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ORIGINAL ARTICLE

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Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals

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Abstract

Aim Certain microRNA molecules (miRNAs) that target genes involved in beta-cell growth and insulin resistance are found deregulated in patients with type-2 diabetes mellitus (T2D) and correlate with its complications. However, the expression profile of miRNAs that regulate genes bearing T2D-related single-nucleotide polymorphisms has been hardly studied. We recently reported that the mRNA patterns of specific T2D-susceptibility genes are impaired in patients, and associate with disease parameters and risk factors. The aim of this study was to explore the levels of miRNAs that target those genes, in peripheral blood of patients versus controls.

Methods A panel of 14 miRNAs validated to target the *CDKN2A*, *CDK5*, *IGF2BP2*, *KCNQ1*, and *TSPAN8* genes, was developed upon combined search throughout the DIANNA TarBase v7.0, miRTarBase, miRSearch v3.0-Exiqon, miRGator v3.0, and miRTarget Link Human algorithms. Specifically developed poly(A)polyadenylation(PAP)-reverse transcription (RT)-qPCR protocols were applied in peripheral blood RNA samples from patients and controls. Possible correlations with the disease, clinicopathological parameters and/or risk factors were evaluated.

Results T2D patients expressed decreased levels of let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, and miR-133a-3p, compared with controls. Moreover, these levels correlated with certain disease features including insulin and % HbA1c levels in patients, as well as BMI, triglycerides' levels and family history in controls.

Conclusions A T2D-specific expression profile of miRNAs that target disease-susceptibility genes is for the first time described. Future studies are needed to elucidate the associated transcription-regulatory mechanisms, perchance involved in T2D pathogenesis, and to evaluate the potential of these molecules as possible biomarkers for this disorder.

Supplementary information The online version of this article (https://doi.org/10.1007/s12020-019-02062-0) contains supplementary material, which is available to authorized users.

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Highlights

- Let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, and miR-133a-3p, which target certain T2D-susceptibility genes, are decreased in peripheral blood samples of patients compared with controls.
- The expression levels of let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, and miR-133a-3p correlate with the mRNA levels of their target T2D-susceptibility genes.
- The levels of these miRNAs correlate with certain disease parameters, including insulin, % HbA1c levels, BMI, triglycerides' levels, and family history.

Keywords MicroRNA (miRNA) · Type-2 diabetes mellitus (T2D) · T2D-susceptibility genes · Peripheral blood · PAP-RT-qPCR

Introduction

Nowadays, type-2 diabetes mellitus (T2D), a chronic metabolic disorder with growing cardiovascular morbidity and mortality, is considered as one of the worldwide epidemics with increasing prevalence [1]. T2D outbreak is tightly linked to the global obesity rise, which, in turn, is highly associated with the adoption of a sedentary lifestyle [2]. Also, it is now well established that the development of the disease is ascribed to the interplay between environmental factors and genetic components [3].

So far, numerous genome-wide association studies have identified a large pool of single-nucleotide polymorphisms related to T2D [4, 5]. Until very recently, the gene-expression signature of those disease-susceptibility genes had been little investigated. Lately, using a blood-based transcriptome analysis, we identified that the expression of specific transcript variants of certain T2D-susceptibility genes, is differentially regulated in patients versus control individuals [6]. Specifically, T2D patients and healthy individuals at risk of developing the disease were suggested to exhibit deregulated mRNA levels of the transcript variants (tv) 1 and tv2 of *CDK5*, tv3 and tv4 of *CDKN2A*, tv7 of *IGF2BP2*, tv5 of *THADA*, tv3 of *CAPN10*, tv1 of *KCNQ1*, and *TSPAN8* compared with controls. This disease-specific mRNA signature probably reflects the transcriptome dynamics taking place in the target tissues of T2D (including the pancreas, muscle, and adipose tissue), and/or peripheral blood cells, further implicated in disease's pathogenetic pathways [6].

Nevertheless, the molecular mechanisms underlying this deregulated expression in T2D patients have not yet been studied. It is known that the expression of the genome can be regulated by various mechanisms, including methylation of DNA, post-translational modification of histones, or activation of microRNAs, which ultimately influence the phenotype [3]. A number of studies have shown that certain microRNAs (miRNAs) are components of pathways triggered by, or contributing to, the pathophysiology of T2D [7, 8]. They have been found to be functionally involved in beta-cell growth and insulin resistance, in liver, fat, and skeletal muscle [9], while many findings confirm that several miRNAs are deregulated both in the affected tissues and blood of patients [10] and proposed them as promising biomarkers for T2D and its complications [11, 12]. Furthermore, certain T2D-susceptibility loci are bioinformatically predicted to be targets of islet-expressing miRNAs, further supporting the possible involvement of these molecules in molecular pathophysiological mechanisms of T2D [13].

However, nothing is yet known about the expression profile of those miRNAs that target the recently described differentially expressed T2D-susceptibilty genes [6]. Following our previous study, we herein investigated the expression patterns of these miRNAs, in peripheral blood samples of T2D versus control subjects. Possible associations with certain disease parameters and risk factors were also explored.

Methods

Study design

We first aimed to develop a panel of miRNAs that are experimentally validated to target the *CAPN10*, *CDK5*, *CDKN2A*, *IGF2BP2*, *KCNQ1*, *THADA*, and *TSPAN8* T2D-susceptibility genes, using appropriate algorithms. For the evaluation of miRNAs' levels, specific poly(A)polyadenylation(PAP)-reverse transcription (RT)-qPCR protocols were developed and applied in RNA extracted from peripheral blood samples of T2D patients and control individuals (CT). Appropriate statistical tests were performed to explore the possible differential expression of these miRNAs in T2D versus CT subjects. Moreover, to examine specific distribution patterns in individuals at high risk of developing the disease, a distinct group of CTs bearing T2D risk factors was included in the total CT group. The two subgroups were analyzed both together and separately. Possible associations with certain disease parameters

were also explored. In addition, correlations between the levels of expression of these miRNAs and those of their T2D-susceptibilty target genes and/or transcript variants, as described previously [6], were examined. Finally, a bioinformatics approach was applied to explore the T2D-related signaling pathways and networks possibly regulated by the differentially expressed miRNAs found herein.

Development of the miRNA panel

The panel of the miRNAs that have been experimentally shown to target the aforementioned T2D-susceptibility genes was developed upon in-depth and combined search in the following algorithms: DIANA-TarBase v7.0, miRTarBase, miRSearch v3.0-Exiqon, miRGator v3.0, and miRTarget Link Human [14–17] (assessed: May 2018).

Patients and samples

The study examined 77 peripheral blood samples, obtained from 40 consecutive T2D patients and 37 controls (CT) with normal glucose metabolism (Table 1), as described previously [6].

RNA extraction, polyadenylation, and quantitative real-time PCR (qPCR)

Total RNA was isolated using the PAXgene Blood miRNA Kit (QIAGEN GmbH, Hilden, Germany), using direct-blood lysis, according to manufacturer's instructions (manual process). A total of 0.5 μg of total RNA was polyadenylated at the 3′end using *E. coli* poly(A)polymerase (New England Biolabs Inc., Ipswich, MA, USA), and reverse transcribed using MMLV Reverse Transcriptase (Invitrogen by Thermo Fischer Scientific, Waltham, MA, USA), following manufacturer's instructions.

Specific SYBR-Green fluorescent-based qPCR assays were developed and applied for the quantification of each of the 14 miRNAs of the panel. The small nucleolar RNA C/D box 48 (SNORD48; RNU48), was used as the endogenous reference control miRNA. Specific forward primers were designed based on published sequences (NCBI Reference Sequence) and upon in-silico specificity analysis. All specific forward as well as the universal reverse primer are reported in Supplementary Table 1. The reaction was performed using the Kapa SYBR® Fast qPCR Master Mix (2×) (Kapa Biosystems, Inc., Woburn, MA, USA), 5 ng of cDNA template and optimized amount (ng) of each primer (Supplementary Table 1). The thermal protocol was: 95 °C for 3 min, 95 °C for 3 sec (40 cycles), 60 °C for 30 s. qPCR reactions were performed in duplicates in a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Details regarding the PAP-RT and qPCR protocols are reported in Supplementary Table 2.

The relative quantification (RQ) levels of the above-mentioned miRNAs in each sample were assessed by the $2^{-\Delta\Delta Ct}$ method [18]. The 1.2B4 human immortalized beta-pancreatic cell line (ECACC, Salisbury, UK) was used as the calibrator sample.

