Original Article
miR-375 is downregulated by promoter hypermethylation in MIN6 insulinoma cells

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Abstract: Epigenetics may affect the susceptibility for type 2 diabetes mellitus (T2DM). Aberrant DNA methylation patterns are nowadays recognized as a key epigenetic hallmark of T2DM. Previously, our studies have shown that the hypomethylation of human miR-375 promoter may contribute to the pathogenesis of T2DM. However, no comprehensive study defines the miR-375 promoter methylation patterns present in the established pancreatic β cell line. To address this matter, we have analyzed the DNA methylation profile of insulinoma MIN6 cells by MassARRAY spectrometry and employed the DNA demethylating drug 5-aza-2’-deoxycytidine (5-aza-CdR) to treat MIN6 cells to explore the methylation patterns of the mmu-miR-375. The expression of mmu-miR-375 in mRNA level was measured by quantitative RT-PCR (qRT-PCR). Methylation analysis reveals that MIN6 cells display hypermethylation at the mmu-miR-375 promoter. Following the decreased methylation of mmu-miR-375, the relative expression of mmu-miR-375 increased gradually after 5-Aza-CdR treatment. In addition, we find that there was an inverse correlation between DNA methylation levels and transcription level of mmu-miR-375. In summary, this is the first report for analyzing mmu-miR-375 promoter methylation using MALDI-TOF MS technology and our results indicate that promoter hypermethylation of the mmu-miR-375 is a common event in MIN6 cells.

Keywords: Type 2 diabetes mellitus, DNA methylation, insulinoma MIN6 cells, 5-aza-2’-deoxycytidine

Introduction
Type 2 diabetes mellitus (T2DM), a complex polygenic disease, accounting for more than 90% of DM, is a chronic metabolic disease, commonly resulting from defects in insulin secretion and/or diminished sensitivity of target tissues to insulin [1] and is a leading cause of death worldwide. However, the exact mechanisms of this dysfunction are not yet fully understood.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs (~22 nucleotides), which are involved in post-transcriptional control of gene expression [2]. Studies showed that miRNAs are involved in the regulation of major cellular activities, such as metabolism, differentiation, proliferation, and apoptosis [3, 4]. Recent studies have identified several miRNAs that have potential roles in pancreas development, islet function, insulin secretion, and diabetic complications [5, 6]. It has been shown that miR-375, a pancreatic islet-specific miRNA, regulates insulin secretion through direct inhibition of insulin exocytosis [7]. In vivo promoter expression studies revealed that it is specifically transcribed in α-, β-, and δ-islet cells of the adult pancreas via the helix-loop-helix Id2 and Id3 factors [8]. One of its identified targets includes V1/myotrophin, whose knockdown mimicked miR-375’s effect in β-cells. A second study, while confirming that miR-375 inhibits insulin secretion, shows that it does so through PDK1-dependent inhibition of insulin synthesis [9]. High levels of miR-375 are found in the pancreatic islet of obese (ob/ob) mice and have been shown to regulate glucose homeostasis [10]. One study demonstrated that upregulated pancreatic miR-375 is a useful biomarker for pathways in the pathogenesis of T2DM associated...
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with islet amyloid deposition and β-cell deficit [11]. These findings suggested that miR-375 could play important roles in the pathogenesis of T2DM, but the function of miR-375 and its regulation mechanism in T2DM remains unclear.

In addition to classical genetic abnormalities in the pathogenesis of T2DM, epigenetic alterations such as DNA methylation play a key role during T2DM pathogenesis. Found in the tumor area, epigenetic regulation of miRNA genes within or near CpG islands in their promoter regions can result in aberrant expression of a number of miRNAs. Interestingly, miR-375 is located in the intergenic region and it has an independent promoter containing CpG islands, which provides a structural basis for regulation of its expression by methylation. Studies to date have suggested the aberrant miR-375 methylation patterns in cancer [12-15]. Our previous studies have shown that the hypomethylation of human miR-375 promoter may contribute to the pathogenesis of T2DM [16, 17] and sex-specific miR-375 methylation patterns have been previously characterized [18].

