Evaluation of Tear Protein Markers in Dry Eye Disease with Different Lymphotoxin-Alpha Expression Levels

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• PURPOSE: To compare tear protein markers between normal subjects and patients with dry eye (DE) and high and low lymphotoxin-alpha (LT-α) levels.
• DESIGN: Prospective cross-sectional study.
• METHODS: Patients with DE were divided into low (≤700 pg/mL) and high (>700 pg/mL) LT-α groups. Twelve protein markers were measured by microsphere-based immunoassay and ocular surface parameters were determined in right eyes (33 high LT-α DE, 27 low LT-α DE, and 20 control eyes) and left eyes (21 high LT-α DE, 39 low LT-α DE, and 20 control eyes).
• RESULTS: In both eyes, tumor necrosis factor–α (TNF-α), interleukin (IL)-10, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-17A, and IL-12/23 p40 levels in high LT-α DE were significantly higher ($P < .01$) than in low LT-α DE. Significant correlations identified in high LT-α DE were: Standard Patient Evaluation Eye Dryness with IL-10 ($R = 0.43$, $P = .013$), IL-1β ($R = 0.48$, $P = .005$), and IL-12/23 p40 ($R = 0.50$, $P = .003$), IL-12/23 p40 with ocular surface disease index ($R = 0.35$, $P = .049$), and epidermal growth factor with corneal fluorescein staining score ($R = -0.36$, $P = .038$). Significant correlations in low LT-α DE were: Standard Patient Evaluation Eye Dryness with IL-10 ($R = -0.39$, $P = .046$), TNF-α ($R = -0.39$, $P = .047$), and IL-17A ($R = -0.48$, $P = .013$), ocular surface disease index with TNF-α ($R = -0.47$, $P = .017$) and IL-17A ($R = -0.46$, $P = .018$), and IL-6 with tear breakup time ($R = -0.40$, $P = .044$). Lastly, IL-1Ra levels significantly increased in DE patients, positively correlated with temporal conjunctival hyperemia index, and negatively correlated with Schirmer I test ($P < .05$).
• CONCLUSIONS: Our study identified tear IL-1Ra level as a potential biomarker to replace the Schirmer I test. Multiple tear protein marker levels increased in high LT-α DE, indicating that high LT-α DE might have a different pathogenesis. (Am J Ophthalmol 2020;217:198–211. © 2020 Elsevier Inc. All rights reserved.)

DRY EYE (DE) IS A MULTIFACTORIAL PROPERTY OF A disease in which the main pathophysiology is the loss of tear film homeostasis. Tear film instability, hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities are key etiologies that lead to the characteristic ocular symptoms.[1] The discomfort, irritation, and blurred vision that patients with DE often experience can interfere with daily activities and work-related tasks, leading to a diminished quality of life.[2] There is increasing evidence that inflammation is involved in the pathophysiology of DE disease (DED); studies have identified inflammatory-related cytokines and other tear protein markers in the ocular surface of patients with DED.[3–5] Increased osmotic pressure in tears is one of the factors that can directly cause ocular surface inflammation, which can lead to abnormalities in tear components, such as an increase in inflammatory factors.[6]

Lymphotoxin-alpha (LT-α), a member of the tumor necrosis factor (TNF) superfamily, is expressed by a variety of cells, including T cells, B cells, and natural killer cells. It has a specific role in the development and function of the immune system, especially in the development of lymphoid organs, the organization and maintenance of lymphatic microenvironments, host defense, and inflammation.[7] While an increasing body of research has shown that LT-α plays an important role in inflammation,[8–10] the published literature is also conflicting. For example, a British study of fatigue symptoms that were central to a recognized proinflammatory mechanism found a negative correlation between blood LT-α levels and fatigue in patients with primary Sjögren syndrome (SS).[11] Therefore, we speculate that LT-α may not simply promote inflammation as most studies have suggested, but in fact may have a more complex mechanism. Using big data analysis, the Seinda Biomedical Corporation found that the expression level of LT-α in the tears of most DE patients was <700 pg/mL; however, some DE patients had levels >700 pg/mL. The pathogenesis of DE is still unclear and there is a lack of research on the relationship between LT-α and DE. Based on this result, we investigated the differences in tear protein markers and ocular

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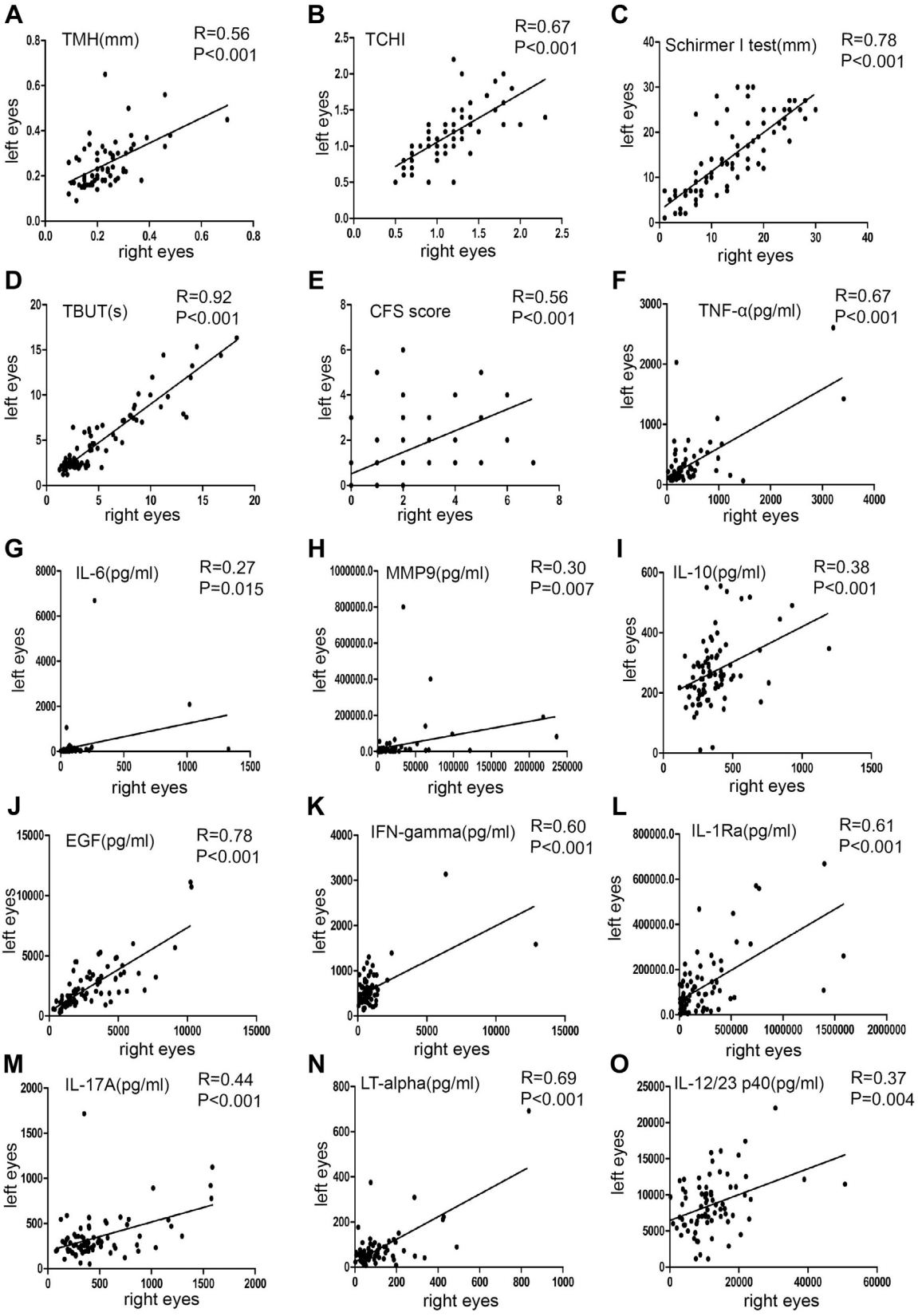

