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MicroRNAs 223-3p and 93-5p in patients with chronic kidney disease before and after renal transplantation

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ABSTRACT

Chronic kidney disease (CKD) is associated with a multifactorial dysregulation of bone and vascular calcification and closely linked to increased cardiovascular mortality and concomitant bone disease. We aimed to investigate specific microRNA (miRNA) signatures in CKD patients to find indicators for vascular calcification and/or bone mineralization changes during CKD and after kidney transplantation (KT).

A miRNA array was used to investigate serum miRNA profiles in CKD patients, then selected miRNAs were quantified in a validation cohort comprising 73 patients in CKD stages 3 to 5, 67 CKD patients after KT, and 36 healthy controls. A spectrum of biochemical parameters including markers for kidney function, inflammation, glucose, and mineral metabolism was determined.

The relative expression of miR-223-3p and miR-93-5p was down-regulated in patients with CKD stage 4 and 5 compared to healthy controls. This down-regulation disappeared after kidney transplantation even when lower glomerular filtration rates (eGFR) persisted. MiR-223-3p and miR-93-5p were associated with interleukin-6 (IL-6) and eGFR levels, and by trend with interleukin-8 (IL-8), C-peptide, hematocrit, and parathyroid hormone (PTH).

This study contributes new knowledge of serum miRNA expression profiles in CKD, potentially reflecting pathophysiological changes of bone and calcification pathways associated with inflammation, vascular calcification, mineral and glucose metabolism. Identified miRNA signatures can contribute to future risk markers or future therapeutic targets in bone and kidney disease.

1. Introduction

Chronic kidney disease (CKD), in particular end-stage renal disease (ESRD), is frequently associated with disorders of mineral and bone metabolism (CKD-MBD) [1,2], and an increased cardiovascular risk [3] with progressive vascular calcification leading to a high cardiovascular mortality [4,5]. Therefore, the identification of biomarkers to predict the risk of vascular calcification and bone diseases is of utmost relevance for CKD patients.

CKD-MBD is a systemic disorder of mineral and bone metabolism due to CKD, manifesting as either one or a combination of abnormalities in the metabolism of calcium, phosphorus, parathyroid hormone (PTH) and vitamin D, as well as abnormalities in bone-turnover, bone mineralization, bone volume, and extra-skeletal calcification relevant to the cardiovascular system [6]. Vascular calcification is highly connected to vascular smooth muscle cells (VSMCs) of the media or intimal vessel layer [7]. Media calcification has been found in the majority of ESRD patients at the time of kidney transplantation (KT) accompanied by an expression of osteogenic factors, which might contribute to a phenotypic switch of VSMCs to osteoblast-like cells, resulting in vessel calcification [8,9]. Also, disturbances of mineral metabolism including hyperphosphatemia, metabolic acidosis, and inflammatory processes are common during CKD, involving parameters such as parathyroid hormone (PTH) [10], C-reactive protein (CRP) [11], interleukin 6 (IL-6) [12], and interleukin 8 (IL-8) [13].

MiRNAs have been proposed to be involved in different aspects of CKD development and deterioration of the disease, but also in systemic effects such as CKD-MBD and vascular calcification [14]. An association of the decrease of miR-125b, -145 and -155 with vascular calcification

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has been shown in ex-vivo and in-vitro experiments [15]. The total amount of miRNAs is generally decreased during CKD, but the renal excretion system is not involved in the excretion of circulating miRNAs [16]. Reduced miRNA levels might potentially occur due to secondary effects of the decreased kidney function, such as accumulation of RNases [16]. On the other hand, miRNA levels are very stable in serum and were shown to be protected from endogenous RNase activity, most likely as a result of a dense packaging into exosomes, an association to protein complexes and other molecules, or potential molecular modifications [17].

Distinctive patterns of circulating miRNA levels have been described as biomarkers for disease, such as coronary artery disease (CAD) and diabetes mellitus (DM) [18]. In type 2 diabetes mellitus (T2DM), a strong confounding element during CKD, miR-126 and other miRNAs were suggested as novel biomarkers for DM risk estimation [19]. The importance of miRNAs in CKD-MBD has been indicated by their role as important regulators of function and differentiation of osteoblasts and osteoclasts during bone development and homeostasis [20] and even first data of miRNAs as biomarkers in bone disease have been published [21]. MiR-550a-5p and miR-382-3p have been suggested as promising circulating biomarkers for diabetic bone disease in postmenopausal osteoporotic women [22]. Another study in patients with recent osteoporotic fractures has indicated different serum levels for several miRNAs compared to healthy controls [23].

The aim of this study was to use human CKD serum samples to identify a pattern of systemic deregulated miRNAs which have a putative involvement in mineral metabolism and calcification and which are changed during CKD progression and potentially after kidney transplantation. These miRNAs are thought to be involved in key pathways of disease-associated complications such as CKD-MBD and may serve as biomarkers and targets for subsequent diagnostic and putative therapeutic studies in CKD patients.

