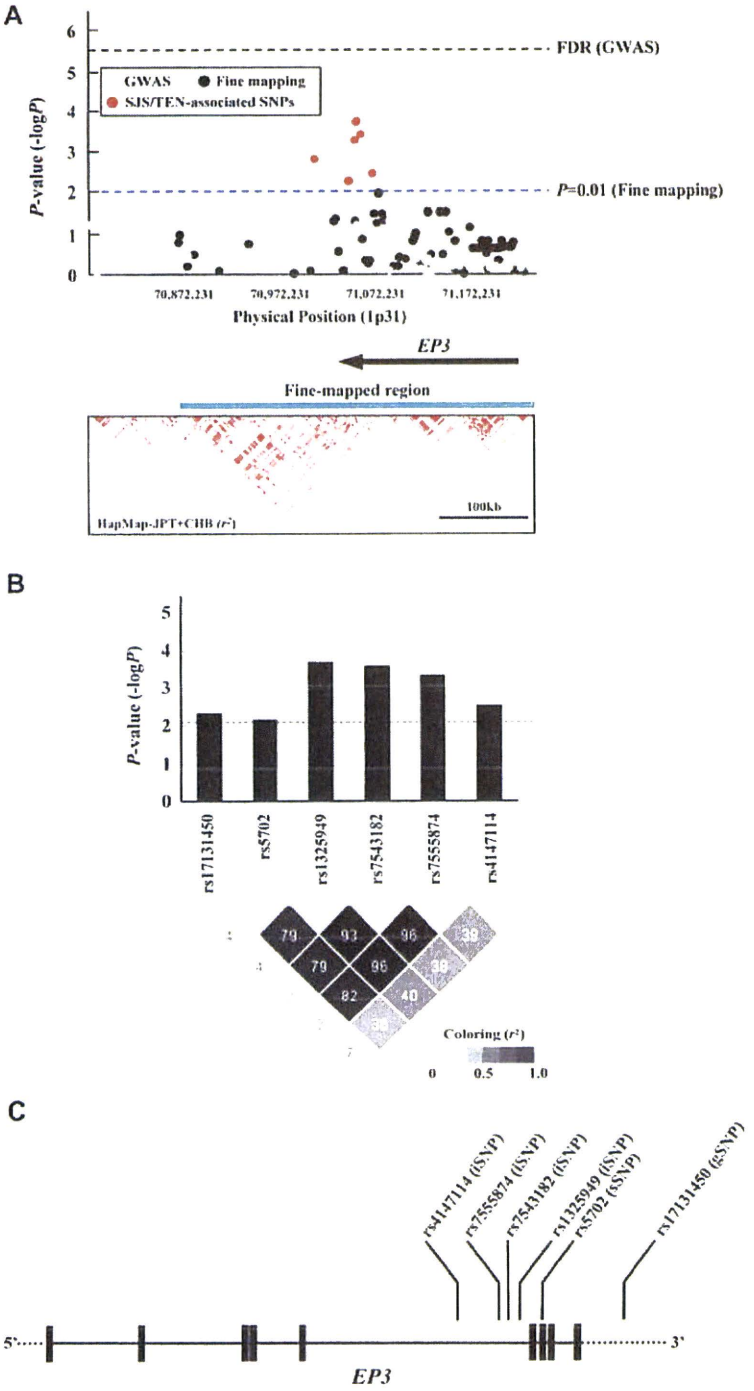

FIG 2. Association of SNPs in the *EP3* gene with SJS/TEN. **A**, Distribution of *P* values from the GWAS and fine-mapping analysis *(horizontal green bar)* of the *EP3* region. We obtained 6 significant SNPs with *P* values of less than .01 (genotype frequency comparison; *red dots*). The *P* values were plotted against the physical position of the 1p31 region and are shown for the GWAS *(gray dots)* and fine-mapping analysis *(black dots)*. *Horizontal lines*, FDR threshold for the GWAS, which was exceeded by rs17131450 (FDR; $P = 4.0 \times 10^{-6}$, *black dotted line*), and the threshold for the fine-mapping analysis ($P = .01$, *blue dotted line*). *Horizontal arrow*, Orientation of *EP3* gene transcription. The LD block of the HapMap-JPT plus HapMap-CHB populations was obtained from the USCS Genome Browser (http://genome.ucsc.edu/; National Center for Biotechnology Information build 35). **B**, Sequencing analysis of the SNPs associated with SJS/TEN. The *P* value of a dominant model for each SNP was calculated (see also Table II). *Dotted line*, Significance threshold for the Bonferroni correction. Pairwise r^2 plots among the SNPs were generated with Haploview software (http://www.broadinstitute.org/haploview/haploview). **C**, Schematic representation of the *EP3* gene structure and the location of the SNPs associated with SJS/TEN. Note that the direction of transcription is the reverse of that shown in Fig 2, *A*.

TABLE II. Genotype frequencies and association results for SJS/TEN-associated SNPs

SNP	Position (chromosome 1)	Genotypes	Frequency of genotypes (%)		Association results		
			Control subjects (n = 160)	Patients with SJS/TEN (n = 100)	Allele 1 vs allele 2 *P* value,* OR (95% CI)	Genotype 11 vs 12+22 *P* value,* OR (95% CI)	Genotype 11+12 vs 22 *P* value,* OR (95% CI)
rs17131450	71,296,002	11 CC	141 (88.1)	75 (75.0)	.00056, 0.36 (0.2-0.7)	.00600, 0.40 (0.2-0.8)	.0092, 0.10 (0.01-0.7)
		12 CT	18 (11.3)	19 (19.0)			
		22 TT	1 (0.6)	6 (6.0)			
rs5702	71,331,430	11 CC	80 (50.0)	67 (67.0)	.0300, 1.6 (1.0-2.4)	.00710, 2.0 (1.2-3.4)	.97, ND (ND)
		12 CT	67 (41.9)	25 (25.0)			
		22 TT	13 (8.1)	8 (8.0)			
rs1325949	71,337,193	11 AA	76 (47.5)	71 (71.0)	.0014, 2.0 (1.3-3.1)	.00020, 2.7 (1.6-4.6)	.61, ND (ND)
		12 AG	70 (43.8)	22 (22.0)			
		22 GG	14 (8.8)	7 (7.0)			
rs7543182	71,339,973	11 GG	80 (50.0)	73 (73.0)	.0023, 2.0 (1.3-3.1)	.00025, 2.7 (1.6-4.6)	.88, ND (ND)
		12 GT	68 (42.5)	20 (20.0)			
		22 TT	12 (7.5)	7 (7.0)			
rs7555874	71,343,960	11 GG	80 (50.0)	72 (72.0)	.0037, 1.9 (1.2-2.9)	.00046, 2.6 (1.5-4.4)	.88, ND (ND)
		12 GA	68 (42.5)	21 (21.0)			
		22 AA	12 (7.5)	7 (7.0)			
rs4147114	71,356,665	11 CC	42 (26.3)	44 (44.0)	.0033, 1.7 (1.2-2.5)	.0031, 2.2 (1.3-3.7)	.09, ND (ND)
		12 CG	82 (51.3)	42 (42.0)			
		22 GG	36 (22.5)	14 (14.0)			

ND, Not determined; *OR*, odds ratio.
**P* value for allele or genotype frequency comparison between cases and control subjects by using the χ^2 test.