FIGURE 1. Correlation analysis between the right and left eyes of protein markers and clinical characteristics based on all study subjects. (A) Tear meniscus height (TMH). (B) Temporal conjunctival hyperemia index (TCHI). (C) Schirmer I test. (D) Tear breakup time (TBUT). (E) Corneal fluorescein staining (CFS). (F) Tumor necrosis factor α (TNF-α). (G) Interleukin-6 (IL-6). (H) Matrix

TABLE 1. Demographics and Clinical Characteristics of the High Lymphotoxin-Alpha Dry Eye Patients, Low Lymphotoxin-Alpha Dry Eye Patients, and Normal Subjects in Right Eyes

				P Value		
Characteristics in Right Eyes	A1	B1	C1	A1 vs B1	A1 vs C1	B1 vs C1
Eyes, n	34	27	20			
Mean age, years ± SD	50.67 ± 14.77	46.59 ± 15.28	44.1 ± 13.44	.302	.104	.556
SPEED score (0-12) ± SD	4.94 ± 3.71	5.81 ± 3.55	1.7 ± 2.08	.356	.0002	<.0001
OSDI score (0-100) ± SD	39.72 ± 16.14	6.36 ± 15.92	3.91 ± 4.71	.422	<.0001	<.0001
CFS score (0-12) ± SD	1.82 ± 1.53	2.44 ± 1.91	0	.174	<.0001	<.0001
TBUT, seconds ± SD	3.34 ± 1.67	3.55 ± 1.73	11.16 ± 3.21	.645	<.0001	<.0001
Schirmer I test, (mm/5 min) ± SD	11.76 ± 6.67	12.44 ± 8.02	17.8 ± 6.83	.72	.003	.017
TMH, mm ± SD	0.20 ± 0.06	0.24 ± 0.13	0.28 ± 0.10	.202	.03	.316
TCHI score (0-4) ± SD	1.28 ± 0.31	1.09 ± 0.42	1.03 ± 0.26	.072	.016	.604

A1 = high lymphotoxin-alpha dry eye in right eye group; B1 = low lymphotoxin-alpha dry eye in right eye group; C1 = control subjects in right eye group; CFS = corneal fluorescein staining; OSDI = Ocular Surface Disease Index (grade estimated by the Oxford scheme); SD = standard deviation; SPEED = Standard Patient Evaluation Eye Dryness; TBUT = tear breakup time; TCHI = temporal conjunctival hyperemia index; TMH = tear meniscus height.

TABLE 2. Demographics and Clinical Characteristics of the High Lymphotoxin-Alpha Dry Eye Patients, Low Lymphotoxin-Alpha Dry Eye Patients, and Normal Subjects in Left Eyes

				P Value		
Characteristics in Right Eyes	A2	B2	C2	A2 vs B2	A2 vs C2	B2 vs C2
Eyes, n	21	39	20			
Mean age, years ± SD	49.33 ± 15.03	48.56 ± 15.19	44.10 ± 13.44	.852	.247	.255
SPEED score (0-12) ± SD	4.90 ± 3.40	5.56 ± 3.78	1.70 ± 2.08	.495	.0009	<.0001
OSDI score (0-100) ± SD	37.72 ± 17.77	38.48 ± 15.19	3.91 ± 4.71	.869	<.0001	<.0001
CFS score (0-12) ± SD	11.24 ± 7.91	14.79 ± 8.97	16.65 ± 0.26	.12	.022	.370
TBUT, seconds ± SD	3.31 ± 2.00	3.26 ± 1.55	10.24 ± 3.07	.919	<.0001	<.0001
Schirmer I test, (mm/5 min) ± SD	1.43 ± 1.57	1.82 ± 1.48	0	.353	<.0001	<.0001
TMH, mm ± SD	0.22 ± 0.07	0.26 ± 0.12	0.28 ± 0.09	.188	.094	.544
TCHI score (0-4) ± SD	1.21 ± 0.45	1.16 ± 0.34	1.09 ± 0.26	.698	.355	.439

A2 = high lymphotoxin-alpha dry eye in right eye group; B2 = low lymphotoxin-alpha dry eye in right eye group; C2 = control subjects in right eye group; CFS = corneal fluorescein staining; OSDI = Ocular Surface Disease Index (grade estimated by the Oxford scheme); SD = standard deviation; SPEED = Standard Patient Evaluation Eye Dryness; TBUT = tear breakup time; TCHI = temporal conjunctival hyperemia index; TMH = tear meniscus height.

surface parameters in DE patients with different LT-α expression levels.

In addition, epidermal growth factor (EGF), interleukin-1 receptor antagonist (IL-1Ra), IL-1β, IL-17A, IL -6, IL-8, IL- 10, IL-12/23 p40, matrix metalloproteinase-9, TNF-α, and interferon-gamma (IFN-γ) in tears, and their clinical correlations, were analyzed to explore potential biomarkers for diagnosis of DE. The results of the present study may deepen our understanding of ocular surface features of DE with different levels of LT-α and normal subjects.

METHODS

THIS PROSPECTIVE CROSS-SECTIONAL STUDY WAS approved by the Institutional Review Board (2018-YK-004) of Southern Medical University. Informed consent was obtained from all subjects. The study was registered at http://www.chictr.org.cn (registry number ChiCTR2000029423).

• **SUBJECTS:** Patients with DE were divided into low (LT-α ≤ 700 pg/mL) and high (LT-α >700 pg/mL) LT-α groups.

metalloproteinase-9 (MMP9). (I) IL-10. (J) Epidermal growth factor (EGF). (K) Interferon-gamma (IFN-gamma). (L) IL-1 receptor antagonist (IL-1Ra). (M) IL-17. (N) Lymphotoxin-alpha (LT-α). (O) IL-12/23 p40.

TABLE 3. Levels of Tear Protein Markers of the Right Eye in High Lymphotoxin-Alpha Dry Eye Patients, Low Lymphotoxin-Alpha Dry Eye Patients, and Normal Subjects

				P Value		
Cytokine, pg/mL	A1	B1	C1	A1 vs B1	A1 vs C1	B1 vs C1
Tumor necrosis factor α	594.47 ± 600.07	156.24 ± 58.59	388.82 ± 699.40	.0002	.282	.154
Interleukin-6	132.78 ± 221.96	54.30 ± 34.15	100.79 ± 221.69	.053	.614	.364
Matrix metalloproteinase-9	33,374.43 ± 46,345.81	6876.97 ± 8886.07	18,969.88 ± 49,373.06	.003	.299	.292
Interleukin-8	2117.20 ± 3953.19	833.28 ± 563.89	2728.45 ± 8106.913	.074	.755	.310
Interleukin-10	428.18 ± 208.63	300.56 ± 95.01	365.22 ± 171.57	.003	.239	.141
Epidermal growth factor	3043.06 ± 2153.85	3273.38 ± 2511.48	2503.21 ± 1653.753	.711	.310	.218
Interleukin-1β	182.20 ± 82.06	90.70 ± 40.34	163.79 ± 200.24	<.0001	.713	.145
Interferon-gamma	1116.04 ± 1059.95	545.20 ± 340.78	1272.36 ± 2921.08	.006	.829	.307
Interleukin-1 receptor antagonist	300,333.03 ± 288,786.25	97,187.50 ± 129,616.57	258,215.1 ± 436,426.70	.0008	.704	.125

A1 = high lymphotoxin-alpha dry eye in right eye group; B1 = low lymphotoxin-alpha dry eye in right eye group; C1 = control subjects in right eye group; SD = standard deviation.

