MicroRNA expression profiling in diabetic GK rat model

Bing Huang, Wenning Qin, Botao Zhao, Yi Shi, Chengguo Yao, Jin Li, Huasheng Xiao, and Youxin Jin

State Key Laboratory of Molecular Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
St Luke’s Hospital, Shanghai 200050, China
National Engineering Center for Biochip at Shanghai, Zhangjiang High-Tech Park, Pudong, Shanghai 201203, China

†These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-54921222; Fax: +86-21-54921011; E-mail: yxjin@sibs.ac.cn

Acta Biochim Biophys Sin (2009) | Volume 41 | Issue 6 | Page 472

MicroRNAs (miRNAs), which are a newly identified class of small single-stranded non-coding RNAs, regulate their target genes via post-transcriptional pathway. It has been proved that miRNAs play important roles in many biological processes. To better understand miRNA function on type 2 diabetes, we used an oligonucleotide microarray to monitor miRNA expression profiles of Goto–Kakizaki (GK) and Wistar rats’ skeletal muscle. It was found that seven miRNAs were down-expressed and two miRNAs were over-expressed in the muscle of GK rats. Among them, miR-24 showed the most prominent change. p38 MAPK, which is a direct target of miR-24, also showed expression difference. All the data give a clue that miR-24 might be associated with diabetes through down-regulation of p38 MAPK.

Keywords microRNA; diabetes; GK (Goto–Kakizaki) rat; Wistar rat; p38 MAPK

Received: December 16, 2008 Accepted: March 3, 2009

Introduction

Diabetes mellitus is a kind of familiar disease that is caused by disorder of energy metabolism and is characterized by insulin resistance and high blood glucose levels [1]. It is becoming so prevalent in the world that it is now taking its place as one of the main threats to human health in the twenty-first century [2]. There are two main kinds of diabetes: type 1 diabetes mellitus is insulin-dependent and it is resulted from absolute insulin deficiency, which is due to auto immune β-cell destruction [3]; type 2 diabetes mellitus (T2DM) is non-insulin-dependent, which is caused by the disorder of β-cell’s function, and it is characterized by insulin resistance and/or abnormal insulin secretion [4]. Type 2 diabetes is far more common than type 1; it accounts for ~90% of patients with diabetes mellitus. Muscle is one of the tissues that are insulin-resistant in type 2 diabetes. Both types of diabetes could lead to severe complications such as heart disease, stroke, kidney failure, blindness, and nerve damage [5].

Goto–Kakizaki (GK) rat is one of the best characterized animal models of spontaneous T2DM. This model was established by selectively breeding of normal Wistar rats with signs of impaired glucose tolerance [6]. It displays hyperglycemia, impaired glucose tolerance, insulin resistance and also defects in insulin secretion. In most of the GK studies, Wistar rats of outbreed origin are used as control animals [7].

MicroRNAs (miRNAs) are a kind of small non-coding RNAs that powerfully regulate gene expression at the post-transcriptional level. Their importance in a variety of biologic processes has become apparent during the last decade [8]. Microarray is a common method to investigate miRNAs’ function. Recent advances in miRNA research have provided evidence that the aberrant expression profile in kinds of diseases, such as cancer, cardiovascular diseases, psychological disorders, and others [9–11]. It has been shown that miRNAs can play roles in insulin secretion and glucose homeostasis. They must have potential roles in type 2 diabetes and related metabolic diseases [12].

In the present study, we analyzed the miRNAs expression profile in muscle tissues from diabetic GK and Wistar rats, and miR-24 showed the most prominent change. p38 MAPK, which is a direct target of miR-24, also showed expression difference. These data give a clue that miR-24 might be associated with diabetes through down-regulation of p38 MAPK.
Materials and Methods

Animals and measurement of blood glucose
In this study, we used male GK rats, which were established by selectively breeding of normal Wistar rats with signs of impaired glucose tolerance, as type 2 diabetes model and male Wistar rats as controls. Male Wistar and diabetic GK rats were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (SLACCAS, Shanghai, China). Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 14 weeks. The blood glucose levels of the rats were measured each other day up to four times before being killed. The rats were killed by decapitation after a brief exposure to diethyl ether. Their skeletal muscles were collected and immediately frozen in liquid nitrogen for later use. All animal experiments were performed according to the instructions of ‘The Animal Care and Welfare Committee’ of the Peking Union Medical College (Beijing, China).

