Expression of Nod-like Receptors and Clinical Correlations in Patients With Dry Eye Disease

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• PURPOSE: To investigate the expression pattern of nucleotide-binding oligomerization domain (Nod)-like receptors that detects "danger" intracellular signaling and its correlation with clinical dry eye (DE) markers.

• DESIGN: Cross-sectional study.

• METHODS: A total of 50 participants with 50 eyes were included: 23 eyes with Sjögren syndrome (SS)-DE, 14 eyes with non-SS-DE, and 13 healthy controls with non-DE. Ocular Surface Disease Index (OSDI) was self-answered and clinical tests including the tear film breakup time (TBUT), Schirmer test, and corneal fluorescein staining (CFS) were performed. Specimens for expression pattern analysis were obtained by conjunctival impression cytology and biopsy. Nod-1, inhibitor kappa B kinase-alpha (IκKα), and nuclear factor kappa B (NF-κB) expression was determined by reverse transcription quantitative real-time polymerase chain reaction and Western blot. Correlations between Nod-1 and ocular surface parameters were determined.

• RESULTS: Patients with SS-DE had significantly higher OSDI and CFS scores and lower TBUT and Schirmer test scores than those with non-SS-DE patients (all $P < .05$). Compared with the control group, both the SS-DE and non-SS-DE groups showed significant upregulation in mRNA expression levels of Nod-1 (relative 3.48-fold and 1.72-fold upregulation, respectively, $P < .01$), IκKα (relative 1.83-fold and 1.24-fold upregulation, respectively, $P < .01$), and NF-κB (relative 1.84-fold and 1.32-fold upregulation, respectively, $P < .01$). Western blot analysis showed that Nod-1 protein expression increased in both the SS-DE and non-SS-DE groups (relative 2.71-fold and 1.64-fold upregulation, respectively, $P < .05$) compared with that in the control group. Similar findings were observed for IκKα and NF-κB. In DE participants, the expression of Nod-1 significantly correlated with the OSDI ($R^2 = 0.61$, $r = 0.78$, $P < .01$), Schirmer test score ($R^2 = 0.44$, $r = -0.66$, $P < .01$), and CFS ($R^2 = 0.46$, $r = 0.68$, $P < .01$) but did not significantly correlate with TBUT ($R^2 < 0.01$, $r = 0.08$, $P = .66$).

• CONCLUSIONS: Nod-1 expression was increased in the conjunctiva of DE, especially SS-DE, and was associated with disease severity. Expression of Nod-like receptors might play an important role in initiating the inflammatory response in DE. (Am J Ophthalmol 2019;200: 150–160. © 2019 Elsevier Inc. All rights reserved.)

DRY EYE (DE) IS ONE OF THE MOST COMMON ophthalmologic diseases that can potentially affect the ocular surface, resulting in a low quality of life. DE is considered to be a multifactorial disorder and although its pathogenesis has not been established, there is increasing evidence that ocular surface inflammation might play a key role.[1–4]

The discovery of pattern recognition molecules such as Toll-like receptors (TLRs), which detect microbial antigens and trigger protective immune responses, has had an enormous impact on the understanding of innate immune pathways.[5–7] Ocular expression of TLRs is known to be associated with ocular infection or allergic diseases.[8–12] Recent studies have demonstrated that the expression of TLRs in the corneal surface was increased in experimental and clinical DE and that TLRs participated in the inflammatory response to ocular surface desiccating stress.[13–17]

Whereas TLRs are transmembrane proteins with an extracellular or luminal binding domain, nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs) are intracellular cytosolic proteins that have recently been identified as key mediators of inflammatory and subsequent immune responses. Among NLRs, it has been shown that Nod-1 and Nod-2 mediate proinflammatory responses through nuclear factor kappa B (NF-κB) activation, detecting characteristic microbial products and danger signals.[18]

Few studies have investigated the role of NLRs in ocular surface diseases. Whereas the mRNA levels of Nod-2 are increased in corneas with herpes simplex keratitis, the expression of Nod-1 and Nod-2 transcripts are decreased in corneas with limbal stem cell deficiency.[19] In cases of

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Chlamydia trachomatis infections, Nod-1 signaling was found to contribute to the induction of corneal neovascularization, which could be suppressed by blocking Nod-1 signaling.[20] A recent study also reported that mRNA and protein expression of NLR pyrin domain–containing 3 (NLRP3) inflammasome was upregulated in human DE, suggesting the involvement of NLRP3 inflammasome in the onset and development of inflammatory DE.[21,22]

Although the role of TLRs and NLRs in ocular surface diseases has been widely investigated, the expression pattern of NLRs in DE has been rarely studied. Therefore, the purpose of this study was to investigate the expression pattern of NLRs and its correlation with ocular surface parameters in DE.

METHODS

THIS WAS A CROSS-SECTIONAL STUDY THAT WAS proceeded from March 2017 to July 2017 at the Department of Ophthalmology at Chonnam National University Hospital, Gwangju, South Korea. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of Chonnam National University Hospital. Written informed consent was obtained from all subjects following an explanation of the purpose and study requirements.

• **SUBJECTS:** A total of 50 participants (50 eyes; 23 eyes with Sjögren syndrome (SS)-DE, 14 eyes with non-SS-DE, and 13 age- and sex-matched non-DE subjects as healthy controls) were recruited. Since it is invasive to conduct conjunctival biopsy as well as conjunctival impression cytology (CIC) for both eyes of subjects including healthy controls, only their right eyes were included in the present study. All participants with DE met the following inclusion criteria: (1) were ≥19 years and <70 years of age, (2) were positive for DE symptoms (>3 months), (3) had a corneal fluorescein staining (CFS) score ≥1, (4) had a tear film breakup time (TBUT) ≤10 seconds, and (5) had a Schirmer test score ≤10 mm. SS-DE patients were selected based on the criteria of the American-European Consensus Group[23] and the American College of Rheumatology.[24] The exclusion criteria were as follows: (1) were <19 years and ≥70 years of age, (2) had systemic medications that could inhibit tear production, (3) had a previous history of punctal plug insertion, (4) used eye drops other than artificial tears, (5) had a previous history of contact lens use, (6) had undergone previous ocular surgery or trauma, and (7) suffered from other ocular and systemic diseases.

