TABLE E1. Primer sets for detecting IK1, Ik2, Ik3, and Ik4 isoforms

Name	Location	Forward or reverse	Sequence
IKZF1, primer A	Exon 2	Forward	GCTGATGAGGGTCAAGACAT
IKZF1, primer B	Exon 4	Forward	TCATTTGCATCGGGCCCAAT
IKZF2, primer C	Exon 6	Reverse	AGCTTCGGCCACAATATCCA
IKZF2, primer D	Exon 8	Reverse	TCGTTCTCCTTCTCGTAGCT

TABLE E2. Replication analysis with Japanese population 2

Patients with SJS, n = 16; control subjects, n = 188				Allele (1 vs 2)		Minor allele frequency	
Gene symbol	**rs no.**	**Minor allele (1)**	**Major allele (2)**	***P* value (χ^2 test)**	**OR (95% CI)**	**Cases**	**Control subjects**
LOC148709	rs10800873	A	C	.407	0.6 (0.2-1.9)	0.125	0.184
IGSF11	rs4687960	C	T	.194	0.4 (0.1-1.7)	0.063	0.145
FUT9	rs11153964	T	G	.029	2.2 (1.1-4.7)	0.438	0.258
FUT9	rs2294839	C	T	.013	2.5 (1.2-5.2)	0.438	0.238
IKZF1	rs897693	C	T	.236	2.1 (0.6-7.7)	0.094	0.046
IKZF1	rs4917014	G	T	.074	0.5 (0.2-1.1)	0.344	0.508
TMCC3	rs4761639	T	C	.583	1.3 (0.6-2.8)	0.281	0.238
TSHZ2	rs4809905	A	G	.752	0.9 (0.4-2.0)	0.281	0.308

TABLE E3. Meta-analysis of the 8 SNPs using samples from Japanese and Korean subjects

Patients with SJS, n = 160; control subjects, n = 967		Minor	Major	Allele (1 vs 2)	
Gene symbol	rs no.	allele (1)	allele (2)	*P* value*	OR (95% CI)
LOC148709	rs10800873	A	C	2.11E-03	1.6 (1.2-2.1)
IGSF11	rs4687960	C	T	5.25E-05	0.4 (0.2-0.6)
FUT9	rs11153964	T	G	2.10E-06	1.8 (1.4-2.3)
FUT9	rs2294839	C	T	3.13E-07	2.0 (1.5-2.5)
IKZF1	rs897693	C	T	5.05E-06	3.0 (1.8-5.0)
IKZF1	rs4917014	G	T	**9.49E-10**	0.5 (0.4-0.6)
TMCC3	rs4761639	T	C	1.66E-04	1.6 (1.3-2.1)
TSHZ2	rs4809905	A	G	5.52E-05	0.6 (0.4-0.7)

Values in boldface indicate statistical significance in the genome-wide association.
*Cochran-Mantel-Haenszel method.

TABLE E4. Results of analysis of the 4 *IKZF1* SNPs: Japanese samples

Patients with SJS, n = 149; control subjects, n = 877			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Minor allele (1)	Major allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	2.16E-06	8.62E-06	3.2 (1.9-5.4)	0	24	125	4	38	828
rs4917014	G	T	2.99E-08	1.20E-07	0.5 (0.4-0.6)	16	62	71	204	449	224
rs4917129	C	T	4.12E-06	1.65E-05	0.5 (0.4-0.7)	15	61	73	172	442	263
rs10276619	G	A	1.28E-06	5.14E-06	1.8 (1.4-2.4)	53	69	27	155	454	267

TABLE E5. Results of analysis of the 4 *IKZF1* SNPs: Korean samples

Patients with SJS, n = 31; control subjects, n = 90			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Allele (1)	Allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	.406	—	1.8 (0.4-7.9)	0	3	27	0	5	85
rs4917014	G	T	**1.20E-03**	4.80E-03	0.4 (0.2-0.7)	2	12	17	20	49	21
rs4917129	C	T	**4.32E-03**	1.73E-02	0.4 (0.2-0.8)	3	10	21	19	39	31
rs10276619	G	A	**1.20E-03**	4.79E-03	2.7 (1.5-4.9)	13	16	2	17	45	28

Values in boldface indicate statistical significance.

TABLE E6. Results of analysis of the 4 *IKZF1* SNPs: Indian samples

Patients with SJS, n = 20; control subjects, n = 56			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Allele (1)	Allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	.891	—	1.1 (0.5-2.2)	4	10	6	9	31	16
rs4917014	G	T	**.016**	.065	0.3 (0.1-0.8)	0	5	15	7	22	27
rs4917129	C	T	.057	—	0.5 (0.2-1.0)	3	6	11	14	25	17
rs10276619	G	A	0.509	—	1.4 (0.5-3.5)	0	8	12	2	13	40

Value in boldface indicates statistical significance.

TABLE E7. Results of analysis of the 4 *IKZF1* SNPs: Brazilian samples

Patients with SJS, n = 39; control subjects, n = 135			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Allele (1)	Allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	.568	—	1.2 (0.6-2.4)	0	13	26	3	32	100
rs4917014	G	T	.581	—	0.9 (0.5-1.5)	2	18	19	12	61	62
rs4917129	C	T	.112	—	0.7 (0.4-1.1)	8	20	11	44	63	27
rs10276619	G	A	.118	—	1.5 (0.9-2.5)	9	19	11	21	58	54

TABLE E8. Replication analysis for Japanese subjects with cold medicine–induced cutaneous adverse reaction (not severe types)

rs no.	Minor allele (1)	Major allele (2)	Cases, no.		Control subjects, no.		Basic allele (1 vs 2)	
			1	2	1	2	*P* value (χ^2 test)	OR (95% CI)
rs897693	C	T	5	137	50	1714	.638	1.3 (0.5-3.2)
rs4917014	G	T	71	71	770	922	.603	1.2 (0.9-1.7)
rs4917129	C	T	69	73	776	988	.288	1.2 (0.9-1.7)
rs10276619	G	A	59	83	775	989	.582	1.1 (0.8-1.5)

TABLE E9. Relation between *HLA-A*02:06* and the 4 IKZF1 SNPs in Japanese population 1: Analysis of *HLA-A*02:06*–positive samples

Patients with SJS, n = 58; control subjects, n = 94			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Minor allele (1)	Major allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	.134	—	2.2 (0.8-6.6)	0	8	50	1	4	89
rs4917014	G	T	**3.53.E-04**	1.41E-03	0.4 (0.3-0.7)	5	25	28	21	54	19
rs4917129	C	T	**5.83.E-04**	2.33E-03	0.4 (0.3-0.7)	6	21	31	19	53	22
rs10276619	G	A	**7.33.E-04**	2.93E-03	2.2 (1.4-3.6)	21	28	9	14	48	32

Values in boldface indicate statistical significance.