However, no comprehensive study defines the miR-375 promoter methylation patterns present in the established pancreatic β cell line.

Therefore, in the present study, we have analyzed DNA methylation profile of insulinoma MIN6 cells by MassARRAY spectrometry. We also treated the MIN6 cells with the cytosine methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-CdR), to determine whether or not modulation of DNA methylation could affect the expression of the miR-375.

Materials and methods

Cell lines and culture

MIN6 cells were originally from the Department of Central Experimental Function of North Sichuan Medical College, and were used for all in vitro studies. NIH3T3 cell lines were purchased from Shanghai Institute of Cell Bank. No human subjects were used in the study. MIN6 cells and NIH3T3 cells were maintained in a 5% CO₂ incubator at 37°C [19]. NIH3T3 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, supplemented with 10% FBS and 1% Penicillin-Streptomycin. MIN6 cells were cultured in the same medium with the addition of 100 umol/L/L β-mercaptoethanol. In experiments to inhibit DNA methylation, cells were incubated in standard culture medium supplemented with 50 µM 5-Aza-CdR (Aza-dC, USA) for 72 hours. Culture medium was changed daily.

Cell proliferation assay

Logarithmic phase MIN6 cells were plated into 96-well culture plates (~1×10³ cells/well). There were six samples in each group for the MTT assay. After 24 h of incubation, the cells were treated with 5-Aza-CdR (0.08, 0.4, 2, 10 and 50 µM), followed by a 24-, 48- and 72-h, then we added 20 ml of MTT into each wells (5 mg/mL), placed in 37°C, 5% CO₂ incubator and cultured for 4 h. We added 150 ml of DMSO into each well, detected the absorbance value of each well at wavelength 490 nm, and calculated the average value.

Quantitative DNA methylation analysis

MIN6 cells were treated with 5-Aza-CdR (0, 0.08, 0.4, 2, 10 and 50 µM), followed by a 24 h, 48 h and 72 h. DNA from these cells and NIH3T3 cells was extracted using a QiAamp DNA Mini Kits (Qiagen). The sequence of CpG...
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island (CGI) was identified by the use of the UCSC genome browser. Quantitative methylation analysis of the CpG islands in the mmu-miR-375 promoter was performed using the MassARRAY platform (Sequenom, San Diego, USA) as described previously [20]. The primers were designed using EpiDesigner (Sequenon) and were as follows: forwards 5'-aggaagagag-GTTTAGAGTTTGAGGGTAGGGTAGG-3' and reverse 5'-cagtaatacgactcactatagggagaaggctAAAAACTCATCCACCAAACACC-3'. A sample of DNA was converted with sodium bisulfite using an EZ DNA methylation kit (Zymo Research) and the modified DNA was amplified by PCR. Briefly, primers for the PTEN CpG island were used to amply bisulfite treated DNA and then the PCR products were spotted on a 384-pad Spectro CHIP (Sequenom, San Diego, USA), followed by spectral acquisition on a MassARRAY Analyzer. Methylation data of individual units (one to three CpG sites per unit) were generated by the EpiTyper v1.0.5 software (Sequenom, San Diego, USA).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The MIN6 cells were treated with 5-Aza-CdR (0, 50 µM), followed by a 24 h, 48 h and 72 h. The control cells were NIH3T3 cells which treated without 5-Aza-CdR. Total RNA was isolated from cell cultures by the miRNeasy Mini Kit and cDNA was prepared from the miScript II RT Kit (Qiagen). qRT-PCR was carried out on an ABI Prism 7300 Fast Real-time PCR System (Applied Biosystems). SYRB-Green for the target gene miR-375 and the internal control U6, which is often used as a normalization control for microRNAs, were obtained from Qiagen. PCR was performed under the following conditions: 50°C for 2 min then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Expression levels of miRNA genes were determined by normalizing the amount of the target message to that of the control U6 transcript.