• **MEASUREMENT OF CLINICAL DRY EYE PARAMETERS:** The Ocular Surface Disease Index (OSDI) score, TBUT, Schirmer test score, and CFS were evaluated in all participants.

The OSDI questionnaire was used to quantify the detailed DE symptom score and includes the following subscales: (1) ocular symptoms (OSDI symptoms), (2) vision-related activities of daily living (OSDI visual function), and (3) environmental triggers (OSDI trigger).[25,26] In this study, the total OSDI score, which ranged from 0 to 100, was analyzed.[26]

TBUT was assessed by using a moistened fluorescein strip (Haag-Streit, Koeniz, Switzerland) and recording the time interval between the last complete blinking and the first appearance of a dry spot or disruption of the tear film. The examination was repeated 3 times, and the mean time was used for the analysis.[27] The Schirmer test score was measured by using a calibrated sterile strip (Color Bar Schirmer Tear Test, Eagle Vision Inc, Memphis, Tennessee, USA) with topical anesthesia (0.5% proparacaine chloride). The sterile strips were placed in the lateral canthus away from the cornea and left for 5 minutes with the eyes closed. Schirmer test scores were taken in millimeters of wetting for 5 minutes.[27]

The CFS was evaluated following the National Eye Institute/Industry Workshop system after using 1% preservative-free fluorescein[28] and was determined for 5 regions (center, temporal, nasal, superior, and inferior). Each region was recorded semi-quantitatively as 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe staining), and the total CFS (maximum of 15 points) was calculated as the sum of the scores of each region.

• **CONJUNCTIVAL IMPRESSION CYTOLOGY AND BIOPSY:** All subjects underwent both CIC and conjunctival biopsy, and each CIC sample was used for gene expression analysis and conjunctival biopsy for Western blot. CIC was performed as follows for the mRNA expression pattern analysis of Nod-1 and downstream mediators. After topical anesthesia with 0.5% proparacaine hydrochloride, strips of cellulose acetate filter paper (MFS membrane filters; Advantec MFS, Dublin, California, USA), 6.2 mm in diameter with 0.45-μm pores (Millipore, Bedford, Massachusetts, USA) were applied, dull side down, to the lower nasal bulbar conjunctiva adjacent to the corneal limbus. The filter strips were pressed gently with blunt and smooth-tipped forceps for 2-3 seconds and then removed. The filter paper collected from the right eye was transferred immediately to a 1.5 mL Eppendorf tube containing 1 mL of RNA stabilization reagent (QIAGEN, Hilden, Germany) for mRNA expression analysis. In addition, a 2 × 3-mm-sized conjunctival biopsy was performed at the lower temporal bulbar conjunctiva for the analysis of protein expression of Nod-1 and downstream mediators. This biopsy sample was placed in an empty 1.5 mL Eppendorf tube for Western blotting. All samples were immediately placed on ice until transferred to -80 C for storage.[21,29–31]

• **mRNA MEASUREMENT BY REVERSE TRANSCRIPTION-QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION:** A tube containing 1 mL of RNA stabilization reagent and CIC sample was thawed at room temperature

TABLE 1. Primer Sequences for Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction of Target Genes

Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Nod-1	ACATCCGCAATACTCAGTGTCTG	ACGCTTTCTCTGAGTGAGCA
NF-κB p65	ACATGGTGGTCGGCTTCGCA	TGCAGAGCTGCTTGGCGGAT
IκKα	TGATCACCAACCAGCCAGAA	TCTCGGAGCTCAGGATCACA
GAPDH	CCAAGGTCATCCATGACAACTTTG	GTCATACCAGGAAATGAGCTTGACA

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IκKα = inhibitor kappa B kinase α; NF-κB = nuclear factor kappa B; Nod-1 = nucleotide-binding and oligomerization domain-containing protein 1.

TABLE 2. Demographic Data, Symptoms, and Ocular Surface Parameters

	Group 1 (N = 23) (SS-DE)	Group 2 (N = 14) (Non-SS-DE)	Group 3 (N = 13) (Non-DE)	*P* Value[a]
Age, y	59.5 ± 9.1	59.2 ± 6.7	58.1 ± 13.8	.93
Sex, M/F	0/23	0/14	0/13	1.00
BCVA, logMAR	0.01 ± 0.10	0.01 ± 0.09	0.00 ± 0.08	.95
OSDI, total	52.7 ± 15.7	21.0 ± 2.7	7.5 ± 1.4	<.01
TBUT, s	3.4 ± 0.8	5.4 ± 1.1	10.6 ± 2.0	<.01
Schirmer test score, mm/5 min	4.3 ± 0.9	7.3 ± 0.9	12.1 ± 1.3	<.01
CFS, score	10.3 ± 2.5	6.9 ± 1.9	0.4 ± 0.5	<.01

BCVA = best-corrected visual acuity; CFS = corneal fluorescein staining; DE = dry eye; logMAR = logarithm of minimal angle of resolution; OSDI = ocular surface disease index; SS = Sjögren syndrome; TBUT = tear film breakup time.

All continuous variables are presented as mean ± standard deviation.

