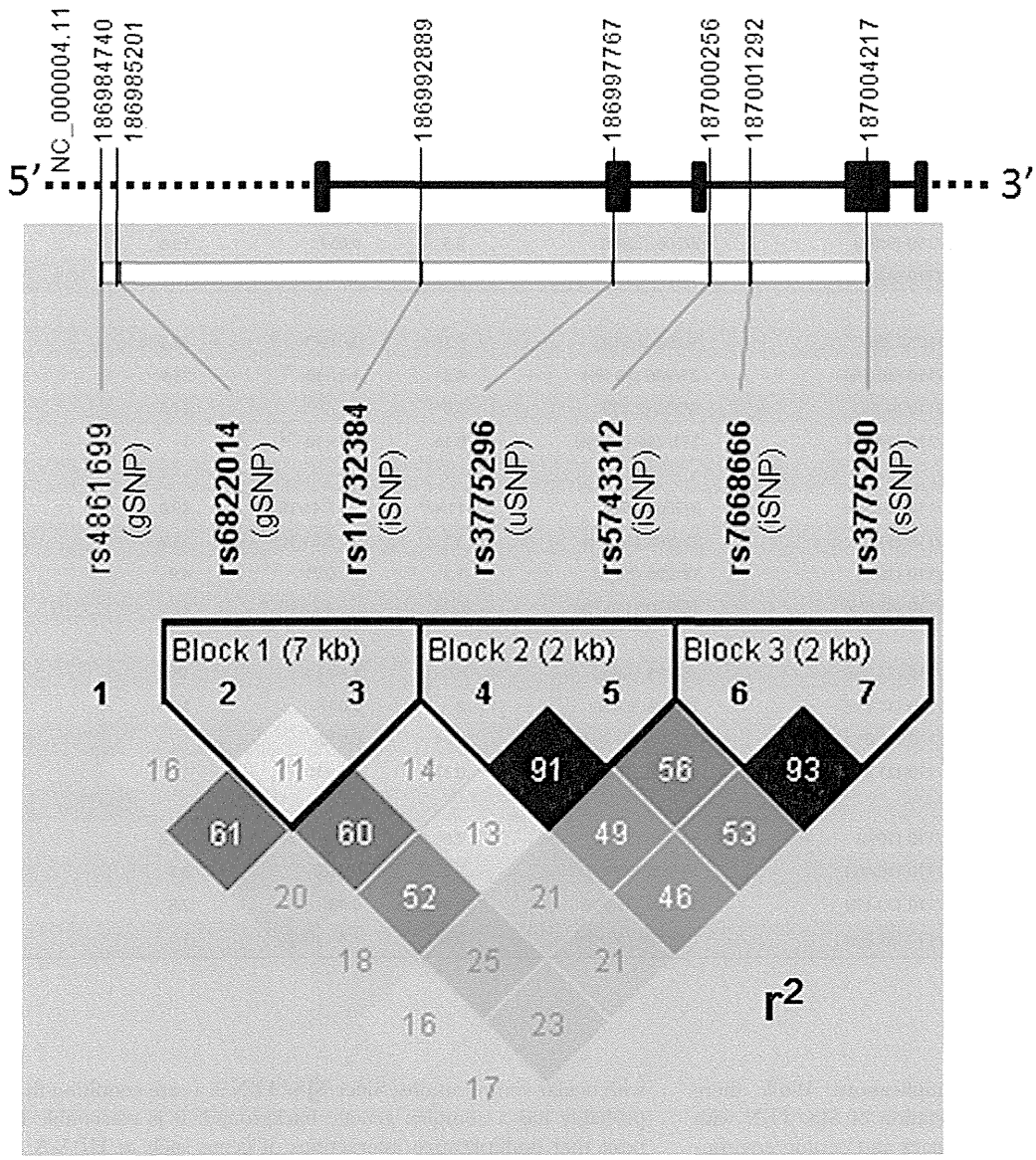

Figure 1. Linkage disequilibria among the 7 *TLR3* SNPs. Strong linkage disequilibrium was observed between rs.3775296 and rs.5743312, and between rs.7668666 and rs.3775290.
doi:10.1371/journal.pone.0043650.g001

with TLR3 rs.7668666, and TLR3 rs4861699G/G SNP exerted additive effects. Moreover, the combination HLA-A*0206 and TLR3 rs3775296T/T was stronger than the combination with TLR3 rs6822014G/G or TLR3 rs3775290A/A, the interactions within the TLR3 gene alone.

HLA-A, a component of HLA class I, alerts the immune system that the cell may be infected with a virus; TLR3 recognizes viral double-stranded RNA [21]. It is worth noting that about 80% of our SJS patients developed SJS after receiving treatment for the common cold with antibiotics, cold remedies, and/or NSAIDs; only about 5% of our SJS patient progressed to SJS after drug treatment to prevent the occurrence of convulsions [11,12]. Moreover, our review of medical records revealed that 9 of the 11 patients with both HLA-A*0206 and TLR3 SNP rs3775296T/T (and rs.5743312T/T) developed SJS after receiving cold medicine, leading us to suspect that they already had a viral infection before taking the cold medicine. Particulars on the other 2 patients are unknown because they developed SJS during childhood.

Although the TLR3 SNPs exerting additive- or more than additive effects with HLA-A*0206 were u-, i-, or gSNPs and without amino acid changes, it is possible that TLR3 SNPs and HLA-A*0206 were involved in the onset of SJS with severe ocular surface complications. Moreover, their interaction might influence the host immune response against viral infection with drug treatments.

Earlier reports indicated regional differences in HLA associations. Although in Japanese SJS patients we were unable to detect the HLA-Bw44 antigen, a subgroup of HLA-B12 [19,23], it was significantly increased in Caucasian SJS patients with ocular involvement [22].

On the other hand, the HLA-A*0206 antigen, which is not found in Caucasians [18,19] was significantly increased in our

Table 3. Interaction analysis between HLA-A*0206 and various TLR3 SNPs.

HLA-A*0206	TLR3 SNP	SJS patients (N = 110)	Controls (N = 206)	OR	p-value	Standardized OR
HLA-A*0206 & TLR3 rs3775296 T/T						
+	+	11/110 (10%)	0/206 (0%)	47.7*	6.5×10^{-6}**	262.7
+	−	40/110 (36.4%)	30/206 (14.6%)	3.4	8.8×10^{-6}	18.5
−	+	10/110 (9.1%)	8/206 (3.9%)	2.5	0.057	13.6
−	−	49/110 (44.5%)	168/206 (81.6%)	0.18	1.4×10^{-11}	1
HLA-A*0206 & TLR3 rs6822014G/G						
+	+	8/110 (7.3%)	3/206 (1.5%)	5.3**	0.019**	32.3
+	−	43/110 (39.1%)	27/206 (13.1%)	4.3	1.2×10^{-7}	25.9
−	+	10/110 (9.1%)	5/206 (2.4%)	4.0**	0.012**	24.5
−	−	49/110 (44.5%)	171/206 (83.0%)	0.16	1.4×10^{-12}	1
HLA A*0206 & TLR3 rs3775290A/A						
+	+	16/110 (14.5%)	3/206 (1.5%)	11.4**	7.4×10^{-6}**	49.0
+	−	35/110 (31.8%)	27/206 (13.1%)	3.1	6.6×10^{-5}	13.2
−	+	11/110 (10%)	18/206 (8.7%)	1.2	0.71	4.9
−	−	48/110 (43.6%)	158/206 (76.7%)	0.24	4.2×10^{-9}	1
HLA A*0206 & TLR3 rs11732384G/G						
+	+	37/110 (33.6%)	16/206 (7.8%)	6.0	4.5×10^{-9}	16.4
+	−	14/110 (12.7%)	14/206 (6.8%)	2	0.077	5.5
−	+	35/110 (31.8%)	87/206 (42.2%)	0.64	0.070	1.7
−	−	24/110 (21.8%)	89/206 (43.2%)	0.37	1.5×10^{-4}	1
HLA A*0206 & TLR3 rs4861699 G/G						
+	+	33/110 (30%)	11/206 (5.3%)	7.6	1.6×10^{-9}	25.7
+	−	18/110 (16.4%)	19/206 (9.2%)	1.9	0.060	6.5
−	+	32/110 (29.1%)	68/206 (33.0%)	0.83	0.48	2.8
−	−	27/110 (24.5%)	108/206 (52.4%)	0.30	1.8×10^{-6}	1

*Woolf's correction,
**Fisher's exact test.
doi:10.1371/journal.pone.0043650.t003

Japanese SJS patients with ocular complications. While there might be ethnic differences in the association of SJS/TEN with HLA,[18,19] specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype. [10,11,12,18,19].

Elsewhere [12] we reported that the epistatic interaction between TLR3 and PTGER3 confers an increased risk for SJS with ocular complications. Since SJS/TEN is a rare condition that probably has a complex genetic background, it is reasonable to posit that multiplicative interactions of genes such as HLA-A & TLR3, and TLR3 & PTGER3, are required for the phenotypic manifestation.

In summary, we show that HLA-A*0206 with TLR3 polymorphisms exerts more than additive effects in SJS with severe

Table 4. Interaction analysis of two SNPs of the TLR3 SNPs (SJS > control and SJS >5).

Combination of 2 TLR3 SNPs		SJS (N = 110)	Controls (N = 206)	OR	p-value
rs3775296 T/T +	rs3775290 A/A +	19/110 (17.3%)	6/206 (2.9%)	7.0	6.6×10^{-6}
rs11732384 G/G +	rs3775290 A/A +	27/110 (24.5%)	21/206 (10.2%)	2.9	7.1×10^{-4}
rs6822014 G/G +	rs3775290 A/A +	15/110 (13.6%)	2/206 (1.0%)	16.1	2.0×10^{-6}
rs4861699 G/G +	rs3775290 A/A +	26/110 (23.6%)	16/206 (7.8%)	3.7	7.5×10^{-5}
rs11732384 G/G +	rs3775296 T/T +	21/110 (19.1%)	8/206 (3.9%)	5.8	8.2×10^{-6}
rs6822014 G/G +	rs3775296 T/T +	17/110 (15.5%)	4/206 (1.9%)	9.2	4.3×10^{-6}
rs4861699 G/G +	rs3775296 T/T +	21/110 (19.1%)	8/206 (3.9%)	5.8	8.2×10^{-6}
rs6822014 G/G +	rs11732384 G/G +	18/110 (16.4%)	8/206 (3.9%)	4.8	1.2×10^{-4}
rs4861699 G/G +	rs6822014 G/G +	18/110 (16.4%)	8/206 (3.9%)	4.8	1.2×10^{-4}

doi:10.1371/journal.pone.0043650.t004

ocular surface complications and we suggest that gene-gene interactions should be considered in addition to major single-locus effects.

