Altered small non-coding RNA expression profiles of extracellular vesicles in the prostatic fluid of patients with chronic pelvic pain syndrome

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Abstract. Chronic pelvic pain syndrome (CPPS) and chronic prostatitis (CP) is difficult to distinguish from each other, herein termed CP/CPPS. The present study aimed at gaining further insight into the change in extracellular vesicles (EVs) in the prostatic fluid of males with CPPS. From December 2019 to November 2020, after clinical screening, 24 patients with CPPS without obvious urinary symptoms and 13 healthy male participants were included. EVs were isolated from expressed prostatic secretion (EPS) of all subjects. The small non-coding ribonucleic acid (sncRNA) expression of EVs was sequenced, analyzed, and validated by quantitative real-time polymerase chain reaction (qPCR) assays. The results showed that numerous sncRNAs were differentially expressed between the patients and healthy participants. Further qPCR assays validated that several chronic pain-related miRNAs, including miR-204-5p, let-7d-3p, let-7b-3p, let-7c-3p, miR-146a-5p, and miR-320a-5p, were differentially expressed. Series sncRNAs including several chronic pain-related miRNAs were altered in EVs in prostatic fluid of patients with CPPS, which may serve as diagnostic markers for CPPS.

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Introduction

Chronic pelvic pain syndrome (CPPS) and chronic prostatitis (CP) are difficult to distinguish from each other; herein termed CP/CPPS or category III prostatitis. CP/CPPS accounts for 90-95% of total prostatitis cases, and has become the most common category of prostatitis (1). According to whether white blood cells (WBCs) are present in expressed prostatic secretions (EPS), CP/CPPS is further divided into category IIIA and category IIIB. Patients with category IIIB prostatitis present similar clinical manifestation as category IIIA prostatitis; however, present with no WBCs in the EPS. The symptoms of pelvic pain are not necessarily linked to concurrent prostate involvement (2). Among patients with CPPS, some present indications of prostatitis, such as urinary symptoms, others present with only the symptoms of pelvic pain. CP/CPPS is considered as a poorly understood medical condition (3). Accordingly, the present study was designed to ascertain whether pathological changes in the prostate are involved in patients with CPPS having no obvious indications of prostatitis, by testing EPS.

Extracellular vesicles (EVs; exosomes, microvesicles and apoptotic bodies) are lipid-enclosed structures that provide clues to the pathogenesis of genitourinary disease (4). Recently, studies have reported that EVs in expressed prostatic secretion (EPS) may serve as a critical biosample for exploring the pathological changes of prostatic diseases, such as prostate cancer (5,6) and male infertility (7). Small non-coding RNAs (sncRNAs) in EVs have been reported to play an important regulatory role in the development of various diseases (8). Zhao *et al* (9) reported that exosomes in the prostatic fluid especially overloaded with microRNA-155 may be involved in the pathogenesis of type IIIA CP/CPPS. However, the sncRNA expression profiles of EVs in the EPS of patients with CPPS remain unknown. Thus, the objective of the present study was to identify whether the expression profiles of sncRNAs of EVs in the EPS (EPS-EVs) of patients with CPPS without obviously

indication of prostatitis are altered and to gain further insight into the molecular mechanisms of CPPS.

Patients and methods

Statement of ethics. This study was approved by the Institutional Ethics Committee of Guangzhou First People's Hospital (K-2020-032-01) (Guangzhou, Guangdong, China). All participants signed consent forms prior to participating in accordance with the Declaration of Helsinki.

Patients. From December 2019 to November 2020, patients suffering from symptoms of pelvic pain, including perineal, testicular, penile, pubic or bladder area discomfort for at least 3 of the previous 6 months were eligible for study. To minimize individual difference, subjects aged 18 to 35 years were chosen. Then, the patients and healthy participants were further screened by the inclusion and exclusion criteria listed at Table I. For the patients, a National Institutes of Health-Chronic Prostatitis Symptom Index (NIH-CPSI) urinary score >4 was used to exclude urinary symptoms (10). A total of 315 outpatients diagnosed with CPPS and 25 healthy male participants who underwent healthy examinations or consultation were screened in this study. All the patients and healthy participants were screened as documented in Fig. 1. Finally, 24 patients with CPSS (mean age, 24.96 years) and 13 healthy participants (mean age, 25.92 years) were included in this study. Three patients with CPSS and 3 healthy participants were randomized for high-throughput sequencing of sncRNAs. Twenty-one patients and 10 healthy participants were utilized to validate 6 chronic pain-related miRNAs by quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays after sequencing and bioinformatics analysis.

EPS and post-massage urine collection. EPS samples were individually collected antiseptically by digital prostatic massage from all subjects, after at least 3 days abstinence. Immediately after EPS collection, the urine was detected for bacterial culture and special infection.

Microscopic examination and bacterial culture for EPS and post-massage urine. Microscopic examination of EPS and bacterial culture of EPS and post-massage urine was performed in the Clinical Laboratory of Guangzhou First People's Hospital.

Examination of special infection in urine samples. Evaluation of infections with *Mycoplasma genitalium* (MG), *Ureaplasma urealyticum* (UU) and *Chlamydia trachomatis* (CT) in the urine samples was conducted following the manufacturer's instructions (RNA Simultaneous Amplification and Testing Kit; Rendu, Shanghai, China) in the Clinical Laboratory of Guangzhou First People's Hospital.