TABLE E10. Relation between *HLA-A*02:06* and the 4 IKZF1 SNPs in Japanese population 1: Analysis of *HLA-A*02:06*–negative samples

Patients with SJS, n = 59; control subjects, n = 595			Basic allele (1 vs 2)			Cases, no.			Control subjects, no.		
rs no.	Allele (1)	Allele (2)	*P* value (χ^2 test)	Corrected *P* value	OR (95% CI)	11	12	22	11	12	22
rs897693	C	T	**1.37E-07**	5.48E-07	5.7 (2.8-11.8)	0	12	47	1	21	570
rs4917014	G	T	**2.04.E-03**	8.16E-03	0.5 (0.4-0.8)	8	23	28	133	304	158
rs4917129	C	T	0.0269	0.108	0.6 (0.4-1.0)	7	25	27	113	293	189
rs10276619	G	A	0.0127	0.0507	1.6 (1.1-2.4)	21	25	13	112	309	174

Values in boldface indicate statistical significance.

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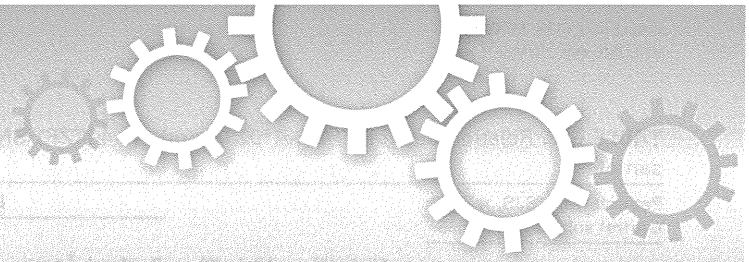

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Correspondence and requests for materials should be addressed to M.U. (mueta@koto.kpu-m.ac.jp)

Trans-ethnic study confirmed independent associations of *HLA-A*02:06* and *HLA-B*44:03* with cold medicine-related Stevens-Johnson syndrome with severe ocular surface complications

Mayumi Ueta[1,2], Chitra Kannabiran[3], Tais Hitomi Wakamatsu[4], Mee Kum Kim[5], Kyung-Chul Yoon[6], Kyoung Yul Seo[7], Choun-Ki Joo[8], Virender Sangwan[9], Varsha Rathi[9], Sayan Basu[9], Almas Shamaila[3], Hyo Seok Lee[6], Sangchul Yoon[7], Chie Sotozono[1], José Álvaro Pereira Gomes[4], Katsushi Tokunaga[10] & Shigeru Kinoshita[1]

[1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan, [3]Prof Brien Holden Eye Research Centre, L V Prasad Eye Institute, Hyderabad, India, [4]Department of Ophthalmology, Federal University of São Paulo, São Paulo, Brazil, [5]Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Korea, [6]Department of Ophthalmology, Chonnam National University, Gwangju, Korea, [7]Department of Ophthalmology, Severance Hospital, Institute of Vision Research, Yonsei University College of Medicine, Seoul, Korea, [8]Department of Ophthalmology & Visual Science, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea, [9]Cornea and Anterior Segment Services, L V Prasad Eye Institute, Hyderabad, India, [10]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory vesiculobullous reactions of the skin and mucous membranes. Cold medicines including non-steroidal anti-inflammatory drugs and multi-ingredient cold medications are reported to be important inciting drugs. Recently, we reported that cold medicine related SJS/TEN (CM-SJS/TEN) with severe mucosal involvement including severe ocular surface complications (SOC) is associated with *HLA-A*02:06* and *HLA-B*44:03* in the Japanese. In this study, to determine whether *HLA-B*44:03* is a common risk factor for CM-SJS/TEN with SOC in different ethnic groups we used samples from Indian, Brazilian, and Korean patients with CM-SJS/TEN with SOC, and investigated the association between CM-SJS/TEN with SOC and *HLA-B*44:03* and/or *HLA-A*02:06*. We found that *HLA-B*44:03* was significantly associated with CM-SJS/TEN with SOC in the Indian and Brazilian but not the Korean population, and that *HLA-A*02:06* might be weakly associated in the Korean- but not the Indian and Brazilian population.

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN) with spots, are acute inflammatory vesiculobullous reactions of the skin and mucous membranes such as the ocular surface, oral cavity, and genitals. They are rare but often associated with inciting drugs and/or infectious agents[1–3].

The association between human leukocyte antigen (HLA) genotypes and drug-induced severe cutaneous adverse reactions (SCARs) including SJS/TEN has been reported. There was a strong association between *HLA-B*58:01* and SCARs, including SJS/TEN and the drug-induced hypersensitivity syndrome (DIHS), induced by the uric acid lowering drug allopurinol. This association was observed in Han Chinese-[4], Caucasian-[5], and Japanese patients[6], suggesting that different ethnic groups share the same risk factor(s) for allopurinol-induced SCARs. *HLA-B*15:02* exhibited a very strong association with carbamazepine-

induced SJS/TEN in Taiwanese Han Chinese patients[7] and *HLA-A*31:01* was strongly associated with carbamazepine-induced SCARs including SJS/TEN in Japanese-[8] and European patients[9]. We recently reported that cold medicine-related SJS/TEN with severe mucosal involvement including severe ocular surface complications (SOC) is associated with *HLA-A*02:06* and *HLA-B*44:03* in Japanese patients[10].

The ophthalmologists Mondino et al.[11] and the dermatologists Roujeau et al.[12,13] reported that *HLA-B12* (*HLA-Bw44*) was significantly increased in Caucasian SJS patients many of whom developed SJS/TEN after taking non-steroidal anti-inflammatory drugs (NSAIDs). *HLA-B12* is primarily coded by *HLA-B*44:02* or *HLA-B*44:03* (http://www.allelefrequencies.net/). The significant association between HLA-B12 and SJS/TEN in Caucasian patients may be attributable to their genetic background.

To determine whether *HLA-B*44:03* is a common risk factor for CM-SJS/TEN with SOC in different ethnic groups we used samples from Indian, Brazilian, and Korean patients with CM-SJS/TEN with SOC, and investigated the association between CM-SJS/TEN with SOC and *HLA-B*44:03* and/or *HLA-A*02:06*.

Methods

Our study was approved by the institutional review boards of the participating institutions. All experimental procedures were conducted in accordance with the principles of the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants, and their prior written informed consent was obtained.

Patients and controls. Ophthalmologists diagnosed SJS/TEN based on a confirmed history of acute-onset high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least two mucosal sites including the ocular surface[1,14,15]. They defined patients with SOC as those who manifested a pseudomembrane and an epithelial defect on the ocular surface in the acute stage, and as patients with ocular sequelae such as dry eye, trichiasis, symblepharon, and conjunctival invasion into the cornea in the chronic stage.

As in our previous study, we focused on SJS/TEN with SOC suspected of having been induced by cold medicines such as multi-ingredient cold medications and NSAIDs. As we found earlier that the genetic predisposition might be different between SJS/TEN with and without severe mucosal involvement including SOC[10] we focused on patients from different ethnic groups who presented with SJS/TEN with SOC.

