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Expression profiles of urine exosomal tRNA-derived small RNAs and their potential roles in calcium oxalate stone disease

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Background and objective: Exosomes have been confirmed to be implicated in the pathogenesis of calcium oxalate (CaOx) stones. tRNA-derived small RNAs (tsRNAs) are among the oldest small RNAs involved in exosome-mediated intercellular communication, yet their role in kidney stones remains unexplored. This pilot study aimed to identify differentially expressed tsRNAs (DEtsRNAs) in urine exosomes between CaOx stone patients and healthy controls and explore their potential roles in nephrolithiasis.
Method: First-morning urine samples were collected from three CaOx stone patients and three healthy controls. Urinary exosomes were isolated and analyzed by high-throughput sequencing to generate the expression profiles of tsRNAs and detect DEtsRNAs. Predicted target genes of DEtsRNAs were subjected to functional enrichment analysis. The authors also combined the public dataset GSE73680 to investigate how DEtsRNAs were related to stone formation.
Results: Four DEtsRNAs were significantly upregulated in CaOx stone patients compared to healthy controls. tRF-Lys-TTT-5005c was the most elevated, followed by tRF-Lys-CTT-5006c, tRF-Ala-AGC-5017b, and tRF-Gly-CCC-5004b. Bioinformatics analysis indicated that these four types of DEtsRNAs might serve distinct biological functions. Combined with data mining from the public dataset GSE73680, the authors assumed that exosomes carrying tRF-Lys-TTT-5005c and tRF-Lys-CTT-5006c could inhibit the expression of SMAD6, FBN1, and FZD1, thereby activating the BMP signaling pathway, which might induce an osteogenic-like transformation in target cells, resulting in the formation of Randall's plaques and CaOx stones.
Conclusion: The authors' findings shed light on the potential roles of tsRNAs in the pathogenesis of CaOx stone disease, highlighting exosomal DEtsRNAs as promising diagnostic biomarkers and therapeutic targets in nephrolithiasis.

Keywords: calcium oxalate stone disease, exosome, randall's plaques, tRNA-derived small RNAs

Introduction

Kidney stones are a prevalent urological disease characterized by high incidence and recurrence rates[1]. The recurrence rate can reach 50% within 5 years and up to 80–90% within 10 years, leading to severe kidney damage and significant health and economic burdens[2,3]. Despite advances in minimally invasive surgical techniques for stone removal, effective medications to prevent recurrence remain lacking due to the unclear mechanisms of stone formation. Thiazide diuretics are commonly used in the treatment of hypercalciuria to reduce urinary calcium excretion and thereby decrease the risk of calcium stone formation, but there are still controversies regarding the effect of thiazide diuretics[4]. Therefore, exploring the etiology and pathogenesis of kidney stones is crucial for their prevention and treatment.

Calcium oxalate (CaOx) stones are the most common type of kidney stones, with Randall's plaques (RPs) considered as the starting point for their formation[5]. RPs are ectopic calcifications in the renal papilla interstitium, primarily composed of hydroxyapatite, and are similar to bone matrix. They progress within the interstitium, eventually breaching the urothelium and serving as attachment sites for CaOx crystals[6]. Studies suggest that RP formation resembles a pathological process of osteogenesis, where renal tubular epithelial cells transform into an osteogenic-like phenotype under lithogenic conditions (hypercalciuria and hyperoxaluria), secreting calcium phosphate-rich matrix vesicles that mineralize to form hydroxyapatite[7,8]. However, the exact mechanism of their formation is not yet fully understood.

HIGHLIGHTS

- The study provides insights into the potential roles of tsRNAs in CaOx stone pathogenesis, highlighting their regulatory functions and impact on BMP signaling.
- Urine exosomal tsRNAs could be promising biomarkers for early diagnosis and targets for therapeutic intervention in CaOx stone disease.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this articles.

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The microstructure of RPs exhibits a substantial presence of extracellular vesicles, which serve as vital mediators in intercellular communication and information exchange[7]. Exosomes, a major type of extracellular vesicles, play a vital role in intercellular communication. They carry proteins, lipids, and nucleic acids, which can regulate biological functions when taken up by target cells[9]. Studies have indicated that exosomes might be closely related to the formation of CaOx stones[10]. Non-coding small RNAs carried within exosomes, including microRNAs (miRNAs), tRNA-derived small RNAs (tsRNAs), small interfering RNAs (siRNAs), have been implicated in various diseases[11]. While exosomal miRNAs have been studied extensively in stone formation[12,13], the roles of other non-coding small RNAs remain underexplored.

tsRNAs are a class of non-coding small RNAs generated through precise processing of tRNA and its precursors[14]. Some studies have found that even in single-celled organisms lacking typical small RNAs such as miRNAs, and siRNAs, a significant abundance of tsRNAs can still be detected. These tsRNAs can accumulate in the extracellular vesicles of single-celled organisms and participate in intercellular communication[15]. This intriguing finding suggests that tsRNAs are among the oldest small RNAs involved in intercellular communication, and as a result, the role of tsRNAs in cell communication mediated by extracellular vesicles is gaining increasing attention.

Urine exosomes are potential biomarkers for kidney diseases due to the non-invasive nature of urine collection[16]. This study is the first to explore tsRNAs in urine exosomes from CaOx stone patients. We conducted sequencing of tsRNAs from urine exosomes of CaOx stone patients and healthy controls, identified differentially expressed tsRNAs (DEtsRNAs), and predicted their potential functions in CaOx stone disease.

Material and methods

Patients recruitment and urine collection

This study was designed as a pilot to obtain preliminary data on the expression profiles of urine exosomal tsRNAs. The choice of three CaOx stone patients and three healthy controls was based on the need to gather initial insights into the variability of urine exosomal tsRNAs, which would guide the design of future studies with larger cohorts. Patients eligible for inclusion in this study were those who had been diagnosed with kidney stones by computed tomography and were experiencing their first episode. These patients presented at our department for percutaneous nephrolithotomy. We excluded patients with hematuria, urinary tract infections, or other organic diseases. Participants meeting these criteria were then requested to provide a 200 ml first-morning urine sample in a sterile urine bag collected prior to their surgical procedure. To ensure the study's focus on CaOx stones, the kidney stones removed during surgery were subjected to chemical analysis to determine their composition. Only patients whose stones were confirmed as CaOx had their urine samples included in the final study cohort. As for the healthy control group, age- and sex-matched individuals coming to our hospital for regular physical examinations were recruited, and they too provided a 200 ml first-morning urine sample. All collected samples were promptly stored at – 80°C and processed within a 24-h timeframe. The study conformed to the provisions of the Declaration of Helsinki. Ethical approval was obtained from the Ethical Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (2019S1147). Informed consent was obtained from all subjects involved in this study.

