LncRNA AK077216 is downregulated in diabetic retinopathy and inhibited the apoptosis of retinal pigment epithelial cells by downregulating miR-383

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Abstract. LncRNA AK077216 is a novel lncRNA with critical role in osteoclastogenesis and bone resorption. Our preliminary RNA-seq data suggested its downregulated expression pattern in diabetic retinopathy (DR). We therefore further investigated the function of AK077216 in DR. Plasma samples used in this study were derived from blood, which was extracted from 60 diabetic retinopathy patients. AK077216 in plasma of DR patients, diabetic patients (DB) and controls (Control) was detected by RT-qPCR. ROC curve analysis was performed to evaluate the diagnostic value of plasma AK077216 for DR. It was found that plasma AK077216 was downregulated only in DR patients but not in diabetic patients (no obvious complications) comparing to healthy controls. Downregulation of AK077216 distinguished DR patients from diabetic patients and healthy controls. High glucose treatment failed to significantly affect AK077216 expression in cells of human retinal pigment epithelial cell line ARPE-19. MiR-383 was inversely correlated with AK077216 in DR patients. AK077216 overexpression caused miR-383 downregulation, but miR-383 overexpression did not significantly alter AK077216 expression. AK077216 overexpression inhibited ARPE-19 cell apoptosis, while miR-383 overexpression played an opposite role and attenuated the effects of AK077216 overexpression. Therefore, AK077216 is downregulated in diabetic retinopathy and inhibited the apoptosis of ARPE-19 cells by downregulating miR-383.

Key words: Diabetic retinopathy, LncRNA AK077216, miR-383, Human retinal pigment epithelial cell, Apoptosis

THE DEVELOPMENT AND PROGRESSION OF DIABETES cause a series of complications. It has been reported that more than 30% percent of diabetic patients have signs of diabetic retinopathy (DR), which is a common and severe diabetic complication [1, 2]. In addition, 10% of diabetic patients experience the development of vision-threatening lesions, such as proliferative retinopathy and macular edema [2]. Hypertension, long-term of high glucose environment, albuminuria and poor glycemic control are risk factors for DR [3]. Genetic studies have revealed several genetic alterations, such as altered expression of VEGF and aldose reductase contributes to DR [4, 5]. However, the complicated pathogenesis cannot be fully explained by the limited number of genetic alterations identified so far.

Long (>200 nt) non-coding RNAs (lncRNAs) are originally considered as the noise of transcriptome, but more and more studies in past 2 decades have showed that lncRNAs actually have critical functions in gene expression regulation [6, 7]. Dysregulation of lncRNAs may cause changes in expression levels of genes involved in human diseases [8]. Therefore, regulation of the expression of lncRNAs may contribute to disease treatment [9]. AK077216 is a novel lncRNA that has been proved to be with critical role in osteoclastogenesis and bone resorption [10]. Our preliminary RNA-seq data suggested its downregulated expression pattern in DR and its inverse correlation with miR-383, which has important functions in the development of DR [11]. We therefore further investigated the function of AK077216 in DR and explored its interaction with miR-383.

Materials and Methods

Plasma collection

Plasma samples used in this study were derived from blood, which was extracted from 60 diabetic retinopathy patients (proliferative stage, DR group, 34 males and 26 females, and 26 healthy controls).
females, 36 to 68 years, 52.5 ± 6.7 years), 60 patients with only diabetic (DB group, no complications, 34 males and 26 females, 33 to 67 years, 49.1 ± 6.3 years), and 60 healthy controls (Control group, 35 males and 25 females, 35 to 68 years, 51.9 ± 6.7 years). Those participants were admitted by Ningbo Medical Center Lihuili Eastern Hospital between January 2016 and January 2018. DR and DB patients were all diagnosed for the first time and no therapies were received before admission. Patients complicated with other medical disorders were excluded from this study. Three groups showed similar gender and age distributions. All healthy controls showed all physiological parameters within normal range. All participants signed informed consent. Before the admission of patients, Ethnic Committee of Ningbo Medical Center Lihuili Eastern Hospital approved this study.

