Letter to the Editor

Urinary extracellular vesicle microRNA profiling for detection in patients with interstitial cystitis

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Interstitial cystitis (IC) is characterized by pelvic pain, pressure, or discomfort related to the urinary bladder, often accompanied by urinary urgency or frequency and relieved by voiding (1). However, diagnosis varies widely depending on the urologist's experience. The identification of a novel objective biomarker for IC could greatly improve diagnostic consistency and expedite timely intervention where needed. Recently, liquid biopsy using microRNA (miRNA) in biofluids shows promise for early cancer detection (2). miRNAs are small noncoding RNAs consisting of about 20 nucleotides. They can be stably present in extracellular vesicles (EVs), and the profiles of miRNA expression in EVs make them good candidates for improving the diagnosis of a variety of diseases. This paper describes our comprehensive analysis of the urinary EV miRNA expression profile and evaluates its potential as a novel biomarker in IC/BPS patients.

Urinary samples were obtained from 8 Hunner type IC (HIC) patients, 2 bladder pain syndrome (BPS) patients, and 10 non-IC/BPS patients (controls) who were diagnosed with stress urinary incontinence and/or pelvic organ prolapse. The definitions of HIC and BPS followed the recent Japanese guideline (1). Briefly, the definition of IC/BPS is the condition with chronic pelvic pain, pressure or discomfort perceived to be related to the urinary bladder accompanied by other urinary symptoms, such as persistent urge to void or urinary frequency in the absence of confusable diseases (1). Additionally, IC/BPS is divided into HIC and BPS. HIC represents IC/BPS with Hunner lesion, and BPS represents IC/BPS without Hunner lesion (1). All HIC and BPS patients were clinically diagnosed by cystoscopy before urine collection. All patients had a urinary analysis to rule out urinary tract infection. All controls did not have any lower urinary tract symptom. Urine was collected through a urethral catheter and immediately centrifuged at 1,500 rpm for 5 min, and the supernatant was frozen at −80 ℃ pending analysis. Cystoscopy and urine collection were performed at least 7 days apart. We collected urinary EV miRNA using a nanowire-based device (CRAIF Inc.) (3,4). Comprehensive miRNA profiles for all samples were analyzed using a 3D-Gene miRNA Labelling kit and 3D-Gene Human miRNA Oligo Chip (Toray Industries). Leave-one-out cross-validation was used to identify the top 20 miRNAs by accuracy of correctly classifying IC/BPS and controls in receiver operating characteristic (ROC) curve analysis; miRNAs with area under the curve (AUC) values ≥0.85 and cross-validated accuracy ≥0.85 were selected. The cut-off levels were selected using Youden index. All HIC and BPS patients completed the O'Leary-Sant score symptom indexes (OSSI), O'Leary-Sant score problem indexes (OSPI), and visual analog scale (VAS) scores. The correlation between these scores and the level of miRNA expression was analyzed from the Pearson correlation. $P<0.05$ was regarded as statistically significant. Details of the methods and statistics are described in Appendix 1.

Characteristics of the study population are detailed in *Table 1*. The cohort of 3 men and 17 women had an average age of 53.3 years (range, 23–73 years). A total of 485 miRNAs passed the quality check criteria and were selected for further study. Principal component analysis (PCA) mapping and heatmap of these miRNAs provided a rough separation of HIC, BPS, and controls (*Figure 1A,1B*). The volcano plot suggested differences in signal intensity

Table 1 Patients' characteristics

Characteristics	HIC (n=8)	BPS (n=2)	Control (n=10)	P value (HIC/BPS *vs.* control)
Female	6 (75.0%)	1 (50%)	10 (100%)	0.06
Age, years	59.9 (10.7)	49 (5.7)	48.9 (18.4)	0.325
BMI, kg/m^2	22.3 (2.2)	19.2 (1.1)	25.7 (3.9)	0.041
OSPI	11.5 (4.0)	10 (0.0)	NA	NA
OSSI	13.3 (3.8)	8.5 (0.7)	NA	NA
VAS score	7.7 (3.1)	7 (0.0)	NA	NA

Values are n (%) or mean (SD). HIC, Hunner type interstitial cystitis; BPS, bladder pain syndrome; BMI, body mass index; OSPI, O'Leary-Sant score problem indexes; NA, not available; OSSI, O'Leary-Sant score symptom indexes; VAS, visual analog scale.