Exosome isolation and purification

Urine exosomes were isolated and purified using ultracentrifugation (Hitachi, CP100MX). First, the urine samples were thawed at 37°C. Next, the samples were subjected to centrifugation at 2000*g*, 4°C, for 30 min. The resulting supernatant was then undergone a second centrifugation at 10 000*g*, 4°C, for 45 min, aimed at removing larger vesicles. Subsequently, the supernatant was filtered through a 0.45 μm membrane to collect the filtrate. This filtrate was subjected to centrifugation at 100 000*g*, 4°C, for 70 min. After removing the supernatant, the exosomes were resuspended in 10 ml phosphate-buffered saline (PBS). To ensure thorough purification, another round of centrifugation at 100 000*g*, 4°C, for an additional 70 min was carried out. The final supernatant was carefully discarded, and the exosomes were resuspended in 100 μl PBS. For further analysis, 10 μl of this suspension was allocated for transmission electron microscopy (TEM), 10 μl for nanoparticle tracking analysis (NTA), and the remaining exosomes were diligently stored at – 80°C for future investigations.

Transmission electron microscopy

10 μl of the exosomes sample was extracted, and was subsequently added dropwise onto a copper grid, allowing it to settle for 1 min. Any excess liquid was carefully removed using filter paper. 10 μl of uranyl acetate was then added dropwise onto the copper grid and left to settle for an additional 1 min. Once again, excess liquid was removed using filter paper. The prepared sample on the grid was left to air-dry at room temperature for several minutes. Finally, electron microscopy imaging (Hitachi, HT-7700) was conducted at 100 kV to observe the morphology of the extracellular vesicles.

Nanoparticle tracking analysis

10 μl of the exosome sample was taken out and diluted to 30 μl. A performance test is conducted on the instrument (NanoFCM, N30E) using a standard sample to ensure its proper functioning before the exosome samples are analyzed. Information about the particle size and concentration of the exosomes can be obtained through the analysis of the samples.

RNA extraction, library preparation and sequencing

Total exosomal RNA was extracted using an exoRNeasy Maxi Kit (Qiagen). The purity and concentration of each RNA sample were assessed through agarose gel electrophoresis and a NanoDrop ND-1000 instrument (Thermo Fisher Scientific). Before library preparation, the total RNA samples were subjected to several essential treatments to remove RNA modifications that interfere with the library construction. These included 3'-aminoacyl (charged) deacylation to 3'-OH for 3'-adapter ligation, 3'-cP (2',3'-cyclic phosphate) removal to 3'-OH for 3'-adapter ligation, 5'-OH (hydroxyl group) phosphorylation to 5'-P for 5'-adapter ligation, and demethylation of m1A and m3C to facilitate efficient reverse transcription. Next, the pretreated total RNA underwent library preparation through the following

sequential steps: (1) ligate 3' adapter; (2) ligate 5' adapter; (3) synthesize cDNA; (4) perform PCR amplification; (5) purify cDNA construct; (6) recover purified cDNA construct of 134–160 base pair. Agilent BioAnalyzer2100 (Invitrogen) was used to qualify and quantify the libraries. The diluted libraries were carefully loaded onto the reagent cartridge and subsequently processed for sequencing on the Illumina HiSeq X Ten platform. Library construction and Illumina sequencing were expertly conducted by OE BioTech Co., Ltd. in Qingdao.

Sequencing data processing

The data obtained from Illumina sequencing was referred to as raw reads. Firstly, it was necessary to remove the adapter sequences from the raw reads, resulting in a collection of small RNA sequences of varying lengths. Subsequently, these sequences underwent quality control, which involves filtering out reads containing N bases and sequences with a low Q20 ratio. Additionally, data falling outside the length range of 15–41 nucleotide were discarded and not included in further analysis. The resulting sequences were referred to as clean reads. The bowtie software and two tsRNAs databases, GtRNAdb (http://gtrnadb.ucsc.edu/index.html) and tRFdb (http://genome.bioch.virginia.edu/trfdb/search.php), were used to align these reads for tRNA classification and annotation. The abundances of tsRNAs were calculated as transcripts per million.

Bioinformatics analysis

The DEtsRNAs between CaOx stone patients and healthy controls were screened by the "DESeq. 2" R package with the threshold of P value less than 0.05 and |log2 fold change (FC)| > 1[17]. The volcano plot representing the DEtsRNAs was created using the "ggplot2" R package. The heatmap displaying the DEtsRNAs was generated using the "pheatmap" R package. The target genes of the DEtsRNAs were predicted by TargetScan and miRanda algorithms[18,19]. A Venn diagram was constructed to take the intersection of both algorithms using the "VennDiagram" R package. Gene Ontology (GO) enrichment analysis was performed to explore potential biological functions of target genes in terms of their involvement in biological processes, cellular components, and molecular functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was applied to elucidate potential signaling pathways of target genes using the "clusterProfiler" R package[20].

In addition, we also utilize the public dataset GSE73680 derived from gene expression omnibus database (http://www.ncbi.nlm.nih.gov/geo/) to explore genes related to CaOx stone disease. The dataset comprises 29 RPs tissues from CaOx stone formers and 6 normal renal papillary tissues from non-stone formers[21]. Differentially expressed genes between two groups were identified using the "limma" R package.

Results

Isolation and identification of urine exosomes

Six participants including three CaOx stone patients and three healthy controls were involved in this study. Detailed information of participants was listed in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/MS9/A602. Exosomes were isolated from morning urine through ultracentrifugation. TEM confirmed that the separated samples were composed of extracellular vesicles with typical exosomal membrane morphology (Supplementary Fig 1A, Supplemental Digital Content 2, http://links.lww.com/MS9/A603). NTA indicated that the majority of vesicles had diameters ranging from 60 to 80 nm, with a peak distribution at 75 nm (Supplementary Fig 1B, Supplemental Digital Content 2, http://links.lww.com/MS9/A603).