Cell line and transient cell transfections
ARPE-19 (a human retinal pigment epithelial cell line) was used in this study to perform all in vitro cell experiments. Cells of this cell line were from ATCC (USA). Full length AK077216 cDNA was inserted into pcDNA3.1 vectors to establish AK077216 expression vector. Negative control miRNA and miR-383 mimic were from Sigma-Aldrich (USA). AK077216 siRNA and siRNA negative control were from RIBOBIO (Guangzhou, China). ARPE-19 were cultivated in DMEM:F12 Medium (10% FBS) at 37°C with 5% CO2 overnight to reach 70–80% confluence. All cell transfections were performed using Lipofectamine 2000 reagent (Invitrogen, USA) with 10 nM vector or 45 nM miRNA or siRNA. Untransfection cells were used as control, and cells transfected with negative control siRNA, negative control siRNA or empty vectors were negative control. Following experiments were performed at 24 h after transfection.

Real-time quantitative PCR
Extraction of total RNA from plasma and ARPE-19 were performed using Ribozol (Thermo Fisher Scientific, Inc.). In cases of high glucose treatment, ARPE-19 cells were treated with 5, 10, 20 and 30 nM D-glucose for 24 h and 48 h before RNA extraction. SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) was used to prepare cDNA samples, and SYBR Green Master Mix (Bio-Rad, USA) was used to prepare all PCR reactions with 18S rRNA as endogenous control to detect the expression of AK077216. This experiment was repeated 3 times and data were processed using 2-ΔΔCT method.

Cell apoptosis assay
ARPE-19 cells were collected at 24 h after transfections to prepare cell suspensions. Cell density was adjusted to 5 × 104 cell/mL. Cells suspensions were transferred to a 6-well plate with 2 mL per well, followed by the addition of 20 nM D-glucose. Cells were cultivated for 48 h and then were digested with 0.25% trypsin. Finally, cells were stained with Annexin V-FITC and propidium iodide (PI) and apoptotic cells were separated through flow cytometry.

Statistical analysis
All experiments were performed three times to obtain solid data. Differences among different groups of participants of different cell treatment groups were analyzed by one-way ANOVA and Tukey test. Linear regression was performed to analyze the correlation between AK077216 and miR-383. ROC curve analysis was performed to evaluate the diagnostic value of plasma AK077216 for DR with DR patients as true positive cases and DB or Control as true negative cases. Differences were statistically significant when p < 0.05.

Results
AK077216 in plasma was specifically downregulated in DR
AK077216 in plasma of DR patients, diabetic patients (DB) and controls (Control) was detected by RT-qPCR. Expression data were analyzed by one-way ANOVA and Tukey test. It was observed that AK077216 was significantly downregulated only in DR patients but not in DB patients comparing to Control (Fig. 1, p < 0.05).

AK077216 distinguished DR patients from DB and Control
ROC curve analysis was performed to evaluate the diagnostic value of plasma AK077216 for DR with DR patients as true positive cases and DB or Control as true negative cases. With DB as true negative cases, area under the curve was 0.86 (Fig. 2A; standard error: 0.032; 95% confidence interval 0.80–0.93; p < 0.001). With Control as true negative cases, area under the curve was 0.88 (Fig. 2B, standard error: 0.83; 95% confidence interval 0.83–0.94; p < 0.001).

High glucose did not significantly affect AK077216 in ARPE-19 cells
ARPE-19 cells were treated with 5 (control), 10, 20
and 30 nM D-glucose for 24 h at 48 h. Analysis of AK077216 expression in ARPE-19 cells was performed by performed RT-qPCR. At 24 h, comparison of AK077216 expression using one-way ANOVA and Tukey test showed no significant differences among different groups of cells (Fig. 3A). At 48 h, high glucose environment slightly downregulated AK077216 in a dose-dependent manner but decreases were not statistically significant (Fig. 3B).

**MiR-383 in DR patients was inversely correlated with AK077216**

MiR-383 in plasma of DR patients was also detected by RT-qPCR. Linear regression was performed to analyze the correlation between AK077216 and miR-383. It was observed that miR-383 inversely correlated with AK077216 in DR patients (Fig. 4).