Materials and Methods

Patients

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine and the University of Tokyo, Graduate School of Medicine. 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.

Diagnosis of SJS/TEN was based on a confirmed history of acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites including the ocular surface [9,11,12,17,18].

To investigate the gene-gene interaction between HLA-A*0206 and TLR3, we enrolled 110 SJS/TEN patients in the chronic or subacute phase; all presented with symptoms of ocular surface complications. None of the patients were relatives. The controls were 206 healthy volunteers. All participants and volunteers were Japanese residing in Japan. The average age of the 110 patients and 206 controls was 43.6±18.0 (SD) and 35.4±11.1 (SD) years, respectively. The male:female ratios in the patient and control groups were 42:68 and 82:124, respectively. Some of the SJS/TEN patients and controls in this study were subjects in our earlier reports [12,17,18,19].

TLR3 SNPs Genotyping

Genomic DNA was isolated from human peripheral blood at SRL Inc. (Tokyo, Japan). Genotyping for 2 SNPs of TLR3 (rs3775290, 3775296) was performed by PCR-direct sequencing as reported previously [17]. For direct sequencing, PCR amplification was conducted with AmpliTaq Gold DNA Polymerase (Applied Biosystems) for 35 cycles at 94°C for 1 min, annealing at 60°C for 1 min, and 72°C for 1 min on a commercial PCR machine (GeneAmp; Perkin-Elmer Applied Biosystems). The PCR products were reacted with BigDye Terminator v3.1 (Applied Biosystems) and sequence reactions were resolved on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Genotyping for 5 SNPs of TLR3 (rs4861699, rs6822014, rs11732384, rs5743312, rs7668666) as performed using DigiTag2 assay [12]. Multiplex PCR was performed in 10 μl of Multiplex PCR buffer containing 25 ng genomic DNA, 25 nM of each multiplex primer mix, 200 μM of each dNTP, 2.25 mM $MgCl_2$, and 0.4 U KAPA2G Fast HotStart DNA polymerase (Kapa Biosystems). Cycling was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 68°C for 2 min. The primers and probes used in this study previously were reported [12,17].

HLA-A Genotyping

For HLA-A genotyping, we performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial bead-based typing kits (WAK Flow, Wakunaga, Hiroshima, Japan), as described previously [18,19].

Statistical Analysis

Statistical significance of the association with each SNP was assessed using Chi-square test or Fisher's exact test on two-by-two contingency tables. When the value obtained for the control was 0 the odds ratio was calculated using Woolf's correction.

Haploview software (ver. 4.2) was used to infer the linkage disequilibrium structure of the 7 TLR3 SNPs and to perform a haplotype analysis of TLR3 gene.

Supporting Information

Table S1 Haplotype analysis of TLR3 gene. Haplotype association analysis with the 7 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs5743312, rs7668666, rs3775290) and the 5 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs3775290)
(DOCX)

Author Contributions

Conceived and designed the experiments: MU. Performed the experiments: MU KT HS. Analyzed the data: MU KT HS GT. Contributed reagents/materials/analysis tools: MU CS TI SK. Wrote the paper: MU.

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The Relation Between Visual Performance and Clinical Ocular Manifestations in Stevens-Johnson Syndrome

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• PURPOSE: To investigate the relation between visual function, clinical findings, and visual symptoms in Stevens-Johnson syndrome (SJS) and to compare the results with Sjögren syndrome (SS) patients and normal subjects.

• DESIGN: Cross-sectional comparative study.

• METHODS: One hundred fifteen eyes of 59 consecutive patients with SJS and toxic epidermal necrolysis (TEN), 208 eyes of 104 healthy normal subjects, and 132 eyes of 66 SS patients were investigated in this multicenter study. All study subjects underwent tear function and ocular surface examinations, Landolt and functional visual acuity examinations, and the Japanese version of the NEI VFQ-25 (National Eye Institute Visual Function Questionnaire).

• RESULTS: The mean ocular surface grading scores were significantly higher and the mean score of all 12 NEI VFQ subscales was significantly lower in the SJS patients compared to the SS patients and the normal subjects ($P < .05$). The conventional and functional logarithm of minimal angle of resolution (logMAR) visual acuities in SJS patients with minimal corneal complications were significantly higher and the mean total composite NEI VFQ scores were lower compared to SS patients. The conventional and functional logMAR visual acuities and the mean ocular surface grading scores in SJS with aqueous deficiency were significantly higher and the mean total composite NEI VFQ scores were lower compared to SS patients. Strong correlations between best-corrected logMAR functional visual acuities and either ocular surface grading scores or the composite NEI VFQ-25 scores were observed.

• CONCLUSIONS: The functional visual acuity examination reflects the severity of clinical ocular surface findings and vision-related quality of life more than the standard conventional visual acuity in SJS. (Am J Ophthalmol 2012;154:499–511. © 2012 by Elsevier Inc. All rights reserved.)

Accepted for publication Mar 28, 2012.

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STEVENS-JOHNSON SYNDROME (SJS) IS AN ACUTE, self-limiting disease of the skin and mucous membranes associated with symblepharon, adhesive occlusion of the lacrimal puncta, and corneal opacification with conjunctivalization and severe dry eyes leading to worsening of the ocular surface health and poor quality of vision.[1–8]

Previous reports have demonstrated that contrast sensitivity, contrast visual acuity, glare disability, and wavefront aberrations are useful to detect quality of vision in everyday life.[9–19] Visual function assessment using these measurement methods has been reported to be useful in keratorefractive surgery, mild cataract, and dry eye diseases.[9–19] To the best of our knowledge, however, there are no reports about visual function assessment in patients with SJS except a previously published report by us.[8]

It has been our experience that SJS patients with good visual acuity and mild ocular surface morbidity may still complain of similar severe eye irritation and visual complaints as patients with Sjögren syndrome (SS). However, the differences in visual symptoms and conventional and dynamic visual acuity between these 2 entities have not been quantified and compared so far. In an attempt to investigate the visual function and ocular surface differences between SJS and SS, we performed this multicenter cross-sectional study, using a previously reported ocular surface morbidity severity questionnaire and functional visual acuity measurement.[20–24]

METHODS

• SUBJECTS: One hundred fifteen eyes of 59 consecutive patients (28 male, 31 female; mean age: 47.5 ± 16.0 years; range: 14–79 years) with SJS, including its more severe variant, toxic epidermal necrolysis (TEN), seen at the Cornea Subspecialty Outpatient Clinic of the Departments of Ophthalmology of Keio University, Tokyo Dental College, Tokyo Medical Center, Kyoto Prefectural University of Medicine, Hokkaido University, Ehime University, and Yamaguchi University were studied in this cross-sectional multicenter study. Clinicians participating

0002-9394/$36.00
http://dx.doi.org/10.1016/j.ajo.2012.03.044

TABLE 1. Clinical Severity Grading Criteria of the Ocular Surface Findings

	Grade 0	Grade 1	Grade 2	Grade 3	Comments
Assessment of Corneal Complications					
SPK	A1D1	A1D2, A2D1	A1D3, A2D2, A3D1	A2D3, A3D2, A3D3	Using fluorescein staining based on the area and density of the lesions as described by Miyata and associates[29]
Corneal epithelial defect	Absent	Less than 1/4 of the corneal surface	1/4 to 1/2 of the corneal surface	More than 1/2 of the corneal surface	
Conjunctivalization	Absent	Less than 1/4 of the corneal surface	1/4 to 1/2 of the corneal surface	More than 1/2 of the corneal surface	
Neovascularization	Absent	Confined to the corneal periphery	Extending beyond the pupil margin	Extending beyond the pupil margin into the central cornea	In eyes where significant opacification or extensive symblepharon formation made it difficult to evaluate corneal neovascularization, a grade of 3 was assigned
Corneal opacification	Clear cornea with easily visible iris details	Partial obscuration of the iris details	Iris details poorly seen with barely visible pupil margins	Complete obscuration of iris and pupil details	
Keratinization	Absent	Less than 1/4 of the corneal surface	1/4 to 1/2 of the corneal surface	More than 1/2 of the corneal surface	
Assessment of Conjunctival Complications					
Conjunctival hyperemia	Absent	Mild or sectoral engorgement of the conjunctival vessels	Moderate or diffuse engorgement of the conjunctival vessels	Severe or significant engorgement of the conjunctival vessels	
Symblepharon	Absent	Involving only the conjunctival surface	Less than 1/2 of the corneal surface	More than 1/2 of the corneal surface	
Assessment of Eyelid Complications					
Trichiasis	Absent	Less than 1/4 of the lid margin	1/4 to 1/2 of the lid margin	More than 1/2 of the lid margin	

Continued on next page

TABLE 1. Clinical Severity Grading Criteria of the Ocular Surface Findings *(Continued)*

MJ involvement	Normal MJ	Mild irregularity of MJ	Moderate irregularity of MJ	Severe irregularity of MJ	Bron grading was employed for the classification of MJ changes.[30] Fluorescein staining of the conjunctiva was performed for evaluating the MJ involvement. In eyes where significant keratinization of the lid margin or extensive symblepharon formation made it difficult to evaluate mucocutaneous junction involvement, Grade 3 was assigned.
MG involvement	Oily expressible secretion	Expressible yellowish-white oily secretion	Expressible thick cheesy material	Inability to express any secretion	The severity was determined clinically by the nature of the meibomian gland secretion expressed manually at the center of the upper lid.
Punctal involvement	Normal	Iatrogenic punctal occlusion	Either superior or inferior punctal occlusion	Both superior and inferior punctal occlusion	

