The Ocular Surface 17 (2019) 160–166

Contents lists available at ScienceDirect

The Ocular Surface

journal homepage: www.elsevier.com/locate/jtos

Innovative Techniques and Technology

A novel and innovative paper-based analytical device for assessing tear lactoferrin of dry eye patients

Hideki Sonobe[a], Yoko Ogawa[a,*], Kentaro Yamada[b], Eisuke Shimizu[a], Yuichi Uchino[a], Mizuka Kamoi[a], Yumiko Saijo[a], Mio Yamane[a], Daniel Citterio[b], Koji Suzuki[b,c], Kazuo Tsubota[a]

[a] Department of Ophthalmology Keio University School of Medicine, Tokyo, Japan
[b] Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan
[c] JSR · Keio University Medical and Chemical Innovation Center, Tokyo, Japan

ARTICLE INFO

Keywords:
Microfluicic paper-based analytical device
Fluorescence detection technique
Lactoferrin
Dry eye disease
ELISA

ABSTRACT

Purpose: To elucidate the correlation between lactoferrin concentration in the tear film and signs and symptoms of severe dry eye disease (DED) using a novel microfluidic paper-based analytical device (μPAD) and enzyme-linked immunosorbent assay (ELISA).

Methods: Twenty-four patients were recruited at the Keio University Hospital. Using a novel μPAD, lactoferrin concentrations were measured in 4 patients with GVHD-related DED, 3 patients with other types of DED and 2 controls (Group A). For validation by ELISA, 22 patients (7 patients from Group A) comprising 9 patients with GVHD-related DED, 6 patients with other types of DED and 7 controls were examined (Group B). The link between lactoferrin concentration and clinical data about the severity of aqueous tear deficient DED was also investigated by both μPAD and ELISA.

Results: The lactoferrin concentration in tear fluid of the DED patients was positively correlated between μPAD and ELISA ($p = 0.006$, $r = 0.886$). The tear fluid of the GVHD patients showed low or undetectable lactoferrin concentration. Analysis by ELISA demonstrated that lactoferrin concentrations in the tear film from the GVHD patients were significantly lower than those from the non-GVHD patients ($p = 0.010576$). ELISA revealed lactoferrin concentration correlated with the value of Schirmer test and tear film breakup time, whereas it was inversely correlated with OSDI, fluorescein and rose bengal scores.

Conclusions: The novel μPAD may pave the way for measuring lactoferrin concentration in tear fluid from DED patients. Our results suggested that lactoferrin concentration in tear fluid reflect the severity of DED.

1. Introduction

Tear film plays an indispensable role in maintaining corneal and conjunctival homeostasis by protecting against foreign body microbial invasion and preserving visual acuity [1]. Tear fluid is composed of a variety of proteins, enzymes, water, lipids, and electrolytes [1–3]. Lactoferrin as well as lysozyme, lipocalin, secretory IgA, phospholipase A, and secretory and membrane-associated mucins are important tear components that protect against invading pathogens [4]. Lactoferrin, a protein secreted from lacrimal gland acini, exerts a bactericidal, anti-tumor, and anti-viral/-fungal effect; exhibits immunomodulatory properties, and maintains homeostasis of ocular surface health [5]. Lactoferrin binds to iron in tear fluid; thus, bacteria cannot colonize the ocular surface due to the lack of this nutrient [4]. Lactoferrin levels in tear fluid are reduced in SS and non-SS dry eye patients [2,4,6–8].

The Tear Film Ocular Surface Society Dry Eye Workshop II (TFOS DEWS II) recently revised the definition of dry eye as “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles” [9]. On the other hand, the Asia Dry Eye Society proposed a new consensus definition of dry eye disease as “a multifactorial disease characterized by unstable tear film causing a variety of symptoms and/or visual impairment, potentially accompanied by ocular surface damage” [10]. Both definitions indicate that an understanding of tear film, including tear dynamics and components, is essential for dry eye disease.

The diagnosis of dry eye disease is based on a combination of signs and symptoms. The ocular surface disease index (OSDI), fluorescein and rose bengal staining, and tear film breakup time (TFBUT) are used as

* Corresponding author.
E-mail address: yoko@z7.keio.jp (Y. Ogawa).

https://doi.org/10.1016/j.jtos.2018.11.001
Received 25 July 2018; Received in revised form 1 November 2018; Accepted 2 November 2018

Table 1
Detailed demographic and clinical data for various types of dry eye disease in Group A using both ELISA and μPAD.