Statistical analysis

The possible differential distribution of the miRNAs' RQ levels between T2D and CTs, or among T2D, CT_{RF+}, and CT_{RF-} individuals, were explored using the nonparametric Mann–Whitney *U* or Jonckheere–Terpstra tests, respectively. Benjamini–Hochberg procedures for adjusting the false discovery rate (FDR = 0.25) in multiple comparisons were also applied. Possible correlations between the expression levels of miRNAs and those of T2D-susceptibility genes/transcript variants (described previously [6]) were analyzed by Spearman's correlation test. Possible associations with binary, ordinal or continuous values of various clinicopathological and laboratory parameters were investigated by Mann–Whitney *U*, Jonckheere–Terpstra, or Spearman's rank correlation coefficient tests, respectively. Binominal logistic regression analysis was performed exploiting the enter model and using the occurrence of T2D as the dependent variable and the miRNA levels, age, and sex, as independent variables. Analyses were performed using the Graph Pad Prism 5.00 or SPSS 21.0 softwares. *P*-values < 0.05 were considered significant.

Bioinformatics analysis

In order to explore which pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) [19] were enriched within the genes regulated by the differentially expressed miRNAs, gene set enrichment analysis was performed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database [20].

Results

The developed miRNA panel

Upon search in appropriate algorithms, the developed panel included 14 miRNAs, namely the: let-7b-5p, let-7g-5p, miR-1-3p, miR-10b-5p, miR-24-3p, miR-29a-3p, miR-29b-3p, miR-34a-5p, miR-98-5p, miR-124-3p, miR-125b-5p, miR-133a-3p, miR-155-5p, and miR-5682 (Supplementary Table 3). No validated miRNAs were found to target the *CAPN10* or *THADA* genes.

Table 1 Characteristics of control individuals (CT) and patients (T2D) included in the study

Features		CT ($n = 37$)	T2D ($n = 40$)
General	Age (years); median (range)	49 (19–69)	59 (35–75)
	Sex (male/female); number (%)	19/18 (51/49)	19/21 (48/52)
	Disease duration (years); median (range)	NA	5 (0–26)
	Family history (yes/no); number (%)	15/22 (41/59)	28/12 (70/30)
	Risk factors[a] (presence/absence); number (%)	21/16 (57/43)	NA
Anthropometric	BMI (body mass index)[b]; median (range)	26.9 (21.3–36.3)	29.3 (21.5–46.5)
	<25: normal weight; number (%)	19 (51)	6 (15)
	25–30: overweight; number (%)	10 (27)	16 (40)
	>30: obese; number (%)	8 (22)	18 (45)
	W/H (waist-to-hip ratio); median (range)	0.89 (0.71–1.09)	0.93 (0.83–1.18)
	Central obesity[c] (yes/no); number (%)	14/23 (38/62)	36/4 (90/10)
Clinical	Hypertension[d] (yes/no); number (%)	5/32 (14/86)	24/16 (60/40)
	Hyperlipidemia[e] (yes/no); number (%)	7/30 (19/81)	31/9 (77/23)
	Metabolic syndrome[f] (yes/no); number (%)	5/32 (14/86)	32/8 (80/20)
Laboratory	HbA1c levels (% or mmol/ml); median (range)	5.6 (5.0–6.1)	6.7 (5.2–12.1)
	<7% or 53; number (%)	37 (100)	25 (63)
	≥7% or 53; number (%)	0 (0)	15 (37)
	Glucose levels (mg/dl); median (range)	85 (68–120)	118 (75–229)
	<130; number (%)	37 (100)	23 (58)
	≥130; number (%)	0 (100)	17 (42)
	Insulin levels (μU/ml); median (range)	9.2 (5.2–19.1)	13.7 (6.9–56.0)
	Cholesterol levels (mg/dl); median (range)		
	Total cholesterol	204 (109–281)	192 (119–256)
	High-density cholesterol (HDL)	48.5 (6–79)	41 (27–125)
	Low-density cholesterol (LDL)	124 (19–192)	113 (66–191)
	Triglycerides levels (mg/dl); median (range)	117 (65–176)	148 (79–363)
T2D therapy	Naïve (prior to treatment); number (%)	NA	7 (17.5)
	Tablets (metformin, vildagliptin, sitagliptin, saxagliptin, glimepiride, and gliclazide); number (%)	NA	18 (45.0)
	Two tablets (metformin + glimepiride, metformin + vildagliptin); or one tablet (metformin) + injectable GLP-1 analog (liraglutide); number (%)	NA	6 (15.0)
	Three tablets (metformin + vildagliptin + pioglitazone or metformin + vildagliptin + glimepiride or metformin + sitagliptin + glimepiride); number (%)	NA	4 (10.0)
	Injectable insulin (±tablets: metformin + sitagliptin); number (%)	NA	2 (5.0)
	Multiple injections of insulin; number (%)	NA	3 (7.5)

[a]Risk factors associated with higher risk of T2D, included: (i) BMI > 25, (ii) prior history of gestational diabetes, (iii) hypertension, (iv) dyslipidemia, (v) cardiovascular disease, or (vi) first-degree family member with T2D [3]

[b]BMI was calculated as weight (kg) divided by the square of height (m^2)

[c]Central obesity was regarded if waist circumference was ≥102 cm (40 in) in men or ≥88 cm (35 in) in women

[d]Hypertension was regarded if blood pressure was ≥130/85 mm Hg (or receiving drug therapy for hypertension)

[e]Hyperlipidemia (defined by the Adult Treatment Panel III of the National Cholesterol Education Program [38]

[f]Metabolic syndrome was diagnosed according to the NCEP-ATP III report [39] requiring at least 3 of the following 5 conditions: (i) fasting glucose ≥ 100 mg/dL (or receiving drug therapy for hyperglycemia), (ii) blood pressure ≥ 130/85 mm Hg (or receiving drug therapy for hypertension), (iii) triglycerides ≥ 150 mg/dL (or receiving drug therapy for hypertriglyceridemia), (iv) HDL-C < 40 mg/dL in men or < 50 mg/dL in women (or receiving drug therapy for reduced HDL-C), (v) waist circumference ≥ 102 cm (40 in) in men or ≥88 cm (35 in) in women

NA not applicable

Differential miRNA expression patterns in patients versus controls

Quantifiable miRNA levels were detected in peripheral blood samples in the cases of the following 10 molecules: let-7b-5p, let-7g-5p, miR-1-3p, miR-24-3p, miR-29a-3p, miR-34a-5p, miR-98-5p, miR-125b-5p, miR-133a-3p, and miR-155-5p. No expression of miR-10b-5p, miR-29b-3p, miR-124-3p, or miR-5682 was detected in the samples of patients or controls.

RQ values (median; range) of the miRNA levels in the groups of T2D patients ($n = 40$) and CTs ($n = 37$) are summarized in Table 2. Mann–Whitney U test revealed that T2D patients expressed significantly lower levels of let-7b-5p ($p = 0.0014$), miR-1-3p ($p < 0.0001$), miR-24-3p ($p = 0.0084$), miR-34a-5p ($p < 0.0001$), miR-98-5p ($p = 0.0013$), and miR-133a-3p ($p = 0.0003$), compared with CTs (Fig. 1a). Although not with statistical significance, lower levels in patients versus controls were detected also in the case of miR-125b-5p ($p = 0.0801$). Further analysis within the group of CTs, revealed that CT_{RF+} individuals ($n = 21$) were characterized by reduced miRNA levels compared with CT_{RF-} ones ($n = 16$) in the cases of miR-1-3p ($p < 0.0001$), miR-34a-5p ($p = 0.008$), miR-98-5p ($p = 0.073$), and miR-133a-3p ($p = 0.001$) (Fig. 1b). Moreover, Jonckheere–Terpstra test revealed a linear trend of decrease in the levels of let-7b-5p ($p = 0.002$), miR-1-3p ($p < 0.0001$), miR-24-3p ($p = 0.011$), miR-34a-5p ($p < 0.0001$), miR-98-5p ($p < 0.0001$), miR-125b-5p ($p = 0.049$), and miR-133a-3p ($p < 0.0001$), among the groups of CT_{RF-}, CT_{RF+}, and T2D patients (Fig. 1b). The differential expression patterns for all the above miRNAs, except for the miR-125b-5p, remained significant upon corrections for multiple comparisons.

As for the levels of let-7g-5p, miR-29a-3p, and miR-155-5p, these were not found to be statistically different in any of the comparisons among the groups of patients and controls (Table 2).

