Original Article Regulation of diabetes-induced inflammation in macrophages by microRNA-26a-5p

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Abstract: Background: Diabetes is one of the most common metabolic disorders and most important health threats. Recently, the risk of inflammation related to diabetes has been recognized. microRNAs (miRNAs) are non-coding small RNAs that regulate various physiological processes at the posttranscriptional level. Methods: In this study, we exanimated the role of diabetes induced miR-26a-5p. Results: miR-26a-5p is up-regulated and PTEN is down-regulated in the macrophages from diabetic mice. Moreover, miR-26a-5p overexpression enhances the expression of IL-1 β , IL-6 and TNF- α . Conclusion: Taken together, we conclude that miR-26a-5p participates in the inflammation process in diabetes and tight regulation of miR-26a-5p may be a potential of therapeutic interventions targeting miRNA in diabetes.

Keywords: Regulation, diabetes, inflammation, macrophages, microRNA-26a-5p

Introduction

Diabetes is a complex, multi-system disease that represents the most common metabolic disorder [1, 2]. Type 1 diabetes (T1D) is characterized by insulin deficiency, and type 2 diabetes (T2D) results from insulin resistance with or without abnormal insulin secretion [3]. Inflammation-related processes have been shown in diabetes development. Inflammation leads to insulin resistance which is the main cause of T2D. In addition, inflammation may also one of the key risk factors of diabetes complications.

Mature microRNAs (miRNAs) are a class of noncoding small RNAs (~21-23 nt) that play various important roles in many biological processes including chronic diseases like diabetes [4-8]. MiRNAs regulate gene and protein expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR) of specific mRNA through inducing mRNA degradation or repressing translation [9, 10]. Previous work has shown that miRNAs performed important roles in diabetes. Overexpression of miR-375 has been approved to impair insulin secretory pathway through suppressing myotrophin (Mtpn) [11, 12]. MiR-96 suppresses insulin secretion by decreasing nucleolar complex protein 2 (Noc2) [13]. MiR-29 family was found to be significantly up-regulated in the context diabetes. Additionally, miR-29a/b/c overexpression plays an important role in insulin resistance by decreasing insulin-induced glucose import via 3T3-L1 adipocytes [14].

Very little is known regarding the role of miRNAs in the inflammation progression of diabetes. In addition, the biological function of miRNAs in inflammatory regulation and their potential key target genes are still less clear. In this study, we identified miR-26a-5p as a diabetic induced miRNA in the mice macrophages related to a reduced phosphatase and tensin homolog (PTEN) expression. Further study revealed that miR-26a-5p overexpression up-regulated expression of IL-1 β , IL-6 and TNF- α . Therefore, our studies extend the previous investigations and provide explanations to the role of diabetes induced miR-26a-5p in the inflammation.

Materials and methods

Cell culture

Macrophages were isolated from wild type (WT) and diabetic mice as described previously [15].



Figure 1. MiR-26a-5p expression in macrophages from wild type (WT) and diabetic mice. Liver macrophages were isolated from WT and diabetic mice. MiR-26a-5p expression was determined by qRT-PCR to verify miR-26a-5p expression pattern from microarray. Data were normalized to U6 snRNA. Results are mean \pm SE (n = 3 cell preparations). *P<0.05 versus WT mice.

Isolated macrophages were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. After then, the isolated macrophages were treated with LPS (055:B5; Sigma-Aldrich, St Louis, MO) at 10 ng/ml for 16 h. MiR-26a-5p mimic and miRNA vector control (40 pmol) were transfected into macrophages from WT mice using lipofectamine RNAiMax (Life Technologies, Carlsbad, CA).

Quantitative real-time PCR

Total RNAs were extracted from macrophages using TRI Reagent (Molecular Research Center, Cincinnati, OH). DNA-free DNase (Ambion, Austin, TX) was used to remove the Residual DNA, cDNA was generated by reverse transcription from RNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and subjected to real-time qPCR analysis using the 2-ADCt method as described elsewhere [16]. GAPDH was used as internal controls for gene expresseion. Quantification of miRNA was analyzed by qRT-PCR as previous description [17]. U6 was used as internal controls for miRNA. Quantitative real-time PCR was performed in triplicate on an ABI 7500 system (Applied Biosystems, Foster City, CA). All TaqMan probes were commercial available from Applied Biosystems.