Identification of DEtsRNAs

tsRNAs can be primarily categorized into two types based on their positional distribution on tRNA: tRNA halves (tiRNAs) and tRNA-derived RNA fragments (tRFs)[22]. tiRNAs are composed of 5'- and 3'-tRNA halves, ~30–35 nucleotide in length, generated by specific cleavage of the mature tRNA under various stress conditions. tRFs, on the other hand, are ~15–30 nucleotide in length and can originate from mature tRNA or tRNA precursors. They can be further subdivided into three types: tRF-5, which correspond to the 5' end of mature tRNA, with cleavage occurring in the D-loop; tRF-3, which correspond to the 3' end of mature tRNA, including the CCA portion, with cleavage occurring in the T-loop; tRF-1 which originate from the 3' tail sequence of tRNA precursors and have a poly-U sequence at the 3' end[23].

We conducted high-throughput sequencing of tsRNAs in the urine exosomes of CaOx stone patients and non-stone healthy controls. The results revealed the identification of a total of 4 types of tiRNAs and 58 types of tRFs. No differentially expressed tiRNAs were observed between the two groups. Four types of tRFs exhibited differential expression between the two groups, including tRF-Lys-TTT-5005c (9.94 fold, $P = 0.0002354$), tRF-Lys-CTT-5006c (7.25 fold, $P = 0.0029189$), tRF-Ala-AGC-5017b (7.11 fold, $P = 0.0031226$), tRF-Gly-CCC-5004b (5.74 fold, $P = 0.0387251$), which were presented in a volcano plot (Fig. 1A). All of them were upregulated in the urine exosomes of CaOx stone patients, suggesting they may play a pathogenic role in stone formation. These four upregulated DEtsRNAs were visualized in a heatmap (Fig. 1B). The detailed information about DEtsRNAs were listed in Table 1.

Prediction of target genes and functional enrichment analysis

tsRNAs have been reported to participate in RNA interference in a manner similar to miRNAs. They can specifically target and bind to the 3'UTR region of mRNA to inhibit its expression[14]. TargetScan and miRanda algorithms were applied to predict the target genes of the DEtsRNAs.

tRF-Lys-TTT-5005c had 1557 target genes predicted by the Targetscan algorithm and 2746 target genes predicted by the miRanda algorithm, and 846 target genes were obtained by taking the intersection of two algorithms (Fig. 2A). GO enrichment analysis showed that potential target genes were related to positive regulation of establishment of protein localization, cardiac septum morphogenesis, and positive regulation of cell development (Fig. 2B). Notably, these target genes were also involved in bone morphogenetic protein (BMP)-related terms, such as cellular response to BMP stimulus and regulation of BMP signaling pathway. KEGG enrichment analysis revealed that target genes were associated with protein digestion and absorption, type II diabetes mellitus, and glycosphingolipid biosynthesis (Fig. 2C).

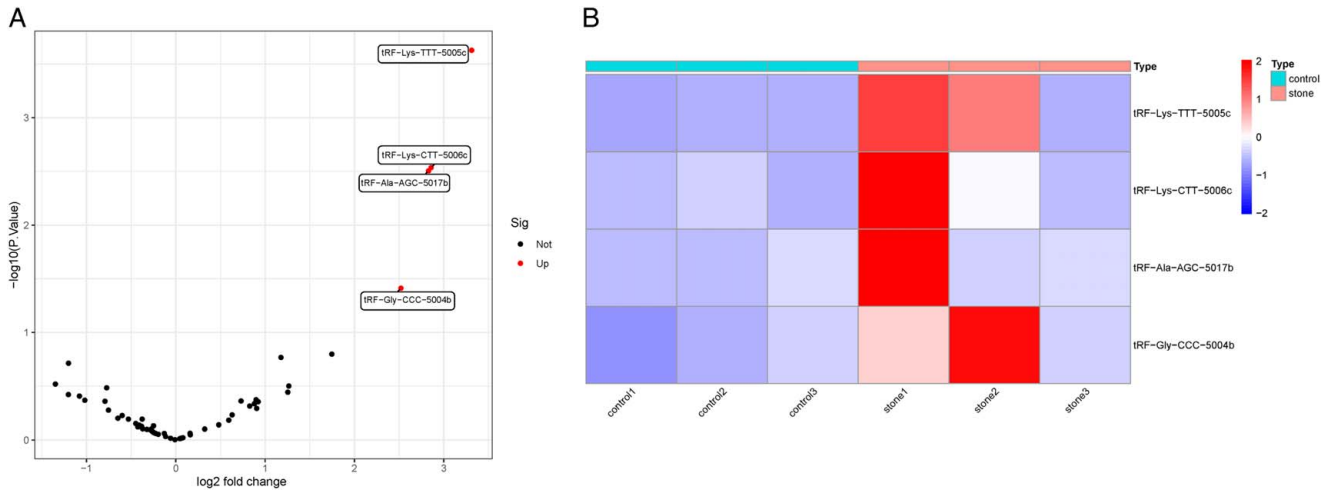

Figure 1. Identification of DEtsRNAs. (A) Volcano plot of DEtsRNAs. (B) Heatmap of DEtsRNAs. DEtsRNAs, differentially expressed tsRNAs.

tRF-Lys-CTT-5006c had 1557 target genes predicted by the Targetscan algorithm and 3789 target genes predicted by the miRanda algorithm, and 1121 target genes were obtained by taking the intersection of two algorithms (Fig. 2D). GO enrichment analysis showed that potential target genes were mainly involved in positive regulation of cell development, Golgi organization, and pattern specification process. Besides, nervous system development-related terms were also associated with target genes (Fig. 2E). KEGG enrichment analysis revealed that target genes were related to protein digestion and absorption, extracellular matrix (ECM)-receptor interaction, and type II diabetes mellitus (Fig. 2F).