Isolation of EVs from EPS. Cells and debris were removed by centrifugation, and the supernatants were used to isolate EVs. EVs were routinely isolated and purified from EPS by differential centrifugation. Briefly, the supernatants were filtered with a 0.22-μm filter Steritop™ (Millipore) to remove the remaining cells and cellular debris, and then ultracentrifuged (Beckman Coulter Optima XE-100 Ultracentrifuge 100Ti; Beckman Coulter) as we previously reported (11). Finally, the EPS-EV pellet was resuspended in 200 μl of PBS, and stored at -80˚C or used for subsequent experiments.

Identification of EPS-EVs. Western blotting was used to detect the positive EV markers CD63, CD81, CD9, Alix and tumor susceptibility gene 101 (TSG101) and the negative EV marker calnexin. Total protein was extracted with radio immunoprecipitation assay lysis buffer (CW2333S; CWBio) supplemented with a protease inhibitor cocktail (CW2200S; CWBio). Protein concentration was determined using bicinchoninic acid assay. Equal amounts of denatured protein (20 μg per lane) were loaded onto 8-10% gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (ISEQ00010; MilliporeSigma). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 1% Tween-20 (v/v) buffer at room temperature for 1 h, and then incubated overnight at 4˚C with mouse anti-CD63 (1:3,000; cat. no. ab59479; Abcam), mouse anti-CD81 (1:3,000; cat. no. ab79559; Abcam), rabbit anti-CD9 (1:1,000; cat. no. ab92726; Abcam), mouse anti-Alix (1:1,000; cat. no. ab117600; Abcam), mouse anti-TSG101 (1:800; cat. no. ab83; Abcam) or rabbit anti-Calnexin (1:500; cat. no. ab133615; Abcam). After washing, the membranes were incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. CW0103S; CWBio) or HRP-conjugated goat anti-mouse secondary antibody (1:5,000; cat. no. CW0102S; CWBio) at room temperature for 1 h. Finally, a ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.) was used to visualize the protein blots. The morphology of the EPS-EVs were assessed with transmission electron microscope (TEM) system (Hitachi, Japan) at x15,000 and x40,000 magnification. The number and size of the EPS-EVs was quantified by using a high sensitivity flow cytometer. Briefly, the size distribution and granular concentration of EPS-EVs was determined by using a flow NanoAnalyzer model type N30 (NanoFCM, China), and data acquisition was subsequently performed with LabVIEW 2012 software (National Instruments Corp.).

RNA extraction from EPS-EVs. TRIzol reagent (TaKaRa, Japan) was used for extraction of total RNA from EPS-EVs. A QIAseq miRNA Library Kit (Qiagen GmbH) was used to establish the small RNA sequencing library.

High-throughput sequencing of sncRNA cargo of EVs. The sequencing of the small RNAs was performed on a NextSeq 500 System (Illumina, Inc.) as previously reported (11). Samples from 3 patients and 3 healthy participants were used for the sequencing.

RT-qPCR assays for 6 screened miRNAs to validate the results of sequencing. EPS-EV samples from 21 patients and 10 healthy participants were validated by RT-qPCR. For RT-qPCR, total RNA was individually extracted from each EPS-EV sample. cDNA was obtained using miRNA First Strand cDNA Synthesis kit (no. B532451; Sangon Biotech Co., Ltd.). All primers are listed in Table SI (purchased from Sangon Biotech Co., Ltd.). The RT-qPCR program,

Table I. Inclusion and exclusion criteria for patients with chronic pelvic pain and healthy participants.

Criteria	Patients with CPPS	Healthy participants
Inclusion criteria	1. Patients complain of pelvic/perineal pain longer than 3 of the previous 6 months in the clinic of Guangzhou First People's Hospital	1. Men underwent health examination in health-check center of Guangzhou First People's Hospital
	2. ≥18 years of age	2. ≥18 years of age
	3. ≤35 years of age	3. ≤35 years of age
		4. Virgin men (never had sex experience)
Exclusion criteria	Symptoms and medical history inquiry:	Symptoms and medical history inquiry:
	1. Previous concurrent urinary tract infection	1. Previous concurrent urinary tract infectionn
	2. Previous urogenital malignancy	2. Previous urogenital malignancy
	3. Urogenital congenital malformation	3. Urogenital congenital malformation
	4. Lithiasis	4. Lithiasis
	5. Neurogenic disease of the bladder	5. Neurogenic disease of the bladder
	6. Diabetes	6. Diabetes
	NIH-CPSI:	NIH-CPSI:
	7. Urinary domain score >4 to exclude prostatitis-related disease	7. Urinary domain score >4 to exclude prostatitis-related disease
	8. Pain domain score ≤7 to exclude mild CPPS	8. Pain domain score >4 to exclude chronic pelvic pain
	Physical examination:	Physical examination:
	9. Urogenital congenital malformation (e.g., hypospadias, urethrostenosis)	9. Urogenital congenital malformation (e.g., hypospadias, urethrostenosis)
	10. Other urogenital disease (e.g., varicocele, hydrocele, epididymitis, orchitis)	10. Other urogenital disease (e.g., varicocele, hydrocele, epididymitis, orchitis)
	Examination of EPS and urine:	Examination of EPS and urine:
	11. Refuse prostate massage	11. Refuse prostate massage
	12. Abnormal routine urine test (e.g., increased WBCs or RBCs)	12. Abnormal routine urine test (e.g., increased WBCs or RBCs)
	13. EPS WBCs/hpf ≥10 in EPS	13. EPS WBCs/hpf ≥10 in EPS
	14. EPS and post-massage urine bacterial culture to exclude urogenital tract infection	14. EPS and post-massage urine bacterial culture to exclude urogenital tract infection
	15. Specific infection of *Mycoplasma genitalium*, *Ureaplasma urealyticum* or *Chlamydia trachomatis*	15. Specific infection of *Mycoplasma genitalium*, *Ureaplasma urealyticum* or *Chlamydia trachomatis*

