# Assessing microRNA-375 Levels in Type 2 Diabetes Mellitus (T2DM) Patients and T2DM Patients Having First-Degree Relatives With T2DM

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**Research article**

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# Abstract

**Background:**

The pancreatic islet specific microRNA-375 (miR-375) is reported to be upregulated in diabetes patients suppressing the glucose-induced insulin secretion. In this clinical study we aimed to assess the significance of miR-375 among type 2 diabetes mellitus (T2DM) patients and their first-degree relatives with normal glucose tolerance (FD-NGT) and those with T2DM (FD-T2DM).

**Methods:**

We included 56 Han Chinese individuals who received medical health check-ups from January 2018 to September 2018 in the Outpatient Department of Endocrinology, The Third Hospital of Yunnan Province, China. They were categorized as normal glucose tolerance (NGT), T2DM, FD-NGT and FD-T2DM. OGTT, C-Peptide and Insulin tests were performed to confirm the diagnosis. The miR-375 levels were determined by Quantitative real-time RT-PCR (qRT-PCR).

**Results:**

The OGTT test showed a significant difference in T2DM and FD-T2DM groups compared with NGT and FD-NGT (p□0.05). Similar results were observed during C-Peptide and insulin tests. Interestingly, the 2-hour insulin test showed FD-NGT group having a significantly higher mean ± standard error of (64.240±12.775) compared to NGT (28.836±10.875). Assessment of miR-375 expression levels in 4 groups showed a significant up-regulation in T2DM and FD-T2DM compared with NGT and FD-NGT group. A slight increase in miRNA expression was observed in FD-NGT compared with NGT group but was not statistically significant.

**Conclusion:**

A significantly higher miR-375 expression was observed in T2DM and FD-T2DM groups compared with NGT and FD-NGT and thus, miR-375 may serve as a stable biomarker for the early prediction of T2DM among high-risk individuals.


# 1. Background

MicroRNAs (miRNAs) are a class of small non-coding RNAs of 20-24 nucleotides which play an important role in regulating gene expression. Most of the miRNAs are transcribed from DNA sequences into primary miRNAs and processed into precursor miRNAs, and finally become mature miRNAs. (1,2). In majority of cases, miRNAs interact with the 3'-untranslated region (UTR) sequences on target genes of target messenger RNAs (mRNAs) to induce mRNA degradation and translational repression.(3) MiRNA can be found both intracellularly and extracellularly in fluids and in the circulation, such as in blood plasma (4) making them a good candidate for blood-based biomarker development.(5,6) Biomarkers are useful as they provide information on early detection of diseases, help in determining individuals at risk of developing complications or subtyping individuals for disease phenotypes. In addition, they may lead to better treatment strategies, personalized therapies, and improved outcome.(7) The studies conducted previously showed the potential role of miRNAs as biomarkers in various diseased conditions like Cancer, cardiovascular conditions and diabetes.(8–10)

Recent studies indicated that miR-375 plays a direct role in insulin secretion(11) and pancreatic islet development(12) by targeting the pancreatic genes; insulin (INS), myotrophin (MTPN) and phosphoinositide dependent protein kinase-1 (PDPK1).(13) Many Studies have confirmed the overexpression of miR-375 in Type 2 Diabetes Mellitus (T2DM) Patients.(14,15) This information may serve as a diagnostic and therapeutic utilities in T2DM.(16) However, the level of miR-375 expression in first degree relatives of T2DM patients is still unknown.

In the present study, we aimed to evaluate the differential expression of miR-375 in T2DM patients and their first-degree relatives with normal glucose tolerance and T2DM individuals by its isolation and characterization using quantitative real-time polymerase chain reaction (qRT-PCR).

# 2. Materials And Methods

### 2.1 Study Design

This was a single center cross-sectional study with a single time point data collection. The study was conducted at The Third Hospital of Yunnan Province, China after receiving approval from the Ethics Committee of the hospital and was conducted in compliance to the Declaration of Helsinki. The written informed consent was obtained from all subjects prior to their enrollment.