TABLE 4. Levels of Tear Protein Markers of the Left Eye in High Lymphotoxin-Alpha Dry Eye Patients, Low Lymphotoxin-Alpha Dry Eye Patients, and Normal Subjects

				P Value		
Cytokine, pg/mL	A2	B2	C2	A2 vs B2	A2 vs C2	B2 vs C2
Tumor necrosis factor α	546.94 ± 467.94	191.57 ± 141.57	299.85 ± 553.37	.003	.132	.390
Interleukin-6	97.33 ± 60.77	225.93 ± 1063.56	194.21 ± 500.41	.456	.400	.877
Matrix metalloproteinase-9	22,034.65 ± 26,676.65	30,599.14 ± 129,168.95	34,305.61 ± 96,093.36	.692	.587	.902
Interleukin-8	3368.62 ± 7535.85	935.59 ± 946.16	1363.75 ± 1843.75	.156	.249	.339
Interleukin-10	344.89 ± 109.94	234.08 ± 81.64	265.03 ± 114.08	.0003	.028	.289
Epidermal growth factor	3448.48 ± 2859.87	2177.44 ± 1412.98	1818.65 ± 1169.26	.067	.023	.305
Interleukin-1β	149.96 ± 62.79	90.12 ± 35.17	128.19 ± 156.20	.0004	.567	.295
Interferon-gamma	766.92 ± 587.75	498.10 ± 264.92	588.04 ± 384.46	.058	.255	.356
Interleukin-1 receptor antagonist	180,482.50 ± 167,875.80	80,457.91 ± 70,350.29	141,968.10 ± 170,760.50	.010	.471	.136

A2 = high lymphotoxin-alpha dry eye in right eye group; B2 = low lymphotoxin-alpha dry eye in right eye group; C2 = control subjects in right eye group; SD = standard deviation.

The left and right eye of the same patient were analyzed separately. In addition, we prospectively analyzed the expression levels of protein markers in tears. Thirty-three right eyes of DE patients with high LT-α, 27 right eyes of DE patients with low LT-α, 21 left eyes of DE patients with high LT-α, and 39 left eyes of DE patients with low LT-α levels were enrolled in the study. The right and left eyes of 20 normal subjects were used as control eyes.

• **DE EXAMINATIONS:** Examinations to evaluate the ocular surfaces and symptoms included the tear breakup time (TBUT), Schirmer I test, corneal fluorescein staining (CFS),[12] the temporal conjunctival hyperemia index (TCHI), tear meniscus height (TMH), Standard Patient Evaluation Eye Dryness (SPEED), and ocular surface disease index (OSDI score) in all patients and control subjects. The Oxford scheme (score 0-12) was used to grade corneal staining. A CFS score ≥1 was considered positive. The SPEED questionnaire,[13] a dry eye symptom evaluation questionnaire, is scored based on ocular symptoms and frequency of occurrence in the past 3 months. The questionnaire examines the following 4 symptoms: eye dryness and grittiness or scratchiness, soreness or irritation, and burning or eye fatigue. Each symptom criterion is scored from 0-3, according to the frequency of each symptom. A score of 0-4 is given according to the severity of each symptom. The scores are summed to calculate the total score, which ranges from 0-28, with higher scores indicating more severe symptoms.

The major exclusion criteria for subjects enrolled in the control group were pregnancy, systemic medication use, a history of contact lens use, and ocular signs, such as corneal erosion or corneal staining. Further, inclusion criteria for the control group subjects were an OSDI score ≤12, a

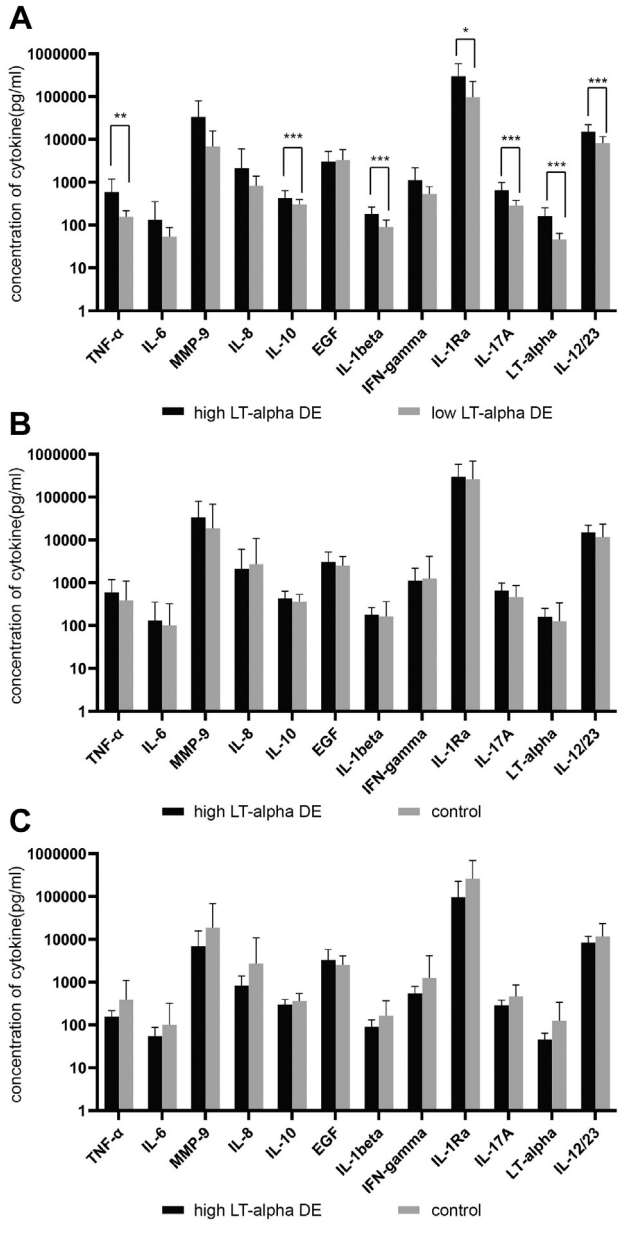

FIGURE 2. Comparison of tear protein makers levels in the right eyes among the 3 groups. (A) Comparison of tear protein maker mean values between the high lymphotoxin-alpha (LT-α) dry eye (DE) group and the low LT-α DE group. (B) Comparison between the high LT-α DE group and the control group. (C) Comparison between the low LT-α DE group and the control group. Data represent means with 95% confidence intervals. *P* values were calculated using 1-way analysis of variance and Tukey post hoc tests for multiple comparisons (P* < .05; ***P* < .01; ****P* < .001).**