CPPS, chronic pelvic pain syndrome; NIH-CPSI, The National Institutes of Health Chronic Prostatitis Symptom Index; EPS, expressed prostatic secretion; WBCs, white blood cells; RBCs, red blood cells.

using a real-time PCR machine (LightCycler 480 II, Roche Diagnostics), was then carried out as follows: initial denaturation at 95˚C for 30 sec and 99 cycles of 95˚C for 5 sec and 60˚C for 20 sec; with a final step melting curve of 95˚C for 5 sec; 60˚C for 1 min and 95˚C for 5 sec. For examining the miRNAs in EPS-EVs, *U6* was used as the internal reference gene. The relative folds were calculated utilizing the 2(-ΔCq) method (12).

Statistical analysis. Data including age, NIH-CPSI and the relative expression level of miRNAs are reported as mean ± standard error of mean (SEM) and were analyzed by SPSS 20.0 (IBM, Corp.). The Student's t test was used to compare the difference in age between the two groups. Mann-Whitney U test was used to compare the difference in NIH-CPSI score.

The DEGseq R package was used for differential expression analysis (13). If the false discovery rate (FDR) was <0.05, the gene was considered significantly differentially expressed. The putative targets of the differentially expressed miRNAs were predicted by miRanda (http://www.microrna.org/microrna/home.do) and RNAhybird (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). The clusterProfiler (14) R package was used to analyze the functional annotation of the miRNA targets. Significantly enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified with a Benjamini-Hochberg adjusted P value <0.05.

Protein-Protein interaction (PPI) network of DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING, http://string-db.org; version 11.0b) with an interaction score ≥0.4.