[a]One-way analysis of variance, Scheffée post hoc multiple comparison; $P < .05$, statistically significant.

and then vortexed for 2 minutes. Total RNA extraction was performed using the RNeasy Mini Kit according to the manufacturer's protocol. The final isolation step was performed with 35 μL RNase-free water. Single-stranded cDNA was reverse-transcribed from 500 ng of total RNA with the Omniscript RT kit (QIAGEN) and subjected to polymerase chain reaction (PCR) with the HiPi PCR Pre-Mix (ELPIS, Daejeon, Korea).

This study focused on the Nod-1-induced NF-κB activation pathway, not on the inflammasome-caspase-interleukin pathway, as has been done elsewhere.[32] Therefore, we evaluated the mRNA expression of Nod-1 and downstream inflammatory mediators including inhibitor kappa B kinase-alpha (IκKα) and NF-κB p65 with the use of a Rotor-Gene 3000 real-time system (Corbett Research, Cambridge, UK). The thermal cycling conditions were as follows: 15 minutes at 95 C, followed by 40 cycles of 95 C for 10 seconds, 55∼60 C for 20 seconds, and 72 C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase was used as a housekeeping gene for the analysis. The primer sequences for gene amplification by quantitative PCR are provided in Table 1. Collected data were analyzed and relative expression was quantified using the comparative CT method ($2^{-\Delta\Delta CT}$).

• **WESTERN BLOT ANALYSIS:** Expression levels of Nod-1, IκKα, and NF-κB p65 proteins were determined by Western blotting. Conjunctival biopsy samples were lysed with radio-immunoprecipitation assay buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate) on ice for 1 hour. The lysates were centrifuged at 12 000 rpm at 4 C for 10 minutes to obtain the supernatant. To each 60-μL cell sample, protein loading buffer was added and degenerated in a heating block for 5 minutes, after which the proteins were isolated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (0.45 μm; Millipore).

The following primary antibodies were used; anti-β-actin (ab95437), anti-CARD4 antibody (anti-Nod-1, ab170547), anti-IκKα (ab38515), and anti-NF-κB p65 (ab207297) (Abcam, Cambridge, Massachusetts, USA). The membrane was blocked in 2% bovine serum albumin (BSA)for 1 hour at room temperature and incubated with the specific primary antibodies (diluted 1:100) overnight at 4 C. After washing with phosphate buffer solution, anti-rabbit horseradish peroxidase–conjugated secondary antibodies (diluted 1:10 000 in 2% BSA) were used as probing markers. β-actin protein level was measured at the same

