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Original Article

Combination of serum HMGB-1 and serum miR-126 achieve high predictive power to detect proliferative diabetic retinopathy: a Chinese population-based study

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Abstract: Objectives: Proliferative diabetic retinopathy (PDR) refers to a more advanced stage of DR, which is one of the main microvascular complications of diabetes mellitus (DM), and in the present study, serum HMGB-1 and serum miR-126 were assessed as possible diagnostic biomarkers in a novel cohort of patients with PDR. Methods: The present study included 119 patients with type 2 DM (T2DM). Among them, 36 had PDR (PDR group), 43 had non-PDR (NPDR group) and 40 had no DR (T2DM group). Additionally, 30 healthy subjects (Control group) were also enrolled. Serum samples were collected from all subjects and HMGB-1 levels were detected by an enzyme-linked immunosorbent assay (ELISA). Reverse transcription-quantitative PCR (qRT-PCR) was conducted to detect serum miR-126 expression. Receiver operating characteristic (ROC) curve analysis was performed to assess the predictive power of the markers. In addition, Pearson correlation analysis was used to study correlations between the variables. Results: The concentrations of HMGB-1 in the serum samples were significantly higher, whereas the levels of serum miR-126 were lower, in patients with PDR as compared with other three groups of participants. ROC curve analysis showed that the combination of serum HMGB-1 and serum miR-126 had greater diagnosis capacity with an AUC (the areas under the ROC curve) of 0.884 (95% confidence interval (CI): 0.811-0.956, P<0.001). Serum HMGB-1 and miR-126 were closely associated with several parameters of PDR patients, including duration of DM, HbA1c, TNF-α, and VEGF levels. Conclusions: Taken together, our findings indicate a potential benefit of using serum HMGB-1 and serum miR-126 in a panel to improve diagnostic accuracy in PDR.

Keywords: Proliferative diabetic retinopathy, HMGB-1, miR-126, serum biomarker, diagnosis

Introduction

It is widely acknowledged that diabetes mellitus (DM) has become an epidemic disease in both developed and developing countries [1]. Diabetic retinopathy (DR), a frequently occurring complication of the late phase DM, is the leading cause of blindness among adults of working age [2]. During the initial stages of DR, patients often do not present any noticeable symptoms, but by the time some vascular lesions are detected in the retina, their vision loss could become irreversible [3, 4]. The incidence and prevalence of DR is increasing currently partially due to the increased life expectancy and lifestyle changes [5]. Accordingly, there is an urgent need to identify novel noninvasive biomarkers with high sensitivity and specificity for the early and accurate diagnosis of this devastating disease.

Chronic, low-grade inflammatory reactions are implicated in the pathophysiology of DR [6]. High-mobility group box-1 (HMGB-1), a member of HMG protein superfamily, was originally regarded as a critical mediator for maintaining the structure and stability of the chromosome in various cells, including eukaryotic cells [7]. As a pro-inflammatory cytokine, extracellular HMGB-1 translocation during inflammatory responses causes significantly elevated in vivo serum levels in patients with inflammatory disorders [8]. DM is also related to inflammatory dysfunction, and HMGB-1 is reportedly associated with DM or high glucose condition in previous studies [9, 10]. HMGB-1 levels are upregulated in vitreous fluid and epiretinal membranes from DR [11, 12].

As a class of small, non-coding RNAs, microRNAs (miRNAs) have been found in several

human body fluids, including blood, in a stable form that is protected from degradation [13]. miRNAs are accessible through non-invasive methods, and can be easily measured. These advantages have led to the proposal that circulating miRNAs may be useful indicators for the investigation of various diseases, including DR [14].

Proliferative diabetic retinopathy (PDR) is a prime manifestation of DR that is responsible for visual loss affecting the life quality of DM patients. In the present work, the utility of serum HMGB-1 and serum miR-126 as biomarkers was determined for diagnosis of PDR. Their associations with relevant clinical parameters were also investigated.

