Plasma miR-126 levels and its genomic polymorphism SNP rs4636297 in Type 2 diabetes

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Abstract. Since the variation of microRNAs expression has been described in type2 diabetes, we aimed to evaluate the plasma levels of miR-126 with known roles in the angiogenesis. Genomic polymorphism of miR-126 (SNP rs4636297) was also assessed as it is involved in the processing of pri-miR-126 to mature microRNA. Genotyping of the SNP rs4636297 was carried out by PCR-RFLP using HaeII enzyme. Real-time PCR assay was applied to assess miR-126 expression, which the fold change in gene expression was calculated by the Relative Expression Software Tool (REST). Data analysis was performed using exact-like logistic regression, Fisher exact test and the *lrm* package with the R software. Our data indicated that the group with BMI greater than 30 was at greater risk of diabetes (odds ratio=14.4, *p* value=0.00), compared with the BMI=19-25. The mean fold decrease in miR-126 gene expression in diabetic samples relative to normal samples was 0.653 (95% confidence interval: 0.012 - 18.765). All patients with the lowest levels of miR-126, had experienced diabetic complications. Considering the AA genotype as the reference, the odds ratio of diabetic complications for the GG and AG genotype were 1.2 and 1.43, respectively. miR-126 is one of the key factors in predisposition to diabetes and diabetic complication. Since the lowest expression of miR-126 leads to the diabetic complications it can be concluded that genetic predisposing factors should be considered in the management and treatment of Type 2 diabetes.

Keywords: human plasma, miR-126, rs4636297, Type 2 diabetes, gene expression, RFLP

INTRODUCTION

Diabetes mellitus consists of heterogeneous metabolic diseases that are characterized by hyperglycemia due to defective insulin secretion, its function, or both (Roder *et al.*, 2016). Type 2 diabetes (T2DM) is the most common type of the disease with an incidence of 90-95% (Chen *et al.*, 2014). Reflected by several studies, microRNAs variations have been implicated in the progress of T2DM and its complications (Deiuliis *et al.*, 2016; Lee *et al.*, 2016; Simpson *et al.*, 2016).

MicroRNAs are small noncoding RNAs composed of 21-23 nucleotides that act as the key regulators of gene expression and biological processes (He *et al.*, 2017). Previous studies have

reported the reduction of miR-126 expression in T2DM patients with or without complications (Kuhnert *et al.*, 2008; Zampetaki *et al.*, 2010). miR-126 is expressed by the vascular endothelial cells and plays an important role in angiogenesis and vascular integrity. This microRNA is a positive regulator of endothelial cell response to vascular endothelial growth factor (VEGF); therefore, its reduction results in resistance to VEGF and defective endothelial cells function. miR-126 gene is located at human chromosome 9 on the seventh intron of the EGFL7 (epidermal growth factor-like protein 7) gene (Meister *et al.*, 2010). This microRNA directly targets phosphoinositide-3-

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kinase regulatory subunit 2 (PI3KR2) and sprouty-related protein-1 (SPRED-1), two negative regulators of the VEGF signaling pathway. Accordingly, it enhances the signaling of phosphoinositide-3-kinase (PI3K) and VEGF-dependent MAP kinase and consequently, affects vascular integrity and angiogenesis (Fish *et al.*, 2008; Liu *et al.*, 2009). One of the main targets of the miR-126 is its host gene; EGFL7. EGFL7 protein is secreted from endothelial cells and is involved in migration and adhesion of endothelial and smooth muscle cells (Kuhnert *et al.*, 2008).

miR-126 also inhibits the expression of vascular adhesion molecule 1 (VCAM-1) in endothelial cells and decreases the leukocytes adhesion which counteract tissue inflammation. Recently, miR-126 was suggested as a therapeutic tool to decrease neuroinflammation via VCAM1reduction (Cerutti *et al.*, 2017).

Given the role of miR-126 in regulating cell migration, vascular adhesion, vascularization and subsequently wound healing, it should be considered as a serious candidate for the treatment of vascular defects, including diabetic complications (Qu *et al.*, 2018).

Genetic variants are involved in MicroRNA expression. Only one single nucleotide polymorphism (SNP) has been identified for mir-126. This SNP is a substitution of the twenty-fourth base, known as the SNP rs4636297. This SNP prevents the processing of pri-microRNA to mature microRNA, resulting in a reduced suppression of the various targets of miR-126. It has been shown that, despite higher G allele expression compared to A allele, the efficacy of G allele processing was only 16% of the A allele processing efficacy. This remarkable difference, roots in pri-mir-126-G condensed structure prevents the access of droscha enzyme. As a result, the amount of mature miR-126 in cells containing A allele is higher than cells bearing G allele (Harnprasopwat *et al.*, 2010).