tRF-Ala-AGC-5017b had 1576 target genes predicted by the Targetscan algorithm and 341 target genes predicted by the miRanda algorithm, and 111 target genes were obtained by taking the intersection of two algorithms (Fig. 2G). GO enrichment analysis showed that potential target genes were responsible for protein tetramerization, regulation of receptor recycling, and kidney development (Fig. 2H). KEGG enrichment analysis revealed that target genes were associated with human T-cell leukemia virus 1 infection, HIV-1 viral life cycle, maturity onset diabetes of the young (Fig. 2I).

tRF-Gly-CCC-5004b had 1331 target genes predicted by the Targetscan algorithm and 23 target genes predicted by the miRanda algorithm, and 7 target genes were obtained by taking the intersection of two algorithms (Fig. 2J). GO enrichment analysis showed that potential target genes were associated with immunity-related terms, such as MHC class II protein complex assembly, antigen processing and presentation of peptide antigen, immunoglobulin mediated immune response (Fig. 2K). KEGG enrichment analysis revealed that target genes were involved in asthma, allograft rejection, and graft-versus-host disease (Fig. 2L).

Potential role of DEtsRNAs in CaOx stone disease based on data mining

Since four DEtsRNAs were upregulated in CaOx stone disease, their target genes might be downregulated due to tsRNAs-mediated gene silencing. We further data mined the public dataset GSE73680 to identify CaOx stone disease-related genes. Differentially expressed genes between 29 RPs tissues and 6 normal renal papillary tissues were screened, and a total of 299 genes were downregulated in CaOx stone diseases. Twenty genes were obtained by taking the intersection of targets genes of DEtsRNAs and downregulated genes in stone disease (Fig. 3A). GO enrichment analysis found that these genes were mainly involved in BMP-related terms, including BMP signaling pathway, response to BMP, cellular response to BMP stimulus, regulation of BMP signaling pathway, negative regulation of BMP signaling pathway (Fig. 3B). KEGG enrichment analysis revealed that these genes were associated with ECM-receptor interaction, human papillomavirus infection, focal adhesion, protein digestion and absorption, PI3K-Akt signaling pathway (Fig. 3C). Notably, these genes were also enriched in the TGF-β signaling pathway, and the BMP signaling pathway was an important component of the TGF-β signaling pathway. We further construct a DEtsRNAs-target genes network using Cytoscape according based on STRING database (Fig. 3D).

Table 1

Four significantly differentially expressed tRNA-derived small RNAs in CaOx stone patients

Differentially expressed tsRNAs	Sequence	Length	Fold change	*P*	Regulation
tRF-Lys-TTT-5005c	GCCCGGCTAGCTCAGTCGGTAGAGCATGAGA	32	9.94	0.0002354	Up
tRF-Lys-CTT-5006c	GCCCGGCTAGCTCAGTCGGTAGAGCATGGGAC	33	7.25	0.0029189	Up
tRF-Ala-AGC-5017b	GGGGGTGTAGCTCAGTGGTAGAGC	25	7.11	0.0031226	Up
tRF-Gly-CCC-5004b	GCATTGGTGGTTCAGTGGTAGA	23	5.74	0.0387251	Up

CaOx, calcium oxalate.

