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ABSTRACT

Background

MiRNAs are small non-coding RNAs participating in genes post-transcriptional regulation. Their key role in modulating the susceptibility to human diseases is now widely recognized, in particular in the context of cardiometabolic disorders. The aim of the present study was to identify miRNAs associated with diabetic nephropathy (DN) in patients with type 2 diabetes (T2D).

Methods

A next generation sequencing-based miRNA profiling was performed in a case-control study for DN in plasma samples of 23 T2D patients with DN (cases) and 23 T2D without (controls). The main associations were confirmed using qRT-PCR and tested for replication in an independent case-control collection of 100 T2D patients, 50 with DN and 50 without.

Results

From the 381 known mature miRNAs that were found highly expressed in the discovery samples, we observed and replicated an association between increased plasma levels of hsa-miR-152-3p and DN ($p = 4.03\ 10^{-4}$ in the combined samples). Hsa-miR-152-3p plasma levels were further found to be positively correlated ($p = 0.003$) to plasma osmolarity, a surrogate marker for solute carrier net activity, whose regulation is controlled by several genes including *SLC5A3*, one of the predicted targets of hsa-miR-152-3p.

Conclusions

We observed strong evidence for the association of hsa-miR-152-3p plasma levels and DN in patients with T2D confirming an association previously observed in patients with T1D.

KEY WORDS: Circulating microRNA; microRNA-Sequencing; Type 2 diabetes; Diabetic nephropathy; biomarkers.

INTRODUCTION

Diabetes is considered as the leading cause of end-stage renal failure, contributing to more than one-third of all end-stage renal disease based on the 2016 report of United States Renal Data System [1]. Diabetic nephropathy (DN) remains incompletely understood and occurs in 30 to 40% of diabetic patients [2], and even in patients with good glycemic control [3]. Abnormalities in several signaling pathways and cytokines have been demonstrated to contribute to DN development, including the renin-angiotensin system, reactive species of oxygen, endoplasmic reticulum stress, pro-inflammatory cytokines and formation of advanced glycation end-products [4,5].

Urinary albumin excretion is widely used as a non-invasive marker for DN although it does not fully reflect the disease process [6]. Histological abnormalities may be present before the detection of microalbuminuria. Similarly, some patients have decreased glomerular filtration rate (GFR) despite normoalbuminuria [7,8]. This phenotype seems to be rather common in type 2 diabetes (T2DM) [9], challenging traditional classification of DN [10]. An important effort is currently undertaken to identify innovative biomarkers of diabetic nephropathy, as illustrated by the recent grant from the UE on biomarkers of DN (https://ec.europa.eu/research/participants/portal/doc/call/h2020/imi2-2015-05-06/1662621-c5_imi2_c5_text_9_july_2015_en.pdf).

Recently, post-transcriptional regulation of mRNA has emerged as a major mechanism modulating gene expression. Non-coding RNAs such as microRNAs (miRNAs) participate in regulating mRNA stability and aberrant miRNA expression has been shown to account for renal fibrosis and podocyte injury [11,12]. In response to various pathophysiological conditions, miRNAs can be released by cells into different extracellular fluids, including blood and could serve as biomarkers of diverse diseases including diabetes and its complications. Circulating miRNAs are more stable due to protection from endogenous RNase activity through their incorporation into lipoprotein complexes or microvesicles such as exosomes [13–15]. Recent reports have demonstrated distinct profiles of miRNAs and exosomal

miRNAs in plasma and urine samples across various stages of diabetic nephropathy [16–20].

Widely used techniques for identification of differentially expressed miRNAs, such as miRNA microarrays, suffer from a low accuracy due to low specificity of probe hybridization and variable sensitivity to detect less abundant miRNAs. The next-generation sequencing has a high-resolution potential, even though limited data is available in the relevant literature on its use as a discovery-based approach to identify enriched or dysregulated miRNAs in DN. The aim of the present study was to perform a comprehensive sequencing of plasma miRNAs in subjects with T2DM to identify candidate miRNAs for DN.