Construction of small RNA cDNA library
Total RNA from muscles was extracted using Trizol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The whole construction was performed as described previously [13]. For each sample, at least two independent reverse transcriptions/amplifications/hybridizations (technical replications) were performed.

miRNA microarray
Microarrays contained 406 capture probes, perfectly matched probes for all human, mouse, and rat miRNAs as registered and annotated in the miRBase [14] at the Wellcome Trust Sanger Institute. Among them, 179 miRNAs were from rats. In addition, 14 negative control probes (complementary to mRNAs, tRNAs, rRNAs, random sequences, and so on) were also included. The whole microarray methods were performed as described previously [13,15] except that the hybridization temperature was adjusted to 45°C.

The miRNA expression data have been submitted to the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under the sample accession numbers GSM350401, GSM350624, GSM350625, and GSM350626 and series accession number GSE13920.

Microarray data analysis
Images were quantified by GenePix Pro6.0 (Axon, Foster City, CA, USA) using the fixed circle quantification methods. Signal intensities of spots were calculated by subtracting local background from total intensities. Further analysis was completed by VBA in Microsoft Excel. Bad spots were picked up using Student’s t-test evaluation of all replicates and were substituted by the mean value of the rest replicates in the array. All hybridizations were normalized by total intensity and then Student’s t-test was performed. For each probe, an arithmetic mean of four replicates from two independent hybridizations was calculated. Those probes whose mean values were <1000 were filtered as undetectable. The probes that changed >2 folds and whose P-value was <0.01 were selected.

RNA extraction and northern blot analysis
RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The total RNA (20 μg) was resolved on a 12% acrylamide/8 M urea gel, then the gel was separated into two parts. The one containing 5S rRNA was stained by ethidium bromide, and the other containing microRNAs transferred onto nylon membranes and cross-linked with UV light at 125 mJ/cm². The DNA probes that were complementary to the miRNA sequences were end-labeled with [γ-32P]ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (Fermentas). The unincorporated [γ-32P]ATP was removed by filtration through a Sephadex G-25 column. Pre-hybridization and hybridization were carried out using PerfectHyb Hybridization Solution (TOYOBO) at 40°C. The blots were washed in 2× saline-sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) according to the user manual. The membrane was measured by Phosphorimager (Amersham). We used 5S rRNA as the loading control for normalization. All probes used were listed as follows: miR-24: 5′-CTGTTTCTGCTGACTAGCCCA-3′ and miR-126: 5′-CGATTATTACTCACGGTACGA-3′.

Protein extraction
After RNA isolation, proteins were precipitated from the phenol–ethanol supernatant (approximate volume 0.8 ml/1 ml of Trizol reagent) with isopropyl alcohol. Initial homogenization was done by adding 1.5 ml of isopropanol/1 ml of Trizol reagent. Samples were stored at room temperature for 10 min, and then centrifuged at 12,000 g for 10 min at 4°C. After removal of the supernatant, the protein pellet was washed three times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. Two milliliters of wash solution per 1 ml of Trizol reagent were used for the initial homogenization.
During each wash cycle, protein pellet in the wash solution was stored at room temperature for 20 min and then centrifuged at 7500 g for 5 min at 4°C. After the final wash, protein pellet was suspended in 2 ml of ethanol and vortexed. Store the protein pellet in ethanol at room temperature for 20 min and then centrifuged at 7500 g for 5 min at 4°C. After removal of the ethanol, protein pellet was vacuum-dried and dissolved in 1% SDS. Complete dissolution of the protein pellet may require incubating the sample at 50°C. Insoluble material was removed by centrifugation at 10,000 g for 10 min at 4°C. Protein concentration was measured by Bradford method. The sample is ready for use in western blotting or may be stored at –5 to –20°C for future use.

**Western blot analysis**

Protein samples were subject to SDS–PAGE on 4–12% gradient gels by loading 20 μg of protein/lane and then transferred to PVDF membrane (Millipore Corporate, Billerica, MA, USA). Blots were probed with mouse monoclonal anti-p38α/β antibody (A-12; Santa Cruz, Santa Cruz, CA, USA) at 1:1000 dilution and with mouse monoclonal anti-GAPDH antibody (Sigma-Aldrich Corp., St Louis, MO, USA) at 1:5000 dilution. The second antibody was anti-mouse IgG (Sigma-Aldrich Corp.) at 1:5000 dilution. The blots were visualized with Pierce SuperSignal detection reagents (Thermo Scientific, Rockford, IL, USA). The relative amounts of the bands were quantified by densitometry using Image software. Results are expressed as the mean ± SEM.