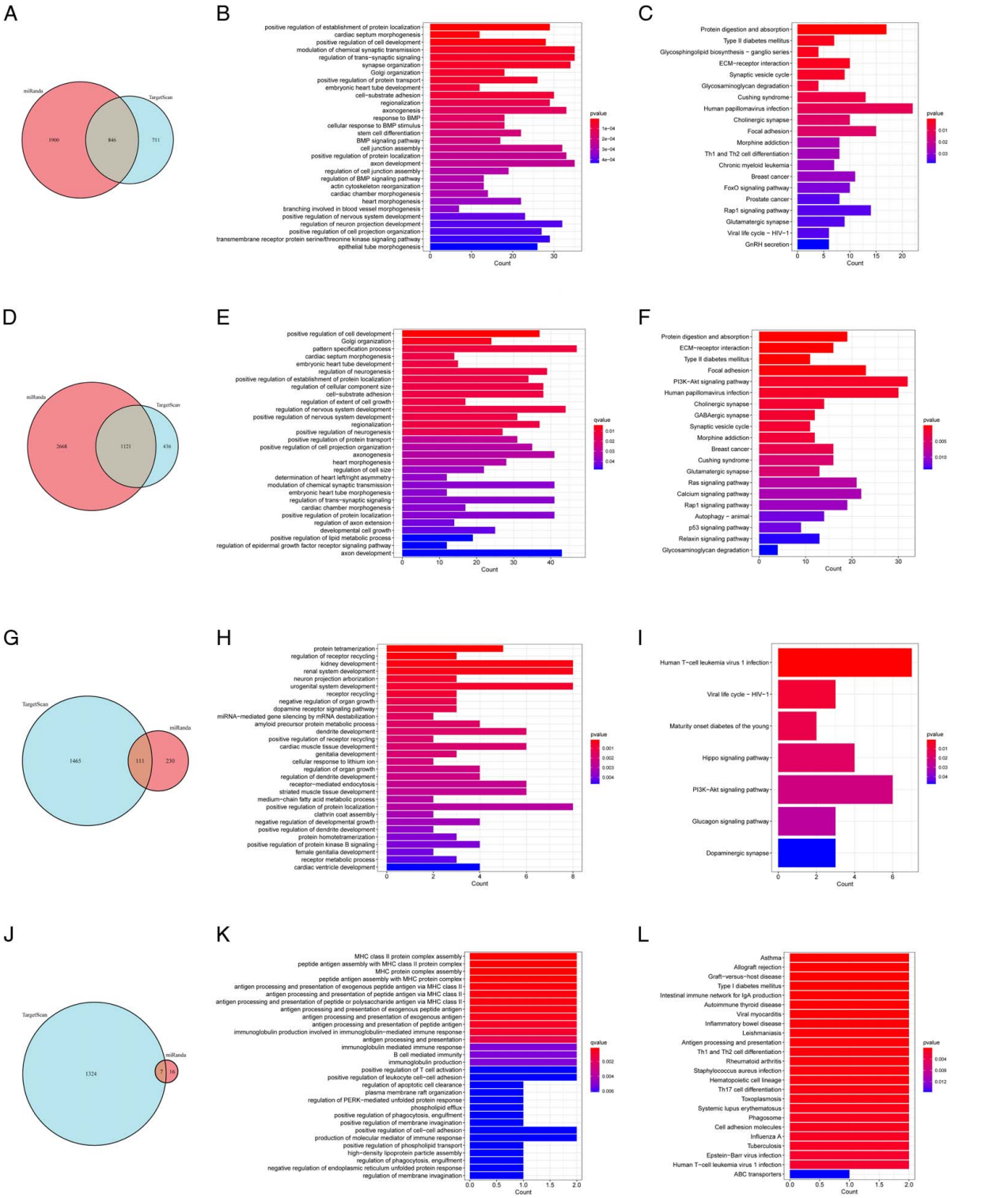

Figure 2. Prediction of target genes and functional enrichment analysis. (A) Target genes of tRF-Lys-TTT-5005c predicted by Targetscan and miRanda algorithms. (B) Gene Ontology (GO) enrichment analysis of the tRF-Lys-TTT-5005c-targeted genes. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the tRF-Lys-TTT-5005c-targeted genes. (D) Target genes of tRF-Lys-CTT-5006c predicted by Targetscan and miRanda algorithms. (E) GO enrichment analysis of the tRF-Lys-CTT-5006c-targeted genes. (F) KEGG enrichment analysis of the tRF-Lys-CTT-5006c-targeted genes. (G) Target genes of tRF-Ala-AGC-5017b predicted by Targetscan and miRanda algorithms. (H) GO enrichment analysis of the tRF-Ala-AGC-5017b-targeted genes. (I) KEGG enrichment analysis of the tRF-Ala-AGC-5017b-targeted genes. (J) Target genes of tRF-Gly-CCC-5004b predicted by Targetscan and miRanda algorithms. (K) GO enrichment analysis of the tRF-Gly-CCC-5004b-targeted genes. (L) KEGG enrichment analysis of the tRF-Gly-CCC-5004b-targeted genes.

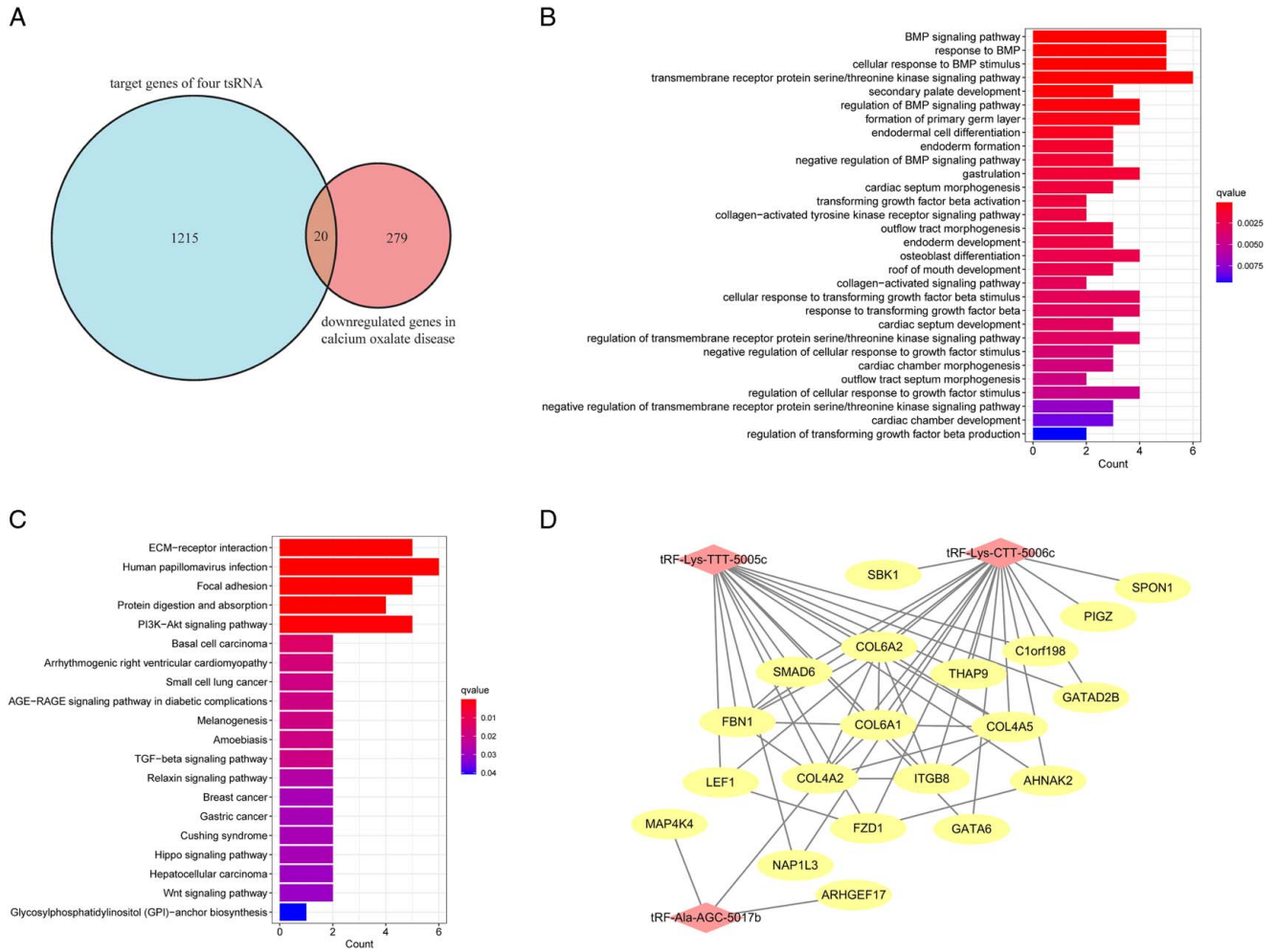

Figure 3. Potential role of differentially expressed tsRNAs (DEtsRNAs) in calcium oxalate stone disease based on data mining. (A) Venn plot showing the intersection of targets genes of DEtsRNAs and downregulated genes in stone disease, and 20 genes were obtained. (B) Gene Ontology enrichment analysis of the obtained 20 genes. (C) Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the obtained 20 genes. (D) Construction of a DEtsRNAs-target genes network.