A = area; D = density; MG = mucocutaneous gland; MJ = mucocutaneous junction; SPK = superficial punctuate keratopathy.

in the study received training to standardize the conduct of examinations performed at each center. The conduct of examinations was checked by trained coordinators at each center for consistency of the examination procedures. The diagnosis of SJS or TEN was based on the history of the presence of cryptogenic fever and acute inflammation of mucosal membranes most commonly after taking cold remedies, antibiotics, or anti-inflammatory drugs, and on the presence of chronic ocular surface complications such as symblepharon, entropion, trichiasis, xerophthalmia, and/or corneal vascularization.[1,3–5] Two hundred eight eyes of 104 healthy normal subjects (30 male, 74 female; mean age: 36.2 ± 12.0 years; range: 20–72 years) without dry eye disease and 132 eyes of 66 SS patients (66 female; mean age: 62.8 ± 11.1 years; range: 28–82 years) who were diagnosed according to Fox criteria were also investigated in this multicenter study.[25] Patients or control subjects with other systemic or ocular diseases, history of ocular surgery within 6 months, history of ocular cicatricial pemphigoid, or chemical, thermal, or radiation injury that would have adverse ocular surface effects were excluded according to the study exclusion criteria. SJS patients with a baseline best-corrected Landolt conventional visual acuity of less than 20/2000 attributable to cataract in both eyes, ocular surface keratinization, glaucoma, or posterior segment disease were excluded from this study, since the functional visual acuity measurement system cannot assess functional visual acuity at such low visual acuity levels.

• SLIT-LAMP EXAMINATIONS: All study subjects underwent slit-lamp examinations observing 12 components of 3 categories of ocular complications, such as corneal complications consisting of superficial punctuate keratopathy (SPK), epithelial defect, conjunctivalization, neovascularization, opacification, and keratinization; conjunctival complications consisting of hyperemia and symblepharon formation; and eyelid complications consisting of trichiasis, mucocutaneous junction involvement, meibomian gland involvement, and punctal damage. Each component was graded on a scale from 0 to 3, depending on the severity of involvement.[26]

The severity gradings and ocular surface tests were performed under the same single protocol by the researchers of all contributing study centers. Table 1 shows the clinical severity grading criteria of the ocular surface findings.

• TEAR FUNCTION AND OCULAR SURFACE EXAMINATIONS: The standard Schirmer test without topical anesthesia was performed as previously reported.[7] A vital staining severity grading was also assigned. A 2-μL volume of 1% fluorescein dye was instilled in the conjunctival sac by a micropipette. The minimum score for corneal fluorescein staining was 0 points and the maximum score was 9 points.[27]

• STANDARD VISUAL ACUITY MEASUREMENTS: Standard visual acuity testing using Landolt charts placed 5 m away from subjects was performed. Landolt visual acuity

TABLE 2. Standard Visual Acuity and Visual Parameters Assessed by Functional Visual Acuity Measurement System in Eyes of Patients With Sjögren Syndrome, Stevens-Johnson Syndrome Patients, and Healthy Normal Subjects

	SJS	SS	Normal
Conventional visual acuity			
logMAR	0.76 ± 0.76	−0.004 ± 0.13	−0.10 ± 0.10
Decimal	0.17	1.01	1.26
Functional visual acuity			
logMAR	0.98 ± 0.62[a]	0.28 ± 0.27[a]	−0.008 ± 0.13
Decimal	0.10	0.52	1.02
Maximal visual acuity			
logMAR	0.83 ± 0.65	0.10 ± 0.26	−0.15 ± 0.12
Decimal	0.15	0.79	1.41
Minimal visual acuity			
logMAR	1.19 ± 0.60	0.53 ± 0.36	0.17 ± 0.19
Decimal	0.06	0.30	0.68
Visual maintenance ratio	0.86 ± 0.12	0.91 ± 0.07[b]	0.98 ± 0.05[c,d]
Reaction time	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
Blink number	11.2 ± 9.3	17.2 ± 9.6[b]	16.4 ± 8.7[c]

logMAR = logarithm of minimal angle of resolution; logMAR = logarithm of minimal angle of resolution; SJS = Stevens-Johnson syndrome; SS = Sjögren syndrome; VA = visual acuity.
[a]$P < .05$ between conventinal VA and functional VA.
[b]$P < .05$ between groups of SJS and SS.
[c]$P < .05$ between groups of SJS and Normal.
[d]$P < .05$ between groups of SS and Normal.

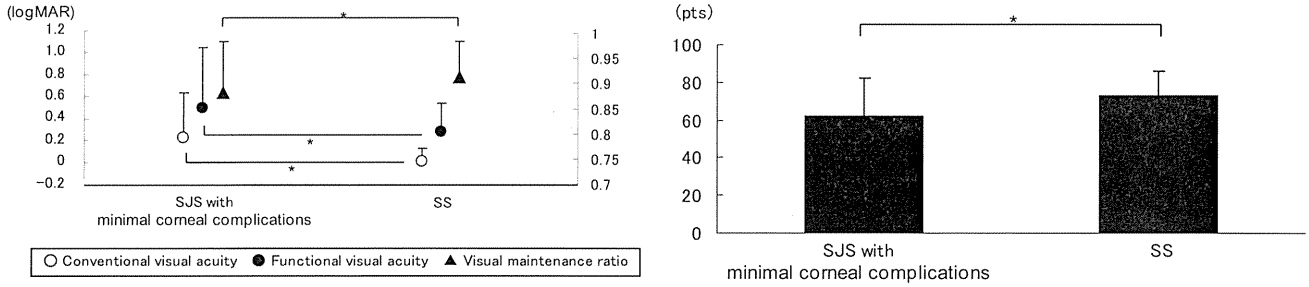

FIGURE 1. Visual function and Visual Function Questionnaire-25 in Stevens-Johnson syndrome (SJS) with minimal corneal complications and Sjögren syndrome (SS) patients. (Left) Conventional and functional visual acuity and visual maintenance ratio in Stevens-Johnson syndrome with minimal corneal complications and Sjögren syndrome patients. (Right) Total composite NEI VFQ-25 scores in Stevens-Johnson syndrome with minimal corneal complications and Sjögren syndrome patients. logMAR = logarithm of minimal angle of resolution.

was employed instead of Snellen chart since it is a standard test in Japan, and because the optotypes in Landolt and functional visual acuity testing are similar.

• FUNCTIONAL VISUAL ACUITY MEASUREMENTS: Continuous visual acuity testing during a 60-second period under natural blinking was performed as previously reported.[24]

• FUNCTIONAL VISUAL ACUITY INDICES: Briefly, the outcome parameters of the functional visual acuity measurement system were functional visual acuity (defined as the average of visual acuities measured during a 60-second testing), visual maintenance ratio (defined as the ratio of logMAR values of the functional visual acuities over the time frame for testing divided by the logarithm of minimal angle of resolution (logMAR) baseline visual acuity),[7] maximal corrected visual acuity, minimal corrected visual acuity, standard deviation of functional visual acuity, mean reaction time (defined as the mean of the response time taken by a subject to respond to an optotype), and blink numbers during a 60-second functional visual acuity test.

• VISUAL FUNCTION QUESTIONNAIRE-25: We used the Japanese version of the NEI VFQ-25 (National Eye Insti-

tute Visual Function Questionnaire 25) to evaluate the vision-related quality of life.[28] NEI VFQ-25 measures the following 12 vision-targeted subscales: general health, general vision, ocular pain, near activities, distant activities, social functioning, mental health, role difficulties, dependency, driving, color vision, and peripheral vision. A scale of 0 to 100 points is used for subscale scores. A score of 100 indicates the best possible score, while 0 indicates the worst possible score.

• STATISTICAL ANALYSIS: A 1-way ANOVA was performed for the comparison of conventional visual acuities, functional visual acuities, visual maintenance ratios, ocular surface grading scores, and VFQ-25 scores among SJS patients, SS patients, and normal control subjects. The Bonferroni test was used for further multiple comparisons. A paired t test was performed for the comparison between conventional and functional visual acuities in SJS patients, SS patients, and normal control subjects alone. To investigate whether the visual disturbance or quality of life are similarly affected in SJS patients compared to SS patients, conventional visual acuities, functional visual acuities, visual maintenance ratios, and VFQ-25 scores were compared among SJS patients with minimal corneal complications and SS patients by paired t test. Minimal corneal complication was defined as a grading score ≤4 points, in relation to keratinization, conjunctivalization, opacification, corneal epithelial defect, neovascularization, and SPK. Severe corneal complication was defined as a grading score >4 points. To investigate the effect of tear functions on the ocular surface complications, visual disturbance, or quality of life in SJS and SS patients, ocular surface grading scores, conventional visual acuities, functional visual acuities, visual maintenance ratios, and VFQ-25 scores were compared in SJS patients with and without aqueous tear deficiency by 1-way ANOVA. Aqueous tear deficiency was defined as a Schirmer test score ≤5 mm. The relation between ocular surface grading scores, conventional visual acuities, and functional visual acuities was analyzed by Pearson correlation analysis. The relation between ocular surface complications and conventional visual acuities, functional visual acuities, visual maintenance ratios, or VFQ-25 scores was also analyzed by Pearson correlation analysis in SJS patients with and without aqueous tear deficiency and SS patients. In the correlation analysis between ocular surface grading scores and conventional visual acuities or functional visual acuities in SJS patients, eyes were divided into 3 visual groups: good conventional visual acuity group (logMAR conventional visual acuity score ≤0), intermediate conventional visual acuity group (0 < logMAR conventional visual acuity ≤ 0.3), and poor conventional visual acuity group (0.3 < logMAR conventional visual acuity score ≤ 2.0). The relation between VFQ-25 score, conventional visual acuity, and functional visual acuity was analyzed by the same methodology, using the eye with better conventional visual acuity. The correlation between clinical findings, conventional visual acuities, and functional visual acuities was also investigated by multiple linear regression analysis. A probability level of $P < .05$ was considered statistically significant. SPSS (SPSS Inc, Chicago, Illinois, USA) was used as the statistical analysis software.

