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ORIGINAL ARTICLE

Comprehensive analysis of vitreous humor chemokines in type 2 diabetic patients with and without diabetic retinopathy

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Abstract

Aims To compare the vitreous levels of chemokines in diabetic patients with and without retinopathy. To find the relationship between stages of diabetic retinopathy (DR) and levels of vitreous chemokines.

Methods The study involved 20 non-diabetic and 20 diabetic patients without clinical signs of DR (NDR) and 40 diabetic patients with proliferative diabetic retinopathy (PDR). The vitreous humor was collected and the levels of 40 chemokines were measured using magnetic color-bead-based multiplex assay.

Results The control group, NDR group, PDR with vitreous hemorrhage (VH) group, and PDR with tractional retinal detachment group comprised 20, 20, 21, and 19 eyes, respectively. Only the concentration of CCL3 was significantly higher in the NDR group compared with the controls ($p=0.038$). Twenty-five types of chemokines were statistically higher in the PDR with VH group in comparison to NDR group (all $p<0.05$). All chemokines were statistically higher in the PDR with TRD group in comparison to NDR group (all $p<0.05$) apart from 3 chemokines: GM-CSF, MIF, and CCL3($p=0.086$, $p=0.109$, $p=0.094$, respectively). The concentration of CCL21, CCL15 in PDR with TRD group was significantly higher compared with PDR with VH group, while other 36 chemokines were not significantly different between PDR with VH group and PDR with TRD group.

Conclusions The inflammation gradually worsen with the progression of DR. CCL3 may be associated with the onset of early diabetic retinal damage, and CCL15 and CCL21 may be closely related to the formation of fibrovascular membrane and the progression of the end stage of DR.

Keywords Diabetic retinopathy · Type 2 diabetes mellites · Chemokines · Vitreous

Introduction

Diabetic retinopathy (DR) is a common microvascular complication of diabetic mellitus (DM). The estimate prevalence of any DR in adult diabetic population is 34.6% (93 million) worldwide [1], and it is a leading cause of blindness in working-age population [2]. Functional and anatomical evidences have confirmed pre-clinical neurovascular dysfunction in diabetic patients even in the absence of visible fundus lesions [3–6]. Thus, damage of the diabetic retinal may occur before the fundus lesions become visible.

Inflammation plays an important role in the pathogenesis and progression of DR, and DR may be considered as a low-grade inflammatory disease [7]. The previous studies have demonstrated the role of IL-1β, IL-6, IL-8, CXCL10, and CCL2 in patients with and without DR, and the increased levels of these factors were correlated with the severity of DR [8–12]. Thus, cytokines associated with inflammation may contribute to the pathogenesis of DR, and chemokines are also closely related to the development of this disease [11].

Chemokines are a family of small heparin-binding proteins, mostly known for their role in immune surveillance

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and inflammation [13]. They are able to guide the cells which migrate to the inflammatory cite. Although increased aqueous or vitreous levels of cytokines and chemokines were reported in diabetic patients with and without retinopathy [10–12], there are limited published studies on cytokine or chemokine levels in patients without the clinical signs of DR (NDR). Apart from the several chemokines mentioned above, we believed that a more complex chemokine network was involved in the inflammation of NDR and DR patients.

Therefore, we designed the current study to find the chemokines related to the pre-clinical stage and end stage of DR by comparing their levels in non-diabetic controls, NDR patients, and proliferative diabetic retinopathy (PDR) patients. Besides, we also aim to provide more evidences on the low-grade inflammation, occurring in NDR patients, and confirm the levels of chemokines in the vitreous using a panel detecting 40 kinds of chemokines in a single test.