2. Materials and methods

2.1. Patient cohorts

Seventy-one patients with CKD stages 3 to 5 (without renal replacement therapy (RRT)), sixty-six CKD patients after KT (mean time since transplantation: 7 years, range 1–28), and 36 healthy controls were included in this cross-sectional study. CKD patients were separated into subgroups according to their estimated glomerular filtration rate (eGFR) (calculated via CKD-EPI creatinine equation [24]). A total of five groups was compared, including CKD stages 3–5 without RRT (eGFR: 30–44, 15–29 and below 15 [ml/min/1.73 m^3]) and patients after KT (eGFR: >15 [ml/min/1.73 m^3]). Blood samples were drawn in the morning after a 12-h overnight fast and serum as well as plasma samples were centrifuged according to a standardized protocol, aliquoted, and stored at −80 °C until analysis.

2.2. Ethical approval

This study was approved by the Research Ethics Committee of the Medical University of Graz, Austria, (23-056 ex 10/11) and is registered at ClinicalTrials.gov (NCT01362569). Written informed consent was obtained from all patients and controls and the study was performed according to the principles laid out in the Declaration of Helsinki.

2.3. MiRNA techniques

MiRNAs were isolated from serum using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). 1200 μl Qiazol lysis reagent were added to 200 μl of serum and incubated at room temperature for 5 min. 2 fmol of non-human mi-RNA (ath-miR-159a) were spiked in as an internal control. After mixing, 200 μl of chloroform were added, followed by 15 s of vortexing. After incubation for 3 min at room temperature, the samples were centrifuged for 15 min at 12000*g* and 4 °C. The upper phase (approximately 800 μl) was transferred to a new tube and 1200 μl 100% ethanol and 20 μl glycerol (Ambion, ThermoFischer, Waltham, USA) were added. The subsequent isolation was done using miRNeasy Kit column tubes and solutions following the manufacturer's instructions. RNA was eluted from the columns in 40 μl RNase-free water and stored at −80 °C.

The digital multiplexed nanoString nCounter human miRNA expression assay (nanoString Technologies, Seattle, USA) was performed with 10–30 ng total RNA isolated from a net volume of 250 μl serum as input material in a separate isolation also using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Small RNA samples were prepared by ligating a specific DNA tag (miR-tag) onto the end of each mature miRNA. Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. Data was processed according to the instructions by nanoString using the nSolver software. In the pilot step, changes in miRNAs in 10 CKD stage 5 patients compared to healthy controls were assessed. Based on the human miRNA expression assay results, covering over 800 human miRNAs, 12 miRNAs were selected for further analysis based on expression differences seen in the array and their putative involvement in pathways relevant to CKD, vascular calcification, bone mineralization, and VSMC biology according to relevant literature and bioinformatics tools (mirPath, TargetScan, miRDB).

The 12 selected miRNAs were analyzed using quantitative real-time PCR (qPCR, polymerase chain reaction) in a discovery group of random samples from all 5 available groups (CKD 3–5, KT, controls). Based on reproducible results for 4 miRNAs (miR-223-3p, miR-93-5p, miR-142-3p, and miR146a-5p), a subsequent qPCR analysis of these miRNAs was performed in all samples. The qPCR was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA). 3 μl isolated RNA was used for reverse transcription to synthesize complementary DNA using TaqMan miRNA-specific primers and the TaqMan reverse transcription kit (Applied Biosystems). After reverse transcription, an amplification step using the TaqMan PreAmp Master Mix was conducted. Real-time PCR amplifications were performed using TaqMan miRNA assays (TaqMan MGP probes, FAM dye-labeled) on the LightCycler® 480 (Roche, Basel, Switzerland). For all qPCRs, a maximum of 40 cycles was performed and the cycle number at which the amplification plot crossed the threshold was calculated (CT) [25]. Relative expression levels were calculated using miRNA levels after normalization to non-human spiked-in miRNA ath-miR-159a. Expression differences were calculated using the mean value of the controls as the factor for normalization. Calculations were done according to the ΔΔct-method [25].

2.4. Biomarkers

Biomarkers were measured in serum and/or plasma according to the different manufacturers' instructions. Levels of IL-6, TNFα, IL-8, and IL-10 were measured using FlowCytomix™ Multiplex Technology (eBioscience, San Diego, CA, USA). IL-6R (human IL-6R Platinum ELISA, eBioscience, San Diego, CA, USA) and MPO (Myeloperoxidase ELISA K6631B, Immundiagnostik, Bensheim, Germany) where measured with standard ELISA technology. bALP, CRP, iron, ferritin, gamma-glutamyl transferase (GGT), glucose, calcium, magnesium, sodium, phosphate, and potassium were measured on an automated system via the Cobas® 8000/6000 modular analyzer series, PTH, OC, and CTX on the Cobas® 4000 analyzer series (Roche Diagnostics, Rotkreuz, Switzerland). C-peptide was determined by the ADVIA Centaur XP Immunoassay System (Siemens Healthcare, Erlangen, Germany). Hematocrit was measured by ABL800 FLEX blood gas analyzer (Radiometer GmbH, Willich, Germany). 25(OH)vitamin D (25(OH)D) levels were assessed via an automated chemiluminescence immunoassay on the iSYS automated system (Immunodiagnostic Systems, Boldon NE35 9PD, UK).