Samples from Indian patients with CM-SJS/TEN were collected at the LV Prasad Eye Institute (n = 20; 12 males, 8 females; age range 7 to 63 years; median age 27.1 ± 13.4 (SD) years). Their age at onset ranged from 3 to 42 years (median age at onset, 19.2 ± 12.2 (SD) years; in 8 patients the age at onset was unknown). The drugs administered to these patients and the HLA type (A and B) of patients with CM-SJS/TEN with SOC are shown in Supplemental Table 1. The specific drug(s) were not known in all patients. Healthy volunteers (n = 55; 29 males, 26 females; median age 36.0 ± 11.6 years) served as the Indian controls.

Samples from Brazilian patients with CM-SJS/TEN were collected at the Federal University of Sao Paulo (n= 39, 15 males, 24 females; age range 13 to 69 years; median age, 37.1 ± 15.9 years; age range at onset, 3 to 69 years; median age at onset, 24.0 ± 17.2 years). The drugs administered, the ethnicity, and the HLA type (A and B) of these CM-SJS/TEN patients with SOC are shown in Supplemental Table 2. Healthy volunteers (n = 134; 55 males, 79 females; median age 41.2 ± 12.8 years) were the Brazilian controls (ethnicity: pardo, n = 66; white, n = 62; black, n = 4, Indian plus white, n = 2).

Samples from Korean patients with CM-SJS/TEN were collected at the Seoul National University College of Medicine, Chonnam National University, Yonsei University, and the Catholic University of Korea. There were 31 patients (12 males, 19 females) ranging in age from 4 to 71 years (median age 33.7 ± 19.0 years). Their age at SJS/TEN onset ranged from 3 to 63 years (median age at onset, 23.0 ± 16.1 years). The drugs used and the HLA type (A and B) of these patients with SOC are presented in Supplemental Table 3. The specific drug(s) were not known in all patients. Healthy volunteers (n = 90; 35 males, 55 females; median age 31.7 ± 7.9 years) were the Korean controls.

Samples from Indian subjects were obtained by extracting DNA from whole peripheral blood with the phenol chloroform method. For Brazilian samples, DNA was extracted from whole peripheral blood using the PAX gene blood DNA kit (Qiagen, Hilden, Germany) or from saliva using Oragene DNA (Kyodou International, Kanagawa, Japan). To obtain the samples from Korean subjects, DNA was extracted from whole peripheral blood using the PAXgene Blood DNA kit (Qiagen).

HLA genotyping. For the analysis of *HLA-A* and *HLA-B* we performed polymerase chain reaction (PCR) assays followed by hybridization with sequence-specific oligonucleotide probes using commercial bead-based typing kits (Wakunaga,

Table 1 | Results of association analyses in patients with CM-SJS/TEN with SOC

ethnic group	HLA genotype	Carrier frequency (%)		Dominant model analysis			Gene frequency (%)		Dominant model analysis			
		CM-SJS/TEN with SOC	Control	P	Pc	Odds ratio (95% CI)	CM-SJS/TEN with SOC	Control	P	Pc	Odds ratio (95% CI)
Indian	*A*02:06*	1/20 (5.0%)	3/55 (5.5%)	0.938	-	0.91 (0.09–9.31)	1/39 (2.5%)	3/110 (2.7%)	0.939	-	0.91 (0.09–9.06)
	*B*44:03*	12/20 (60.0%)	6/55 (10.9%)	**1.07.E-05**	**2.14.E-05**	**12.25 (3.57–42.01)**	17/40 (42.5%)	7/110 (6.4%)	**9.37.E-08**	**1.87.E-07**	**10.88 (4.04–29.3)**
Brazilian	*A*02:06*	0/39 (0.00%)	0/134 (0.00%)	-	-	-	0/78 (0.00%)	0/268 (0.00%)	-	-	-
	*B*44:03*	10/39 (25.6%)	15/134 (11.2%)	**0.0239**	**0.0478**	**2.74 (1.12–6.71)**	11/78 (14.1%)	15/268 (5.60%)	**0.0121**	**0.0242**	**2.77 (1.22–6.31)**
Korean	*A*02:06*	11/31 (35.5%)	14/90 (15.6%)	**0.0181**	**0.0362**	**3.00 (1.18–7.57)**	12/62 (19.4%)	16/180 (8.9%)	**0.0263**	0.0526	**2.46 (1.09–5.54)**
	*B*44:03*	6/31 (19.4%)	18/90 (20.0%)	0.938	-	0.96 (0.34–2.69)	7/62 (11.3%)	19/180 (10.6%)	0.872	-	1.07 (0.43–2.70)

P: P values obtained with the χ^2-test (Pearson), CI: Confidence interval.
Pc: P values corrected for the multiplicity of testing by the number of comparisons 2 [HLA-A*02:06 + HLA-B*44:03].
CM-SJS/TEN: cold medicine-related SJS/TEN, SOC: severe ocular surface complications.

Hiroshima, Japan). Briefly, the target DNA was PCR-amplified with biotinylated primers specifically designed for amplified exons 2 and 3 of HLA-A, and -B genes. Then the PCR amplicon was denatured and hybridized to complementary oligonucleotide probes (72 probes for HLA-A, 93 probes for HLA-B) immobilized on fluorescent-coded microsphere beads. At the same time, the biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with Luminex 100 (Luminex, Austin, TX, USA). Genotype determination and data analysis were performed automatically using the WAKFLOW typing software (Wakunaga, Hiroshima, Japan) according to the manufacturer's instructions.

Statistical analysis. We compared the carrier frequency and gene frequency of individual HLA alleles in the patients and controls with the χ^2-test (Pearson) (JMP version 11 software; SAS Institute Japan Ltd., Tokyo, Japan).

Results

Strong association between *HLA-B*44:03* and CM-SJS/TEN with SOC in Indian patients. We genotyped HLA-A and HLA-B in samples from Indian subjects (20 CM-SJS/TEN with SOC patients and 55 controls). Although the number of Indian subjects was small, we found a strong and significant association between their CM-SJS/TEN with SOC and *HLA-B*44:03* (carrier frequency: $p = 1.07 \times 10^{-5}$, odds ratio (OR) = 12.25, gene frequency: $p = 9.37 \times 10^{-8}$, OR = 10.88) but not *HLA-A*0206* (Table 1).

Significant association between *HLA-B*44:03* and CM-SJS/TEN with SOC in Brazilian patients. Next we genotyped HLA-A and HLA-B in samples from Brazilian subjects (39 CM-SJS/TEN with SOC patients and 134 controls). Although the number of Brazilian subjects was small we found a significant association between Brazilian patients with CM-SJS/TEN with SOC and *HLA-B*44:03* (carrier frequency: p = 0.0239, OR = 2.74, gene frequency: p = 0.0121, OR = 2.77) but not *HLA-A*0206* which is absent in the Brazilian population (Table 1). Interestingly, in Caucasians in the Brazilian samples (Brazilian Caucasian CM-SJS/TEN with SOC patients: n = 15, Brazilian Caucasian controls: n = 62), the association with *HLA-B*44:03* was stronger (carrier frequency: p = 0.0037, OR = 6.22, gene frequency: p = 0.0011, OR = 5.99).