Methods

This study was performed in accordance to the tenets of the Declaration of Helsinki and approved by the local Research Ethics Committee of the Guangdong Provincial People's Hospital (Number 2016232A). Informed consent was obtained from the subjects. Type 2 diabetes mellitus was diagnosed by endocrinologist using the diagnostic criteria of American Diabetes Association [14]. Diagnosis and classification of DR were confirmed according to the international clinical diabetic retinopathy and diabetic macular edema disease severity scales [15]. Normal controls comprised patients with idiopathic pre-retinal membranes, idiopathic macular holes, or rhegmatogenous retinal detachment without diabetes mellitus. Diabetic patients with idiopathic pre-retinal membranes, idiopathic macular holes, or rhegmatogenous retinal detachment without DR were classified as NDR group. PDR subjects were sub-divided into two groups according to the severity of the disease: a group for patients with vitreous hemorrhage (VH) and the other group for the patients with tractional retinal detachment (TRD). The exclusion criteria were as follows: (1) patients with other ocular conditions affecting the relatively normal state of the eye (glaucoma, uveitis et al.); (2) history of ocular surgery; (3) patients who received anti-VEGF treatment; (4) patients with a history of severe systemic inflammatory diseases. All the subjects underwent a complete ocular examination and measured the blood pressure, glycated hemoglobin (HbA1c), creatinine, blood urea nitrogen levels before surgery. 20 non-diabetic and 60 diabetic patients were recruited, including 46 males and 34 females. The control group, NDR group, PDR with VH, and PDR with TRD consisted of 20, 20, 21, and 19 eyes, respectively. All the patients underwent pars plana vitrectomy in accordance with the standardized operation procedures using the 23-gauge trocar and cannula system (Alcon Laboratories, Inc. Fort Worth, Tex. USA). About 0.2–0.4 ml of vitreous humor was aspirated into a sterile syringe before intraocular infusion. The vitreous samples were then immediately centrifuged at 3000 rpm at 4 °C for 10 min. The supernatants were aspirated and subsequently stored at − 80 °C until further analysis.

Bio-Plex Pro™ human chemokine panel 40-plex kit (Bio-Rad Laboratories, Inc., Hercules, CA. USA) was used to measure the concentrations of 40 human chemokines, including C-C motif ligand (CCL)21, C-X-C motif ligand (CXCL)13, CCL27, CXCL5, CCL11, CCL24, CCL26, CX3CL1, CXCL6, granulocyte macrophage colony-stimulating factor (GM-CSF), CXCL1, CXCL2, CCL1, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, CXCL10, CXCL11, CCL2, CCL8, CCL7, CCL13, CCL22, macrophage migration inhibitory factor (MIF), CXCL9, CCL3, CCL15, CCL20, CCL19, CCL23, CXCL16, CXCL12, CCL17, CCL25, and TNF-α. The experimental procedures were conducted according to the instructions of manufacturer and 50 µl of undiluted vitreous humor sample were used in the reaction. The final step of the assay is to analyze the fluorescence intensity from the immunoassay using the Bio-Plex™ 200 System (software version 6.1, Bio-Rad Laboratories). The concentration of chemokine factor lower than the limit of detection was regarded as non-measurable.

Statistical methods

The statistical analyses were performed using IBM SPSS statistics version 19.0 (IBM SPSS Statistics; IBM Corporation, Chicago, IL, USA). One-way ANOVA analysis was performed if the numerical variables were in parametric distribution, and Kruskal–Wallis *H* test was performed for evaluation of differences in chemokine levels and Bonferroni correction was used to adjust *p* values for multiple pairwise comparison between subgroups. Spearman's rank correlation coefficient was used to analyze the associations between levels of chemokines and stages of the disease. Statistical significance was accepted at $p < 0.05$.

Results

The clinical characteristics of the control group, NDR group, PDR with VH group, and PDR with TRD group are presented in Table 1. In comparison to the control group, the HbA1c levels of diabetic groups were significantly increased. The serum creatinine and BUN levels were both significantly higher in PDR with VH group and PDR with TRD group when compared with both the NDR group and the controls. There were no significant differences in the

Table 1 Clinical characteristics of the subjects

	Controls ($n=20$)	NDR ($n=20$)	PDR with VH ($n=21$)	PDR with TRD ($n=19$)	p value
Age (years)	58.40 ± 12.03	61.85 ± 11.49	56.28 ± 8.73	55.63 ± 7.64	0.218
Male/female	9/11	10/10	11/10	9/10	0.971
Duration of DM (years)	N/A	7.94 ± 2.74	10.15 ± 2.17	10.87 ± 3.18	N/A
SBP (mmHg)	130.55 ± 17.11	131.20 ± 13.85	133.00 ± 15.39	141.21 ± 15.19	0.127
DBP (mmHg)	76.65 ± 8.52	75.15 ± 8.15	79.81 ± 9.58	77.84 ± 1083	0.438
HbA1c (%)	5.65 ± 0.36	6.46 ± 0.85	8.05 ± 1.64	7.18 ± 1.02	< 0.001*
Serum creatinine (μmol/L)	74.91 ± 9.28	80.09 ± 14.54	114.56 ± 62.44	143 ± 76.04	< 0.001*
BUN (mmol/L)	5.23 ± 1.00	5.47 ± 1.22	9.38 ± 4.42	11.87 ± 7.11	< 0.001*

NDR diabetic with no retinopathy, *PDR* proliferative diabetic retinopathy, *VH* vitreous hemorrhage, *TRD* tractional retinal detachment, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *DM* diabetes mellitus, *BUN* blood urea nitrogen

*Statistically significant p value by one-way ANOVA

clinical characteristics of age, gender, and systolic and diastolic blood pressure among all the four groups.