According to the abovementioned data, the panel of the T2D-specific miRNAs finally included the: let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, miR-125b-5p, and miR-133a-3p. Among them, binomial multivariate analysis corrected for age and sex revealed that miR-24-3p and miR-133a-3p can predict T2D among participants of the current study ($p = 0.042$, OR = 0.390 and $p = 0.025$, OR = 30.86, respectively).

Correlations between miRNA and target-mRNA expression levels

Possible correlations between the levels of the differential expressed miRNAs and the levels of expression of their target mRNAs (T2D-susceptibility genes or transcript variants that show a T2D-specific expression pattern), as evaluated previously [6] in the same cohort, were explored (Table 3), for those miRNAs that displayed statistically significant differential expression between patients and controls, or among patients, controls with and controls without risk factors, namely the miR-24-3p, miR-34a-5p, miR-125b-5p, let-7b-5p, miR-98-5p, miR-1-3p, and miR-133a-3p.

Spearman's rank correlation test revealed significantly negative correlations between the levels of miRNAs that target the *CDKN2A* gene and the levels of the tv3 of *CDKN2A*, in CT_{RF+} individuals ($r = -0.6313$; $p = 0.0021$ for miR-24-3p, $r = -0.6988$; $p = 0.0004$ for miR-34a-5p, $r = -0.4421$; $p = 0.0448$ for miR-125b-5p) and T2D patients ($r = -0.5371$; $p = 0.0004$ for miR-24-3p, $r = -0.4578$; $p = 0.0030$ for miR-34a-5p). MiR-34a-5p levels also correlated with *CDKN2A* tv4 levels in CTs ($r = -0.3636$; $p = 0.0270$) and tended to correlate with the last, in T2D patients ($r = -0.2802$; $p = 0.0799$).

On the other hand, positive correlations were detected between the levels of miRNAs that target *KCNQ1* and those of the *KCNQ1* tv1 in T2D patients ($r = 0.5902$; $p < 0.0001$ for miR-1-3p, $r = 0.4867$; $p = 0.0015$ for miR-34a-5p, and $r = 0.6124$; $p < 0.0001$ for miR-133a-3p). Similar correlations were also observed in the CT group ($r = 0.5000$; $p = 0.0016$ for miR-1-3p, $r = 0.4047$; $p = 0.0130$ for miR-34a-5p, and $r = 0.3992$; $p = 0.0144$ for miR-133a-3p). This was mainly attributed to the CT_{RF-} participants, as suggested by the tendencies of significance revealed ($r = 0.4794$; $p = 0.0624$ for miR-1-3p, $r = 0.4412$; $p = 0.0889$ for miR-34a-5p, and $r = 0.4676$; $p = 0.0698$ for miR-133a-3p), rather than the CT_{RF+} participants, where no correlation was detected. Also, the levels of these miRNAs correlated positively with the levels of total *KCNQ1* mRNA, in the total CT group ($r = 0.3037$; $p = 0.0676$ for miR-1-3p, $r = 0.4116$; $p = 0.0114$ for miR-34a-5p, and $r = 0.3772$; $p = 0.0214$ for miR-133a-3p) and in the CT_{RF-} subgroup ($r = 0.6294$; $p = 0.0106$ for miR-1-3p, $r = 0.5000$; $p = 0.0508$ for miR-34a-5p, $r = 0.4618$; $p = 0.0738$ for miR-133a-3p).

No correlations were revealed between the levels of miRNAs that target *IGF2BP2* or *TSPAN8* and those of their mRNAs. Correlations between the levels of miR-155-5p and those of its target *CDK5* mRNA, were not explored, since the first did not exhibit any significant difference among the samples of patients and controls.

Associations of the levels of miRNAs with clinicopathological data

Possible associations between the expression levels of the differentially expressed miRNAs and certain clinicopathological and laboratory characteristics of the disease were further evaluated in the groups of CTs and T2D patients (Table 4).

Table 2 Relative quantification (RQ) expression levels of the miRNAs-of-interest

Gene/Variant	RQ levels, median (range)				Statistical significance, *p*				
	CT_{TOTAL} ($n = 37$)	CT_{RF-} ($n = 16$)	CT_{RF+} ($n = 21$)	T2D ($n = 40$)	CT_{TOTAL} vs. T2D	CT_{RF-} vs. T2D	CT_{RF+} vs. T2D	CT_{RF-} vs. CT_{RF+}	Linear trend following the $CT_{RF-} \rightarrow CT_{RF+} \rightarrow$ T2D order
miRNAs									
let-7b-5p	**561.6** (53.55–6363)	**508.29** (89.60–4911.16)	**573.05** (53.55–6363.20)	**212.4** (43.26–2008)	**0.0014**	**0.005**	**0.018**	0.868	**0.002**
let-7g-5p	**19.13** (4.232–171.7)	**11.77** (4.23–171.74)	**30.95** (4.39–153.30)	**24.34** (2.571–389.3)	0.627	0.095	0.504	0.115	0.333
miR-1-3p	**20.36** (1.549–383.1)	**50.62** (5.08–206.13)	**9.082** (1.55–383.06)	**4.183** (0.683–211.2)	**<0.0001**	**<0.0001**	**0.008**	**<0.0001**	**<0.0001**
miR-24-3p	**2.827** (0.740–22.67)	**2.616** (1.09–22.67)	**2.858** (0.74–17.61)	**1.826** (0.444–10.04)	**0.0084**	**0.026**	**0.042**	0.868	**0.011**
miR-29a-3p	**0.174** (0.034–0.905)	**0.158** (0.04–0.91)	**0.186** (0.03–0.55)	**0.133** (0.032–0.478)	0.301	0.384	0.412	0.868	0.353
miR-34a-5p	**0.296** (0.032–2.568)	**0.733** (0.03–2.30)	**0.166** (0.03–2.57)	**0.077** (0.016–1.402)	**<0.0001**	**<0.0001**	**0.018**	**0.008**	**<0.0001**
miR-98-5p	**0.998** (0.078–14.84)	**1.334** (0.09–12.25)	**0.879** (0.08–14.84)	**0.356** (0.049–3.226)	**0.0013**	**0.001**	0.052	0.073	**<0.0001**
miR-125b-5p	**0.288** (0.058–1.195)	**0.302** (0.08–1.19)	**0.279** (0.06–1.05)	**0.211** (0.043–1.631)	0.0801	0.075	0.261	0.370	**0.049**
miR-133a-3p	**1.545** (0.219–14.86)	**3.289** (0.50–11.09)	**0.975** (0.22–14.86)	**0.476** (0.114–9.176)	**0.0003**	**<0.0001**	0.095	**0.001**	**<0.0001**
miR-155-5p	**0.132** (0.027–0.514)	**0.104** (0.03–0.51)	**0.152** (0.03–0.49)	**0.120** (0.034–0.981)	0.479	0.758	0.187	0.229	0.772

The power of the sample size for the comparison of CT versus T2D with Type I error probability $\alpha = 0.05$ is: 0.82 for miR-34a-5p, 0.73 for miR-1-3p, 0.71 for let-7b-5p, 0.70 for miR-24-3p, 0.70 for miR-133a-3p, and 0.58 for miR-98-5p, as calculated using the PS: Power and Sample Size Calculation v3.1.6 software

NE not expressed, *NA* not applicable, *NS* not significant, CT_{TOTAL} the total cohort of controls, CT_{RF-} controls with risk factors for T2D, CT_{RF+} controls without risk factors for T2D, *T2D* patients

Data are expressed as median values (range). The between-group differential distribution and the $CT_{RF-} \rightarrow CT_{RF+} \rightarrow$ T2D-ordered linear trend were evaluated by Mann–Whitney *U* and Jonckheere–Terpstra non-parametric tests, respectively

