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Original Article

Histamine H₁ and H₄ receptor expression on the ocular surface of patients with chronic allergic conjunctival diseases

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ACD, allergic conjunctival diseases; AC, allergic conjunctivitis; AKC, atopic keratoconjunctivitis; ECP, eosinophil cationic protein; H₁R, Histamine H₁ receptor; H₄R, Histamine H₄ receptor; MBP, eosinophil major basic protein; PAC, perennial allergic conjunctivitis; RT-PCR, reverse transcription polymerase chain reaction; SAC, seasonal allergic conjunctivitis; VKC, vernal keratoconjunctivitis

ABSTRACT

Background: This study investigated the histamine H₁ and H₄ receptors mRNA (*H1R* and *H4R*, respectively) expression on the ocular surface of patients with chronic forms of allergic conjunctival diseases to determine whether they can serve as biomarkers for allergic inflammation in the conjunctiva.

Methods: We examined 19 patients with vernal or atopic keratoconjunctivitis (AKC/VKC group) and 15 healthy volunteers (control group). The AKC/VKC group was divided into active and stable stage subgroups. Specimens were obtained from the upper tarsal conjunctiva of each participant using a modified impression cytology method. *H1R*, *H4R*, and *eotaxin-1*, *-2*, and *-3* mRNA (*eotaxin-1*, *eotaxin-2*, *eotaxin-3*, respectively) expression was determined by real-time RT-PCR. Immunohistochemical analysis for eosinophil cationic protein (ECP), eosinophil major basic protein (MBP), *eotaxin-2*, and histamine H₄ receptor (H₄R) were performed using conjunctival smears.

Results: The number of H₄R-positive patients was higher in the active than the stable stage subgroup and control group, whereas no difference was observed for *H1R*. *H1R* levels were higher in the active than in the stable stage subgroup, while those of H₄R were higher in the active stage subgroup than in the control group. *H1R* and H₄R levels were correlated with *eotaxin-2* level. In immunohistochemical analysis, H₄R revealed their expression on eosinophils in conjunctival smears of patients with AKC/VKC.

Conclusions: H₄R is useful as biomarkers of allergic inflammation on ocular surfaces. Most notably, H₄R expressed on eosinophils is useful as a biomarker of eosinophilic inflammation of the ocular surface.

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Introduction

Allergic conjunctival diseases (ACDs) are inflammatory disorders of the conjunctiva that are provoked by IgE-mediated immediate hypersensitivity and include various clinical conditions, such as seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), atopic keratoconjunctivitis (AKC), and vernal keratoconjunctivitis (VKC).^{1,2} Severe and chronic forms of ACD, including AKC and VKC, are predominantly eosinophil-mediated, whereas SAC and PAC involve immediate hypersensitivity with a severe early phase reaction. The most common mediator of the

early phase of immediate hypersensitivity is histamine derived from mast cell degranulation; thus, a histamine H₁ receptor antagonist is used for the treatment of SAC and PAC.³ The concentration of histamine produced by basophils in the tears of ACD patients reportedly increases during the late phase of immediate hypersensitivity.⁴ However, the role of histamine in the pathophysiology of severe/chronic forms of ACD is not well understood.

Histamine acts through its cognate receptors, of which there are four subtypes: H₁R, H₂R, H₃R, and H₄R.⁵ H₁R plays a critical role in itching, conjunctival edema, and hyperemia in ACD patients by increasing vasodilation and vascular permeability and stimulating C-nerve fibers and proinflammatory cytokine production by immune cells.^{6–8} H₄R is predominantly expressed by immune cells and mediates inflammatory responses in the allergic reaction by selectively recruiting allergy-associated immune cells to the site of inflammation.^{8,9} Infiltration by H₄R-expressing inflammatory cells

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was observed in the conjunctival tissue of VKC patients.¹⁰ Therefore, the effectiveness of drugs for chronic ACDs, such as AKC and VKC, can be assessed by analyzing H₁R and H₄R expression patterns in the conjunctiva.

To establish an ocular surface clinical test to noninvasively detect changes in H₁R and H₄R mRNA (*H1R* and *H4R*, respectively) expression over time, we quantitatively analyzed the expression levels of these two receptors on the ocular surface in AKC and VKC patients using a modified impression cytology method and real-time reverse transcription polymerase chain reaction (RT-PCR).

Methods

The study protocol was approved by the institutional review board of the Nihon University School of Medicine and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Subjects

In total, 19 consecutive patients diagnosed with AKC or VKC at the Department of Ophthalmology of Nihon University Itabashi Hospital, Tokyo, Japan, between August 2015 and April 2016 were included in the study (AKC/VKC group). Demographic data for the subjects are shown in Table 1. Only patients who had not received treatment or were treated with anti-allergic ophthalmic solutions, including mast cell stabilizers, H₁R antagonists, corticosteroids, and immunosuppressive agents such as cyclosporine and tacrolimus, were included in the study (Table 1); patients who used oral medicines, or received injections for the treatment of allergic disease or immunotherapy were excluded. Patients with ocular surface diseases other than ACD—including lagophthalmos, blepharospasm, conjunctival chlamydia, dry eye, infectious conjunctivitis, infectious keratitis, Stevens–Johnson syndrome, and ocular pemphigoid—and those who could not provide a sufficient amount of tear sample were also excluded. AKC and VKC were diagnosed according to the Japanese ACD guidelines.² Healthy volunteers without any allergic diathesis or history of wearing contact lenses were recruited as controls (n = 15).