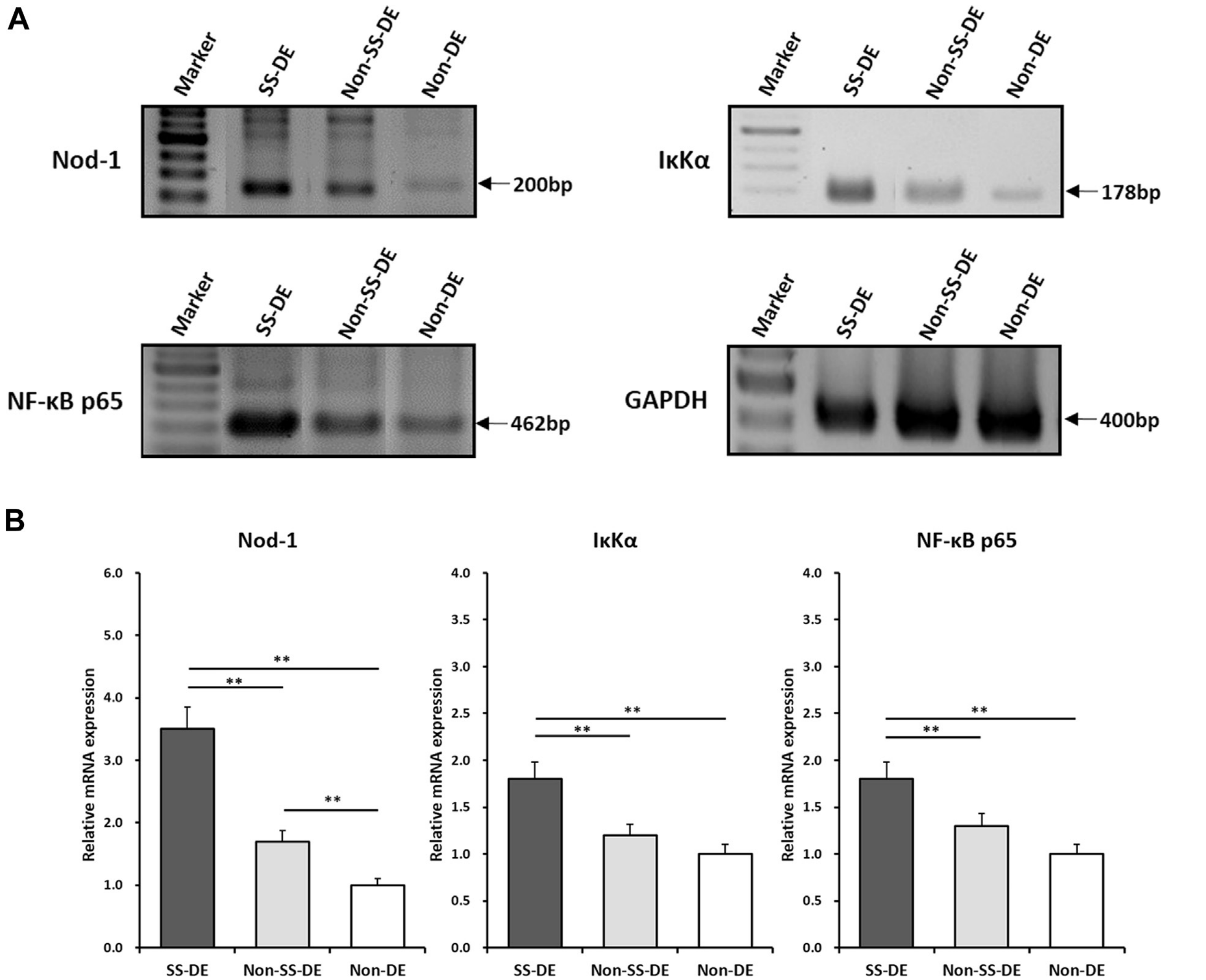

FIGURE 1. Reverse-transcription quantitative real-time PCR (A) and mRNA expression of Nod-1, IκKα, and NF-κB p65 (B) in the conjunctival impression cytology samples of study subjects. Significant upregulation of Nod-1, IκKα, and NF-κB p65 was shown in both the Sjögren syndrome dry eye (SS-DE) (n = 23) and non-SS-DE (n = 14) groups compared with that in the non-DE (n = 13) group. Additionally, mRNA expression of Nod-1, IκKα, and NF-κB p65 was significantly upregulated in the SS-DE group compared with that in the non-SS-DE group (**$P < .01$, compared with the groups).

time as an endogenous control. The results were quantified by analyzing for grayscale characteristics using the image analysis software ImageJ (Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA).

• **STATISTICAL ANALYSIS:** Statistical analysis was performed using SPSS 18.0 for Windows (SPSS Inc, Chicago, Illinois, USA) and Excel (Microsoft Corporation, Santa Rosa, California, USA). The normal distribution for all continuous variables was assessed using the Kolmogorov-Smirnov test. Data are presented as mean ± standard deviation. Differences between groups for continuous variables were assessed using the 1-way analysis of variance test and the Scheffée post hoc multiple-comparison method. Differences between groups for categorical variables were assessed using the χ^2 tests. Pearson correlation coefficient analysis was used to analyze the correlation between Nod-1 expression and DE indices. A P value of less than .05 was considered to have statistical significance.

RESULTS

• **DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF SUBJECTS:** A total of 50 participants were enrolled in this study. Demographic and ophthalmologic characteristics of the subjects are summarized in Table 2. Significant differences were found in total OSDI, TBUT, Schirmer test score, and CFS score among the 3 groups (all $P < .05$).