The level of CCL17 was non-measurable in more than 50% samples of each group, so it was not included in the analysis. All the other chemokines in each group are summarized in Table 2. There were significant differences in the levels of all the 39 chemokines among the 4 groups (all $p < 0.001$). The subsequent comparison between the four groups are summarized in Table 3.

Only the concentrations of CCL3 were significantly higher in the NDR group compared with the controls ($p = 0.038$). However, there were no significant differences in the levels of CCL3 between diabetic groups (NDR group VS PDR with VH group, NDR group VS PDR with TRD group, PDR with VH group VS PDR with TRD group, $p = 1.000$, $p = 0.094$, $p = 0.103$, respectively, Fig. 1A). All the concentrations of the 39 chemokines were significantly higher in the PDR with VH group and PDR with TRD group in comparison to the controls (all $p < 0.05$).

The levels of 25 chemokines were statistically higher in the PDR with VH group in comparison to NDR group (all $p < 0.05$), including CXCL13, CCL27, CXCL5, CCL11, CCL24, CCL26, CXCL2, IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-16, CXCL11, CCL7, CCL13, CCL22, CXCL9, CCL19, CCL23, CXCL16, CXCL12, CCL25, and TNF-α. All chemokines were statistically higher in the PDR with TRD group in comparison to NDR group (all $p < 0.05$) apart from three chemokines: GM-CSF, MIF, and CCL3 ($p = 0.086$, $p = 0.109$, $p = 0.094$, respectively). The concentration of CCL21, CCL15 in PDR with TRD group was significantly higher compared with PDR with VH group (Fig. 1b, c), while other 37 chemokines were not significantly different between PDR with VH group and PDR with TRD group. Moreover, we found that increased CCL15 and CCL21 were positively associated with the stages of the disease ($r = 0.717$, $p < 0.001$ and $r = 0.719$, $p < 0.001$).

Discussion

The identification and evaluation of chemokines in the vitreous humor of diabetic patients may provide a better insight of the pathogenesis of DR. These chemotactic cytokines could be the future targets for further investigation and treatment. Most studies in NDR were based on aqueous humor or serum but not vitreous, since NDR patients usually do not need to be surgically intervened [10–12]. Cytokines and growth factors levels in the aqueous humor of diabetic patients do not reliably correlate with their counterparts in the vitreous, reflecting that vitreous protein levels must be tested for each protein biomarker of interest [16]. In our study, we revealed a network of chemokines using the vitreous humor of diabetic patients with severe retinopathy and without retinopathy. PDR patients (including VH and TRD) showed significantly increased levels of 39 chemokines. Out of the 39 chemokines, only CCL3 was significantly higher in the NDR group compared with the controls. Twenty-five chemokines were statistically higher in the PDR with VH group in comparison to NDR group, while 36 chemokines were statistically increased in the PDR with TRD group in comparison to NDR group. With the progression of DR, more and more chemokines are elevated, indicating that the inflammation gradually worsens. These findings in chemokines suggested that they were related to the early retinal damage and the progression of DR.

The previous studies have reported the functions and effects of angiogenic and inflammatory factors in patients with or without DR. For cytokines or chemokines, prior studies showed the significant increase of CX3CL1, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, CXCL10, CCL2, MIF, CXCL9, CXCL12, and TNF-α levels in patients with DR, and these biomarkers might be used to diagnose or prognose DR [8–12]. To our knowledge, most of the chemokines in our panel are seldom investigated in the vitreous of diabetic patients. There