Clinical scoring of VKC and AKC with the 5-5-5 exacerbation grading scale for ACDs

Clinical severity of AKC/VKC was scored using the 5-5-5 exacerbation grading scale for ACDs.¹¹ In this method, 100 points are assigned for each of five severe clinical findings; 10 points are

assigned for each of five moderate clinical findings; and 1 point is given for each of five mild clinical findings. The clinical findings were finally converted into severity order by 5-5-5 exacerbation grading scale for ACDs, and a difference between each group was evaluated by a distribution-free test.

According to the results of the 5-5-5 exacerbation grading scale for ACDs, the AKC/VKC group was divided into two subgroups: active stage (n = 12; with clinical score of ≥100 points) and stable stage (clinical score < 100 points; n = 7).

Sample collection by modified impression cytology and conjunctival smear

Modified impression cytology was performed by using the 5 mm tip of Schirmer's test paper (Schirmer Tear Production Measuring Strips, Showa Yakuhin Kako, Tokyo, Japan) instead of a nitrocellulose membrane. Schirmer's test paper was applied to the upper tarsal conjunctiva without local anesthesia or washing the eye, pressed gently using a glass rod, then removed, and preserved in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) until real-time RT-PCR analysis.

We performed scrapings of the upper tarsal conjunctiva using a sterile cotton swabs to obtain conjunctival smear specimens. Conjunctival smears from the upper tarsal conjunctiva were air-dried and fixed using cold acetone for immunofluorescence staining.

Detection of *H1R*, *H4R*, and *eotaxin-1*, *-2*, and *-3* mRNA on the ocular surface

H1R, *H4R*, and *eotaxin-1*, *-2*, and *-3* mRNA (*eotaxin-1*, *eotaxin-2*, *eotaxin-3*, respectively) expression on the ocular surface of control subjects and AKC/VKC patients was evaluated with a modified impression cytology test using Schirmer's test paper. Modified impression cytology specimens were obtained from the affected eye in unilateral cases or from the more severely affected eye in bilateral cases of AKC and VKC and from the right eye of control subjects.

To detect *H1R*, *H4R*, *eotaxin-1*, *-2*, and *-3* expression by real-time RT-PCR, total RNA from each specimen was extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, and used to synthesize cDNA with the High-Capacity cDNA Reverse Transcription kit (Life Technologies Japan, Tokyo, Japan). Real-time RT-PCR was carried out using the TaqMan gene expression assay (Life Technologies Japan) and the predesigned primers/probes Hs00185542_m1 (*H1R*), Hs00222094_m1 (*H4R*), Hs00237013_m1 (*eotaxin-1*), Hs00171082_m1 (*Eotaxin-2*), and Hs00171146_m1 (*eotaxin-3*) (Life Technologies Japan) on a Step One Plus™ system (Life Technologies Japan). Target Ct values were normalized to those of GAPDH (Hs99999905_m1) from the same sample. Expression levels were determined by the $\Delta\Delta$ CT method.

Immunohistochemical analysis for ECP, MBP, *eotaxin-2*, and *H4R*

Conjunctival smears were stained using the double staining method of immunofluorescence staining for eosinophil major basic protein (MBP) and *H4R*, or eosinophil cationic protein (ECP) and *eotaxin-2*.

For double-immunofluorescence staining for MBP and *H4R*, conjunctival smears were blocked for 30 min at room temperature with 5% normal donkey serum (Vector Laboratories, Burlingame, CA, USA) in phosphate-buffered saline (PBS). After blockade, the slides were incubated overnight at 4 °C with an anti-human *H4R* rabbit polyclonal antibody (GeneTex, Texas, USA), which was detected with Alexa Fluor® 488-labeled donkey anti-rabbit IgG (Life Technologies Japan). Then, conjunctival smears were incubated for 90 min at 30 °C with a mouse anti-human MBP monoclonal

Table 1
Demographic data of study subjects.

	Control	AKC/VKC		P value
		Stable stage	Active stage	
No. of patients (cases)	15	7	12	
Age [years (mean ± SD)]	27.6 ± 3.6	25.9 ± 14.6	22.0 ± 12.2	0.316
Gender (male: female)	9:4	5:2	11:1	0.355
Clinical score [†] [points (median)]	0	23	223	0.000000171
(5-5-5 points)	(0-0-0)	(0-2-3)	(2-2-3)	
Medical treatment with ophthalmic solution at initial investigation				
Anti-allergy drugs		7/7	10/12	0.253
Corticosteroids		0/7	0/12	0.779
Immunosuppressive agents (C:T ratio [‡])		5/7 (1:4)	8/12 (1:7)	0.829
None		0/7	2/12	0.253

AKC, atopic keratoconjunctivitis; VKC: vernal keratoconjunctivitis.

Bold value is statistically significant.