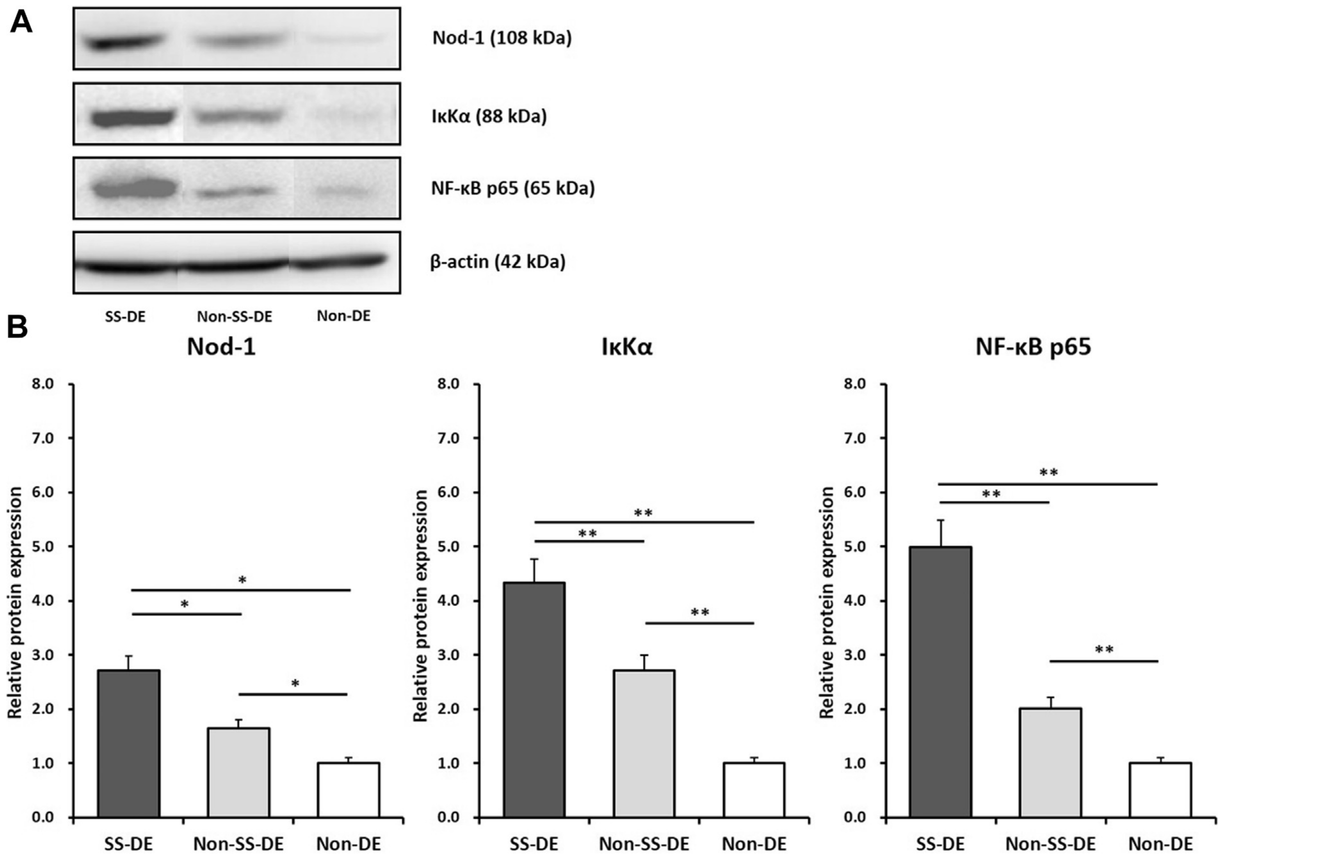

FIGURE 2. Western blot (A) and densitometric analysis of Nod-1 (108 kDa), IκKα (88 kDa), and NF-κB p65 (65 kDa) protein expression in the conjunctiva of study subjects (B). Significant upregulation of Nod-1, IκKα, and NF-κB p65 protein was shown in both the Sjögren syndrome dry eye (SS-DE) (n = 23) and non-SS-DE (n = 14) groups compared with the non-DE (n = 13) group. Additionally, relative protein expression of Nod-1, IκKα, and NF-κB p65 was significantly upregulated in the SS-DE group compared with that in the non-SS-DE group ($P < .01$, *$P < .05$, compared with the groups).**

• **mRNA EXPRESSION FROM CONJUNCTIVAL IMPRESSION CYTOLOGY SAMPLES OF THE SUBJECTS:** Relative mRNA expression of Nod-1, IκKα, and NF-κB p65 was acquired using reverse transcription quantitative real-time polymerase chain reaction (Figure 1). Nod-1 mRNA expression levels were significantly higher in the SS-DE (relative 3.48-fold upregulation, $P < .01$) and non-SS-DE (relative 1.72-fold upregulation, $P < .01$) groups than in the non-DE group. In addition, relative Nod-1 mRNA expression was significantly higher in the SS-DE group than in the non-SS-DE group ($P < .01$).

The mRNA expression of IκKα was significantly upregulated in the SS-DE (relative 1.83-fold upregulation, $P < .01$) and non-SS-DE (relative 1.24-fold upregulation, $P = .03$) groups than in the non-DE group. There was a significant mRNA upregulation of IκKα in the SS-DE group compared with that in the non-SS-DE group ($P < .01$).

NF-κB p65 mRNA expression also demonstrated significant upregulation in the SS-DE (relative 1.84-fold upregulation, $P < .01$) and non-SS-DE (relative 1.32-fold upregulation, $P = .02$) groups compared with that in the non-DE group. Relative mRNA expression of NF-κB p65 showed significant upregulation in the SS-DE group compared with that in the non-SS-DE group ($P < .01$).

• **PROTEIN EXPRESSION AND DENSITOMETRIC ANALYSIS FROM THE CONJUNCTIVA SAMPLES OF THE SUBJECTS:** Western blot and densitometric analyses of Nod-1, IκKα, and NF-κB p65 are shown in Figure 2. Nod-1 protein expression levels in both the SS-DE and non-SS-DE groups showed a significant increase in comparison with the non-DE group, with 2.71-fold ($P = .01$) and 1.64-fold ($P = .04$) upregulations, respectively. The SS-DE group also had significantly higher Nod-1 expression than the non-SS-DE group ($P = .02$).

IκKα protein expression was significantly upregulated in both the SS-DE and non-SS-DE groups in comparison with the non-DE group, with upregulation levels of 4.33-fold ($P < .01$) and 2.72-fold ($P < .01$), respectively. The SS-DE group had higher IκKα protein expression than the non-SS-DE group ($P < .01$).