Association between *HLA-A*02:06* and Korean patients with CM-SJS/TEN with SOC. We also genotyped HLA-A and HLA-B in samples from Koreans (31 patients with CM-SJS/TEN with SOC and 90 controls). Although the number of Korean patients was small we found a significant association between patients with CM-SJS/TEN with SOC and *HLA-A*0206* (carrier frequency: p = 0.0362, OR = 3.00, gene frequency: p = 0.0263, OR = 2.46) but not *HLA-B*44:03* (Table 1).

Discussion

We previously reported that in the Japanese, CM-SJS/TEN with severe mucosal involvement including SOC was associated with *HLA-A*02:06* and *HLA-B*44:03*[10]. In the present study we investigated whether the association with these alleles is shared by other ethnic groups. We found that *HLA-B*44:03* was strongly associated with CM-SJS/TEN with SOC in the Indian population which is genetically close to European populations[16] and significantly associated in the Brazilian population which is comprised of individuals with different ethnic backgrounds. There was no association between *HLA-B*44:03* and CM-SJS/TEN with SOC in the Korean population. *HLA-A*02:06* was weakly associated in the Korean population which is genetically close to the Japanese, but not in the Indian and Brazilian population.

HLA-B12 (*HLA-Bw44*) was significantly increased in Caucasian SJS patients many of whom developed SJS/TEN after taking NSAIDs[11–13]. Because *HLA-B12* is primarily coded by *HLA-B*44:02* or *HLA-B*44:03* (http://www.allelefrequencies.net/), the significant association of *HLA-B12* with SJS/TEN in Caucasian patients may be attributable to the association with the *HLA-B*44:03* genotype.

We also found that in Brazilian Caucasian patients with CM-SJS/TEN with SOC, the significant association with *HLA-B*44:03* was stronger than in the entire study population of Brazilians with CM-SJS/TEN with SOC. To determine whether *HLA-B*44:03* is a common marker for CM-SJS/TEN with SOC in Caucasian, HLA analysis of European patients with CM-SJS/TEN with SOC is needed.

Although *HLA-A*02:06* was strongly associated with the Japanese CM-SJS/TEN with SOC, and the Korean and Japanese population is genetically close[16], in Korean patients CM-SJS/TEN with SOC was not strongly associated with *HLA-A*02:06*. To determine whether *HLA-A*02:06* is a common marker for CM-SJS/TEN with SOC in East Asian populations further investigations using a larger number of samples are needed.

We also performed a meta-analysis by adding our previously-reported samples[10]. We used Cochran-Mantel-Haenszel statistics and found that both HLA-A*02:06 and HLA-B*44:03 are significantly associated with CM-SJS/TEN with SOC (Supplemental Table 4).

SCARs including SJS/TEN and DIHS induced by allopurinol were commonly and strongly associated with *HLA-B* 58:01* in patients of different ethnic backgrounds including Han Chinese-[4], Caucasian-[5], and Japanese patients[6]. This observation suggests that different ethnic groups share the same risk factor(s) for allopurinol-induced SCARs.

With respect to carbamazepine-induced SJS/TEN, different HLA alleles are associated. *HLA-B*15:02* is associated in Taiwanese Han Chinese patients[7] and *HLA-A*31:01* in Japanese-[8] and European patients[9].

In CM-SJS/TEN with SOC, the associated alleles we identified are *HLA-A*02:06* in Japanese and Korean patients and *HLA-B*44:03* in Indian-, Brazilian-, and Japanese patients. Studies are underway to determine whether other HLA alleles are associated with CM-SJS/TEN with SOC in other populations.

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Author contributions

M.U. wrote the main manuscript text and prepared the tables. M.U., C.K., T.W., M.K., K.Y., K.S., C.J., V.S., V.R., S.B., A.S., H.L., S.Y., C.S., J.G., K.T. and S.K. contributed to material of the research and reviewed the manuscript.

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Suppression of polyI:C-inducible gene expression by EP3 in murine conjunctival epithelium

Keywords:
Prostaglandin E receptor 3 (EP3)
Toll-like receptor 3 (TLR3)
GeneChip
Conjunctival epithelium

To the Editor,

We previously reported that EP3, a subtype of prostaglandin E_2 receptors (EP1-EP4), negatively regulates eosinophilic infiltration in murine experimental allergic conjunctivitis (EAC) induced by TLR3, which causes reduced eosinophilic conjunctival inflammation in TLR3/EP3 double knock-out (DKO) mice although in EP3-KO mice eosinophilic conjunctival inflammation is pronounced [1]. We also documented that in human conjunctival epithelial cells, the EP3 agonist suppressed the production of cytokines such as CXCL10, CXCL11, IL6, CCL5, TSLP, and MCP-1 induced by polyI:C, a TLR3 ligand [2]. EP3 was dominantly expressed in conjunctival epithelial cells [3], airway epithelial cells [4], and keratinocytes [5].

To examine the effects of EP3 against polyI:C-inducible gene expression in conjunctival epithelium we performed gene expression analysis of the polyI:C-stimulated conjunctival epithelium in wild-type, EP3-KO-, and EP3/TLR3 DKO mice.

Balb/c mice were purchased from CLEA (Tokyo, Japan). EP3/TLR3 DKO mice were produced by interbreeding EP3-KO- and TLR3-KO mice at Kyoto Prefectural University of Medicine [1]. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan.

For the *in vivo* analysis of murine conjunctival epithelial cells we prepared a 100 μg/ml polyI:C solution in 50% VISCOAT® (Alcon Laboratories Ltd, Fort Worth, TX)/PBS [6]. The polyI:C solution (each about10 μl) was injected subconjunctivally and dropped into the eyes as described elsewhere [6]. At 6 h after the injection, murine conjunctival tissues were resected and then murine conjunctival epithelium were detached and collected (Supplemental methods). Collected murine conjunctival epithelium almost consisted of epithelial cells (Supplemental Fig. 1). Quantitative RT-PCR was on an ABI-prism 7000 instrument (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers for the murine samples are shown in Supplemental Table 1. Microarray analysis was with Affymetrix GeneChip® mouse gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). Throughout the process we followed Affymetrix instructions (Supplemental Methods).

