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MicroRNAs miR-21a and miR-93 are down regulated in peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes

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ABSTRACT

Introduction: It is well established that type 1 diabetes (T1D) is an autoimmune disease. Controversial data exists regarding the differential control of the immune system in T1D patients compared to unaffected individuals. MicroRNAs (miRNAs) are involved in the control of gene expression (by negative regulation of gene expression at post-transcriptional level, by mediating translational repression or degradation of the mRNA targets). Their potential role in T cell activation and autoimmunity is controversial.
Aim: We investigated the expression profile of miR-21a and miR-93 in PMC samples of 20 T1D patients and 20 healthy controls by means of qPCR in different glucose concentrations (basal, 11 nM and 25 mM), and we analyzed the possible relationship of this expression pattern with autoimmunity.
Results: MiR-21a was significantly underexpressed in T1D samples (media values expression 0.23 ± 0.05, $p < 0.01$) compared to controls (values less than 1 indicate a decrease in gene expression). When the PMCs were incubated with glucose 11 mM and 25 mM, miR-21a expression decreased in controls and increased in T1D samples (0.506 ± 0.05, $p < 0.04$). MiR-93 was underexpressed in T1D patients (0.331 ± 0.05, $p < 0.02$) compared to control samples. However, when the PBMCs were incubated with glucose, no changes were observed. No association with autoimmunity was observed.
Conclusion: We demonstrated that miRNAs have a differential expression in PBMCs from T1D patients compared to controls, suggesting that these miRNAs or others could be involved in T cell regulation.

Introduction

Type 1 diabetes (T1D) is a hereditary predisposition disease whose common features are: genetic heterogeneity and lower penetrance in its familial transmission. The affected individuals will develop the disease, in accordance with genetic susceptibility mainly influenced by certain haplotypes linked to the major histocompatibility complex (HLA class II molecules), plus another group of genes classified as non-HLA (Noble and Valdes 2011). Its etiology is extremely complex where environmental factors interact with genetic predisposition, leading to an irreversible autoimmune attack against insulin-producing cells located in the pancreas. Environmental factors include viral infections toxins and diet (Todd 2010; Jaidane et al. 2012; Issazadeh-Navikas et al. 2012). The genetic basis of T1D does not have a Mendelian inheritance pattern; therefore, the best model to explain the susceptibility locus involves a unique and important number of other genes that confer small effects on risk (Polychronakos and Li 2011). Furthermore, due to the heterogeneity, some loci may have stronger effects on particular populations or a subset of families. A higher level of complexity can be given for variations in genes encoded by specific miRNAs (Sebastiani et al. 2011a,b). miRNAs are a class of non-coding single strand RNAs of 21–25 nt that are transcribed from DNA, but are not translated into protein, and which are believed to function, at least in animals, by inhibiting the effective mRNA translation through imperfect base pairing in the 3′ untranslated region (3′ UTR) of target mRNAs. A comparison between the chromosomal locations of 530 miRNAs in loci associated with susceptibility to T1D showed that 27 miRNAs are located in the human loci associated with the disease (Zhou et al. 2008). Interestingly, the predicted targets for these miRNAs include genes related to autoimmunity and β cells, such as inducible T cell costimulator and CD28 (miR-16-2), INFγ and FasL (miR-551b), autoimmune regulator (miR-877) and miR-375, which are involved in the regulation of insulin secretion (Sebastiani et al. 2011a,b). Because each miRNA can target multiple mRNAs, often in combination with other miRNAs, these molecules create complex regulatory networks of gene expression (Jeker and Bluestone 2010; Pandey et al. 2009; Pauley and Chan 2008).

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Table 1
Clinical, immunological and inflammatory profile in T1D cases and healthy control subject.

	T1D cases ($n=20$)	Controls ($n=20$)
Glycemia at diagnosis (mmol/L)	33.2 ± 6.3	–
Disease duration (year)	3.9 ± 1.4	–
Age at diagnosis (year)	8.4 ± 4.3	–
Positive anti-GAD65 (%)	17 (85%)	1 (5%)
Positive anti-IA-2 (%)	14 (70%)	1 (5%)
Positive anti-ZnT8 (%)	12 (60%)	0 (0%)
Positive anti-TTG (%)	3 (15%)	1 (5%)
Levels VCAM (ng/mL)	373.4 (78–1750.2)*	212.9 (115–633.9)
Levels usPCR (mg/mL)	2.48 (0.88–7.88)**	1.45 (0.63–3.35)
Levels IL-6 (ng/mL)	1.7 (0.6–2.9)*	0.8 (0.8–1.4)

* $p<0.05$.
** $p<0.02$.