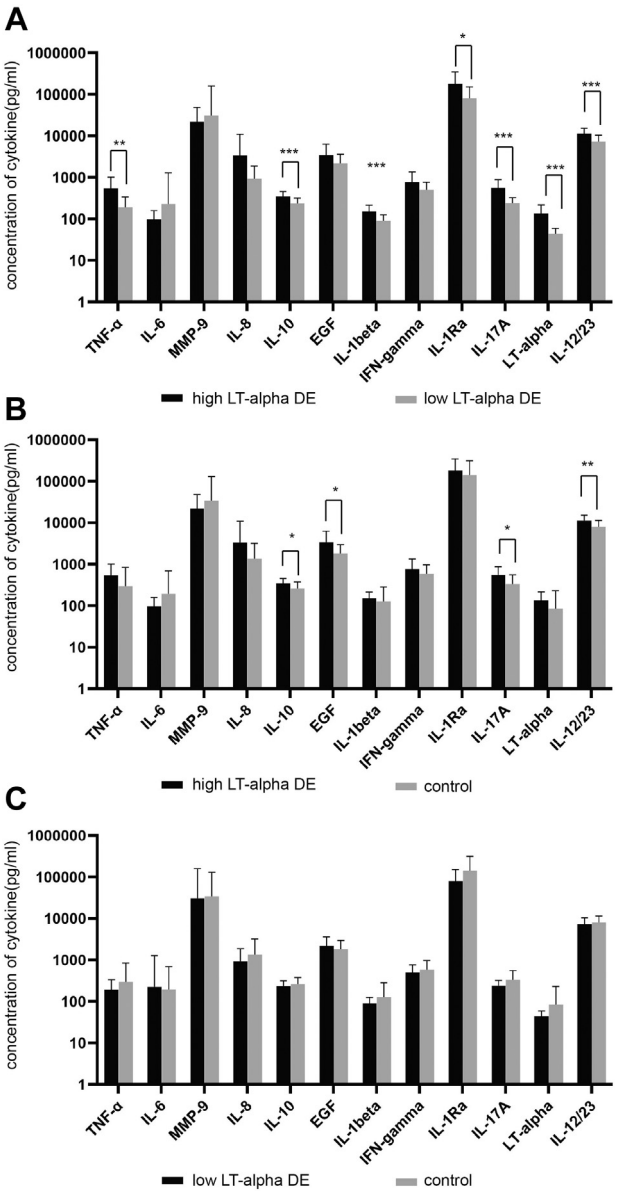

FIGURE 3. Comparison of tear protein makers levels in the left eyes among the 3 groups. (A) Comparison of tear protein maker mean values between the high lymphotoxin-alpha (LT-α) dry eye (DE) group and the low LT-α DE group. (B) Comparison between the high LT-α DE group and the control group. (C) Comparison between the low LT-α DE group and the control group. Data represent means with 95% confidence intervals. *P* values were calculated using 1-way analysis of variance and Tukey post hoc tests for multiple comparisons (P* < .05; ***P* < .01; ****P* < .001).**

TBUT >7 seconds, and a Schirmer I test >7 mm. All DE patients had experienced symptoms of DE for >1 month and had an OSDI score >12. Inclusion criteria in the DE group included either a TBUT ≤3 seconds, a Schirmer I test ≤5 mm/5 minutes, 3 second <TBUT ≤10 seconds and positive fluorescein staining, or 5 mm/5 minutes < Schirmer I test ≤10 mm/5 minutes and positive fluorescein staining. Subjects were excluded from the study if they: 1) had active ocular infection or inflammation, such as active allergic conjunctivitis, active trachoma, endophthalmitis, etc; 2) had inflammation of the eyelid or the skin surrounding

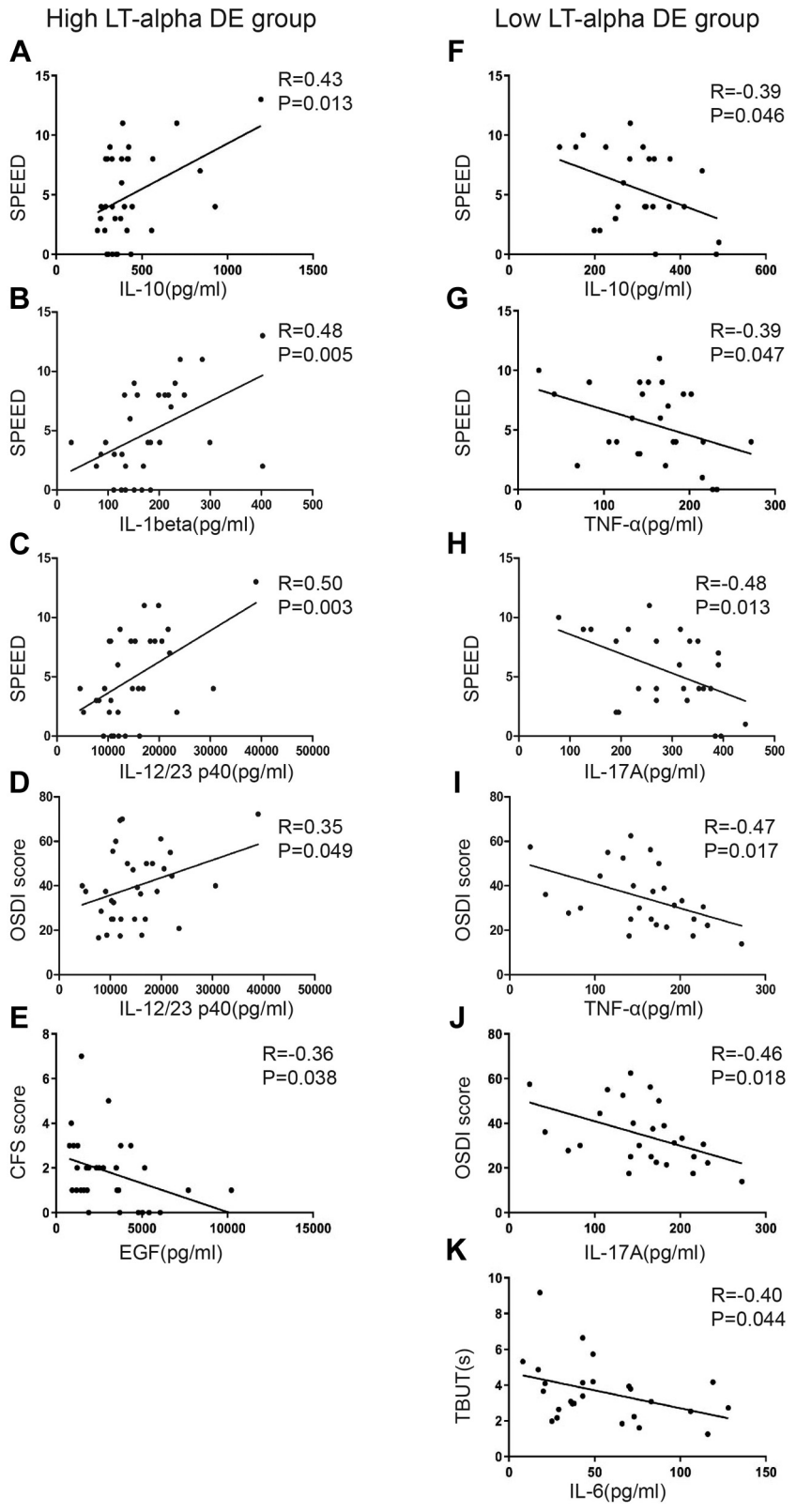

FIGURE 4. Correlation between tear protein makers and ocular surface parameters for the high and low lymphotoxin-alpha (LT-α) dry eye (DE) group in the right eyes. In the high LT-α DE group, Standard Patient Evaluation Eye Dryness (SPEED) was significantly correlated with tear interleukin-10 (IL-10) level (A), tear IL-1β level (B), and tear IL-12/23 p40 level (C). Tear IL-12/23 p40 level was significantly correlated with ocular surface disease index (OSDI) score (D). There was also significant correlation between tear epidermal growth factor (EGF) with grade in the high LT-α DE group (E). In the low LT-α DE group, SPEED was significantly