**AK077216 regulated ARPE-19 cell apoptosis through miR-383**

AK077216 expression vector, AK077216 siRNA and miR-383 mimic were transfected into ARPE-19 cells. Comparing to negative control (C) and control (NC), AK077216 and miR-383 were significantly altered (Fig. 5A) at 24 h after transfection ($p < 0.05$). In addition, AK077216 overexpression caused miR-383 downregulation and AK077216 siRNA silencing caused miR-383 upregulation (Fig. 5B and C, $p < 0.05$), but miR-383 overexpression did not significantly alter AK077216 expression (Fig. 5C). Moreover, analysis of cell apoptosis data showed that, under 20 nM D-glucose, AK077216 overexpression inhibited ARPE-19 cell apoptosis, while miR-383 overexpression and AK077216 siRNA silencing played an opposite role and miR-383 overexpression attenuated the effects of AK077216 overexpression (Fig. 5D, $p < 0.05$).

**Discussion**

AK077216 is a novel lncRNA that has been proved to be with critical role in osteoclastogenesis and bone resorption [10]. However, the involvement of AK077216 in other types of human disease is still unknown. Our study reported that AK077216 was downregulated in DR, and overexpression of AK077216 may inhibit human retinal pigment epithelial cell apoptosis by down-regulating miR-383.

Retinal pigment epithelium is nourishes retinal visual cells [12]. Therefore, apoptosis of retinal pigment epithelial cells contributes to vision loss in DR patients [13, 14]. How to inhibit the apoptosis of retinal pigment epithelial cells is a major task in the prevention of DR...
among diabetic patients. It has been reported that the apoptosis of retinal pigment epithelial cells can be regulated by certain lncRNAs [15]. In the present study we found that AK077216 was downregulated in DR, and overexpression of AK077216 inhibited human retinal pigment epithelial cell apoptosis under high glucose treatment. Therefore, AK077216 may serve as a potential therapeutic target for DR. However, AK077216 expression in retinal pigment epithelial cells was not significantly affected by high glucose treatment. So, the altered expression of AK077216 in DR patients may be induced by long-term of high glucose treatment (not as short as 24 h in this study) or AK077216 dysregulation is induced by the development of eye lesions.

It has been reported that miR-383 can target peroxiredoxin 3 to induce the apoptosis of retinal pigment epithelial cells under high glucose treatment [11], thereby contributing to the development of DR. Our study confirmed the enhancing effects of miR-383 on retinal pigment epithelial cell apoptosis. However, pathogenesis of DR is unclear and the contribution of retinal pigment epithelial cell apoptosis to DR remains to be further analyzed. Our study also proved that AK077216 was likely an upstream inhibitor of miR-383 in regulating cell apoptosis. However, the mechanism that mediates the interaction between these two factors is unclear. It is known that lncRNAs may serve as miRNA sponge to inhibit their functions [16, 17]. However, no promising binding site of miR-383 was found on AK077216. Our future study will further explore the mechanism.

In a recent study Yan et al. reported that miR-383 inhibited colorectal cancer by directly targeting paired box 6 [18], which is a critical player in eye development [19]. Therefore, AK077216 may regulate paired box 6 through miR-383 to participate in physiological and pathological processes in eye. Our future studies will explore the interactions between AK077216 and paired box 6.

Our knowledge on the expression pattern of AK077216 is still limited. A recent study reported that AK077216 can be expression in primary bone marrow macrophages [12]. Our study showed that AK077216 can be expressed in RPEC. Its expression in other types of tissues is unknown. However, our apoptosis assay performed under high-glucose treatment may support the functional contribution of AK in DR.

In conclusion, AK077216 is downregulated in DR and overexpression of AK077216 may inhibit human retinal pigment epithelial cell apoptosis by downregulating miR-383.

Fig. 3  High glucose did not significantly affect AK077216 in ARPE-19 cells
ARPE-19 cells were treated with 5, 10, 20 and 30 nM D-glucose for 24 h (A) and 48 h (B). It was observed that high glucose did not significantly affect AK077216 in ARPE-19 cells.

Fig. 4  MiR-383 in DR patients was inversely correlated with AK077216
Linear regression analysis showed that miR-383 in DR patients was inversely correlated with AK077216.
Conflict of Interests

The authors declare they have no conflict of interests.
References