The search for genes associated with T1D has identified a few candidate genes, so there is the possibility that non-coding regulatory sequences which have not been previously considered may have significant importance. The possibility that miRNAs regulate risk genes of T1D can be the basis of various findings in linkage studies, given that polymorphisms that alter genes are not usually found in T1D. Dysregulation of translation of a normal gene can be miRNA abnormal expression of a gene which is similar to the interruption of the gene itself. Therefore, an inappropriate or untimely expression of a functional protein may occur either by an interruption of the DNA coding sequence, leading to a dysfunctional protein, such as an abnormal regulation of certain miRNA target genes (Zhou et al. 2008; Sebastiani et al. 2011a,b). Previous data shows that the apoptotic mechanism in PBMC of T1D patients under high glucose conditions are altered and this is proved by the decreased expression of the pro-apoptotic genes *fas* and *bax* and by the increased expression of the anti-apoptotic gene *xiap* (Valencia et al. 2012). Several miRNAs like miR-375, miR-9 and miR-34a (Pandey et al. 2009) are involved in T1D regulating certains genes importants in insulin synthesis or glucose metabolism, so is valid think that other miRNAs can regulate genes involved in apoptosis or it's expression can be modulated under high glucose conditions. According to these antecedents, we analyzed here the expression levels of miR-21, a molecule overexpressed in various cancer types (Satzger et al. 2012) and miR-93, a miRNA that high glucose decreases it's expression in T2D models (Long et al. 2010), in PBMCs from T1D patients in relationship with ongoing islet autoimmunity.

Patients and methodology

Subjects

An informed consent was obtained from all subjects enrolled in this study, in compliance with the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki). T1D patients ($n=20$) age 8.5 ± 4.3 years old and healthy controls ($n=20$) age 18.5 ± 10 years old were enrolled into the study from the Maternal and Child Research Institute (IDIMI), San Borja Arriarán Hospital, Santiago de Chile. Diagnosis of T1D was performed based on the criteria used by the American Diabetes Association (ADA). Healthy control subjects had no family history of type 1 or type 2 diabetes or of other autoimmune diseases (see Table 1).

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMC samples from T1D patients and healthy controls were drawn and collected in sterile EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). PBMCs were isolated by centrifugation over Histopaque 1077 density gradient (Sigma Chemical, St Louis, MO). Briefly, blood was diluted 1:1 in PBS, overlaid onto lymphocyte separation media (Lymphocyte Separation Medium), centrifuged at 2200 rpm for 30 min at room temperature and plasma was removed. Mononuclear cell fraction was harvested and washed twice in phosphate buffer saline (PBS) (Sigma, St. Louis, MO). Final pellet was re-suspended in Tri-Reagent solution (Molecular Research Center Inc., Cincinnati, OH).

RNA extraction and RT-PCR

The cells from each patient or control were exposed in duplicate to 11 and 25 mM of glucose during 20 h. The recovery of cells was performed by collecting and centrifuging the medium at 3000 rpm for 5 min and gentle treatment with trypsin-EDTA at 37 °C for 5 min to adhere the cells. Finally, the cell population was washed extensively in PBS and preserved at −80 °C in TRIzol (Invitrogen) for subsequent RNA extraction. Total RNA extraction was performed with the TRIZOL® method (Invitrogen) according to manufacturer's instructions. Its integrity was verified by 1.5% agarose gel plus ethidium bromide and the ratio of optical density (OD) at 260 nm and 280 nm was also measured. RNA was quantified using a NanoDropND-1000 Spectrophotometer (ThermoScientific) and single-stranded cDNA was synthesized from 300 ng of RNA in each sample, using the Promega Kit (ImProm-IITM Reverse Transcriptase, Promega Corporation, Madison, USA) with 1 L of oligo-dT as a starter and magnesium to a final concentration of 3 mM in 20 μL total reaction volume. As a control for integrity of cDNA, samples were amplified with the β-actin primers 5′-ATTGCCGACAGGATGCAGAA-3′ Fw and 5′-AAGCATTTGCGGAGGACGAT-3′ Rv.