### 2.2 Research subjects

The study population consisted of Han Chinese individuals who received medical health check-ups from January 2018 to September 2018 in the Outpatient Department of Endocrinology, The Third Hospital of Yunnan Province, China. Individuals with malignant tumor, cardiovascular disease, nephropathy or other chronic diseases which pose latent effect on miRNAs expression and who had previously been diagnosed with diabetes mellitus or had any history of medication for 6 months prior to the study were excluded.

All the diagnoses were confirmed by Oral Glucose Tolerance Test on the basis of fasting plasma glucose (FPG) and plasma glucose (PG) levels at 30 minutes, 1, 2, and, 3 hours Among 56 individuals enrolled, 21 were healthy individuals with normal glucose tolerance (NGT), 10 were diagnosed with T2DM, first degree relatives of T2DM patients with normal glucose tolerance (FD-NGT) were 13 in number and 12 individuals were first degree relatives of T2DM patients with T2DM (FD-T2DM).

### 2.3 Laboratory analyses

#### OGTT (Oral Glucose Tolerance Test)

A 3-hour OGTT (75 g of glucose) was performed in the laboratory department of The Third Hospital of Yunnan Province, China. The samples for plasma glucose (PG) were drawn at 0, 30 minutes, 1, 2 and, 3 hours Diagnosis was based on OGTT recommended by American Diabetes Association and evaluated as follows Patients with (FPG<5.6 mmol/L and 2-hour PG<7.8 mmol/L) were considered as normal glucose tolerance (NGT) and those with (FPG 5.6-6.9 mmol/L and 2-hour PG 7.8-11.0 mmol/L) were considered as T2DM.(17)

#### C-peptide Test

The Connecting peptide (C-peptide) is produced in equal amounts to insulin and is considered a measure of endogenous insulin secretion. (18) Hence, C-peptide levels were analyzed using the automated Roche diagnostics (Manheim, Germany) E170 immuno-analyser (limit of detection 3.3 pmol/l, inter- and intra-assay coefficients of variation < 4.5% and < 3.3%, respectively). Fasting C –peptide level was: 1.1 to 4.4, 1 hour after meal it was5 to 10 times the fasting level and 3 hours after meal it was back to the level of fasting.

#### Insulin Test

Insulin was measured by chemiluminescent immunometric assay (Siemens Healthcare Diagnostics B.V., Breda, the Netherlands). The intra-assay variation was 6% at 47 pmol/L and 3% at 609 pmol/L. The inter-assay variation was 4% at 91 pmol/L and 6% at 120 pmol/L. The detection limit was 15 pmol/L. The fasting insulin level was 2.6 to 24.9 mIU/L, 1 hour after meal it was 5 to 10 times (13 to 249 mIU/L) the fasting level and 3 hours after meal it was back to the level of fasting.

### 2.4 RNA isolation and characterization

Peripheral blood was obtained by venipuncture and then coagulated at room temperature for 0.5-2 h. Following centrifugation at 3000g for 5 min, serum was collected and centrifuged again at 12,000g for 15 min to completely remove cell debris. It was then aliquoted and stored at -80℃ until miRNA detection. Total RNA containing small RNA was extracted from 500 µl of serum using mirVana isolation kit (Ambion, Austin, USA) according to the manufacturer's protocol. The final elution volume was 100 µl. The concentration of all RNA samples was quantified by NanoDrop 1000 (Nanodrop, Wilmington, USA), and 20 ng of serum RNA containing miRNA was reverse transcribed to cDNA using TaqMan MicroRNA Reverse Transcription Kit (Applied BioSystems, Foster, USA) and miRNA-specific primers provided by the manufacturer in an Applied BioSystems 9300 Thermocycler (Applied Biosystems, Foster, USA). All Complementary DNAs (cDNAs) were stored at -20℃ until Quantitative real-time polymerase chain reaction (qRT-PCR) analysis. After 1:2 dilution, 4.5 µl was used as template in a 10 µl qPCR.