		ELISA [pg/μL]	μPAD [pg/μL]	Age [years of age]	Sex	OSDI [points]	Fluo [points]	RB [points]	TFBUT [second]	Schirmer [mm]	**LogMAR [−]**
GVHD	GVHD-01	0.02357	0.00000	44	M	29	2.83	2.00	2.00	1.00	−0.050
	GVHD-02	0.00245	0.00000	49	M	9	0.33	0.00	5.00	6.50	−0.100
	GVHD-03	0.01522	0.00000	38	F	15	1.00	0.50	4.00		−0.100
	GVHD-04	0.26075	0.00000	58	F	13	1.00	0.67	3.00	5.00	−0.100
Non-GVHD	Non-GVHD-01	0.27244	0.16667	49	M	9	0.00	0.17	10.00	13.00	−0.100
	Non-GVHD-02	1.30000	1.25000	56	F	0	0.00	0.00	8.00	14.50	−0.100
Dry eye	Simple-01	0.29393	0.20000	59	M						
	Simple-02	0.21213	0.20000	74	F	7	0.00	0.00	4.00	13.00	−1.00
	Simple-03	0.42488	0.45000	57	F	27	0.33	0.33	10.00	5.00	0.100

ELISA = Enzyme-linked Immunosorbent Assay; μPAD = Microfluidic Paper-based Analytical Device; OSDI = Ocular Surface Disease Index; Fluo = Fluorescein staining score; RB = Rose Bengal staining score; TFBUT = Tear Film Breakup Time, Schirmer = Schirmer test value without anesthesia, logMAR = Logarithm of the Minimum Angle of Resolution, GVHD = Graft-versus-Host Disease; M = Male; F = Female; Dry eye = Simple dry eye. Non-GVHD-01 and Non-GVHD-02 = non dry eye controls.

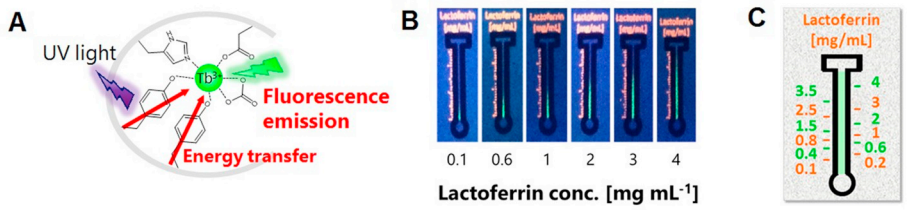

Fig. 1. An antibody-free microfluidic paper-based analytical device (μPAD) for the determination of tear fluid lactoferrin. (A) Terbium-lactoferrin fluorescence emission is utilized by energy transfer to terbium through tyrosine at the binding site. (B) Visualization of transported lactoferrin on μPAD. The length of the fluorescent line increased in proportion to the lactoferrin concentration in the tear fluid samples. (C) Outline of the μPAD for distance-based lactoferrin measurement.

Table 2
Detailed demographic and clinical data for various types of dry eye disease for Group B using ELISA.

		ELISA [pg/μL]	Age [years of age]	Sex	OSDI [points]	Fluo [points]	RB [points]	TFBUT [second]	Schirmer [mm]	**LogMAR [−]**
GVHD	GVHD-01	0.02357	44	M	29	2.83	2.00	2.00	1.00	−0.050
	GVHD-02	0.00245	49	M	9	0.33	0.00	5.00	6.50	−0.100
	GVHD-03	0.01522	38	F	15	1.00	0.50	4.00		−0.100
	GVHD-04	0.26075	58	F	13	1.00	0.67	3.00	5.00	−0.100
	GVHD-05	0.35884	56	M	3	0.50	0.67	2.00	1.50	−0.100
	GVHD-06	0.09363	48	M	25	0.83	0.83	1.50	0.00	−0.100
	GVHD-07	0.07130	22	F	11	1.00	1.67	2.00	6.50	−0.100
	GVHD-08	0.00090	60	M	11	1.00	1.00	3.00	5.00	−0.100
	GVHD-09	0.30079	33	M	0	1.17	0.50	5.00	7.00	−0.100
Non-GVHD	Non-GVHD-01	0.27244	49	M	9	0.00	0.17	10.00	13.00	−0.100
	Non-GVHD-02	1.30000	56	F	0	0.00	0.00	8.00	14.50	−0.100
	Non-GVHD-03	0.63636	52	M	0	0.00	0.00	6.50		0.025
	Non-GVHD-04	0.79000	20	F	3	0.00	0.00	10.00	11.50	−0.100
	Non-GVHD-05	0.69000	55	M	4	0.00	0.00	6.50	25.00	−0.100
	Non-GVHD-06	0.17000	44	F	6	0.00	0.00	10.00	12.00	−0.100
	Pre-BMT	0.67000	49	M	0	0.00	0.00	9.00	2.00	−0.100
Dry eye	OCP	0.00538	78	F	18	1.50	1.17	4.00	2.00	0.125
	SS	0.13615	40	F	24	0.83	1.33	2.00	1.00	−0.100
	Simple-03	0.42488	57	F	27	0.33	0.33	10.00	5.00	0.100
	Simple-04	0.17953	65	F	15	0.67	0.33	4.00		−0.100
	Simple-05	0.34993	59	M	32	1.00	0.83	2.00	4.50	−0.100
	Simple-06	0.21022	67	F	5	1.17	0.83	1.00	2.00	−0.100

