

## Differential Expression of MiR-18b in PBMC from T1D Patients in Isfahan Population

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### Abstract

**Background:** Type 1 diabetes (T1D) is caused by cell-mediated autoimmune attack on pancreatic beta cells. Previous studies highlight the role of microRNAs (miRNAs) in the pathogenesis of T1D. MiRNAs are small non-coding RNAs involved in the regulation of gene expression post-transcriptionally. In this work, microRNA-18b (miR-18b) was chosen and its differential expression was measured between T1D patients and healthy controls from Isfahan population.

**Materials and Methods:** miR-18b was selected according to bioinformatic studies using miRWalk software. 22 T1D patients and 18 healthy controls from Isfahan population were enrolled in this study. Total RNA of the peripheral blood mononuclear cell (PBMC) samples was extracted. After cDNA synthesis, the expression profile of miR-18b was quantified by means of qPCR method in patients and controls. Finally, the results were statistically analyzed.

**Results:** In this study despite our hypothesis, the expression levels of miR-18b didn't show any significant difference between T1D patients and healthy controls (p value: 0.145).

**Conclusion:** Due to the results of our experimental analysis, it seems that miR-18b doesn't have any association with T1D disease in Isfahan population.

**Keywords:** microRNA, miR-18b, miRWalk, qPCR, Type 1 diabetes (T1D)

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### Introduction

Type 1 diabetes (T1D) results from the autoimmune destruction of pancreatic islet- $\beta$  cells which produce insulin (1). In this disease, genetics, epigenetics and environmental factors interact with each other and make it a multifactorial complex disease (2). T1D can occur at any ages, but it is known as one of the most common chronic diseases in children (3). The incidence of T1D has been shown to increase for decades and the number of new T1D cases is increasing at the rate of about 3% per year in different countries worldwide. This increase indicates the importance of environmental factors (4-6). Genetic susceptibility plays a critical role in the pathogenesis of T1D, in which genome-wide association studies (GWAS) have linked more than 50 genetic variants to the T1D susceptibility (6, 7). The major susceptibility locus is related to HLA class II genes which are located on chr6p21. In addition,

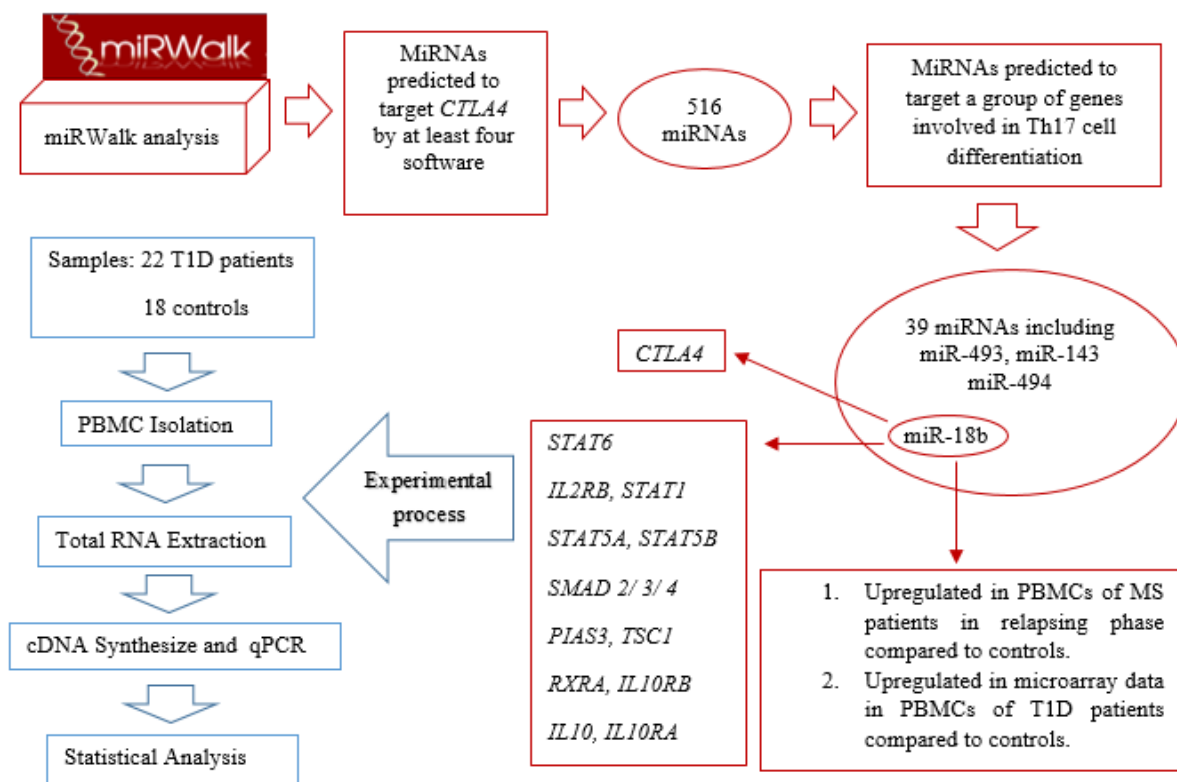
multiple non-HLA loci play effective role in the T1D risk, including INS, PTPN22, CTLA4, IL2RA, etc. (8-10). The CTLA4 (cytotoxic T lymphocyte-associated protein 4) gene participates in the regulation of T cell activation. This gene is a potential candidate for autoimmune diseases like T1D (10). Different types of T cells have a vital role in the pathogenesis of T1D (11). Among them, Th17 cells (a subpopulation of CD4+ T cells) are considerable. Several reports highlight the role of the plasticity of Th17 cells and the balance between them and T regulatory cells or Tregs (inhibitors of immune responses), in the pathogenesis of various autoimmune diseases including T1D (1, 12). miRNAs are 20-22 nucleotide non-coding RNAs regulating gene expression after translation (13). These molecules are involved in various physiological processes, including cell proliferation, cell differentiation, apoptosis,