### 2.5 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time RT-PCR (qRT-PCR) was performed to assess the levels of miR-375. Essential MicroRNA-specific data are presented in **Table 1**. Each reaction was performed in a final volume of 10µl containing 4.5µl cDNA, 5µl TaqMan 2 × Universal PCR Master Mix (No AmpErase) and 0.5µl TaqMan miRNA Assay (Applied BioSystems). The thermal cycle was set as start with 10 min template denaturation at 95℃, 40 cycles of denaturation at 95℃ for 15 s and combined primer annealing/elongation at 60℃ for 1 min. Each sample was run in triplicate for analysis. As the internal control gene, non-coding small RNA RNU6B was used according to the Applied Biosystems Application Note. RNU6B has demonstrated both stable and abundant expression in different human tissues and organs. It is regarded as one of the control genes with the least variability for miRNAs assays and has been widely used in different fields including diabetic research

### 2.6 Statistical Analysis

Data were presented as means ± standard error. For qRT-PCR data, the difference of threshold cycle (Ct) between miR-375 and RNU6B (△Ct) which was equivalent to the ratio of log2-transformed absolute copy numbers was employed to show the relative expression levels of miR-375. The one-way ANOVA followed by a post hoc multiple comparison test was used to compare the concentration of miR-375 among the four individual groups Statistical analysis was performed by using SPSS software. P value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1 Clinical data

A significant difference was observed in T2DM and FD-T2DM groups compared to NGT and FD-NGT in terms of OGTT, C-Peptide, and insulin test results. The 3-hour OGTT result showed a mean ± standard error of 3.844±0.732 for NGT group whereas T2DM group showed a value of 13.810±0.982 (p□0.05). In the FD-T2DM group, the 3-hour value was observed as 10.583±0.897 in comparison to NGT (3.844±0.732) and FD-NGT (4.130±0.861) showing statistical significance with p□0.05. **Table 2, Fig 1A**. Similar results were obtained from the C-Peptide test. The C-Peptide, mean ± standard error of 2.854±1.351 was observed in NGT group after 3 hours whereas T2DM group showed a value of 6.875±1.728 and was statistically significant with p□0.05. FD-T2DM showed a value of 12.218±1.728 and p□0.05 when compared to NGT (2.854±1.351), T2DM (6.875±1.728) and FD-NGT (6.704±1.590) **Table 3, Fig 1B**. The 3-hour insulin test showed a mean ± standard error value of 9.802±5.835, 23.543+7.829, 22.523±6.866, and 42.619±7.147 in NGT, T2DM, FD-NGT, and FD-T2DM respectively. A significantly higher value of p□0.05 was observed in T2DM compared to NGT and in FD-T2DM also when compared to FD-NGT. Interestingly, FD-NGT showed a significantly higher value compared to NGT group. **Table 4, Fig 1C.**

These results also signify that individuals in FD-NGT group are at a higher risk of developing T2DM compared with individuals in NGT group.

### 3.2 Expression levels of miR-375 were significantly elevated in T2DM and FD- T2DM

MiR-375 assessment showed significant up-regulation in T2DM group compared with NGT group (**Fig. 2**) similarly, a significant up-regulation was observed in FD-T2DM group compared with FD-NGT group. P value of □0.001 was observed in T2DM and FD-T2DM groups showing its statistical significance. Interestingly, even though there was no significant difference between NGT and FD-NGT groups, we observed a slight increase in miRNA expression in FD-NGT compared with NGT group.

## 4. Discussion

The current study is the first clinical study to assess the differential expression of miR-375 in first degree relatives of T2DM patients in Han Chinese population. The results of the study revealed a significant difference in terms of OGTT, C-Peptide and insulin test results in T2DM and FD-T2DM groups compared with NGT and FD-NGT. The OGTT test showed a significantly higher plasma glucose in T2DM compared with NGT and FD-T2DM compared with FD-NGT. The findings of our study were consistent with previous clinical study which showed a significantly higher stepwise trends in FPG and 2-h PG from T2D-susceptible individuals with normal glucose tolerance (s-NGT) to pre-diabetes and the newly diagnosed T2D patients (n-T2D).(14)