[†] 5-5-5 exacerbation grading scale for allergic conjunctival diseases.¹¹

[‡] Cyclosporine ophthalmic solution 0.1%; tacrolimus ophthalmic suspension 0.1% ratio.

antibody (clone: BMK-13, Bio-Rad, CA, USA), which was detected with Alexa Fluor®-647-labeled donkey anti-mouse IgG (Life Technologies Japan). Finally, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI)-fluoromount-G (Southern Biotech; Birmingham, AL, USA). Images were recorded with a BIO-REVO digital camera (BZ-9000; KEYENCE Japan; Osaka, Japan).

For double-immunofluorescence staining for ECP and eotaxin-2, we used a combination of anti-human eotaxin-2 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was detected with Alexa Fluor®-488-labeled donkey anti-goat IgG and anti-human ECP rabbit polyclonal antibody (Bioss Inc., Boston, USA) detected with Alexa Fluor®-647-labeled donkey anti-rabbit IgG.

Conjunctival smears stained by double-immunofluorescence staining without primary antibodies were used as negative controls.

Statistical analyses

Statistical analyses were carried out using MAC Toukei-Kaiseki v.2 software (Esumi, Tokyo, Japan). Differences among active and stable stage subgroups of the AKC/VKC group and the control group were evaluated with the χ^2 and Kruskal–Wallis H tests. *H1R*, *H4R*, and *eotaxin-2* expression levels were compared among the AKC/VKC stable stage subgroup, the AKC/VKC active stage subgroup, and the control group with the Steel–Dwass test. Differences between AKC/VKC and control groups were evaluated using Fisher's exact test and the Mann–Whitney *U*-test. Differences between AKC and VKC groups were also evaluated using Fisher's exact test and the Mann–Whitney *U*-test. Assessments of the relationship among *H1R*, *H4R*, and *eotaxin-2* expression levels were performed using a partial correlation coefficient test. Correlations between the expression levels of *H1R* and *eotaxin-2*, or *H4R* and *eotaxin-2* in the AKC/VKC group were analyzed by Spearman's rank correlation coefficient. *P* values < 0.05 were considered statistically significant.

Results

H1R and *H4R* mRNA expression in AKC/VKC patients

Positive ratios of *H1R* and *H4R* expression on the ocular surface in stable and active stage subgroups of AKC/VKC patients and control subjects are shown in Table 2. The expression ratios of *H4R*

($P < 0.001$, χ^2 test) but not *H1R* ($P = 0.317$) differed significantly among the three groups. The expression levels [median, (range)] of *H1R* on the ocular surface in the stable and active stage subgroups of AKC/VKC patients and the control subjects were 1.0 (0.3–2.8), 5.4 (0.6–111.6), and 3.3 (0.3–25.6), respectively. The *H1R* level was higher in the active than in the stable stage subgroup of AKC/VKC patients ($P < 0.05$, Steel–Dwass test) but was not higher than the level in control subjects (Fig. 1a). Ocular surface *H4R* expression levels in stable and active stage subgroups of AKC/VKC patients and control subjects were 33.7 (3.5–71.2), 366.6 (10.7–5065.8), and 1.5 (1.0–6.1), respectively. The *H4R* level was higher in the active stage subgroup of AKC/VKC patients than in control subjects ($P < 0.05$, Steel–Dwass test) but was not higher than the level in the stable stage subgroup of AKC/VKC patients (Fig. 1b).

Comparison of *H1R* and *H4R* mRNA expression between the AKC and VKC groups

A comparison of the number of patients within the active stage subgroup, positive ratios of *H1R* expression, and positive ratios of *H4R* expression between the AKC and VKC groups are shown in Table 3. There were no significant differences between the AKC and VKC groups. The median values (range) of *H1R* expression levels of the AKC and VKC groups were 1.9 (0.3–33), and 3.1 (0.6–1.1 × 10²), respectively. There were no significant differences in *H1R* expression levels between the AKC and VKC groups (Fig. 2a). The median values (range) of *H4R* expression levels in the AKC and VKC groups were 44 (11–1.2 × 10³), and 3.6 × 10² (3.5–5.1 × 10³), respectively. There were no significant differences in *H4R* expression levels between the AKC and VKC groups (Fig. 2b).

Eotaxin-1, *-2*, and *-3* mRNA expression in AKC/VKC patients

Expression ratios of *eotaxin-1*, *-2*, and *-3* are shown in Figure 3a. No subject in the control group expressed *eotaxin-1* on the ocular surface. Expression ratios of *eotaxin-1* in the AKC/VKC group were significantly higher than those in the control group (Fig. 3a; $P < 0.05$, Fisher's exact test). Detected expression of *eotaxin-1* was nearly the same as the patients belonging to the AKC/VKC active stage subgroup, where the expression levels in the AKC/VKC active stage subgroup were 0.3 (0.08–1.0) [median (range)] (Fig. 3c).

Expression ratios of *eotaxin-2* in the AKC/VKC group were significantly higher than those in control group (Fig. 3a; $P < 0.05$, Fisher's exact test). Detected expression levels of *eotaxin-2* in the control group, AKC/VKC stable stage, and AKC/VKC active stage subgroups were 0.3 (0.07–2.6), 4.2 (0.5–70), and 94.9 (13–3.6 × 10²) [median (range)], respectively (Fig. 4b). *Eotaxin-2* expression levels in the AKC/VKC stable and active stage subgroups are significantly higher than those in control group. Moreover, *eotaxin-2* levels in the AKC/VKC active stage subgroup were higher than those in the AKC/VKC stable stage subgroup, and control group (Fig. 3d).