Bold values indicate median values and the statistical significance $p < 0.05$

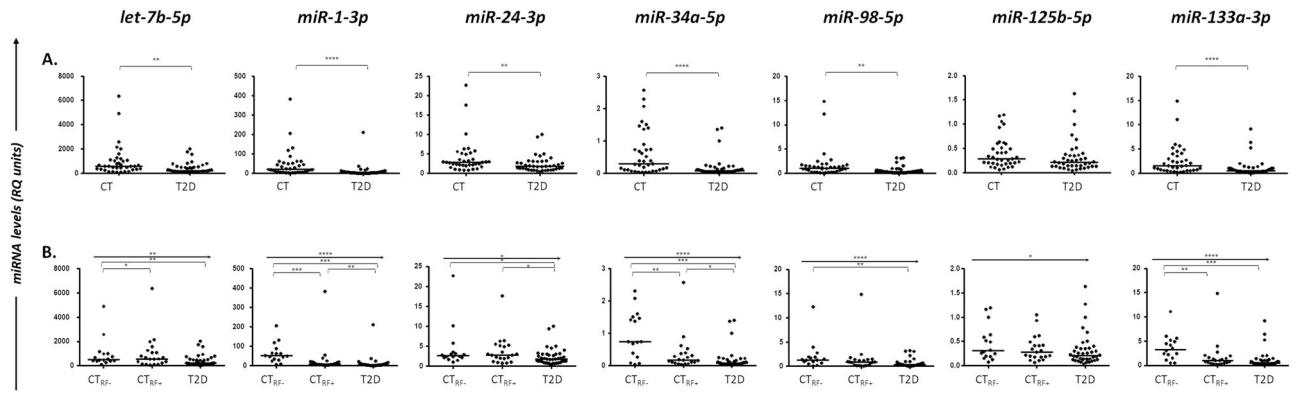

Fig. 1 **a** Dot-plots depicting the differential distribution of miRNA levels (RQ units) in controls (CT) and T2D patients (T2D), as attested by appropriate nonparametric tests. Mann–Whitney analysis revealed that T2D patients are characterized by lower levels of the miRNAs: let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, miR-125b-5p, and miR-133a-3p, compared with CTs. **b** Dot-plots depicting the differential distribution of miRNA levels (RQ units) in controls without T2D risk factors (CT_{RF-}), controls with T2D risk factors (CT_{RF+}) and T2D patients. Jonckheere–Terpstra test showed a stepwise decrease in the levels of the abovementioned miRNAs among the CT_{RF-}, CT_{RF+}, and T2D groups. *P*-values are designated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$), whereas horizontal bars represent the median value of the group

More specifically the levels of: (1) miR-125b-5p correlated negatively with the levels of insulin (μU/ml) ($p = 0.0157$) and % HbA1c in the serum of T2D patients ($p = 0.0198$), (2) let-7b-5p and (3) miR-98-5p also negatively with insulin levels in patients ($p = 0.0241$ and $p = 0.0441$, respectively), (4) miR-1-3p negatively with % HbA1c levels ($p = 0.0115$) and with family history for T2D ($p = 0.0077$) in the CTs subgroup, (5) miR-34a-5p negatively with triglycerides' levels (mg/dl) in the total group of CTs ($p = 0.0390$) and % HbA1c levels in the CT_{RF+} subgroup ($p = 0.0402$), and (6) miR-24-3p positively with body mass index (BMI) in CT_{RF+} individuals ($p = 0.0168$).

Pathway analysis of the proteins encoded by target genes of the differentially expressed miRNAs

To further explore the involvement of the differentially expressed miRNAs in the regulation of T2D-associated molecular mechanisms, we explored their validated target genes (including, but also other than, the T2D-susceptibility genes). The panel of the target genes was developed upon search in the miRTarget Link Human algorithm [17] and included 635 molecules (Supplementary Table 4). This gene/protein set was further analyzed in terms of network interactions, using STRING-DB tools and KEGG database. The analysis revealed that numerous proteins of the above set are significantly involved in T2D-related pathways including PI3K-Akt, HIF-1, MAPK, FoxO, and insulin-signaling pathways (false discovery rate <5E−06 for all) (Fig. 2). All the regulated pathways revealed by this approach are included in Supplementary Table 5.

Discussion

Herein, we described impaired levels of certain miRNAs that target the T2D-susceptibility genes *CDKN2A*, *IGF2BP2*, *KCNQ1*, and *TSPAN8*, previously found deregulated in peripheral blood of patients and controls with risk factors for the disease [6]. More specifically, the levels of let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, miR-125b-5p, and miR-133a-3p were detected to be lower in the peripheral blood of T2D patients compared with control individuals; of them, miR-24-3p and miR-133a-3p displayed independent prognostic values. Furthermore, among controls, those bearing risk factors for T2D displayed decreased miRNA levels compared with those without.

These findings are in line with previous data reporting decreased levels of miR-24 and miR-125b in plasma samples [21–23] and of miR-133a and miR-98-5p in skeletal-muscle biopsies of T2D patients [24]. Furthermore, the levels of certain let-7 family members have been reported to be reduced in T2D patients compared with controls; the first exhibit lower levels of let-7a and let-7f in plasma exosomes [25] and let-7i in the serum [26]. As for miR-1-3p, its levels have been found decreased in the heart of STZ-induced diabetic mice [27].

Moreover, in this study, paired analysis between the expression levels of miR-24-3p or miR-34a-5p and the levels of specific transcript variants (tv3 and tv4) of their target T2D-susceptibility gene *CDKN2A* (previously found upregulated in T2D peripheral blood [6]), revealed a significant negative association, in the groups of patients and CT_{RF+} individuals, while not in CT_{RF-} individuals. Also, the levels of miR-125b-5p were negatively associated with

Table 3 Correlations between the expression levels of the differentially expressed miRNAs and those of their validated mRNA targets, as these were previously analyzed in the same cohort [6]

miRNA/target-mRNA		CDKN2A														
		CDKN2A (total variants)					CDKN2A transcript variant 3					CDKN2A transcript variant 4				
		All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D	All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D	All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D
miR-24-3p	p	0.4835	0.3717	0.3158	0.5556	0.1827	**<0.0001**	0.1573	0.3188	**0.0021**	**0.0004**	0.1640	0.9334	0.8412	0.9110	0.2378
	r	−0.0810	0.1512	0.2666	0.1364	−0.2150	**−0.4709**	−0.2373	0.2646	**−0.6313**	**−0.5371**	−0.1602	0.0142	−0.0530	0.0259	−0.1910
miR-34a-5p	p	0.0573	0.0953	0.5991	0.9377	0.8039	**<0.0001**	**0.0017**	0.8342	**0.0004**	**0.0030**	**0.0010**	**0.0270**	0.4340	0.9465	0.0799
	r	−0.2176	−0.2783	−0.1399	0.0181	−0.0405	**−0.5665**	**−0.4982**	0.0422	**−0.6988**	**−0.4578**	**−0.3684**	**−0.3636**	−0.2077	−0.0155	−0.2802
miR-125b-5p	p	0.9174	0.5842	0.1550	0.7626	0.9076	**0.0312**	0.2910	0.5163	**0.0448**	0.3246	0.0808	0.8165	0.7900	0.5051	0.1591
	r	0.0120	0.0929	0.3726	0.0701	0.0189	**−0.2458**	−0.1783	0.1737	**−0.4421**	−0.1598	−0.2002	−0.0394	0.0721	−0.1540	−0.2269
		IGF2BP2														
		IGF2BP2 (total variants)					IGF2BP2 transcript variant 7									
		All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D	All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D					
let-7b-5p	p	0.4503	0.3820	0.7139	0.5219	0.2336	0.9748	0.9972	0.9781	0.8866	0.2336					
	r	0.0937	0.1752	0.2000	0.1481	0.1927	−0.0036	0.0005	0.0088	−0.0331	0.1927					
miR-98-5p	p	0.8053	0.4848	0.7139	0.6868	0.8146	0.4869	0.7692	0.9258	0.7370	0.8146					
	r	0.0306	0.1404	0.2000	0.0935	0.0382	−0.0804	−0.0499	0.0264	−0.0779	0.0382					
		KCNQ1														
		KCNQ1 (total variants)					KCNQ1 transcript variant 1									
		All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D	All	CT_{TOTAL}	CT_{RF-}	CT_{RF+}	T2D					
miR-1-3p	p	**0.0186**	0.0676	**0.0106**	0.3218	0.3651	**<0.0001**	**0.0016**	0.0624	0.1134	**<0.0001**					
	r	**0.2678**	0.3037	**0.6294**	0.2273	0.1471	**0.5532**	**0.5000**	0.4794	0.3558	**0.5902**					
miR-34a-5p	p	**0.0045**	**0.0114**	0.0508	0.1563	0.4577	**<0.0001**	**0.0130**	0.0889	0.4856	**0.0015**					
	r	**0.3202**	**0.4116**	0.5000	0.3208	0.1208	**0.4967**	**0.4047**	0.4412	0.1610	**0.4867**					
miR-133a-3p	p	**0.0336**	**0.0214**	0.0738	0.0949	0.7155	**<0.0001**	**0.0144**	0.0698	0.3570	**<0.0001**					
	r	**0.2424**	**0.3772**	0.4618	0.3740	0.0594	**0.5543**	**0.3992**	0.4676	0.2117	**0.6124**					
		TSPAN8														
		All		CT_{TOTAL}		CT_{RF-}		CT_{RF+}		T2D						
miR125b-5p	p	0.2250		0.4487		0.5682		0.3121		0.7426						
	r	−0.1399		−0.1284		0.1533		−0.2318		−0.0535						