The miR-375 level showed a significant up-regulation in T2DM compared with NGT group. A pilot cross-sectional study conducted to assess the miRNA levels in the circulation of subjects with diabetes showed similar results. The levels of miRNAs were significantly elevated in subjects with various forms of diabetes compared to healthy controls.(19) A previous study on miR-375 up-regulation in T2DM patients showed its association with pancreatic islet amyloid formation and β-cell deficit. Owing to this, microRNA-375 may serve as a biomarker in the pathogenesis of type 2 diabetes related to islet amyloid deposition and β-cell dysfunction. (20) Additionally, our study results revealed a significant up-regulation in FD-T2DM compared with FD-NGT group. Previous studies conducted on familial aggregation of T2DM showed a higher risk of developing diabetes and pre-diabetes progression in first degree relatives. (21,22) The inclusion of first-degree relatives and assessing miRNA levels in them provides soundness to our study. A small sample size and consideration of only miR-375 in the analysis can be considered as the limitations of our study. Besides, this study was conducted only in Chinese population, and hence the results observed might not be generalizable.

In future studies, influences on circulating miRNA by gender, age and other factors (other diseases, medications, lifestyle factors) should be investigated. These studies will help to predict diabetes in the general population. Further, amassing data samples, standardizing miRNA

detection technologies, and tracking and validating correlations with disease states will improve their predictive and diagnostic efficacy for developing strategies of therapeutic intervention.

To conclude, the present study demonstrated a significantly higher miR-375 expression in T2DM compared to NGT and FD-T2DM compared to FD-NGT group, and thus, miR-375 may serve as a stable biomarker for the early prediction of T2DM among high-risk individuals.

# Declarations

### Ethics approval and consent to participate

The study was conducted at The Third Hospital of Yunnan Province, China after receiving approval from the Ethics Committee of the hospital and was conducted in compliance to the Declaration of Helsinki. The written informed consent was obtained from all subjects prior to their enrollment.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

All authors declare that they have no competing financial interests.


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### Authors' contributions

WX and LY analyzed and interpreted the patient data. MB and LD were major contributors in writing the manuscript. All authors read and approved the final manuscript.


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## Tables

**Table 1:** Information about detected miRNA

| Assay name | Assay type | AB assay ID | miRBase accession |
|---|---|---|---|
| hsa-miR-375 | Mature miRNA | 000564 | MIMAT0000728 |
| RNU6B | Control miR | 001093 | NR_002752 |

**Table 2: Oral Glucose Tolerance Test**

| | Time for 75-g oral glucose | | | | |
|---|---|---|---|---|---|
| Groups | FPG | 30 min | 1 h | 2 h | 3 h |
| NGT | 4.541±0.490 | 5.293±0.543 | 4.763±0.677 | 4.649±0.595 | 3.844±0.732 |
| T2DM | 7.605±0.657* | 10.336±00.729* | 14.604±0.908* | 15.781±0.799* | 13.810±0.982* |
| FD-NGT | 4.928±0.576 | 7.547±0.639* | 7.193±0.796* | 6.150±0.701 | 4.130±0.861 |
| FD-T2DM | 8.141±0.600*△ | 11.646±0.665*△ | 14.550±0.829*△ | 13.846±0.729*△ | 10.583±0.897*△ |

**Notes**: * p□0.05 in comparison to NGT, △ p□0.05 in comparison to FD-NGT, Data are expressed as mean ± standard error.

**Abbreviations**□NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; FD-NGT, in first-degree relatives of type 2 diabetes mellitus individuals with normal glucose tolerance; FD- T2DM, first-degree relatives of type 2 diabetes mellitus individuals with type 2 diabetes mellitus.

**Table 3: The C-Peptide Test**

| | Time for meal | | | | |
|---|---|---|---|---|---|
| Groups | FPG | 30 min | 1 h | 2 h | 3 h |
| NGT | 2.002±0.414 | 5.692±0.720 | 6.362±1.301 | 5.249±1.686 | 2.854±1.351 |
| T2DM | 2.241±0.529 | 3.495±0.921 | 5.598±1.665 | 7.505±2.157 | 6.875±1.728 |
| FD-NGT | 3.594±0.487* | 8.858±0.847* | 10.793±1.531* | 11.628±1.984* | 6.704±1.590* |
| FD-T2DM | 4.285±0.529*# | 6.383±0.921# | 10.722±1.665*# | 14.526±2.157*# | 12.218±1.728*#△ |

**Notes**: * p□0.05 in comparison to NGT, △ p□0.05 in comparison to FD-NGT, # p□0.05 in comparison toT2DM. Data are expressed as mean ± standard error.