MATERIAL & METHODS

Study populations

Studied participants were patients with T2DM selected from the cross-sectional DIAB2NEPHROGENE study, which aimed to compare genetic and non-genetic determinants of renal complications in T2D patients. This study design was previously reported [21,22]. The DIAB2NEPHROGENE study was approved by the Poitiers University Ethics Committee and was in accordance with the Helsinki Declaration of 1975, as revised in 2000. All participants gave written informed consent.

Cases were patients with T2DM and DN defined as increased urinary albumin (micro or macroalbuminuria) with or without altered renal function (defined as estimated glomerular filtration rate eGFR below 60 ml/min per 1.73m^2, according to CKD-EPI equation [23]) and no clinical and/or biological suspicion of non-diabetic kidney disease. Micro and macroalbuminuria were diagnosed according to urinary albumin determined on two out of three sterile urine collections: microalbuminuria range $\geq$ 20 - 199 mg/l or 30-299 mg/24 hrs, and macroalbuminuria range $\geq$ 200 mg/l or 300 mg/24 hrs. Controls were patients with T2DM with normal urinary albumin (range < 20 mg/l or 30 mg/24 hrs) and eGFR above 60 ml/min/1.73m^2.

DN patients and controls were matched for the following criteria: recruiting center, age (+/- 5 years), sex. Although not strictly considered as a matching criterion, a special effort was made to have cases and controls not largely differing for diabetic retinopathy (absent, simple, severe non-proliferative or proliferative retinopathy) in order to focus on DN rather than on microvascular risk in general. Biological samples were stored at -80°c at the CHU Poitiers biobanking facility (CRB Poitiers BB0033-00068) until use (first freeze-thawing cycle).

Biological determinations

Biological determinations of classical biological variables are briefly reported here : HbA1c by HPLC (Adams A1c HA-8160 analyzer; Menarini, Florence, Italy); creatinine by colorimetry on an automated analyzer (Kone Optima; Thermo Clinical Labsystems, Vantaa, Finland) for plasma determinations and on a Hitachi 911 automatic analyzer (Roche Diagnostics, Meylan, France) for urinary determinations; urinary albumin by nephelometry on a Modular System P (Roche Diagnostics GmbH) and osmolarity by the measure of the point of congelation of aqueous solutions with an automatic micro-osmometer (Fisher Bioblock Scientific, Illkirch, France).

Experimental protocol for miRNA sequencing

Plasma miRNA profiling was conducted in a sample of 46 T2D patients, half of which being patients with DN and the remaining half being free of DN. Total RNA containing miRNA was extracted from EDTA plasma sample (400 µl) with Qiagen miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany). Plasma miRNA libraries were then prepared from 6µl of total RNA using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs France, Evry) according to manufacturer's instructions with a specific dilution of adaptors to 1/10 and 15 cycles for PCR amplification. Specific small RNAs bands were then selected by a 3-steps optimized AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA) protocol in 96-well plate

(200 µl), without QIAQuick PCR purification: 1- a 1.5X AMPure XP beads purification step was first performed on 100 µl of amplified library to remove smaller fragments and the bead product was resuspended with H2O (42 µl); 2- 1.1X AMPure XP beads were added to the first purification products (42 µl); 3- After discarding beads with the smallest fragments, the resulting supernatant was kept and purified with 1.8X AMPure XP beads. The beads product, resuspended in H2O (15 µl), served as the final purified library. Two plasma samples were analyzed in duplicate. A pool of equal quantity of 24 purified libraries was constructed, each library being tagged with a different index. Two pools of 24 libraries were constituted in order to analyze the 46 plasma samples, each pool being loaded on 2 lanes of a rapid flowcell. A 55bp single read sequencing of miRNAs pool libraries was performed on an Illumina Hiseq 1500 instrument (Illumina, San Diego, California, USA) at the Post-Genomic Platform of the Pitié-Salpêtrière Hospital.