ELISA = Enzyme-linked Immunosorbent Assay; μPAD = Microfluidic Paper-based Analytical Device; OSDI = Ocular Surface Disease Index; Fluo = Fluorescein staining score; RB = Rose Bengal staining score; TFBUT = Tear Film Breakup Time, Schirmer = Schirmer test value without anesthesia, logMAR = Logarithm of the Minimum Angle of Resolution, GVHD = Graft-versus-Host Disease; M = Male; F = Female; Dry eye = Simple dry eye. From Non-GVHD-01 to Non-GVHD-06 and Pre-BMT = non dry eye controls.

standard methods of evaluating dry eye disease [11]. The difficulty in diagnosis and evaluation of the severity of dry eye disease is potentially attributed to an inconsistent correlation between reported symptoms and observed signs [12]. Analysis of tear proteins, including lactoferrin, is an important tool to diagnose various types of ocular surface diseases and many studies have evaluated the relationship between dry eye and lactoferrin protein concentration [2,4,6–8]. Recently, InflammaDry® is used to detect MMP-9 concentrations in tear fluid as a complementary diagnostic method to detect dry eye disease [13].

In this study, we showed the evaluation by a novel and innovative microfluidic paper-based analytical device for measuring lactoferrin concentration in tear fluid of patients with aqueous tear deficient DED [14,15]. A majority of previous lactoferrin quantification methods are based on immunoassays, including a conventional enzyme-linked immunosorbent assay (ELISA) [16]. ELISA involves an antibody-based immunoassay. Given that the ELISA protocol requires washing and

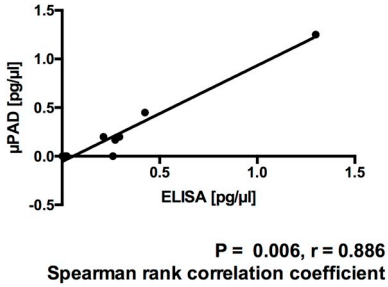

Fig. 2. Comparison of a microfluidic paper-based analytical device (μPAD) and ELISA for lactoferrin assays using tear fluid from dry eye patients. Y-axis; lactoferrin concentration measured by μPAD. X-axis; lactoferrin concentration measured by ELISA.

incubation steps, the process is rather time consuming. In addition, ELISA reagents, including enzymes and antibodies, are expensive.

Therefore, we recently developed a cheap, simple, easy, rapid and accurate analytical method that can be used in clinical settings [14,15]. Terbium produces fluorescence emission upon interacting with lactoferrin. The terbium-lactoferrin fluorescence emission occurs via energy transfer to terbium through tyrosine at the binding site [14]. We used this fluorescence emission as a signal. We used a novel and innovative antibody-free μPAD for the determination of tear fluid lactoferrin levels for aqueous tear deficient DED patients in this study.

The technique conserves the use of small samples and expensive reagents because this device has a microfluidic structure. Moreover, the technique is inexpensive based on the use of μPAD. Because it utilizes microcapillary flow, a pump is not needed. Therefore, we developed this device that saves cost and time.

The purpose of this study was to validate the usefulness of a novel and innovative paper-based microfluidic device and to elucidate the correlation between lactoferrin concentrations in the tear film and signs and symptoms of aqueous tear deficient DED using a novel μPAD and ELISA.

2. Methods

2.1. Subjects

The Ethics Research Committee at Keio University Hospital approved this study (#20090277). Written informed consent was obtained from all patients. This study was conducted at the dry eye outpatient clinic of Keio University hospital between June 2014 and May 2017. Twenty-four subjects were recruited to this study. Using a novel μPAD lactoferrin concentrations were measured in 4 patients with GVHD-related dry eye disease and 3 simple dry eye patients and 2 non dry eye controls (Group A) (Table 1). To validate these results using ELISA, 22 patients (7 patients from Group A), comprising 9 patients with GVHD-related dry eye disease, 6 patients with other types of dry eye disease, and 7 non dry eye controls were examined (Group B).

2.2. Diagnostic criteria for dry eye disease

Patients were diagnosed with DED using the Japanese diagnostic criteria revised in 2006 if all of the 3 criteria were met: (1) DED symptoms, (2) positive fluorescein staining (≥ 3 on a scale of 0–9) and/or rose bengal staining (≥ 3 on a scale of 0–9), and (3) a Schirmer I test value of 5 mm or less and/or tear film breakup time (TFBUT) of 5 s or less [17,18].

2.3. Methods for collecting tear samples and analysis

The tear fluid was collected using a microcapillary tube (HIRSCHMANN ringcaps; Hirschmann Laborgeräte GmbH & Co.; Eberstadt, Germany) with 10 μL of saline (0.9% NaCl; Otsuka Pharmaceutical Co.; Tokyo, Japan) placed into the conjunctival sac using a micropipette for ringcaps (Hirschmann Laborgeräte GmbH & Co. Eberstadt, Germany). Tear samples were stored at −80°C until analysis. Lactoferrin concentration was determined using classical ELISA [16] and a newly developed μPAD [14,15]. The human lactoferrin ELISA kit was purchased from EMD Chemicals, Inc. (San Diego, USA). Additionally, we analyzed the correlation between lactoferrin concentration and clinical variables, including symptoms measured by ocular surface disease index (OSDI), fluorescein and rose bengal staining scores of corneal and conjunctival epithelia and tear dynamic abnormalities.