One subject expressed *eotaxin-3* on the ocular surface in the control group. Expression ratios of *eotaxin-3* in the AKC/VKC group were significantly higher than those in the control group (Fig. 3a; $P < 0.0001$, Fisher's exact test). Detected expression levels of *eotaxin-3* in the AKC/VKC stable stage and AKC/VKC active stage subgroups were 0.3 (0.3–2.6) and 16 (0.2–1.5 × 10²) [median (range)], respectively (Fig. 3e). *Eotaxin-3* expression in the AKC/VKC active stage subgroup was higher than those in the AKC/VKC stable stage subgroup (Fig. 3e).

Correlation between *eotaxin-2* and *H1R* or *H4R* mRNA expression

The partial correlation between *eotaxin-2*, *eotaxin-3*, *H1R*, and *H4R* in the AKC/VKC group is shown in Figure 3b. A positive

Table 2
Positive ratios of *H1R* and *H4R* mRNA expression.

a	<i>H1R</i> expression			<i>P</i> value*
	Control	AKC/VKC: stable stage	AKC/VKC: active stage	
Positive	12	7	11	0.317
Negative	3	0	1	
Total	15	7	12	
Positive ratio (%)	80	100	92	
b	<i>H4R</i> expression			<i>P</i> value*
	Control	AKC/VKC: stable stage	AKC/VKC: active stage	
Positive	3	4	12	0.0000320
Negative	12	3	0	
Total	15	7	12	
Positive ratio (%)	20	57	100	

Fisher's exact test with Bonferroni's correction, Control vs AKC/VKC stable stage: NS, AKC/VKC stable stage vs AKC/VKC active stage: NS, Control vs AKC/VKC active stage: $p < 0.001$. AKC, atopic keratoconjunctivitis; VKC, vernal keratoconjunctivitis; *H1R*, histamine H1 receptor mRNA; *H4R*, histamine H4 receptor mRNA; NS, not significant.

* Chi-square test.

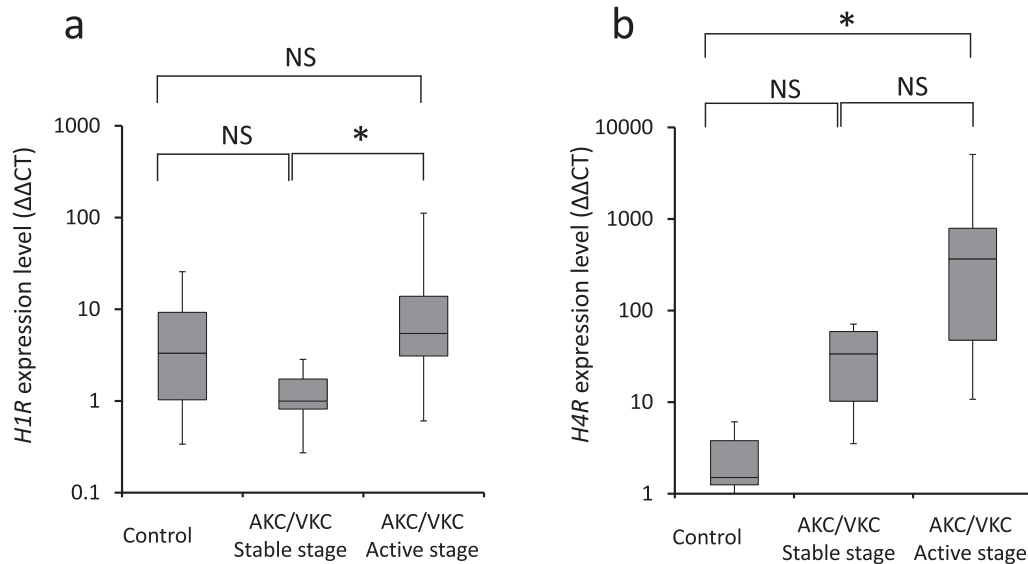


Fig. 1. (a) *H1R* expression levels in AKC/VKC and control groups. *H1R* expression was higher in the active than in the stable stage subgroup of AKC/VKC patients. * $P < 0.05$ (Steel–Dwass test). NS, not significant; *H1R*, histamine H_1 receptor mRNA. (b) *H4R* expression levels in AKC/VKC and control groups. *H4R* expression was higher in the active stage subgroup of AKC/VKC patients than in the control group. * $P < 0.05$ (Steel–Dwass test). NS, not significant; *H4R*, histamine H_4 receptor mRNA.

Table 3
Comparison of active stage ratios, and positive ratios of *H1R* and *H4R* expression on ocular surface in VKC and AKC groups.

	VKC	AKC	<i>P</i> value*
Active stage ratios			
Active stage	7	5	0.960
Stable stage	4	3	
Positive ratios of <i>H1R</i>			
<i>H1R</i> positive	11	7	0.526
<i>H1R</i> negative	0	1	
Positive ratios of <i>H4R</i>			
<i>H4R</i> positive	10	6	0.348
<i>H4R</i> negative	1	2	

VKC, vernal keratoconjunctivitis; AKC, atopic keratoconjunctivitis; *H1R*, histamine H_1 receptor mRNA; *H4R*, histamine H_4 receptor mRNA.