Table 2 Summary of chemokine in the measured vitreous

Chemokine (pg/ml)	Control ($n=20$)		NDR ($n=20$)		PDR with VH ($n=21$)		PDR with TRD ($n=19$)		p value
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
CCL21	114.68	40.40	178.35	147.09	248.00	110.83	576.49	563.14	<0.001*
CXCL13	0.45	0.14	0.58	0.26	1.05	0.40	1.57	2.02	<0.001*
CCL27	1.42	0.48	3.06	2.73	7.12	5.06	18.62	45.94	<0.001*
CXCL5	171.80	17.42	154.90	26.26	259.41	36.97	309.29	340.29	<0.001*
CCL11	1.88	0.52	2.07	0.67	3.58	1.12	3.98	3.24	<0.001*
CCL24	4.39	0.60	3.90	0.51	7.46	2.36	9.91	7.72	<0.001*
CCL26	1.23	0.11	1.36	0.21	2.28	0.70	2.88	5.04	<0.001*
CX3CL1	6.15	1.65	9.25	6.10	11.89	7.13	21.56	21.70	<0.001*
CXCL6	0.23	0.63	0.86	0.50	2.18	0.76	3.26	3.92	<0.001*
GM-CSF	4.28	1.13	3.58	0.54	5.81	1.68	5.64	6.96	<0.001*
CXCL1	6.31	3.00	8.53	4.37	14.46	6.42	17.73	28.15	<0.001*
CXCL2	7.95	0.59	7.95	0.66	11.54	1.96	14.61	12.60	<0.001*
CCL1	4.42	0.58	5.08	1.27	7.11	1.34	8.65	5.45	<0.001*
IFN-γ	1.43	0.41	1.83	0.42	3.03	0.98	3.43	4.93	<0.001*
IL-1β	0.26	0.06	0.32	0.03	0.48	0.13	0.71	0.83	<0.001*
IL-2	0.53	0.02	0.63	0.17	1.23	0.54	1.44	1.48	<0.001*
IL-4	1.24	0.74	2.05	1.09	5.68	5.47	6.36	5.42	<0.001*
IL-6	8.20	9.94	8.66	19.90	22.09	39.05	77.60	147.28	<0.001*
IL-8	0.96	0.67	2.07	2.58	3.34	1.89	9.38	24.38	<0.001*
IL-10	0.83	0.15	0.97	0.38	2.12	0.69	3.30	2.33	<0.001*
IL-16	12.14	2.21	16.45	12.80	28.45	11.84	57.66	79.77	<0.001*
CXCL10	17.13	10.97	30.23	13.86	42.30	24.67	126.42	160.45	<0.001*
CXCL11	0.09	0.03	0.10	0.04	0.20	0.08	0.33	0.39	<0.001*
CCL2	63.57	34.95	87.72	48.13	133.23	73.36	192.56	841.34	<0.001*
CCL8	0.26	0.15	0.64	0.61	1.22	2.50	2.37	8.74	<0.001*
CCL7	8.74	1.50	8.74	0.02	14.39	4.09	17.08	14.44	<0.001*
CCL13	0.20	0.06	0.23	0.09	0.46	0.19	0.74	0.96	<0.001*
CCL22	3.84	0.60	4.51	1.23	7.12	2.20	10.79	22.34	<0.001*
MIF	166.77	177.09	366.13	351.78	502.44	1006.05	603.62	2815.21	<0.001*
CXCL9	5.22	1.47	11.27	8.81	26.23	21.38	90.34	221.12	<0.001*
CCL3	0.78	0.51	1.32	0.58	1.28	0.91	2.84	2.51	<0.001*
CCL15	70.57	31.16	121.22	46.13	176.79	332.08	423.80	431.99	<0.001*
CCL20	0.41	0.11	0.56	0.26	0.88	0.46	1.84	3.22	<0.001*
CCL19	3.61	1.71	6.79	8.30	20.21	14.43	45.44	68.04	<0.001*
CCL23	2.21	0.55	2.89	3.45	7.57	4.09	9.09	10.20	<0.001*
CXCL16	89.11	9.82	86.53	17.26	151.21	47.69	214.62	365.95	<0.001*
CXCL12	45.11	9.35	57.32	23.75	87.45	22.76	142.69	151.83	<0.001*
CCL25	7.56	2.38	8.90	3.99	20.76	7.18	33.77	82.19	<0.001*
TNF-α	0.82	0.49	1.71	1.59	5.10	3.68	6.65	9.26	<0.001*

IQR interquartile range, *NDR* diabetic with no retinopathy, *PDR* proliferative diabetic retinopathy, *VH* vitreous hemorrhage, *TRD* tractional retinal detachment, *CCL* C-C motif ligand; *CXCL* C-X-C motif ligand, *GM-CSF* granulocyte macrophage colony-stimulating factor, *IFN-γ* interferon-gamma, *IL* interleukin, *MIF* macrophage migration inhibitory factor, *TNF-α* tumor necrosis factor-alpha