2.4. A newly developed μPAD

This novel μPAD utilizes terbium as a reagent to detect fluorescence emission. $TbCl_3$ is deposited in the center of a microfluidic channel patterned on filter paper, and a scale indicating the lactoferrin concentration is printed next to the microfluidic channel. In this setting, we can measure the concentration of lactoferrin by reading the length of the fluorescent line. [14,15] (Fig. 1).

After 2 μL of a lactoferrin sample, was dropped on the μPAD, the length of the fluorescent line increased in proportion to the lactoferrin levels in the samples. High reproducibility and correlation among several observers were confirmed by the results of repeated experiments and measurements by several independent observers [15]. Given the antenna function of two tyrosine groups in the metal binding site of lactoferrin, trivalent terbium (Tb^{3+}) becomes fluorescent in the presence of lactoferrin [15]. The green fluorescence emission from the $Tb^{3+}-$lactoferrin complex is utilized as the detection signal [14]. For the quantification of lactoferrin based on the distance, $TbCl_3$ was deposited in a straight microfluidic channel on filter paper by means of an inkjet printer. As the sample travels along the channel, lactoferrin is continuously consumed by the formation of fluorescent $Tb^{3+}-$lactoferrin complexes until the analyte is completely depleted from the sample liquid. As a result, the concentration-dependent portion of the green fluorescent line is observed in the channel under UV illumination. Molecular diagnostic test such as MMP-9 could not be used as external control in this study, because MMP9 is not able to bind terbium which binds with lactoferrin to produce fluorescence emission for measuring lactoferrin concentration.

2.5. Statistical analysis

The data were analyzed using Prism software (ver. 6.04 for Mac; GraphPad Software, Inc., San Diego, CA, USA). The D'Agostino-Pearson omnibus normality test was used to assess whether the data exhibited a normal distribution [19]. To compare multiple differences between several data, Spearman's rank correlation coefficient was used. To compare differences between two data points, Student's t-test was used. The data are expressed as the mean ± standard deviation (SD). A P-value < 0.05 indicates statistical significance.

3. Results

Demographics and clinical data of patients are presented in Tables 1 and 2.

Group A was analyzed using the new μPAD and ELISA and consisted of 9 patients, comprising 4 patients with GVHD-related dry eye disease (2 males and 2 females, median age 46.5 years of age, range 38–58) and 5 controls (2 males and 3 females, median age 57 years of age, range 49–74, 3 simple dry eye and 2 non-GVHD recipients) (Table 1).

Group B was exclusively analyzed by ELISA and consisted of 14 patients, including 9 patients with GVHD-related dry eye disease (6 males and 3 females, median 48 years of age, range 22–60), and other types of dry eye disease (ocular cicatricial pemphigoid (1 female, 78 years of age); Sjögren's syndrome (1 female, 40 years of age); simple dry eye disease (1 male, 59 years of age and 3 female patients, median 61 years of age, range 57–67) (Table 2).