Quantification of the expression of miR-21a and miR-93 genes was performed using Agilent equipment Mx3005P (Agilent Technologies). The relative quantification method was used, where we compared the gene expression in each patient with the gene expression in the control group. We used two endogenous control genes of small RNAs RNU48 and RNU6B. The primer sets for each gene were designed by AmplifX.1.5.4 program. Gene expression values were expressed as Ct, Ct being the point at which the fluorescence rises significantly above baseline or background fluorescence, and comparing the Ct of the genes in patients with the Ct of the genes in controls. The qPCR reaction was performed in a total volume of 20 μL, with 2 μL cDNA synthesized from 300 ng of RNA, 10 μL master mix containing SYBR GreenII (Stratagene), $MgCl_2$, dNTPs, polymerase enzyme and its cofactors, 1 μL 5 μM stock of primers (250 nM) and 7 μL nuclease-free water. The program used was: 10 min of initial denaturation and enzyme activation at 95 °C, followed by 40 cycles composed of 15 s at 95 °C, 15 s at 60 °C for annealing and 15 s at 72 °C for elongation. Subsequently, for the melting curve, a program of 5 s at 95 °C, 1 s at 25 °C, 15 s at 70 °C and 1 s at 95 °C was used. Two points of measurement of fluorescence were set: the elongation phase of each cycle and the final stage of the melting curve.

Serum analysis

Screening for serological anti-GAD65, anti-IA2 and anti-ZnT8 auto-antibodies was performed in duplicate by Enzyme Immunoassay (ELISA) from Medizym® Diagnostic (Berlin, Germany), anti-TTG was determined by ELISA (ALPCO, Immunoassays, USA). Serological levels of Protein C reactive (usPCR) were determined by means of ELISA (BioVendor, Czech Republic). IL6 and VCAM were performed by ELISA (R&D System, UK).

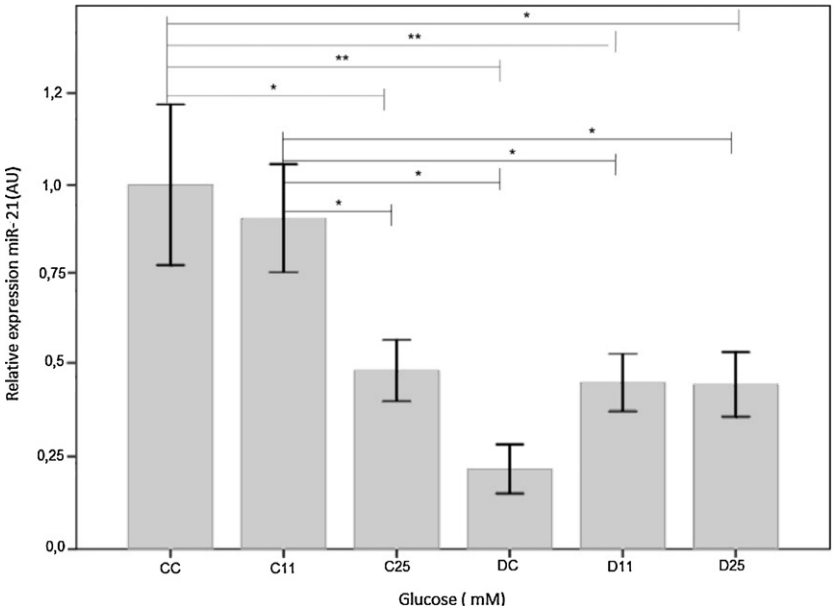

Fig. 1. Glucose effect on microRNA-21 expression. Relative expression of microRNA-21 in T1D patients ($n = 20$) in comparison to control group ($n = 20$). We determined the relative expression through qPCR in RNA samples from T1D patients and healthy controls, isolated from PBMC submitted to different glucose stimuli (Basal concentration, 11 mM and 25 mM). Normalization control: RNU6 and RNU48; one-way ANOVA, post hoc Bonferroni $^{*}p = 0.05$, $^{**}p = 0.01$, and $^{***}p = 0.001$ (C: Healthy controls, D: T1D patients).

Statistical analysis

We used the REST© (Relative Expression Software Tool) program (Pfaffl et al., 2002), designed especially for analyzing results of qPCR using the Pfaffl equation. Afterwards, tests were performed to evaluate the statistical significance or non significance of the results, regarding the variations in expression observed between patients and controls. All subsequent calculations were performed using the SPSS 15.0.1 software package (SPSS Inc, Chicago, IL) and Graph Pad Prism 5 (Graph Pad Software, Inc. San Diego CA, USA). The Shapiro–Wilk normality test was used; the effect of glucose was studied in GraphPad using the Kruskal–Wallis test. To determine the relationship between gene expression and clinical records, the bivariate correlation test was used. A p value <0.05 was considered as statistically significant.