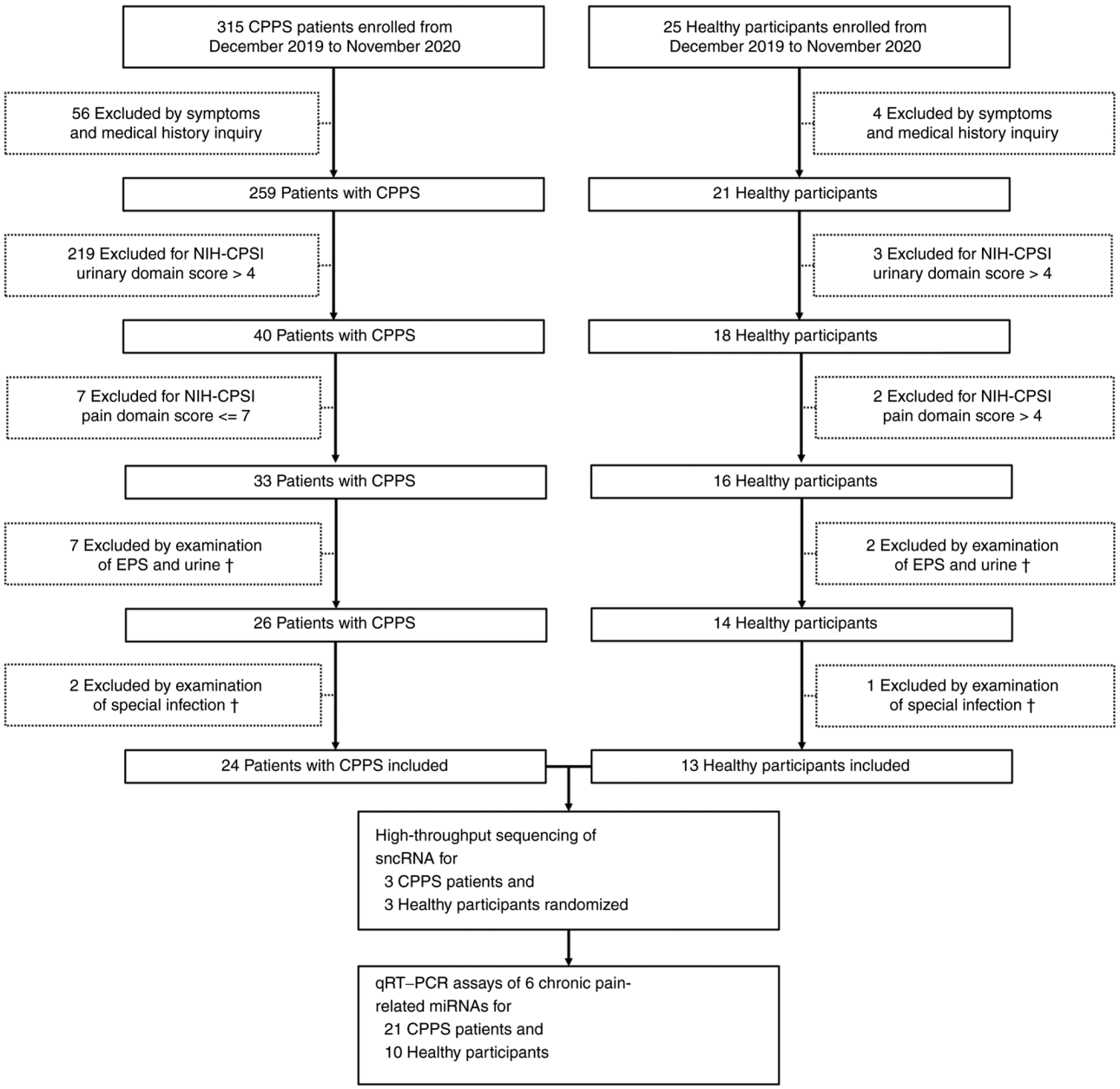

Figure 1. Flow diagram of the participant screening and identification of the candidate RNAs in EPS-EVs. CPPS, chronic pelvic pain syndrome; NIH-CPSI, The National Institutes of Health Chronic Prostatitis Symptom Index; EPS, expressed prostatic secretion; EVs, extracellular vesicles. † According to exclusion in Table I.

Correlation Network diagram was performed using the OmicStudio tools at https://www.omicstudio.cn/tool.

Results

Patients enrolled. After series clinical screening (Fig. 1), 24 patients with CPPS and 13 healthy participants were enrolled in this study. Table II lists the information of the 3 patients and 3 healthy participants who were randomized for sncRNA sequencing. Table III displays the information of subjects for validation by RT-qPCR assays.

Characteristics of EVs in prostatic fluid. Western blot analysis showed the positive expression of the exosome-specific surface markers, including CD63, CD81, CD9, Alix, and TSG101, and the negative expression of the endoplasmic reticulum-specific marker calnexin in the collected EPS-EVs (Fig. 2A). The TEM displayed the collected EPS-EVs were homogeneous spherical vesicles (Fig. 2B). The mean diameter of the EPS-EVs was 74.8±16.2 nm as detected by high-sensitivity flow cytometry (Fig. 2C).

High-throughput sequencing for sncRNA cargo of the EVs. The sequencing confirmed that the EPS-EVs were abundant in regards to miRNAs, PIWI-interacting RNAs (piRNAs), and tRNA-derived small RNAs (tsRNAs). Volcano plot (Fig. 3A) shows the differentially expressed RNA numbers between the CPPS patients and healthy participants. Fig. 3B shows that 63 miRNAs, 35 piRNAs and 898 tsRNAs were differentially expressed. Among these three categories, sncRNA, miRNA

Table II. Information of the CPPS patients and healthy participants for sncRNA sequencing.

Variables	P1	P2	P3	N1	N2	N3
Age (years)	22	25	20	21	22	20
Total NIH-CPSI score	29	19	25	1	0	2
Total pain score	11	9	12	0	0	0
Total urination score	3	2	2	1	0	2
Quality of life score	9	9	12	0	0	0

CPPS, chronic pelvic pain syndrome; P, patient; N, healthy participant; NIH-CPSI, The National Institutes of Health Chronic Prostatitis Symptom Index.

Table III. Information of the CPPS patients and healthy participants for validation by RT-qPCR.

Variables	Patients with CPPS	Healthy participants	P-value
Total participants (n)	21	10	/
Age (years)	25.33±0.957	24.3±0.932	0.673
Total NIH-CPSI score	24.71±0.787	0.4±0.163	0.000
Total pain score	14.38±0.571	0.1±0.1	0.000
Total urination score	2.62±0.176	0.3±0.153	0.000
Quality of life score	7.71±0.325	0.9±0.180	0.000

Data are shown as mean ± SEM (standard error of the means). CPPS, chronic pelvic pain syndrome; NIH-CPSI, National Institutes of Health Chronic Prostatitis Symptom Index.