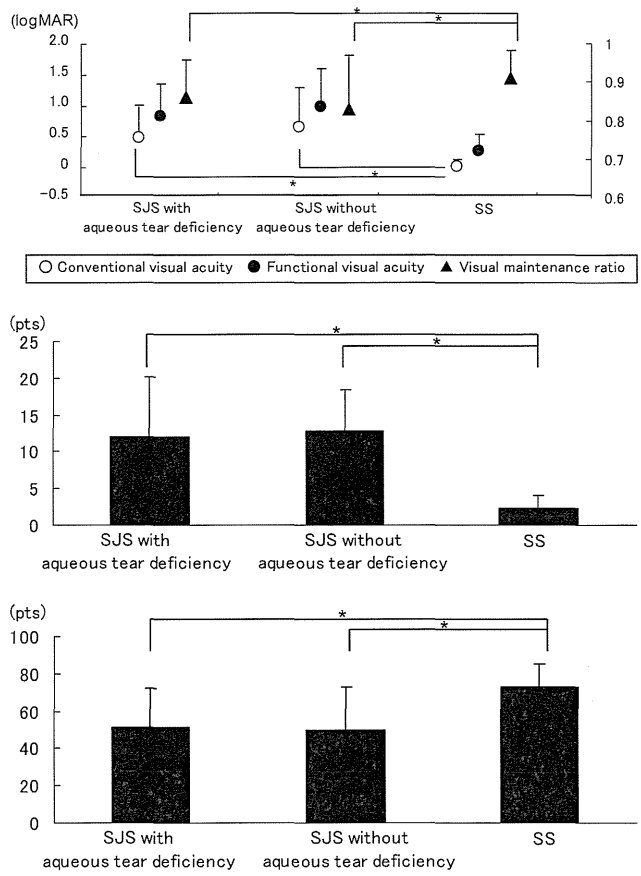

FIGURE 2. Visual function, ocular surface grading score, and Visual Function Questionnaire-25 in Stevens-Johnson syndrome (SJS) patients with and without aqueous tear deficiency and Sjögren syndrome (SS) patients. (Top) Conventional and functional visual acuity and visual maintenance ratio in Stevens-Johnson syndrome patients with and without aqueous tear deficiency and Sjögren syndrome patients. (Middle) Total ocular surface grading scores in Stevens-Johnson syndrome patients with and without aqueous tear deficiency and Sjögren syndrome patients. (Bottom) Total composite NEI VFQ-25 scores in Stevens-Johnson syndrome patients with minimal corneal complications and Sjögren syndrome patients. logMAR = logarithm of minimal angle of resolution.

RESULTS

• TEAR FUNCTION TESTS: The mean Schirmer test values were 9.1 ± 9.3 mm in SJS patients, 4.6 ± 4.5 mm in SS patients, and 18.6 ± 9.5 mm in healthy control subjects, respectively. The Schirmer test values were significantly higher in SJS patients compared to SS patients

TABLE 3. Percentages of Ocular Surface Grading Score in Sjögren Syndrome Patients, Stevens-Johnson Syndrome Patients, and Healthy Normal Subjects

	SJS				SS				Normal			
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3
Assessment of corneal complications												
SPK	15.1%	25.9%	25.2%	33.8%	31.1%	25.6%	20.2%	23.3%	99.0%	1.0%	0	0
Corneal epithelial defect	92.2%	2.8%	0	5.0%	100%	0	0	0	100%	0	0	0
Conjunctivalization	32.1%	14.3%	12.9%	40.7%	95.4%	4.6%	0	0	100%	0	0	0
Neovascularizarion	25.7%	27.1%	24.3%	22.9%	97.7%	2.3%	0	0	98.1%	1.9%	0	0
Opacification	28.4%	50.4%	13.5%	7.8%	99.2%	0.8%	0	0		0	0	0
Keratinization	88.7%	4.3%	3.4%	3.4%	100%	0	0	0	100%	0	0	0
Assessment of conjunctival complications												
Conjunctival hyperemia	24.1%	58.9%	14.9%	2.1%	82.3%	16.9%	0.8%	0	100%	0	0	0
Symblepharon	38.8%	48.2%	7.9%	5.0%	99.2%	0.8%	0	0	100%	0	0	0
Assessment of eyelid complications												
Trichiasis	40.3%	25.2%	14.4%	20.1%	100%	0	0	0	99.0%	1.0%	0	0
MJ involvement	15.6%	42.6%	27.0%	14.9%	86.2%	12.3%	1.5%	0	100%	0	0	0
MG involvement	17.0%	22.0%	17.7%	43.3%	81.5%	14.6%	0	3.8%	100%	0	0	0
Punctal involvement	27.7%	19.9%	9.9%	42.6%	75.4%	23.1%	0.8%	0.8%	100%	0	0	0

MG = meibomian gland; MJ = mucocutaneous junction; SJS = Stevens-Johnson syndrome; SPK = superficial punctuate keratopathy; SS = Sjögren syndrome.

($P < .05$). A total of 49.6 % of the patients with SJS had Schirmer test values greater than 5 mm.

• STANDARD CONVENTIONAL VISUAL ACUITY: Table 2 shows the mean logMAR conventional visual acuity in SJS and SS patients and the normal subjects. The mean logMAR conventional visual acuity in SJS patients was significantly lower compared to the mean logMAR conventional visual acuity in SS patients and normal controls ($P < .05$).

The mean logMAR conventional visual acuity in SJS patients with severe corneal complications was 0.74 ± 0.57. The mean logMAR conventional visual acuity in SJS patients with minimal corneal complications and SS patients was 0.21 ± 0.42 and −0.001 ± 0.12, respectively. The logMAR conventional visual acuities in SJS patients were significantly higher compared to SS patients (Figure 1, Left).

The mean logMAR conventional visual acuity in SJS patients with and without aqueous tear deficiency and SS patients was 0.47 ± 0.53, 0.65 ± 0.63, and −0.004 ± 0.13, respectively. The logMAR conventional visual acuities in SJS patients were significantly higher compared to SS patients (Figure 2, Top).

• FUNCTIONAL VISUAL ACUITY INDICES: Table 2 shows the results of all indices measured by the functional visual acuity measurement system. The mean logMAR functional visual acuity was significantly lower compared to the mean logMAR conventional visual acuity in patients with SJS and SS ($P < .05$). The mean logMAR standard deviation of functional visual acuity was significantly greater in patients with SJS and SS compared to normal subjects ($P < .05$). The mean visual maintenance ratio in the SJS patients was significantly lower than in SS patients, and the mean visual maintenance ratio in SS patients was significantly lower than in normal subjects ($P < .05$). There were no significant differences in reaction times among SJS patients, SS patients, and normal subjects. The mean blink number in the SJS patients was significantly lower compared to SS patients and normal subjects ($P < .05$).

The mean logMAR functional visual acuity in SJS patients with severe corneal complications was 1.16 ± 0.45. The mean logMAR functional visual acuity in SJS and SS patients with minimal corneal complications was 0.50 ± 0.55 and 0.28 ± 0.27, respectively. The functional visual acuities in SJS patients were significantly higher compared to in SS patients (Figure 1, Left). The mean visual maintenance ratio in SJS and SS patients with minimal corneal complications was 0.88 ± 0.10 and 0.91 ± 0.07, respectively. Visual maintenance ratios in SJS patients were significantly lower compared to SS patients (Figure 1, Left).

The mean logMAR functional visual acuity in SJS patients with and without aqueous tear deficiency and SS patients was 0.83 ± 0.54, 0.99 ± 0.63, and 0.28 ± 0.27, respectively. The functional visual acuities in SJS patients with and without aqueous tear deficiency were significantly

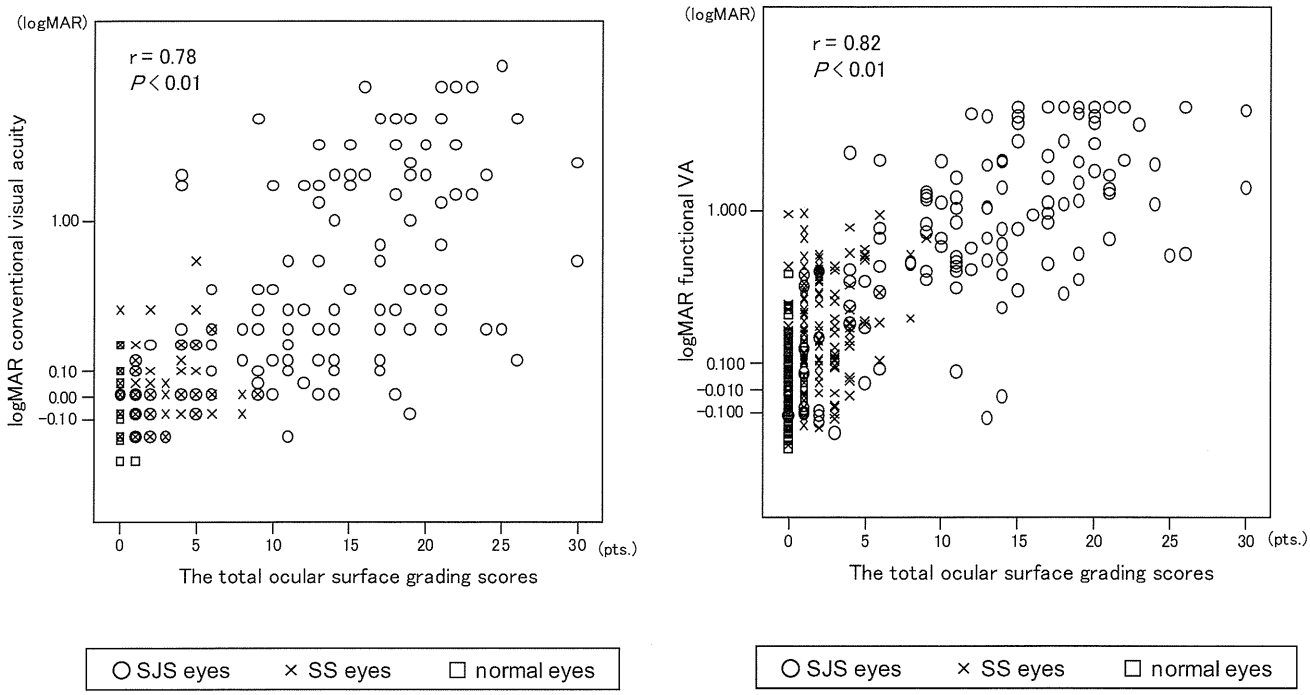

FIGURE 3. Correlation between visual function and ocular surface grading score. (Left) Correlation between logMAR conventional visual acuity scores and total ocular surface grading scores. (Right) Correlation between logarithm of minimal angle of resolution (logMAR) functional visual acuity scores and total ocular surface grading scores. SJS: Stevens-Johnson syndrome; SS: Sjögren syndrome.