Western blotting

Protein was extracted by lysis buffer (Thermo Scientific) and western blot analysis was per-



Figure 2. LPS enhances miR-26a-5p expression in macrophages from WT mice. Macrophages were stimulated with 10 ng/ml LPS for 16 h after isolation. MiR-26a-5p expression was analyzed by qRT-PCR. Data were normalized to U6 snRNA. Results are mean \pm SE (n = 3 cell preparations). *P<0.05 versus Control.

formed as previous instruction [16]. The antibodies used were PTEN (1:500) (Abcam, Cambridge, MA), GAPDH (1:1000) (Abcam) and HRP-conjugated secondary Abs (1:1000) (Bio-Rad). Target proteins were visualized using the ECL detection system.

ELISA

Culture medium was collected and centrifuged at 1000 rpm for 5 min at 4°C to remove residual cell. Concentrations of IL-1 β , IL-6 and TNF- α using and ELISA (Thermo Scientific, Waltham, MA) as previously described [18].

Statistical analysis

All data were analyzed with SPSS 17.0 software. Student's t test was used to analyze the difference of results between diabetic and WT mice. All experiments were performed at least 3 independent experiments and P<0.05 was considered as significant. And, in all panels, data are presented as mean ± SEM.

Results

MiR-26a-5p was up-regulated in macrophages from diabetic mice than macrophages from WT

Based on our preliminary microRNA microarray studies, we found miR-26a-5p is 3.5-fold enhanced in macrophages from diabetic mice compared with WT mice *in vitro* (**Figure 1**). qRT-PCR analysis also revealed that miR-26a-5p

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Figure 3. PTEN mRNA and protein level are down-regulated in macrophages from diabetic mice compared to those of WT mice. A. The relative PTEN mRNA levels were measured by qRT-PCR using GAPDH as endogenous control. Results are mean \pm SE (n = 3 cell preparations). *P<0.05 versus WT mice. B. Western blot for PTEN in macrophages from WT and diabetic mice.

expression was increased in macrophages from diabetic mice than from WT mice (**Figure 1**).

MiR-26a-5p expression in macrophages from WT mice has increased under diabetic conditions

LPS induces the rapid production and release of inflammatory cytokines and chemokines that are known to be involved in inflammation related to diabetes [19]. And, miR-26 families have been reported to have the function of inflammation enhancement [17]. Next, we treated macrophages from WT mice with LPS and found a significant up-regulation of miR-26a-5p expression (**Figure 2**). This result indicates that diabetic miR-26a-5p induction in macrophages may be likely induced in response to inflammatory mediators increased during diabetes.

PTEN is repressed by miR-26a-5p in relation to diabetes

PTEN is considered as a target of miR-26 families and involved in the NF-κB signaling pathway related to inflammation [17]. In this study, we also exanimated the expression of PTEN in macrophages from WT and diabetic mice. As shown in **Figure 3**, PTEN mRNA (**Figure 3A**) and protein (**Figure 3B**) levels are remarkably downregulated in macrophages from diabetic mice compared with that of WT mice. Therefore, miR-26a-5p likely promotes the inflammation related to diabetes mainly through the down-regulation of PTEN. Increased inflammatory activation in miR-26a-5p overexpressed macrophages from WT mice

In order to explore whether diabetes induced miR-26a-5p regulates inflammatory cytokine and chemokine mRNA levels in macrophage, we further overexpressed miR-26a-5p or VC plasmid in the macrophages from WT mice. The blank control (BC) group was macrophages without transfection. As seen in **Figure 4**, the IL-1 β , IL-6 and TNF- α

mRNA levels were greatly increased after miR-26a-5p overexpression.