Discussion

CaOx stones are the most common type of kidney stones, forming on RPs on the renal papillary surfaces[5]. The formation of RPs is believed to be a process of ectopic calcification similar to osteogenesis. Khan and colleagues proposed that in a high calcium or high oxalate urinary environment, the expression of osteogenesis-related molecules in renal tubular epithelial cells is upregulated. This prompts a phenotypic transformation of these cells into an osteogenic-like state, secreting calcium phosphate-rich matrix vesicles that mineralize to form hydroxyapatite in the renal interstitium, ultimately resulting in the formation of RPs[7,8]. Jia and colleagues found that rats with hypercalciuria, compared to normal rats, had a significant increase in the expression levels of osteogenesis-related molecules such as Runx2, Osterix, OPN, and BMP2 in the kidney. Similarly, renal tubular epithelial cells exposed to high calcium stimulation underwent similar osteogenic-like changes[24,25]. Zhu and colleagues have extensively studied the osteogenic-like transformation in renal fibroblasts in CaOx stone formation. They discovered that lncRNA NEAT1 can induce EGR1 binding to the BMP2 promoter region to promote its expression, thereby mediating the osteogenic-like transformation of renal fibroblasts[26]. Similarly, lncRNA MALAT1 can act as an endogenous competing RNA to bind with miR-320a-5p, leading to the upregulation of Runx2 expression and thus promoting the osteogenic-like transformation of renal fibroblasts and the formation of RPs[27]. Furthermore, they discovered that the α-Klotho protein secreted by renal tubular epithelial cells can inhibit the osteogenic-like transformation of renal fibroblasts and thus suppress stone formation, confirming the existence of intercellular communication between these two cell types[28]. Despite the increasing focus on this research direction, the role of osteogenic-like transformation in RPs and CaOx stone formation is not yet fully elucidated.

The presence of numerous extracellular vesicles in renal pelvis urobiome has highlighted the role of exosomes in stone formation[7]. Yang and colleagues discovered that exosomes secreted by renal tubular epithelial cells under CaOx crystal stimulation could promote oxidative stress in the same cell types, further activating the MAPK signaling pathway. This upregulation leads to the expression of osteogenesis-related molecules

such as BMP2, OPN, and OCN, mediating an osteogenic-like transformation that ultimately promotes stone formation[29]. Yan *et al*. found that macrophage-derived exosomes exposed to CaOx crystals accelerated stone formation by promoting autophagy and apoptosis in renal tubular epithelial cells[30]. Besides, research on urine exosomes as biomarkers for kidney stones has also been conducted. Wang and colleagues identified urine exosomal S100 proteins (S100A8, S100A9, and S100A12) as potential biomarkers for kidney stones[31]. Urine exosomal miR-223-3p also showed potential as a biomarker[13]. Notably, studies have shown that tsRNAs can accumulate in the extracellular vesicles of unicellular organisms[15], suggesting that tsRNAs are among the most ancient small RNAs involved in intercellular communication. Thus, increasing research is focusing on exosomal tsRNAs in human diseases.

tsRNAs primarily regulate various cellular functions through two main mechanisms: they can act similarly to miRNAs by specifically inhibiting target gene mRNAs or directly bind to RNA-binding proteins, thereby influencing changes in relevant molecules and signaling pathways[14]. For example, tRF-Gln-CTG in vascular smooth muscle cells downregulated the expression of FAS mRNA by targeting its 3'UTR region, inhibited the apoptosis pathway, promoted cell proliferation, and further led to intimal hyperplasia[32]. In thyroid cancer, tiRNA-Gly could directly bind to the RBM17 protein, regulate alternative splicing of MAPK pathway-related genes, and promote tumor proliferation and invasion[33]. Although research on exosomal tsRNAs is still evolving, it has already been confirmed to serve as a biomarker for predicting disease prognosis[34]. Moreover, through intercellular communication, they can influence the biological effects of target cells and participate in the development and progression of diseases. Dou *et al*.[35] found that tsRNA-21109 from mesenchymal stem cell-derived exosomes, once taken up by macrophages, can inhibit their polarization toward the M1 pro-inflammatory phenotype, thus slowing down the progression of systemic lupus erythematosus. However, research on exosomal tsRNAs has not been reported in the field of kidney stones.

In this study, we isolated exosomes from the urine of CaOx stone patients and healthy controls and conducted tsRNAs sequencing for the first time. We found four exosomal tsRNAs significantly increased in stone patients, with tRF-Lys-TTT-5005c being the most significantly upregulated, followed by tRF-Lys-CTT-5006c, tRF-Ala-AGC-5017b, and tRF-Gly-CCC-5004b. Through target gene prediction and functional enrichment analysis, we observed that tRF-Lys-TTT-5005c and tRF-Lys-CTT-5006c share similar functions, such as positive regulation of cell development, positive regulation of protein transport, and cell-substrate adhesion. The function of tRF-Ala-AGC-5017b is primarily related to renal system development, while tRF-Gly-CCC-5004b is mainly associated with immune responses. To further investigate the roles of DEtsRNAs in CaOx stone formation, we intersected the predicted target genes with downregulated genes screened from the public dataset GSE73680 and performed another round of functional enrichment analysis. Interestingly, we found that DEtsRNAs are primarily associated with the BMP signaling pathway.

Numerous studies have indicated that the BMP signaling pathway played a crucial role in regulating cell osteogenic differentiation[36]. The involvement of the BMP signaling pathway was also confirmed in regulating the osteogenic-like transformation of renal tubular epithelial cells[24,25]. BMPs bind to BMP receptors (BMPRs) on the cell membrane, leading to signal transduction into the cytoplasm, where it acts through various SMAD proteins. The SMAD protein family, as the primary signal effectors, is functionally categorized into three classes: receptor-activated SMAD (R-SMAD, SMAD1/5/9), common SMAD (Co-SMAD, SMAD4), and inhibitory SMAD (I-SMAD, SMAD6/7). Upon phosphorylation and activation by BMPRs, R-SMADs form complexes with Co-SMAD and translocate into the nucleus, where they, along with other transcription factors and co-activators, collectively influence the sequences of osteogenic specific transcription factors like Runx2 and Osterix, ultimately upregulating the expression of osteogenesis-related genes to promote osteogenic differentiation[37]. On the other hand, I-SMADs negatively regulate the BMP signaling pathway through various mechanisms: they can inhibit R-SMADs phosphorylation, competitively bind to R-SMADs to prevent their formation of complexes with Co-SMAD and entry into the nucleus, and even recruit ubiquitin ligases to facilitate the degradation of R-SMADs and Co-SMAD, thereby suppressing the BMP signaling pathway[38].