miRNA sequencing data analysis

After demultiplexing, reads that did not pass Illumina filters were removed with fastq_illumina_filter[24]. Cutadapt [25] was used to trim 3' and 5' adapters and remove low-quality bases (quality <28) from the 3' and 5' ends. Reads with a length between 15 and 30 bases, i.e. reads that could correspond to miRNAs, were selected for further alignment. Bowtie v1.1.1 [26] was used to align reads against the Homo Sapiens mature miRNA sequences from the miRBase 21 database [27]. Only reads that uniquely aligned without any mismatch were kept for the present study. miRNA abundance was expressed as the number of reads (i.e counts) that aligned to a given miRNA. To avoid any background noise due to very low expressed miRNAs, only miRNAs with counts higher than 10 in more than 5 subjects were kept for analysis. To normalize abundances, we first applied the size factor correction as proposed in the Deseq method [28] followed by the application of the variance stabilizing transformation [29]. Impact of the normalization procedure can be evaluated by inspecting the miRNA expression profiles that are more comparable across samples after normalization (**Supplementary**

Figure S1), without impacting on the reproducibility of the technical replicates (**Supplementary Figure S2**). For the subsequent differential analysis, replicates with the lower count variability were used.

Association between detected miRNAs and DN was performed using linear regression analysis adjusted for age, sex, and counts of 3 let7 controls (let7d/7g/7i) miRNAs in order to avoid any uncontrolled bias in libraries preparation [30]. Beforehand, miRNAs were normalized using the normal inverse quantile transformation [31].

qRT-PCR validation

Candidate miRNAs showing suggestive statistical evidence for association with DN in the miRNA sequencing analysis were re-quantified for technical validation by qRT-PCR after a new extraction. miRNA qRT-PCR was performed by specific TaqMan miRNA assays using the TaqMan microRNA Reverse Transcription kit and TaqMan Universal PCR Master Mix no UNG (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions (**Supplementary Methods**). MiRNA levels were normalized to the miRNA content of a mix of 3 miRNAs -let7d, let7g and let7i- and the relative miRNA levels were calculated using the $2^{-\Delta CT}$ method. The same methodologies were applied to the candidate miRNAs further measured in an independent sample of 100 T2D patients composed of 50 patients with DN and 50 without. Linear regression analysis, adjusting for age and sex, were employed to test for the association between the qRT-PCR miRNA levels and DN status, in the discovery and replication samples, separately.

RESULTS

miRNA identification results

Before pre-processing steps, the average number of reads per sample was 10.5 10^6, ranging from 7.5 10^6 to 13.8 10^6. After quality controls, a per-sample average of 64.67% of reads (min = 46.55%, max

= 86.59%) was kept for alignment analysis. The percentage of reads that uniquely matched to human mature miRNAs ranged from 2.4% to 16.44%, with a mean of 6.47%, which led to the detection in the 46 studied plasma samples of 1,424 known mature miRNAs. From these miRNAs, 384 miRNAs (including the 3 housekeeping miRNAs serving as positive controls - see Methods) were considered as expressed and entered into the differential expression analysis with respect to DN.

Discovery step

Clinical and biological characteristics of cases and controls in both the discovery and replication cohorts are presented in **Table 1**. Differential expression analysis between cases and controls, with the full list of results given in **Supplementary Table S3**, did not reveal any significant association that satisfied the statistical threshold of $1.3\ 10^{-4}$ corresponding to the Bonferroni correction for the number of tested miRNAs (n = 381). The lowest p-value was observed at $p = 1.57\ 10^{-3}$ for hsa-miR-362-5p. Nevertheless, 4 miRNAs exhibited a marginal association with DN at $p < 0.01$. Boxplot representation of their expression levels in cases and controls are shown in **Figure 1**. Among these 4 miRNAs, three were over-expressed in cases compared to controls, hsa-miR-362-5p ($p = 1.57\ 10^{-3}$), hsa-miR-152-3p ($p = 4.34\ 10^{-3}$) and hsa-miR-196b-5p ($p = 4.47\ 10^{-3}$) while the remaining hsa-miR-140-3p ($p = 6.28\ 10^{-3}$) demonstrated opposite pattern of association. These 4 miRNAs were selected for further technical validation.