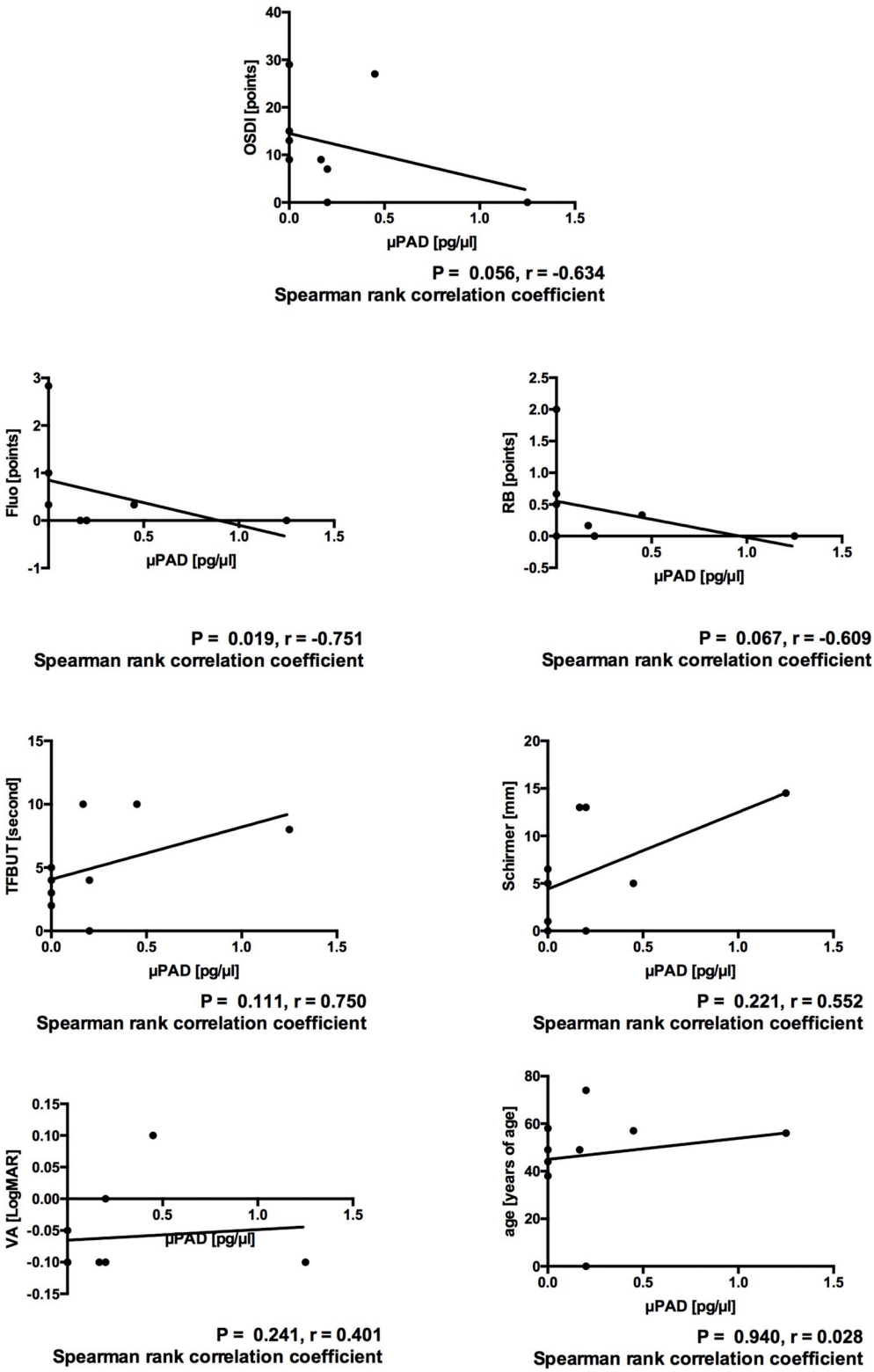

Fig. 3. Relationship between lactoferrin concentration and clinical parameters regarding dry eye using a microfluidic paper-based analytical device (μPAD). Y-axis; value of ocular surface disease index (OSDI), fluorescein staining scores (Fluo), rose bengal staining scores (RB) staining, tear film breakup time (TFBUT), Schirmer test (Schirmer), visual acuity, and age. X-axis; lactoferrin concentration measured using μPAD.

3.1. Comparison of the microfluidic paper-based analytical device and ELISA for lactoferrin assays in tear fluid

The lactoferrin concentration determined using the new μPAD was reproducible in cases other than GVHD when compared with the ELISA method. A positive correlation was noted between the lactoferrin concentration results measured using ELISA and μPAD (P = 0.006, r = 0.886) (Fig. 2). The fluorescein staining score and lactoferrin concentration were inversely correlated (P = 0.019, r = - 0.751), and negative correlations were noted between lactoferrin concentration and OSDI or rose bengal staining score (P = 0.056, r = - 0.634 and P = 0.067, r = - 0.609, respectively). TFBUT and Schirmer test values