TABLE III. Haplotypes of the SNPs in *EP3* associated with SJS/TEN

Types	SNPs				Frequency (%)	
	rs5702	rs1325949	rs7543182	rs7555874	Control subjects (n = 160)*	Patients with SJS/TEN (n = 100)*
1	C/C	A/A	G/G	G/G	46.3	67.0
2	C/T	A/G	G/T	G/A	40.0	19.0
3	T/T	G/G	T/T	A/A	7.5	7.0
4	Other combinations				6.3	8.0

*Number of subjects analyzed.

expression of *EP3* in human CE. First, we used RT-PCR to examine the expression of *EP3* mRNA and obtained PCR products of the expected length (622 bp) from the human CE samples (Fig 3, *A*, *a*). PCR products were isolated and sequenced to confirm their identity. The sequences were identical to that of the human *EP3* cDNA (data not shown).

Immunohistochemistry of control human conjunctival tissue (using conjunctival tissues from a patient with conjunctivochalasis as a normal conjunctival sample) showed obvious EP3 protein expression in the CE (Fig 3, *A*, *b*).

Suppression of cytokine production by an EP3 agonist

We previously reported that prostaglandin E_2 (PGE_2) is a ligand for EP3 in murine CE and that it downregulates the progression of murine experimental allergic conjunctivitis.[35] We also reported that *TLR3* polymorphisms are associated with SJS/TEN in ethnic Japanese subjects,[7] that the human ocular surface epithelium expresses TLR3, and that cytokine production is upregulated by polyI:C, a TLR3 ligand.[6,34] On the basis of these findings, we examined the function of EP3 in polyI:C-stimulated PHCjE cells using an EP3 agonist, ONO-AE248. PHCjE cells that were untreated or pretreated with 10 μg/mL ONO-AE248 were incubated for 24 hours with 10 μg/mL polyI:C. As early as 24 hours after adding polyI:C, we found high levels of CXCL11, CCL20, IL-6, and IL-8 in the supernatants from the polyI:C-treated, but ONO-AE248-untreated, PHCjE cultures (Fig 3, *B*, *a*). Cultures pretreated with ONO-AE248 produced significantly lower levels of CXCL11, CCL20, and IL-6, but the level of IL-8 was not affected (Fig 3, *B*, *a*). The mRNA levels for *CXCL11*, *CCL20*, and *IL6* were also significantly less in the PHCjE cultures pretreated with ONO-AE248 compared with those seen in the untreated cultures (Fig 3, *B*, *b*).

Taken together, these results show that cytokine production by the CE in response to polyI:C stimulation can be suppressed through the activation of EP3.

Reduced EP3 expression in the CE of patients with SJS/TEN

Next we examined the expression of EP3 in the CE of patients with SJS/TEN by means of immunohistochemistry. Unlike in the control CE samples from patients with conjunctivochalasis or pterygium, we could not detect EP3 protein in the CE samples from patients with SJS/TEN (Fig 3, *C*). These results suggest that EP3, which is a receptor for PGE_2, was downregulated in the CE of patients with SJS/TEN.