*Statistical significant p value by the Kruskal–Wallis test

are limited studies on CCL20, CCL22, CCL25, CCL23, CXCL11, and CXCL16 in the ocular humor of diabetic patients. According to the data, we have known about the 39 chemokines in all studies (including studies on other diseases or situations), a complex chemokine network based on the STRING database is demonstrated in Fig. 2. The STRING database (http://string-db.org) could provide a critical assessment and integration of protein–protein

Table 3 Adjusted *p* values of DR groups and the control group

Chemokine	Adjusted *p* values					
	Control-NDR	Control-PDR with VH	Control-PDR with and TRD	NDR-PDR with VH	NDR-PDR with TRD	PDR with VH-PDR with TRD
CCL21	0.131	<0.001*	<0.001*	0.279	<0.001*	0.026*
CXCL13	0.153	<0.001*	<0.001*	0.014*	0.003*	1.000
CCL27	0.202	<0.001*	<0.001*	0.022*	<0.001*	0.846
CXCL5	1.000	<0.001*	<0.001*	0.002*	0.001*	1.000
CCL11	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CCL24	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CCL26	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CX3CL1	0.270	<0.001*	<0.001*	0.135	0.001*	0.568
CXCL6	0.904	<0.001*	<0.001*	0.01*	<0.001*	1.000
GM-CSF	0.609	0.020*	<0.001*	0.320	0.086	1.000
CXCL1	0.413	<0.001*	<0.001*	0.075	0.020*	1.000
CXCL2	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CCL1	0.172	<0.001*	<0.001*	0.095	0.006*	1.000
IFN-γ	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
IL-1β	0.150	<0.001*	<0.001*	0.007*	<0.001*	1.000
IL-2	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
IL-4	0.418	<0.001*	<0.001*	0.007*	0.001*	1.000
IL-6	1.000	0.001*	<0.001*	0.114	<0.001*	0.572
IL-8	0.161	<0.001*	<0.001*	0.486	0.070*	0.685
IL-10	0.386	<0.001*	<0.001*	0.012*	<0.001*	1.000
IL-16	0.576	<0.001*	<0.001*	0.033*	<0.001*	1.000
CXCL10	0.146	<0.001*	<0.001*	0.487	0.020*	0.375
CXCL11	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CCL2	0.785	0.001*	<0.001*	0.099	0.001*	0.916
CCL8	0.059	<0.001*	<0.001*	0.105	0.004*	1.000
CCL7	1.000	<0.001*	<0.001*	0.001*	<0.001*	1.000
CCL13	1.000	<0.001*	<0.001*	0.003*	<0.001*	1.000
CCL22	1.000	<0.001*	<0.001*	0.003*	<0.001*	0.916
MIF	0.300	0.008*	<0.001*	1.000	0.109	1.000
CXCL9	0.097	<0.001*	<0.001*	0.021*	<0.001*	1.000
CCL3	0.014*	0.013*	<0.001*	1.000	0.091	0.111
CCL15	0.765	0.022*	<0.001*	1.000	<0.001*	0.002*
CCL20	0.255	<0.001*	<0.001*	0.103	<0.001*	0.399
CCL19	0.104	<0.001*	<0.001*	0.033*	0.001*	1.000
CCL23	0.794	<0.001*	<0.001*	0.001*	<0.001*	1.000
CXCL16	1.000	<0.001*	<0.001*	<0.001*	<0.001*	1.000
CXCL12	0.997	<0.001*	<0.001*	0.009*	<0.001*	0.644
CCL25	1.000	<0.001*	<0.001*	0.001*	<0.001*	1.000
TNF-α	0.403	<0.001*	<0.001*	0.003*	<0.001*	1.000

PDR proliferative diabetic retinopathy, *NDR* diabetic without DR, *VH* vitreous hemorrhage, *TRD* tractional retinal detachment

*Statistical significant

interactions, including direct (physical) as well as indirect (functional) associations [17]. One of the functions of the chemokines is to mediate leukocytic activation and migration, which may lead to leukostasis, eventually lead to capillary occlusion and retinal hypoxia. Our findings provide a more comprehensive insight on the roles of inflammation in DR.

