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ORIGINAL ARTICLE

Impact of miR-302b on Calcium-phosphorus Metabolism and Vascular Calcification of Rats with Chronic Renal Failure by Regulating BMP-2/Runx2/Osterix Signaling Pathway

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Objective. To investigate how miR-302b affect the calcium-phosphorus metabolism and vascular calcification (VC) of rats with chronic renal failure (CRF) via the regulation of bone morphogentic proteins 2/Runt-related transcription factor 3/Osterix (BMP-2/Runx2/Osterix) signaling pathway.

Methods. SD rats were selected to establish CRF rat models and assigned into Sham, CRF, CRF + miR-302b, and CRF + miR-NC groups. The biochemical indexes of rats were detected at 8th and 12th week. Besides, HE staining and Von Kossa staining were performed to monitor renal structural changes and VC respectively; and quantitative real-time PCR (qRT-PCR) and Western blotting to evaluate the expressions of miR-302b and BMP-2/Runx2/Osterix signaling pathway separately.

Results. HE and Von Kossa staining showed evident vascular calcification in rats from CRF and CRF + miR-NC groups with a large number of black granules deposited in renal artery compared with Sham group, but was improved in rats in the CRF + miR-302b group compared to those in the CRF group. Besides, rats in the CRF group had elevated levels of Scr, BUN, P, Cys C, and PTH, as well as the mRNA and protein expression of BMP-2, Runx2, and Osterix, and reduced serum Ca and miR-302b levels in a time-dependent manner (all $p < 0.05$), which was in a completely opposite tendency in the CRF + miR-302b group (all $p < 0.05$).

Conclusion. miR-302b may improve calcium-phosphorus metabolism, and inhibit VC to alleviate the condition of CRF rats possibly associated with the BMP-2/Runx2/Osterix pathway, opening a new idea for CRF therapy.

Key Words: miR-302b, BMP-2, Runx2, Osterix, Chronic renal failure, Calcium-phosphorus metabolism, Vascular calcification.

Introduction

Chronic renal failure (CRF), as the final stage of the chronic kidney disease (CKD), poses a serious threat to human health with its increasing morbidity and mortality year by year (1). Cardiovascular disease (CVD) has been regarded as the main cause for the death of CKD patients, which resulted in over 50% of all CKD deaths (2). To the best of our knowledge, vascular calcification (VC) is one of the most serious complications in CRF patients and becomes more common with the worsening of cardiac and renal function, ultimately leading to an increased risk of cardiovascular mortality (3). As documented, dysregulations of mineral metabolism, such as calcium (Ca) and phosphate (P), and the deposition of Ca and P, are possibly implicated in the VC development, which could occur in the blood vessels, myocardium, and so on (4). Of note, the presence and severity of VC has been reported as a valuable indicator of cardiovascular dysfunction in patients with end-stage renal disease (5). Therefore, to clarify the pathogenesis of VC in CRF is of great significance for reducing the incidence and mortality rate of cardiovascular events.

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It is gradually clear that the regulation of VC is actively mediated by the vascular smooth muscle cells (VSMCs), which is a process similar to the mineralization of osteoblasts (6). While bone morphogentic proteins 2 (BMP-2), and its downstream regulator runt-related transcription factor 3 (Runx2), as well as the osteoblast-specific transcription factor (Osterix), play important roles in the bone formation, to accelerate VC process by promoting the differentiation of osteoblasts (7,8). In recent studies, a set of miRNAs have been reported to provide a molecular signature for the cardiovascular calcification (9), which could effectively regulate VSMC phenotypes (10), calcium-phosphorous balance (11) and extracellular vesicles (12). The miR-302/367 family, including miR-302a/b/c/d and miR-367 (13), exerts essential functions in the maintenance of self-renewal or pluri-potency, and specifically affects many biological processes, like cell cycle, epithelial-mesenchymal transition (EMT) and vesicular transport (14,15). There was evidence described by Lipchina et al. that miR-302 could promote the activation of BMP signaling pathway in undifferentiated human embryonic stem cells (16). Besides, Kang et al. revealed that BMP can reduce the expression of miR-302 in many body systems, and in turn miR-302 can also suppress BMP signaling via the targeted regulation of BMPRII transcription (17), implying a complex mutual regulation relationship between BMP and miR-302 in various cell types and systems. Nevertheless, there was really no clear evidence that whether miR-302b can regulate BMP signaling pathway in CRF patients.

Hence, this study established the CRF rat models to investigate whether miR-302b can affect the calcium-phosphorus metabolism and VC in CRF through the regulation of BMP-2//Osterix signaling pathway.

Materials and Methods

Ethics Statement

The design of animal experiments was approved by the Ethics Committee for Laboratory Animals in Dongying People's Hospital and all procedures were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the National Institutes of Health (NIH) (18).

Animal Models Establishment and Grouping

As for the experimental animals, 40 male clean-grade Sprague-Dawley rats with the weigh of 180−220 g were bought from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and then maintained the normal circadian rhythm in an animal room at a temperature of 22−25°C with available food or water. Rats were randomly assigned into four groups (n = 10): Sham group, CRF group (rats with chronic renal failure), CRF+miR-302b group (CRF rats received miR-302b lentivirus plasmid), and CRF+miR-NC group (CRF rats received lentivirus empty plasmid). The establishment of CRF rat models was conducted by referring to a previous study (19). To be specific, the rats were anesthetized via intraperitoneal injection of 2% pentobarbital sodium (0.2 mL/100 g). Next, the left kidney was exposed from the retroperitoneum via a small flank incision under the sterile condition. After the renal capsule was separated, about 2/3 branches of the left renal artery was ligated (with the posterior and anterior branches being ligated separately) and sutured, and the right kidney was removed a week later. In the Sham group, rats only went through the same incision, kidney exposure, and wound suturing. Rats in the CRF+ miR-302b and CRF+ miR-NC groups were given intravenous injection of 10 μL miR-302b lentiviral plasmid and empty lentiviral plasmid respectively. Lentivirus was bought from Shanghai GenePharma Co., Ltd. At the 8th week and 12th week, blood samples were obtained from the tail vein and determined for levels of serum creatinine (Scr), blood urea nitrogen (BUN), serum calcium (Ca) and phosphorus (P) with the Unicel Dxc800 automatic biochemical analyzer (Beckman). Besides, enzyme-linked immunosorbent assay (ELISA) Kit (R&D Company) was used for the determination of serum cystatin C (Cys-C) and parathyroid hormone (PTH) levels. Finally, rats were killed and the tissue samples were preserved for subsequent experimental detection.

Hematoxylin and Eosin (HE) Staining

After routine fixation with formalin, kidney specimens were embedded in paraffin, sliced into sections. Then sections were dewaxed conventionally with xylene for two times, rinsed with running water and stained with hematoxylin for 1 min. After washing with tap water for 10 min, sections were then re-stained with eosin for 2 min, washed once again with running water for 2 min, and mounting with neutral resin. Later, four visual fields were randomly selected in each section for observation and photograph under the optical microscope (Olympus). This experiment was repeated for 3 times.

Von Kossa Staining

Renal arterial tissues were taken for formalin fixation, alcohol dehydration, xylene hyalinization, wax dipping, and paraffin embedment before being made into paraffin sections of 4 um in thickness. Next, sections were soaked in 2% silver nitrate for 20 min under ultraviolet light, washed with distilled water for 5 min, treated with 5% sodium thiosulfate solution for 1 min, washed with tap water for 5 min, stained with eosin for 2 min, and rinsed with distilled water for 10 s, and finally mounted with neutral resin. At last, four visual fields were randomly selected from each section for observation and photographing under