IL-1 β , IL-6 and TNF- α protein production in culture media of macrophages were quantified by ELISA. In the macrophages transfected with miR-26a-5p, we observed a significant increase in IL-1 β , IL-6 and TNF- α protein in the culture media compared with the levels of these cytokines in the VC group (**Figure 5**).

Discussion

MiRNA expression patterns and functions are cell and tissue specific. The connection between miRNAs and disease was obvious. Obesity is known to be associated with a state of chronic, low-grade inflammation. Inflammation is a major contributor to the pathogenesis of diabetes. Macrophages are among the major inflammatory cells to create a pro-inflammatory conditions through infiltrating into the kidney under diabetic conditions [20]. The role of diabetes induced miRNA in the response of macrophages has not been investigated in detail. In this study, miR-26a-5p was found to be diabetes induced miRNA in the macrophages. miR-26a-5p increases the expression of IL-1 β , IL-6 and TNF- α as well as the secreted production. In addition, PTEN mRNA and protein levels are down-regulated in the macrophages from diabetic mice. Taken together, these data show that miR-26a-5p may play in the inflammatory responses in diabetes. It raises the possibility that miR-26a-5p is a potential novel therapeutic strategy in diabetes.





Figure 4. MiR-26a-5p overexpression up-regulated IL-1 β , IL-6 and TNF- α mRNA levels in macrophages isolated from WT mice. Liver macrophages from WT mice were transfected with miR-26a-5p mimic or miRNA vector control (VC) (40 pmol) lipofectamine RNAiMax. After transfection, the mRNA expressions of IL-1 β (A), IL-6 (B) and TNF- α (C) were analyzed by qRT-PCR using GAPDH as internal control. Data are expressed as the fold change relative to BC control. Results are mean \pm SE (n = 3 cell preparations). *P<0.05 versus BC; **P<0.05 versus VC. BC control: liver macrophages without transfection.

Expression of diabetes induced miRNAs has been determined in different diabetic complications [21, 22]. miR-26 family is involved in many biological processes. It was suggested that miR-26b enhances inflammatory response in in alveolar macrophages by regulating PTEN after



Figure 5. MiR-26a-5p overexpression enhances IL-1 β , IL-6 and TNF- α protein expression in macrophages from WT mice. Liver macrophages from WT mice were transfected with miR-26a-5p mimic or miRNA vector control (VC) (40 pmol) lipofectamine RNAiMax. After transfection, the protein levels of IL-1 β (A), IL-6 (B) and TNF- α (C) in the culture media were analyzed with specific ELISA. Results are mean \pm SE (n = 3 cell preparations). *P<0.05 versus VC.

LPS challenge [17]. miR-26b may regulate obesity-related insulin sensitivity and inflammatory

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responses [23]. Despite all of these studies, there are very few functional studies of miR-26a-5p in diabetes associated with inflammation. We found that miR-26a-5p enhances diabetes inflammation by up-regulating mRNA levels of IL-1 β , IL-6 and TNF- α . Moreover, secreted cytokine protein levels mirrored mRNA pattern.

Several studies reported that PTEN works as a tumor-suppressor gene and is frequently mutated in various human cancers. Recently, PTEN was found to be involved in the inflammatory response in macrophages after LPS stimulation [17]. However, it is unknown whether diabetes induced miRNAs acting through PTEN play any role in the inflammation of diabetes. In this study, we found PTEN expression is downregulated in macrophages from diabetes mice. This leaves us with an interesting direction to follow in future studies.

Collectively, miR-26a-5p promotes inflammatory responses in vitro in macrophages from diabetic mice. Consequently, PTEN expression is down-regulated as well in macrophages of diabetic mice. Therefore, this study has indicated the connections between miR-26a-5p expression and inflammation in diabetes and may be exploited as therapeutic targets in the management of inflammation in diabetes.

Disclosure of conflict of interest

None.

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References

- [1] Adams KF, Schatzkin A, Harris TB, Kipnis V, Mouw T, Ballard-Barbash R, Hollenbeck A, Leitzmann MF. Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old. N Engl J Med 2006; 355: 763-778.
- [2] Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010; 87: 4-14.
- [3] Kahn SE. Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 2001; 86: 4047-4058.