maturation and inflammation (14, 15). Many miRNAs have been reported to be expressed differently in T1D and may have a critical role in the pathogenesis of this disease and autoimmunity through the regulation of key genes. So these molecules can be used as useful biomarkers in the early detection and cure of T1D (16-19). For this purpose, bioinformatic databases were used in order to find an appropriate miRNA that could be related to T1D and finally miR-18b was chosen. In addition, miR-18b was previously reported to be upregulated

in the PBMCs of T1D patients by microarray analysis (20). Here, the expression levels of miR-18b were quantified in the PBMCs of T1D patients from Isfahan population and compared to healthy controls by qPCR method. The expression levels of this miRNA didn't show any significant difference between T1D patients and healthy controls.

### Materials and methods

The methods used in this study are summarized in Figure 1.



**Figure 1.** Workflow of the Bioinformatics and experimental processes in this study. Bioinformatics process indicated in red borders and experimental processes shown in blue borders.

#### *In silico studies*

In this study, miRWalk 2.0 software was used for prediction of miRNAs and only miRNAs which were predicted by at least four software were considered (21, 22). First, miRNAs predicted to target *CTLA4* gene were noted, then a list of about 54 validated genes involved in Th17 cell differentiation was prepared and miRNAs which also targeted some of these genes were chosen from the previous list. The final list consisted of miRNAs which had differential expression in autoimmune diseases in addition to mentioned properties. Finally, miR-18b which was predicted to target *CTLA4* gene and several key involved in Th17 cell differentiation was selected.

#### *Sample collection and isolation of peripheral blood mononuclear cells (PBMCs)*

An informed consent form was signed by all subjects enrolled in this study and was approved by the University of Isfahan Ethical Committee. The whole blood samples (5 ml in EDTA containing tube) were drawn from 22 T1D patients and 18 healthy controls. All the subjects were between the ages of 13-30 years old and from Isfahan state population. The disease duration of patients was between 4-25 years. The patient group involved 13 females and 9 males and the control group consisted of 13 females and 5 males. The healthy controls had no family history of T1D and other autoimmune diseases. Briefly, the

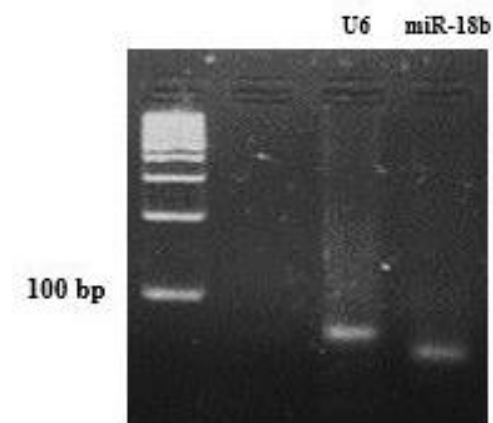
whole blood was diluted in 1:1 PBS and layered over a Hypaque-Ficoll (Innotrain, Germany) medium with half the volume of blood in a falcon tube. Fresh PBMCs were isolated using Hypaque-Ficoll density gradient centrifugation following phosphate buffered saline (PBS) washing.