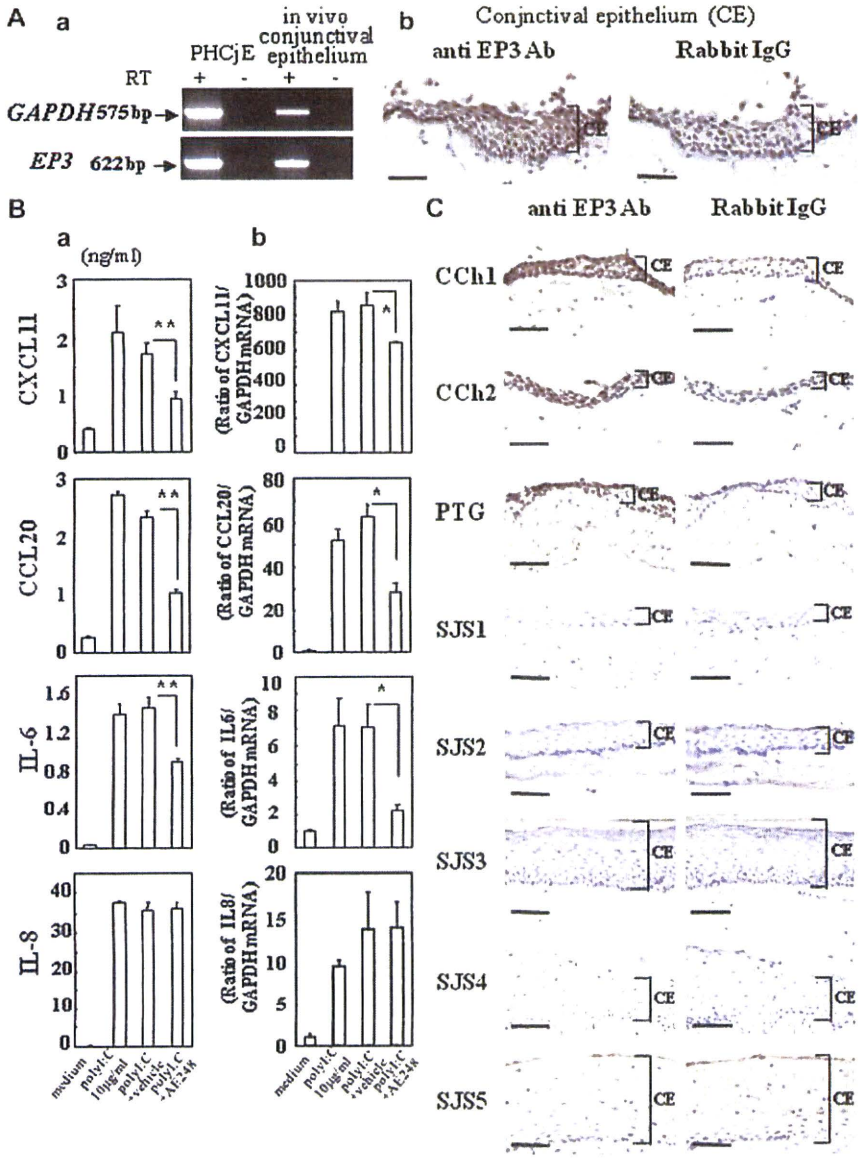

FIG 3. Expression and functional analyses of EP3 in human CE cells. **A**, *EP3* mRNA and EP3 protein expression in the human CE. *a*, RT-PCR analysis of *EP3* mRNA in normal human CE. *b*, Immunohistological analysis of EP3 in normal human conjunctival tissues. *Bars* = 50 μm. **B**, Suppression of cytokine production by an EP3 agonist. Pretreatment with ONO-AE248 significantly suppressed the protein *(a)* and mRNA *(b)* levels of CXCL11, CCL20, and IL-6 but not IL-8. Data are representative of 6 separate experiments for proteins and 2 separate experiments for mRNA. Data show the mean ± SEM from an experiment carried out in 6 wells for protein and 4 wells for mRNA per group. $*P < .05$, $**P < .01$. **C**, Reduced EP3 protein expression in the CE of patients with SJS/TEN. *CCh*, Conjunctivochalasis; *PTG*, pterygium; *SJS*, SJS/TEN. Each *bar* represents 50 μm.

DISCUSSION

In this study we performed a GWAS to identify genetic markers associated with SJS/TEN. Because of the extremely low prevalence of the disease, our GWAS was quite challenging to perform because of the sample size, which directly affects the statistical power to detect significant SNPs. As expected from the phenotype of SJS/TEN (which has been considered to be multifactorial), the low power of the study design, or both, we could not obtain genome-wide significant SNPs associated with the disease (see Fig E3 in this article's Online Repository at www.jacionline.org). However, we were able to demonstrate that as long as functional evidence could be obtained, it was worthy to perform a GWAS to list-up the candidate gene or genes or region or regions to choose and carry out follow-up functional studies. Therefore the concept of this study should shed light on future studies aimed at identifying genetic markers associated with diseases with low prevalence.

We successfully identified 3 significant SNPs in the GWAS that passed the FDR threshold, and one of them was from the *EP3* region (Fig 2, *A*). We focused on that region and discovered 5 more SNPs located in *EP3*, suggesting that *EP3* might code for a functional determinant of SJS/TEN pathogenesis (Fig 2, *A* and *C*). Further functional studies revealed that EP3 suppressed the polyI:C-induced cytokine production (Fig 3, *B*), suggesting that

EP3 might function in downregulating inflammation in the CE. Indeed, EP3 protein expression in the CE was greatly reduced in patients with SJS/TEN, but it was clearly detectable in control subjects with noninflammatory ocular surface diseases (chalasis and pterygium; Fig 3, *C*). However, although significant reduction in EP3 protein expression was observed in the tissue derived from patients with SJS/TEN, the patients did not always possess the risk allele of the identified SNPs (data not shown), suggesting that EP3 levels in CE cells of the patients might be affected by not only *EP3* SNPs but also other factors, such as ocular surface inflammation. Because the SNPs in *EP3* were intronic or silent (Fig 2, *C*), they could be involved in regulating the transcription of *EP3*. Therefore we assumed that the significant reduction in EP3 expression in the CE of the patients with SJS/TEN might be due to EP3 downregulation at the transcriptional level. Such a reduction in EP3 expression in the CE of patients with SJS/TEN could contribute to ocular surface inflammation, which is a major characteristic of the disease, and therefore the polymorphisms in *EP3* might contribute to the pathophysiology of SJS/TEN.

We also found that 4 of the 6 SNPs (rs5702, rs1325949, rs7543182, and rs7555874) were in strong LD (see the Results section). According to the International HapMap project, the 6 SNPs (rs17131450, rs5702, rs1325949, rs7543182, rs7555874, and rs4147114) identified in the current study are found in both ethnic Japanese and white populations, indicating that it is important to examine these *EP3* SNPs in non-Japanese populations.

Prostanoids (ie, the prostaglandins and the thromboxanes) are a group of lipid mediators that form in response to various stimuli. They include prostaglandins D_2, E_2, $F_{2\alpha}$, and I_2 and thromboxane A_2. They are released extracellularly immediately after their synthesis, and they act by binding to a G protein–coupled rhodopsin-type receptor on the surface of target cells. Eight types of prostanoid receptors are conserved in mammals from mice to human subjects: the prostaglandin D receptor (DP), 4 subtypes of the prostaglandin E receptor (EP1, EP2, EP3, and EP4), the prostaglandin F receptor (FP), the prostaglandin I receptor (IP), and the thromboxane A receptor (TP).[36,37] PGE_2–EP3 signaling is reported to inhibit keratinocyte activation and exert anti-inflammatory actions in murine contact hypersensitivity.[38] We also previously reported that PGE_2 acts as a ligand for EP3 in the CE and downregulates the progression of murine experimental allergic conjunctivitis.[35] Here we demonstrated that an EP3 agonist suppressed the production of CXCL11, CCL20, and IL-6 by human CE cells in response to polyI:C stimulation (Fig 3, *B*). Thus EP3 in the CE might downregulate ocular surface inflammation, an idea that is supported by our finding that EP3 was strongly downregulated in the CE of patients with SJS/TEN with ocular involvement (Fig 3, *C*).

Drugs are probably the most widely accepted causative factor for SJS/TEN.[3,9,10,12] Many patients with SJS/TEN with ocular involvement have had the disease after taking remedies for the common cold or NSAIDs. Given the association between the onset of SJS/TEN and various infections, we have considered the possibility that susceptibility to SJS/TEN is related to a disordered innate immune response.[5-7,24] In this study we showed that EP3 suppressed the cytokine production elicited by stimulation with polyI:C (which mimics viral double-stranded RNA) in the human CE (Fig 3, *B*), which might suggest that EP3 is involved in innate immunity.

Of our 100 patients, 76 had SJS after being treated for the common cold with medications that included NSAIDs. NSAIDs inhibit the production of the EP3 ligand PGE_2.[39] When we analyzed the association between the 6 SNPs identified here and their frequency in the 76 cold remedy–related SJS/TEN cases, the associations remained strongly significant (data not shown). These data support the idea that EP3 is involved in the development of SJS/TEN.

