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.

Table 4 | Results of association analyses using combined SJS/TEN patients' data

a. Comparison between CM-SJS/TEN with SOC (Group 1a and Group 2a) and combined healthy volunteers' data

	Carrier frequency (%)		Dominant model analysis	
HLA genotype	CM-SJS/TEN with SOC (Group 1a and Group 2a)	Control (Combined healthy controls)	p	Odds ratio (95% CI)
*A*02:06*	71/151 (47.0%)	87/639 (13.6%)	2.72E-20	5.63 (3.81–8.33)
*B*44:03*	39/151 (25.8%)	95/639 (14.9%)	0.00125	1.99 (1.30–3.05)

b. Comparison between CM-SJS/TEN with SOC (Group 1a and Group 2a) and without SOC (Group 2c)

	Carrier frequency (%)		Dominant model analysis	
HLA genotype	CM-SJS/TEN with SOC (Group 1a and Group 2a)	CM-SJS/TEN without SOC (Group 2c)	p	Odds ratio (95% CI)
*A*02:06*	71/151 (47%)	2/16 (12.5%)	0.00812	6.21 (1.36–28.28)
*B*44:03*	39/151 (25.8%)	0/16 (0%)	0.02023	11.59* (0.68–197.7)

c. Comparison of CM unrelated SJS/TEN with SOC and combined healthy volunteers' data

	Carrier frequency (%)		Dominant model analysis
HLA genotype	CM unrelated-SJS/TEN with SOC (Group 1d and Group 2d)	Control (Combined healthy controls)	p
*A*02:06*	7/52 (13.5%)	87/639 (13.6%)	0.975
*B*44:03*	6/52 (11.5%)	95/639 (14.9%)	0.514

d. Comparison between Acetaminophen-SJS/TEN with SOC (Group 1b and Group 2b) and combined healthy volunteers' data

	Carrier frequency (%)		Dominant model analysis	
HLA genotype	Acetaminophen-SJS/TEN with SOC (Group 1b and Group 2b)	Control (Combined healthy controls)	p	Odds ratio (95% CI)
*A*02:06*	37/73 (50.7%)	87/639 (13.6%)	2.54E-15	6.52 (3.91–10.88)
*B*44:03*	20/73 (27.4%)	95/639 (14.9%)	0.0059	2.16 (1.27–3.78)

*Woolf's correction.
P: P values obtained by χ^2-tests.
CM-SJS/TEN: cold medicine related SJS/TEN who had taken cold medicine.
SOC: severe ocular surface complications.
CI: Confidence interval.

patients with AR-SJS/TEN with SOC, we found a significant association with both alleles (*HLA-A*02:06*, $p = 2.5 \times 10^{-15}$, OR = 6.5; *HLA-B*44:03*, p = 0.0059, OR = 2.2) (Table 4d).

Discussion

In this study we examined possible HLA risk factors for CM-SJS/TEN with SOC using two independently collected data sets of Japanese SJS/TEN patients.

The carrier frequency of *HLA-A*02:06*, which we reported to have a very strong association with causative drug-unspecified SJS/TEN with SOC[15,19], was significantly higher in CM-SJS/TEN with SOC patients than in the healthy controls. This significant association was maintained in AR-SJS/TEN with SOC.

On the other hand, the carrier frequency of *HLA-A*02:06* in the 16 CM-SJS/TEN without SOC patients of Group 2c and the 52 CM-unrelated SJS/TEN with SOC patients from Groups 1d and 2d did not significantly differ from that in our healthy controls. These results suggest that *HLA-A*02:06* is a risk factor for CM-SJS/TEN with SOC but not for CM-SJS/TEN without SOC or CM-unrelated SJS/TEN with SOC.

Moreover, *HLA-A*02:06* and *HLA-B*44:03* might not be primarily associated with only infection related SJS/TEN, because drug-unrelated SJS/TEN with SOC in KPUM, which seemed to be only infectious agents-related SJS/TEN, was not associated with *HLA-A*02:06* and *HLA-B*44:03* in our preliminary study (Supplemental Table 1).