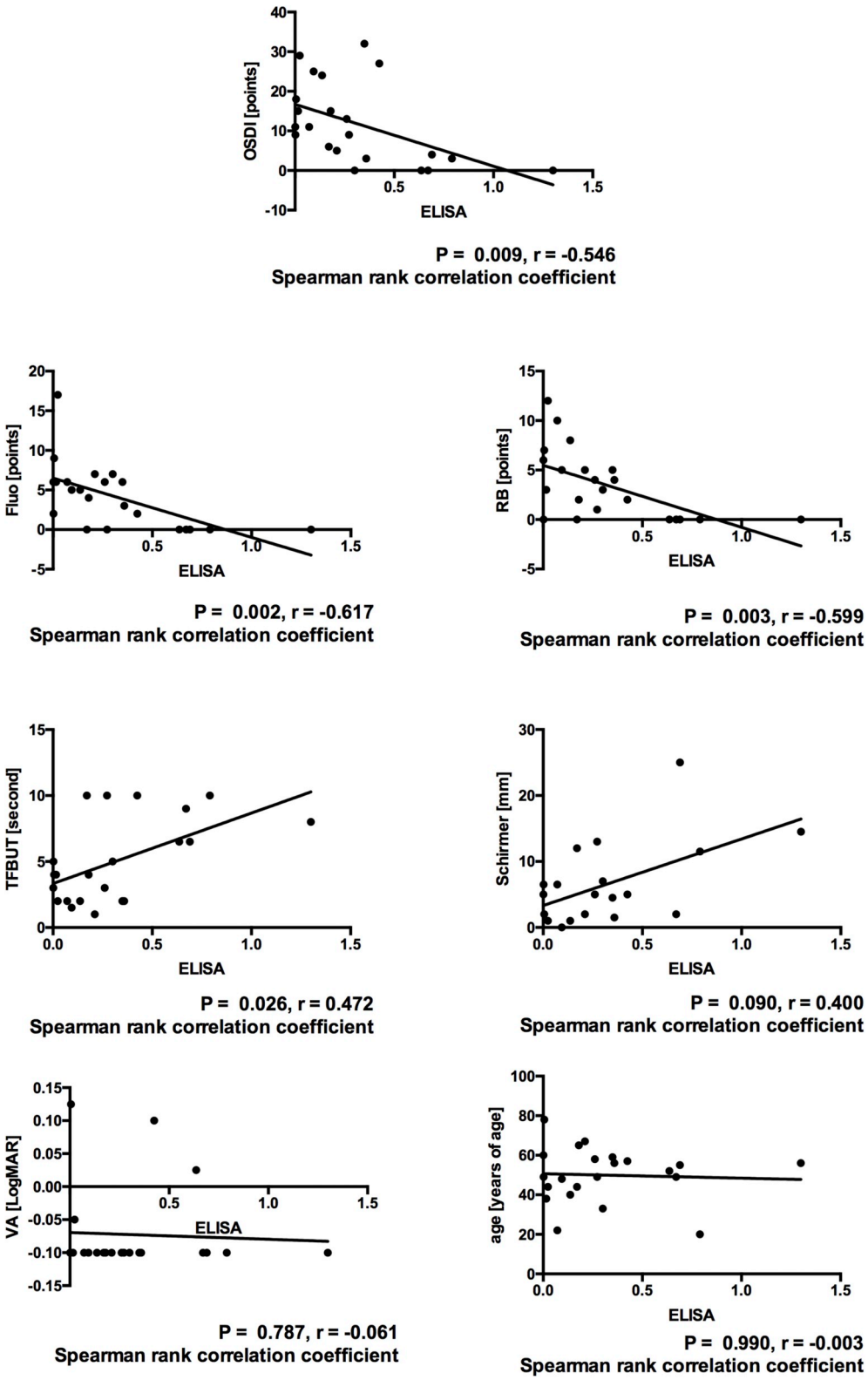

Fig. 4. Relationship between lactoferrin concentrations and clinical parameters regarding dry eye using ELISA. Y-axis; value of ocular surface disease index (OSDI), fluorescein staining scores (Fluo), rose bengal staining scores (RB), tear film breakup time (TFBUT), Schirmer test (Schirmer), visual acuity, and age. X-axis; lactoferrin concentration measured by ELISA.

tended to have a positive correlation with lactoferrin concentrations (P = 0.111, r = 0.750; P = 0.221, r = 0.552, respectively). No correlations were noted between lactoferrin concentration and visual acuity, or age (Fig. 3).

3.2. Study of the relationship between lactoferrin concentration and clinical parameters regarding dry eye (ELISA method)

Regarding the lactoferrin concentration measured by the ELISA method, the lactoferrin concentration in the tear fluid was significantly

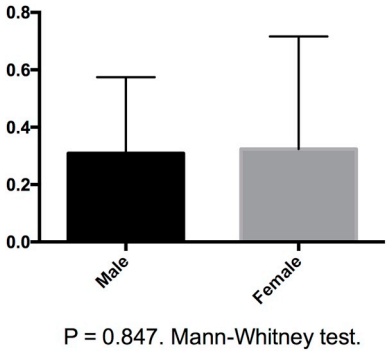

Fig. 5. Comparison of lactoferrin concentration between males and females. Y-axis; lactoferrin concentration measured by ELISA.

decreased in the GVHD group when compared with the non-GVHD group (p = 0.010576).

Overall, our ELISA method revealed low lactoferrin concentrations in tear fluid from GVHD and OCP patients, slightly low lactoferrin concentrations in Sjögren's syndrome patients, and high lactoferrin concentrations in simple dry eye and healthy subjects (Table 1).

Positive correlations were noted between lactoferrin concentrations and TFBUT or Schirmer test values (P = 0.026, r = 0.472; P = 0.090, r = 0.400, respectively). Negative correlations were noted between OSDI score (P = 0.009, r = - 0.546), fluorescein staining score (P = 0.002, r = - 0.617) or rose bengal staining score (P = 0.003, r = - 0.559) and lactoferrin concentration. No obvious correlation was noted between lactoferrin concentration and visual acuity, age, and gender (Fig. 4). Lacroferrin concentration was not affected by gender (0.30899 ± 0.26551 pg/μg versus 0.32395 ± 0.39210 pg/μg; male versus female). There was no statistically significant differences of lactoferrin concentrations between genders (P = 0.847) (Fig. 5).

4. Discussion

In this study, we demonstrate that a novel and innovative μPAD is useful in measuring lactoferrin concentrations in human tear fluid affected by immune-mediated dry eye disease. Compared with the lactoferrin concentration obtained using the standard ELISA method, the concentration obtained using the new μPAD was reproducible in patients suffering from various types of immune-mediated dry eye disease. In addition, we could discriminate the differences between GVHD-related dry eye as aqueous tear deficient DED and non-GVHD without dry eye by measuring lactoferrin levels in tear fluid. Although several methods are available for measuring lactoferrin levels [16,20], a standardized method is required to examine lactoferrin concentrations in tear fluid to diagnosis or monitor dry eye in the clinical setting [11]. Measuring lactoferrin using this newly developed μPAD may become a useful tool for the standardized method because it is cheap without high cost instruments, antibody-free, rapid for quantification, and easy to perform.