Notably, there were three target genes enriched in negative regulation of BMP signaling pathway, including SMAD6, FBN1, and FZD1, which were targeted by tRF-Lys-TTT-5005c and tRF-Lys-CTT-5006c. SMAD6 is a member of the I-SMAD family, which has the ability to specifically inhibit the BMP pathway. Wang *et al*.[39] showed that miR-186, through its targeting effect on SMAD6, activated the BMP pathway to promote osteogenic differentiation and accelerate the healing of femoral fractures. Li *et al*.[40] found that overexpression of SMAD6 could inhibit the BMP pathway, suppress osteogenic differentiation, and thus prevent valve calcification. FBN1 is a major component of extracellular microfibrils, which plays an important role in the BMP signaling pathway through its interactions with ECM components and modulators of BMP signaling. For example, FBN1 could interact with BMP ligands, such as BMP2 and BMP4, and sequester them within the ECM. This interaction can affect the local concentration and availability of BMP ligands to cells and negatively modulate the BMP signaling pathway[41]. Besides, FBN1 could interacts with proteins like BMP-binding endothelial regulator (BMPER). BMPER is a BMP antagonist with ability to inhibit BMP2- and BMP4-dependent osteogenic differentiation[42]. FZD1, as a member of the Frizzled family of cell surface receptors, is primarily associated with the Wnt signaling pathway[43]. There may be crosstalk between BMP and Wnt signaling pathways, and Wnt signaling can antagonize BMP signaling in specific contexts[44]. Based on this, we speculate that after exosomes are taken up by target cells, the carried tRF-Lys-TTT-5005c and tRF-Lys-CTT-5006c inhibit the expression of SMAD6, FBN1, and FZD1 specifically, thereby activating the BMP signaling pathway and promoting their osteogenic-like transformation. The osteogenic-like phenotype secretes matrix vesicles rich in calcium phosphate, which mineralize in the renal interstitium to form hydroxyapatite crystals, ultimately leading to the formation of Randall's plaques and CaOx stones.

There are some limitations in our study. First, the number of study subjects is too small, and we need to expand the sample size to validate the sequencing results. Next, we have to admit that our study is merely descriptive. Experimental studies should be performed to investigate the biological functions and specific mechanisms of exosomal DEtsRNAs in RPs and CaOx stone formation.

Conclusion

Our study revealed a landscape of urine exosomal tsRNAs expression profiles, identified four upregulated DEtsRNAs in CaOx stone diseases, including tRF-Lys-TTT-5005c, tRF-Lys-CTT-5006c, tRF-Ala-AGC-5017b, and tRF-Gly-CCC-5004b, and predicted their target genes and biological functions. tRF-Lys-TTT-5005c and tRF-Lys-CTT-5006c might activate the BMP signaling pathway to induce cellular osteogenic-like transformation, further contributing to RPs and CaOx stone formation. The findings provide a novel insight into the pathogenesis of CaOx stone disease and hold great promise for offering potential diagnostic biomarkers and therapeutic targets.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (2019S1147).

Consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

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Author contribution

All authors participated in the conception and design of the study. S.Y.H. and S.G.W. conceived and drafted the manuscript. L.T.M. collected samples and clinical information from the patients. Y.Y.Y. participated in the data process, analysis and interpretation. Y.Y.Y. and S.G.W. supervised the project and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare no conflicts of interest.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Shao-Gang Wang.

Data availability statement

Sequencing data from this study have been deposited in the GenBank Sequence Read Archive under Accession number PRJNA1010196 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1010196/).

Provenance and peer review

My paper was not invited.