* Fisher's exact test.

correlation was detected between the expression of *eotaxin-2* and *H1R*, *eotaxin-2* and *H4R*, and *eotaxin-2* and *eotaxin-3*, while a negative correlation was detected between *H1R* and *H4R* levels.

H1R and *H4R* expression levels were significantly correlated with those of *eotaxin-2* in the AKC/VKC group ($r_s = 0.645$, $P < 0.01$ and $r_s = 0.832$, $P < 0.01$, respectively; Spearman's correlation coefficient) (Fig. 4a, b).

Immunohistochemical analysis of ECP, MBP, *eotaxin-2*, and *H4R* expression

In conjunctival smears in the AKC/VKC active stage subgroup ($n = 3$), the double staining of ECP and *eotaxin-2* showed the presence of both ECP and *eotaxin-2* expression in granulocytes with segmented nuclei (Fig. 5a). Moreover, the double staining of MBP and *H4R* revealed the expression of both MBP and *H4R* in granulocytes with segmented nuclei (Fig. 5b).

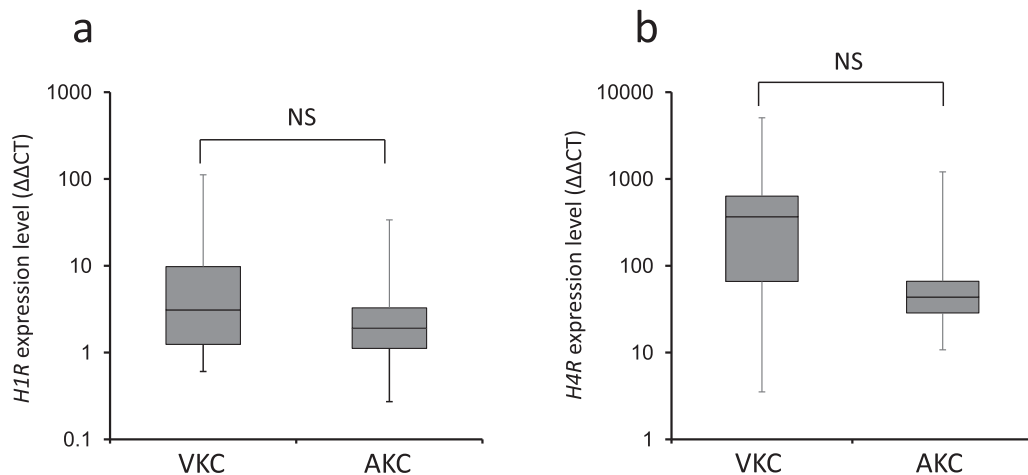


Fig. 2. Comparison of *H1R* and *H4R* expression levels on the ocular surface between the VKC and AKC groups. Regarding *H1R* (a) and *H4R* (b) expression levels on the ocular surface, there are no significant differences between the AKC and VKC groups. NS, not significant; *H1R*, histamine H_1 receptor mRNA; *H4R*, histamine H_4 receptor mRNA; VKC, vernal keratoconjunctivitis; AKC, atopic keratoconjunctivitis.