TABLE 4. Multivariable Regression Analyses Between Ocular Surface Grading Score, logMAR Conventional Visual Acuity, and logMAR Functional Visual Acuity

	logMAR Conventional Visual Acuity[a]		logMAR Functional Visual Acuity[b]	
Complication	Standard Partial Regression	*P* value	Standard Partial Regression	*P* value
Neovascularization	0.509	<.001	0.229	<.001
Opacification	0.385	<.001	0.308	<.001
Keratinization	−0.088	.002	−0.131	<.001
SPK	0.054	.061	0.193	<.001
Symblepharon	0.059	.103	0.162	<.001
Conjunctivalization	−0.058	.262	0.168	<.001
Corneal epithelial defect	0.034	.198	0.027	.319

logMAR = logarithm of minimal angle of resolution; SPK = superficial punctuate keratopathy.

[a]Conditioned multiple correlation coefficient for logMAR conventional visual acuity =0.81.

[b]Conditioned multiple correlation coefficient for logMAR functional visual acuity =0.84.

higher compared to SS patients (Figure 2, Top). The mean visual maintenance ratios in SJS patients with and without aqueous tear deficiency and SS patients were 0.86 ± 0.10, 0.83 ± 0.14, and 0.91 ± 0.07, respectively. The visual maintenance ratios in SJS patients both with and without aqueous tear deficiency were significantly lower compared to SS patients (Figure 2, Top).

• CLINICAL FINDINGS: Table 3 shows the mean ocular surface grading scores in SS and SJS patients and normal subjects. The mean ocular surface grading scores in all 12 components of clinical findings was significantly higher in SJS patients compared to SS patients and normal subjects ($P < .05$).

The mean ocular surface grading scores in SJS patients with and without aqueous tear deficiency and SS patients were 12.0 ± 8.1, 12.8 ± 5.7, and 2.3 ± 1.8, respectively. The total ocular surface grading scores in SJS both with and without aqueous tear deficiency were significantly higher compared to SS patients (Figure 2, Middle).

• CORRELATION BETWEEN VISUAL FUNCTION AND CLINICAL FINDINGS: Figure 3 shows the correlation between visual function and ocular surface grading score in SJS patients, SS patients, and normal subjects overall. A strong significant correlation was observed between total ocular-surface grading scores and best-corrected logMAR Landolt conventional visual acuities ($r = 0.78$, $P < .001$), as well as best-corrected logMAR Landolt functional visual acuities ($r = 0.82$, $P < .001$).

Table 4 shows the correlation of visual function and ocular surface grading scores. The results of multiple linear regression analysis between the clinical findings and log-

TABLE 5. Correlations Between Ocular Complications and Visual Function or the Composite National Eye Institute Visual Function Questionnaire Scores in Stevens-Johnson Syndrome Patients With Aqueous Tear Deficiency and Sjögren Syndrome Patients

	SJS With Aqueous Tear Deficiency				SS			
	Pearson CC				Pearson CC			
	Log Conventional Visual Acuity	Log Functional Visual Acuity	Visual Maintenance Ratio	NEI VFQ-25	Log Conventional Visual Acuity	Log Functional Visual Acuity	Visual Maintenance Ratio	NEI VFQ-25
Trichiasis	0.09	0.08	0.16	−0.02	—	—	—	—
Symblepharon	0.43[b]	0.53[b]	−0.30[a]	−0.46[a]	0.08	0.15	−0.07	−0.12
Punctal involvement	0.55[b]	0.57[b]	−0.28	−0.49[b]	0.09	0.13	−0.13	0.01
MG involvement	0.48[b]	0.44[b]	−0.23	−0.55[b]	0.06	0.07	−0.04	−0.42[b]
MJ involvement	0.25	0.33[b]	−0.26	−0.39[a]	−0.06	0.20[a]	−0.32[a]	−0.05
Conjunctival hyperemia	0.28[a]	0.31[a]	−0.23	−0.48[b]	0.22[b]	0.11	0.01	−0.24
Keratinization	0.06	0.09	−0.03	−0.03	—	—	—	—
Conjunctivalization	0.53[b]	0.52[b]	−0.19	−0.53[b]	0.29[b]	0.41[b]	−0.28[b]	−0.22
Opacification	0.59[b]	0.67[b]	−0.47[b]	−0.69[b]	0.17	0.20	−0.19	—
Corneal epithelial defect	0.06	0.16	−0.15	−0.11	—	—	—	—
Neovascularization	0.64[b]	0.63[b]	−0.20	−0.63[b]	0.21[a]	0.23[b]	−0.14	−0.2[a]
SPK	0.35[a]	0.35[a]	−0.08	−0.41[a]	0.04	0.212	−0.11	−0.22
Total ocular complications	0.55[b]	0.58[b]	−0.26	−0.61[b]	0.12	0.25[b]	−0.15	−0.40[b]

CC = correlation coefficient; MG = meibomian gland; MJ = mucocutaneous junction; NEI VFQ-25 = National Eye Institute visual function questionnaire; SJS = Stevens-Johnson syndrome; SPK = superficial punctuate keratopathy; SS = Sjögren syndrome.

[a] $P < .05$.

[b] $P < .01$.

TABLE 6. Correlations Between Visual Function and Ocular Surface Grading Scores or Composite National Eye Institute Visual Function Questionnaire Scores in Good, Intermediate, or Poor Conventional Visual Acuity Group of Stevens-Johnson Syndrome Patients

	All Groups		Good Conventional Visual Acuity Group		Intermediate Conventional Visual Acuity Group		Poor Conventional Visual Acuity Group	
	Pearson CC	*P* Value	Pearson CC	*P* Value	Pearson CC	*P* Value	Pearson CC	*P* Value
Conventional visual acuity vs clinical finding scores	0.59[b]	.001	0.37	.08	0.24	.15	0.40[b]	.001
Functional visual acuity vs clinical finding scores	0.63[b]	.001	0.56[b]	.005	0.49[b]	.002	0.34[b]	.007
Conventional visual acuity vs composite NEI VFQ-25 scores	−0.74[b]	.001	−0.44	.06	−0.25	.25	−0.56[a]	.03
Functional visual acuity vs composite NEI VFQ-25 scores	−0.74[b]	.001	−0.55[b]	.02	−0.20	.37	−0.57[a]	.03

CC = correlation coefficient; NEI VFQ-25 = National Eye Institute Visual Function Questionnaire.

[a] $P < .05$.

[b] $P < .01$.

MAR conventional visual acuity showed a significant and strong correlation with neovascularization, opacification, and keratinization grades. Clinical findings such as SPK, symblepharon, and conjunctivalization also had a significant and strong correlation with the functional visual acuities. The multiple regression equation of logMAR conventional visual acuity was expressed as follows: logMAR conventional visual acuity = −0.084 + neovascularization × 0.509 + opacification × 0.385 + keratinization × −0.088. Likewise, the multiple regression equation of logMAR functional visual

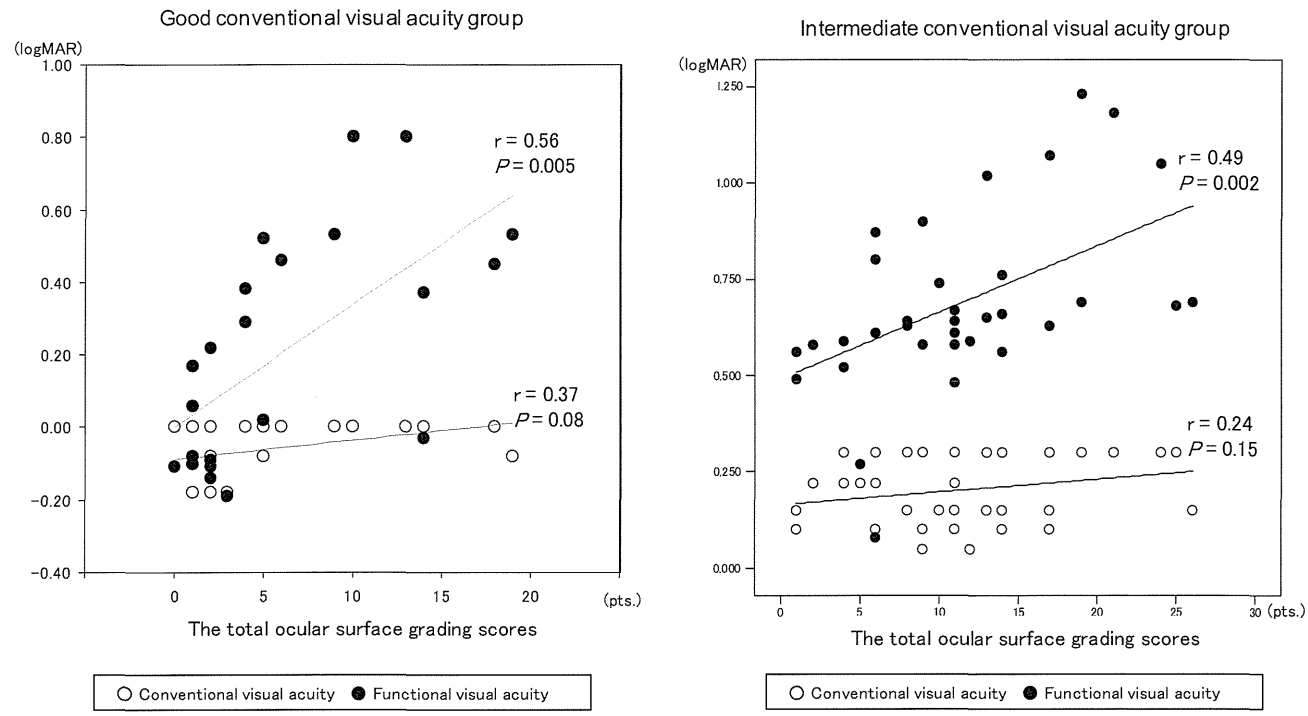

FIGURE 4. Correlations between visual function and ocular surface grading score in the good and intermediate conventional visual acuity group of Stevens-Johnson syndrome patients. (Left) Correlation in the good conventional visual acuity group of Stevens-Johnson syndrome patients. (Right) Correlation in the intermediate conventional visual acuity group of Stevens-Johnson syndrome patients. logMAR = logarithm of minimal angle of resolution.