In the present study, we evaluated the frequency of genomic SNP rs4636297 and the miR-126 gene expression in T2DM patients in the Iranian population for the first time. We have also studied the association between the T2DM complications and miR-126 plasma levels.

MATERIALS AND METHODS

Participants

T2DM patients were selected according to the latest criteria of the World Health Organization. Briefly, patients have considered T2DM if: 1) fasting plasma glucose (FPG) was equal to or higher than 126 mg percent, 2) hyperglycemia symptoms present along with plasma glucose in a casual testing, equal to or greater than 200 mg percent, 3) two-hour, 75-gram oral glucose tolerance test (OGTT) equal to or greater than 200 mg percent. Based on the mentioned criteria 34 T2DM and 38 non-diabetic individuals aged 45-64 years were selected among referrals to Imam Reza Hospital, Tehran, Iran.

In order to exclude the pre-diabetic subjects, the control group was precisely evaluated by a specialist in the field. Individuals were enrolled in the study as the controls who had no symptoms of diabetes in the two previous clinical and laboratory examinations and none of their first-degree relatives has a medical history of diabetes.

The information on age, sex, weight, height, and the family history of diabetes was collected. Applying a questionnaire, clinical and laboratory characteristics and complications including nephropathy, neuropathy, retinopathy, and cardiovascular disorder were also recorded in case group. BMI of all participants calculated by $\text{kg} (\text{weight})/\text{m}^2 (\text{height})$ formula.

Following informed consent obtaining, 2 ml of venous blood samples were collected in EDTA-containing tubes. Blood samples were centrifuged for 5 min at 1000 xg and plasma was transferred to RNase free tubes. Using spectrophotometer (Biochrom, WPA), oxyhemoglobin adsorption was calculated at 414 nm to determine the purity of the plasma and then the samples were stored at -80°C until further investigation.

Genotyping by PCR- RFLP

DNA extraction was done by salting out method on blood cells obtained during centrifuge steps for plasma isolation. Specific primers were designed using the Gene Runner software and verified using BLAST (Basic Local Alignment Search Tool) website. The final volume of the PCR was 20 μl , containing 10 μl of master mix

(Taq DNA polymerase 2X Master Mix RED, PishgamBiotech, IRAN), 1 μ l of each primers, 8 μ l of double distilled water and 1 μ l of DNA. PCR program was then performed in 30 cycles at 95°C for 5 minutes, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, 72°C for 10 minutes followed by visualization of DNA by 1.5% agarose gel. To identify SNP rs4636297 genotypes, RFLP method was applied using HaeII enzyme (Thermo Scientific FastDigest HaeII (BfoI), PishgamBiotech, IRAN). The reaction mixture consisted of 2 μ l buffer, 0.5 μ l HaeII enzyme, 8.5 μ l double distilled water and 10 μ l PCR product in a final volume of 20 μ l, which was incubated at 37°C for 4-5 hours.

MicroRNAs extraction and real-time PCR

Total RNA extraction from plasma was performed using RNX plus (Sinaclon, IRAN) according to the manufacturer's instructions. cDNA was reverse transcribed from 10 ng of total RNA using the miR-126 specific stem-loop primer. miR-16 was selected as the intrinsic reference gene for the normalization of miR-126 expression. Reverse transcription was performed with the BioFact™ RT Series (Noavaranteb, Iran). MicroRNAs specific forward primer and universal reverse primer were employed for quantitative real-time PCR amplification. Amplification of miR-126 and miR-16 was performed on StepOne Real-Time PCR System using 10 μ l of the Biofact Syber-green high-Rox master mix (Noavaranteb, Iran), 1 μ L of forward primer, 1 μ l of reverse primer, 6 μ l of double distilled water and 2 μ l of cDNA in the final volume of 20 μ l. The program was defined as 95°C for 15 minutes, 95°C for 20 seconds, and 60°C for 40 seconds. No Template Control (NTC) reactions were also included in the experiments. All qRT-PCR reactions were performed in duplicate.

Statistical analysis

Fold change in gene expression was determined using the Relative Expression Software Tool (REST®). To investigate the association of SNP rs4636297 genomic polymorphism with the risk of T2DM and T2DM complications due to the low sample size and subgroups, exact-like logistic regression and fisher exact test were applied by R software, using the elrm package. The regression

model was adjusted based on age and gender variables. As smoking and blood pressure influence T2DM, the analyses were adjusted for these confounding factors in the exact logistic model. However, the results remained unchanged, suggesting that our analysis is not markedly affected by these risk factors.