The carrier frequeny of *HLA-A*02:06* in all of our healthy controls was 13.6% (Tables 2 and 3), indicating that *HLA-A*02:06* is a very common allele in the Japanese. However, as it is very rare in Caucasians and less frequent in Southern Han Chinese[20], in these populations, this allele might not be a major risk factor for CM-SJS/TEN with SOC. We also found a significant association between *HLA-B*44:03* and CM-SJS/TEN with SOC (including AR-SJS/TEN with SOC). This association was not detected in CM-SJS/TEN without SOC patients nor in CM-unrelated SJS/TEN with SOC patients. This again suggests *HLA-B*44:03* as a risk factor for CM-SJS/TEN with SOC. Data on our controls (Tables 2 and 3) indicate that *HLA-B*44:03* is a common *HLA-B* type in the Japanese population. Unlike *HLA-A*02:06*, *HLA-B*44:03* is observed in Asians, Caucasians and Africans[21]. Reports from the USA[22] and France[23,24] showed that the *HLA-B12* (*HLA-Bw44*) antigen was significantly increased in Caucasian SJS patients. The *HLA-B12* antigen is mainly coded by *HLA-B*44:02* or *HLA-B*44:03* (http://www.allelefrequencies.net/).

Cold medicines were reported to be major causative drugs in SJS/TEN in Europe[4] and in its drug safety communications, the U.S. Food and Drug Administration (http://www.fda.gov/Drugs/DrugSafety/ucm363041.htm) alerted to the possibility of serious skin reactions to acetaminophen. The significant association of HLA-B12 with SJS/TEN in European patients may be attributable to their genetic backgrounds. To determine whether *HLA-B*44:03* is a common risk

factor for CM-SJS/TEN with SOC in various populations, independent association studies in divergent ethnic groups are needed.

Because *HLA-A*02:06* is rarely a haplotype with *HLA-B*44:03* (http://www.allelefrequencies.net/), these two HLA alleles might be independent genetic risk factors that render the host susceptible to severe mucosal disorders and to severe sequelae such as visual disturbance when SJS/TEN develops after the administration of cold medicines including NSAIDs. In our study, 96 of 151 patients (63.6%) with CM-SJS/TEN with SOC (group 1, n = 131; group 2, n = 20) harbored either *HLA-A*02:06* or *HLA-B*44:03*. On the other hand, only 177 of our 639 controls (27.7%) had one of these *HLA* alleles.

Forman et al.[25] and Leaute-Labreze[26] reported other infectious agents as triggers of SJS/TEN. Elsewhere[27] we showed that rs3775296T/T, a SNP of *Toll-like receptor 3 (TLR3)*, was a risk factor for SJS/TEN with SOC and that the interaction between rs3775296T/T and *HLA-A*02:06* exerted more than additive effects. TLR3 is a pattern-recognizing receptor related to innate immunity after viral infections that often produce common cold symptoms. Moreover, cold medicines such as acetaminophen and NSAIDs, including ibuprofen and loxoprofen, commonly down-regulate the production of prostanoid including PGE_2. We also reported earlier that in our study population, EP3, which is one of the PGE_2 receptors, polymorphisms were strongly associated with SJS/TEN with SOC[14] and that the EP3 protein levels were much lower in the conjunctival epithelial cells of SJS/TEN patients than in the control subjects[14,28]. It is noteworthy that in our earlier study of SJS/TEN with SOC patients[14] about 80% had CM-SJS/TEN with SOC. It might be possible that not only cold medicine but cold medicine with infectious agent could cause CM-SJS/TEN with SOC, because the patients develop CM- SJS/TEN with SOC by taking cold medicines after having common cold induced by infectious agents. We believe that interactions between *HLA* risk factors detected in the current study and *TLR3*, and/or *EP3* might be keys in the pathogenesis of CM-SJS/TEN with SOC.

In summary, we reported the association between certain *HLA* types and CM-SJS/TEN with SOC. We propose that *HLA-A*02:06* and *HLA-B*44:03* be considered as strong risk factors for CM-SJS/TEN with SOC. Our findings may help to elucidate the pathogenesis of CM-SJS/TEN with SOC.

Methods

Our study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan, the National Institute of Health Sciences, Tokyo, Japan, and the Faculty of Medicine, University of Tokyo, Tokyo, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the study and the experimental protocols were explained to all participants and their prior written informed consent was obtained.

