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Association between circulating microRNA-122, microRNA-126-3p and microRNA-146a and inflammatory markers in patients with pre-diabetes and type 2 diabetes mellitus

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ABSTRACT

Ninety individuals were included in this case-control study:30 type 2 diabetes mellitus, 30 pre-diabetes and 30 healthy individuals (males and females, age: 25-65, body mass index: 25-35). MicroRNAs expression was determined by real-time polymerase chain reaction. Furthermore, plasma concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 6 (Il-6) and fasting insulin was measured by enzyme-linked immunosorbent assay. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as an indicator of insulin resistance.

EXTERNAL LINK

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KEYWORDS

Type2 diabetes mellitus, Pre-diabetes, MicroRNA-122, MicroRNA-126, MicroRNA-146a, Interleukin 6, Tumor necrosis factor alpha, Insulin resistance.

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GUIDELINES

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C)
samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

MATERIALS TEXT

MATERIALS

Nuclease-free Water **Contributed by users**

Chloroform **Contributed by users**

Vortex Mixer **Contributed by users**

Large volume 8-channel pipette tips **Contributed by users**

Disposable gloves, nitrile **Contributed by users**

Aerosol (filter) barrier tips (RNase/DNase free) **Contributed by users**

Primer **Contributed by users**

Distilled Water **Thermo**

Fisher Catalog #15230261

Sterilin™ Clear Microtiter™ Plates, Microtiter Plates, Clear, Lid for Microtiter plate, IRR **Thermo**

Fisher Catalog #642000

Reagent Reservoirs, 25mL **Thermo**

Fisher Catalog #95128095

SAFETY WARNINGS

SAFETY WARNINGS

Laboratory gloves should be worn at all times.
Avoid any skin contact with H2SO4 and TMB. In case of contact, wash thoroughly with water. • Do not eat, drink, smoke or apply cosmetics where kit reagents are used. • Do not pipette by mouth. • When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels. • All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use. • Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration. • Cover or cap all reagents when not in use. • Do not mix or interchange reagents between different lots. • Do not use reagents beyond the expiration date of the kit. • Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H2SO4 and substrate solution, avoid pipettes with metal parts. • Use a clean plastic container to prepare the washing solution. • Thoroughly mix the reagents and samples before use by agitation or swirling. • All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells. • The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly. • If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay. • When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells. • Follow incubation times described in the assay procedure. • Dispense the TMB solution within 15 min of the washing of the microtiter plate.

BEFORE STARTING

1. Fasting blood (10 ml) was taken from participants in the medical diagnostic laboratory of the Yazd Diabetes Research Center: 4 ml in tubes containing ethylene diamine tetra acetic acid(EDTA) for RNA extraction, 2 ml in a CBC tube containing EDTA to measure HbA1c and 4 ml in a test tube containing a clot activator for serum separation.
2. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.
3. Preparation of Wash Buffer Dilute the (200X) wash buffer concentrate 200 fold with distilled water to give a 1X working solution. Pour entire contents (10 ml) of the Wash Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-25˚C.
4. Preparation of Standard Diluent Buffer Add the contents of the vial (10X concentrate) to 225 ml of distilled water before use. This solution can be stored at 2-8°C for up to 1 week.

RNA extraction

1 RNA extraction http://tribioscience.com/files/315-150.pdf

1.1 Prepare 750 ul RiboExTM LS in a 1.5 ml microcentrifuge tube (not provided).

1.2 Add 250 ul blood sample to the 1.5 ml microcentrifuge tube and vortex vigorously. If sample volume is 100 ul, sample should be adjusted to 250 ul with PBS or RNase-free water. Be sure to confirm the applicable minimum volume, which is 100 ul.

1.3 Incubate 2 min at room temperature. This step allows leukocytes to completely be collapsed.

1.4 Add 0.2 ml of chloroform. Shake vigorously for 15 seconds and let it stand for 2 minutes at room temperature. Alternatively, 0.1 ml of BCP (1-bromo-3-chloropropane) can be used in place of

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chloroform.

1.5 Centrifuge at 12,000 x g for 15 minutes at 4°C. The mixture will be separated into three phases; a lower layer, an interphase, and a colorless upper aqueous layer. The upper aqueous volume is about 450 ul. Centrifugation at temperatures >8°C may cause some DNA to partition in the aqueous phase.