In summary, we have demonstrated, using both genetic and functional analyses, that EP3 could be a key player in the pathogenesis of SJS/TEN accompanied by ocular complications.

We thank all of the patients and volunteers who enrolled in our study. We also thank Dr Natsue Omi, Ms Sayaka Ohashi, Ms Naoko Saito, and Ms Yuko Konoshima for processing blood samples and performing genotyping; Ms Hiromi Yamada for assistance in clinical information analysis; and Mr Ryuichi Sato and Ms Fumiko Sato (World Fusion, Tokyo, Japan) for management of the genotype data.

Clinical implications: EP3 could be a key player in the pathogenesis of SJS/TEN accompanied by ocular complications, and EP3 might be a target for the prevention or treatment of this disease.

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METHODS

GWAS

We used the Affymetrix GeneChip Mapping 500K Array Set in the GWAS. In brief, 2 aliquots of approximately 250 ng of genomic DNA were digested with *Nsp*I and *Sty*I. Adaptor oligonucleotides specific to each digested end were ligated to the fragments, and the resulting molecules were amplified by means of PCR with adaptor-specific primers. The PCR products were fragmented, labeled, and hybridized with the corresponding *Nsp*I or *Sty*I arrays. After hybridization, the arrays were stained and scanned with a GeneChip Scanner 3000. The scanned data were managed with GeneChip Operating Software. Intensity data provided by the CEL files were used for SNP genotyping. Detailed methods for the genotype-calling algorithms, SNP genotyping, and criteria for SNP selection are described below.

SNP genotyping. The SNPs were initially genotyped with the Dynamic Model algorithm with GeneChip Genotyping Analysis Software (GTYPE, Affymetrix) to check the quality of each array. Arrays that did not pass a call rate of 93% at a confidence threshold of 0.33 were rehybridized with the stored hybridization cocktail. To confirm that no samples were mixed up, we checked the genotypes of 50 common SNPs placed on both the *Nsp*I and *Sty*I arrays. We checked for sex mismatch by comparing clinical records and genotyping results for the X-chromosome. For the association analysis, we genotyped the SNPs by using the Bayesian Robust Linear Model with a Mahalanobis distance classifier (BRLMM) algorithm with a BRLMM Analysis Tool. The multiple sample classification was performed by clustering 60 case and 301 control samples, separately. Because 60 samples were not enough for accurate clustering, we added 286 population-matched samples from another project (data not shown). After excluding a sample from the control group that yielded low-quality data (see the Results section), we used 60 case and 300 control samples for the association analysis.

Criteria for SNP selection. From 500,568 SNPs (262,264 and 238,304 SNPs in the *Nsp*I and *Sty*I arrays, respectively), a total of 313,924 autosomal SNPs were selected for association analysis based on our stringent QC filter, which had the following criteria: (1) 90% or greater call rate per SNP in cases and control subjects, (2) 5% or less call rate difference between cases and control subjects for each SNP, and (3) 5% or greater minor allele frequency (MAF) in cases and control subjects. After the association analysis, we visually checked the 2-dimensional cluster plots of the genotypes for the 25 SNPs that passed the FDR threshold to remove the SNPs that clustered poorly. Using our custom tool, we selected the SNPs with good 2-dimensional cluster plots, as described below. The cluster for each SNP was given an acceptability score of 0 (reject), 1 (acceptable), or 2 (accept), and this was done separately for the case and control data. The clusters were scored in random order by 3 independent observers (M. N., T. T., and K. T.). The score given by at least 2 observers had to agree to be accepted and was expressed as a total acceptability score of the summed case and control scores, ranging from 0 to 4. We excluded poorly clustered SNPs, which were given a total score of 0 to 3. We ultimately selected 3 SNPs from the GWAS as candidates (Table I).

Fine-mapping of the SNPs in the *EP3* region

Genotyping of the SNPs in *EP3* was performed by using the iSelect Custom Infinium Genotyping system (iSelect, Illumina). Briefly, 150 to 300 ng of genomic DNA was denatured and amplified by using the manufacturer-provided reagents. The samples were then fragmented, precipitated, and resuspended completely. After being denatured, the samples were hybridized with iSelect Genotyping BeadChips, and the BeadChips were then reacted to detect single-base or allele-specific extensions. After being stained, the BeadChips were scanned with a BeadArray Reader. The intensity data from each chip were entered for analysis by using the BeadStudio 3.0 software, which converts fluorescence intensities into SNP genotyping results. Detailed methods for the SNP selection, SNP genotyping, and criteria for SNP selection are described below.

SNP selection. We genotyped the SNPs in the *EP3* gene using 75 case samples, including 60 from the GWAS population, and 455 control samples from different subjects than in the GWAS by using the iSelect Custom Infinium Genotyping system (iSelect, Illumina). We first selected the SNPs on the *EP3* gene together with adjacent SNPs on the LD block of the HapMap-JPT and HapMap-CHB populations derived from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Of these, we selected the non-monomorphic SNPs in the HapMap-JPT and HapMap-CHB populations. We then validated the suitability of selected SNPs for constructing custom chips by using the Assay Design Tool. Finally, 163 SNPs were chosen for the custom chip and used for the subsequent analysis. To check sex mismatches between the clinical records and the genotyping results, we also analyzed 13 SNPs on the X-chromosome.

SNP genotyping. To check the quality of each data point, the SNPs were initially genotyped by clustering 530 (75 cases plus 455 control subjects) samples at a no-call threshold of 0.15. For these SNPs, we analyzed the call rate per sample and the QC indices (staining, extension, target removal, hybridization, stringency, nonspecific binding, and nonpolymorphic) using the BeadStudio software. Because 8 samples showed a lower call rate than the others (<95%), we reprocessed them starting with the sample preparation, as described in the manufacturer's technical note (Infinium Genotyping Data Analysis). All the reprocessed samples showed a higher call rate than was seen in the initial results. We excluded 7 samples (see the Results section) and then clustered the results from 75 case and 448 control samples separately by using our standard cluster file (data not shown). Three independent observers (M. N., T. T., and T. Y.) visually checked the 2-dimensioanl cluster plots of the genotypes for all of the SNPs.

Criteria for SNP selection. From the 163 SNPs, 86 were selected for the association analysis based on our QC filter: (1) 90% or greater call rate per SNP and (2) 5% or greater MAF for both cases and control subjects.