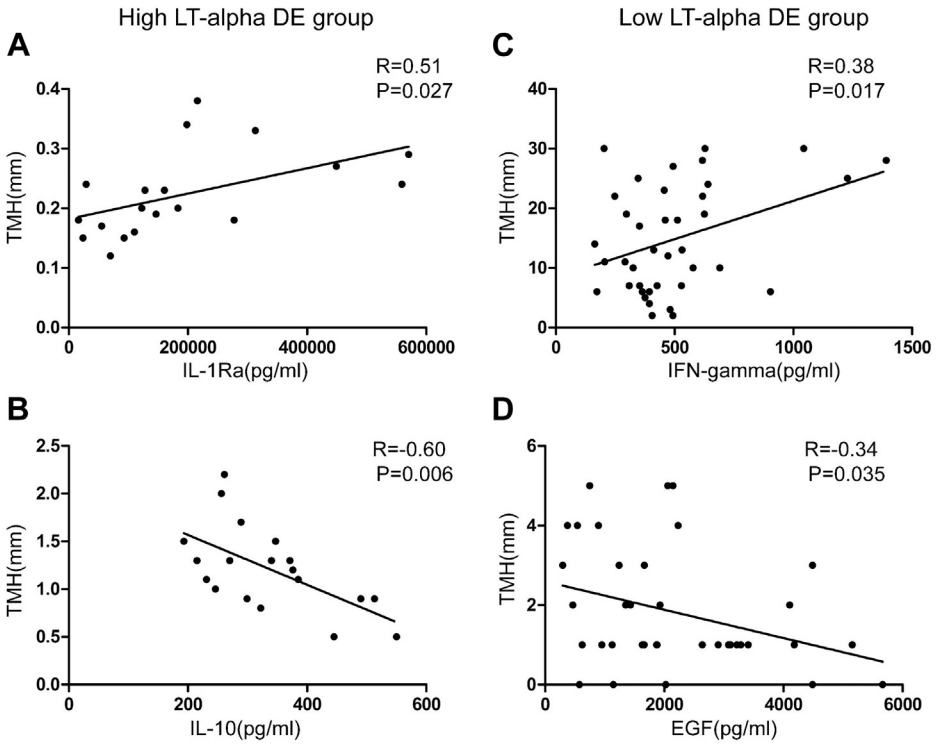

FIGURE 5. Correlation between tear protein makers and ocular surfaces parameters for the high and low lymphotoxin-alpha (LT-α) dry eye (DE) group in the left eyes. In the high LT-α DE group, only interleukin-10 (IL-10) (A) and IL-1 receptor antagonist (IL-1Ra) (B) levels significantly correlated with ocular surface parameters. In the low LT-α DE group, only interferon-gamma (IFN-gamma) (C) and epidermal growth factor (EGF) (D) levels significantly correlated with ocular surface parameters.

the eye, such as anterior blepharitis, etc; 3) had eyelid abnormalities, such as eyelid varus, ectropion, eyelid tumor, eyelid insufficiency, severe trichiasis, or severe ptosis, etc; 4) had previous severe corneal damage, such as corneal chemical injuries or corneal dystrophy, etc; 5) had other systemic diseases, such as Stevens-Johnson syndrome, graft versus host disease, vitamin A deficiency, Wegener granulomatosis, SS, etc; 6) had an allergy to sodium fluorescein; 7) used anti-inflammatory eye drops within 1 week before examination or artificial tears on the day of examination; or 8) had a history of previous ocular surgery within 3 months of examination. SS is a systemic disease that affects tear production. Moreover, some studies have shown that the tear cytokines in SS DEs and non-SS DEs are different.[14] Therefore, cytokines in SS DE tears may need to be further analyzed separately at different levels of LT-α. In addition, we also ruled out other major systemic diseases affecting tear secretion, including autoimmune diseases, such as psychiatric diseases and malignant tumors, ensuring the accuracy of the research results.

• **TEAR SAMPLE COLLECTION AND INFLAMMATORY BIOMARKER ANALYSIS:** Nonstimulated tear fluids were collected from the tear lake inside the lateral conjunctival sac by a capillary tear collector (2.2 μL; Seinda Biomedical Corporation, Guangzhou, China), dispensed into a 0.5-mL centrifuge tube, and frozen immediately at −80 C. All tests were performed on each eye separately, with at least a 5-minute interval between each eye to minimize the impact of the same researcher's experiments. The tear reflex was carefully avoided as much as possible. At the end of sample acquisition, all tear samples were analyzed for concentrations of 12 protein analytes using a microsphere-based immunoassay (Luminex, Austin, Texas, USA) in 3 multiplex panels and a lysozyme assay.

• **STATISTICAL ANALYSIS:** Statistical analyses were performed using SPSS software (v 15.0; IBM Corp, Chicago, Illinois, USA). Tear protein marker concentrations among the 6 study groups were compared by analysis of variance (ANOVA) with Tukey post hoc testing. Corre-

correlated with tear IL-10 level (F), tear tumor necrosis factor α (TNF-α) level (G) and tear IL-17A level (H). OSDI was significantly correlated with tear TNF-α level (I) and tear IL-17A level (J). IL-6 was significantly correlated with tear breakup time (TBUT) (K). Pearson correlation coefficients were calculated to analyze correlations.