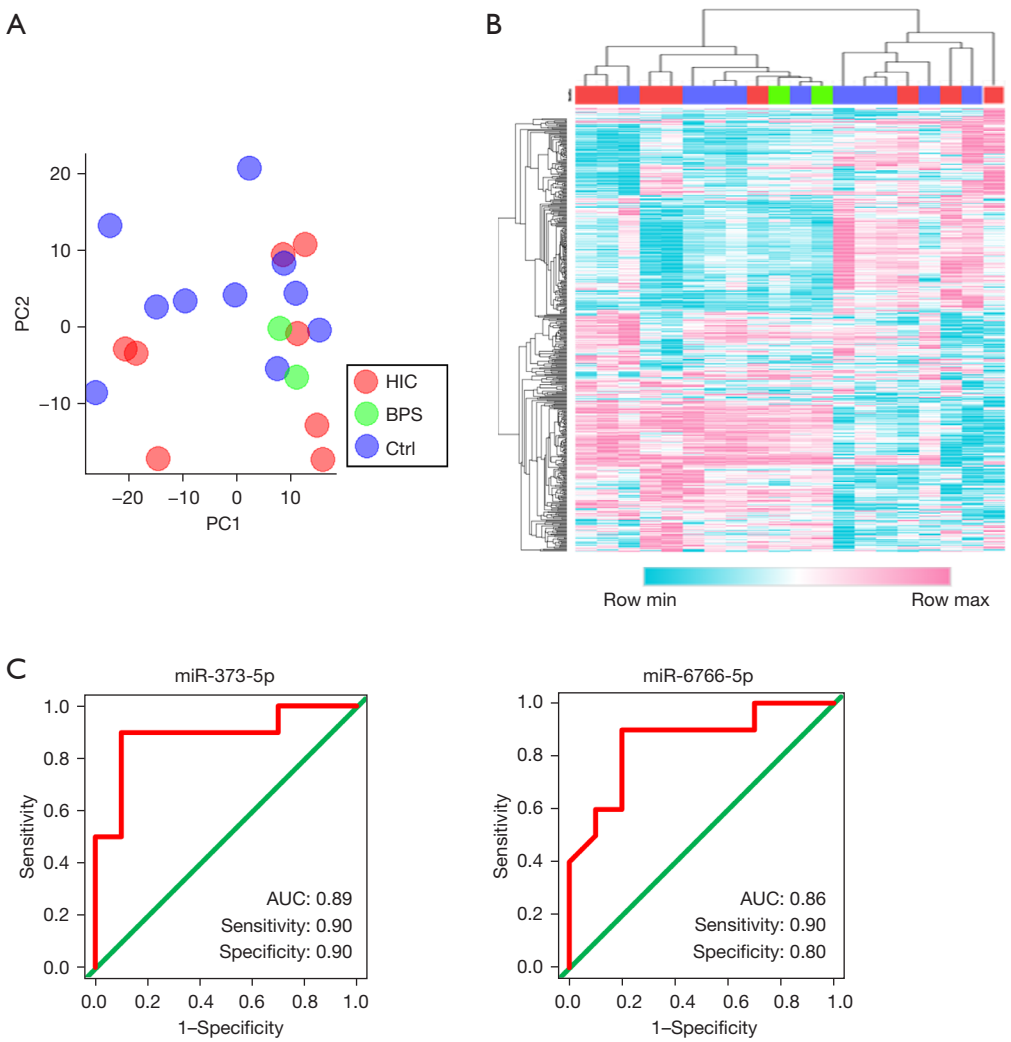

Figure 1 Analyses of urinary miRNAs. (A) A PCA map for HIC, BPS, and Ctrl samples using 485 miRNAs. (B) An unsupervised hierarchical clustering analysis with a heatmap showing HIC, BPS, and Ctrl samples with 485 miRNAs. (C) ROC curve analysis of miR-373-5p and miR-6766-5p. HIC, Hunner type interstitial cystitis; BPS, bladder pain syndrome; Ctrl, control; AUC, area under the curve; miRNA, microRNA; PCA, principal component analysis; ROC, receiver operating characteristics.

Table 2 Urinary miRNAs detecting IC

Rank	miRNA	AUC	Cross-validated accuracy
1	miR-373-5p	0.89	0.90
2	miR-6766-5p	0.86	0.85
2	miR-6813-5p	0.79	0.85
3	miR-1181-3p	0.82	0.80
3	miR-4638-5p	0.85	0.80
3	miR-4648	0.80	0.80
3	miR-4730	0.77	0.80
3	miR-4731-5p	0.84	0.80
3	miR-6090	0.81	0.80
3	miR-6778-5p	0.80	0.80
3	miR-6872-3p	0.79	0.80
4	miR-204-3p	0.76	0.75
4	miR-2392	0.73	0.75
4	miR-3188	0.74	0.75
4	miR-371a-5p	0.80	0.75
4	miR-375-5p	0.70	0.75
4	miR-4447	0.83	0.75
4	miR-4454	0.73	0.75
4	miR-4476	0.77	0.75
4	miR-4675	0.77	0.75

IC, interstitial cystitis; AUC, area under the curve.

between IC/BPS and controls (Figure S1). miR-375-5p showed an AUC of 0.89 and cross-validated accuracy of 0.90, and miR-6766-5p showed an AUC of 0.86 and cross-validated accuracy of 0.85 (*Figure 1C*, *Table 2*). The signal intensity of miR-6766-5p was significantly higher, and of miR-373-5p was significantly lower, in HIC patients than in the control patients (Figure S1B,S1C). In addition, we evaluated diagnostic performance of miR-375-5p and miR-6766-5p between HIC patients and controls, which also showed high accuracy, respectively (miR-375-5p: AUC, 0.86; sensitivity, 0.88; specificity, 0.90; and miR-6766-5p: AUC, 0.86; sensitivity, 0.88; specificity, 0.80) (Figure S2). Although some trends were noted for miR-373-5p, no significant association between signal intensity and quality of life (QOL) scores was detected for either miRNA (Figure S3). As the body mass index (BMI) was significantly higher in controls, we also evaluated the association between the two miRNAs and BMI. There was no significant association between them (Figure S4).