Quantitative measurements of lactoferrin concentrations in human tear fluid from normal subjects has been reported using μPAD [15]. Identical lactoferrin concentrations within 6% error using μPAD and ELISA were reported in that study. In our study, a positive correlation was noted between ELISA and a novel paper-based microfluid method even in immune-mediated dry eye disease patients. The μPAD is a simple, rapid and considerable method for lactoferrin detection in tear fluid, and results are secured within 15 min of a single application of 2.5 μL of sample [15]. Slight discrepancies were noted between the significance of the relationship between lactoferrin concentration and clinical variables in analyses of TFBUT, Schirmer test values, and rose bengal staining scores. However, the overall tendency was similar between the two methods. The limited number of patients analyzed using μPAD may cause the differences in statistically significant values.

Regarding GVHD-related severe DED, lactoferrin was drastically reduced in tear film based on the low level of lactoferrin measured using ELISA methods. One possibility is that the μPAD method may not detect the extremely low concentration of lactoferrin in cGVHD. However, cGVHD lacrimal glands are significantly destroyed by excessive inflammation and fibrosis [21,22]; therefore, it is likely that a severely damaged cGVHD lacrimal gland cannot effectively produce lactoferrin. Based on the fluorescence emission distance, the μPAD successfully analyzed lactoferrin in human tear film with a lower limit of detection of 0.1 mg/mL [14]. Further studies are needed to improve the ability of μPAD to detect extremely low levels of lactoferrin in the tear film layer. The duration after the onset of immune-mediated DED, progression and perpetuation of DED, severity of DED, systemic condition, and local treatment may influence lactoferrin concentrations in patients with various types of immune-mediated DED. In addition, hematopoietic stem cell transplant recipients received irradiation as conditioning regimen before transplantation, which might reduce corneal sensitivity and result in a lower OSDI score than expected [19,23]. Although more studies are needed, (1) the timing of examination, (2) the severity of dry eye and (3) systemic and local treatment of DED may affect lactoferrin concentrations in tear fluid given that signs and symptoms fluctuate over time and vary significantly within different levels of dry eye disease severity [24].

The following study limitations are noted. 1) The methods of collecting human tear samples must be improved. 2) For severe cGVHD-related dry eye patients, lactoferrin is not detectable by μPAD because the lactoferrin concentration in severe DED related to cGVHD is extremely low according to the ELISA results for the same samples. The low limitation of measurement by μPAD is between 0.21 pg/μl and 0.26 pg/μl according to our data (Table 1). We need to determine the lowest limitation of measurement and improve detection in low-concentration samples. 3) According to our data, lacroferrin concentration was not affected by gender. Because there is small number of patients in this study, the gender differences may not be conclusive. Further study will be required to confirm it. In conclusion, we can measure the lactoferrin concentration in tear fluid of dry eye patients using the newly developed μPAD, which is cheap and easy to perform without the use of any antibodies or enzymes [25]. Assays performed on μPAD require a small sample volume, which is useful for samples with limited availability, such as tear film, especially from DED patients [26]. In particular, the concentration of lactoferrin in tear fluid from severe DED patients with cGVHD is extremely low, suggesting that this condition is related to the development of a severely damaged ocular surface in this intractable disease.

Further investigations revising our methods for more quantitative measurements of lactoferrin concentrations in tear fluid are clearly warranted. We need to perform further detailed analyses to assess the correlation our data with the underlying disease mechanisms. This device may be useful for dry eye diagnoses, monitoring the severity of dry eye and treatment strategies.

Funding

This work was partially supported by the Japanese Ministry of Education, Science, Sports, Culture and Technology, Japan (#26462668 and #18K09421) (YO); the Medical Research and Development Programs Focused on Technology Transfer: Development of Advanced Measurement and Analysis Systems (SENTAN) from the Japan Agency for Medical Research and Development (AMED), Japan (DC); and a Research Fellowship of the Japan Society for the Promotion of Science (JSPS) for Young Scientists, Japan (KY).

Conflicts of interest

KY, KS, and DC have a patent application for the microfluidic paper-

based analytical device. Application number: JP20140037523 20140227. The other authors have no financial and scientific conflicts of interest.

References

[1] Willcox MDP, Argueso P, Georgiev GA, Holopainen JM, Laurie GW, Millar TJ, et al. TFOS DEWS II tear film report. Ocul Surf 2017;15:366–403.

[2] Versura P, Giannaccare G, Vukatana G, Mule R, Malavolta N, Campos EC. Predictive role of tear protein expression in the early diagnosis of Sjogren's syndrome. Ann Clin Biochem 2018:4563217750679.