Bold values indicate statistical significance $p < 0.05$

Table 4 Analysis of correlations between the levels of miRNAs with differential distribution among the CT_{RF-}, CT_{RF+}, and T2D groups, and certain clinical and laboratory features

Variable	Type		*let-7b-5p*				*miR-1-3p*				*miR-24-3p*				*miR-34a-5p*			
			CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D
BMI	*ordinal*	*p*	NA	0.482	0.706	0.383	NA	0.574	0.279	0.161	NA	0.140	0.888	0.328	NA	0.725	0.814	0.153
		<25	NA	339.195 (108.28–6363.2)	864.78 (108.28–6363.2)	157.95 (56.05–460.77)	NA	29 (1.55–383.06)	44.475 (1.55–383.06)	3.335 (1.44–11.78)	NA	0.955 (0.74–17.61)	4.14 (0.74–22.67)	1.845 (0.64–2.77)	NA	0.125 (0.05–2.57)	0.26 (0.03–2.57)	0.06 (0.02–0.12)
		25–30	NA	618.695 (53.55–2144.91)	450.505 (53.55–2144.91)	181.735 (64.93–1562.05)	NA	8.64 (2.19–18.12)	8.64 (2.19–18.12)	3.48 (0.86–211.22)	NA	2.95 (0.89–5.59)	2.58 (0.89–5.59)	1.555 (0.79–9.39)	NA	0.21 (0.03–0.61)	0.14 (0.03–0.61)	0.07 (0.03–1.36)
		>30	NA	676.125 (138.64–1987.14)	676.125 (138.64–1987.14)	291.81 (43.26–2007.8)	NA	11.83 (3.63–39.46)	11.83 (3.63–39.46)	6.71 (0.68–36.11)	NA	3.865 (1.92–6.43)	3.865 (1.92–6.43)	1.89 (0.44–10.04)	NA	0.215 (0.04–0.89)	0.215 (0.04–0.89)	0.11 (0.02–1.4)
	continuous	*p*	0.1028	0.0951	0.8125	0.3807	0.0583	0.2595	0.4359	0.0574	0.1028	0.0168	0.4957	0.3173	0.5639	0.2096	0.6216	0.1245
		r	−0.7714	0.3835	0.0488	0.1443	−0.8286	0.2647	−0.1597	0.3069	−0.7714	0.5278	0.1398	0.1644	−0.3143	0.2932	0.1015	0.2502
W/H ratio	*continuous*	*p*	>0.9999	0.1758	0.3312	0.8088	>0.9999	0.4752	0.8887	0.7736	>0.9999	0.5254	0.6927	0.7282	>0.9999	0.4960	0.4994	0.8701
		r	−0.5000	0.3339	0.2230	−0.0417	−0.5000	0.1798	0.0325	−0.0496	−0.5000	0.1602	0.0916	−0.0599	0.5000	0.1716	0.1560	−0.0282
Metabolic syndrome	*categorical*	*p*	NA	0.7355	0.7283	0.0980	NA	0.3713	0.2268	0.2683	NA	>0.9999	0.8283	0.2268	NA	0.5129	0.5422	0.3833
		ab	NA	618.7 (108.3–6363)	611.1 (89.60–6363)	130.2 (56.05–490.4)	NA	10.39 (1.549–383.1)	14.43 (1.549–383.1)	2.540 (1.123–11.78)	NA	2.948 (0.7401–17.61)	2.948 (0.7401–22.67)	1.290 (0.6405–3.112)	NA	0.1742 (0.0413–2.568)	0.1600 (0.0338–2.568)	0.0789 (0.0166–0.2449)
		pr	NA	333.2 (53.55–2145)	333.2 (53.55–2145)	264.9 (43.26–2008)	NA	5.457 (2.188–24.85)	5.457 (2.188–24.85)	4.701 (0.6837–211.2)	NA	2.106 (1.036–6.433)	2.106 (1.036–6.433)	1.863 (0.4443–10.04)	NA	0.1537 (0.0320–0.6132)	0.1537 (0.0320–0.6132)	0.0775 (0.0244–1.402)
Central obesity	*categorical*	*p*	NA	0.9628	0.7335	0.2514	NA	0.4929	0.1172	0.9036	NA	0.9000	0.5208	0.1917	NA	0.9000	0.8491	0.1578
		ab	NA	617.5 (120.5–6363)	617.5 (89.60–6363)	140.1 (56.05–266.2)	NA	14.72 (2.955–383.1)	24.63 (2.955–383.1)	4.438 (1.445–11.78)	NA	3.295 (0.7773–17.61)	3.295 (0.7773–22.67)	0.8276 (0.6405–2.631)	NA	0.1600 (0.1069–2.568)	0.1600 (0.0338–2.568)	0.0418 (0.0166–0.1233)
		pr	NA	618.7 (53.55–2145)	618.7 (53.55–2145)	225.7 (43.26–2008)	NA	9.949 (2.188–39.46)	9.949 (2.188–39.46)	4.183 (0.6837–211.2)	NA	2.843 (0.8886–6.433)	2.843 (0.8886–6.433)	1.863 (0.4443–10.04)	NA	0.2079 (0.0320–0.8932)	0.2079 (0.0320–0.8932)	0.0842 (0.0244–1.402)
Family history	*categorical*	*p*	NA	0.4019	0.6985	0.7908	NA	0.9656	0.0077	0.8132	NA	0.2224	0.4149	0.5986	NA	0.2532	0.2101	0.6712
		ab	NA	557.6 (120.5–1087)	557.6 (89.60–4911)	225.7 (56.05–749.6)	NA	7.350 (2.955–55.04)	44.89 (2.955–206.1)	4.183 (0.6837–17.41)	NA	2.242 (0.7773–5.195)	2.459 (0.7773–22.67)	1.589 (0.6405–3.284)	NA	0.1254 (0.0707–0.1817)	0.5315 (0.0338–2.295)	0.1100 (0.0166–0.2449)
		pr	NA	677.2 (53.55–6363)	677.2 (53.55–6363)	182.1 (43.26–2008)	NA	11.69 (1.549–383.1)	11.69 (1.549–383.1)	4.180 (0.8637–211.2)	NA	3.055 (0.7401–17.61)	3.055 (0.7401–17.61)	1.908 (0.4443–10.04)	NA	0.2777 (0.0320–2.568)	0.2777 (0.0320–2.568)	0.0725 (0.0244–1.402)
Hypertension	*categorical*	*p*	NA	NA	NA	0.6246	NA	NA	NA	0.4461	NA	NA	NA	0.8918	NA	NA	NA	0.4141
		ab	NA	NA	NA	199.9 (56.05–2008)	NA	NA	NA	3.640 (1.123–36.11)	NA	NA	NA	1.800 (0.6405–10.04)	NA	NA	NA	0.0725 (0.0166–1.000)
		pr	NA	NA	NA	225.7 (43.26–1736)	NA	NA	NA	5.460 (0.6837–211.2)	NA	NA	NA	1.826 (0.4443–9.392)	NA	NA	NA	0.0842 (0.0244–1.402)
Hyperlipidemia	*categorical*	*p*	NA	0.8503	0.8342	0.0575	NA	0.6328	0.2181	0.1027	NA	0.8785	0.6968	0.1027	NA	0.4243	0.0617	0.2039
		ab	NA	610.8	559.5	125.2	NA	7.789	26.67	1.722	NA	2.948	2.843	1.261	NA	0.2015	0.3873	0.0450
			NA	(108.3–6363)	(89.60–6363)	(56.05–490.4)	NA	(1.549–383.1)	(1.549–383.1)	(1.118–11.78)	NA	(0.7401–17.61)	(0.7401–22.67)	(0.6405–3.112)	NA	(0.0413–2.568)	(0.0333–2.568)	(0.0166–0.2449)
		pr	NA	573.1	573.1	291.8	NA	16.92	16.92	5.161	NA	2.302	2.302	1.886	NA	0.1666	0.1666	0.0807
			NA	(53.55–2145)	(53.55–2145)	(43.26–2008)	NA	(2.188–55.04)	(2.188–55.04)	(0.6837–211.2)	NA	(0.7773–6.433)	(0.7773–6.433)	(0.4443–10.04)	NA	(0.0320–0.6132)	(0.0320–0.6132)	(0.0244–1.402)
HbA1c (%)	*categorical*	*p*	NA	NA	NA	0.6779	NA	NA	NA	0.2705	NA	NA	NA	0.7805	NA	NA	NA	0.4217
		<7%	NA	NA	NA	194.7	NA	NA	NA	4.339	NA	NA	NA	1.678	NA	NA	NA	0.0775
			NA	NA	NA	(43.26–2008)	NA	NA	NA	(0.8637–36.11)	NA	NA	NA	(0.444–10.04)	NA	NA	NA	(0.0244–1.402)
		>7%	NA	NA	NA	211.3	NA	NA	NA	2.831	NA	NA	NA	1.839	NA	NA	NA	0.0603
			NA	NA	NA	(52.24–1562)	NA	NA	NA	(0.6837–211.2)	NA	NA	NA	(0.6405–9.392)	NA	NA	NA	(0.0166–1.357)
	continuous	*p*	NA	0.0739	0.0739	0.2936	NA	0.0115	0.0115	0.2970	NA	0.0802	0.0802	0.6275	NA	0.0402	0.0402	0.1582
		r	NA	−0.4350	−0.4350	−0.1827	NA	−0.5936	−0.5936	−0.1814	NA	−0.4263	−0.4263	−0.0849	NA	−0.4945	−0.4945	−0.2438
Glucose (mg/dl)	*categorical*	*p*	NA	NA	NA	0.7766	NA	NA	NA	0.9310	NA	NA	NA	0.6890	NA	NA	NA	0.8216
		<130	NA	NA	NA	279.0 (43.26–2008)	NA	NA	NA	4.282 (1.118–36.11)	NA	NA	NA	1.826 (0.4443–10.04)	NA	NA	NA	0.0893 (0.0166–1.402)
		>130	NA	NA	NA	193.6 (83.58–1562)	NA	NA	NA	4.081 (0.6837–211.2)	NA	NA	NA	1.678 (0.7859–9.392)	NA	NA	NA	0.0674 (0.0292–1.357)
	continuous	*p*	0.9500	0.3959	0.4902	0.6841	0.6833	0.2678	0.2037	0.9293	0.9500	0.6517	0.7160	0.6020	0.6833	0.4068	0.5929	0.5418
		r	−0.1000	−0.2008	−0.1447	0.0682	−0.3000	−0.2602	−0.2632	−0.0148	−0.1000	−0.1076	−0.0765	0.0873	−0.3000	−0.1963	−0.1124	0.1021
Insulin (μU/ml)	*continuous*	*p*	0.5167	0.2539	0.2399	0.0241	0.6833	0.4503	0.1510	0.5430	0.5167	0.6912	0.4228	0.0658	0.7833	0.1163	0.1983	0.1541
		r	−0.4000	−0.2677	−0.2439	−0.3861	−0.3000	−0.1789	−0.2959	−0.1081	−0.4000	−0.0947	−0.1678	−0.3191	−0.2000	−0.3624	−0.2663	−0.2499