Material and methods

Subjects

A total of 149 Chinese subjects (age ≥18 years), including 119 patients with T2DM and 30 healthy controls, were recruited from No.1 People's Hospital of Jining City (Jining, China) from May 2016 to December 2016. All subjects underwent a general physical examination and a complete ophthalmic examination. The presence of T2DM was confirmed based on the guidelines by the World Health Organization criteria (WHO) [15]. After undergoing ophthalmic examination, DR patients were categorized into a non-PDR (NPDR) group and a PDR group according to the international clinical classification of DR [16]. In brief, DR was classified as NPDR based on the presence of at least one definite retinal hemorrhage and/or microaneurysm, whereas patients were assigned to PDR when there was neovascularization and/or vitreous/pre-retinal hemorrhages. The severity of DR was graded based on the worst eye. Patients with T2DM and no evidence of retinopathy were recruited in the T2DM group. Exclusion criteria: [1] patients with systemic diseases, including cancers and cardiovascular diseases; [2] patients with acute complications, including diabetic ketoacidosis and diabetic nephropathy. No subjects received any treatment prior to enrollment in the study. All experimental protocols were performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of No.1 People's Hospital of Jining City. Prior to participation, all subjects gave written informed consent.

Determination of serum HMGB-1 levels

A total of 5 mL of whole blood was collected from the patients and healthy controls at their first visit. Serum samples were obtained after centrifugation at 800 × g for 10 minutes, aliquoted, and stored at -80°C until further analysis. Serum levels of HMGB-1 was determined by commercially available ELISA kits (Quantikine, XiTang, Inc., Shanghai, China), according to the manufacturers' instructions.

RNA isolation and miRNA detection by qRT-PCR

Total RNA was isolated using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) from 200 μL serum. The concentration and purity of RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (2 μg) was reverse transcribed to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using the SYBR Green I PCR Master Mix (TaKaRa, Japan) on Applied Biosystems 7500 Real Time PCR system (Applied Biosystems). For serum samples, no internal control was appropriate for normalization, and accordingly, 2 μL of 25 fmol synthetic Caenorhabditis elegans miR-39-3p (Qiagen) was spiked-in to all samples before RNA isolation for normalization. The relative expression level of hsa-miR-126 was calculated using the $2^{-\Delta\Delta Ct}$ method [17]. The ΔCt was obtained by subtracting the cycle threshold (Ct) values of cel-miR-39-3p from the Ct values of hsa-miR-126. The sequences of primers are listed as follows: hsa-miR-126, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCGTA-3', forward primer: 5'-CATTATTACTTTTGG-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3'; cel-miR-39-3p, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCT-3', forward primer: 5'-TCACCGGGTGTAAATC-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3'.

Statistical analysis

All analyses were performed with SPSS 13.0 software (Chicago, Ill., USA) and GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data have been summarized and are

Table 1. Demographic and clinical characteristics of the study subjects

Characteristic	Control (n=30)	T2DM (n=40)	NPDR (n=43)	PDR (n=36)
Age (yr)	53.6±9.1	54.2±8.9	53.3±8.2	52.7±8.4
Gender (M/F)	17/13	25/15	26/17	21/15
Duration of DM (yr)	0.0±0.0	6.1±3.4	12.2±5.9	16.1±6.6
BMI (kg/m^2)	24.5±2.1	23.1±2.8	23.6±2.6	24.0±2.8
Systolic BP (mmHg)	123.5±8.4	121.5±9.0	122.9±8.5	123.8±9.2
Diastolic BP (mmHg)	81.9±4.1	83.5±3.8	82.2±4.3	83.3±4.5
HbA1c (%)	5.5±0.6	7.8±0.9	9.2±1.3	9.5±1.5
TNF-α (ng/L)	11.5±1.6	20.6±3.6	24.7±3.3	26.2±4.3
IL-6 (ng/L)	86.3±19.6	199.8±28.3	243.2±29.5	246.5±27.4
VEGF (ng/L)	69.5±8.2	88.6±9.9	111.3±15.6	148.1±23.0