RESULTS

Patient's data

In order to cluster the data in case and control groups, the groups were matched in terms of gender and age. The mean age in the cases and controls were 58 ± 5 and 57 ± 6.086 , respectively. The study of the link between BMI and the risk of T2DM revealed that the group with BMI greater than 30 was at greater risk of T2DM (OR = 14.4; CI: 2.34-446) compared to the reference group (BMI 19-25) which was statistically significant (p value, 0.00). There were significant differences between cases and controls in terms of fasting glucose and hemoglobin A1c rates, however triglycerides and cholesterol measurements showed no important differences between groups (Table1).

Genotyping of the SNP rs4636297

The HaeII enzyme has RG↓CGCY restriction site and G allele substitution with A allele removes the restriction site. As a result, AA genotype had only one fragment of 335 bp and those of the genotype AG displayed three fragments at 225 bp, 110 bp and 335 bp. GG genotype produced two distinct bands of 225 bp and 110 bp. In order to confirm the accuracy of RFLP results, randomly selected samples with AA, AG and GG genotypes were sequenced (Figure 1). Using the information obtained from RFLP, the frequency of A allele and G allele were calculated 0.43 and 0.57 respectively. The frequency of A allele and G allele in healthy subjects were 0.45 and 0.55, respectively. Regarding the link between genotypes and the general risk of diabetes, the odds ratio for GG genotype was 1.13 (CI: 0.256-4.53) and the odds ratio for AG genotype was 1.26 (CI: 0.311-5.91) compared with the AA reference genotype. The risk of diabetic complications for the GG and AG genotype showed a slight

increase, considering the AA genotype as the reference group (OR=1.12, CI: 0.117-11 and OR=2.13, CI: 0.22-27.1, respectively). However,

the differences were not statistically significant for both genotypes (p-value> 0.05).

Table 1. Clinical characteristics of the study subjects.

	Normal controls	T2DM	P value
	Mean±SD	Mean±SD	
Number of subjects	38	24	-
Age (year)	57± 6.086	58± 5	0.521
BMI (kg/m ²)	26±3	29±3	0.001
Duration of diabetes (month)	-	76±96	-
Familial history (complicated)	-	44.11%	-
Biochemical tests			
TG* (mmol/L)	121 ± 50	183 ± 253	0.267
FG** (mmol/L)	91 ± 6	144 ± 48	0.000
Cholestrol (mmol/L)	188 ± 45	178 ± 32	0.408
HbA1c*** (%)	5 ± 0	7±1	0.000
Complications			
Complicated	-	47.1%	-
Nephropathy	-	0%	-
Neuropathy	-	8.8%	-
Retinopathy	-	11.8%	-
Neuropathy + Retinopathy	-	5.9%	-
Cardiovascular	-	20.6%	-

*Triglycerides, **Fasting Glucose, ***Hemoglobin A1c

Figure 1. Electropherograms obtained from the three possible genotypes. Above AG, middle AA, and below GG genotypes.

miR-126 gene expression

Melting curve analysis showed a single specific peak for each microRNAs. Raw amplification data were transferred to LinReg software and used to generate linear standard curves (Figure 2). The mean fold decrease of miR-126 gene expression in T2DM samples relative to normal samples was 0.653 (95% confidence interval: 0.012 - 18.765; standard error: 0.093 - 4.257). The overall profile of the gene expression variations remained unchanged when the data were adjusted to age and gender. Delta Ct (Δ CT) value for each sample was calculated using the formula (Ct target gene - Ct reference gene). Then miR-126 expression was categorized into 3 groups; Δ CT<-3 with the highest expression, Δ CT between -3 to +3 and Δ CT >+3 with the lowest expression. As indicated in Figure 3, the frequency of AG genotype in the group with the lowest expression of miR-126 (Δ CT >+3) was higher than AA genotype; while, there was no GG genotype in this group. Furthermore, the results showed that 44% of uncomplicated T2DM patients had miR-126 expression in the Δ CT range of <-3 (highest

expression), whereas only 19% of subjects with T2DM complications expressed miR-126 in $\Delta CT < -3$ (Figure 4). On the other hand, all of the

patients in $\Delta CT > +3$ group with the lowest expression of miR-126 had experienced a kind of T2DM complications.

Figure 2. Calculation of the real-time PCR efficiencies. Raw amplification data were transferred to LinReg software and used to generate linear standard curves. The PCR efficiencies were calculated for miR-16 and miR-126 gene expression reactions.

Figure 3. Correlations between genotypes and the expression of miR-126.