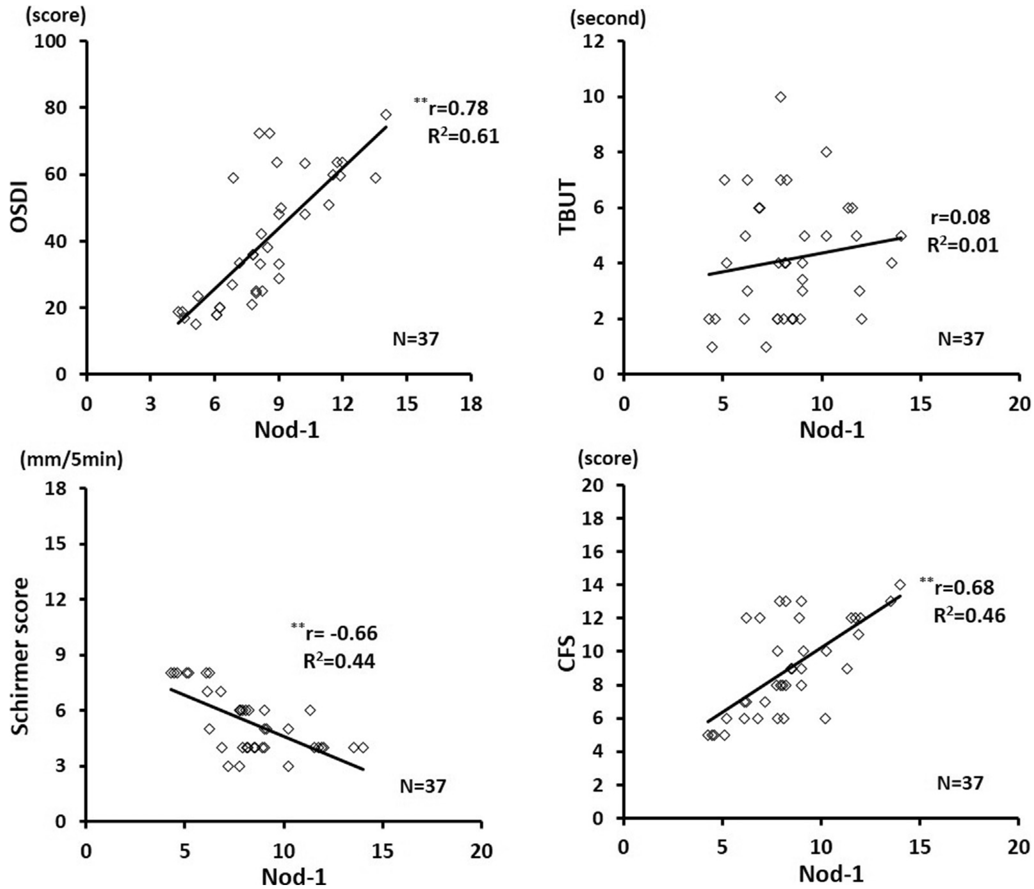

FIGURE 3. Correlations between Nod-1 protein expression and ocular surface parameters in all dry eye patients (n = 37). The level of Nod-1 expression showed positive correlations with the Ocular Surface Disease Index (OSDI) and corneal fluorescein staining (CFS) score. A negative correlation was observed between the Nod-1 expression and the Schirmer test score ($P < .01$, *$P < .05$, Pearson correlation coefficient).**

NF-κB p65 protein expression was significantly upregulated in both the SS-DE and non-SS-DE groups in comparison with the non-DE group, with increases of 4.99-fold ($P < .01$) and 2.01-fold ($P < .01$), respectively. Higher expression levels of NF-κB p65 protein were found in the SS-DE group than in the non-SS-DE group ($P < .01$).

• **CORRELATIONS BETWEEN NOD-1 EXPRESSION AND OCULAR SURFACE PARAMETERS IN DRY EYE:** Quantified data of Nod-1 protein expression levels from Western blots were used to investigate the correlations between Nod-1 expression and each ocular surface parameter (Figures 3-5). Among DE participants (n = 37), the level of Nod-1 expression correlated significantly with the OSDI score ($R^2 = 0.61$, r = 0.78, $P < .01$), the Schirmer test score ($R^2 = 0.44$, r = -0.66, $P < .01$), and the CFS ($R^2 = 0.46$, r = 0.68, $P < .01$) (Figure 3).

We also evaluated correlations in the SS-DE group (n = 23) and found the OSDI score ($R^2 = 0.31$, r = 0.55, $P < .01$) and CFS ($R^2 = 0.19$, r = 0.44, $P = .04$) to be significantly correlated with the level of Nod-1 expression (Figure 4). In addition, in the group of non-SS-DE (n = 14), we observed significant correlations between Nod-1 expression and the OSDI score ($R^2 = 0.58$, r = 0.76, $P < .01$), Schirmer test score ($R^2 = 0.57$, r = -0.75, $P < .01$), and the CFS ($R^2 = 0.34$, r = 0.58, $P = .03$) (Figure 5). The correlations of IκKα and NF-κB p65 protein expression with OSDI, TBUT, Schirmer score and CFS were shown in the Supplemental Table (Supplemental Material available at AJO.com).

DISCUSSION

OCULAR SURFACE INFLAMMATION IS NOW CONSIDERED TO be the main pathophysiological factor in the development of DE. Hallmarks of inflammation are observed in DE disease, as evidenced by increased proinflammatory cytokines in the tear film,[2,33–36] upregulation of mitogen-activated protein kinase pathways on the ocular surface,[37] induced Th1-type immune response expressing high levels of CXCR3

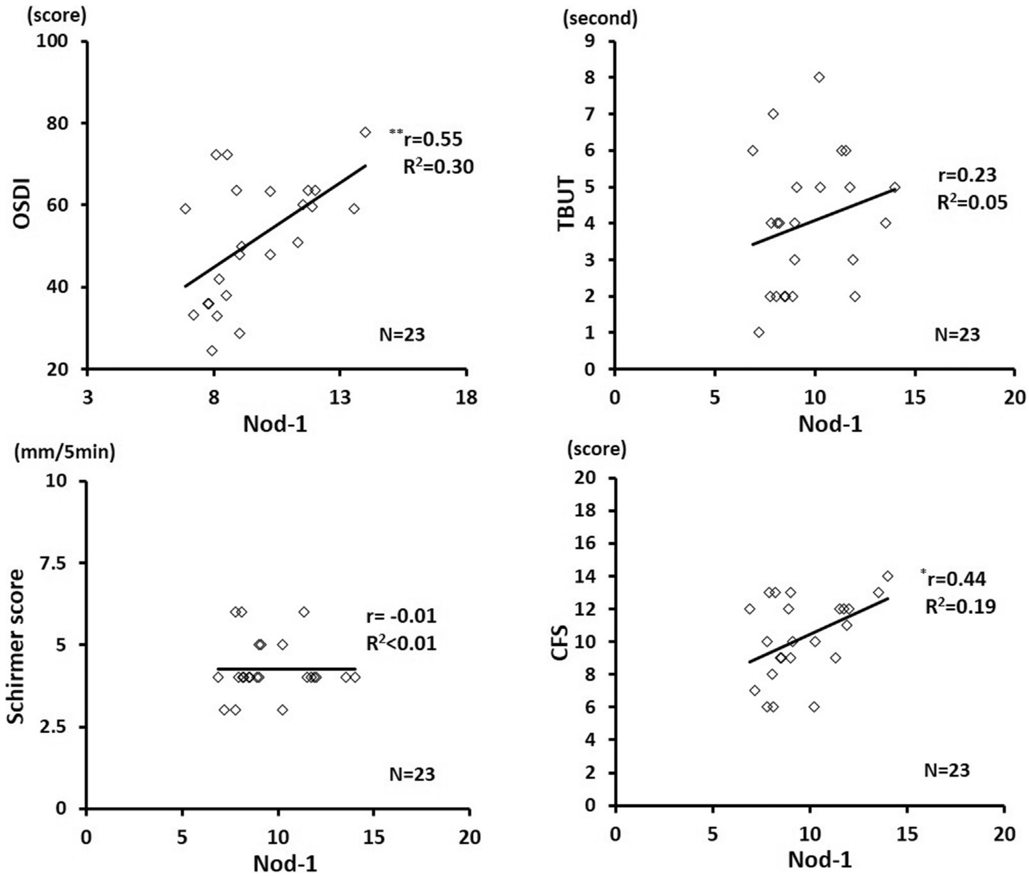

FIGURE 4. Correlations between Nod-1 protein expression and ocular surface parameters in dry eye patients with Sjögren syndrome (n = 23). Nod-1 expression was found to have a significant positive correlation with the Ocular Surface Disease Index (OSDI) and corneal fluorescein staining (CFS) score ($P < .01$, *$P < .05$, Pearson coefficient correlation).**