#### RNA extraction and cDNA synthesis

Total RNA, including miRNAs, was extracted with Trizol reagent (Invitrogen, USA) from isolated PBMC samples according to manufacturer's instruction and afterwards the quantity and quality of extracted RNAs were verified by 1.5% agarose gel and 260/280 absorbance ratio. cDNA synthesis of 2µg isolated RNA was conducted for miR-18b and RNU6B using miRNAs complementary DNA (cDNA) Synthesis Kit (ParsGenome, Iran) based on manufacturer's instruction.

#### Real time PCR (qPCR)

The qPCR reaction was carried out in a total volume of 15µl, involved cDNA synthesized of 2 µg RNA, added to a master mix containing 10 pmol/µl of miR-18b specific primer (Parsgenome, Iran) and 7.5µl SYBR Green (TAKARA, Japan). The sequence of miR-18b is UAAGGUGCAUCUAGUGCAGUUAG (www.miRbase.org). The qPCR method was performed using Bio-Rad equipment with 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 63 °C for 20 s and 72 °C for 30 s. In this study miRNA expression was normalized by the endogenous control gene of small RNA, RNU6B (U6), as the reference gene (Figure 2).



**Figure 2** Visualization of miR-18b and RNU6B (U6) on agarose gel 4%. Both miRNAs have a single band and are located beyond the 100 bp band of ladder. Marker in here is a 100bp DNA ladder.

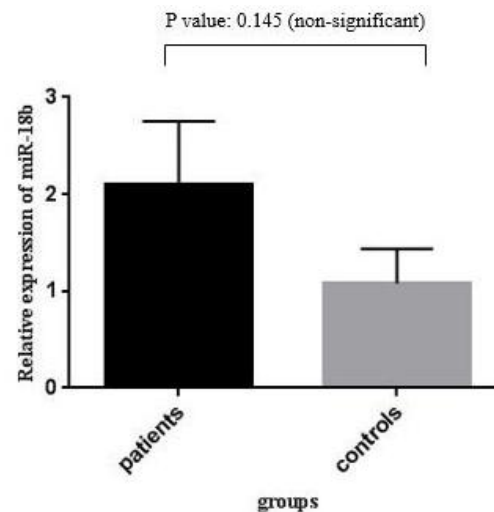
#### Statistical analysis

The REST (Relative Expression Software Tool) program was used for data analysis (23). Subsequently, nonparametric Mann-Whitney U test was performed

in order to determine the significance or non-significance of variations between controls and patients using SPSS 16 software (SPSS Inc. 2007. Chicago) and GraphPad Prism 6 (GraphPad prism Software, Inc. San Diego CA, USA). The expression levels of this miRNA were also analyzed in association with gender, disease duration, the age at disease onset and HbA1c levels. As mentioned before, U6 gene which was confirmed as an appropriate reference gene in previous studies, was used in order to normalize final data (17, 19). P value < 0.05 was considered statistically significant.

#### Results

In this study, 516 miRNAs were predicted to target CTLA4 gene by at least four software using miRWalk 2.0. Among these, 39 miRNAs had differential expression in autoimmune diseases and were predicted to target some genes of our list which were involved in Th17 cell differentiation.



**Error! Reference source not found.** The expression level of miR-18b in T1D patients (n: 22) compared to healthy controls (n: 18). Results normalized with RNU6B gene. P value < 0.05 was considered statistically significant. There is no significant difference between T1D patients and healthy controls (p value: 0.145, non-parametric Mann-Whitney U test).

Finally, miR-18b was chosen as an appropriate candidate related to T1D. This miRNA was predicted to target STAT6, IL2RB, STAT1, STAT5A, STAT5B, SMAD2/3/4, PIAS3, TSC1, RXRA, IL10RB, IL10, IL10RA and FOXO3 genes in addition to CTLA4. The expression levels of miR-18b and U6 (as reference gene) were measured by the qPCR method in the PBMCs of 22 patients and 18 healthy controls from Isfahan population. Next, data was normalized with U6 and the statistical analysis was performed by REST, SPSS and GraphPad Prism 6 software. The statistical results show no significant

changes in miR-18b expression levels between patients and controls (p value: 0.145) (Figure 3). In addition, no significant changes were found in the expression levels of this miRNA in correlation with gender, disease duration, the age at disease onset and HbA1c levels.