- [4] Foster PS, Plank M, Collison A, Tay HL, Kaiko GE, Li J, Johnston SL, Hansbro PM, Kumar RK, Yang M, Mattes J. The emerging role of microR-NAs in regulating immune and inflammatory responses in the lung. Immunol Rev 2013; 253: 198-215.
- [5] O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol 2012; 30: 295-312.
- [6] Zhao Y, Schetter AJ, Yang GB, Nguyen G, Mathé EA, Li P, Cai H, Yu L, Liu F, Hang D, Yang H, Wang XW, Ke Y, Harris CC. microRNA and inflammatory gene expression as prognostic marker for overall survival in esophageal squamous cell carcinoma. Int J Cancer 2013; 132: 2901-2909.
- [7] Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. Diabetes 2011; 60: 1832-1837.
- [8] Argyropoulos C, Wang K, McClarty S, Huang D, Bernardo J, Ellis D, Orchard T, Galas D, Johnson J. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PLoS One 2013; 8: e54662.
- [9] Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 2010; 79: 351-379.
- [10] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008; 9: 102-114.
- [11] Li Y, Xu X, Liang Y, Liu S, Xiao H, Li F, Cheng H, Fu Z. miR-375 enhances palmitate-induced lipoapoptosis in insulin-secreting NIT-1 cells by repressing myotrophin (V1) protein expression. Int J Clin Exp Pathol 2010; 3: 254-264.
- [12] Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004; 432: 226-230.
- [13] Lovis P, Gattesco S, Regazzi R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. Biol Chem 2008; 389: 305-312.
- [14] He A, Zhu L, Gupta N, Chang Y, Fang F. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. Mol Endocrinol 2007; 21: 2785-2794.
- [15] Reddy MA, Chen Z, Park JT, Wang M, Lanting L, Zhang Q, Bhatt K, Leung A, Wu X, Putta S, Sætrom P, Devaraj S, Natarajan R. Regulation of inflammatory phenotype in macrophages by a diabetes-induced long noncoding RNA. Diabetes 2014; 63: 4249-4261.
- [16] Deshpande SD, Putta S, Wang M, Lai JY, Bitzer
 M, Nelson RG, Lanting LL, Kato M, Natarajan
 R. Transforming growth factor-beta-induced

cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy. Diabetes 2013; 62: 3151-3162.

- [17] Zhang L, Huang C, Guo Y, Gou X, Hinsdale M, Lloyd P, Liu L. MicroRNA-26b Modulates the NF-kappaB Pathway in Alveolar Macrophages by Regulating PTEN. J Immunol 2015; 195: 5404-5414.
- [18] Subramanian S, Han CY, Chiba T, McMillen TS, Wang SA, Haw A 3rd, Kirk EA, O'Brien KD, Chait A. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2008; 28: 685-691.
- [19] Bhatt K, Lanting LL, Jia Y, Yadav S, Reddy MA, Magilnick N, Boldin M, Natarajan R. Anti-Inflammatory Role of MicroRNA-146a in the Pathogenesis of Diabetic Nephropathy. J Am Soc Nephrol 2016; 27: 2277-88.

- [20] Tesch GH. Macrophages and diabetic nephropathy. Semin Nephrol 2010; 30: 290-301.
- [21] Liang G, Zhu Y, Sun B, Shao Y, Jing A, Wang J, Xiao Z. Assessing the survival of exogenous plant microRNA in mice. Food Sci Nutr 2014; 2: 380-388.
- [22] Wang HJ, Huang YL, Shih YY, Wu HY, Peng CT, Lo WY. MicroRNA-146a decreases high glucose/thrombin-induced endothelial inflammation by inhibiting NAPDH oxidase 4 expression. Mediators Inflamm 2014; 2014: 379537.
- [23] Xu G, Ji C, Shi C, Fu H, Zhu L, Zhu L, Xu L, Chen L, Feng Y, Zhao Y, Guo X. Modulation of hsamiR-26b levels following adipokine stimulation. Mol Biol Rep 2013; 40: 3577-3582.