**Statistical analysis**

All results were repeated in at least three separate experiments. The date are expressed as the means ± SD. The differences in miR-375 methylation level between MIN6 cells and controls with NIH3T3 cells were assessed using the Kruskal-Wallis tests. Using the two-way hierarchical cluster analysis, the most variable CpG units for mmu-miR-375 were clustered based on pair-wise Euclidean distances and linkage algorithm for all studied sample. Statistics comparisons were carried out using one-way ANOVA. Student’s t-test was used to reveal differences between two sample means.
We calculated the Pearson correlation between DNA methylation levels and mRNA levels of mmu-miR-375. Statistical analyses were carried out using SPSS version 17.0 software. \( P < 0.05 \) was considered to indicate significance.

Results

Effect of 5-Aza-CdR on MIN6 cells proliferation

To investigate the effect of 5-Aza-CdR on cell growth, MIN6 cells were cultured with 0, 0.08, 0.4, 2, 10, and 50 umol/L 5-Aza-CdR for 24, 48, and 72 h. Cell proliferation was determined by the MTT assay. As demonstrated in Figure 1, 5-Aza-CdR was shown to inhibit the growth of the cells in a dose- and time-dependent manner.

CpG methylation of mmu-miR-375 in MIN6 cells and NIH3T3 cells

In order to known the impact of 5-Aza-CdR on MIN6 cells and NIH3T3 cells in CpG methylation of mmu-miR-375, methylation patterns of mmu-miR-375 from different concentrations of 5-Aza-CdR treated cells were quantitatively analyzed using MassARRAY spectrometry. Figure 2 shows the promoter region of miR-375 according to UCSC Genome Browser’s CpG island annotations and miRbase release 13.0. As illustrated in Figure 3, methylations were present from +199 bp to +491 bp relative to the transcription start site of mmu-miR-375 in MIN6 cells and NIH3T3 cells. Hierarchical clustering showed substantial differences in quantitative methylation profiling of these groups (Figure 3). Eighteen CpG units (comprising a
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A total of 29 CpG sites spanning 293 bp on the specified promoter region of mmu-miR-375 were analyzed in these groups (a total of 551 CpG sites in all analyzed samples). Among these units, 2 CpG units (CpG_2 and CpG_15) did not yield successful measurements. The overall methylation of mmu-miR-375 promoter was found to be >85% for the MIN6 cells treated without 5-Aza-CdR. MIN6 cells were cultured with 50 umol/L 5-Aza-CdR for 24, 48, and 72 h. The overall methylation of miR-375 promoter was less than 0 umol/L group; the differences were statistically significant for 24 h (Figure 4A). However, we found that the amplicon of the mmu-miR-375 was uncovered in NIH3T3 cells. We next examined the methylation levels of mmu-miR-375 promoters in MIN6 cells which cultured with 50 umol/L 5-Aza-CdR for 24, 48, and 72 h. MassARRAY (Sequenom, San Diego, CA, USA) was used to determine the overall methylation levels. The mean methylation level of the 27 CpG residues in these groups were used to represent the promoter methylation levels. Data are presented as the mean ± standard deviation (SD). B. The average methylation of the CpG units of amplicon is presented for in 24 h, 48 h, and 72 h groups. Error bars represent standard error. C. The levels of mmu-miR-375 expression in NIH3T3 cells and MIN6 cells. The levels of mmu-miR-375 in NIH3T3 cells and MIN6 cells were determined by qRT-PCR as described in Materials and Methods. The relative expression values were defined as the expression ratio of mmu-miR-375 to U6. The values represented average expression levels with standard deviation (SD) of three independent experiments and the significant level was determined by Student’s t-tests between the indicated groups (*P<0.05). D. The level of mmu-miR-375 expression in MIN6 cells which cultured with 50 umol/L 5-Aza-CdR for 0, 24, 48, and 72 h. The levels of mmu-miR-375 in MIN6 cells were determined by qRT-PCR as described in Materials and Methods. The relative expression values were defined as the expression ratio of mmu-miR-375 to U6. The values represented average expression levels with standard deviation (SD) of three independent experiments and the significant level was determined by Student’s t-tests between the indicated groups (*P<0.05).
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<table>
<thead>
<tr>
<th>Group (MIN6)</th>
<th>miR-375 expression</th>
<th>DNA Methylation (%)</th>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 umol/L</td>
<td>0.760±0.0842</td>
<td>0.859±0.1896</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 umol/L (24 h)</td>
<td>1.003±0.1018</td>
<td>0.667±0.1473</td>
<td>0.010</td>
<td>-0.710</td>
</tr>
<tr>
<td>50 umol/L (48 h)</td>
<td>1.003±0.1018</td>
<td>0.730±0.1522</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 umol/L (72 h)</td>
<td>1.003±0.1049</td>
<td>0.751±0.1509</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