SNP analysis by means of direct sequencing

The 6 SJS/TEN-associated SNPs that showed significant associations ($P < .01$) in the Custom Genotyping BeadChip assay were sequenced from both sides (ie, the forward and reverse directions) for rigorous assessment of our genotyping results. For the *EP3* SNPs, the PCR and sequence primers were as shown in Table E2. Genomic DNA was isolated from human peripheral blood at SRL, Inc (Tokyo, Japan). PCR amplification was performed with DNA polymerase (Takara, Shiga, Japan) for 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute on a commercial PCR machine (GeneAmp, Perkin-Elmer Applied Biosystems). The PCR products were reacted with BigDye Terminator v3.1 (Applied Biosystems), and the sequence reactions were resolved on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

PHCjE cells

PHCjE cells were cultured for use in ELISAs. Conjunctival tissues were washed and immersed for 1 hour at 37°C in 1.2 U/mL purified dispase (Roche Diagnostic Ltd, Basel, Switzerland). The epithelial cells were detached, collected, and cultured in low-calcium k-SFM medium supplemented with 0.2 ng/mL human recombinant epidermal growth factor (Invitrogen, Carlsbad, Calif), 25 mg/mL bovine pituitary extract (Invitrogen), and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within 3 to 4 days. After reaching 80% confluence in 7 to 10 days, the cells were seeded into culture dishes and used for experiments once they had reached subconfluence.

Immunohistochemistry

Sections 6 μm thick were cut and fixed in 100% acetone at 4°C for 10 minutes. They were then blocked for 30 minutes with 10% normal donkey serum in PBS. The anti-EP3 antibody was a rabbit polyclonal antibody (Cayman Chemical Co, Ann Arbor, Mich). A nonspecific rabbit IgG (Abcam Ltd, Cambridge, United Kingdom) was used as the negative control. The secondary antibody (Biotin-SP–conjugated AffiniPure F[ab′]$_2$ Fragment Donkey Anti-Rabbit IgG[H+L], 1:500 dilution; Jackson ImmunoResearch, Baltimore, Md) was applied for 30 minutes. Vectastain ABC Reagent (Vector Laboratories, Inc, Burlingame, Calif) was used to amplify the signal from the DAB substrate (DAB substrate kit, Vector Laboratories).

RESULTS

Power estimation

As described in the introduction, it is extremely difficult to recruit patients with SJS accompanied by ocular complications because of the low annual rate of incidence. Therefore we decided to complete the GWAS using the first set of subjects (60 cases vs 300 control subjects) and then move on to the fine-mapping analysis by adding the new samples. In support of that decision, we estimated the statistical power of both situations: 100 cases versus 756 control subjects, which would be the maximum number of subjects throughout the study, and 60 cases versus 300 control subjects (Fig E3). If we expected to detect SNPs with an MAF of 0.1 and an odds ratio of 1.5 at a less than .05, the power of both situations were 0.46 (blue line) and 0.30 (grey line), respectively. The results suggested that even if we performed the GWAS with 100 cases and 756 control subjects, we could gain a statistical power of no more than twice that of the sample size we used. Therefore our decision was fairly reasonable considering the circumstances surrounding the collection of our samples.

Genotyping for GWAS

We first genotyped 60 case and 301 control samples using the Affymetrix GeneChip Mapping 500K Array Set. Using the Dynamic Model algorithm, we found no mix-ups of the samples between the *Nsp*I and *Sty*I arrays. We observed no inconsistent results for sex between the clinical records and the genotyping results. The final genotyping results for 500,568 SNPs were called by using the BRLMM algorithm. Because the value of the raw intensity of 1 control sample was out of the accepted range, this sample was excluded from the analysis. Ultimately, we used 60 cases and 300 control subjects for the association study. The mean call rate per sample was more than 98% for the case and control samples. Our stringent QC filter for the call rate and MAF (see the Methods section) permitted 313,924 autosomal SNPs to be used in the subsequent analysis.

Quantile-quantile plot

According to the quantile-quantile plot (Fig E4, *A*), the observed P value deviated from the expected P value between 10^{-2} and 10^{-3}, which might be reflecting the imbalanced sample number of our case-control population. When we analyzed the distribution with and without 44 SNPs from the *EP3* region (Fig E4, *B*), we were able to see a slight difference in the deviation (enlarged box, red open circle to black-filled circle), suggesting the contribution of the *EP3* region to the SJS trait. However, because the magnitude of the difference was small, the genetic contribution of *EP3* SNPs to the trait seemed to be shared by other unidentified variants, which should also be explained from the result of the GWAS that we could not obtain genome-wide significant SNPs possessing strong effects to the trait.

Population stratification of the subjects used in the GWAS

According to the population stratification analyses using STRUCTURE software, our case plus control samples showed a similar stratification with those of the HapMap-JPT population (Fig E5, *A*) and clearly differed from those of the HapMap-CEU and HapMap Yoruba in Nigeria populations (Fig 5, *A*). The analysis also showed no significant difference in population stratification between the case and control samples used in the GWAS (Fig E5, *B*). Moreover, Yamaguchi-Kabata et al[E1] reported that the Japanese population stratification mainly divided into 2 clusters, the main islands of Hondo and Ryukyu in Okinawa. They suggested that the false-positive rates in the GWAS would be acceptable when the samples were collected from Hondo island, indicating that the population stratification within that region was relatively small. In this study we collected all of the samples at the single institute in the middle part of Hondo. Consequently, we concluded that there was no significant difference in population stratification between the case and control samples used in the GWAS.

SNP density of the fine-mapping analysis

After applying our QC filter (≥90% call rate per SNP and ≥5% MAF in the case and control samples), the SNPs that we used for the fine-mapping analysis resulted in 52.8% (86/163) of the total number of SNPs for which we initially designed. The major component of eliminated SNPs (76/77) belonged to an MAF of less than 0.05 in our population. Of the passed 86 SNPs, 75 were on the *EP3* gene, with the rest being from the adjacent region. The mean and median lengths between the SNPs on the *EP3* gene were 2.5 and 1.9 kb, respectively. In contrast, the mean and median lengths of the dbSNPs on the *EP3* gene (in total, 76 SNPs with MAFs > 0 based on HapMap-JPT + HapMap-CHB data) were 2.5 and 2.0 kb, respectively, indicating the dense distribution of analyzed SNPs on our custom chip through the gene (Fig E6). Therefore although the success rate was low, the actual coverage of the *EP3* gene by the SNPs that passed the filter was reasonably high enough to satisfy our purpose.