Table 4 (continued)

Variable	Type		*let-7b-5p*				*miR-1-3p*				*miR-24-3p*				*miR-34a-5p*			
			CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D
Cholesterol total (mg/dl)	*continuous*	*p*	>0.9999	0.0835	0.2042	0.6381	>0.9999	0.4916	0.6063	0.7105	>0.9999	0.1257	0.2331	0.9128	>0.9999	0.3887	0.5690	0.5306
		r	−0.5000	0.4331	0.2966	−0.0989	−0.5000	0.1779	0.1227	−0.0781	−0.5000	0.3865	0.2793	−0.0230	−0.5000	0.2221	0.1355	−0.1316
HDL (mg/dl)	*continuous*	*p*	>0.9999	0.4589	0.9523	0.9307	>0.9999	0.6957	0.6584	0.7894	>0.9999	0.4707	0.8009	0.4651	>0.9999	0.6540	0.2465	0.6557
		r	0.5000	−0.1902	0.0143	−0.0187	0.5000	−0.1006	0.1054	−0.0575	0.5000	−0.1853	0.0602	−0.1565	0.5000	0.1166	0.2717	−0.0959
LDL (mg/dl)	*continuous*	*p*	0.3333	0.3111	0.6472	0.4931	0.3333	0.7781	0.8923	0.8369	0.3333	0.4888	0.8625	0.5489	0.3333	0.7530	0.9950	0.4403
		r	−1.000	0.2600	0.1091	−0.1470	−1.000	0.0735	−0.0323	−0.0443	−1.000	0.1790	0.0413	−0.1287	−1.000	0.0821	0.0015	−0.1653
Triglycerides (mg/dl)	*continuous*	*p*	0.3333	0.7729	0.2297	0.9505	0.3333	0.7084	0.2145	0.7841	0.3333	0.9284	0.2567	0.6110	0.3333	0.2390	0.0390	0.5803
		r	−1.000	−0.0759	−0.2812	−0.0130	−1.000	−0.0980	−0.2902	0.0576	−1.000	−0.0245	−0.2662	0.1069	−1.000	−0.3015	−0.4647	0.1162