Using GeneChip® we first examined the comprehensive effects of gene expression in polyI:C-stimulated conjunctival epithelium of wild-type mice. We found that after 6-h stimulation, 31 transcripts were up-regulated more than 10-fold (Supplemental Table 2). Quantitative RT-PCR confirmed that 21 of the 31 transcripts (Cxcl10, Rsad2, Ifi205, Mx1, Cmpk2, Iigp1, Mx2, Iigp2, Ifit3, Gbp5, Cxcl11, H28, Slfn8, Plscr2, Slfn4, Usp18, Sectm1a, Oas2, Dhx58, Ccl5, Isg15) were significantly (>3-fold) up-regulated. Next, to identify the transcripts regulated by EP3 we compared the gene expression of these 21 transcripts in polyI:C stimulated conjunctival epithelium of wild-type and EP3-KO mice by quantitative RT-PCR. We found that all 21 transcripts were expressed significantly stronger in polyI:C stimulated conjunctival epithelium of EP3-KO mice (Fig. 1A). We also confirmed that the mRNA expression of these 21 transcripts was significantly reduced in polyI:C stimulated conjunctival epithelium of EP3/TLR3 DKO- compared to EP3-KO mice (Fig. 1A). *Ptger3* was almost undetectable in EP3-KO and EP3/TLR3-DKO mice as was TLR3 in EP3/TLR3-DKO mice (Fig. 1B).

GeneChip® analysis also showed that the number of 4 transcripts was more than 5 times greater in polyI:C stimulated conjunctival epithelium of EP3-KO- than wild-type mice although in wild-type mice these 4 transcripts were not significantly up-regulated after 6-h polyI:C stimulation (data not shown). Quantitative RT-PCR confirmed that the number of 2 of the 4 transcripts (Oas1g and Oas1a) was more than 100-fold higher in polyI:C stimulated EP3 KO- than wild-type mice (Fig. 1C).

We found that EP3 suppresses polyI:C-inducible genes in murine polyI:C stimulated conjunctival epithelium.

Of the 21 transcripts down-regulated by EP3, 13 (Cxcl10, Rsad2, Ifi205, Mx1, Iigp1, Mx2, Iigp2, Ifit3, Cxcl11, H28, Usp18, Oas2, and Isg15) are IFN-inducible genes. Our observations on EP3-KO mice suggest that Oas1g and Oas1a are markedly suppressed by EP3; they also are IFN-inducible genes and we posit that EP3 regulates the IFN-related response. It is of interest that there was no significant difference between wild-type and EP3/TLR3-DKO mice with respect to many of the 21 transcripts that were significantly up-regulated in EP3-KO mice. This suggests that polyI:C-inducible genes are regulated not only by TLR3 but also by other molecules such as MDA5 and RIG-I. We now know that EP3 suppresses

Abbreviations: EP3, prostaglandin E receptor 3; TLR3, toll-like receptor 3; EAC, experimental allergic conjunctivitis; DKO, double knock-out; TSLP, thymic stromal lymphopoietin; MCP-1, monocyte chemoattractant protein-1; polyI:C, polyinosinic:polycytidylic acid; Cxcl10, chemokine (C-X-C motif) ligand 10; Rsad2, radical S-adenosyl methionine domain containing 2; Ifi205, interferon activated gene 205; Mx1, myxovirus (influenza virus) resistance 1; Cmpk2, cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial; Iigp1, interferon inducible GTPase 1; Mx2, myxovirus (influenza virus) resistance 2; Iigp2, interferon inducible GTPase 2; Ifit3, interferon-induced protein with tetratricopeptide repeats 3; Gbp5, guanylate binding protein 5; Cxcl11, chemokine (C-X-C motif) ligand 11; H28, histocompatibility 28; Slfn8, schlafen 8; Plscr2, phospholipid scramblase 2; Slfn4, schlafen 4; Usp18, ubiquitin specific peptidase 18; Sectm1a, secreted and transmembrane 1A; Oas2, 2′-5′ oligoadenylatesynthetase 2; Dhx58, DEXH (Asp-Glu-X-His) box polypeptide 58; Ccl5, chemokine (C–C motif) ligand 5; Isg15, ISG15 ubiquitin-like modifier; Oas1g, 2′-5′ oligoadenylatesynthetase 1G; Oas1a, 2′-5′ oligoadenylatesynthetase 1A.

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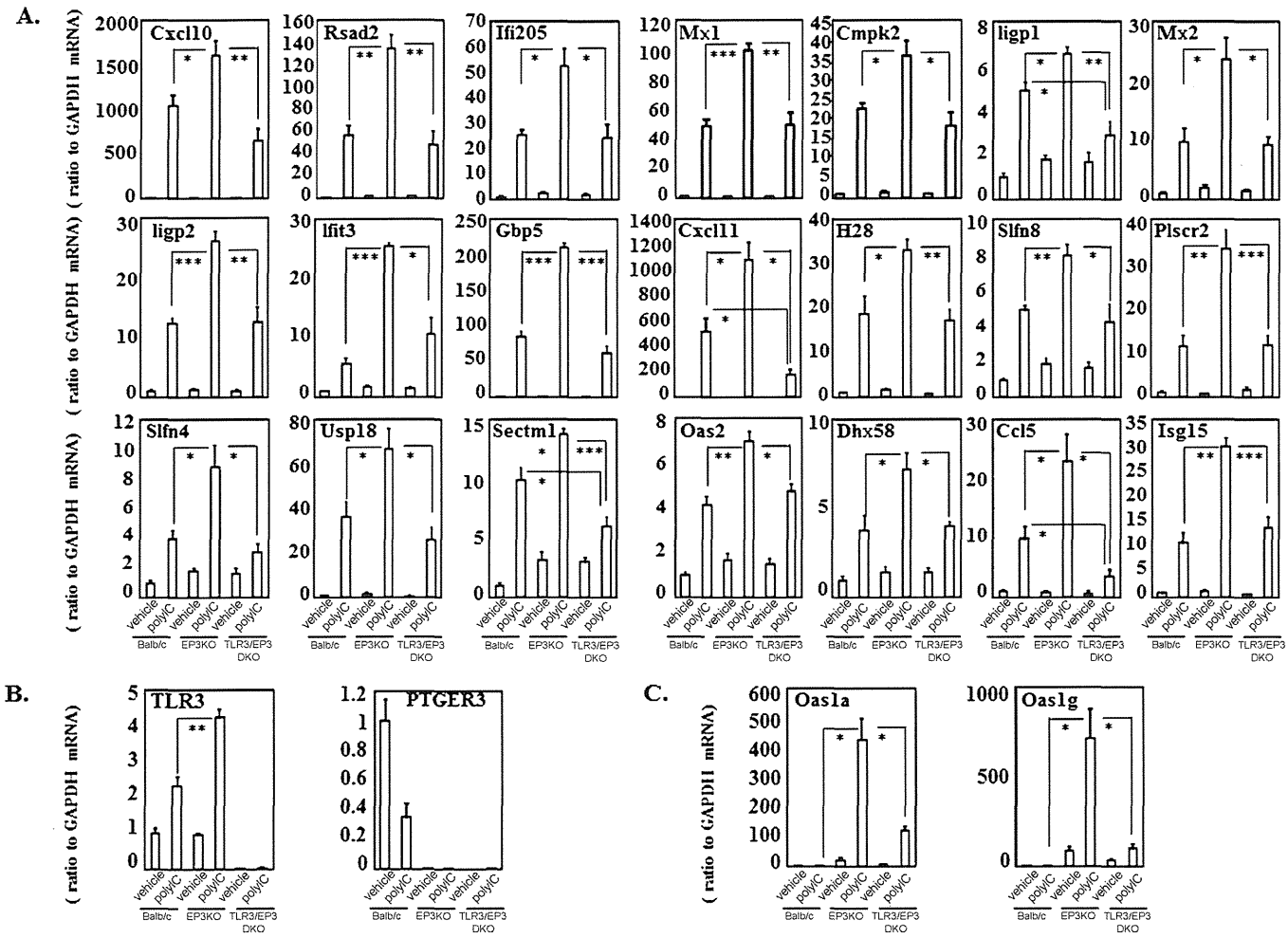

Fig. 1. Expression of transcripts induced by the polyI:C stimulation of conjunctival epithelium of wild-type-, EP3-KO-, and EP3/TLR3-KO mice. Quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y*-axis shows the increase in specific mRNA over unstimulated samples from wild-type mice. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 4 mice per group (*$p < 0.05$, **$p < 0.005$, ***$p < 0.005$).

polyI:C-inducible genes in polyI:C, a TLR3 ligand, stimulated conjunctival epithelium.