DISCUSSION

The overall pattern of T2DM worldwide indicates that developing countries are at higher risk of the disease. The Tehran Lipid and Glucose Study, which is the most significant national study over the past 15 years in the field of diabetes, indicate that the incidence of T2DM is rapidly increasing due to the lifestyle (Hadaegh *et al.*, 2007). The results of our study also revealed that BMI is significantly associated with an increased risk of T2DM. According to a previous study in Iran, BMI is an important factor for progression from healthy to pre-diabetes status especially in men (Khaloo *et al.*, 2018). Interestingly, a new study has shown that increased BMI is associated with lower miR-126. This finding is also true in diabetic patients and it can be expected that with increasing weight, the level of miR-126, which has anti-inflammatory function, will be drastically reduced and pave the way for vascular inflammation (Hijmans *et al.*, 2018).

To provide elements to explain inter population variation we focused on miR-126 plasma levels and its genomic SNP rs4636297. Based on our results the A and G allele frequency were 0.43 and 0.57 in T2DM group. A previous study that has been conducted on the association of miR-126 genomic SNP rs4636297 with diabetic

Figure 4. Correlations between the diabetic complications and the expression of miR-126

retinopathy, has reported the A and G allele frequency, 0.40 and 0.60 respectively (McAuley *et al.*, 2015). In different populations the frequency of A allele varies from 0.1 to 0.4 based on the information provided at ENSEMBELE browser, with the maximum A allele frequency of 0.45. Taken together these results indicate that the Iranian population can be among the populations with the highest frequency of A allele. Of course, given the sample size, we can't conclude on the allele frequency in Iranian population for sure and this result should be considered as a preliminary. McAuley *et al.* also reported a significant relationship between the presence of A allele and STDR (sight-threatening diabetic retinopathy) (McAuley *et al.*, 2015).

We observed an increased odds ratio for GG and AG genotypes compared with AA genotype for the risk of T2DM and T2DM complications, interestingly with a greater susceptibility for AG genotype. To provide elements to explain this observation we analyzed the miR-126 expression in correlation with genotypes. Our finding indicates that among patients with the lowest plasma levels of miR-126, the frequency of AG genotype was higher than AA and GG genotypes. As mentioned before, it has been shown that despite higher expression of the G allele compare to A allele, the processing efficacy of G allele for mature miR-126 synthesis is much lower than cells that express A allele. It can be assumed that, in heterozygote AG genotype, A allele causes lower primary expression and G allele causes reduced processing efficiency of pri-microRNA that simultaneously leads to lower expression of mature miR-126. However, the genotype of SNP rs4636297 was not related to mature miR-126 plasma levels as the majority of AG genotype showed normal expression levels. Functional cell study of the heterozygous AG genotype is needed to reveal whether miR-126 processing efficiency is more profoundly affected than homozygous genotypes.

Several studies have been conducted concerning the expression levels of miR-126 in T2DM compared to healthy subjects (Meng *et al.*, 2012; Olivieri *et al.*, 2015; Zhang *et al.*, 2015). In the present study, the results from real-time PCR showed 0.653 mean fold decrease in miR-126 gene expression which confirmed previous studies. According to the results, we observed that

all patients in $\Delta CT > +3$ group which represents the lowest expression of miR-126 had experienced diabetic complications.

Since miR-126 is a positive regulator of angiogenesis in normal endothelial cells, it could be considered as a key factor in predisposition to T2DM complications. miR-126 promotes VEGF activity and vessel formation by down-regulation of SPRED-1 which is an intracellular inhibitor of angiogenic signals (Wang *et al.*, 2008). Furthermore, one of the main targets of the miR-126 is EGFL7 which plays a pivotal role in smooth muscle contraction and endothelial cells migration (Kuhnert *et al.*, 2008). Inactivation of EGFL7/miR-126 robustly impairs developmental angiogenesis and vascular integrity via PI3K and VEGF-dependent MAP kinase pathway (Fish *et al.*, 2008). In addition, PI3K regulation occurs by miR-126 mediated activation of angiopoietin-1 signaling which results in vessel stabilization and maturation (Sessa *et al.*, 2012).

CONCLUSION

By enhancing VEGF signaling, miR-126 is one of the key factors in predisposition to T2DM and its complications. Reduction in the expression of miR-126 interferes with cell homeostasis, vascular coherence, and wound healing, all of which are related to T2DM complications. Further understanding of the genetic factors such as noncoding RNAs involved in T2DM will provide preventive strategies as well as effective therapeutic methods leading to appropriate management of the disease. It can be concluded that with considerable healthcare costs and resources allocated to T2DM, in addition to nutrition and lifestyle, other contributors such as genetic factors should be considered in the management of the disease.

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