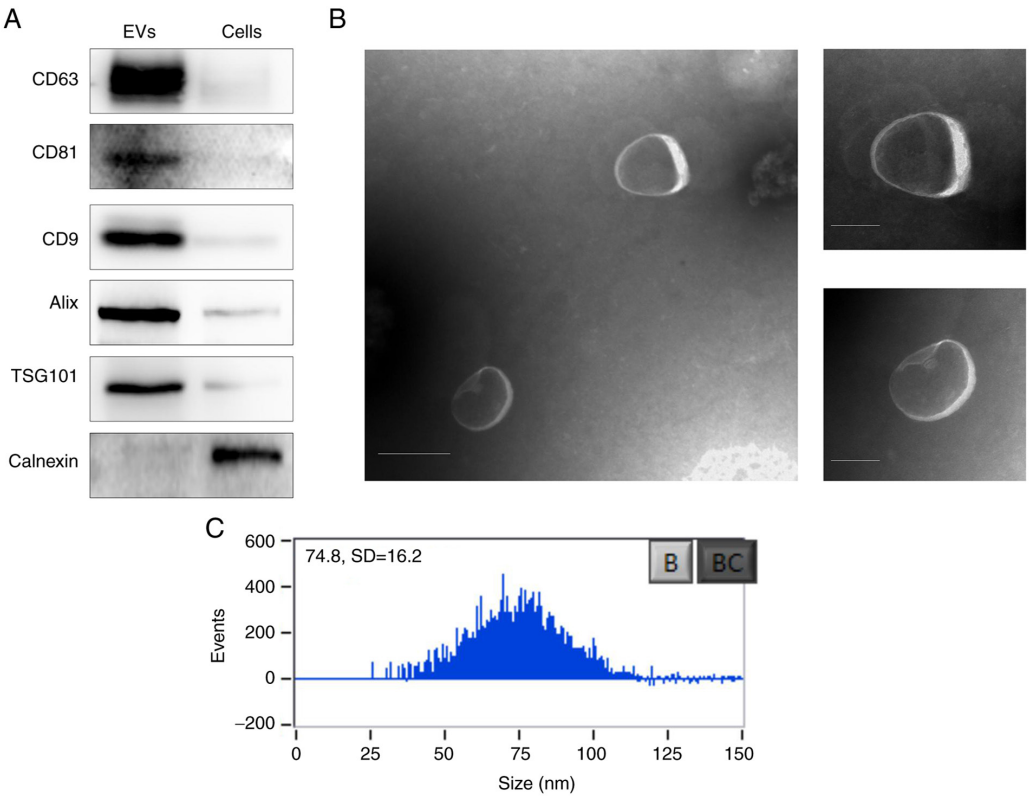

Figure 2. Characterization of isolated EVs in prostatic fluid. (A) Western blot results showing the presence of the positive EV markers CD63, CD81, CD9, Alix and TSG101, as well as the absence or underrepresentation of negative EV marker calnexin in isolated EVs. (B) The size and spheroid morphology of EVs are shown under TEM. Original scale bar, 100 nm. (C) The diameter quantitation of EVs was performed with high sensitivity flow cytometry, EPS-EVs had a mean size of 74.8±16.2 nm. EPS, prostatic secretion; EVs, extracellular vesicles; TSG101, tumor susceptibility gene 101.