EP3 negatively regulates the eosinophilic infiltration of TLR3-induced murine EAC [1] and, EP3 and TLR3 were dominantly expressed in conjunctival epithelial cells [3,7]. In conjunctival epithelium EP3 suppresses polyI:C, a TLR3 ligand, inducible genes, suggesting that the conjunctival epithelium plays a critical role in the regulation of allergic conjunctivitis. Okuma et al. [8] recently reported that dysfunction of epithelial cells by the disruption of IκBζ induction elicits ocular surface inflammation via the activation of self-reactive lymphocytes, indicating that epithelial cells have an important role in the regulation of inflammation.

Elsewhere [1,8,9] we suggested that the pathogenesis of ocular surface inflammation such as Stevens–Johnson syndrome with severe ocular surface complications is associated with anomalies in innate immune reactions, especially reactions that involve epistatic interactions between TLR3 and EP3. We think that a lack of balance between TLR3 and EP3 is involved in triggering ocular surface inflammation [9].

In summary, we found that EP3 suppressed polyI:C, a TLR3 ligand, inducible genes in polyI:C stimulated murine conjunctival epithelium. Our findings suggest that EP3 and TLR3 in conjunctival epithelium play a critical role in regulating ocular surface inflammation.

Contributors

Material contributions to the research: Mayumi Ueta, Katsura Mizushima, Yuji Naito, Shuh Narumiya, Katsuhiko Shinomiya, Shigeru Kinoshita.

Writing and review contributions to the manuscript: Mayumi Ueta.

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None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2013.08.010.

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Mayumi Ueta [a,b,*]

[a] *Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

[b] *Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan*

Katsura Mizushima
Yuji Naito
Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Shuh Narumiya
Department of Pharmacology and Faculty of Medicine, Kyoto University, Kyoto, Japan

Katsuhiko Shinomiya
Shigeru Kinoshita
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

* Corresponding author at: Department of Ophthalmology, Kyoto Prefectural University of Medicine, Hirokoji, Kawaramachi, Kamigyo-ku, Kyoto 602-0841, Japan. Tel.: +81 75 251 5578; fax: +81 75 251 5663.
E-mail address: mueta@koto.kpu-m.ac.jp (M. Ueta)

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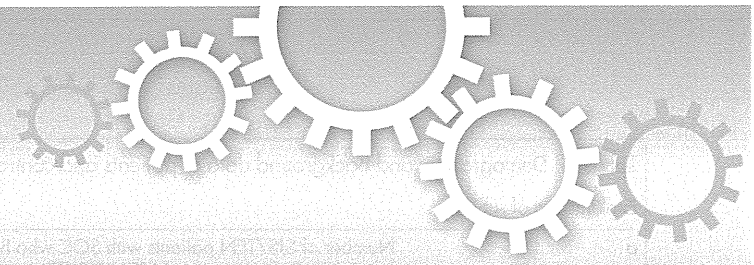

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Correspondence and requests for materials should be addressed to M.U. (mueta@koto.kpu-m.ac.jp)

Independent strong association of *HLA-A*02:06* and *HLA-B*44:03* with cold medicine-related Stevens-Johnson syndrome with severe mucosal involvement

Mayumi Ueta[1,2], Nahoko Kaniwa[3], Chie Sotozono[1], Katsushi Tokunaga[4], Yoshiro Saito[3], Hiromi Sawai[4], Hiroko Miyadera[4], Emiko Sugiyama[3], Keiko Maekawa[3], Ryosuke Nakamura[3], Masaki Nagato[5], Michiko Aihara[6], Kayoko Matsunaga[7], Yukitoshi Takahashi[8], Hirokazu Furuya[9], Masaaki Muramatsu[10], Zenrou Ikezawa[11] & Shigeru Kinoshita[1]

[1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan, [3]Division of Medicinal Safety Science, National Institute of Health Sciences, Tokyo, Japan, [4]Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, [5]Molecular Diagnostics Division, Wakunaga Pharmaceutical Co., Hiroshima, Japan, [6]Department of Environmental Immuno-Dermatology, Yokohama City University Graduate School of Medicine, Kanagawa, Japan, [7]Department of Dermatology, Fujita Health University School of Medicine, Aichi, Japan, [8]National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders, Shizuoka, Japan, [9]Department of Neurology, Faculty of Medicine, Kochi University, Kochi, Japan, [10]Molecular Epidemiology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan, [11]Department of Dermatology, International University of Health and Welfare (IUHW) Atami Hospital, Shizuoka, Japan.

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory vesiculobullous reactions of the skin and mucous membranes. Cold medicines including non-steroidal anti-inflammatory drugs (NSAIDs) and multi-ingredient cold medications are reported to be important inciting drugs. We used two sample sets of Japanese patients to investigate the association between HLA genotypes and cold medicine-related SJS/TEN (CM-SJS/TEN), including acetaminophen-related SJS/TEN (AR-SJS/TEN) with severe mucosal involvement such as severe ocular surface complications (SOC). *HLA-A*02:06* was strongly associated with CM-SJS/TEN with SOC and AR-SJS/TEN with SOC. *HLA-B*44:03* was also detected as an independent risk allele for CM-, including AR-SJS/TEN with SOC. Analyses using data obtained from CM-SJS/TEN patients without SOC and patients with CM-unrelated SJS/TEN with SOC suggested that these two susceptibility alleles are involved in the development of only CM-SJS/TEN with SOC patients.

Stevens-Johnson syndrome (SJS) is an acute inflammatory vesiculobullous reaction of the skin and mucous membranes such as the ocular surface, oral cavity, and genitals. It is rare but often associated with inciting drugs and/or infectious agents[1–3]. In patients with extensive skin detachment and a poor prognosis the condition is called toxic epidermal necrolysis (TEN)[4]. The annual incidence of SJS and TEN has been reported as 1–6 and 0.4–1.0 cases per million persons, respectively[3,5] and the mortality rate as 3% and 27%, respectively[6].