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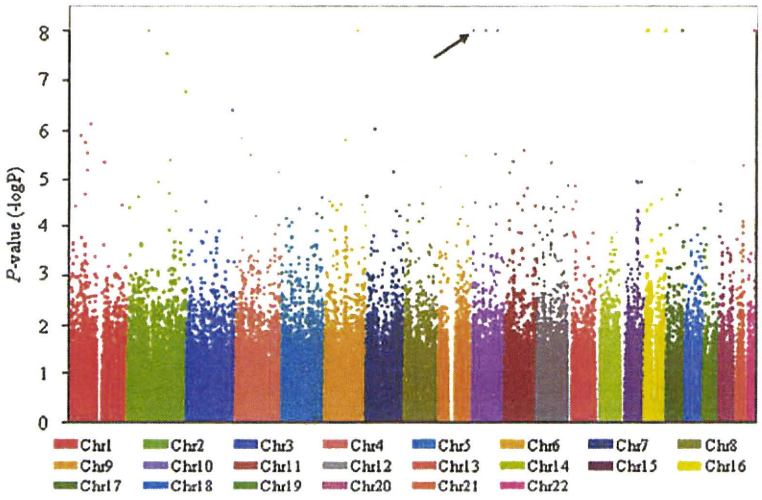

FIG E1. Manhattan plot of the SNPs of the GWAS. Distribution of *P* values obtained from the result of the GWAS is shown. *Horizontal line,* FDR threshold; *arrow,* 2 adjacent SNPs with a similar *P* value were overlapped.

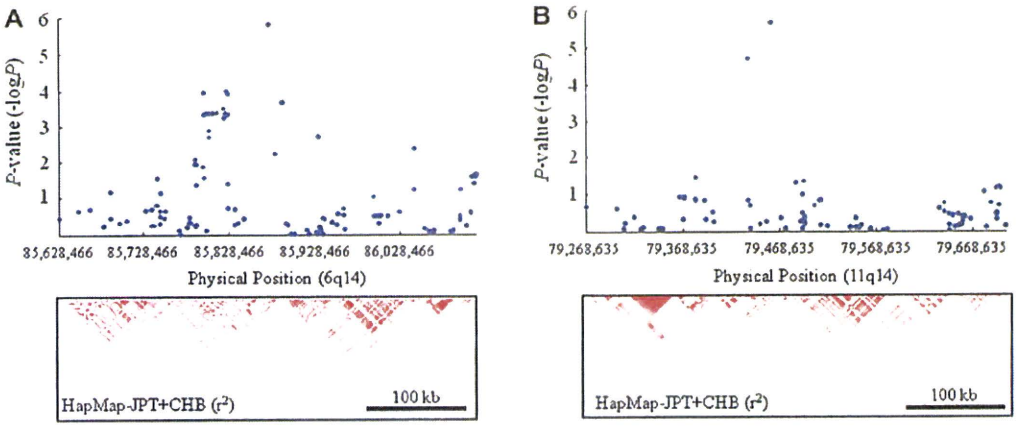

FIG E2. Two other candidate regions associated with SJS/TEN identified by the GWAS. Two other significant SNPs were identified from chromosomes 6 **(A)** and 11 **(B)**. Distribution of *P* values obtained from the result of the GWAS is shown. The LD block for the HapMap-JPT plus HapMap-CHB population based on the r^2 value was obtained from the USCS Genome Browser (http://genome.ucsc.edu/). Physical coordinates refer to National Center for Biotechnology Information Build 35 of the human genome. There was no annotated gene within 500 kb in these regions.

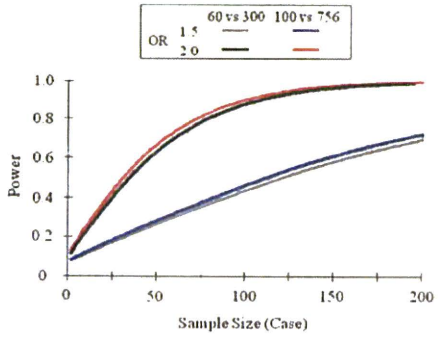

FIG E3. Power calculation. Power calculation was performed by using Power and Sample Size Calculation software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). Power simulations of the maximum number of subjects (100 cases vs 756 control subjects) and the subjects used in the GWAS (60 cases vs 300 control subjects) were performed. The parameters entered were as follows: statistical significance, $P < .05$; MAF, 0.1; and control/case ratio, 5 and 7.56, respectively. *OR,* Odds ratio.

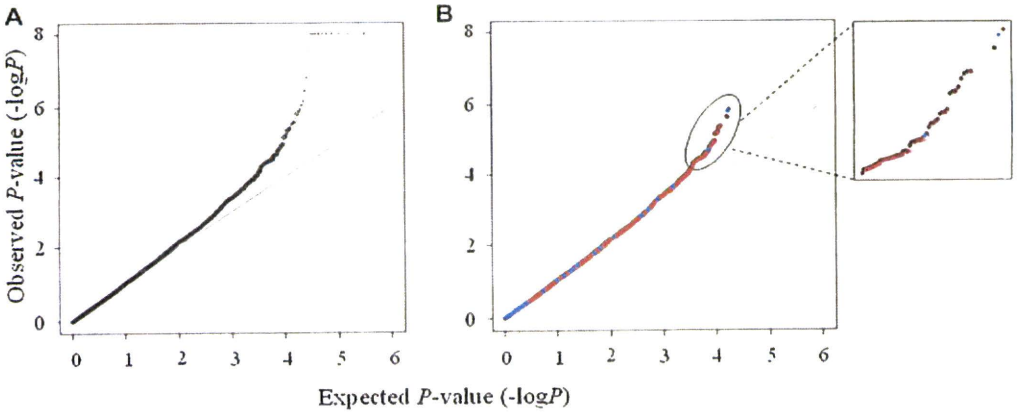

FIG E4. Quantile -quantile plot of the SNPs that passed the stringent filter. **A**, Quantile-quantile plot of the 313,924 SNPs that passed the stringent QC filter in the GWAS and the SNPs selected after the visual check of 2-dimensional cluster plots *(open circles)*. **B**, Distribution of the plot with *(solid black circles)* and without *(open red circles)* the 44 SNPs *(filled blue circles)* derived from the *EP3* region. The distribution of the expected *P* values of genotype frequency comparison plotted against the observed *P* values is shown. Under the null hypothesis, with no disease association, the points lie on the *solid line*. Observed SNP *P* values smaller than 10^{-8} are plotted as 10^{-8}.