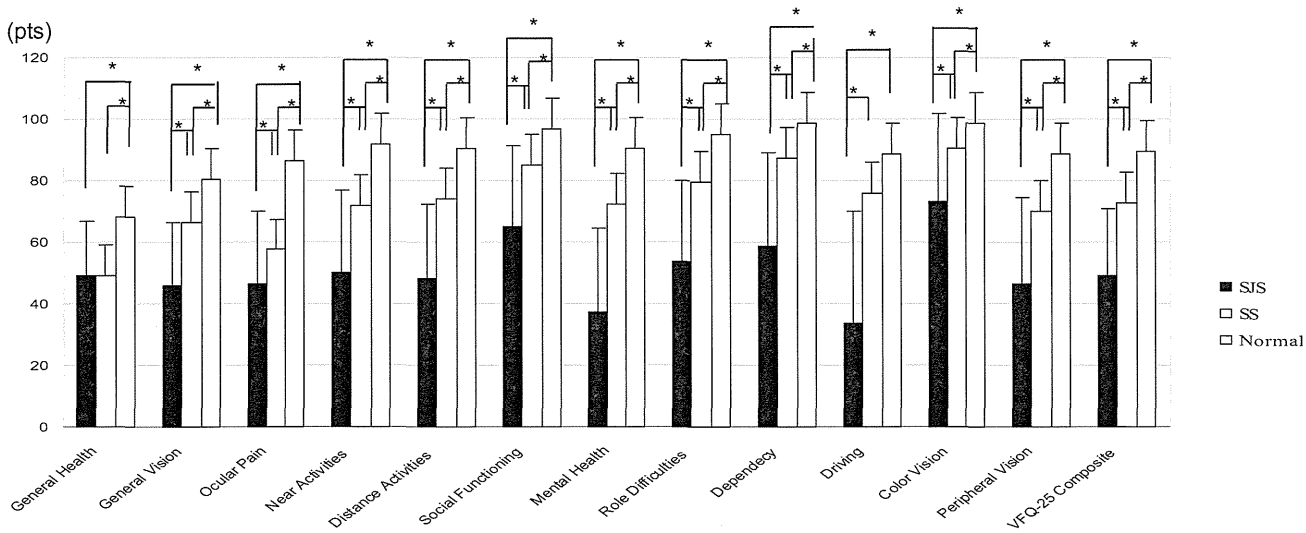

FIGURE 5. Visual Function Questionnaire-25 results in patients with Stevens-Johnson syndrome (SJS), patients with Sjögren syndrome (SS), and healthy normal subjects.

acuity was expressed as follows: logMAR functional visual acuity = −0.061 + neovascularization × 0.229 + opacification × 0.308 + keratinization × −0.131 + SPK × 0.193 + symblepharon × 0.162 + conjunctivalization × 0.168.

Table 5 shows the correlation between ocular complications and visual function in SJS patients with aqueous tear deficiency and SS patients. Strong significant correlations were observed between total ocular surface grading score and logMAR conventional visual acuities or logMAR functional visual acuities in SJS patients with aqueous tear deficiency, and similar strong significant correlations in SJS patients without aqueous tear defi-

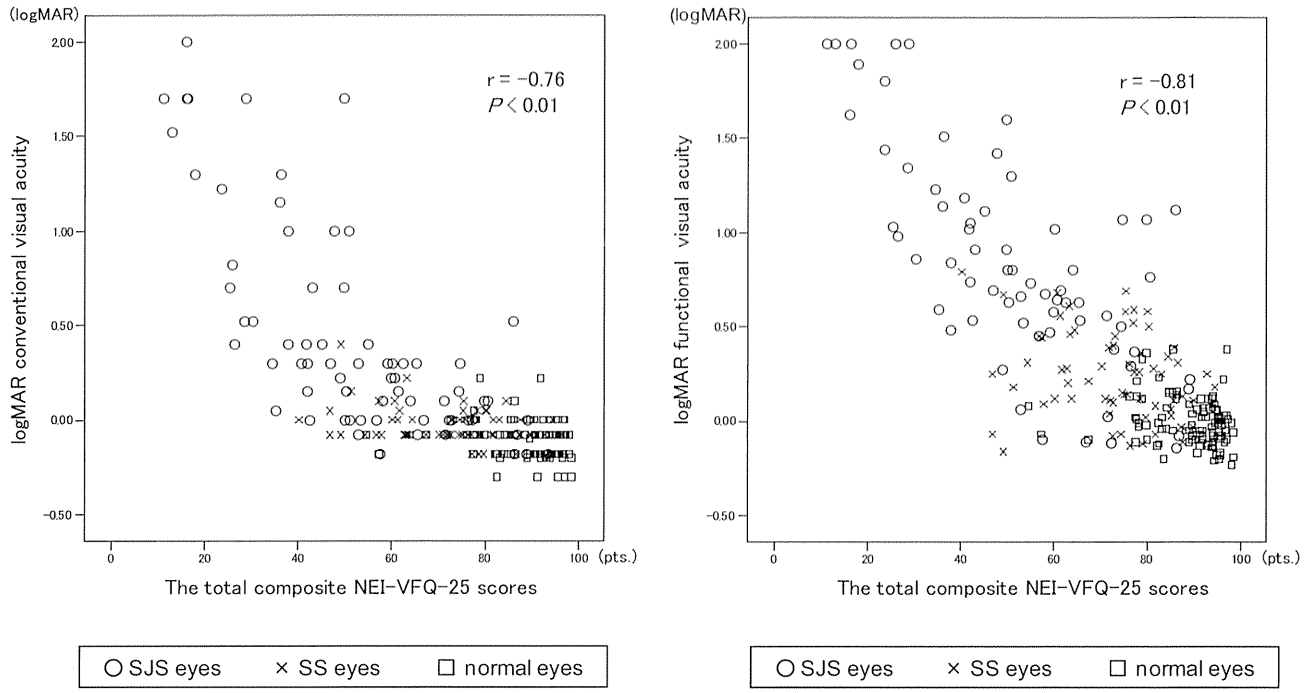

FIGURE 6. Relation between visual function and composite NEI VFQ-25 scores. (Left) Correlation between logMAR conventional visual acuity scores and total composite NEI VFQ-25 scores. (Right) Correlation between logarithm of minimal angle of resolution (logMAR) functional visual acuity scores and total composite NEI VFQ-25 scores. SJS: Stevens-Johnson syndrome; SS: Sjögren syndrome.

ciency (r = 0.66, $P < .001$) (data not shown), while significant correlations were observed only between total ocular surface grading score and logMAR functional visual acuities in SS patients (Table 5).

Table 6 shows the correlations between visual function and ocular surface grading scores in the good, intermediate, and poor conventional visual acuity groups of SJS patients. A strong positive significant correlation was observed between total ocular surface grading scores and logMAR Landolt functional visual acuities in the good conventional visual acuity group (r = 0.56, $P = .005$) and intermediate conventional visual acuity group (r = 0.49, $P = .002$), while no correlation was observed between total ocular surface grading scores and logMAR Landolt conventional visual acuities in these groups (Figure 4).

• VISUAL FUNCTION QUESTIONNAIRE-25: Mean subscale and composite NEI VFQ scores for SJS and SS patients and normal subjects are presented in Figure 5. All 12 subscale NEI VFQ scores were significantly lower in the SJS patients compared to the normal subjects ($P < .05$). Likewise, all subscale scores were significantly lower in the SS patients compared to the normal subjects ($P < .05$). The subscale of "ocular pain" was remarkably low in SS patients, while all subscale scores were remarkably lower in SJS patients. The mean composite NEI VFQ scores of the 12 subscales were 49.1 ± 21.6 in SJS patients, 72.8 ± 12.8 in SS patients, and 89.4 ± 8.1 in the normal subjects.

The mean total composite NEI VFQ score in SJS patients with severe corneal complications was 45.2 ± 20.9. The mean total composite NEI VFQ scores in SJS patients with minimal corneal complications and SS patients were 62.2 ± 19.8 and 73.0 ± 12.8, respectively. The total composite NEI VFQ scores in SJS patients were significantly lower compared to SS patients (Figure 1, Right).

The mean total composite NEI VFQ scores in SJS patients with and without aqueous tear deficiency were 51.6 ± 20.2, 49.4 ± 23.5, and 72.8 ± 12.8, respectively. The total composite NEI VFQ scores in SJS both with and without aqueous tear deficiency were significantly lower compared to SS patients (Figure 2, Right).

• CORRELATION OF VISUAL FUNCTION AND NEI VFQ-25 SCORES: Figure 6 shows the correlation between visual function and the composite NEI VFQ-25 scores in SJS patients, SS patients, and normal subjects overall. A strong negative correlation was detected between the composite NEI VFQ-25 scores and best-corrected logMAR Landolt conventional visual acuities (r = −0.76, $P < .01$), and best-corrected logMAR Landolt functional visual acuities (r = −0.81, $P < .01$).