and CCR5,[1,38,39] elevated expression of lipid peroxidation markers (4-hydroxy-2-nonenal and malondialdehyde),[29] and epithelial apoptosis by leukocyte infiltration.[40,41]

Recently, the importance of innate immunity and its balance with adaptive immunity in the pathogenesis of DE have been highlighted. TLR and NLR have been demonstrated to play a key role in triggering the innate immune system, which results in the production of proinflammatory cytokines and chemokines.[42] Among NLRs, Nod-1 promotes ocular inflammation in a dose- and time-dependent manner.[43] Similar to other mucosal barriers, the epithelial NF-κB pathway acts as a pivotal regulator of downstream immune reactions in several corneal and conjunctival diseases, including DE.[44–48] Particularly, chlamydial ocular infections activate NF-κB through Nod-1.[49] In addition, it has been reported that the canonical Nod-1-IκKα (also called NF-κB essential modulator)-NF-κB axis contributes to initiating the innate immune cascade.[32] However, the role of Nod-1 in the pathogenesis of DE is still unclear.

We hypothesized that Nod-1-associated innate immune pathways might be involved in the development of DE. Therefore, we aimed to evaluate the expression of Nod-1 and its downstream inflammatory mediators, IκKα and NF-κB, and determine any correlations between Nod-1 expression and ocular surface parameters in patients with DE.

Our results showed a significant upregulation of Nod-1 mRNA and protein expression in SS-DE and non-SS-DE patients compared with that in controls. In addition, Nod-1 mRNA and protein expression was significantly higher in SS-DE patients than in non-SS-DE patients. These results imply that Nod-1 may be associated with ocular surface inflammation in the development of DE. Our results also showed that the TBUT, Schirmer test score, and CFS were significantly worse in SS-DE patients than in non-SS-DE patients and healthy controls, indicating that the level of Nod-1 expression is related to the severity of ocular surface inflammation. According to a study by Niu and associates,[21] the expression of the NLRP3 inflammasome and its downstream inflammatory factors caspase-1, IL-1β, and IL-18 were upregulated in the tears and ocular surface of DE patients. These findings are consistent with our results in that the increased expression of NLR was observed in the ocular surface of DE.

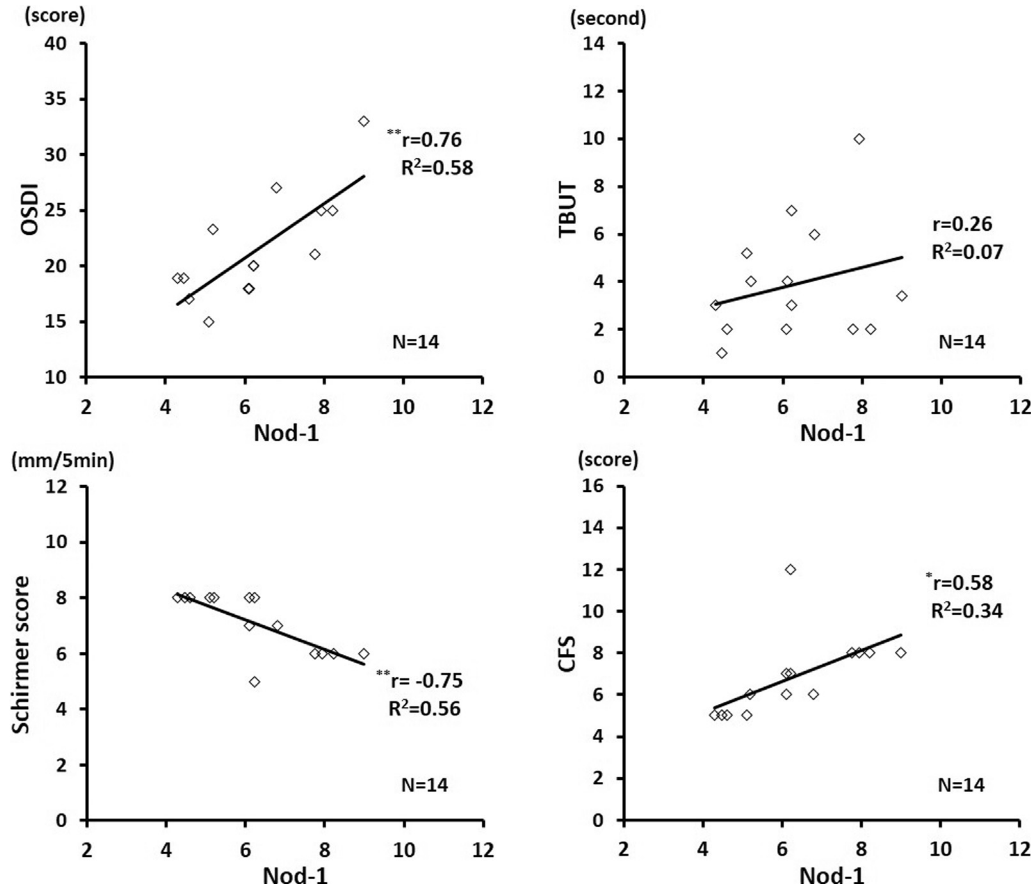

FIGURE 5. Correlations between Nod-1 protein expression and ocular surface parameters in dry eye patients without Sjögren syndrome (n = 14). Nod-1 expression had a significant positive correlation with the Ocular Surface Disease Index (OSDI) and corneal fluorescein staining (CFS) score, and a negative correlation with the Schirmer test score ($P < .01$, *$P < .05$, Pearson correlation coefficient).**