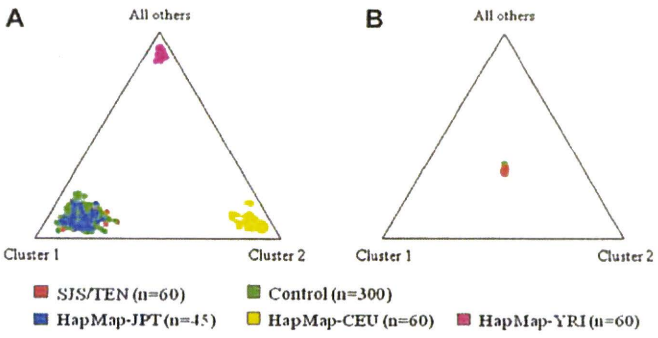

FIG E5. Analysis of population stratification. Population stratification analysis for the subjects used in the GWAS analyzed by using STRUCTURE software is shown. Data are shown in triangle plots (assumed number of populations = 3). **A**, The analysis shows the populations used in the GWAS (case and control groups) and the HapMap-JPT population separated from the HapMap-CEU and HapMap Yoruba in Nigeria (HapMap-YRI) populations with a highest log likelihood of an assumed number of populations of 3. **B**, When the analysis was restricted to the DNA samples used in the GWAS, plots showed a single tight cluster with a highest log likelihood of an assumed number of populations of 1. *Red* and *green dots* correspond to case and control samples, respectively. *Blue*, *yellow*, and *pink dots* correspond to the HapMap-JPT, HapMap-CEU, and HapMap-YRI samples, respectively.

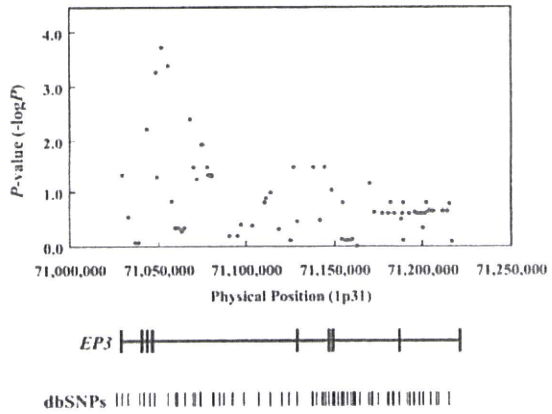

FIG E6. SNP density on the *EP3* gene for the fine-mapping analysis. Density of the *EP3* SNPs (the QC filter passed 75 SNPs) on our custom chip is shown by indicating the distribution of *P* values obtained from the result of the fine-mapping analysis. The exon-intron structure of the *EP3* gene and the dbSNPs on the gene (76 SNPs with MAF > 0 based on HapMap-JPT+CHB data) are shown at the bottom.

TABLE E1. Clinical characteristics of cases and control subjects*

	GWAS		Fine-mapping analysis		Sequencing analysis	
	Cases†	Control subjects	Cases†	Control subjects	Cases†	Control subjects
No. of subjects for case-control analyses	60	300	75	448	100	160
Ratio of female subjects/male subjects	1.1 (60)	1.3 (300)	1.0 (75)	1.8 (448)	1.5 (100)	1.8 (160)
Age‡ (y) at:						
Blood sampling	44.9 ± 17.3 (60)	51.1 ± 13.9 (300)	44.0 ± 16.6 (75)	55.2 ± 14.7 (448)	44.2 ± 17.8 (100)	36.2 ± 11.5 (160)
Onset	29.1 ± 16.7 (60)	—	27.4 ± 17.2 (75)	—	28.0 ± 18.2 (100)	—

*Numbers in parentheses are the total number of subjects whose samples were used for the analysis.
†Many of the case samples were shared in the analyses. We added samples from 15 patients for the fine-mapping analysis to those used in the GWAS, and samples from 25 additional patients were used along with samples from the other 75 patients for the sequencing analysis.
‡Data show the mean ± SD.

TABLE E2. Primers for PCR and sequencing to detect SJS/TEN-associated SNPs

SNPs	Strand	Primers (5′-3′)
rs17131450	Sense	TTTTATGCAGCTTTCGGTCA
	Antisense	CCCCTCCAGGCTGATAACTC
rs5702	Sense	CAAGTAGCAGTTGGCAGCAA
	Antisense	TGCAATCAGACAGGCAAGAG
rs1325949	Sense	AATTGCAAGTCCAGCTCAGG
	Antisense	AGGCCTCAGGGAGCTTTTAC
rs7543182	Sense	TGTGAGGCAAGAACCAGACA
	Antisense	AGGACCTGGGAGGGAAGATA
rs7555874	Sense	AAGCCAGCAAAGGACAAGAA
	Antisense	TGTTGTGTGTCTGCCAGGTT
rs4147114	Sense	TGCTGGAAGCTCATGGTCTA
	Antisense	TGCATGGTTCGTCTAACCTTAT

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BRIEF COMMUNICATION

*HLA-B*1511* is a risk factor for carbamazepine-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese patients

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Summary

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare but life-threatening severe cutaneous adverse reactions. Recently, strong associations of *HLA-B*1502* with carbamazepine-induced SJS/TEN have been found in Han Chinese patients. These associations have been confirmed in several Asian populations, excluding Japanese. SJS patients carrying *HLA-B*1508*, *HLA-B*1511*, or *HLA-B*1521*, which are members of the HLA-B75 type along with *HLA-B*1502*, were detected in studies in India and Thailand. In the current study, we genotyped the *HLA-B* locus from 14 Japanese typical and atypical SJS/TEN patients in whom carbamazepine was considered to be involved in the onset of adverse reactions. Although there were no *HLA-B*1502* carriers, four patients had *HLA-B*1511*. Our data suggest that *HLA-B*1511*, a member of *HLA-B75*, is a risk factor for carbamazepine-induced SJS/TEN in Japanese.

KEY WORDS: **HLA-B*1502, HLA-B75, Serotype.**

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe adverse drug reactions (ADRs) with mucosal and cutaneous disorders, and often are accompanied by high fever and systemic complications. Although incidence is low, SJS and TEN are life-threatening and their mortalities are estimated at 5% and 30%, respectively. On the basis of summarized spontaneous reports of severe ADRs to the Ministry of Health, Labor and Welfare (MHLW) from 2006 to 2008, the incidence of SJS/TEN in Japan can be calculated as 3.4 patients per million per year (approximately 430 cases annually), and major causative drugs are allopurinol and carbamazepine.