### Discussion

T1D is a complex multifactorial disease affecting millions of people around the world. There are at least 20 regions of the genome noted to link with the susceptibility to T1D (9). PTPN22, UBASH3A, and CTLA4 loci are mapped to have gene variants, which downregulate TCR activation response (2). CTLA4 gene is located on chromosome 2q33 and codes an important molecule for proper negative regulation of immune responses, which have reported severe lymphoproliferative disorders in knockout mice (24, 25). Treg cells expressing the transcription factor Foxp3 have a critical role in suppression of inflammation and maintenance of immunological self-tolerance. A specific deficiency of CTLA4 in Tregs of mice shows these cells may critically require CTLA4 in order to suppress immune responses (26, 27). The 3'-UTR rare variants in the CTLA4 gene may affect T1D pathogenesis by modification of miRNA-binding sites and thus gene regulation through miRNAs (28). The importance of environment in the pathogenesis of T1D highlights the role of epigenetic mechanisms which link the environment and gene function (29). Epigenetics involves mechanisms which cause stable and heritable changes in gene expression without alterations in DNA sequence, which can have an influence on autoimmunity development (30). Among epigenetic processes DNA methylation, histone modifications, chromatin accessibility and miRNAs can be noted (31). The differential expression of miRNAs can affect the particular targets and pathways which cause autoimmune diseases (32, 33). Previous studies show T1D is associated with differential expression of specific miRNAs in the blood and according to the role of these molecules in the immune homeostasis, they can be proper candidates for exploring the mechanism of pathogenesis, early detection and cure of T1D (16, 17, 19). In this study, miRWalk was used as a bioinformatic tool for the prediction of appropriate miRNAs which target CTLA4 as an important gene in T1D susceptibility. hsa-miR-18b was selected as a proper candidate to target CTLA4 by screening 516 miRNAs using at least four software. According to miRWalk results, miRWalk, miRMap, RNAhybrid and TargetScan software predicted CTLA4 as a target of miR-18b. This target was predicted for miR-18b by 90 percent confidence using TargetScan software. In addition, it is predicted

that this miRNA targets, IL2RB, STAT1, STAT6, STAT5A, STAT5B, SMAD2/3/4, PIAS3, TSC1, RXRA, IL10RB, IL10, IL10RA and FOXO3 which all are negative regulators of Th17 cell differentiation. So, it has been predicted that miR-18b would be overexpressed in T1D and by inhibition of CTLA4, negative regulators of Th17 cell differentiation and therefore disruption of the balance between TReg/Th17 cells, may have a role in T1D pathogenesis. This miRNA is a member of miR-106a/363 cluster, which is located on chrX.q26.2 and expressed in CD4+ and CD8+ T cells (34, 35). Despite our prediction by bioinformatic tools, the expression levels of miR-18b didn't show any significant difference between T1D patients and healthy controls, so it seems there is no correlation between the expression levels of miR-18b and T1D in Isfahan population. However, our *in silico* analyses suggest mentioned genes as predicted targets of miR-18b which needs to be experimentally validated. The relative expression levels of miR-18b also seem not to be associated with gender, disease duration, the age at disease onset and HbA1c levels in Isfahan population. It was reported that miR-18b is overexpressed in relapsing groups with multiple sclerosis compared to controls and may be associated with the relapse phase of multiple sclerosis (36). The miRNA expression profile of 11 T1D patients compared to 9 healthy controls by microarray slides showed that miR-18b is significantly upregulated in the PBMCs of T1D patients. In addition, their functional annotation studies showed this miRNA may be involved in various pathways, including apoptosis, chemokine signaling pathway, infection, focal adhesion, gap junction, hedge hug signaling pathway, insulin signaling pathway, MAPK signaling pathway, P53 signaling pathway, T cell receptor signaling pathway, TGFβ signaling pathway and Wnt signaling pathway. Furthermore HORMAD2, CLEC16A, ORMDL3, MTMR3 and LIF genes were predicted to be the targets of miR-18b (20). In the present study, we quantified the expression levels of miR-18b in the PBMCs of T1D patients and controls by the qPCR method which is also used for validation of microarray data (37). As mentioned before, miRNAs are a group of epigenetic modifications which are linked to the environment, so different environments and populations may affect on the expression pattern of miR-18b. On the other hand, using more samples and applying qPCR method which is more sensitive than microarray method, can be considered for the contradictory results of this study compared with microarray results.

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#### Authors' contributions

AZ experimental design, collection and assembly of data, data analysis, interpretation and manuscript writing. HZ conception and design of the study, data analysis, interpretation, manuscript writing and final approval of the manuscript.

All authors contributed to and approved the manuscript

#### Conflict of interest

The authors declare no conflict of interest.

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