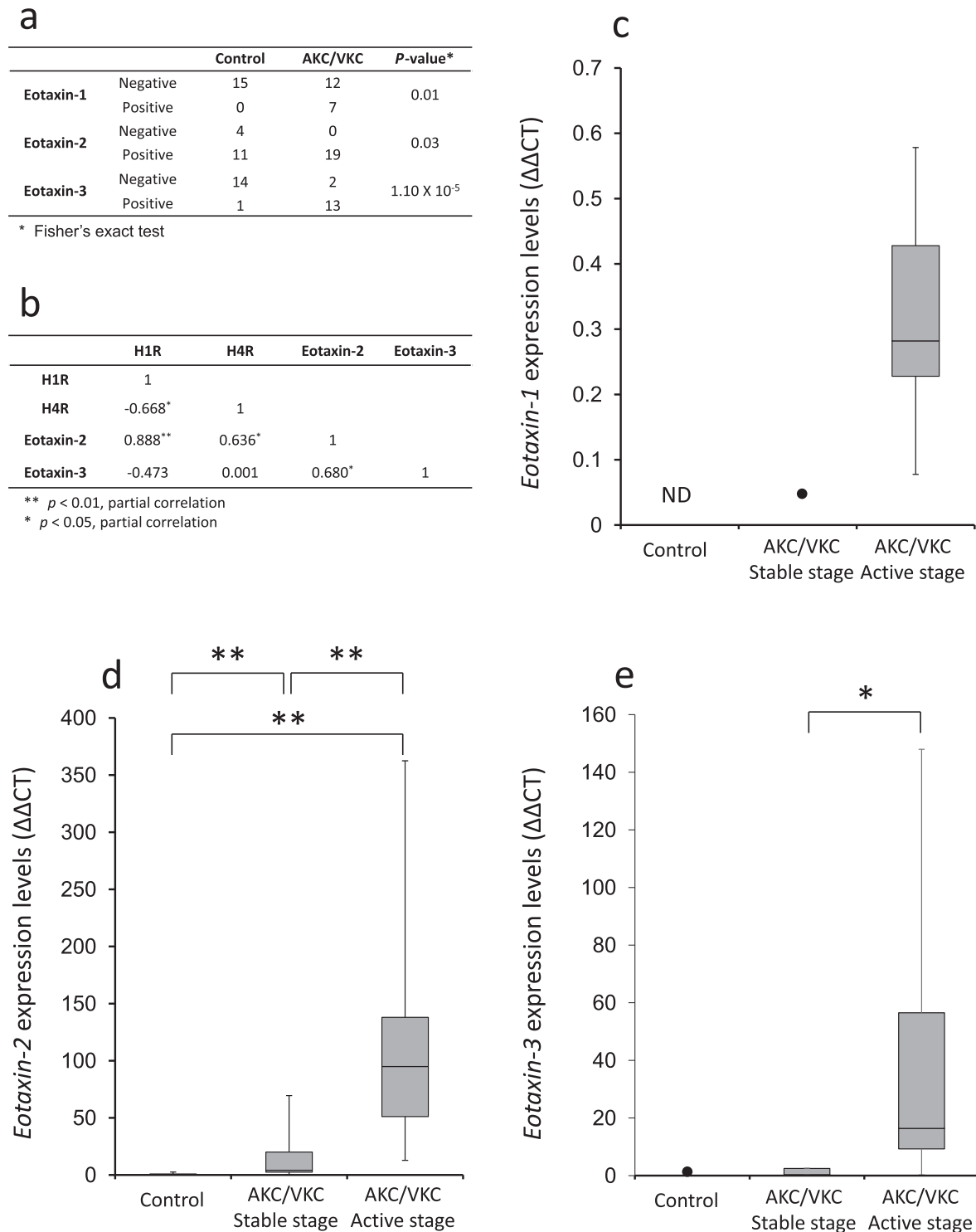


Fig. 3. (a) Expression ratios of eotaxin-1, -2, and -3 mRNA on the ocular surface. (b) Partial correlation coefficients between *eotaxin-2*, *eotaxin-3*, *H1R*, and *H4R*. Expression levels of *eotaxin-1*, -2, and -3 on the ocular surface in control, stable stage AKC/VKC, and active stage AKC/VKC subgroups (3c–3e). (c) *Eotaxin-1* detection: No expression is detected (ND) in control group subjects, expression was detected in only one patient (0.045) in the AKC/VKC stable stage subgroup, and expression was detected in 6 out of 12 patients in the AKC/VKC active stage subgroup (median value: 0.28). (d) *Eotaxin-2* detection: *eotaxin-2* expression levels in the AKC/VKC stable and active stage subgroups are significantly higher than those of the control group. Moreover, levels of *eotaxin-2* expression in the AKC/VKC active stage subgroup are high in comparison to those in the AKC/VKC stable stage subgroup. ** $P < 0.01$, Steel–Dwass test. (e) *Eotaxin-3* detection: levels of *eotaxin-3* expression in the AKC/VKC active stage subgroup are high in comparison to those in the AKC/VKC stable stage subgroup. Expression was detected in only one patient (0.1) in the control group. * $P < 0.05$, Mann–Whitney *U*-test. AKC, atopic keratoconjunctivitis; VKC, vernal keratoconjunctivitis; *Eotaxin-1*, eotaxin-1 mRNA; *Eotaxin-2*, eotaxin-2 mRNA; *Eotaxin-3*, eotaxin-3 mRNA; *H1R*, histamine H₁ receptor mRNA; *H4R*, histamine H₄ receptor mRNA.

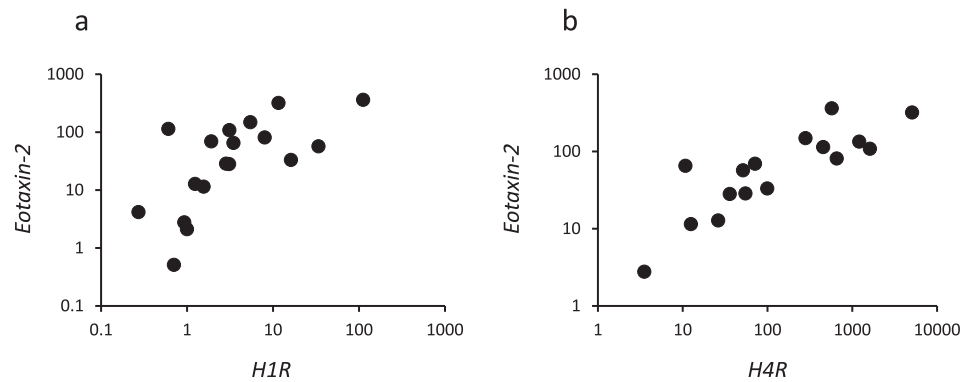


Fig. 4. Correlation between *H1R* or *H4R* and *Eotaxin-2* expression at the ocular surface. **(a)** Correlation between *H1R* and *eotaxin-2*. *H1R* and *eotaxin-2* levels were significantly correlated ($r_s = 0.645$, $P < 0.01$, Spearman's correlation coefficient). **(b)** Correlation between *H4R* and *eotaxin-2*. *H4R* and *eotaxin-2* levels were significantly correlated ($r_s = 0.832$, $P < 0.01$, Spearman's correlation coefficient). *H1R*, histamine H_1 receptor mRNA; *H4R*, histamine H_4 receptor mRNA.