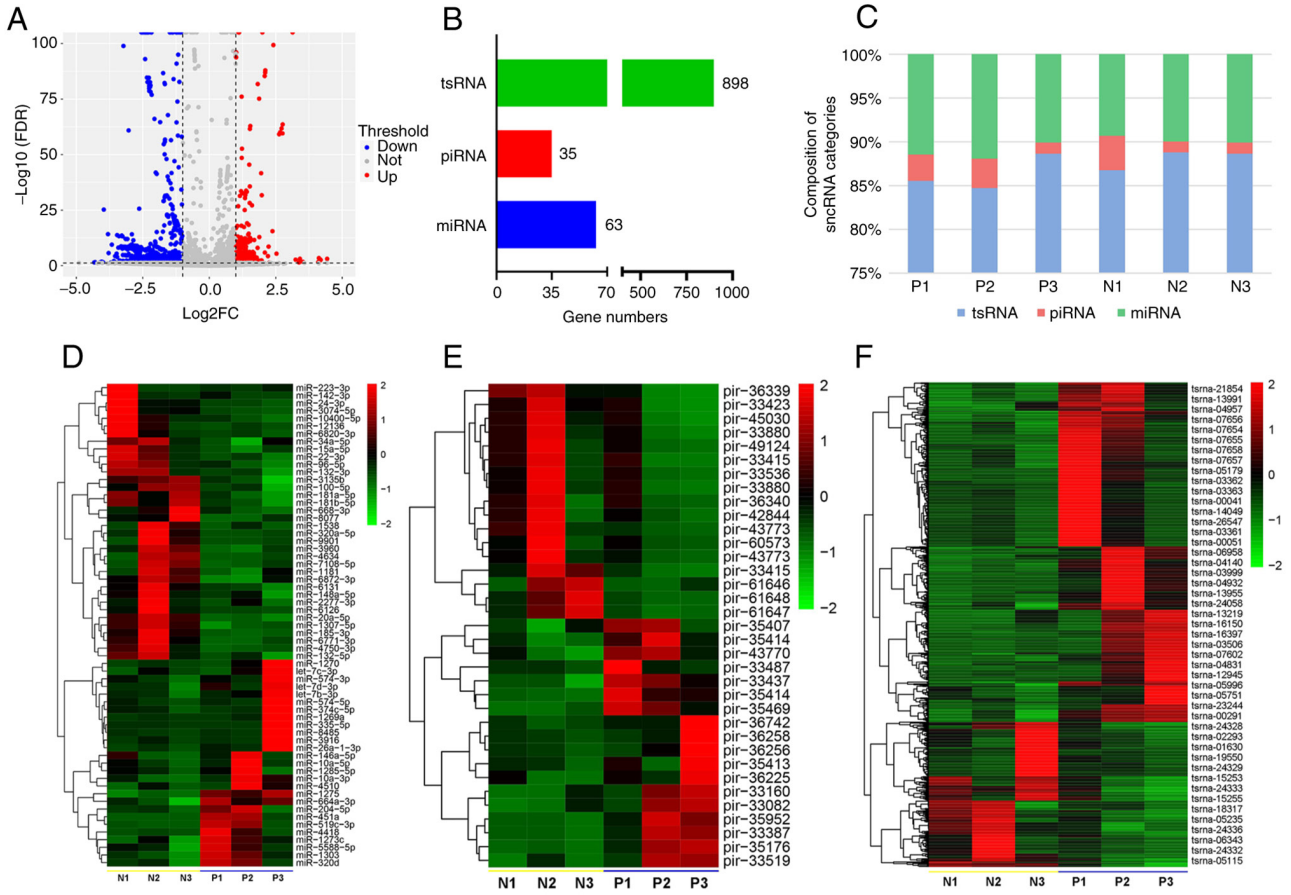

Figure 3. Comparative analysis of sncRNAs in CPPS patients and health participants. (A) Volcano plot illustrating of differentially expressed sncRNA. (B) Differentially expressed gene numbers. (C) Composition of sncRNA categories in EVs. (D) Heat map of 63 differentially expressed miRNAs; (E) Heat map of 35 differentially expressed piRNAs. (F) Heat map of top 47 differentially expressed tsRNA. P, patients; N, healthy participants; Each column represents a certain sample and lines represent different genes. CPPS, chronic pelvic pain syndrome; EVs, extracellular vesicles; sncRNA, small non-coding RNA; piRNA, PIWI-interacting RNAs; tsRNA, tRNA-derived small RNAs.

and tsRNA were more abundant than piRNA (Fig. 3C). Based on the sequencing data, the differentially expressed miRNAs (Fig. 3D, Table SII; n=63), piRNAs (Fig. 3E, Table SIII; n=35), and tsRNAs (Fig. 3F, Table SIV; n=47) were further analyzed and displayed as heat maps.

Analysis of miRNA target genes. GO analysis was performed using miRNA sequencing data. The data was deeply mined from three categories: molecular function, biological process and cellular component, and the corresponding functional categories and cell positioning were clearly defined. The categories included neurotransmitter transport, regulating synaptic plasticity, regulating chemical synaptic transmission, and regulating antisynaptic signals (Fig. 4A). Fig. 4B displays the PPI network of the differentially expressed miRNAs in the process of neurotransmitter transport. Moreover, KEGG analysis of the miRNA target genes revealed that the target genes corresponding to the miRNAs in the EVs may participate in the calcium signaling pathway, Hedgehog signaling pathway, and MAPK signaling pathway (Fig. 4C). Fig. 4D shows the PPI network of the differentially expressed miRNAs in the calcium signaling pathway.

RT-qPCR assays for 6 chronic pain-related miRNAs. As all the patients mainly complained of pelvic pain, we screened the function of the differentially expressed 63 miRNAs at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov), and filtered out 6 chronic pain-related miRNAs (Table SV). Subsequently, RT-qPCR assays were performed for the 6 chronic pain-related miRNAs in EPS-samples of 21 patients with CPPS and 10 healthy participants. Compared to the healthy participants, miR-320a-5p was markedly decreased, and 5 miRNAs (miR-204-5p, let-7d-3p, let-7b-3p, let-7c-3p, miR-146a-5p) were significantly increased in the patients with CPPS (Fig. 5).

Discussion

Extracellular vesicles (EVs) in prostatic fluid can help explore the pathological changes of prostatic diseases and serve as a critical biosample for chronic pelvic pain syndrome (CPPS)/chronic prostatitis (CP) (15). However, studies of prostatic fluid of CP/CPPS have reported only a few changes, such as decreased citrate level (16), higher prostaglandin E2 (17), lower β-endorphin, and elevated monocyte chemoattractant protein-1 (18), were observed in the prostatic fluid. Therefore, further research concerning the pathological changes of CPPS is necessary. In the present study, miRNAs, tsRNAs and piRNAs were differentially expressed in the CPPS patients