**Abbreviations**□NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; FD-NGT, first-degree relatives of type 2 diabetes mellitus individuals with normal glucose tolerance; FD- T2DM, first-degree relatives of type 2 diabetes mellitus individuals with type 2 diabetes mellitus.

**Table 4: The Insulin Test**

| | Time for meal | | | | |
|---|---|---|---|---|---|
| Groups | FPG | 30 min | 1 h | 2 h | 3 h |
| NGT | 9.073±1.275 | 50.660±6.294 | 43.932±8.189 | 28.836±10.875 | 9.802±5.835 |
| T2DM | 6.540±1.711 | 14.789±8.444* | 28.961±10.987 | 30.773±14.566 | 23.543±7.829 |
| FD-NGT | 12.988±1.500 | 69.371±7.406 | 65.078±9.636 | 64.240±12.775* | 22.523±6.866 |
| FD-T2DM | 12.502±1.562# | 31.137±7.709△ | 55.045±10.030△ | 76.580±13.297*# | 42.619±7.147*△ |

**Notes**: * p□0.05 in comparison to NGT, △ p□0.05 in comparison to FD-NGT, # p□0.05 in comparison toT2DM. Data are expressed as mean ± standard error.

**Abbreviations**□NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; FD-NGT, n first-degree relatives of type 2 diabetes mellitus individuals with normal glucose tolerance; FD- T2DM, first-degree relatives of type 2 diabetes mellitus individuals with type 2 diabetes mellitus

## Figures

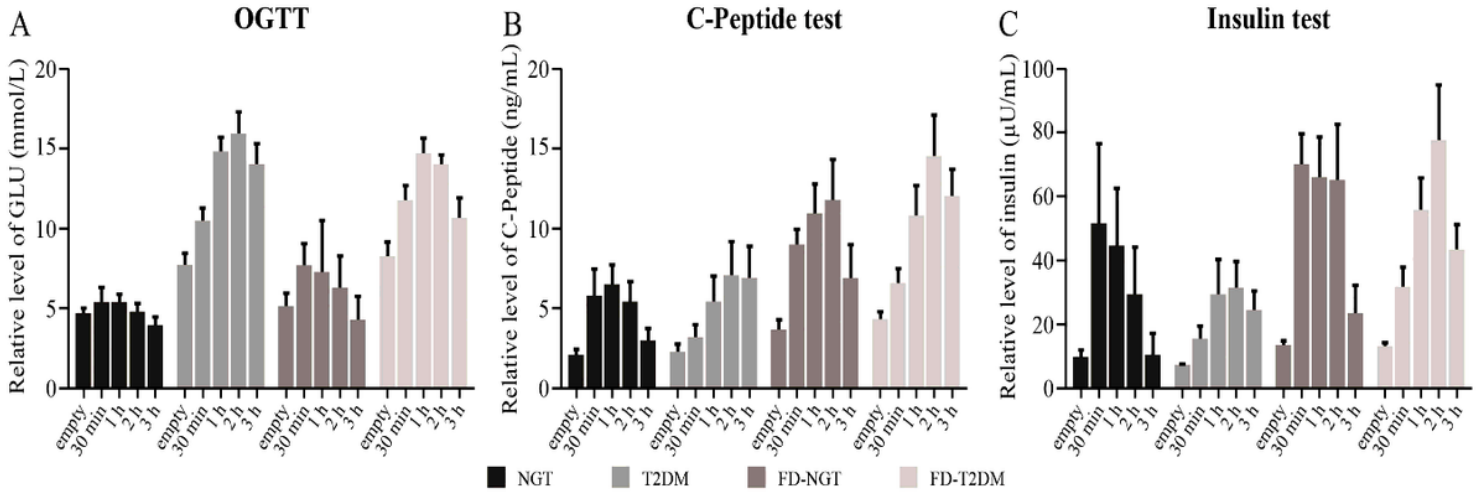


Figure 1

Clinical data a. OGTT (Oral Glucose Tolerance Test). b. C-Peptide test. c. Insulin test.