Although cells positive for double staining were detected in conjunctival smears from the AKC/VKC stable subgroup ($n = 3$), similar to the AKC/VKC active stage subgroup, the frequency of detection was considerably lower than that in the AKC/VKC active stage subgroup.

Discussion

This study investigated *H1R* and *H4R* expression levels on the ocular surface using a modified impression cytology method to evaluate their clinical utility as biomarkers for chronic forms of ACD, including AKC and VKC. We modified the conventional impression cytology method in order to reduce the invasiveness and potential side effects of the procedure, including irritation and pain after the examination, by using filter paper instead of a methylcellulose membrane, and used this procedure to monitor biomarkers that would normally require several examinations in clinical practice. In theory, our modified method can be applied to

the real-time PCR analysis of several biomarkers, such as cytokines, chemokines, and growth factors and their receptors, in tear fluid, mucin, epithelial cells, and inflammatory cells on the ocular surface.

In the conjunctival immediate hypersensitivity reaction (type 1 allergy), histamine is released from degranulated mast cells and basophils in the early and late phases.⁴ Histamine levels in the tears of VKC patients were reportedly higher than those in control subjects.¹² The major histamine receptor associated with the pathogenesis of ACDs is thought to be H_1R ; clinical manifestations, such as conjunctival hyperemia and chemosis, are elicited by H_1R activation, and H_1R antagonists are used for the therapeutic treatment of ACDs.³ H_1R is mainly expressed in vascular endothelial cells, epithelial cells and nerve fibers at the ocular surface,¹⁰ but is also expressed in immune cells, including mast cells, basophils, eosinophils, type 2 helper T (Th2) cells, lymphocytes, macrophages, and dendritic cells.⁸ Therefore, our modified impression cytology method can potentially be used to evaluate *H1R* expression in conjunctival epithelial and inflammatory cells. Our results showed

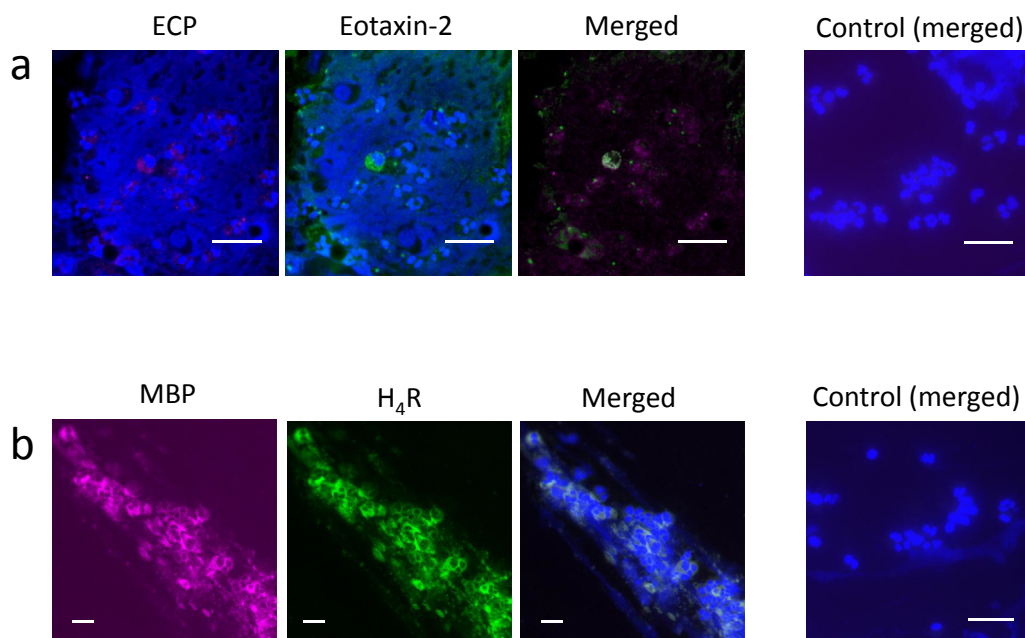


Fig. 5. Immunohistochemical analysis. **(a)** Immunofluorescence detection for ECP and eotaxin-2. Both ECP-positive (Alexa Fluor[®]647; pink) and eotaxin-2-positive (Alexa Fluor[®]488; green) cells can be seen in granulocytes with segmented nuclei in the conjunctival discharge. Scale bar: 30 μ m. ECP, eosinophil cationic protein. **(b)** Immunofluorescence detection for MBP and histamine H_4 receptor. Both MBP-positive (Alexa Fluor[®]647; pink) and H_4R -positive (Alexa Fluor[®]488; green) cells can be seen in granulocytes with segmented nuclei in the conjunctival discharge. Scale bar: 30 μ m. MBP, major basic protein; H_4R , histamine H_4 receptor.

that the positive ratio of *H1R* expression on the ocular surface was similar between the stable stage subgroup of the AKC/VKC group and the control group. However, *H1R* expression was higher in the active than the stable stage subgroup of AKC/VKC patients, indicating that *H1R* is normally expressed on the ocular surface but that the expression levels are increased in activated AKC/VKC.