Technical validation

We checked the consistency between miRNA levels measurements obtained by sequencing and qRT-PCR for the 4 miRNAs with p-values <0.01, selected at the discovery step. The correlation between qRT-PCR levels and sequencing data was satisfactory for hsa-miR-152-3p (r = 0.64), hsa-miR-196b-5p (r = 0.53) and hsa-miR-362-5p (r = 0.42) but not for hsa-miR-140.3p (r = 0.19).
Nevertheless, using the qRT-PCR measurements, only the association of hsa-miR-152-3p with DN

was still significant (p = 0.002) with increased expression in cases compared to controls (**Table 2**), leading us to select hsa-miR-152-3p for replication in an independent sample.

Replication step

Following these observations, the association of miRNA hsa-miR-152-3p with DN was tested in another sample of 100 T2D patients made of 50 cases and 50 controls, whose characteristics are detailed in **Table 1**. Consistently with the results observed in the discovery cohort, the hsa-miR-152-3p showed increased plasma levels in DN patients compared to non-DN patients (0.061 vs 0.055) with association reaching borderline significance (p = 0.053) (**Table 2**).

In the combined discovery and replication samples, the statistical evidence between hsa-miR-152-3p plasma levels and DN was $p = 4.03\ 10^{-4}$.

Intermediate phenotype

Interestingly, the identified hsa-miR-152-3p is computationally predicted [32] to target the *SLC5A3* gene previously suspected to be involved in acute renal failure [33] of which DN is an important contributing factor [34]. As the SLC5A3 protein is involved in the regulation of osmolarity, we examined the relationship between hsa-miR-152-3p plasma levels and plasma osmolarity, which is a surrogate marker for solute carrier net activity. Plasma osmolarity did not differ between cases and controls (**Table 1**). In the total samples, we observed a positive correlation (r = 0.26, p = 0.003) between hsa-miR-152-3p plasma levels and plasma osmolarity **(Supplementary Figure S4)**. This correlation was observed both in the discovery (r = 0.20) and the replication (r = 0.28) samples, and in cases (r = 0.16) and controls (r = 0.24).

Of note, we did not evidence any correlation between hsa-miR-152-3p plasma levels and urinary albumin/creatinine ratio nor eGFR (data not shown).

DISCUSSION

To our knowledge, the pilot work reported here is the first to apply an agnostic next-generation sequencing technology to plasma samples of T2D patients in order to identify candidate miRNAs for DN. In addition, our study included a technical validation step of the main results observed in a discovery phase as well as a statistical replication in an independent sample. This work revealed that hsa-miR-152-3p plasma levels were increased in DN cases compared to controls, both in the discovery and replication cohorts. Interestingly, this miRNA had been previously found associated with DN in type 1 diabetes patients by a candidate miRNA approach[35]. These results strongly suggest that the hsa-miR-152-3p is involved in the DN pathophysiology in the population of patients with diabetes. Computational prediction includes SLC5A3 gene as one of the hsa-miR-152-3p's targets. *SLC5A3* encodes the SMIT1 (Sodium/myo-inositol transporter) protein, an inositol transported contributing to maintaining the osmotic balance in different tissues/organs, including the kidney [36], whose evidence for its role in diabetes-associated metabolism is accumulating[33,37,38]. Besides, we evidenced an association of hsa-miR-152-3p plasma levels with plasma osmolarity, an intermediate phenotype for the activity of solute carrier transporters genes, including SLC5A3. Of note, SLC5A3 has been reported to associate with acute renal failure [33]. Unfortunately, we were not able to detect SLC5A3 mRNA in our T2D plasma samples (**Supplementary Methods**) which prevented us from testing any association between DN, SLC5A3 mRNA and hsa-miR-152-3p levels. We did not observe any correlation between hsa-miR-152-3p plasma levels and markers of DN (eg eGFR, urinary ACR) suggesting the identification of a potential pathway not involved in glomerular pathway but more likely related to tubular ion transfer. A recent study [39] has highlighted the role of hsa-miR-152-3p in the hepatic regulation of glucose metabolism through a mechanism involving the PTEN pathway. Of note, Kato *et al* [40] have demonstrated in mice that the PTEN pathway is also a key target for several glomerular miRNAs associated with DN. In the same mice model, glomerular levels of hsa-miR-152-