CCL3 was increased in NDR group, PDR with VH group, and PDR with TRD group in comparison to the controls, suggesting that CCL3 may be associated with the early

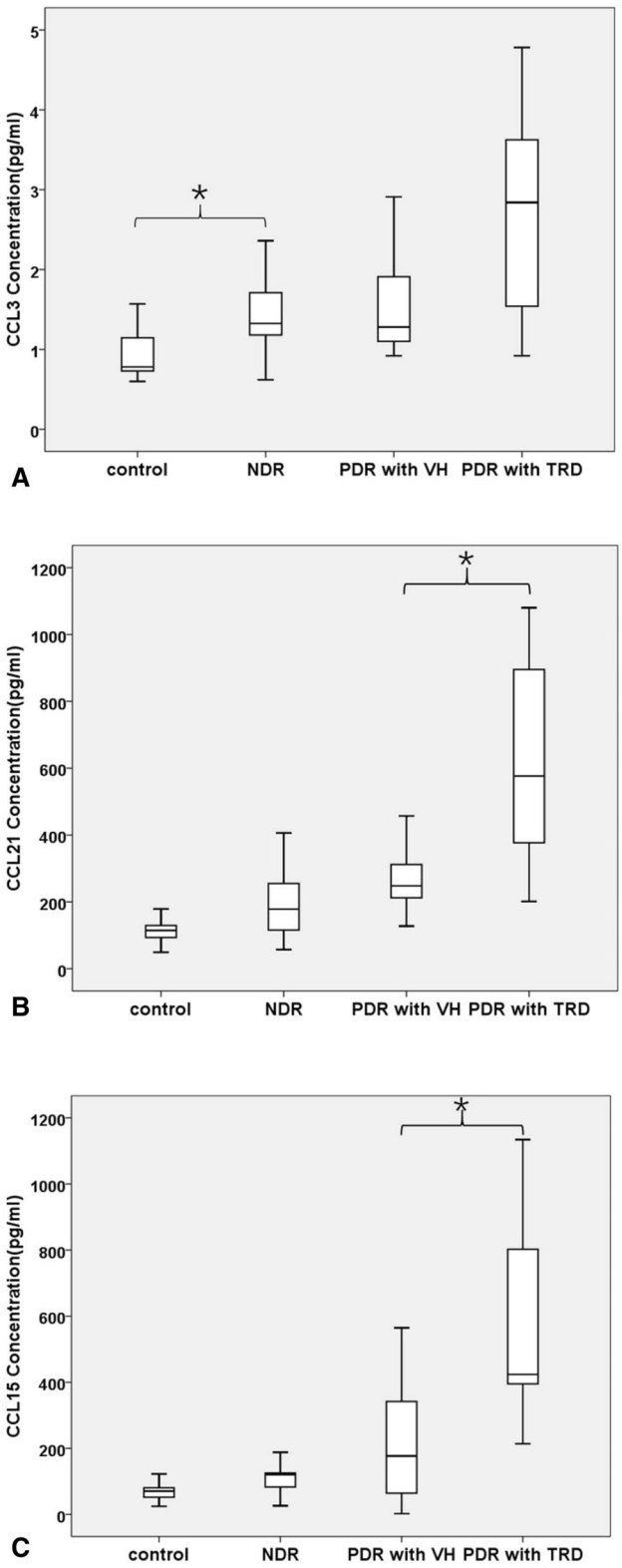

Fig. 1 Levels of CCL3 (1A), CCL21 (1B), and CCL15 (1C), and comparisons between subgroups. CCL3 was significantly higher in the NDR group compared with the controls ($p=0.038$); the concentration of CCL21 and CCL15 in PDR with TRD group was significantly higher compared with PDR with VH group; *statistically significant. *NDR* diabetic with no retinopathy, *PDR* proliferative diabetic retinopathy, *VH* vitreous hemorrhage, *TRD* tractional retinal detachment, *CCL* C-C motif ligand; *Statistically significant *p* value by Kruskal–Wallis test

retinal damage and progression of DR. Vujosevic et al. found that there were significantly elevated IFN-γ, IL-1α, IL-3, and CCL8 in the aqueous humor of NDR patients compared to non-diabetic controls [18]. The previous study had successfully detected CCL3 in the serum of NDR patients using multiplex array, but it was not significantly different from non-diabetic controls [19]. The levels of CCL3 in the vitreous of PDR patients were undetectable using enzyme-linked immunosorbent assay [20, 21]. These controversial findings may result from different samples and methods. In the current study, the duration of DM was relatively shorter in NDR group than that in PDR group, and we could see a rising trend in CCL3 level from NDR to PDR with TRD (median of 1.31 pg/ml and 2.84 pg/ml in NDR group and PDR with TRD group), although not significant in intergroup comparison. Therefore, we assumed that the CCL3 might be associated with the onset of early diabetic retinal damage. Kohno et al. found that CCL3 production by microglial cells modulated disease severity in mouse model of Stargardt disease and mouse model of retinitis pigmentosa, implicating that CCL3 may be a critical regulator of retinal inflammation and degeneration [22]. In diabetes, retinal microglia are also involved in diabetic retinal inflammatory changes, and retinal neurodegeneration might precede microvascular changes in the early DR [23]. Taken together, the proinflammatory chemokine CCL3 may be related to the early neurovascular impairment of DR. Therefore, CCL3 is potential target for therapeutic intervention in early DR.