Eotaxin-1, -2, and -3 is a ligand of C–C chemokine receptor type 3 and is involved in eosinophil infiltration in allergic diseases. Our previous investigation demonstrated that among the three subclasses of eotaxin, eotaxin-2 was the most commonly detected and showed the highest expression on the ocular surface. Given its correlation with eosinophil cationic protein levels, eotaxin-2 is considered an excellent biomarker for evaluating eosinophilic inflammation in the conjunctiva of ACD patients.^{13,14} Therefore, in this study, we used eotaxin-2 as an ocular surface biomarker of eosinophilic inflammation and investigated the relationship between eosinophilic inflammation and the expression of *H1R* and *H4R* on the ocular surface. Our observation that *H1R* expression was correlated with that of eotaxin-2 indicates that *H1R* levels are modulated by allergic inflammation in AKC/VKC. Consistent with this supposition, *H1R* expression in the nasal mucosa was upregulated in patients with allergic rhinitis¹⁵ and *H1R* was found to be involved in the late phase reaction of allergic conjunctivitis in an *H1R*-deficient mice model.¹⁶

H4R is expressed by various inflammatory cells, including eosinophils and Th2 cells, in allergic disorders.^{8,9} Histamine modulates eosinophil, mast cell, and bone marrow-derived basophil chemotaxis and thymus and activation regulated chemokine (CCL17/TARC) production via *H4R*, which is important for Th2-type immunity mediated by bone marrow-derived mast cells. Thus, *H4R* plays important roles in inflammation in patients with allergic disorders. Infiltrating inflammatory cells in subconjunctival tissues of VKC patients strongly expressed *H4R*.¹⁰ We found that the positive ratio of *H4R* on the ocular surface in the AKC/VKC group was higher compared to that in the control group, and the levels of *H4R* expression on the ocular surface in the active stage subgroup of AKC/VKC patients increased significantly compared with those in the control group. Moreover, *H4R* and eotaxin-2 expression levels on the ocular surface were strongly correlated, suggesting that *H4R* is involved in allergic inflammation of the conjunctiva in chronic ACDs and that eosinophil infiltration may affect the *H4R* level on the ocular surface in patients with AKC/VKC. In our results of immunofluorescence double staining, MBP-positive eosinophils were stained positive for *H4R*, and ECP-positive eosinophils were also stained positive for eotaxin-2. Similar results have been reported in patients with active eosinophilic esophagitis (i.e., *H1R*, *H2R*, and *H4R* expression was upregulated in esophageal biopsies), while tissue eosinophil counts were correlated with mucosal histamine receptor expression.¹⁷ It was also demonstrated that *H4R* mediated allergic inflammation in ovalbumin-induced atopic dermatitis-like skin lesions in a mouse model.¹⁸

In patients with the stable stage of AKC/VKC, the *H4R* expression level was in the normal range. These patients were treated with immunosuppressive drugs, such as tacrolimus ophthalmic suspension, which suppress the infiltration of eosinophils and cluster of differentiation 4 (CD4)-positive lymphocytes into the conjunctiva.¹⁹ Since *H4R* is mainly expressed by immune cells, down-regulation of *H4R* may lead to decreased infiltration of eosinophils into the conjunctival tissue. Therefore, the *H4R* expression level on the ocular surface may be a useful biomarker for AKC/VKC in clinical examinations; therapeutic strategies that target both *H1R* and *H4R* should therefore be investigated for the treatment of chronic ACDs.

This study had some limitations. The expression levels of *H1R*, and *H4R* in CD4-positive T lymphocytes—another critical factor in allergic inflammation—was not evaluated. In addition, this

investigation of the expression of *H1R* and *H4R* on the ocular surface included AKC/VKC patients treated with ophthalmic solutions, and a significant difference was shown between the active stage subgroup and the stable stage subgroup of AKC/VKC patients. However, there was no detailed investigation of the therapeutic significance of *H1R* and *H4R* upregulation in AKC/VKC patients. Future large-scale intervention studies will examine the therapeutic efficacy of anti-allergy drugs and newly developed *H4R* antagonists in ACD patients using expression tests for the biomarkers *H1R* and *H4R*.

In conclusion, *H1R* and *H4R* are useful biomarkers of allergic inflammation on the ocular surface. Most notably, *H4R* expressed on eosinophils is useful as a biomarker of eosinophilic inflammation of the ocular surface. The evaluation of the expression levels of *H1R* and *H4R* on the ocular surface may lead to novel therapeutic approaches for ACD.

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Conflict of interest

NI received research funds from Santen Pharmaceutical and Otsuka Pharmaceutical. JS received honorarium for his lecture from Santen Pharmaceutical and Alcon Japan. SY received research funds from Hoya Corporation. The rest of the authors have no conflict of interest.

Authors' contributions

Designing and conducting the study: NI and JS; collecting data: JS, NI and HA; analyzing and interpreting data: NI, JS, YS, HA and SY; providing statistical expertise: JS; searching literature: NI and JS; writing the manuscript: NI and JS; critically revising the manuscript: SY. All the authors approved the final manuscript.

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