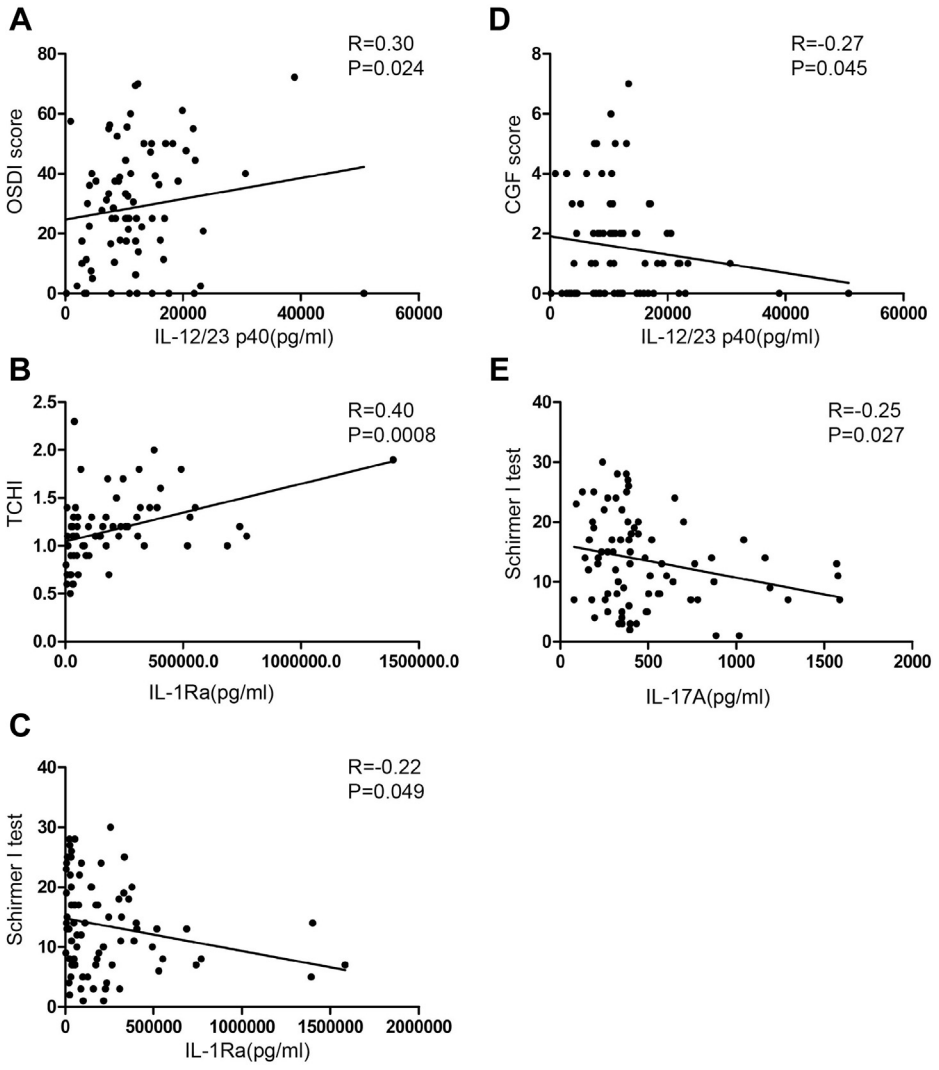

FIGURE 6. Correlation between tear protein makers and ocular surfaces parameters for the high and low lymphotoxin-alpha (LT-α) dry eye (DE) group in the left eyes. The tear interleukin-12/23 (IL-12/23) p40 level was significantly correlated with ocular surface disease index (OSDI) score (A). Tear IL-1 receptor antagonist (IL-1Ra) level was significantly correlated with the temporal conjunctival hyperemia index (TCHI) (B) and the Schirmer I test (C). There was also significant correlation between tear IL-12/23 p40 level and the corneal staining score (D) and between IL-17A and the Schirmer I test (E). Pearson correlation coefficients were calculated to analyze correlations.

lations between tear protein marker concentrations and clinical parameters (OSDI score, SPEED, TCHI, TMH, TBUT, Schirmer I test, and CFS score) were analyzed by Pearson correlation coefficient.

RESULTS

RESULTS FOR CORRELATION ANALYSIS BETWEEN THE RIGHT and left eyes for protein markers and clinical characteristics based on all study subjects are shown in Figure 1. The demographics and clinical features of the high LT-α DE group, the low LT-α DE group, and the control group are shown in Tables 1 and 2. There were no significant differences in mean sex among the 3 groups. Between the 2 DE groups, there were also no significant differences in mean OSDI score, SPEED, TCHI, TMH, CFS score, TBUT, or Schirmer I test ($P > .05$).

The mean tear protein makers values in right and left eyes are shown in Tables 3 and 4. Figures 2 and 3 show the differences in tear protein maker concentrations of the right and left eyes among the 3 groups. All the mean concentrations of tear protein makers in the high LT-α DE group were higher than those in the other 2 groups. In the right eyes, the mean concentrations of TNF-α ($P = .0002$), matrix metalloproteinase-9 ($P = .003$), IL-10 ($P = .003$), IFN-γ ($P = .006$), IL-1β ($P < .0001$), IL-1Ra ($P =$

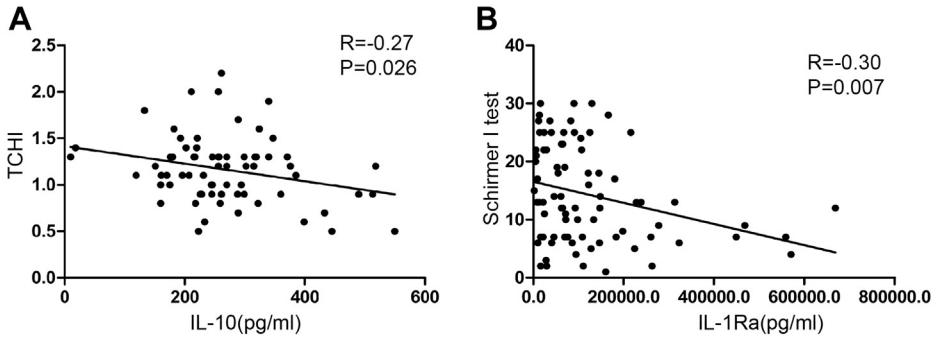

FIGURE 7. Correlation between tear protein maker and clinical parameters of all subjects in the left eyes. The tear interleukin-10 (IL-10) level was significantly correlated with the temporal conjunctival hyperemia index (TCHI) (A). There was also significant correlation between tear IL-1 receptor antagonist (IL-1Ra) level and the Schirmer I test (B) in the left eyes. Pearson correlation coefficients were calculated to analyze correlations.

.0008), IL-17A ($P < .0001$), LT-α ($P < .0001$), and IL-12/23 p40 ($P < .0001$) in the high LT-α DE group were significantly higher than those in the low LT-α DE group. There were no significant differences in tear protein markers between the high LT-α DE group and the control group. There were also no differences in tear protein markers between the low LT-α DE group and the control group.

In the left eyes, the mean concentrations of TNF-α ($P = .003$), IL-10 ($P = .0003$), IL-1β ($P = .0004$), IL-1Ra ($P = .010$), IL-17A ($P = .0001$), LT-α ($P < .0001$), and IL-12/23 p40 ($P = .0003$) in the high LT-α DE group were significantly higher than those in the low LT-α DE group. There were also significant differences in IL-10 ($P = .028$), EGF ($P = .023$), IL-17A ($P = .011$), and IL-12/23 p40 ($P = .007$) between the high LT-α DE group and the control group. However, there were no significant differences in tear protein markers between the low LT-α DE group and the control group.

We next investigated correlations between tear protein markers and parameters of ocular surfaces including OSDI score, SPEED, TCHI, TMH, TBUT, Schirmer I test, and CFS score for the 2 DE groups. In the right eyes, in the high LT-α DE group, IL-10 and IL-1β levels significantly correlated with SPEED, IL-12/IL-23 p40 significantly correlated with OSDI score, and EGF significantly correlated with CFS score (Figure 4; IL-10 with SPEED: $R = 0.43$, $P = .013$; IL-1Ra with SPEED: $R = 0.48$, $P = .005$; IL-12/IL-23 p40 with OSDI: $R = 0.35$, $P = .049$; EGF with CFS score: $R = -0.36$, $P = .038$). In the low LT-α DE group, IL-17A and TNF-α level significantly correlated with OSDI score and SPEED, IL-10 also significantly correlated with SPEED, and IL-6 significantly correlated with TBUT (Figure 4; IL-17A with OSDI score: $R = -0.46$, $P = .018$; IL-17A with SPEED: $R = -0.48$, $P = .013$; TNF-α with SPEED: $R = -0.47$, $P = .017$; TNF-α with OSDI score: $R = -0.47$, $P = .017$; IL-10 with SPEED: $R = -0.39$, $P = .046$; IL-6 with TBUT: $R = -0.40$, $P = .044$). In the left eyes, in the high LT-α DE group, only IL-10 and IL-1Ra levels significantly correlated with ocular surface parameters. (Figure 5; IL-10 with TCHI: $R = -0.60$, $P = .006$; IL-1Ra with TMH: $R = 0.51$, $P = .027$). In the low LT-α DE group, only IFN-γ and EGF levels significantly correlated with ocular surface parameters (Figure 5; IFN-γ with Schirmer I test: $R = 0.38$, $P = .017$; EGF with corneal staining CFS score: $R = -0.34$, $P = .035$). The other tear protein markers were not significantly correlated with ocular surface parameters.

We investigated the correlations between tear protein markers and parameters of ocular surfaces in all subjects, including OSDI score, SPEED, TCHI, TMH, TBUT, Schirmer I test, and CFS score. As shown in Figure 6, in the right eyes, the expression level of IL-12/23 p40 positively correlated with the OSDI score ($R = 0.30$, $P = .024$) and CFS score ($R = -0.27$, $P = .045$). The Schirmer I test negatively correlated with the level of IL-1Ra ($R = -0.22$, $P = .049$) and IL-17A ($R = -0.25$, $P = .027$) in the tears, and the level of IL-1Ra also correlated with the TCHI ($R = 0.40$, $P = .0008$). As shown in Figure 7, the expression level of IL-10 in the left eyes positively correlated with the TCHI ($R = -0.27$, $P = .026$). The Schirmer I test negatively correlated with the level of IL-1Ra ($R = -0.30$, $P = .007$).