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As for carbamazepine-induced SJS/TEN, involvement of *HLA-B*1502* in Han Chinese SJS/TEN patients has been reported (Chung et al., 2004), and has been confirmed in Asians in Hong Kong (Man et al., 2007), Europe (Lonjou et al., 2006), Thailand (Locharernkul et al., 2008), and India (Mehta et al., 2009). However, no association between *HLA-B*1502* and carbamazepine-related SJS/TEN was detected in our previous study with seven Japanese SJS/TEN patients (Kaniwa et al., 2008). Therefore, we extended the investigation to explore other biomarkers in Japanese SJS/TEN patients who were administered carbamazepine.

Methods

Patients

The ethics committee of each participating institute of the JSAR (Japan Severe Adverse Reactions) research group approved this study. Written informed consent was obtained from each patient. Fifteen unrelated Japanese patients who were prescribed carbamazepine before the onset of SJS/TEN were recruited from participating institutes or through

a nationwide blood sampling network in Japan operated by the National Institute of Health Sciences in cooperation with the MHLW and the Federation of Pharmaceutical Manufacturers' Association of Japan. Patient characteristics are summarized in Table 1. Seven patients were included in our previous report (Kaniwa et al., 2008), and two patients were in another study (Ikeda et al., 2009). Twelve patients were diagnosed as definite SJS or TEN and three patients were diagnosed as probable SJS due to atypical or mild symptoms by the JSAR research group experts. This diagnosis was based on criteria proposed by Bastuji-Garin et al. (1993) using a standardized case report form including medicinal records, disease progress, and involvement of systemic complications as well as treatment. Severity of ocular complication was scored as follows: 0, no involvement; 1, only hyperemia of bulbar and palpebral conjunctiva; 2, pseudomembrane formation; 3, defect of conjunctival or corneal epithelia.

HLA-B typing

High-resolution *HLA-B* typing was performed by a sequence-based method using SeCore B Locus Sequencing kit (Invitrogen Corp., Brown Deer, WI, U.S.A.) and an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Genomic DNA (250 ng) was used for PCR amplification and sequencing exons 2, 3, and 4. HLA-B haplotype was estimated with the Assign SBT software (version 3.2.7b; Conexio Genomics, Applecross, WA, Australia).

Statistical analysis

*HLA-B*1511* allele frequency reported by Tanaka et al. was used as the control frequency (Tanaka et al., 1996). Fisher's exact test was conducted using JMP ver. 7.0.1 (SAS Institute Japan, Co., Ltd., Tokyo, Japan) to calculate the odds ratio and its 95% confidence interval (CI).

Results

Demographics, symptomatic state, coadministered drugs with carbamazepine, and *HLA-B* diplotypes of 15 patients are summarized in Table 1. However, Patient 12 was excluded from the following statistical analyses because zonisamide was a more likely causative drug. Involvement of carbamazepine in the onset of SJS/TEN could not be excluded for the remaining 11 definite SJS/TEN patients and three probable SJS patients.

In contrast to data on Han Chinese (Chung et al., 2004) and Thai populations (Locharernkul et al., 2008), *HLA-B*1502* was not detected in this work. However, two patients with definite SJS/TEN and two patients with probable SJS carried *HLA-B*1511*. The allele frequencies of *HLA-B*1511* in the SJS/TEN groups were compared with the allele frequency in a Japanese population reported by Tanaka et al. (1996) (n = 493) instead of that in carbamazepine-tolerant patients, because the incidence of SJS/TEN in Japan is very low (three per million/year). Allele frequencies of *HLA-B*1511* increased significantly in the SJS/TEN group regardless of the exclusion or inclusion of probable SJS patients [0.0909 (2 of 22) and 0.143 (4 of 28), respectively) than in the Japanese population (0.01), and the odds ratios were 9.76 (p = 0.0263, CI 2.01–47.5) and 16.3 (p = 0.0004, CI 4.76–55.6), respectively. No patients with *HLA-B*1511* had severe ocular complications.

Discussion

Recently, *HLA-B*1502* involvement has been reported in carbamazepine-induced SJS/TEN in Southern Asian patients (Chung et al., 2004; Man et al., 2007; Locharernkul et al., 2008; Mehta et al., 2009) and patients of Asian ancestry living in Europe (Lonjou et al., 2006). Although we did not detect SJS/TEN patients receiving carbamazepine who carried *HLA-B*1502*, we did find four patients carrying *HLA-B*1511*. *HLA-B*1511* and *HLA-B*1502* belong to the same HLA-B75 serotype. Other major members of HLA-B75 are *HLA-B*1508*, *HLA-B*1515*, and *HLA-B*1521*. Mehta et al. (2009) have investigated the association between *HLA-B*1502* and carbamazepine-induced SJS using eight Indian patients. Although in their study most patients (six of eight) did carry *HLA-B*1502*, one patient was homozygous *HLA-B*1508*. Tassaneeyakul et al. (2010)have also performed a case–control study using 42 CBZ-induced SJS/TEN patients and 42 carbamazepine-tolerant controls in a Thai population. In their study, 37 SJS/TEN patients carried *HLA-B*1502* and the very strong association of *HLA-B*1502* with SJS/TEN was again confirmed. Although the statistical significance was not examined, two patients carrying heterozygous *HLA-B*1521* and one patient carrying heterozygous *HLA-B*1511* were detected, suggesting that not only *HLA-B*1502* but also some subfamilies of serotype HLA-B75 are involved in the onset of carbamazepine-induced SJS/TEN.

Allele frequencies of individual HLA genotypes in worldwide populations obtained from various studies are shown at Allelefrequencies.net (Middleton et al., 2003). Table 2 summarizes the population allele frequencies of representative types of HLA-B75 in various ethnic groups. In Han Chinese, Thai and Indians, carriers of *HLA-B*1502*, *HLA-B*1521*, and *HLA-B*1508* are at high risk of carbamazepine-induced SJS/TEN, although *HLA-B*1502* is mainly involved. A comparable allele frequency of *HLA-B*1511* (higher than 3.8%) to that of *HLA-B*1502* in Han Chinese in Beijing has been reported recently by Yang et al. (Yang et al., 2010). Because the allele frequency of *HLA-B*1511* is higher than that of *HLA-B*1502* in Japanese and Koreans, carriers of the former may more easily be detected in association studies than carriers of the latter in northeast Asian populations. *HLA-B*1521* can be a risk