[3] Uchino Y, Mauris J, Woodward AM, Dieckow J, Amparo F, Dana R, et al. Alteration of galectin-3 in tears of patients with dry eye disease. Am J Ophthalmol 2015;159:1027–1035.e3.

[4] Narayanan S, Redfern RL, Miller WL, Nichols KK, McDermott AM. Dry eye disease and microbial keratitis: is there a connection? Ocul Surf 2013;11:75–92.

[5] Flanagan JL, Willcox MD. Role of lactoferrin in the tear film. Biochimie 2009;91:35–43.

[6] Grus FH, Podust VN, Bruns K, Lackner K, Fu S, Dalmasso EA, et al. SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye. Invest Ophthalmol Vis Sci 2005;46:863–76.

[7] Ohashi Y, Ishida R, Kojima T, Goto E, Matsumoto Y, Watanabe K, et al. Abnormal protein profiles in tears with dry eye syndrome. Am J Ophthalmol 2003;136:291–9.

[8] Danjo Y, Lee M, Horimoto K, Hamano T. Ocular surface damage and tear lactoferrin in dry eye syndrome. Acta Ophthalmol 1994;72:433–7.

[9] Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo CK, et al. TFOS DEWS II definition and classification report. Ocul Surf 2017;15:276–83.

[10] Tsubota K, Yokoi N, Shimazaki J, Watanabe H, Dogru M, Yamada M, et al. New perspectives on dry eye definition and diagnosis: a consensus report by the Asia dry eye society. Ocul Surf 2017;15:65–76.

[11] Wolffsohn JS, Arita R, Chalmers R, Djalilian A, Dogru M, Dumbleton K, et al. TFOS DEWS II diagnostic methodology report. Ocul Surf 2017;15:539–74.

[12] Clayton JA. Dry eye. N Engl J Med 2018;378:2212–23.

[13] Sambursky R, Davitt 3rd WF, Latkany R, Tauber S, Starr C, Friedberg M, et al. Sensitivity and specificity of a point-of-care matrix metalloproteinase 9 immunoassay for diagnosing inflammation related to dry eye. JAMA Ophthalmol 2013;131:24–8.

[14] Yamada K, Henares TG, Suzuki K, Citterio D. Distance-based tear lactoferrin assay on microfluidic paper device using interfacial interactions on surface-modified cellulose. ACS Appl Mater Interfaces 2015;7:24864–75.

[15] Yamada K, Takaki S, Komuro N, Suzuki K, Citterio D. An antibody-free microfluidic paper-based analytical device for the determination of tear fluid lactoferrin by fluorescence sensitization of Tb^{3+}. Analyst 2014;139:1637–43.

[16] Hetherington SV, Spitznagel JK, Quie PG. An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin. J Immunol Methods 1983;65:183–90.

[17] He J, Ogawa Y. In vivo confocal microscopy evaluation of ocular surface with graft-versus-host disease-related dry. Eye Disease 2017;7:10720.

[18] Uchino M, Yokoi N, Uchino Y, Dogru M, Kawashima M, Komuro A, et al. Prevalence of dry eye disease and its risk factors in visual display terminal users: the Osaka study. Am J Ophthalmol 2013;156:759–66.

[19] D'Agostino R, Belanger A, D'Agostino RBJ. A suggestion for using powerful and informative tests of normality. Am Statistician 1990;44:316–21.

[20] Abe T, Nakajima A, Matsunaga M, Sakuragi S, Komatsu M. Decreased tear lactoferrin concentration in patients with chronic hepatitis C. Br J Ophthalmol 1999;83:684–7.

[21] Ogawa Y, Yamazaki K, Kuwana M, Mashima Y, Nakamura Y, Ishida S, et al. A significant role of stromal fibroblasts in rapidly progressive dry eye in patients with chronic GVHD. Invest Ophthalmol Vis Sci 2001;42:111–9.

[22] Ogawa Y, Shimmura S, Kawakita T, Yoshida S, Kawakami Y, Tsubota K. Epithelial mesenchymal transition in human ocular chronic graft-versus-host disease. Am J Pathol 2009;175:2372–81.

[23] Wang Y, Ogawa Y, Dogru M, Tatematsu Y, Uchino M, Kamoi M, et al. Baseline profiles of ocular surface and tear dynamics after allogeneic hematopoietic stem cell transplantation in patients with or without chronic GVHD-related dry eye. Bone Marrow Transplant 2010;45:1077–83.

[24] Sullivan BD, Crews LA, Sonmez B, de la Paz MF, Comert E, Charoenrook V, et al. Clinical utility of objective tests for dry eye disease: variability over time and implications for clinical trials and disease management. Cornea 2012;31:1000–8.

[25] Martinez AW, Phillips ST, Whitesides GM, Carrilho E. Diagnostics for the developing world: microfluidic paper-based analytical devices. Anal Chem 2010;82:3–10.

[26] Yetisen AK, Akram MS, Lowe CR. Paper-based microfluidic point-of-care diagnostic devices. Lab a Chip 2013;13:2210–51.