Patients and controls. Japanese SJS/TEN patients (n = 236) were independently recruited at Kyoto Prefectural University of Medicine (KPUM)(Group 1, n = 162) and by the Japan Severe Adverse Reactions Research Group, mainly conducted by the National Institute of Health Sciences (NIHS) (Group 2, n = 74).

Between October 2004 and May 2013, 162 SJS/TEN with SOC were treated at Kyoto Prefectural University of Medicine; of these, 71 were included in our previous study[15]. The diagnosis of SJS/TEN with SOC was based on a confirmed history of acute-onset high fever, serious mucocutaneous illness with skin eruptions, and the involvement of at least 2 mucosal sites including the oral cavity and ocular surface. Some of the patients had developed SJS/TEN many years before recruitment for this study. Of the 162 patients in Group 1, 131 patients had taken cold medicines such as NSAIDs and multi-ingredient cold medications for a few ~ several days before disease onset for common-cold symptoms; they were classified as CM-SJS/TEN with SOC (Group 1a). Although the specific drugs were not identified by all 131 CM-SJS/TEN with SOC patients, 59 of 131 CM-SJS/TEN with SOC patients (45%) reported taking medicines containing acetaminophen (AR-SJS/TEN with SOC, Group 1b). Among the 162 of SJS/TEN with SOC patients (Group 1), 14 patients (Group 1d) were classified as CM unrelated-SJS/TEN with SOC, because they manifested anticonvulsants-related SJS/TEN with SOC (n = 10) or SJS/TEN with SOC after being treated with antimalarial-, anticancer-, or anti-depressive agents or steroids n = 4). We also excluded 17 patients; in 9 SJS/TEN with SOC the drugs were unknown and in 8 SJS/TEN with SOC were not related to drugs.

Group 2 (n = 74) consisted of patients with newly-developed SJS/TEN; they were recruited between June 2006 and May 2013 by participating institutes or via a nationwide blood sampling network operated by the NIHS in cooperation with the Ministry of Health, Labour and Welfare, the Pharmaceutical and Medical Devices Agency, and the Federation of Pharmaceutical Manufacturers' Association of Japan. The criteria proposed by Bastuji-Garin et al.[16] were used for a diagnosis of SJS/TEN in this group.

Ocular surface complications were judged to be severe ocular complications (SOC) when pseudo-membrane formation and/or conjunctival or corneal epithelial defects were observed in the acute phase. As shown in Table 1, Group 2 (n = 74) consisted of 20 patients with CM-SJS/TEN with SOC (Group 2a), all but 6 of these presented with AR-SJS/TEN with SOC (Group 2b). Group 2 also included 16 patients with CM-SJS/TEN without SOC (Group 2c), and 38 patients with CM-unrelated-SJS/TEN with SOC (Group 2d). The background of the 236 patients with SJS/TEN in group1 and group2 is summarized in Table 1.

Healthy Japanese volunteers (n = 639) served as the controls. They were independently recruited by the University of Tokyo (n = 419)[17] and by Kyoto Prefectural University of Medicine (n = 220)[18] and served for comparison studies of patient groups 1 and 2, respectively. In this study we enrolled only mainland Japanese.

HLA genotyping. We analyzed *HLA-A, -B, and -C* of all 162 group 1 patients, which consist of 131 CM-SJS/TEN with SOC (group 1a), 14 CM-unrelated (other medicine related) SJS/TEN with SOC (group 1d), and 17 SJS/TEN with SOC excluded because of being drug-unrelated and detail unknown. We performed polymerase chain reaction (PCR) assays followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial bead-based typing kits (Wakunaga, Hiroshima, Japan). In group 2 (n = 74) we performed high-resolution HLA typing with a sequence-based method using SeCoreA, -B, and -C, locus sequencing kits (Invitrogen Corp., Brown Deer, WI, USA) and ABI 3730 and 3130 DNA sequencers (Applied Biosystems, Foster City, CA, USA). HLA genotypes were assigned using Assign SBT- or Assign ATF software (versions 3.2.7b and 1.0.2.41; respectively, Conexio Genomics, Western Australia, Australia). We also genotyped all volunteers for *HLA-A, -B, and -C* using PCR-SSO and commercial bead-based typing kits (Wakunaga or One Lambda, CA, USA).