Variable	Type		*miR-98-5p*				*miR-125b-5p*				*miR-133a-3p*			
			CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D
BMI	*ordinal*	*p*	NA	0.440	0.851	0.526	NA	0.399	0.346	0.146	NA	0.440	0.572	0.398
		<25	NA	0.69 (0.1–14.84)	1.29 (0.1–14.84)	0.355 (0.07–0.41)	NA	0.165 (0.06–0.93)	0.615 (0.06–1.19)	0.145 (0.04–0.35)	NA	0.66 (0.22–14.86)	1.69 (0.22–14.86)	0.645 (0.12–1)
		25–30	NA	0.86 (0.08–1.76)	0.525(0.08–1.76)	0.325 (0.05–3.23)	NA	0.275 (0.1–1.05)	0.275 (0.08–1.05)	0.205 (0.07–0.51)	NA	0.99 (0.23–2.07)	0.75 (0.23–2.07)	0.375 (0.13–9.18)
		>30	NA	0.995 (0.23–2.47)	0.995 (0.23–2.47)	0.49 (0.05–3.15)	NA	0.295 (0.18–0.62)	0.295 (0.18–0.62)	0.26 (0.08–1.63)	NA	1.1 (0.3–3.99)	1.1 (0.3–3.99)	0.49 (0.11–6.39)
	continuous	*p*	0.1028	0.1038	0.7463	0.5061	0.0583	0.0645	0.5889	0.1636	0.1361	0.1317	0.9881	0.4728
		r	−0.7714	0.3744	0.0666	0.1097	−0.8286	0.4211	−0.1111	0.2275	−0.7143	0.3489	0.0030	0.1184
W/H ratio	*continuous*	*p*	>0.9999	0.2169	0.3458	0.5468	>0.9999	0.3553	0.9176	0.3329	>0.9999	0.2848	0.5400	0.5127
		r	−0.5000	0.3059	0.2165	−0.1038	−0.5000	0.2315	0.0240	−0.1661	−0.5000	0.2667	0.1417	−0.1127
Metabolic syndrome	*categorical*	*p*	NA	0.7956	0.8795	0.1478	NA	0.6200	0.4585	0.5673	NA	0.5652	0.4585	0.4208
		ab	NA	0.8980 (0.1013–14.84)	0.8778 (0.0934–14.84)	0.2178 (0.0742–0.5590)	NA	0.3089 (0.0581–0.9295)	0.4073 (0.0581–1.195)	0.1802 (0.0437–1.631)	NA	0.9863 (0.2193–14.86)	1.058 (0.2193–14.86)	0.4067 (0.1235–1.136)
		pr	NA	0.6012 (0.0783–1.758)	0.6012 (0.0783–1.758)	0.4173 (0.0497–3.226)	NA	0.2002 (0.1048–1.054)	0.2002 (0.1048–1.054)	0.2118 (0.0568–1.266)	NA	0.7409 (0.2302–2.068)	0.7409 (0.2302–2.068)	0.5129 (0.1142–9.176)
Central obesity	*categorical*	*p*	NA	0.8012	>0.9999	0.3491	NA	0.4434	0.0945	0.1917	NA	0.9628	0.5208	0.8255
		ab	NA	0.8778 (0.1686–14.84)	0.8778 (0.0934–14.84)	0.3536 (0.0742–0.3572)	NA	0.3785 (0.0581–0.9295)	0.5546 (0.0581–1.195)	0.0873 (0.0437–0.3523)	NA	0.9572 (0.3768–14.86)	1.145 (0.3768–14.86)	0.9240 (0.1235–1.000)
		pr	NA	0.9166 (0.0783–2.474)	0.9166 (0.0783–2.474)	0.4091 (0.0497–3.226)	NA	0.2470 (0.1048–1.054)	0.2470 (0.1048–1.054)	0.2118 (0.0568–1.631)	NA	0.9863 (0.2302–3.985)	0.9863 (0.2302–3.985)	0.4762 (0.1142–9.176)
Family history	*categorical*	*p*	NA	0.4916	0.4840	0.7685	NA	0.7534	0.6485	0.8357	NA	0.4453	0.0850	0.3500
		ab	NA	0.8401 (0.1686–1.212)	1.105 (0.0934–12.25)	0.3885 (0.0742–0.9992	NA	0.2187 (0.0581–0.6195)	0.2732 (0.0581–1.195)	0.1662 (0.0437–1.631)	NA	0.6881 (0.5250–1.273)	2.346 (0.4975–11.09)	0.4213 (0.1235–1.452)
		pr	NA	0.9540 (0.0783–14.84)	0.9540 (0.0783–14.84)	0.3562 (0.0497–3.226)	NA	0.3145 (0.1048–1.054)	0.3145 (0.1048–1.054)	0.2343 (0.0568–0.9954)	NA	1.172 (0.2193–14.86)	1.172 (0.2193–14.86)	0.6071 (0.1142–9.176)
Hypertension	*categorical*	*p*	NA	NA	NA	0.5866	NA	NA	NA	0.0738	NA	NA	NA	0.8918
		ab	NA	NA	NA	0.3219 (0.0742–3.151)	NA	NA	NA	0.1607 (0.0437–0.9954)	NA	NA	NA	0.5009 (0.1235–5.247)
		pr	NA	NA	NA	0.4615 (0.0497–3.226)	NA	NA	NA	0.2581 (0.0655–1.631)	NA	NA	NA	0.4542 (0.1142–9.176)
Hyperlipidemia	*categorical*	*p*	NA	0.7391	0.5625	0.0575	NA	0.3834	0.2648	0.7330	NA	0.8225	0.0807	0.1817
		ab	NA	0.8198	1.092	0.2135	NA	0.3263	0.3157	0.2261	NA	0.8690	2.111	0.3493
			NA	(0.1013–14.84)	(0.0934–14.84)	(0.0742–0.5590)	NA	(0.1088–0.9295)	(0.0750–1.195)	(0.0437–1.631)	NA	(0.2193–14.86)	(0.2193–14.86)	(0.1235–1.136)
		pr	NA	0.9169	0.9169	0.4206	NA	0.2002	0.2002	0.2022	NA	0.9755	0.9755	0.5376
			NA	(0.0783–1.758)	(0.0783–1.758)	(0.0497–3.226)	NA	(0.0581–1.054)	(0.0581–1.054)	(0.0568–1.266)	NA	(0.2302–2.068)	(0.2302–2.068)	(0.1142–9.176)
HbA1c (%)	*categorical*	*p*	NA	NA	NA	0.6125	NA	NA	NA	0.0925	NA	NA	NA	0.9592
		<7%	NA	NA	NA	0.3562	NA	NA	NA	0.2581	NA	NA	NA	0.4776
			NA	NA	NA	(0.0497–3.151)	NA	NA	NA	(0.0568–1.631)	NA	NA	NA	(0.1142–6.394)
		>7%	NA	NA	NA	0.2980	NA	NA	NA	0.1825	NA	NA	NA	0.4251
			NA	NA	NA	(0.0701–3.226)	NA	NA	NA	(0.0437–0.3974)	NA	NA	NA	(0.1142–9.176)

Table 4 (continued)

Variable	Type		*miR-98-5p*				*miR-125b-5p*				*miR-133a-3p*			
			CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D	CT_{RF-}	CT_{RF+}	CT	T2D
	continuous	*p*	NA	0.1205	0.1205	0.4608	NA	0.0840	0.0840	0.0198	NA	0.0580	0.0580	0.8453
		r	NA	−0.3804	−0.3804	−0.1288	NA	−0.4213	−0.4213	−0.3921	NA	−0.4598	−0.4598	−0.0342
Glucose (mg/dl)	*categorical*	*p*	NA	NA	NA	0.7990	NA	NA	NA	0.2805	NA	NA	NA	0.2962
		<130	NA	NA	NA	0.3789 (0.0497–3.151)	NA	NA	NA	0.2624 (0.0437–1.631)	NA	NA	NA	0.4226 (0.1142–6.394)
		>130	NA	NA	NA	0.3048 (0.1251–3.226)	NA	NA	NA	0.1638 (0.0568–1.266)	NA	NA	NA	0.4762 (0.1363–9.176)
	continuous	*p*	0.9500	0.3388	0.5079	0.5620	0.6833	0.8378	0.7686	0.4376	0.6833	0.4777	0.6134	0.1970
		r	−0.1000	−0.2256	−0.1389	0.0970	0.3000	−0.0488	−0.0619	−0.1297	−0.3000	−0.1685	−0.1062	0.2140
Insulin (μU/ml)	*continuous*	*p*	0.5167	0.3227	0.3433	0.0441	0.6833	0.4899	0.1183	0.0157	>0.9999	0.2120	0.2078	0.0869
		r	−0.4000	−0.2331	−0.1978	−0.3474	−0.3000	−0.1639	−0.3205	−0.4113	0.0	−0.2917	−0.2609	−0.2980
Cholesterol total (mg/dl)	*continuous*	*p*	>0.9999	0.1516	0.2561	0.9287	>0.9999	0.0762	0.0913	0.5966	>0.9999	0.2315	0.3812	0.6713
		r	−0.5000	0.3632	0.2665	−0.0188	0.5000	0.4429	0.3877	−0.1112	−0.5000	0.3055	0.2070	0.0892
HDL (mg/dl)	*continuous*	*p*	>0.9999	0.5932	0.7985	0.9179	0.3333	0.5541	0.8377	0.4489	>0.9999	0.9905	0.3620	0.8476
		r	0.5000	−0.1374	0.0609	0.0222	1.000	−0.1521	0.0489	0.1622	0.5000	−0.0024	0.2153	−0.0340
LDL (mg/dl)	*continuous*	*p*	0.3333	0.4920	0.7671	0.6131	>0.9999	0.2707	0.4937	0.3987	0.3333	0.5195	0.8526	0.5904
		r	−1.000	0.1778	0.0707	−0.1087	−0.5000	0.2820	0.1625	−0.1805	−1.000	0.1668	0.0433	−0.1157
Triglycerides (mg/dl)	*continuous*	*p*	0.3333	0.6053	0.1582	0.9070	>0.9999	0.4264	>0.9999	0.5528	0.3333	0.5214	0.0923	0.8782
		r	−1.000	−0.1348	−0.3278	0.0246	−0.5000	0.2059	0.0	−0.1246	−1.000	−0.1667	−0.3865	0.0323

Analysis was applied in the CT_{RF-} and CT_{RF+} subgroups separately, the total CT group and the T2D group, using appropriate non-parametric tests as required: differential expression levels in the cases of categorical variables [namely metabolic syndrome, central obesity, family history, hypertension, hyperlipidemia (presence; *p* or absence; *ab* of the manifestation), HbA1c levels (<7% = 53 mmol/mol or > 7% = 53 mmol/mol) and glucose levels (<130 mg/dl or > 130 mg/dl)] were evaluated by the Mann–Whitney test, in the cases of ordinal variables [namely BMI levels (< 25, 25–30, > 30)] by the Jonckheere–Terpstra test, while correlations with continuous variables [namely levels of BMI, W/H ratio, HbA1c, glucose, total cholesterol, HDL, LDL, and triglycerides] were evaluated by the Spearman's rank correlation test. The statistical significance (*p*), the miRNA levels [median (range)] in the groups of categorical and ordinal variables, and the regression (*r*) for continuous variables examined, are displayed