Lastly, we analyzed the correlation of tear protein markers and found a highly positive correlation among the expression level of TNF-α, LT-α level, and IL-17A in both eyes (in the right eyes—Figure 8, TNF-α with LT-α: $R = 0.83$, $P < .0001$; TNF-α with IL-17A: $R = 0.79$, $P < .0001$; IL-17A with LT-α: $R = 0.87$, $P < .0001$; in the left eyes—Figure 9, TNF-α with LT-α: $R = 0.91$, $P < .0001$; TNF-α with IL-17A: $R = 0.83$, $P < .0001$; IL-17A with LT-α: $R = 0.83$, $P < .0001$.).

DISCUSSION

OUR PROSPECTIVE STUDY WAS DESIGNED TO ASSESS THE abundance of protein markers in the tears of patients

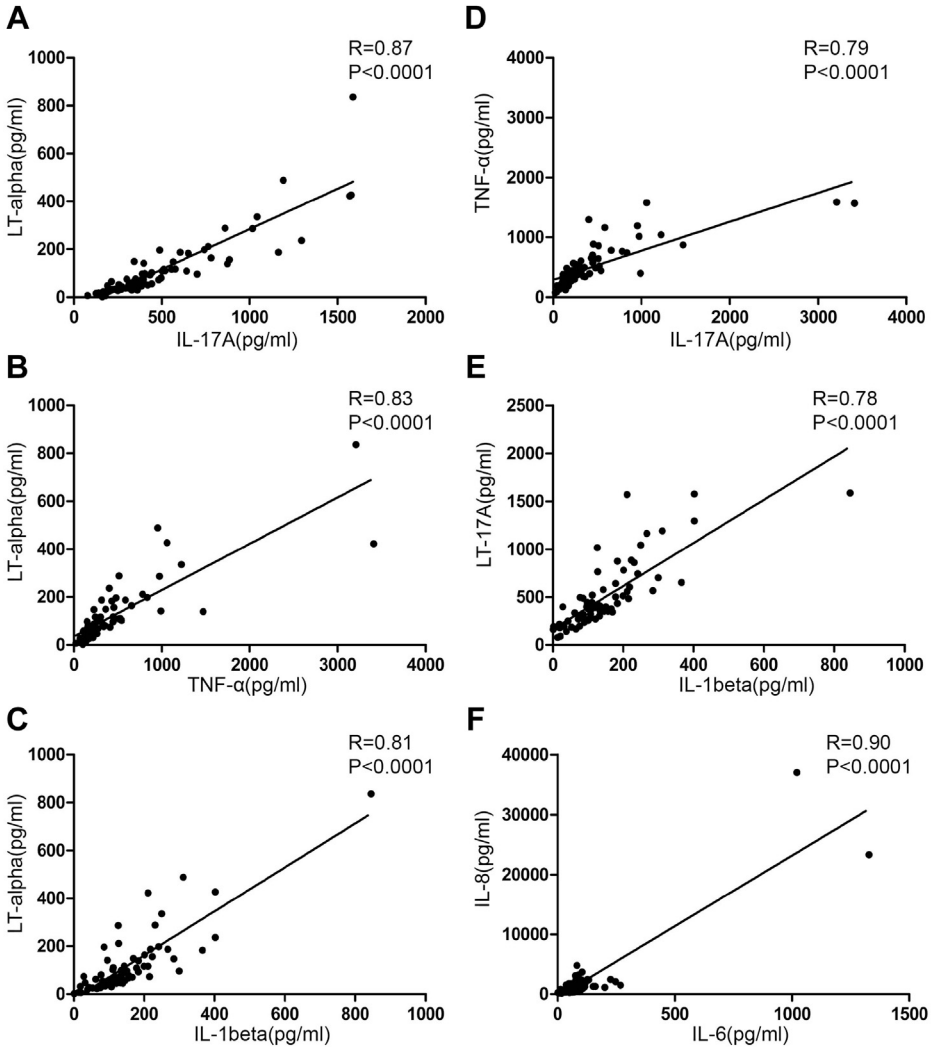

FIGURE 8. Correlation between tear protein makers of all subjects in the right eyes. The tear interleukin-17A (IL-17A) level was significantly correlated with tear lymphotoxin-alpha (LT-α) (A), tear tumor necrosis factor α (TNF-α) was significantly correlated with LT-α (B), and tear IL-1β was significantly correlated with LT-α (C). There was also significant correlation between tear IL-17A and TNF-α (D), IL-1β and IL-17A (E), and IL-6 and IL-8 (F). Pearson correlation coefficients were calculated to analyze correlations.

with DE with high vs low levels of LT-α, as well as in the tears of control subjects. The objective of this assessment was to determine which of these markers was associated with DED ocular signs and disease severity. Clinical parameters of DED showed a significant correlation with the content of certain tear protein markers.

First, we analyzed the correlation of the data between the left and right eye of the same subject. Our results found that the clinical parameters for the left and right eye of the same subject were positively correlated, in particular with the TBUT. The expression levels of the left and right eye tear protein markers in the same subject were also positively correlated, except with IL-8 and IL-1β. The IL-8 and IL-1β results were not identical with the results from Huang and associates,[15] which is likely related to differences in the number of study cases. The correlation between the left and right eye data provided a basis for analyzing the left and right eyes of the same subject separately, and further suggested that the clinical signs and tear protein expression levels in the left and right eyes of the same subject may be at the same level.

In the high LT-α DE group, TNF-α, IL-10, IL-1β, IL-1Ra, IL-17A, and IL-12/23 p40 were significantly increased in comparison with the low LT-α level DE group. This tear protein marker difference suggested that DED with high vs low LT-α may have different pathological mechanisms. Moreover, the differences in IL-10, EGF, IL-17A, and IL-12/23 p40 levels between the LT-α high-expression DE

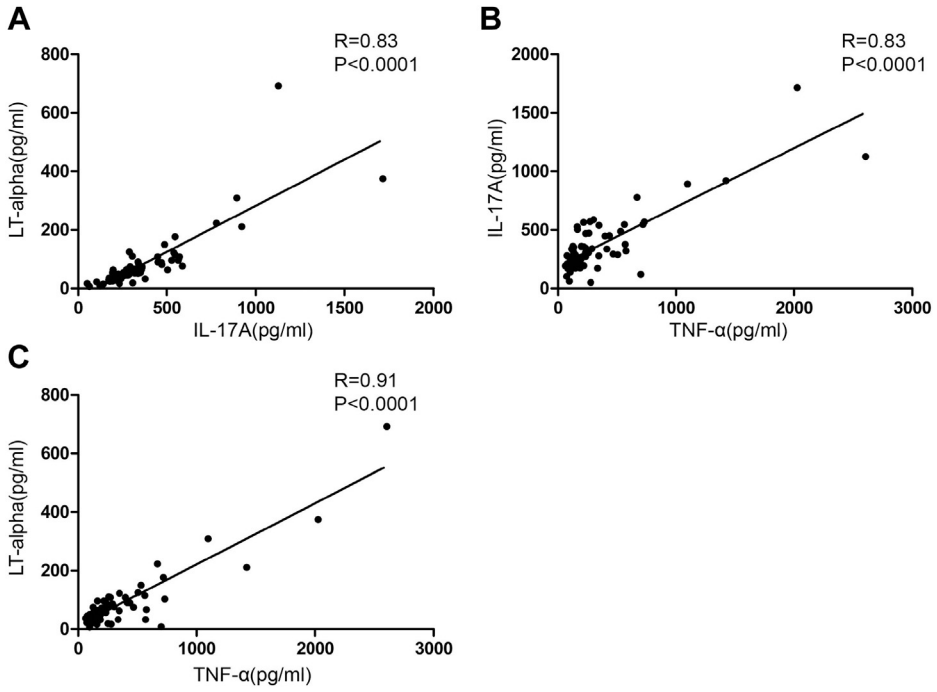

FIGURE 9. Correlation between tear protein makers of all subjects in the left eyes. The tear interleukin-17A (IL-17A) level was significantly correlated with tear lymphotoxin-alpha (LT-α) (A) and tear tumor necrosis factor α (TNF-α) was significantly correlated with LT-α (B). There was also significant correlation between tear TNF-α and IL-17A (C) in the left eyes. Pearson correlation coefficients were calculated to analyze correlations.