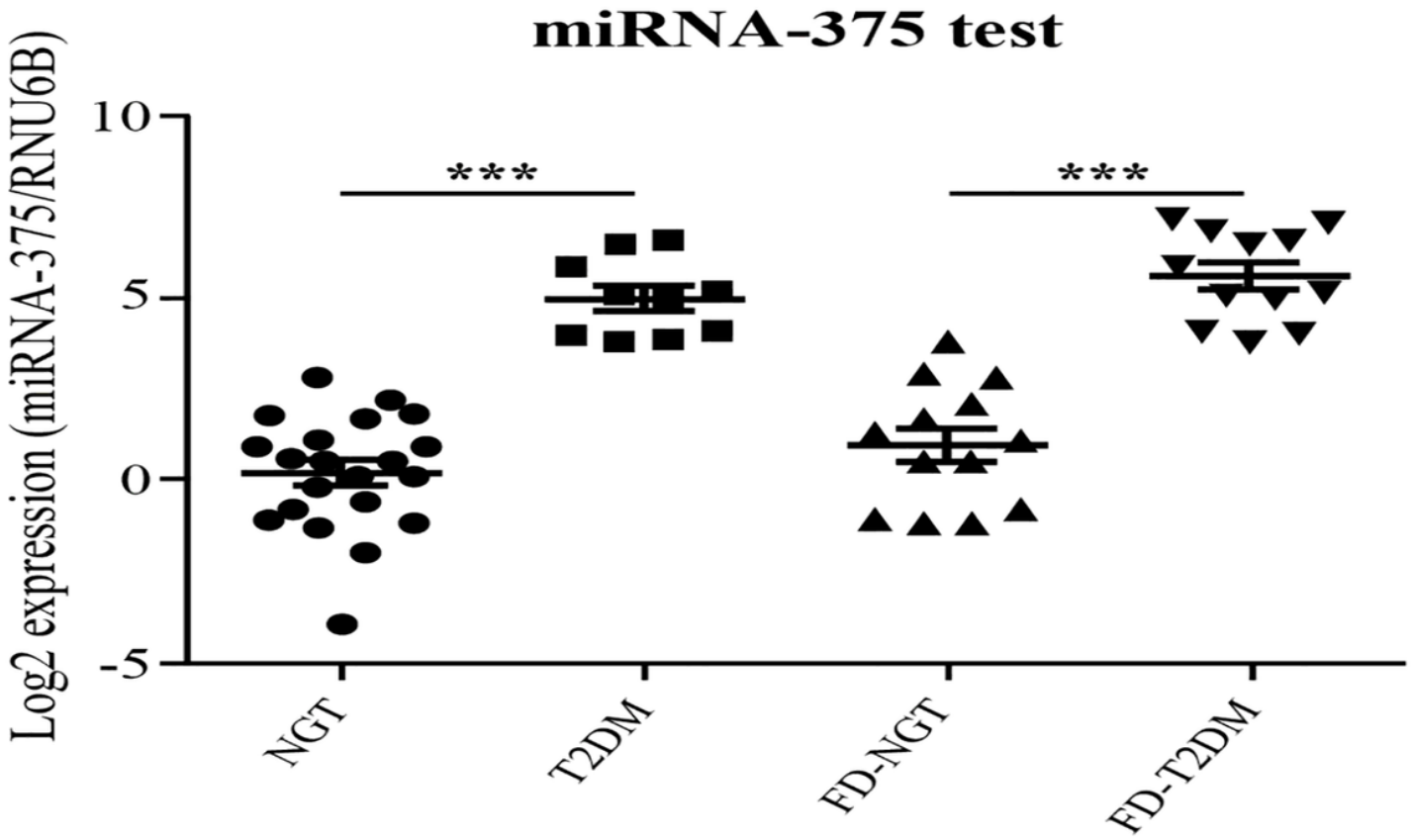


Figure 2

Expression levels of miRNA-375