Table 6 shows the correlations between visual function and the composite NEI VFQ-25 scores in the good, intermediate, and poor conventional visual acuity groups in SJS patients. A positive significant correlation was

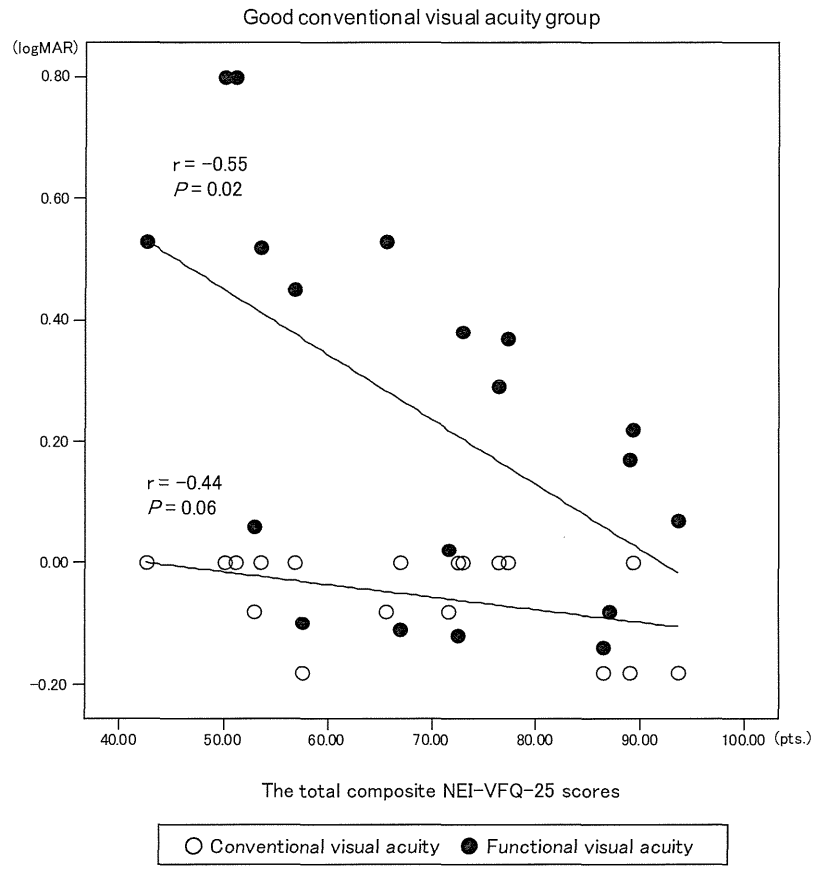

FIGURE 7. Correlations between visual function and the total composite NEI VFQ-25 scores in the good conventional visual acuity group of Stevens-Johnson syndrome patients. logMAR = logarithm of minimal angle of resolution.

observed between the composite NEI VFQ-25 scores and logMAR Landolt functional visual acuities in the good conventional visual acuity group ($r = 0.55$, $P = .02$), while no correlation was observed between the composite NEI VFQ-25 scores and logMAR Landolt conventional visual acuities in this group ($r = 0.44$, $P = .06$) (Figure 7).

• CORRELATION BETWEEN NEI VFQ-25 SCORES AND CLINICAL FINDINGS: Table 5 shows the correlation between ocular complications and the total composite NEI VFQ-25 scores in SJS patients with aqueous tear deficiency and SS patients. Strong significant correlations were observed between total ocular surface grading score and the composite NEI VFQ-25 scores in SJS patients with aqueous tear deficiency and SS patients (Table 5), and similarly in SJS patients without aqueous tear deficiency ($r = 0.51$, $P = .002$) (data not shown).

DISCUSSION

SEVERE OCULAR SURFACE DISEASE ASSOCIATED WITH SJS has been reported to cause visual deterioration. However, quantifying visual acuity in SJS patients has not been assessed, although an interest in the quantitative interpretation of visual function has been rising over the last few years, especially in the fields of refractive surgery, cataract, and dry eyes, through analyses by contrast sensitivity, contrast visual acuity, and wavefront analysis.[9–19] In this report, we measured the functional visual acuity in addition to conventional visual acuity testing and evaluated the relations between visual functions, ocular surface clinical findings, and the vision-related quality of life in SJS patients. We chose functional visual acuity testing for the assessment of the visual function, which has been shown to be efficient in the detection of "masked impairment of visual function" in dry eye patients, since SJS is known to be associated with severe dry eyes.[20–24]

Visual function testing revealed several interesting findings. First, visual acuities measured by conventional Landolt visual acuity testing were low in the SJS patients as compared with SS patients and normal subjects. When we focused on the visual function of the SS patients and normal subjects, the functional visual acuity scores in the SS patients were significantly lower than in the normal subjects, although there were no differences in the conventional visual acuities. In addition, the mean visual maintenance ratios in the SJS patients were significantly lower than in the SS patients, indicating that ability to

maintain the best visual acuity in SJS patients had deteriorated more than in the SS patients.

The functional visual acuity examination has been shown to be useful for the assessment of visual function related to dry eyes in our previous reports.[20–22] A previous report has also suggested the possibility that the functional visual acuity examination might reflect the effect of ocular surface findings and dry eye states on visual functions.[8] In this report, we analyzed the relation of ocular surface findings with visual function and quality of life in detail by grading the severity of ocular surface findings. The clinical severity scores of the examined ocular surface findings were much higher in the SJS patients. We analyzed whether visual disturbance and quality of life were similarly affected in SJS and SS patients without corneal complications or only with minimal corneal complications. Interestingly, we observed that visual function and quality of life were deteriorated in SJS patients with minimal corneal complications compared with SS patients. Moreover, we noted more visual dysfunction and declined quality of life in SJS patients with similar aqueous tear deficiency compared to SS patients. According to the multiple linear regression analysis, neovascularization, opacification, and keratinization involving the optical axis appeared to have a significant effect on the logMAR conventional visual acuities. SPK, symblepharon, and conjunctivalization also had a significant effect on the logMAR functional visual acuities.

Our findings that a stronger correlation existed between ocular surface grading score and logMAR functional visual acuity compared to logMAR conventional visual acuity suggest that functional visual acuity testing can indeed reflect the effect of clinical complications of ocular surface disease on visual function in SJS. In the correlations between visual function and ocular surface grading scores in the good, intermediate, and poor conventional visual acuity groups in SJS patients, a strong positive significant correlation was observed between total ocular surface grading scores and logMAR Landolt functional visual acuities in the good conventional visual acuity group and intermediate conventional visual acuity group, while no correlation was observed between total ocular surface grading scores and logMAR Landolt conventional visual acuities in these groups. These results suggest that functional visual acuity reflects the effect of ocular surface complications on visual function more sensitively in SJS patients with good and intermediate conventional visual acuities. The strong correlation of logMAR functional visual acuity with ocular surface grading score also suggests that functional visual acuity may be detecting the effect of ocular surface disease severity on other visual functions such as contrast, glare, or higher-order aberrations (compared to conventional visual acuity testing), which needs to be investigated in future studies employing the above-mentioned methodologies in conjunction with functional visual acuity testing.

The mean of all VFQ-25 subscale scores was remarkably worse in the SJS patients compared to normal subjects. Likewise, the mean of all subscale scores in SS patients was significantly lower than in the normal subjects. When analyzed in detail, only the subscale scores of "general health" and "ocular pain" were worse, without marked changes in other subscale scores. In SJS patients, as compared with normal subjects, all VFQ-25 subscale scores, especially "ocular pain," "near activities," "distance activities," "mental health," "role difficulties," and "driving," were very low. These findings suggest that SJS patients suffer from an actual limitation of vision-related daily activity rather than a sense of decreased visual performance and health decline.

A strong negative correlation was observed in the relation between logMAR conventional visual acuities and the VFQ-25 composite scores in this study, with a strong correlation detectable for the relation between the VFQ-25 composite scores and the logMAR functional visual acuities.

We had noteworthy observations that patients with SJS had significantly worse dry eye and visual symptom scores compared to SS patients. We believe these observations owe to the presence of a higher incidence of ocular surface complications in SJS such as symblepharon, corneal opacification, and SPK.

One of the weak points of the current study is that SJS patients, SS patients, and normal subjects were not age-matched. However, it was actually difficult to recruit subjects with age matching in the current study. In fact, the age of onset of SS is usually beyond middle age, while individuals have a risk to be involved with SJS at any age. Moreover, recruitment of elderly individuals with normal tear functions as normal control subjects is another challenging task. It should be noted that the VFQ-25 subscale scores might have been affected by sex and age differences. Another weakness was the lack of definitive diagnosis of SJS/TEN by skin biopsy. We diagnosed SJS or TEN on the history of the presence of cryptogenic fever and acute inflammation of mucosal membranes, most commonly after taking cold remedies, antibiotics, or anti-inflammatory drugs, and on the presence of the chronic ocular surface complications.

Overall, although standard visual acuity testing is a good measurement of one aspect of visual function, the functional visual acuity examination provided other important and detailed information on visual functions related with clinical findings and vision-related quality of life. In conclusion, SJS patients with good or intermediate visual acuity scores measured by conventional visual acuity testing were found to suffer from lower vision-related quality of life, as assessed by functional visual acuity testing and VFQ scores.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF Interest. None of the authors received lecture fees or equity payments from Nidek. Drs Kazuo Tsubota and Minako Kaido both hold patent rights for the method and the apparatus for the measurement of functional visual acuity (US patent no: 7470026). All study centers received and shared an official grant from the Japanese Ministry of Health and Welfare, Tokyo, Japan during the conduct of the study. Involved in conception and design (M.K., C.S., S.K., K.T.); analysis and interpretation (M.K., C.S., K.T.); writing the article (M.K.); critial revision of the article (M.Y., C.S., S.K., J.S., Y.T., Y.H., T.C., K.T.); final approval of the article (M.K., M.Y., C.S., S.K., J.S., Y.T., Y.H., T.C., K.T.); data collection (M.K., M.Y., C.S., J.S., Y.T., Y.H., T.C.); provision of materials, patients, or resources (M.K., M.Y., C.S., J.S., Y.T., Y.H., T.C.); statistical expertise (M.K.); obtaining funding (M.K., M.Y., C.S.); lierature search (M.K., C.S.); and administrative, technical, or logistical support (C.S., S.K., K.T.). Ethics committee approvals for the examination procedures and study protocol were obtained at each center for this prospective study (IRB approval number: 17–129, Keio University School of Medicine, 20. 6, 2006). Written informed consent was obtained from each patient to participate in this study.