In comparison to NDR group, 25 chemokines were statistically higher in the PDR with VH group, while there were 36 in PDR with TRD group. The levels of GM-CSF, MIF, and CCL3 in PDR with TRD group were comparable with PDR with VH group. The previous study found that the levels of adhesion molecules in peripheral blood were correlated with the stages of DR [24]. PDR with TRD is the most severe complication of diabetic eye disease, and our study supported that the levels of proinflammatory factors in the vitreous are positively correlated to the severity of DR. Furthermore, the elevated chemokines are likely to be associated with the transition from the early stage of PDR to the end stage of PDR.

IL-10 and IL-4 were believed to be an anti-inflammatory factor in diabetic retinopathy [25, 26]. However, there were controversial evidences on the roles of IL-10 and IL-4. Some studies found that the levels of IL-10 and IL-4 were increased in DR, while some found the opposite result [10, 12, 27]. There might be a differential expression of IL-10 and IL-4 in different situation of DR. However, increased

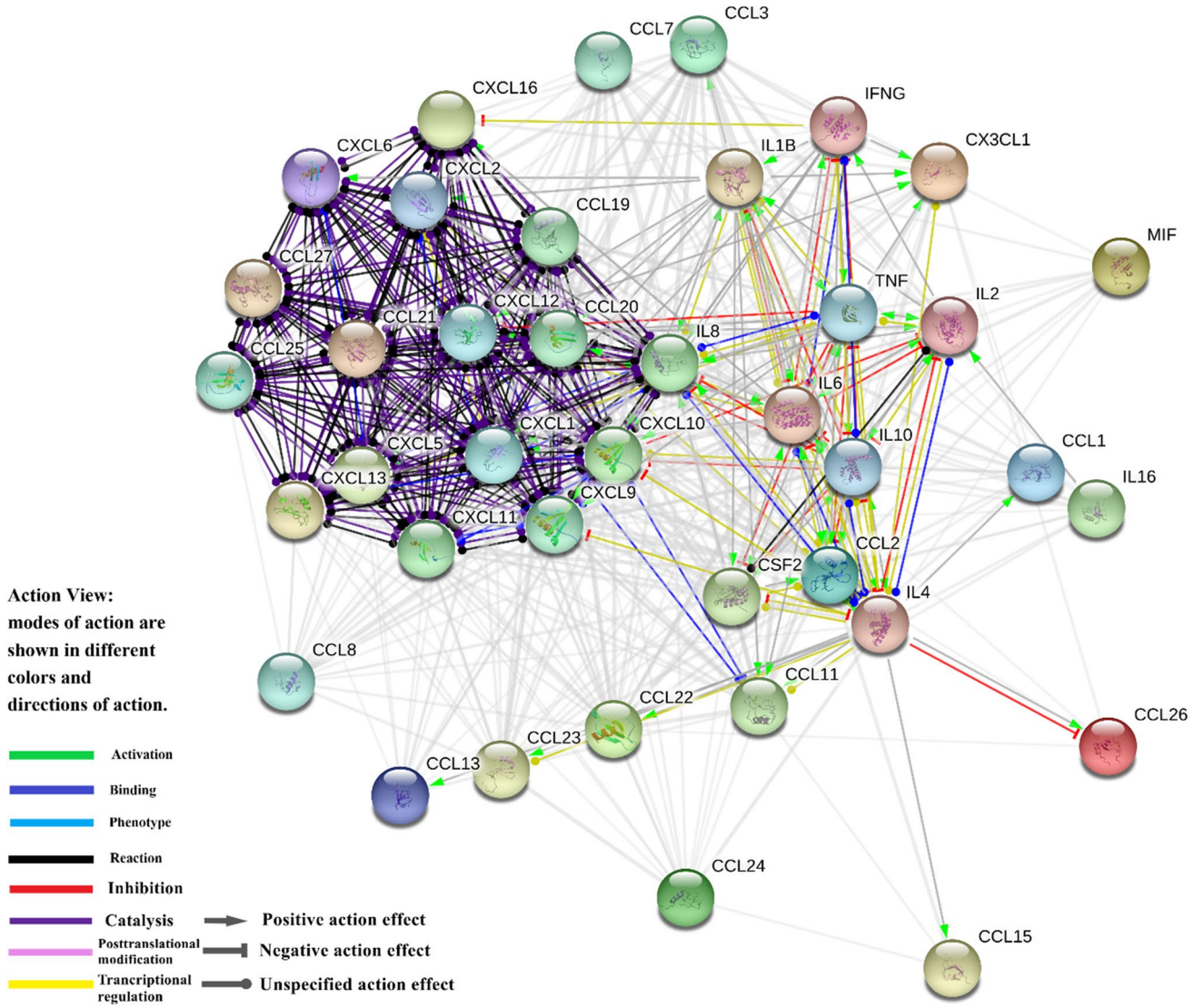

Fig. 2 A string pathway demonstrating the relationships of the 39 chemokines. *IFN-γ* interferon-gamma, *IL* interleukin, *MIF* macrophage migration inhibitory factor, *CCL* C-C motif ligand, *CXCL* C-X-C motif ligand, *CSF-2=GM-CSF* granulocyte macrophage colony-stimulating factor, *TNF-α* tumor necrosis factor-alpha

levels of IL-10 and IL-4 might be a compensatory mechanism of inflammation. Study found that transplantation of IL-10-transfected endothelial progenitor cells (EPCs) significantly improved EPC-mediated retinal vascular repair and subsequently suppressed NPDR progression [26]. Another study showed that IL-4 ameliorated high glucose and interleukin-1β stimulated inflammatory reaction in human retinal endothelial cells and retinal pigment epithelial cells [28]. Nevertheless, more investigations are needed to confirm the exact roles of IL-10 and IL-4 in DR.

The concentration of CCL15 and CCL21 was significantly higher in PDR with TRD group compared to PDR with VH group, suggesting that these two chemokines may be related to the progression of PDR. There were limited researches on CCL15 in diabetic retinopathy, and we found that CCL5 was relatively isolated in Fig. 2. CCL15 has the ability to stimulate the migration and differentiation of the chemotactic endothelial cell, and it has in vitro and in vivo angiogenic activity, suggesting its role