cDNA synthesis

2 cDNA synthesis

2.1 Prepare the following reaction mixture in a microcentrifuge tube to the total volume of 12µl:

Total RNA	1-5 µg
BON RT adaptor	1 µl
RNase free water	Up to 12 µl

2.2 Incubation in thermocycler at 75 °C for 5 minute. 5m

2.3 Place on ice immediately and add following component in bellow table.

RT enzyme	1 µl
dNTP	2 µl
5×RT Buffer	4 µl
RNase free water	Up to 20 µl

2.4 Incubation the mixture in thermocycler according to following temperature and duration and cycle:

25 °C	10 min	1 cycle
42 °C	60 min	1 cycle
70 °C	10 min	1 cycle

Real time PCR

3 Real time PCR

3.1 After cDNA synthesis, add the following components:

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cDNA	1 µl
miRNA specific forward primer	0.5 µl
universal reverse primer	0.5 µl
2×miRNA QPCR master mix	6.5 µl
Nuclease free PCR grade H2O	Up to 13 µl

3.2 Incubation the mixture in thermocycler according to following temperature and duration and cycle:

95 °C	2 min	1 cycle
95 °C	5 sec	
60 °C	30 sec	40 cycle

IL6 measurement

4

IL6 measurement by ELISA kit
https://www.diaclone.com/documents/protocole/950.030_Human_IL-6_ELISA_kit_insert_v11.pdf

4.1 Addition: Prepare Standard curve

4.2 Addition :Add 100µl of each Sample, Control and zero (Standard diluent) in duplicate to appropriate number of wells

4.3 Addition: Add 50µl of diluted biotinylated anti-IL-6 to all wells

4.4 Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 1 hour 1h

4.5 Wash: Remove the cover and wash the plate as follows:
a) Aspirate the liquid from each well
b) Dispense 0.3 ml of 1x washing solution into each well
c) Aspirate the contents of each well
d) Repeat step b and c another two times

4.6 Addition: Add 100µl of Streptavidin-HRP solution into all wells

4.7 Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 min 30m

4.8 Wash: Repeat wash step 4.5.

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4.9 Addition: Add 100µl of ready-to-use TMB Substrate Solution into all wells

4.10 Incubation: Incubate in the dark for 12-15 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil. 15m

4.11 Addition :Add 100µl of H2SO4:Stop Reagent into all wells Read the absorbance value of each well (immediately after step 4.11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

TNF alpha measurement

5

TNF alpha measurement by ELISA kit
https://www.diaclone.com/documents/protocole/950.090_Human_TNFa_ELISA_kit_version_9.pdf

5.1 Addition: Prepare Standard curve

5.2 Addition: Add 100µl of each Sample, Standard, Control and zero (appropriate standard diluent) in duplicate to appropriate number of wells

5.3 Addition: Add 50µl of diluted biotinylated anti-TNFα to all wells

5.4 Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 3 hours 3h

5.5 Wash: Remove the cover and wash the plate as follows:
a) Aspirate the liquid from each well
b) Dispense 0.3 ml of 1x wash buffer into each well
c) Aspirate the contents of each well
d) Repeat step b and c another two times

5.6 Addition: Add 100µl of Streptavidin-HRP solution into all wells

5.7 Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 min 30m

5.8 Wash: Repeat wash step 5.5.

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5.9 Addition: Add 100µl of ready-to-use TMB Substrate Solution into all wells

5.10 Incubation: Incubate in the dark for 12-15 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

5.11 Addition: Add 100µl of H2SO4:Stop Reagent into all wells

5.12 Read the absorbance value of each well (immediately after step 5.11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

Fasting insulin

6 Determining serum levels of fasting insulin by ELISA kit

6.1 Addition: Prepare Standard curve

6.2 Addition: Add 50µl of each Sample, Standard, Control and zero (appropriate standard diluent) in duplicate to appropriate number of wells

6.3 Addition: Add 100µl of diluted conjugate Insulin-R to all wells

6.4 Shake the plate for 20-30 seconds in room temperature 30s

6.5 Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 1 hour 1h

6.6 Wash: Remove the cover and wash the plate as follows:
a) Aspirate the liquid from each well
b) Dispense 0.35 ml of 1x wash buffer into each well
c) Aspirate the contents of each well
d) Repeat step b and c another two times

6.7 Addition: Add 100µl of substrate solution into all wells

Citation: Hassan Mozaffari-Khosravi, Fahime Zeinali, Seyed Mohsen Aghaei Zarch, Alireza Jahan-mihan, Shima Abdollahi, Seyed Mehdi Kalantar, Mohammad Yahya Vahidi Mehrjardi, Hossein Fallahzadeh, Mahdieh Hosseinzadeh-Shamsi-Anar, Masoud Rahmanian (06/03/2021). Association between circulating microRNA-122, microRNA-126-3p and microRNA-146a and inflammatory markers in patients with pre-diabetes and type 2 diabetes mellitus.
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6.8 Incubation: Incubate in the dark for 15 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil. 15m

6.9 Addition: Add 100µl of Stop Reagent into all wells. Put the plate on the table without any shake for 15-20 seconds. 20s

6.10 Read the absorbance value of each well (immediately after step 6.9.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length.

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