Results

The clinical, immunological and inflammatory profile is summarized in Table 1. Patients with T1D showed a marked autoimmunity and a high inflammatory profile. The control subject that showed an altered profile of autoimmunity was excluded from the study out to be a patient with undiagnosed celiac disease. Among the 20 patients with T1D, 15 of them had a debut with diabetic ketoacidosis and 5 showed no ketoacidosis.

The expression of miR-21 was quantified by qPCR in PBMC samples that were cultured in different glucose concentrations (11 mM and 25 mM) and also in a baseline condition, without glucose insult. Fig. 1 shows the relative expression of miR-21 with different glucose insults, both in T1D patients and controls. It was observed that there is increased expression of miR-21 in control subjects in all conditions compared with T1D patients, but with increasing glucose stimulation this expression tends to decrease significantly. Moreover, patients with T1D appear to show an increase in the expression of miR-21 (D11 and D25) in respect of controls, but this increase is not statistically significant.

The expression of miR-93 in PBMCs was determined under different concentrations of glucose (11 mM and 25 mM) and also in a baseline condition, without glucose insult. Fig. 2 shows the relative expression of miR-93 with various insults of glucose in samples of T1D patients compared with control subjects. It was noted that controls in a baseline condition showed higher expression levels of miR-93 than patients with T1D in the different conditions (DC: T1D without glucose, D11: T1D with 11 mM, D25: T1D with 25 mM). Regarding the samples from the diabetic patients, they had lower levels of expression of miR-93 and its expression is not affected by glucose levels, showing an apparent insensitivity to the stimulus.

Finally, the analysis of the relationship between the relative expression of miR-21 and miR-93 and the profile of auto-antibodies was performed comparing different categories: antibody-positive individuals; individuals positive for 2 antibodies or individuals positive for the 3 antibodies. When performing statistical analyses by one-way ANOVA and Bonferroni post hoc, no correlations were found between a special autoimmunity pattern or inflammatory pattern and the expression of miR-21 or miR-93 (data not shown).

Discussion

T1D is a disease whose etiology is complex and the mechanisms underlying this pathology have not been fully described. In recent years, epigenetic modifications have accounted for much of the research in complex diseases, and among them, the analysis of microRNAs has been the fastest growing (Sebastiani et al. 2011a,b).

One of the miRNAs quantified was miR-21, the gene encoding the primary miR-21 (the primary transcript containing miR-21) is located within the intron region of the TMEM49 gene (Kumarswamy et al. 2011). Unlike other miRNAs, the function of miR-21 has been clarified to a large extent, its over-expression patterns in cancer have generally been well established, and many of its bioinformatically predicted targets have been confirmed. However, instead of providing more answers, this wealth of information has served to raise new questions. One of them concerns what is behind the complex mechanism that establishes the relationship between miR-21 and NF-κB (Young et al. 2010). In MCF-10A, miR-21 is characterized as part of the positive feedback loop between inflammation and cell transformation mediated by STAT3 (Iliopoulos et al. 2010). STAT3 inhibition causes a lower level of expression of miR-21. PTEN, which is a target of miR-21 and a known inhibitor of

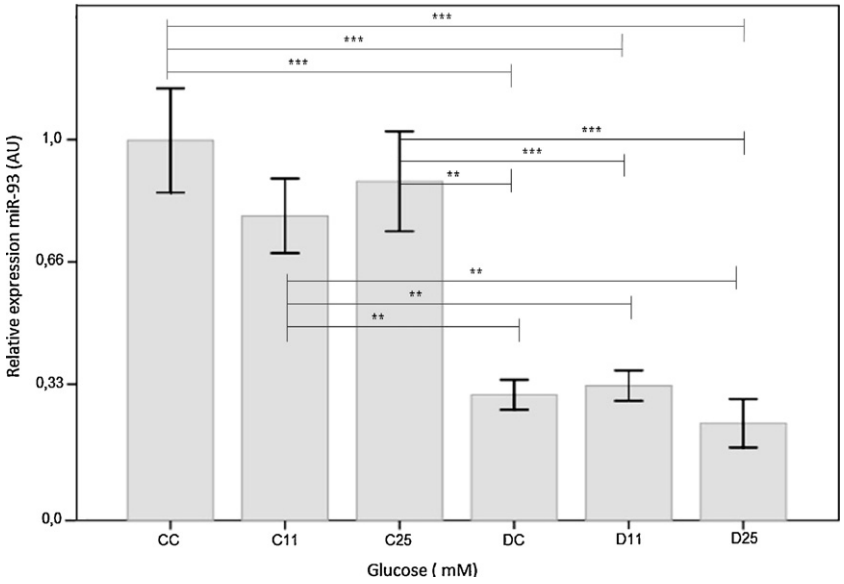

Fig. 2. Glucose effect on microRNA-93 expression. Relative expression of microRNA-93 in T1D patients ($n = 20$) in comparison to control group ($n = 20$). We determined the relative expression through qPCR in RNA samples from T1D patients and healthy controls, isolated from PBMC submitted to different glucose stimuli (Basal concentration, 11 mM and 25 mM). Normalization control: RNU6 and RNU48. One way ANOVA, post hoc Bonferroni $^{*}p = 0.05$, $^{**}p = 0.01$, and $^{***}p = 0.001$ (C: Healthy controls, D: T1D patients).