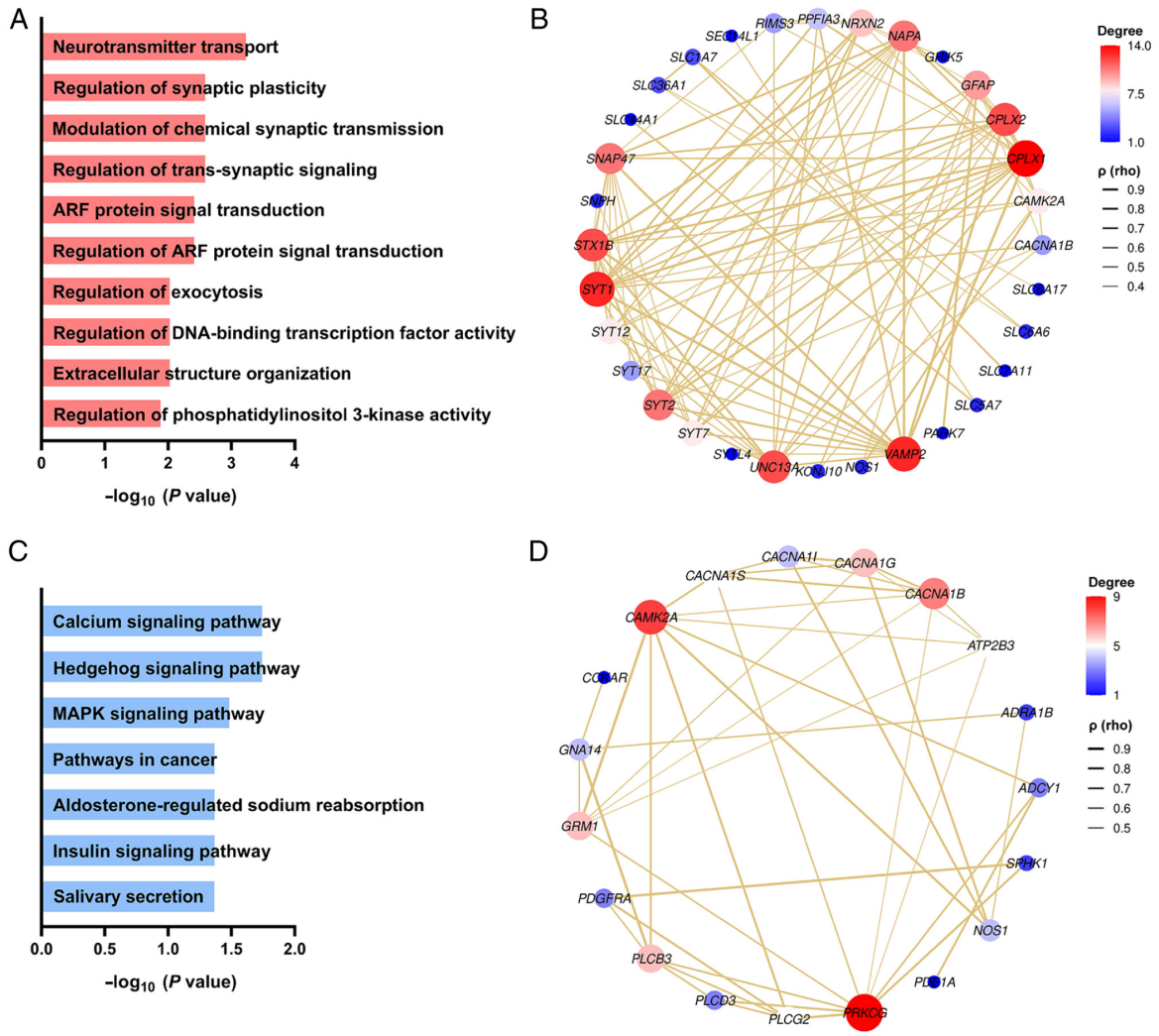

Figure 4. Bioinformatics Analysis. (A) Gene Ontology (GO) enrichment of miRNA target genes. Most genes were enriched for the GO term regulation process as a biological process. (B) Protein-protein interaction network of the differentially expressed miRNAs in the process of the neurotransmitter transport. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of miRNA target genes. Most genes were enriched for the pathways. (D) Protein-protein interaction network of the differentially expressed miRNAs in the calcium signaling pathway.

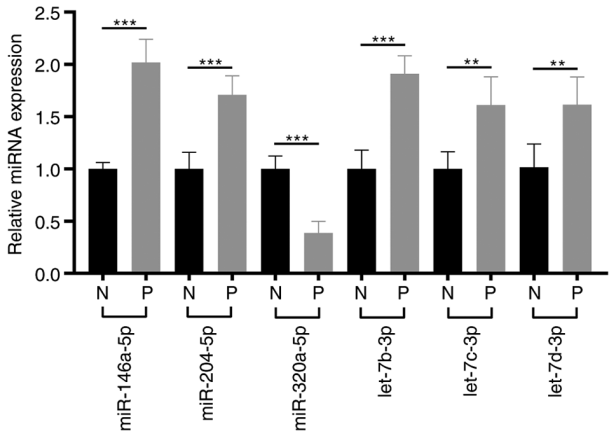

Figure 5. Validation of 6 chronic pain-related miRNAs by RT-qPCR assays in the CPPS patients (P) and the healthy subjects (N). **P<0.01, ***P<0.001. CPPS, chronic pelvic pain syndrome.

and healthy participants. Among these three categories of sncRNAs, miRNAs have been the most extensively studied, and some of them have been reported to be closely related to chronic pain. Therefore, we compared these altered miRNAs with pain-related miRNAs, and filtered out 6 chronic pain-related miRNAs. miR-320a-5p was found to be downregulated, which is consistent with a previous study on bladder pain syndrome (19). Expression of the miR-320 family was found to be downregulated in bladder tissues of patients with bladder pain syndrome (19). The miR-320 family may suppress inflammation by downregulating the expression of the nucleotide-binding oligomerization domain which inhibits the inflammatory response (20). In the present study, 5 pain-related miRNAs were elevated (miR-204-5p, hsa-let-7d-3p, hsa-let-7b-3p, hsa-let-7c-3p, miR-146a-5p). miR-204-5p was also elevated in spinal cord injury-related neuropathic pain (21). It was reported that miR-204-5p may suppress the inflammatory response by targeting the interleukin (IL)-6 receptor (22). miR-146a-5p was demonstrated to alleviate TNF-α- or LPS-induced mechanical allodynia (23). In addition, circulating miRNA-146a-5p was found to be decreased in patients with knee osteoarthritis who were responders to treatment with celecoxib (24). These results

indicate that elevated miR-204-5p and miR-146a-5p may be self-protective mechanisms and serve as markers of prostatic pathology. The let-7 family of miRNAs plays an important role in the regulation of μ opioid receptor function (25) and was found to be highly expressed in chronic neuropathic pain (26). These data indicate that altered miRNAs are critical for the pathological changes of pelvic pain in CPPS.