The association between human leukocyte antigen (HLA) genotypes and drug-induced severe cutaneous adverse reactions (SCAR) including SJS/TEN has been reported. In Taiwanese Han Chinese patients the *HLA-B*15:02* allele exhibited a very strong association with carbamazepine-induced SJS/TEN[7]. Similarly, in Japanese-[8] and European individuals[9] the *HLA-A*31:01* allele was strongly associated with carbamazepine-induced SCAR including SJS/TEN and drug-induced hypersensitivity syndrome (DIHS). Allopurinol, a uric acid-lowering drug, often induced SCAR including SJS, TEN and DIHS, and allopurinol-induced SCARs were strongly associated with *HLA-B* 58:01* in Han Chinese-[10], Caucasian-[11], and Japanese patients[12], suggesting that different ethnic groups may share the same risk factor for allopurinol-induced SCARs. Mockenhaupt et al.[13] reported that

Table 1 | Demographic and background data of patients and controls

Explaination of subjects		Group 1 (KPUM)	Group 2 (NIHS)
a	Number of SJS/TEN patients with SOC who had taken cold medicines for treatment of common cold (CM-SJS/TEN with SOC group)	131	20
	Female/Male	80/51	14/6
	Age of onset (years, mean ± SD)	26.6 ± 17.5	54.0 ± 17.7
b (which are included in a)	Number of SJS/TEN patients with SOC who had taken acetaminophen for treatment of common cold (Acetaminophen-SJS/TEN with SOC group)	(59)	(14)
	Female/Male	37/22	9/5
	Age of onset (years, mean ± SD)	31.1 ± 15.8	35.2 ± 16.9
c	Patients with SJS/TEN without SOC who had taken cold medicines for treatment of common cold (CM-SJS/TEN without SOC group)		16
	Female/Male	-	9/7
	Age of onset (years, mean ± SD)		62.0 ± 25.0
d	Patients with SJS/TEN with SOC who had taken medicines not for treatment of common cold (CM unrelated-SJS/TEN with SOC group)	14	38
	Female/Male	11/3	19/19
	Age of onset (years, mean ± SD)	44.8 ± 19.3	57.4 ± 23.1
the samples excluded because of drug unrelated or detail unknown		17	-
total number of the SJS/TENpatients		162	74
Controls	Healthy volunteers	419	220
	Female/Male	350/69	131/89
	Age (years, mean ± SD)	-	35.5 ± 11.0

CM-SJS/TEN: Cold medicine-related SJS/TEN.
SOC: severe ocular surface complications.
KPUM: Kyoto Prefectural University of Medicine, NIHS: National Institute of Health Sciences.

allopurinol and anticonvulsants such as carbamazepine are the main inciting drugs for SJS/TEN; we[14] and others[2,4] found that cold medicines including non-steroidal anti-inflammatory drugs (NSAIDs) and multi-ingredient cold medications are also major causative drugs for SJS/TEN. However, there have been no reports on the association between HLA genotypes and cold medicines in patients with SCAR.

Many SJS/TEN survivors suffer severe sequelae such as visual disturbance due to severe ocular surface complications (SOC) in the acute phase of the disease. In our earlier study of 71 Japanese SJS/TEN patients we reported the strong association between *HLA-A*02:06* and SJS/TEN with SOC[15]. We found that a considerable number of these patients used cold medicines to treat the common cold[14]. Therefore, in this study we focused on a possible association between HLA genotypes and cold medicine (NSAIDs and analgesics)-related SJS/TEN (CM-SJS/TEN) with severe mucosal involvement including SOC.

Results

HLA-type associated with CM-SJS/TEN with SOC. First we compared the carrier frequencies of HLA alleles in the 131 CM-SJS/TEN with SOC patients and in 419 controls. The results are summarized in Table 2.

*HLA-A: HLA-A*02:06* was strongly associated with CM-SJS/TEN with SOC ($p = 2.8 \times 10^{-16}$, $Pc = 4.8 \times 10^{-15}$, odds ratio (OR) = 5.7). *HLA-A*24:02* was inversely associated with CM-SJS/TEN with SOC ($p = 3.9 \times 10^{-4}$, Pc = 0.0066, OR = 0.5). *HLA-A*03:01* was weakly associated with the risk for- and *HLA-A*11:01* was weakly associated with resistance to CM-SJS/TEN with SOC; the association was not significant after Bonferroni correction.

*HLA-B: HLA-B*13:01, HLA-B*44:02, HLA-B*44:03*, and *HLA-B*46:01* were weakly associated with CM-SJS/TEN with SOC; the association was not significant after correction. *HLA-B*15:01, HLA-B*52:01* and *HLA-B*54:01* were weakly inversely associated with CM-SJS/TEN with SOC; the association was not significant after correction.

*HLA-C: HLA-C*03:04* and *HLA-C*05:01* were weakly associated- and *HLA-C*12:02* was weakly and inversely associated with CM-SJS/TEN with SOC; the association was not significant after correction.

Next, to confirm these associations we compared the carrier frequency of HLA alleles with p values less than 0.05 before Bonferroni correction in the 131 CM-SJS/TEN with SOC of Group 1a, in another 20 CM-SJS/TEN with SOC patients (Group 2a) and 220 healthy controls of Group2.

In Group 2a (n = 20), *HLA-A*02:06* and *HLA-B*44:03* were significantly associated with CM-SJS/TEN with SOC ($p = 0.0014$, $Pc = 0.0056$, OR = 5.2 and $p = 0.0058$, $Pc = 0.0406$, OR = 4.22, respectively) (Table 3). However, the other HLA alleles examined were not significantly associated. Although the patient backgrounds were a little bit different in Groups 1a and 2a (1a: CM-SJS/TEN with SOC as sequelae, 2a: CM-SJS/TEN with SOC in the acute phase), we identified the same HLA types, *HLA-A*02:06* and *HLA-B*44:03*, as risk factors for CM-SJS/TEN with SOC.

As we observed the same tendency in Groups 1a and 2a, we combined the 151 CM-SJS/TEN with SOC patients (Group 1a, n = 131; Group 2a, n = 20) to compare the carrier frequencies of *HLA-A*02:06* and *HLA-B*44:03* with the frequencies in the 639 combined healthy controls. (Group 1, n = 419; Group 2, n = 220). The combined data revealed a strong association of *HLA-A*02:06* and *HLA-B*44:03* with CM-SJS/TEN with SOC (*HLA-A*02:06*, $p = 2.7 \times 10^{-20}$, OR = 5.6; *HLA-B*44:03*, $p = 1.25 \times 10^{-3}$, OR = 1.99) (Table 4a).

Comparison between CM-SJS/TEN with and without SOC. Among 16 CM-SJS/TEN without SOC patients (Group 2c), 2 carried *HLA-A*02:06* and none carried *HLA-B*44:03* (Table 4b). These carrier frequencies did not differ significantly from the Group 2 controls (p = 1.000 and p = 0.2324, respectively). These results suggest that *HLA-A*02:06* and *HLA-B*44:03* are not common risk factors for both CM-SJS/TEN with and without SOC, but were risk factors for only CM-SJS/TEN with SOC.