References

[1] Zeng G, Mai Z, Xia S, *et al*. Prevalence of kidney stones in China: an ultrasonography based cross-sectional study. BJU Int 2017;120:109–16.
[2] Skolarikos A, Straub M, Knoll T, *et al*. Metabolic evaluation and recurrence prevention for urinary stone patients: EAU guidelines. Eur Urol 2015;67:750–63.
[3] Gambaro G, Croppi E, Bushinsky D, *et al*. The risk of chronic kidney disease associated with urolithiasis and its urological treatments: a review. J Urol 2017;198:268–73.
[4] Albelal D, Gilani A, Yousefichaijan P, *et al*. The role of thiazide diuretics in treating idiopathic hypercalciuria complications in children. J Pediatr Nephrol 2024;11:114–5.
[5] Howles SA, Thakker RV. Genetics of kidney stone disease. Nat Rev Urol 2020;17:407–21.
[6] Daudon M, Bazin D, Letavernier E. Randall's plaque as the origin of calcium oxalate kidney stones. Urolithiasis 2015;43(Suppl 1):5–11.
[7] Khan SR, Rodriguez DE, Gower LB, *et al*. Association of Randall plaque with collagen fibers and membrane vesicles. J Urol 2012;187:1094–100.
[8] Khan SR, Canales BK, Dominguez-Gutierrez PR. Randall's plaque and calcium oxalate stone formation: role for immunity and inflammation. Nat Rev Nephrol 2021;17:417–33.
[9] Kourembanas S. Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy. Annu Rev Physiol 2015;77:13–27.
[10] Thongboonkerd Roles V. for exosome in various kidney diseases and disorders. Front Pharmacol 2019;10:1655.
[11] Wang J, Yue BL, Huang YZ, *et al*. Exosomal RNAs: novel potential biomarkers for diseases—a review. Int J Mol Sci 2022;23:2461.
[12] Shi J, Duan J, Gong H, *et al*. Exosomes from miR-20b-3p-overexpressing stromal cells ameliorate calcium oxalate deposition in rat kidney. J Cell Mol Med 2019;23:7268–78.
[13] Yang Y, Wang Q, Xun Y, *et al*. The preliminary exploration of what role miRNAs derived from urinary exosomes play in kidney stone formation. Urology 2022;166:104–10.
[14] Kim HK, Yeom JH, Kay MA. Transfer RNA-derived small RNAs: another layer of gene regulation and novel targets for disease therapeutics. Mol Ther 2020;28:2340–57.
[15] Lambertz U, Oviedo Ovando ME, Vasconcelos EJ, *et al*. Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Leishmania providing evidence for conserved exosomal RNA Packaging. BMC Genom 2015;16:151.
[16] Street JM, Koritzinsky EH, Glispie DM, *et al*. Urine exosomes: an emerging trove of biomarkers. Adv Clin Chem 2017;78:103–22.
[17] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq. 2. Genome Biol 2014;15:550.
[18] Agarwal V, Bell GW, Nam JW, *et al*. Predicting effective microRNA target sites in mammalian mRNAs. Elife 2015;4:e05005.
[19] John B, Enright AJ, Aravin A, *et al*. Human MicroRNA targets. PLoS Biol 2004;2:e363.
[20] Yu G, Wang LG, Han Y, *et al*. clusterProfiler: an R package for comparing biological themes among gene clusters. Omics 2012;16:284–7.
[21] Taguchi K, Hamamoto S, Okada A, *et al*. Genome-wide gene expression profiling of Randall's plaques in calcium oxalate stone formers. J Am Soc Nephrol 2017;28:333–47.
[22] Zong T, Yang Y, Zhao H, *et al*. tsRNAs: Novel small molecules from cell function and regulatory mechanism to therapeutic targets. Cell Prolif 2021;54:e12977.
[23] Dhahbi JM. 5' tRNA Halves: the next generation of immune signaling molecules. Front Immunol 2015;6:74.
[24] Jia Z, Wang S, Tang J, *et al*. Does crystal deposition in genetic hypercalciuric rat kidney tissue share similarities with bone formation? Urology 2014;83:509.e7–14.

[25] Jia Z, Wang S, He D, *et al*. Role of calcium in the regulation of bone morphogenetic protein 2, runt-related transcription factor 2 and Osterix in primary renal tubular epithelial cells by the vitamin D receptor. Mol Med Rep 2015;12:2082–8.
[26] Zhu Z, Zhang X, Jiang Y, *et al*. NEAT1 functions as a key mediator of BMP2 to promote osteogenic differentiation of renal interstitial fibroblasts. Epigenomics 2021;13:1171–86.
[27] Zhu Z, Huang F, Xia W, *et al*. Osteogenic differentiation of renal interstitial fibroblasts promoted by lncRNA MALAT1 may partially contribute to Randall's plaque formation. Front Cell Dev Biol 2020;8:596363.
[28] Zhu Z, Ruan S, Jiang Y, *et al*. α-Klotho released from HK-2 cells inhibits osteogenic differentiation of renal interstitial fibroblasts by inactivating the Wnt-β-catenin pathway. Cell Mol Life Sci 2021;78:7831–49.
[29] Yang Y, Hong S, Wang Q, *et al*. Exosome-mediated crosstalk between epithelial cells amplifies the cell injury cascade in CaOx stone formation. J Biol Eng 2023;17:16.
[30] Yan L, Chen J, Fang W. Exosomes derived from calcium oxalate-treated macrophages promote apoptosis of HK-2 cells by promoting autophagy. Bioengineered 2022;13:2442–50.
[31] Wang Q, Sun Y, Yang Y, *et al*. Quantitative proteomic analysis of urinary exosomes in kidney stone patients. Transl Androl Urol 2020; 9:1572–84.
[32] Zhu XL, Li T, Cao Y, *et al*. tRNA-derived fragments tRF(GlnCTG) induced by arterial injury promote vascular smooth muscle cell proliferation. Mol Ther Nucleic Acids 2021;23:603–13.
[33] Han L, Lai H, Yang Y, *et al*. A 5'-tRNA halve, tiRNA-Gly promotes cell proliferation and migration via binding to RBM17 and inducing alternative splicing in papillary thyroid cancer. J Exp Clin Cancer Res 2021; 40:222.
[34] Zhu L, Li J, Gong Y, *et al*. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. Mol Cancer 2019;18:74.
[35] Dou R, Zhang X, Xu X, *et al*. Mesenchymal stem cell exosomal tsRNA-21109 alleviate systemic lupus erythematosus by inhibiting macrophage M1 polarization. Mol Immunol 2021;139:106–14.
[36] Rosen V. BMP2 signaling in bone development and repair. Cytokine Growth Factor Rev 2009;20:475–80.
[37] Wu M, Chen G, Li YP. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res 2016;4:16009.
[38] Itoh S, ten Dijke P. Negative regulation of TGF-beta receptor/Smad signal transduction. Curr Opin Cell Biol 2007;19:176–84.
[39] Wang C, Zheng GF, Xu XF. MicroRNA-186 improves fracture healing through activating the bone morphogenetic protein signalling pathway by inhibiting SMAD6 in a mouse model of femoral fracture: an animal study. Bone Joint Res 2019;8:550–62.
[40] Li X, Lim J, Lu J, *et al*. Protective role of Smad6 in inflammation-induced valvular cell calcification. J Cell Biochem 2015;116:2354–64.
[41] Nistala H, Lee-Arteaga S, Smaldone S, *et al*. Fibrillin-1 and -2 differentially modulate endogenous TGF-β and BMP bioavailability during bone formation. J Cell Biol 2010;190:1107–21.
[42] Correns A, Zimmermann LA, Baldock C, *et al*. BMP antagonists in tissue development and disease. Matrix Biol Plus 2021;11:100071.
[43] Flahaut M, Meier R, Coulon A, *et al*. The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway. Oncogene 2009;28:2245–56.
[44] Pinto C, Cárdenas P, Osses N, *et al*. Characterization of Wnt/β-catenin and BMP/Smad signaling pathways in an in vitro model of amyotrophic lateral sclerosis. Front Cell Neurosci 2013;7:239.