It has been reported that suppression of IκBζ (a negative regulator of NF-κB) in a murine model resulted in a marked inflammatory phenotype with loss of goblet cells and CD4+ cell infiltration.[50] In the circumstances of desiccating stress, topical inhibition of NF-κB activity markedly ameliorates DE signs, suggesting the important role of the NF-κB pathway in DE progression.[51] In addition, Peng and associates[52] demonstrated that IκBα-mediated feedback regulation of NF-κB has an essential role in controlling T-cell development in a murine SS model. In the present study, we confirmed that expression of NF-κB and IκKα was upregulated in the SS-DE and non-SS-DE groups compared with that in the control group and that the SS-DE group showed higher expression levels of NF-κB and IκKα than the non-SS-DE group. These findings suggest that increased IκKα and NF-κB expression levels might be regulated by Nod-1 in DE.

The activation of the innate immune system results in antigen presentation, T-cell activation, and the release of proinflammatory cytokines, consequently activating the adaptive immune system. Strikingly, there is increasing evidence that DE is a localized self-antigen-driven autoimmune disease.[4] Although no known autoantibodies are known to be involved in DE, our results confirm the upregulation of Nod-1 in the ocular surface of DE. Interestingly, chemical inhibitors of Nod-1 were described in previous studies.[53,54] Suppression of Nod-1 deactivates not only NF-κB, but also IFN-γ, which are key mediators of balancing the innate and adaptive immune systems. Taken together, changes in the expression of innate immune molecules such as Nod-1 in DE may represent a compromised innate immune system, causing a subsequent imbalance in the adaptive immune response.

NLR dysfunction has been described in a variety of diseases, including chronic inflammation, autoimmune diseases, and cancers.[18,55] For instance, polymorphisms of Nod-1 gene may be associated with several types of malignancy, including gastric, ovarian, prostate, and lung cancer as well as lymphoma.[56] To our knowledge, this is the first study to investigate the correlation between Nod-1 expression and ocular surface parameters in DE. We found that the OSDI and CFS had a significant positive correlation with Nod-1 expression in each subject group, indicating that the level of Nod-1 expression is associated with the severity of ocular inflammation and with the grade of DE symptoms. Importantly, the Schirmer test score showed a significant negative correlation with Nod-1 in both the DE and non-SS-DE groups, but no correlation with Nod-1 in the SS-DE group. This result could be owing to the severe chronic inflammation of the conjunctiva and lacrimal glands of SS-DE,[57–59] resulting in very low tear production regardless of Nod-1 expression levels. In addition, correlation coefficient values of the SS-DE group were lower than those of the non-SS-DE group. This appears to be owing to more wide distribution of ocular parameters in the non-SS-DE group than in the SS-DE group, and the ocular parameters of the SS-DE group being concentrated on the severe side than the non-SS-DE group. In other words, we speculate that SS-DE is a more severe, chronic autoimmune, and adaptive immune-biased disorder that results from an imbalance in innate immunity than non-SS-DE. Another interesting finding is that there were no significant correlations between Nod-1 expression levels, meaning innate immunity, and TBUT, indicating tear film instability, in all groups. This result indirectly suggests that there are 2 different axes, ocular surface inflammation and tear film instability, in the pathogenesis of DE.[60,61]

The current study has several limitations. First, the sample size was small. Second, the study only showed a parallel increase in both Nod-1 and IκKα and NF-κB p65. According to Kim and associates,[32] we hypothesized that Nod-1 induces IκKα and NF-κB expression. Since many pathways can upregulate IκKα-NF-κB p65, and gene knockout study to demonstrate that Nod-1 directly induces IκKα and NF-κB p65 expression could not be applied to human subjects, so it is necessary to pay attention to interpretation. Third, both CIC membranes and conjunctival biopsy specimens might contain conjunctival epithelial cells as well as immune cells. Therefore, when interpreting our results, it should be noted that individual contributions of each cell type cannot be separated. Despite these limitations, it is meaningful that this study sheds new light on the pathogenesis of DE.

In conclusion, based on CIC and conjunctival biopsy samples, our findings showed that the expressions of Nod-1, IκKα, and NF-κB p65 are upregulated in DE patients, especially in SS-DE. The level of Nod-1 expression was significantly correlated with DE symptom grade and ocular surface inflammatory parameters. These findings suggest the involvement of Nod-1 in the development of ocular surface inflammatory response in DE. Understanding the expression pattern of NLRs including Nod-1 in DE may elucidate its pathophysiology and lead to the development of NLR-targeted therapies in DE. Although experimental Nod-1 inhibitors have been shown to predispose a knockout mouse model to adverse events related to infection risk,[62,63] pharmacologic Nod-1 inhibitory agents only reduce but rarely totally suppress their targets, providing the possibility that Nod-1 inhibition might be a safe therapeutic strategy.[64] Therefore, future studies should focus on the regulatory mechanisms of Nod-1 in ocular surface inflammation, the homeostasis between NLRs and inflammatory cytokines of the adaptive immune response in DE, the role of other NLRs other than Nod-1 in DE, and whether inhibition of NLRs can be a viable therapeutic target in DE.

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