group and control group demonstrated that the participation of those cytokines in the high LT-α DE group was stronger. However, there were no significant differences between the low LT-α DE group and the control group. This may mean that the pathological mechanisms of this kind of DE is independent from LT-α or mediated by a tear protein marker other than the 12 protein markers that we studied.

Yoon and associates[16] reported that IL-6 levels in the tears of DE patients were associated with TBUT. Comparing previous studies and our study, it is thought that IL-6 exhibited a reliable correlation with TBUT in the low LT-α DE group. However, the fact that IL-6 did not significantly correlate with clinical parameters in DE patients with high LT-α indicated that tear protein markers exert different influences in high and low LT-α DE groups. Another proof of this conclusion is the relationship between EGF and corneal staining scores; we found that EGF exhibited a negative correlation with CFS scores in the high LT-α DE group. This is the same conclusion as previous studies, which suggested that EGF can promote the proliferation and migration of human corneal cells under both physiological and disease conditions. The regeneration of posterior corneal epithelium shortens the covering time of corneal epithelial defects,[17,18] and the insufficient expression of EGF may also be one of the reasons for ocular surface damage in DE. Although we can confirm some correlations mentioned above, and correlation analysis demonstrated different reactions between the 2 DE groups, additional studies are necessary to assess the correlation among tear protein marker concentrations, clinical parameters, and DE severity.

IL-1Ra is a naturally occurring and specific inhibitor of IL-1 activity.[19] IL-1Ra can bind to IL-1 receptor I tightly without triggering inflammatory signaling and, thus, antagonize the inflammatory effects induced by IL-1β and IL-1α. In ocular tissues, IL-1Ra is abundantly expressed in normal corneal and conjunctival epithelium; the strongest expression is in the superficial apical layer of the corneal epithelium.[20–23] Numerous studies have shown anti-inflammatory and tissue protective benefits from IL-1Ra in different diseases or inflammatory conditions.[19,24–27] We found that IL-1Ra levels negatively correlated with the Schirmer I test in both eyes of the all subjects, and positively correlated with the TCHI. However, studies have reported a lack of precision in the Schirmer I test objective measures, poor correlation between the Schirmer I test and symptoms, and the significant heterogeneity of the DED patient population remain major challenges in DED clinical research and drug development.[27–30] Indeed, the advantage of Schirmer strips is that they are widely available and relatively cheap. However, the Schirmer I test was removed from the diagnostic criteria during the

TFOS Dry Eye Workshop II Executive Summary Report,[1] possibly because the results of repeated Schirmer I tests are inconsistent and easily affected by the external environment. IL-1Ra levels in tears may more stably indicate lacrimal function than the Schirmer I test. Tear Luminex assays for protein markers in tears are also highly replicable when testing the same patient for a week.[12] In addition, relevant clinical kits only require 2 μL of tear fluid, which is more convenient. Although tear Luminex assays are not yet universal, we believe that quick assay kits will be developed to detect cytokines in tears, which will be used clinically in the future.

We found that the expression levels of TNF-α and IL-17A, LT-α and IL-17A, and TNF- α and LT-α were highly positively correlated in tears. The highly positive correlation between these three factors reminded us of the biological relationship between TNF-α, IL-17A, and LT-α. LT-α was considered to be the closest homolog to TNF-α, with the two being 30% homologous in their primary amino acid sequence, but also have some distinct molecular and biological differences.[31,32] The N terminus of LT-α, unlike that of TNF-α, enables effective conversion into a soluble form. Thus, LT-α is not found at the cell surface, which is a unique feature among TNF superfamily members.[33,34] In addition, the outer surfaces of the molecules have little similarity, so they can interact with different receptors. Despite the considerable sequence homology, the 2 proteins exhibit different biological behaviors. LT-α and TNF-α differ in terms of receptor interaction. Cross-linking experiments with 125I-rh LT-α result in the presence of 100- and 120-kDa bands, while 125I-rh TNF-α produces a single 100-kDa band.[35] TNF-α exerts its effects through both TNF receptors, but LT-α only interacts with TNF receptor 1. However, the same cells can express both LT-α and TNF-α. Knockout mice for either cytokine show different phenotypes, indicating that the 2 cytokines have overlapping but different functions.[10] Finally, patients cross-reacting with LT-α have different outcomes from patients treated with anti–TNF-α monoclonal antibody.[36] These previous results support our finding that LT-α and TNF-α have distinct functions. In our study, Luminex assays identified the expression difference between LT-α and TNF-α in tears, though the two were highly correlated. Many studies support the theory that LT-α somehow controls the expression of TNF-α and the absence of LT-α could interfere with the production of this cytokine.[10] The highly correlated expression level of TNF-α and LT-α in tears indicates that there is a deeper relationship between them, in addition to their mutual regulation. The high correlation between TNF-α and IL-17A is potentially related to the relationship between LT-α and TNF-α. However, there are some deficiencies in the research in this field, and thus it deserves further investigation and discussion.

There are some limitations in this study. First, the time for tear collection was not unified, and we do not know whether there are differences in the expression of tear proteins at different time periods, which may lead to data bias. Second, subjects with systemic diseases that might affect DE, such as patients with SS, psychiatric diseases, and malignant tumors, were ruled out, and thus, it is unclear whether cytokines have different effects between normal subjects and patients with DE with high and low LT-α levels. This may require further studies. Finally, our study suggested that there are no significant differences between the low LT-α DE group and the control group. This may mean that the pathological mechanisms of this kind of DE is independent of LT-α or mediated by a tear protein marker other than the 12 protein markers that we studied. This in part may be a result of the small sample size and the quantitative restriction of cytokines. A larger study should be conducted in the future to evaluate more cytokines and to determine whether there are any differences or similarities between them.

In summary, our study showed that the levels of multiple tear protein markers were elevated in the high-LT-α DE group. The elevated levels of tear proteins indicated that the tear protein markers played distinctive roles in DE with different LT-α expression levels; this suggests the involvement of contrasting inflammatory processes, and the potential use of the level of IL-1Ra in tears as a biomarker to replace the Schirmer I test.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

HAIYAN CHEN: FORMAL ANALYSIS, DATA CURATION, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Huijie Chen:** Software, Data curation, Investigation, Validation, Writing - original draft, Writing - review & editing. **Lifang Liang:** Investigation, Data curation, Validation. **Yanyan Zhong:** Formal analysis, Validation. **Yingying Liang:** Software, Investigation. **Ying Yu:** Formal analysis, Methodology. **Shuxin Huang:** Project administration, Resources. **Xiaohe Lu:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

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