**Construction of 3′UTR-luciferase plasmid and reporter assays**

The predicted binding site sequence on 3′UTR of mitogen-activated protein kinase 14 (MAPK14, also known as p38α kinase) was cloned into the EcoRI–XbaI sites of pGL3 (Promega, Madison, WI, USA) and named Luc-p38. For reporter assays, Renilla were transiently co-transfected with Luc-p38 or pGL3 reporter plasmid and miR-24 or miR-24-ASO (anti-sense oligonucleotide) using Lipofectamine 2000 (Invitrogen). Reporter assays were performed 24 h after transfection using the Dual-luciferase-assay system (Promega).

**Results**

**Microarray analysis of microRNAs in muscle of GK and Wistar rats**

Before the microarray experiment, the blood glucose levels of the rats were measured each other day up to four times. GK rats’ blood glucose levels were obviously higher than those of Wistar rats, which showed a characteristic phenotype of diabetes (Fig. 1).

Then we used microarray analysis to examine the differential expression of miRNAs in the muscles of GK and Wistar rats. The data indicated that there were significant changes in the miRNA expression between GK and Wistar rats. In comparison with the expression levels in the muscle of GK rats, seven miRNAs in the Wistar rats exhibited at least 2-fold up-regulation, whereas two miRNAs in the Wistar rats exhibited at least 2-fold down-regulation (Table 1).

**Confirmation of miRNA microarray data**

In order to confirm the results from the miRNA microarray experiments, we chose these nine miRNAs for northern blot analysis. But only miR-24 and miR-126 had much better signal intensity, the other seven could not produce good signal intensity (data not shown). And the target of miR-24 had already been proven [16]. So we chose the miR-24 to do further research. As shown in Fig. 2, both miR-24 and miR-126 showed down-expression in the muscle of GK rats, which were consistent with the microarray data (we used three GK rats and three Wistar rats).

**p38 MAPK might also be a direct target of miR-24 in rats**

Among the changed miRNAs, miR-24 is the most prominent. It has been proven that p38 MAPK is a direct target of miR-24 in human and mice [16], and the validated single binding site locates in the 3′UTR of p38.
Subsequently, we analyzed the sequence similarities of miRNA and p38 among rat, mice, and human. We found that the miRNA sequences among the three species are the same, and the binding site for p38 is quite conserved [Fig. 3(A,B)]. Besides this conserved miR24-p38 pairing site, another binding site was predicted in the 3′UTR of rat p38 MAPK mRNA [Fig. 3(C)].

To demonstrate the direct interaction between miR-24 and p38 MAPK mRNA in rats, we cloned the two predicted binding site sequences into the 3′UTR of luciferase in PGL3 plasmid. Because 293T cell shows a low endogenous expression level of miR-24, we co-transfected this reporter vector with random RNA oligonucleotide as a control, miR-24 mimics or 2′-OMe-ASO-miR24 into this cell line. The relative luciferase activity was markedly diminished after miR-24 co-transfection and was increased after ASO-miR-24 co-transfection compared with the control RNAs [Fig. 3(D)]. These in vitro experiments indicated that rat p38 MAPK might be a direct target of miR-24.

We also measured the expression levels of p38 MAPK in GK and Wistar rats’ muscles. The proteins from skeletal muscle tissues were extracted and used for p38 MAPK western blot analysis. GAPDH served as a loading control. Results showed that the protein level was ~3-fold higher in GK muscle tissue than that in Wistar rats, which indicated that miR-24 might down-regulate rat p38 MAPK in vivo (Fig. 4). This further confirmed that p38 MAPK was a direct target of miR-24.

### Discussion

MicroRNA has recently emerged as one of the most important new classes of cellular regulators. It has been shown that miR-375, a pancreatic islet-specific microRNA, regulates insulin secretion and homeostasis [17]. But few studies have been carried out on the relationship between miRNA and diabetes. In this paper, we employed GK and Wistar rat models to investigate the differential expression of microRNAs. Microarray analysis demonstrated that nine miRNAs were

<table>
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<th>Name</th>
<th>P-value</th>
<th>Fold difference a</th>
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<tr>
<td>rno-miR-24</td>
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<tr>
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<td>2.34</td>
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</table>

aGK versus Wistar rats. rno, Rattus norvegicus.