Statistical analysis. We compared the carrier frequency of individual HLA alleles between our patients and controls based on the dominant model using the χ^2-test (Labo Server software;World Fusion, Tokyo, Japan) or Fisher's exact test (JMP version 7.0.1 software; SAS Institute Japan Ltd., Tokyo, Japan). Significance levels were corrected with the Bonferroni correction for multiple comparisons.

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Author contributions

M.U., N.K. and K.T. wrote the main manuscript text and made Table, M.U., N.K., C.S., K.T., Y.S., H.S., H.M., E.S., K.M., R.N., M.N., M.A., K.M., Y.T., H.F., M.M., Z.I. and S.K. contributed to material of the research and reviewed the manuscript.

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Upregulation of Toll-like receptor 5 expression in the conjunctival epithelium of various human ocular surface diseases

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ABSTRACT

Aims To examine the expression of Toll-like receptor (TLR) 5 in the conjunctival epithelium of patients with severe ocular surface diseases.

Methods Immunohistochemical study of TLR5 was performed on conjunctival tissues obtained from patients undergoing surgical reconstruction of the ocular surface to treat Stevens-Johnson syndrome (SJS) (n=4), ocular cicatricial pemphigoid (OCP) (n=3), chemical eye burn (n=3), and pterygium (n=2), and on nearly normal conjunctival tissues obtained during surgery for four cases of conjunctivochalasis as a control.

Results TLR5 protein was consistently and abundantly expressed in the conjunctival epithelium and detected only at the basal and wing cells. However, in the conjunctival epithelium obtained from the patients with SJS, OCP and chemical eye burns, the TLR5 protein was detected at not only the basal and wing cells but also at the superficial cells. TLR5 protein detected in the pterygium patients mirrored that detected in the controls.

Conclusions Although TLR5 was normally present on the basal and wing cells of conjunctival epithelium with spatially selective presence, it was expressed on not only the basal and wing cells but also the superficial cells in the conjunctival epithelium of patients with SJS, OCP or chemical eye burns, suggesting that TLR5 might be upregulated in the conjunctival epithelium of these diseases.

INTRODUCTION

The ocular surface epithelium serves as the defensive front line of the innate immune system, and Toll-like receptors (TLRs) are known to be one of the key receptors of the innate immune system. Reportedly, TLRs are pattern-recognition receptors that sense conserved pathogen-associated molecular patterns (PAMPs), and are the key receptors for the recognition of microbes.[1] TLRs are a type of transmembrane protein composed of three major domains, and are characterised by LRRs (leucine-rich repeats) in the ectodomain which mediate the recognition of their respective PAMPs. TLR5 recognises bacterial flagellin, a component protein of bacterial flagella.[2 3] Flagella are present in both gram-positive and gram-negative bacteria and are essential for bacterial motility, invasion and chemotaxis.[4] We previously reported that human ocular surface epithelial cells, both corneal and conjunctival, express TLR5-specific mRNA and TLR5 proteins, and TLR5 proteins were detected at basal and wing site cells.[5 6] The purpose of this present study was to examine the expression of TLR5 in the conjunctival epithelium of patients with severe ocular surface disorders.

METHODS

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan, and all experiments were conducted in accordance with the tenets set forth in the Declaration of Helsinki. The purpose of the research and the experimental protocol were explained to all patients, and their informed consent was obtained before their involvement in the study.