3p were found elevated in diabetic animals compared to controls [40]. Of note the main glomerular DN-associated miRNA identified in their work, miR-379, was not among those showing strong association with DN in our plasma samples (not shown data). Finally, obesity, a risk factor for chronic kidney disease [41] has been reported to associate with increased serum levels of hsa-miR-152-3p in non-diabetic patients [42]. We thus questioned if obesity could be a confounding factor between hsa-miR-152-3p and DN. In our study population with T2DM, we did not observe any association between hsa-miR-152-3p and body mass index (data not shown).

Several limitations must be acknowledged. We were not able to find any significant association between miRNA and DN that passed the study-wise statistical threshold in our discovery cohort. This is likely to be a power issue due to the small size of the T2D samples processed for miRNA sequencing. As an illustration, the power of our discovery cohort to detect, at the $p = 1.3\ 10^{-4}$ threshold, any association observed with the hsa-miR-152-3p was only 50%. We were also limited by the available quantity of mRNA material we could have access to in order to validate all suggestive associations observed in the miRNA sequencing phase. As a consequence, we cannot exclude we have missed some additional miRNAs that could associate with DN. In addition, a very stringent bioinformatics pipeline was applied to miRNA sequenced data where mismatch and multiple alignments were not taken into account in this study. This could have limited our chance to discover additional relevant miRNAs. Finally, even though cases and controls were well-matched for important determinants of DN such as age, gender [43] but also diabetic retinopathy [44], the phenotype between discovery and replication steps was slightly different for cases, with only proteinuric patients in the discovery and a combination of microalbuminuric and proteinuric patients in the replication population. In that way, the replication step could be considered as a replication and an extension step rather than a mere replication with strictly concordant inclusion criteria. These phenotypic differences might explain why the hsa-miR-152-3p plasma levels tend to be higher in cases from the discovery group than in those from the replication phase. However, we found no confounding factors for the

association between hsa-miR-152-3p levels and patients' characteristics (see **Table** 1). The lower association observed in the replication compared to the discovery step could also be due to the well-known winner curse effect [45,46].

Although our research strategy was not aiming to validate candidate miRNAs, we were able to find 23 miRNAs with nominal p-values <0.05 **(Supplementary Table S3)** and some of them including hsa-miR-132p, hsa-miR-192-5p and hsa-miR-326 were already described in previous work[35]. The association observed with hsa-miR-132p was however in the opposite direction as that observed in type 1 patients. Finally, our research design was cross-sectional and led to the identification of an association between DN and plasma levels of hsa-miR-152-3p. In this context, an important caveat must be kept in mind as hsa-miR-152-3p dysregulation could be a consequence of DN rather than causally implicated. The positive correlation between plasma osmolarity and hsa-miR-152-3p plasma levels adds support to the predicted relation between hsa-miR-152-3p and *SLC5A3* regulation. But the lack of association of plasma osmolarity and DN tends to exclude that the association of hsa-miR-152-3p plasma levels with DN could be mediated via a direct influence on plasma osmolarity.