AKT phosphorylation, promotes activation of NF-κB and promotes tumorigenesis. Therefore, miR-21 participates in the positive feedback loop between inflammation and processing by reducing the expression of PTEN to increase the activity of NF-κB, a very important transcription regulator.

In making the quantification of miR-21 in PMCs, it was observed that patients with T1D had lower levels of miR-21 in relation to controls, and that these did not vary significantly under different glucose stimuli. This contradicts the hypothesis, which stated that this molecule would be increased in T1D patients mainly because, returning to the idea of lymphocytes resistant to apoptosis, it would be the similar resistance seen in cancer cells where high levels of miR-21 have been detected. This confirms the wide diversity of responses and the different regulatory pathways that are reported for miR-21, establishing a highly complete picture in which it is not yet possible to establish a definition of its mechanisms of control and action (Mohri et al. 2009).

Also we determined the expression levels of miR-93, a miRNA described as participating in angiogenesis and tumor formation in endothelial cells. In PBMCs of T1D patients, there is a significant lower expression of miR-93 and its expression is not affected by glucose levels showing that in this cell type, miR-93 have a minor impact in this regulation, unlike what was observed in animal models of type 2 diabetes mellitus. MiR-93 negatively regulates the vascular endothelial growth factor (VEGF), a dimeric glycoprotein (Long et al. 2010). High levels of VEGF have been associated with the pathogenesis of a number of inflammatory diseases and particularly microvascular diabetic complications. As there is a lower level of miRNA-93, there will be a high level of VEGF that stimulates the expression of VCAM and, in conjunction with an exacerbated inflammatory profile given by high levels of IL-6, increases the risk of developing microvascular complications in T1D patients.

On the other hand, both miR-21 and miR-93 have other bioinformatically proposed target genes whose expression can be negatively regulated, such as Caspase 8, Caspase 7 and IL-8 in the case of miR-93. For miR-21, it has been proposed that it could negatively regulate the expression of Fas ligand, MTPN, APAF1, IL12A, IL22 and IL-1B genes. Interestingly, miR-93 may negatively regulate the expression of STAT3, which belongs to a family of proteins involved in maintaining immune tolerance and tumor survival (Ho et al. 2012). However, the fact that certain miRNAs present a differential pattern of expression in certain pathologies, needs to be validated by identifying the levels of proteins that these molecules are able to negatively regulate, for determining whether a lower expression of a miRNA can be directly related to the expression of a particular gene. In the case of miR-21 and miR-93, both miRNAs converge in the NKκB pathway, NKκB being a transcription factor that is modulated by pro-inflammatory cytokines which stimulate the production of free radicals by nitric oxide synthase (iNOS) and another means of generating free radicals under conditions of hyperglycemia, which is the situation of cells from T1D patients (Ali and Sultana 2012). Therefore, there is a multiplicity of factors in which a condition of hyperglycemia and the development of a pro-inflammatory environment sustained over time, are capable of modulating the expression of NKκB (Dai and Ahmed 2011).

An important aspect to consider in interpreting our results relates to the possible influence of age on miRNAs. In our study, patients with T1D are younger than controls, that because the search for greater control is needed to exclude the presence of T1D. However, this age difference might be affecting the expression of miRNAs, although there are no reports on the subject. In other cellular models (cultured cardiomyocytes, endothelial progenitor cells and muscle) has been observed that the expression and concentration of caspase 3 for example, is increased, and the activity tends to increase with age (Kakarla et al. 2010; Kushner et al. 2011). This background is important and although there is no registry of miRNAs variation in PBMCs related with age, this result, should be interpreted with caution.

Finally, patients with T1D have a pattern of decreased expression of miR-21 and miR-93 which can be translated into alterations in apoptosis signaling pathways, which may be reflected in a differential pattern of apoptosis in PBMCs of T1D patients. The latter is consistent with the hypothesis of an apoptosis resistant lymphocyte that is capable of maintaining a pro-inflammatory environment, which in the long run would result in the development of complications in T1D.

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