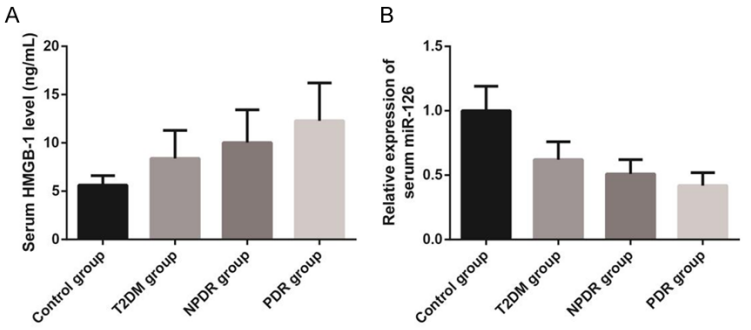

Figure 1. A. Comparison of serum HMGB-1 levels in different groups of subjects. B. Comparison of serum miR-126 levels in different groups of subjects. The data are presented as mean ± SD.

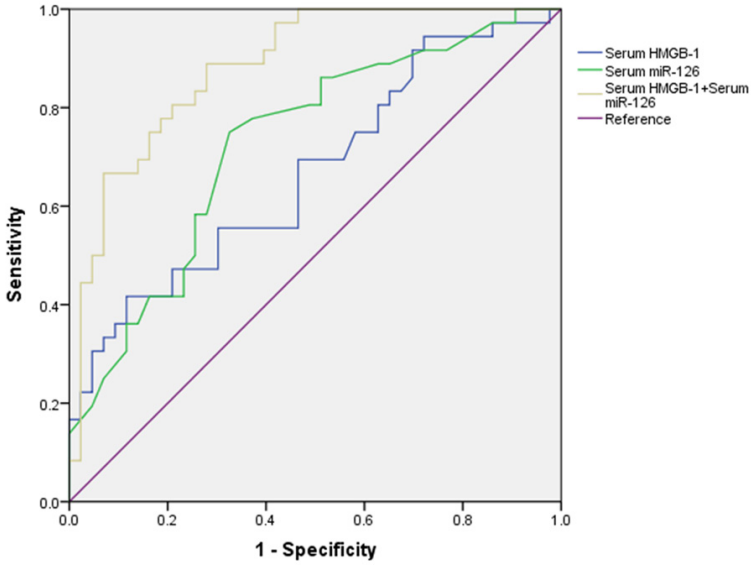

Figure 2. Receiver-operating characteristic (ROC) curves showing prognostic values of serum HMGB-1 and serum miR-126 to predict PDR.

presented as the mean ± standard deviation (SD). Receiver-operator characteristic (ROC) curves and the areas under the ROC curve (AUC) were established to evaluate the diagnostic values of serum HMGB-1 and serum miR-126. Pearson correlation analysis was used to study correlations between the variables. All comparisons were two sided, and a value of $P<0.05$ was considered statistically significant.

Results

This study enrolled 119 patients of T2DM, with 40 in T2DM group, 43 in NPDR group and 36 in PDR group. 30 healthy subjects were enrolled in the control group. **Table 1** summarizes the demographic, clinical, and laboratory characteristics of the four study groups. PDR patients had a longer duration of DM, and higher serum TNF-α and VEGF levels compared to NPDR patients.

As showed in **Figure 1A**, there was significantly higher serum HMGB-1 concentration in PDR patients than in the other three groups, and NPDR patients exhibited higher serum HMGB-1 concentrations compared with diabetic patients without DR and healthy controls. Furthermore, the levels of serum miR-126 were significantly reduced in PDR patients compared to the other three groups (**Figure 1B**).

ROC curve analysis also suggested that the combination of serum HMGB-1 and miR-126 could achieve high predictive power for discriminating PDR patients from NPDR patients, with an AUC of 0.884 (95% confidence interval (CI): 0.811-0.956, P<0.001; **Figure 2**).