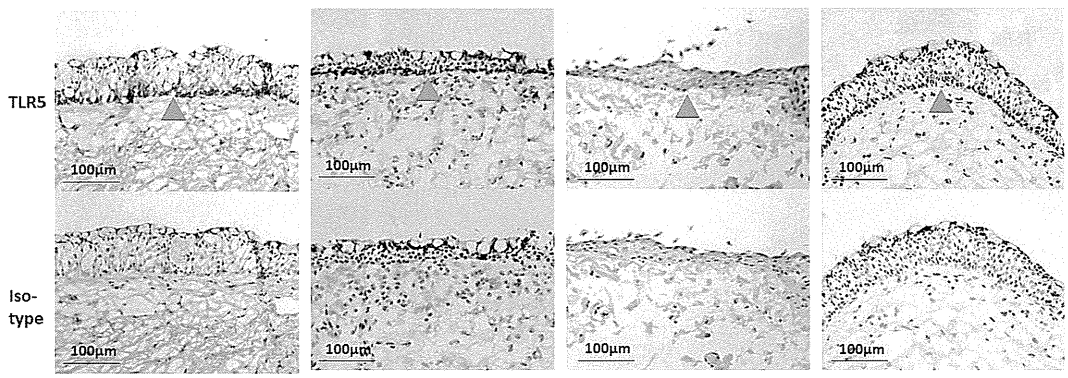

Figure 1 Immunohistological analysis of Toll-like receptor (TLR) 5 in the conjunctival epithelium of the controls. Localisation of TLR5 in human conjunctival epithelium as control tissue, from four conjunctivochalasis cases which underwent surgery. The conjunctival tissue was incubated with anti-TLR5 antibody. Isotype control incubation was used as the negative control.

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For the immunohistochemical study, the control samples were nearly normal human conjunctival tissues obtained at the time of surgery for the treatment of conjunctivochalasis (four cases). Conjunctivochalasis is a normal ageing related change, and it is an isolated bilateral condition in which redundant bulbar conjunctival tissue interposes between the globe and the lower eyelid.[7] Cicatricial conjunctival tissues were also obtained from patients undergoing surgical reconstruction of their ocular surface that had been devastated due to Stevens-Johnson syndrome (SJS) (four cases), ocular cicatricial pemphigoid (OCP) (three cases), and chemical eye burns (three cases). In addition, the conjunctival tissues of patients with pterygium (two cases) were obtained. Pterygium is reportedly a benign growth of the conjunctiva that is thought to be caused by exposure to ultraviolet light.[8]

For TLR5 staining, we used mouse antihuman TLR5 monoclonal antibody (mAb; Abcam, Cambridge, UK) (TLR5: TLR5 antibody (19D759.2)) in a 0.5 μg/μL dilution with blocking solution. As isotype controls, mouse IgG2a X0943 (Dako Cytomation, Kyoto, Japan) in a 0.1 μg/μL dilution was used. After incubation overnight at 4°C in a moist chamber, the sections were thoroughly washed with 0.01 M phosphate buffered saline. Next, the sections