**Fig. 2 Northern blot analysis verified the microarray results of miR-24 and miR-126 in rats** 5S rRNA served as a loading control. The relative amounts of the bands were quantified by densitometry using Image software. Results are expressed as the mean ± SEM (n = 3). GK, Goto–Kakizaki rat; WS, Wistar rat; rno, Rattus norvegicus.
differentially expressed in skeletal muscle tissue with the fold change of GK/Wistar \(2\). And we used northern blot analysis to validate two miRNA (miR-24 and miR-126) expression profiles. But our microarray results were not consistent with a previous study [18], except the miR-130a. In our study, the miR-29 had no significant up-regulation in GK rats. It is possible that the methodology of tissue harvesting could impact miRNA expression. It has been shown that hypoxia [19] and other cell stresses [20] can impact miRNA expression. Our tissues were harvested after a brief exposure to diethyl ether, whereas those of He et al.’s group were harvested after a brief exposure to carbon dioxide. In a similar manner, adjuvant treatments might potentially impact miRNA expression.

Insulin resistance and hyperglycemia are important characteristics of T2DM. Muscle is one of the important insulin resistance tissues, so these changed miRNAs might have important function in hyperglycemia resistance. Background studies support the existence of several pathways linking with insulin resistance and hyperinsulinemia. Insulin per se stimulates the production of some important growth factors such as insulin-like growth factor-1 and transforming growth factor \(\beta\) (TGF-\(\beta\)) [21]. miR-23, miR-24, and miR-27 constitute an miRNA cluster in the rat genome. In our study, we found that all the three miRNAs showed lower expression in GK rats. Previous studies showed that TGF-\(\beta\) inhibits the transcription of miR-24 and clustered genes [22]; whereas non-fasting plasma insulin levels in GK rats from all colonies have been found similar or somewhat increased when compared with age-matched controls [6]. So further experiments are required to verify the specific relationship between the TGF-\(\beta\) and miR-24 in hyperglycemia.

p38 MAPK activity is increased in glomeruli from streptozotocin-induced type 1 diabetes model rats and high glucose condition has been shown to activate p38 MAPK activity [23,24]. Previous studies have also

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**Fig. 3** p38 is a direct target of miR-24 in rats

(A) Potential base paired between miR-24 and p38 (MAPK14) mRNA. Numbers refer to nucleotide position after the stop codon. (B) The predicted binding site was quite conserved in human, rat, and mouse. (C) Another novel base-paring site was also predicted in the rat p38 mRNA. Numbers refer to nucleotide position after the stop codon. (D) Luciferase assay in 293T cell indicated that rat miR-24 can bind to the putative p38 3’UTR site and diminish the luciferase activity.

**Fig. 4** p38 expression level is higher in the muscle of GK rats compared to Wistar rats in vivo

The proteins from skeletal muscle tissues were extracted and used for western blot analysis. Results are expressed as the mean ± SEM (n = 3). GK, Goto–Kakizaki rat; WS, Wistar rat.
concluded that p38 MAPK is involved in the pathogenesis of early-stage diabetic nephropathy in T2DM [25], and Imai et al. [26] showed that p38 MAPK expression was higher in the male Wistar fatty rats, a type 2 diabetic model, than the Wistar lean rats; our results also showed that p38 MAPK protein was over-expressed in GK rats’ muscles than Wistar rats’, which provided the evidence that p38 MAPK had important function in diabetes and high blood glucose tolerance; however, the mechanism through which the p38 MAPK was finely tuned was largely unknown. So further experiments are required to verify the specific relationship among the TGF-β, p38 MAPK and miR-24 in hyperglycemia.

In conclusion, using microRNA microarray, we found that several miRNAs were differentially expressed between GK and Wistar rats’ muscle. miR-24, with significantly changed expression levels, might be associated with diabetes. p38 MAPK was also down-regulated in diabetes. So miR-24 might be associated with glucose through down-regulation of p38 MAPK. But the mechanism through which the p38 MAPK was finely tuned still need further study.

Funding

This work was supported by the grants from the National Key Basic Research and Development Program (No. 2005CB724602) and the Chinese Academy of Sciences (KSCX1-YW-R-64, KSCX2-YW-R-096).

References