For further confirmation we compared the carrier frequency of both HLA alleles in the 151 combined CM-SJS/TEN with SOC patients (Group 1a, n = 131, Group 2a, n = 20) and in the 16 CM-SJS/TEN without SOC patients in Group 2c. The carrier frequencies of both alleles were significantly higher in the CM-SJS/TEN with SOC (Group 1a + Group 2a) than in the CM-SJS/TEN without

Table 2 | Results of association analysis for HLA types and CM-SJS/TEN with SOC in Group 1 (KPUM)

HLA genotype	Carrier frequency (%)		Dominant model analysis		
	Case (n = 131)	Control (n = 419)	P	Pc	Odds ratio (95% CI)
HLA-A					
*A*02:06*	62/131 (47.3%)	57/419 (13.60%)	**2.79.E-16**	**4.75E-15**	**5.71 (3.666-8.881)**
*A*03:01*	5/131 (3.82%)	4/419 (0.95%)	**0.0242**	0.412	4.12 (1.089-15.564)
*A*11:01*	10/131 (7.6%)	71/419 (16.95%)	**8.67.E-03**	0.147	0.405 (0.202-0.811)
*A*24:02*	57/131 (43.5%)	256/419 (61.10%)	**3.89.E-04**	**6.60.E-03**	**0.490 (0.330-0.730)**
HLA-B					
*B*13:01*	10/131 (7.6%)	13/419 (3.10%)	**0.0237**	0.807	2.58 (1.104-6.032)
*B*15:01*	11/131 (8.4%)	69/419 (16.47%)	**0.0222**	0.755	0.465 (0.238-0.908)
*B*44:02*	5/131 (3.82%)	5/419 (1.19%)	**0.0498**	1.69	3.29 (0.936-11.532)
*B*44:03*	31/131 (23.7%)	66/419 (15.75%)	**0.0381**	1.29	1.66 (1.024-2.682)
*B*46:01*	22/131 (16.8%)	38/419 (9.07%)	**0.0133**	0.453	2.02 (1.148-3.566)
*B*52:01*	12/131 (9.2%)	79/419 (18.85%)	**9.16.E-03**	0.311	0.434 (0.228-0.825)
*B*54:01*	10/131 (7.6%)	61/419 (14.56%)	**0.0391**	1.33	0.485 (0.241-0.976)
HLA-C					
*C*03:04*	42/131 (32.1%)	98/419 (23.39%)	**0.0467**	0.841	1.55 (1.00-2.38)
*C*05:01*	5/131 (3.82%)	5/419 (1.19%)	**0.0498**	0.897	3.29 (0.936-11.532)
*C*12:02*	13/131 (9.9%)	80/419 (19.09%)	**0.0145**	0.262	0.467 (0.251-0.870)

P: P values obtained with χ^2-tests.
Pc: P values corrected for the multiplicity of testing by the number of comparisons (17, 34, and 18 for *HLA-A*, *HLA-B* and *HLA-C*, respectively).
CM-SJS/TEN: cold medicine related SJS/TEN who had taken cold medicine.
SOC: severe ocular surface complications.
CI: confidence interval.

SOC (Group 2c) (*HLA-A*02:06*, p = 0.00812, OR = 6.2; *HLA-B*44:03*, p = 0.02023, OR = 11.59) (Table 4b).

Analysis of CM unrelated-SJS/TEN with SOC. As shown in Table 1, Group 1d contained 14- and Group 2d contained 38 patients with CM unrelated (other medicine related) -SJS/TEN with SOC. Among the 14 CM unrelated-SJS/TEN with SOC patients from Group 1d, 3 carried *HLA-A*02:06* and 4 carried *HLA-B*44:03*. Among the 38 CM unrelated SJS/TEN with SOC patients from Group 2d, 4 manifested *HLA-A*02:06* and 2 had *HLA-B*44:03*. To obtain higher power, we combined the data from the 52 CM unrelated -SJS/TEN with SOC patients from Groups 1d (n = 14) and 2d (n = 38) and compared their carrier frequency with that of combined healthy volunteers (n = 639). As shown in Table 4c, the carrier frequencies of *HLA-A*02:06* and *HLA-B*44:03* were comparable in the 2 groups (52 CM unrelated -SJS/TEN with SOC patients and 639 controls) and the difference was not statistically significant.

Analysis of acetaminophen-SJS/TEN with SOC (AR-SJS/TEN with SOC). Acetaminophen is contained as an analgesic in most cold medicines. At least 59 patients in Group 1b and 14 in Group 2b were known to have taken acetaminophen for a few ~ several days before the onset of SJS/TEN. Therefore we examined the association of *HLA-A*02:06* and *HLA-B*44:03* with acetaminophen-related SJS/TEN (AR-SJS/TEN) with SOC using the combined data (73 AR-SJS/TEN with SOC from 59 in Group 1b and 14 in Group 2b). In all 73

Table 3 | Results of association analysis between HLA types and CM-SJS/TEN with SOC in Group 2 (NIHS)

HLA genotype	Carrier frequency (%)		Dominant model analysis		
	Case (n = 20)	Control (n = 220)	P	Pc	Odds ratio (95% CI)
HLA-A					
*A*02:06*	9/20 (45.0%)	30/220 (13.6%)	**0.0014**	**0.00560**	**5.18 (1.98–13.56)**
*A*03:01*	0/20 (0%)	19/220 (8.6%)	0.3804		
*A*11:01*	2/20 (10.0%)	39/220 (17.7%)	0.5408		
*A*24:02*	14/20 (70.0%)	132/220 (60.0%)	0.4770		
HLA-B					
*B*13:01*	2/20 (10%)	6/220 (2.7%)	0.1364		
*B*15:01*	2/20 (10%)	39/220 (17.7%)	0.5408		
*B*44:02*	0/20 (0%)	4/220 (1.8%)	1.0000		
*B*44:03*	8/20 (40.0%)	30/220 (13.6%)	**0.0058**	**0.0406**	**4.22 (1.59–11.19)**
*B*46:01*	2/20 (10%)	18/220 (8.2%)	0.6764		
*B*52:01*	1/20 (5.0%)	48/220 (21.8%)	0.0857		
*B*54:01*	5/20 (25%)	33/220 (15.0%)	0.3316		
HLA-C					
*C*03:04*	6/20 (30%)	43/220 (19.5%)	0.2573		
*C*05:01*	0/20 (0%)	4/220 (1.8%)	1.0000		
*C*12:02*	1/20 (5.0%)	47/220 (21.4%)	0.1388		

P: p-values obtained by Fisher's exact tests are shown.
Pc: p-values corrected for the mutiplicity of testing by the number of comparisons: (4, 7 and 3 for HLA-A, HLA-B and HLA-C, respectively).
CM-SJS/TEN: cold medicine related SJS/TEN who had taken cold medicine.
SOC: severe ocular surface complications.
CI: Confidence interval.