Prediction of miRNA target genes can be performed by computational prediction tools (27). Therefore, miRNA-target prediction was also performed in the present study. A series of biological processes associated with synapses, including neurotransmitter transport, regulation of synaptic plasticity, regulation of trans-synaptic signaling, and modulation of chemical synaptic transmission, were identified by an analysis of significant Gene Ontology (GO) terms. These signaling molecules may act on their receptors, such as α1 adrenoceptors (28) and cholinergic receptors, which in turn mediate the development of chronic pelvic pain and urinary symptoms. Both α1 adrenoceptors and cholinergic receptors are highly expressed in the muscular tissue of the prostate (29), which is the mainly therapeutic target of male lower urinary tract symptoms at present (30). α-adrenoceptor antagonists and muscarinic receptor antagonists are widely used in CP/CPPS. α-adrenoceptor antagonists have been shown to relieve pain and improve symptom scores in patients with CP/CPPS (31). α1-adrenoceptors are a type of postsynaptic receptor and play a pivotal role in prostate biofunction (32). Thus, we propose that the altered miRNAs regulate α1 adrenoceptors and cholinergic receptors by synapse-associated pathways resulting in pain and urinary symptoms.

A total of 35 piRNAs were altered in this study. PIWI-interacting RNAs (piRNAs) are small non-coding RNAs expressed mainly in the gonads. piRNAs play an important role in maintaining gametogenesis by regulating the activity of transposons (33). Many piRNAs have been identified to be highly expressed in semen, and play a role in semen quality (34,35). piR-61648 has been reported to be highly expressed in semen and vaginal secretions (34), and was also elevated in patients with CPPS in the present study. The differential expression of piRNAs between patients with CPPS and the healthy participants indicates the substantial value of piRNAs as biomarkers for CPPS.

In the present study, 898 tsRNAs (269 elevated and 629 declined) were altered between the two groups. tRNA-derived small RNAs (tsRNAs) are novel sncRNAs that are generated from diverse tRNAs and are present in many tissues and body fluid and function by gene expression regulation (36). tsRNAs are also expressed in sperm and can be altered by a high fat diet. A study on mice found that the altered expression of sperm tsRNAs can influence embryonic gene expression and mediate intergenerational inheritance (8). The influence of sperm tsRNAs on embryonic gene expression and embryonic quality have also been detected in humans (37). Sperm quality are also demonstrated to be negatively affected by CP/CPPS (38). However, the mechanism of the negative effect of CP/CPPS on sperm is unclear. The prostatic contribution to an average ejaculate (3.5 ml) is usually 0.5-1.0 ml (39). Therefore, the altered tsRNA expression profile in EVs of prostatic fluid may help elucidate the effect of CPPS on sperm quality.

A series of miRNAs were also reported to be altered in the EPS of patients with category IIIA CP/CPPS (40). However, there are many differences in this study. In the present study, instead of direct isolation from EPS, the RNAs detected were isolated from the EVs of EPS. EVs from the prostate are also defined as prostasomes and believed to play many roles in sperm that promote fertilization (41). However, the term prostasomes usually refers to all EVs isolated from semen, which may mix with other EVs from the reproductive tract (42). Prostasomes isolated from different sources have a similar size distribution (41), which indicates that it is difficult to distinguish EVs from the prostate from those from the semen. Therefore, we chose EPS-EVs in this study to explore the biological functions in the prostate.

Although the present study makes significant contributions to understanding the altered sncRNA expression profiles and several chronic pain-related miRNAs of EPS-EVs in patients with CPPS, it was limited in some ways. As these miRNAs are closely related to chronic pain, these chronic pain-related miRNAs were speculated to serve as diagnostic markers in CPPS. However, the number of samples used in this study was extremely small that they were not sufficient for calculating the diagnostic efficacy using receiver operating characteristic analysis. In future research, we would like to expand the sample size to clarify the diagnostic value and compare these miRNAs with other diagnostic markers in CPPS. Furthermore, CPPS is a multifactorial disorder in which pain may originate in any of the urogynecological, gastrointestinal, pelvic musculoskeletal, or nervous systems (43). Although a series of sncRNAs were altered in EPS-EVs of patients with CPPS, lesions of the pelvic organs other than the prostate cannot be excluded. Only patients with CPPS and healthy men were included. The expression levels of these miRNAs in prostatic fluid of patients only with chronic prostatitis and patients with category IIIA prostatitis require further exploration. Further investigation is warranted to explore the miRNA expression of patients with chronic prostatitis, which may be used to differentiate chronic prostatitis, chronic pelvic pain syndrome and category IIIA prostatitis. In addition, sncRNAs of prostatic tissue could be studied, if feasible.

In summary, a series of sncRNAs, including 6 chronic pain-related miRNAs, were differentially expressed in EPS-EVs of patients with CPPS without obvious indication of prostatitis and healthy participants, which may serve as diagnostic markers for CPPS.

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Availability of data and materials

The high-throughput sequencing data was submitted to the GEO datasets (http://www.ncbi.nlm.nih.gov/geo) with accession no. GSE195766.

Authors' contributions

JB and SH conceived and designed the study. BO, DH, ZG, WL and LH performed the experiments. JD, JL and ZC analyzed and checked the data. LH, JL and ZC prepared the figures. BO and DH drafted the manuscript. BO, JB and SH edited and revised manuscript. BO and SH confirm the authenticity of all the raw data. All authors read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of Guangzhou First People's Hospital (K-2020-032-01) (Guangzhou, Guangdong, China). All participants signed consent forms prior to participating in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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