Furthermore, the result of Pearson correlation analyses showed that in PDR patients, serum HMGB-1 was positively correlated with the

Table 2. Pearson correlation analysis between serum HMGB-1/miR-126 levels and various indexes in the PDR patients

Characteristic	HMGB-1		miR-126	
	r	*P*	r	*P*
Age (yr)	0.079	0.645	-0.174	0.311
Duration of DM (yr)	0.395	0.017	-0.319	0.058
BMI (kg/m^2)	0.248	0.144	-0.256	0.131
Systolic BP (mmHg)	-0.081	0.638	0.078	0.651
Diastolic BP (mmHg)	0.033	0.848	-0.101	0.558
HbA1c (%)	0.340	0.042	-0.274	0.106
TNF-α (ng/L)	0.372	0.025	-0.267	0.115
IL-6 (ng/L)	0.315	0.061	-0.319	0.058
VEGF (ng/L)	0.276	0.103	-0.491	0.002

duration of DM (r=0.395; P=0.017), HbA1c (r=0.340; P=0.042) and TNF-α (r=0.372; P=0.025) (**Table 2**) and that serum miR-126 was negatively correlated with VEGF (r=-0.491; P=0.002) (**Table 2**).

Discussion

Vision loss from DR is preventable with early intervention, however early detection of DR remains a difficult problem [18]. The current gold standard for diagnosing DR is stereoscopic slit lamp ophthalmoscopy/fundus photographic screening, but this method demands expensive equipment and highly specialized medical expertise. Body fluid-based markers are relatively non-invasive and therefore can be easily used to monitor patients. Currently, a growing number of circulating biomarkers have been indentified for the diagnosis and evaluation of DR.

It was well-known that PDR refers to a severe stage of DR and is featured by vitreous hemorrhage, retinal hemorrhage, and neovasculatization originating from the retina [19]. In the current study, the diagnostic values of serum HMGB-1 and serum miR-126 for PDR were evaluated. As a result, serum HMGB-1 levels were elevated, whereas serum miR-126 levels were markedly decreased in PDR patients. Furthermore, ROC analysis displayed that the combination of serum HMGB-1 and miR-126 had a potential function to distinguish PDR patients from NPDR patients, supporting their use in PDR diagnostics.

Elevated HMGB-1 mRNA and protein levels were previously found in retinal ganglion cells under high glucose environment [20] and diabetic rat retinal tissues [21]. Furthermore, the significant upregulation of HMGB-1 has been demonstrated to be associated with the progression of a series of DM complications, including diabetic cardiomyopathy [22, 23] and diabetic nephropathy [10, 24]. Thus serum HMGB-1 as a diagnostic biomarker for DR needs improvement. However, it is difficult to acquire a sensitive biomarker for a single disease. The diagnosis of a disease should be made based on comprehensive information including the clinical manifestations and symptoms.

miR-126 has a well-established link with DM complications. As an angiogenesis-related miRNA, miR-126 was found to be under-expressed in the retina tissues of streptozotocin-induced diabetic rats [25], and reduced miR-126 levels might be implicated in the development and progression of diabetic vascular complications [26]. miR-126 may suppress inflammation and ROS production in high glucose-treated endothelial cells through targeting HMGB1 [27]. Recently, several studies suggested that miRNAs from serum and other body fluids contained abundant biological information as well as offered diagnostic value [28]. Zampetaki and Zhang have reported a prognostic value of circulating miR-126 in identification of T2DM [29-31]. miR-126 exerts an inhibitory effect on VEGF, a potent angiogenic factor in carcinomas and retinal neovascularization [25, 32]. Our study also revealed a close correlation of serum miR-126 expression with VEGF levels in PDR patients.

To the best of our knowledge, this study is the first to identify the combination of serum HMGB-1 and miR-126 as potential biomarkers for the diagnosis of PDR. Nevertheless, there are still several inherent shortcomings in the present study. First, it involved a relatively low number of participants from a single center. The study is cross-sectional, accordingly dynamic changes in the levels of serum HMGB-1 and miR-126 could not be detected to explain the causalities among increased serum HMGB-1 levels, decreased serum miR-126 levels, and the development of PDR.

In conclusion, this study found that the levels of serum HMGB-1 and serum miR-126 were significantly changed and closely associated with

several clinical characteristics of PDR patients. Their combination is a better non-invasive biomarker to diagnosis PDR. Further investigation is necessary to confirm the results with a larger population from heterogeneous ethnicities for their general application in the future.

Disclosure of conflict of interest

None.

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