In conclusion, our pilot study identified the hsa-miR-152-3p as a miRNA that associate with the risk of DN in patients affected with T2D and that can be measured in plasma material. This miRNA has been previously shown to associate with DN in a cross-sectional study in patients with T1D. Large prospective studies are now required to validate plasma hsa-miR-152-3p as a biomarker for DN and to establish its prognostic value beyond urinary albumin. In addition, functional experiments are also mandatory to deeply characterize the underlying pathophysiological mechanisms.

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CONFLICT OF INTEREST STATEMENT None declared.

The results presented in this paper have not been published previously in whole or part, except in abstract format.

AUTHORS' CONTRIBUTION

MR was in charge of all bioinformatic and statistical analyses of the biological data under the supervision of DAT. MR drafted the manuscript that was further reviewed by EF, DAT and SH. miRNA extraction and libraries preparation for sequencing were conducted by CPe. EF, PJS and CPr participated to clinical and biological data collection and measurements. Sequencing of the miRNA data was performed by BMO. The study was designed by DAT and SH. All authors approved the submitted version.

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Table 1 Characteristics of the study population

	Discovery Sample		Replication Sample	
	Cases N = 23	Controls N = 23	Cases N = 50	Controls N = 50
Age (years)	64.56 (8.35)	64.74 (7.29)	65.50 (8.11)	64.70 (7.65)
Sex (Male/Female)	17/6	17/6	37/13	37/13
eGFR (ml/min/1.73m^2)	50.74 (19.11)	82.35 (13.24)	64.64 (22.89)	80.04 (12.93)
uACR (mgl/mmol)	132.0 [26.8 - 189.0]	0.67 [0.47 - 1.13]	12.8 [3.84 - 40.98]	0.99 [0.64 - 1.55]
Normo/micro/macroalbuminuria	0/0/23	23/0/0	0/27/23	50/0/0
Diabetic Retinopathy (absent/simple/severe non proliferative/proliferative)	0/17/2/4	2/15/4/2	0/31/16/3	5/32/8/5
Systolic Blood Pressure (mmHg)	164.2 (22.83)	140.6 (18.69)	143.6 (18.98)	136.2 (18.03)
Diastolic Blood Pressure (mmHg)	83.9 (11.73)	78.8 (8.83)	76.1 (8.29)	75.2 (10.74)
BMI (kg.m^{-2})	30.88 (5.69)	29.75 (5.24)	30.67 (5.34)	30.53 (6.06)
HbA1c (%)	7.77 (1.20)	7.70 (1.00)	8.11 (1.74)	7.78 (1.18)
Known Diabetes duration (years)	22.56 (5.83)	22.26 (5.16)	18.52 (9.06)	19.52 (10.04)
Current Smoker (%)	0%	13%	17%	6%
Plasma Osmolarity (mOsm)	308.5 (8.29)	301.5 (5.07)	303.8 (9.12)	300.5 (7.52)

eGFR: estimated Glomerular Filtration Rate determined according to the CKD EPI formula.
uACR: Urinary albumin:creatinine ratio
Data shown represent mean (standard deviation) except for smoking (% of smokers); sex, albuminuria and diabetic retinopathy (counts); uACR (median [1st quartile - 3rd quartile]).

Table 2 Association of hsa-miR-152-3p with DN

	Discovery			Replication		
	Cases (n = 23)	Controls (n = 23)	P value[a]	Cases (n = 50)	Controls (n = 50)	P value[a]
hsa-miR-152-3p	0.072 (0.026)	0.052 (0.018)	p = 0.002	0.061 (0.017)	0.055 (0.018)	p = 0.053

Shown values correspond to mean (standard deviation) of miRNA levels measured by qRT-PCTR and normalized to let7 controls miRNAs using the $2^{-\Delta CT}$ method. [a]Adjusted for age and sex.

Figure 1

Association of top 4 miRNAs with DN status.

Boxplot representation of top miRNAs plasma levels in T2D patients with (cases) and without (controls) DN. Association testing was performed on normalized plasma levels adjusted for age, sex, and counts of the 3 controls (let7d, let7g and let7i) miRNAs.