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Biosketch

Minako Kaido graduated from the Medical University of Occupational and Environmental Health, Fukuoka, Japan in 1991. She joined Dr Tsubota's dry eye and cornea team at Tokyo Dental College Ichikawa Hospital in 1995 and has been a pivotal member of Dr Tsubota's team at Keio University School of Medicine, Tokyo Japan since 2004. She received her PhD degree in 2012. Dr Kaido's work is focused on the treatment of dry eyes and functional visual acuity technology.

Molecular Vision 2012; 18:1727-1739 <http://www.molvis.org/molvis/v18/a178>
Received 29 January 2012 | Accepted 23 June 2012 | Published 27 June 2012

A selective inhibitor of the Rho kinase pathway, Y-27632, and its influence on wound healing in the corneal stroma

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Purpose: Our study examined the effect of a selective Rho kinase inhibitor, Y-27632, on corneal wound healing and potential stromal scarring after superficial keratectomy.

Methods: Rabbit keratocytes were induced into myofibroblasts by transforming growth factor β1 (TGFβ1) either with or without Y-27632. Then α-smooth muscle actin (α-SMA) was examined by immunohistochemistry and western blotting, and the contractility of the seeded collagen gels was measured. Y-27632 eye drops (or vehicle only) were administered to eyes after a superficial keratectomy, and the tissue was examined by immunohistochemistry for α-SMA, collagen types I, II, and III, and keratan sulfate. Electron microscopy was conducted with and without histochemical contrasting of sulfated proteoglycans.

Results: Spindle-like cells in culture constituted 99.5±1.1% with TGFβ1 stimulation, but 3.5±1.0% after TGFβ1 and Y-27632 treatment ($p<0.01$, $n=6$). α-SMA was seen in 4% of TGFβ1-treated cells, but in only 0.3% of cells with Y-27632 added ($p<0.01$, $n=6$), which was confirmed by western blotting. Y-27632 also inhibited the TGFβ1-induced contraction of seeded collagen gels. After superficial keratectomies, collagen type I and keratan sulfate were unchanged by Y-27632 application. Collagen type II was not detected in Y-27632 or vehicle-only corneas. With Y-27632 treatment, α-SMA expression increased and the collagen type III signal became in the weaker subepithelial area. Interestingly, bundles of aligned and uniformly spaced collagen fibrils were more prevalent in keratocytes in Y-27632-treated corneas, which is reminiscent of fibripositor-like structures that have been proposed as a mechanism of matrix deposition in embryonic connective tissues.

Conclusions: Y-27632 inhibits keratocyte-to-myofibroblast transition, and its topical application after a superficial lamellar keratectomy elicits an altered wound healing response, with evidence of an embryonic-type deposition of collagen fibrils.

Keratocytes are quiescent in mature healthy cornea, but after an injury or surgery, they differentiate into myofibroblasts and migrate to the wound site [1-4]. This phenotypic transformation is identified by the presence of microfilament bundles or stress fibers in myofibroblasts, which are associated with 1) the expression of α-smooth muscle actin (α-SMA) and 2) the spindle-like morphology of myofibroblasts compared to dendritic keratocytes [5-8]. The expression of α-SMA during corneal wound healing is important for cell migration and wound contraction [9]. However, the presence of excess numbers of myofibroblasts in wounded tissue is undesirable because of the risk of fibrotic scar formation. Thus, investigations into possible regulators of keratocyte-to-myofibroblast transformation offer significant scope for future intervention strategies for modulating wound healing in the cornea.

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A key factor in the keratocyte-to-myofibroblast transition is transforming growth factor β (TGFβ) [10-12]. *TGFβ1* mRNA and protein are present in the corneal epithelium and corneal stroma, and both paracrine and autocrine TGFβ1 response pathways are involved in the induction of keratocyte transformation [13-16]. Multiple signaling cascades are activated when TGFβ binds to its cognate receptor. These include Smad [17], RhoA-related signals [18], mitogen-activated protein kinase (MAPK)-Erk-1 and −2 [19], stress kinases (i.e., c-Jun N-terminal kinase [JNK]) [20,21], p38 mitogen-actiated protein kinase (p38MAPK) [22,23], phosphatase 2A [24], and phosphoinositide 3-kinase/AKT (PI3K/AKT) [25,26]. The pathways involved in cellular differentiation or transformation are Smad, Rho proteins, and PI3-kinase.

It is known that assembly and organization of actomyosin filaments to transform keratocytes into myofibroblasts are regulated by Rho GTPases. One of the downstream effectors of Rho is Rho-associated coiled-coil containing protein kinase (ROCK), which is a serine/threonin protein kinase that contains an NH_2-terminal catalytic kinase domain and plays an important role in the activation of actin/myosin interactions

and smooth muscle cell contraction by maintaining the activity of myosin light chain kinase (MLCK). Previous investigations showed that ROCK inhibitor (Y-27632) inhibited keratocyte fibrosis in vitro [27]. Other research has shown that Y-27632 has potential beneficial effects via its inhibition of apoptosis [28] and invasive carcinoma [29], the stimulation of cell proliferation in primate corneal endothelial cells [30], the suppression of kidney fibrosis [31], and the regulation of cell differentiation in embryonic stem cells [32]. In the current study, we focus on the Rho signaling pathway, which we attempted to block using a selective Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, Y-27632 [33], both in vitro and in vivo to suppress the differentiation of keratocytes into myofibroblasts and modulate cell-driven wound healing.

METHODS

Rabbit corneas and isolated cells were used as the model system for our study of wound healing [34,35].

Cell culture: Rabbit corneas were incubated with 1.2 U/ml Dispase (Life Technologies Japan Ltd, Tokyo, Japan) for 1 h at 37 °C, after which the corneal epithelium and endothelium were removed by mechanical scraping. The stroma was then cut into small, approximately 1 cm^2 pieces, which were incubated overnight at 37 °C in DMEM/F12 containing 1 mg/ml collagenaseA (Roche Diagnostics K.K., Tokyo, Japan) and 1% penicillin-streptomycin. After centrifugation at 440× g for 3 min, the cells were sub-cultured in serum-free medium (DMEM/F12 containing with 10 μg/ml insulin, 1 mM ascorbic acid, and 1% penicillin-streptomycin) for 48 h. They were then induced into myofibroblasts by TGFβ1 (3 ng/ml; R&D systems, Minneapolis, MN) with or without a 2 h pre-incubation with 10 μM Y-27632 (Wako, Osaka, Japan). After 48 h, cell phenotype was observed by phase contrast light microscopy (Leica CTR 4000; Leica Microsystems GmbH, Wetzlar, Hesse, Germany), and examined by immunofluorescence and western blotting for the myofibroblast marker α-SMA. To calculate the percentage of spindle-like cells, micrographs were taken at six different areas in each well. The total number of cells and the number of spindle-like cells was counted.

Immunohistochemistry for α-SMA: Cells were fixed by immersion in 4% paraformaldehyde for 10 min, after which they were washed three times with phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100, blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then incubated with α-SMA (1:400; Thermo Fisher Scientific K.K, Yokohama, Kanagawa, Japan) antibody or mouse immunoglobulin G 2a (IgG2a) isotype control for 2 h at room temperature. This was followed by incubation with AlexaFluor 488-conjugated secondary antibody (Invitrogen) in a 1:2000 dilution. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA).

Western blotting: Cells were washed with PBS and extracted in lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 0.15 M NaCl, 1% TritonX-100, pH 8.0) containing protease inhibitor and phosphate inhibitor. Lysed cells were centrifuged at 90× g for 10 min at 4 °C, after which the supernatant was collected and stored at −80 °C until required. Protein assay was performed using a BCA™ protein assay kit (Thermo Fisher Scientific) and protein concentration was measured at 562 nm. Equal amounts of protein were resolved by SDS–PAGE (4% to 12% tri-acetate mini gel; Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% skimmed milk dissolved in tris buffered saline Tween (TBS-T; 50mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20), before incubation overnight at 4 °C with α-SMA (1:1,000) and β-actin (1:3,000) primary antibody diluted in 1% skimmed milk. After the blots were washed with TBS-T, they were incubated with horseradish peroxidase conjugated secondary IgG (GE Healthcare, Bucks, UK). The reacted proteins were revealed by an enhanced chemiluminescence system (GE Healthcare).

Collagen gel contraction assays: Fibroblast-mediated gel contraction with or without Y-27632 was measured. Type I collagen gels (AteloCell®; Koken, Tokyo, Japan) were produced in the form of a viscous liquid as described previously [36] to achieve a final concentration of collagen of 1.9 mg/ml. These were seeded with keratocytes to a final cell density of 2×10^5 cells/ml, after which 0.25 ml of the resultant mixture was added to a 48-multiwell plate coated with 1% BSA. This was incubated for 1 h at 37 °C to induce gelation. Serum-free medium was then added to each well for 48 h followed by the addition of 30 ng/ml TGFβ1, with or without 100 μM Y-27632. The area of the collagen gels was measured every 24 h for three days using ImageJ software.

Surgical procedures: Four adult male rabbits (Japanese White) each weighing 2.5 kg to 3.0 kg underwent bilateral superficial keratectomies 7.5 mm in diameter and approximately 150 μm deep. At all times the animals were treated according to full ethical approval. A quarter turn with a BARRON radial vacuum trephine (Katena Products, Denville, NJ) was used to achieve approximately standard depth, with all surgeries conducted by the same surgeon. The keratectomy was achieved by a freehand lamellar dissection, and the thickness of the residual stromal bed was measured using a TOMEY ultrasonic pachymeter (Tomey Corporation, Nagoya, Japan). After surgery, topical antibacterial agent (0.3% ofloxacin eye drops) was applied. Postoperatively, Y-27632 (10 mM) eye drops were administered to the right eyes of all rabbits four times daily for three weeks, with vehicle only added to the left eyes, which acted as controls. Two non-operated rabbits also received this daily application of Y-27632 in one eye and vehicle in the other. Fluorescein staining was used to monitor epithelial healing.

Immunohistochemistry for matrix components: After three weeks of eye drop treatment, the animals were