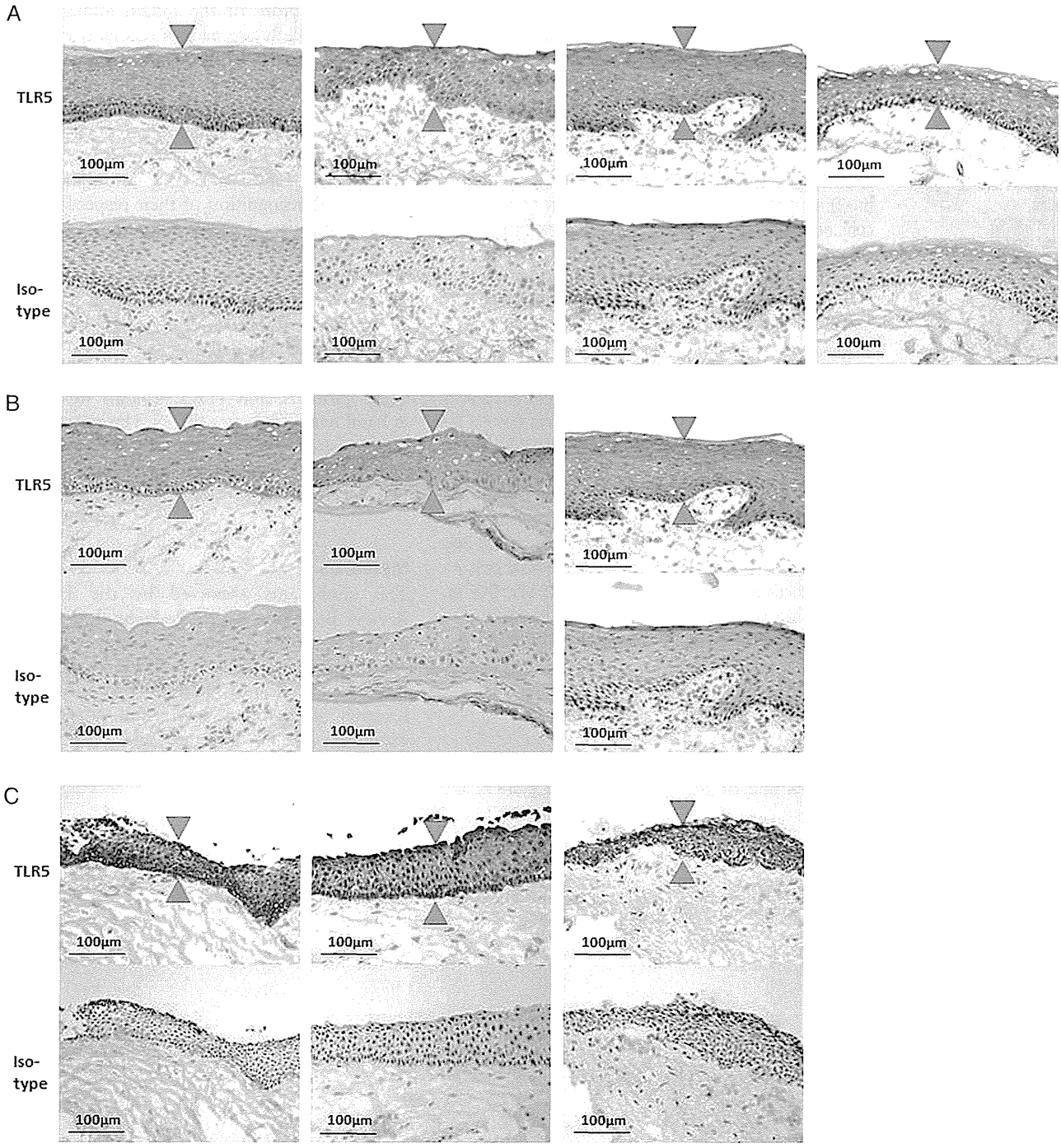

Figure 2 Immunohistological analysis of Toll-like receptor (TLR) 5 in the conjunctival epithelium of various ocular surface disorders. Localisation of TLR5 in the conjunctival epithelium of various conjunctival disorders; four Stevens-Johnson syndrome cases in the chronic stage (A), three ocular cicatricial pemphigoid cases (B), and three chemical burn cases (C). The conjunctival tissue was incubated with anti-TLR5 antibody. Isotype control incubation was used as the negative control.

were subjected to secondary antibody reactions with biotin conjugated donkey anti-mouse IgG Fab fragments in a 0.5 μg/μL dilution. Avidin and biotinylated horseradish peroxidase macromolecular complex reagent (VECTASTAIN ABC reagent; Vector Laboratories, Inc, Burlingame, California, USA) was then applied to the section for 30 min and 3,3′-diaminobenzidine peroxidase substrate solution (DAB substrate kit; Vector Laboratories) was added to the slide sections as a chromogenic substrate. Finally, the sections were counterstained using haematoxylin.

RESULTS

Conjunctival tissues obtained at the time of ocular surface reconstruction surgery were subjected to immunohistochemical study to determine the presence and localisation of TLR5 expression in stratified conjunctival epithelium. In human conjunctival epithelium of the control conjunctiva, TLR5 protein was consistently and abundantly expressed; however, it was detected only at the basal and wing cells (figure 1). These results are consistent with those of our previous report.[6] On the other hand, in the epithelium of the conjunctival tissues obtained from the four SJS patients (figure 2A), the three OCP patients (figure 2B), and the three patients being treated for chemical eye burns (figure 2C), the TLR5 protein was detected in all layers from the basal and wing cells to the superficial cells. However, in the epithelium of the conjunctival tissues obtained from the two pterygium patients (figure 3), the TLR5 protein was detected in its basal and wing cells, but not the superficial cells, the same as with the controls.

DISCUSSION

The findings of this study showed that in devastating ocular surface disorders such as SJS, OCP and chemical eye burns, TLR5 is expressed not only on the basal and wing cells but also on the superficial cells, although TLR5 is expressed on only the basal and wing cells in normal human conjunctival epithelium as we previously reported.[6] In the conjunctival epithelium obtained from the two pterygium patients, TLR5 protein was also consistently and abundantly expressed, yet detected only at the basal and wing cells, the same as with normal human conjunctival epithelium. The above findings suggest that TLR5 might be upregulated in the ocular surface of patients with these devastating ocular surface disorders.