miR-375 promoter may contribute to the pathogenesis of T2DM [16, 17]. However, the role of miR-375 methylation in MIN6 cells has not been described.

In the present study, using the high-throughput robust T-cleavage assay on MALDI-TOF MS, we first showed the hypermethylation patterns of mmu-miR-375 in MIN6 cells and non-pancreatic cell line NIH3T3 cells. The mean levels of miR-375 methylation, calculated from the methylation levels of the 18 CpG residues, were 85.9% in MIN6 cells. In this study, we found that the amplicon of the mmu-miR-375 was uncovered in NIH3T3 cells, which may be due to the MALDI-TOF-MS MassCLEAVE™ software having a default useable mass window of 1500-7000 Da and fragments that fall outside this window will not be analyzed [20]. The mmu-miR-375 promoter shows a much lower level of activity in NIH3T3 cells than in MIN6 cells [21].

5-Aza-CdR is currently acknowledged as a demethylation drug, and causes a certain degree of demethylation in a variety of cancer cells. 5-Aza-CdR can affect pancreatic cancer cells (Capan-2 cells, PANC-1 cells) [22, 23], nasopharyngeal carcinoma cells (CNE-1, CNE-2, 5-8F, 6-10B and NP69 cells) [24], Human Prostate Cancer Cells (LNCaP and PC3 cells) [25] and other cancer cells with regard to proliferation. This study demonstrated that 5-Aza-CdR can inhibit MIN6 cells proliferation in a time- and concentration-dependent manner, consistent with previous research results. In order to determine whether or aberrant methylation could affect the expression of the mmu-miR-375. We also treated the MIN6 cells with the cytosine methylation inhibitor, 5-aza-CdR. Here we found that the miR-375 promoter methylation levels were significantly lower in MIN6 cells with 50 umol/L 5-Aza-CdR for 24 h, 48 h, and 72 h. Meanwhile, the relative expressions of mmu-miR-375 are downregulated after treating the cells with the different 5-Aza-CdR concentrations. We observed that the relative expression of mmu-miR-375 in MIN6 cells without 5-Aza-CdR treatment was lower than that of NIH3T3 cells. Following the decreased methylation of mmu-miR-375, the relative expression of mmu-miR-375 increased gradually after

Discussion

miR-375 is a important regulator of insulin secretion [7]. Because of the special role of miR-375, it may be a potential target to treat diabetes. The aberrant DNA methylation patterns are nowadays recognized as a key epigenetic hallmark of T2DM. Our previous studies have shown that the hypomethylation of human
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5-Aza-CdR treatment. There is a negative correlation between DNA methylation levels and relative expression levels of mmu-miR-375. Although MIN6 cells belong to the tumor cells can proliferate indefinitely, and cannot completely replace the normal human islets β cells, it is reasonable to speculate that the miR-375 promoter showed hypermethylation in human pancreatic β cells. Recent research may use different types of pancreatic β cell lines for the study, such as INS-1, NIT-1 and RINm5F cells, exploring miR-375 methylation and expression features in different types of pancreatic β cell lines.

In conclusion, this is the first report for analyzing mmu-miR-375 promoter methylation using MALDI-TOF MS technology. Our results indicate that promoter hypermethylation of the mmu-miR-375 is a common event in MIN6 cells which may provide important insights into the pathogenesis of β-cell failure in type 2 diabetes. Our data provide a novel link between epigenetic alterations and increase in the transcription level of mmu-miR-375 in MIN6 cells. These epigenetic differences may explain the mechanism by which the aberrant DNA methylation may cause mmu-miR-375 silencing.

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Disclosure of conflict of interest

None.

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