Bold values indicate median values and statistical significance $p < 0.05$

NA not applicable

	Pathway description	Observed gene count	False discovery rate	Matching proteins in the network
	PI3K-Akt signaling pathway	43	1.23E-19	AKT1,AKT2,BCL2,BCL2L1,BCL2L11,BRCA1,CASP9,CCND1,CCND2,CCND3,CCNE2,CDK4,CDK6,CDKN1B,COL1A1,COL3A1,CSF1R,EGFR,EIF4EBP1,EPO,EPOR,FGFR2,FGFR3,FN1,GNB2,GYS1,HRAS,IFNAR1,IFNB1,IGF1R,IL6R,ITGB4,KIT,MAP2K1,MAPK1,MCL1,MET,MYB,MYC,NOS3,NRAS,PDGFRA,PDGFRB
	HIF-1 signaling pathway	19	7.78E-12	AKT1,AKT2,BCL2,CAMK2G,CDKN1B,CYBB,EDN1,EGFR,EIF4EBP1,EPO,ERBB2,HK2,HMOX1,IFNG,IGF1R,IL6R,MAP2K1,MAPK1,NOS3
	MAPK signaling pathway	21	6.93E-07	AKT1,AKT2,BDNF,CACNA1C,CACNA2D1,CDC42,DUSP6,EGFR,FGFR2,FGFR3,FLNA,FLNB,FLNC,HRAS,MAP2K1,MAPK1,MAPK14,MYC,NRAS,PDGFRA,PDGFRB
	FoxO signaling pathway	14	1.68E-06	AKT1,AKT2,BCL2L11,CCND1,CCND2,CDKN1B,EGFR,HRAS,IGF1R,IRS2,MAP2K1,MAPK1,MAPK14,NRAS
	Insulin signaling pathway	14	4.83E-06	AKT1,AKT2,CALM1,CBL,EIF4EBP1,FASN,FLOT2,GYS1,HK2,HRAS,IRS2,MAP2K1,MAPK1,NRAS
	Type 2 diabetes mellitus	4	0.0391	CACNA1C,HK2,IRS2,MAPK1

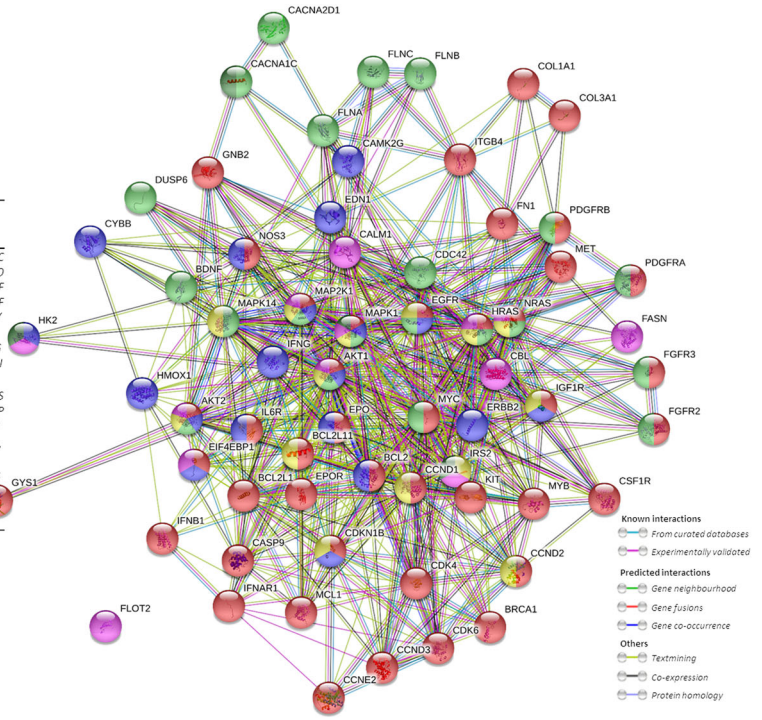

Fig. 2 Network of interactions among the proteins encoded by the set of genes targets of the T2D-specific miRNA panel developed in this study, focusing on molecular pathways involved in T2D pathogenesis. Network analysis was performed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. Different genes/proteins are involved in different (one or more) T2D-related pathways, as this is designated by the differently colored nodes. Edges represent protein–protein associations; either known interactions, predicted interactions or other associations. The incorporated table reports the number and the name of the target genes observed in each pathway and the false discovery rate of significance of the pathway in the analysis

those of the tv3 of *CDKN2A* in the CT_{RF+} group. On the other hand, miR-133a-5p, miR-1-3p, or miR-34a-5p levels were positively associated with *KCNQ1* tv1 levels (recently found to be decreased in T2D patients and individuals at risk of T2D [6]).

CDKN2A genetic variants were previously implicated in the negative regulation of beta-cell mass, proliferation, and insulin secretory function [28], while in human islets the locus can be affected by epigenetic factors [29]. The decreased levels of miR-24-3p, miR-34a-5p, and miR-125b-5p observed in this study, associated with T2D, and their negative correlation with the impaired levels of *CDKN2A* tv 3, may be linked to miRNA transcriptional-regulation events involved in T2D pathogenesis. On the other hand, *KCNQ1* encodes for the potassium voltage-gated channel, implicated in insulin secretion. The positive association between the decreased levels of miR-133a-5p or miR-1-3p with the decreased levels of *KCNQ1* tv1 observed in patients and predisposed individuals, may be implicated in molecular pathways associated with inhibition of repression/disturbance of feedback loops, related to impaired insulin secretion.

Nevertheless, some of the above miRNAs have been already reported to be involved in T2D-related pathophysiology. In vitro experiments in human and murine blood samples and endothelial cells showed that miR-24 is involved in the thrombotic complications of diabetes and atherosclerotic plaque progression [21, 30]. Also, miR-1, and miR-133, the so-called 'myo-miRs' (they are clustered on the same chromosomal locus and transcribed together in a tissue-specific manner [31, 32]), are among the miRNA molecules that perchance play a key role in the cardiovascular complications of diabetes [33–35].

In an attempt to further support our suggestion on the possible involvement of these differentially expressed miRNAs in T2D pathogenesis, we searched for T2D-related molecular pathways regulated by these miRNAs, following a bioinformatics approach. Our data revealed enhanced regulation of PI3K-Akt, HIF-1, MAPK, FoxO, and insulin-signaling pathways, all pivotally involved in T2D pathogenesis. However, gene-set analysis did not reveal any of the T2D-susceptibility genes; this is probably to be expected since their functional involvement in T2D-related pathways is not yet fully elucidated and deposited in any database [36]. Though, by combining the qPCR data and results of the pathway analysis, one could speculate that the differentially expressed miRNAs can be involved in pathogenetic mechanisms and regulate both the genes in the

abovementioned pathways and T2D-susceptibility genes, and not exclude that the last may be also implicated in these pathways. It is also of note, that other pathways regulated by the T2D-related miRNA panel (Supplementary Table 5), are crucially implicated in cancer development, which may imply the common pathogenetic mechanisms shared by these two human disorders [37].

Lastly, the levels of the differentially expressed miRNAs were found to correlate with certain clinicopathological parameters, in a different manner in T2D versus control subjects. MiR-125b-5p, let-7b-5p, and miR-98-5p levels associated negatively with serum insulin levels, exclusively in the group of patients, suggesting possibly their involvement in insulin homeostasis in T2D. MiR-34a-5p levels associated negatively with serum triglycerides levels in the total group of controls and with % HbA1c levels in predisposed individuals, indicating probably their implication in regulatory mechanisms, underlying T2D pathogenesis and/or cholesterol metabolism. Nevertheless, one should be particularly concerned on the interpretation of these results, since, in the current study, the group of patients consisted of individuals both prior and under treatment, and thus miRNA expression as well as biochemical parameters may be regulated also by these anti-diabetic agents. Clearer evidence could arise from corresponding analysis on samples from newly-diagnosed T2D patients before the initiation of any treatment. This was not able to be performed in this study, since the cohort included only seven naïve patients.

Despite its limitations, including the relatively small number of participants and that only a single population is examined, this study describes for the first time to the best of our knowledge, a disease-specific expression profile of miRNAs validated to target T2D-susceptibility genes, in peripheral blood of patients and predisposed individuals. Power analysis suggests that this approach provides with promising data for further evaluation in larger cohorts. The impaired miRNA expression described herein may be linked to pathophysiological mechanisms underlying T2D development; the fact that these patterns correlated with certain disease parameters, differently in patients versus controls, may further support their involvement in disease's pathogenesis. Also, associations between the expression levels of these miRNAs and those of certain transcript variants of their target T2D-susceptibility genes exclusively in T2D and CT_{RF+} subjects, propose a transcript variant specific and disease-related regulation of transcription. Large-scale perspective clinical studies in diverse populations are essential to evaluate the potential of these miRNAs to serve as possible biomarkers for T2D diagnosis, prognosis, and/or monitoring.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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