It should be noted that previous reports have investigated the expression of TLR5 in other human disorders. Recent findings have revealed that both in rheumatoid arthritis (RA) and osteoarthritis, TLR5 immunostaining is significantly higher on synovial tissue lining, sublining macrophages and endothelial cells compared with controls.[9] The findings of that study also suggest that the expression of TLR5 is upregulated in RA disease progression, that RA is reportedly a chronic autoimmune disorder in which the innate immune system plays an important role, and that intestinal epithelium reportedly expresses TLR5.

Rhee *et al*[10] reported that the flagellin/TLR5 response is confined to the basal cells, not the superficial cells, of intestinal mucosa, indicating that the intact colonic mucosa is not responsive to luminal bacterial flagellin. We also reported that on ocular surface epithelium, TLR5 was expressed on only the basal and wing cells, not the superficial cells, thus suggesting that in the normal condition, the intact ocular surface could not be responsive to commensal bacterium of the ocular surface.[11] In a very recent study, it was reported that TLR5 mRNA tends to be upregulated in the active phase of ulcerative colitis (UC) compared to UC quiescent disease, and that there are positive correlations between TLR5 mRNA and endoscopic and histological activity.[12] Moreover, in irritable bowel syndrome (IBS) of dysfunctional colitis that partly results from low grade mucosal inflammation, it was reported that TLR5 mRNA was upregulated in colonic biopsies from active IBS patients.[13] Therefore, TLR5 expressions may be upregulated in the diseases associated with chronic inflammations or in an active inflammation phase.

In chronic phase SJS and OCP patients, it was reported that the number of neutrophil elastase-positive cells and CD4-positive cells was increased in the epithelium and substantia propria of the pannus tissue. In addition, CD3-positive cells in the substantia propria of the pannus were reportedly slightly increased in both OCP and SJS patients, yet other kinds of infiltrating cells were not increased.[14] Therefore, upregulation of TLR5 in the epithelium of the conjunctival tissues of SJS, OCP, and chemical eye burns might be involved in the chronic inflammation of these respective diseases. Although it has been reported that there are chronic inflammatory cells in pterygium,[15] the difference of the extent of TLR5 expression between pterygium and the devastating ocular surface disorders might suggest the different quality of the chronic inflammation.

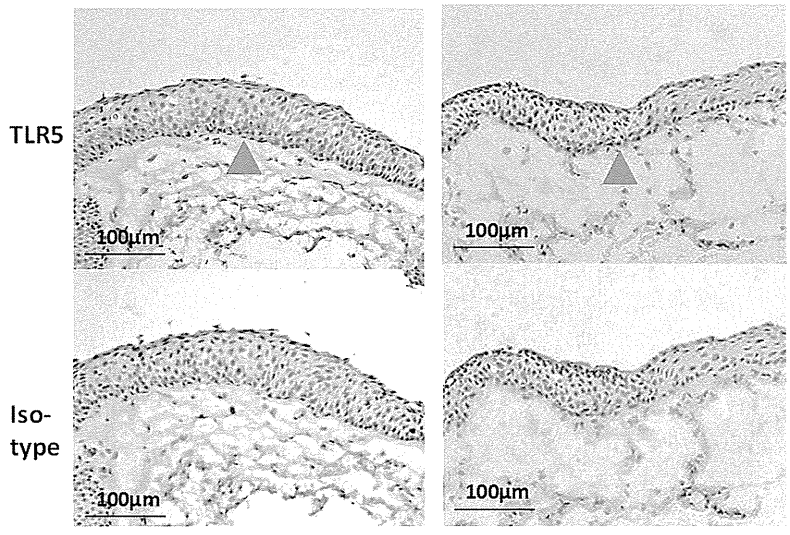

Figure 3 Immunohistological analysis of Toll-like receptor (TLR) 5 in the conjunctival epithelium obtained from two pterygium patients. Localisation of TLR5 in the conjunctival epithelium from the two pterygium cases which underwent surgery. The TLR5 protein was detected only at the basal and wing sites, the same as with the control. Isotype control incubation was used as the negative control.

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Competing interests None.

Patient consent Obtained.

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