in angiogenesis [29]. However, the studies on the role of CCL15 in PDR are very limited. To our knowledge, there was even fewer research on CCL21 on diabetic retinopathy. CCL21 exerts its function by binding to the C–C chemokine receptor type 7(CCR7). Shan et al. found that CCR7 and its ligands are important in the recruitment of T cells into inflamed pancreatic islets and, thus, in the pathogenesis of type I diabetes mellitus [30]. Moreover, studies had found that CCL21 had an important role in regulating the functions of fibrocyte, and might

contribute to hepatic fibrosis, idiopathic pulmonary fibrosis, and renal fibrosis [31–33]. Therefore, CCL5 and CCL21 may be related to the progression of PDR, facilitating the formation of fibrovascular membrane in the diabetic retina.

There were several limitations in the current analysis. The sample size should be larger. It would be more reasonable if we had vitreous sample of non-proliferative DR patients. However, we cannot get such samples in patients who do not need surgical treatment. Besides, we did not collect the serum sample, because the previous studies had showed that circulating cytokines correlated poorly with ocular cytokines. We could not study the systemic circulating chemokines and compare with ocular chemokines.

In summary, the concentrations of chemokines were elevated in patients with PDR and the levels of proinflammatory factors in the vitreous may indicate the progression of inflammation. CCL3 is significantly increased in the absence of any visible fundus lesions in diabetic eyes, suggesting that CCL3 is associated with the pathogenesis of early retinal damage in diabetic patients without DR. Considering the finding that the levels of CCL15 and CCL21 was significantly higher in PDR with TRD group compared to PDR with VH group and their roles in angiogenesis and fibrosis, we believe that CCL5 and CCL21 are related to the formation of fibrovascular membrane in the diabetic retina and the progression of PDR. Further investigations are needed to verify the relationships and roles of CCL3, CCL15, and CCL21 in diabetic retinopathy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical standard statement This study was performed in accordance to the tenets of the Declaration of Helsinki and approved by the local Research Ethics Committee of the Guangdong Provincial People's Hospital (Number 2016232A).

Informed consent Informed consent was obtained from the subjects.

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