Our study identified two miRNAs (miR-373-5p and miR-6766-5p) in EVs that will be useful in diagnosing IC/BPS patients. Previously, Liu *et al.* (5) evaluated the diagnostic value of miR-19a-3p and long noncoding RNA (MEG3) in urinary EVs, but they focused on previously studied RNA (6). To the best of our knowledge, this is the first study that has comprehensively investigated the diagnostic value of urinary EV miRNAs in IC/BPS patients. One strength of this study is that we examined the expression profiles of 2,632 miRNAs, constituting all the human miRNA identified to date according to miRbase release 22. One weakness is the omission of functional analysis in patients with IC/BPS of the two miRNAs (miR-373-5p and miR-6766-5p), which we selected as candidate miRNAs for diagnosis. The sample size was small, and the samples were collected in a single institution. Additionally, only the difference of the miRNA profiles between IC/BPS and controls were evaluated. In clinical, the differences of the profiles between IC/BPS and hyper sensitive bladder might be more useful. Further investigation of clinical applications will thus be required. However, our pilot study showed that urinary EV miRNAs are potentially useful for diagnosing IC. This point may prove beneficial to urologists in daily clinical practice in the near future.

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Footnote

Provenance and Peer Review: This article was a standard submission to the journal. The article has undergone external peer review.

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.

com/article/view/10.21037/tau-22-240/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The protocol for this study was approved by The Jikei University Institutional Review Board [No. 32-284(10366)]. Informed consent was obtained from all patients.

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Appendix 1

Extracting miRNAs in urine using the nanowire device

We extracted miRNAs from 500 µL urine sample using a nanowire-microfluidic device (3,4). We fabricated the nanowire device by assembling ZnO nanowire scaffolds, COP resin microfluidic substrate, COP resin substrate, two stainless steel holders, and PEEK tubes. After assembling the device, the inlet PEEK tube was connected to a syringe pump (KDS-200, KD Scientific Inc.) to introduce urine and lysis buffer. On the other hand, the outlet PEEK tube was put in an RNase-free microfuge tube (Eppendorf AG.) to collect the flow-through urine and miRNA-containing solution. The miRNA-containing solution extracted with lysis buffer was purified using Wako microRNA Extractor SP Kit (FUJIFILM Wako Chemical Corporation) according to the manufacturer's instructions. We profiled miRNA expression using microarray.

Microarray analysis of miRNA expression

Comprehensive miRNA expression analysis was performed using the 3D Gene miRNA Labeling Kit and the 3D-GeneHumanmiRNA Oligo Chip (Toray Industries, Inc.), which was designed to detect 2,632 miRNAs registered in miRBase release 22 (http://www.mirbase.org/; ref. 22). Fluorescent signals for each spot on the microarray were obtained using the 3D-Gene Microarray Scanner (Toray Industries, Inc.) and digitized using the accessory digitizing application "Extraction" (Toray Industries, Inc.) Background (BG) signals were subtracted from the raw signals by statistically inferring the true signal based upon the assumption of normal distribution for the BG signal and exponential distribution for the true signal (7). miRNAs with (BG-subtracted signal) $>2^6$ with at least 50% of the samples were selected, and the other miRNAs were removed from the down-stream analyses. Quantile normalization was applied after BG subtraction and low-expression miRNAs removal. Log2 transformation was applied after quantile normalization.

Data accessibility

The microarray data have been deposited in the GEO database under accession codes GSE196156.

Statistical analysis

The urinary samples were analyzed to identify IC/BPS in the following way: the top 20 miRNAs by accuracy to correctly classify HIC/BPS and controls in ROC curve analysis were identified by leave-one-out cross-validation; miRNAs with AUC value ≥0.85 and cross-validated accuracy ≥0.85 were selected. Categorical and continuous variables of patients' characteristics were compared using chi-square test and Mann-Whitney U test, respectively. Statistical analysis were performed using R version 3.4.3 (R Foundation for Statistical Computing, http://www.R-project.org), compute.es package version 0.2-4, hash package version 2.2.6.1, MASS package version 7.3-51.3, mutoss package version 0.1-12, and pROC package version 1.14.0. Heat maps were created using the online tool Morpheus (https://software.broadinstitute.org/morpheus). Other statistical analyses were performed using STATA version 14 (StataCorp). For all analyses, a two-sided P value less than 0.05 was considered statistically significant.

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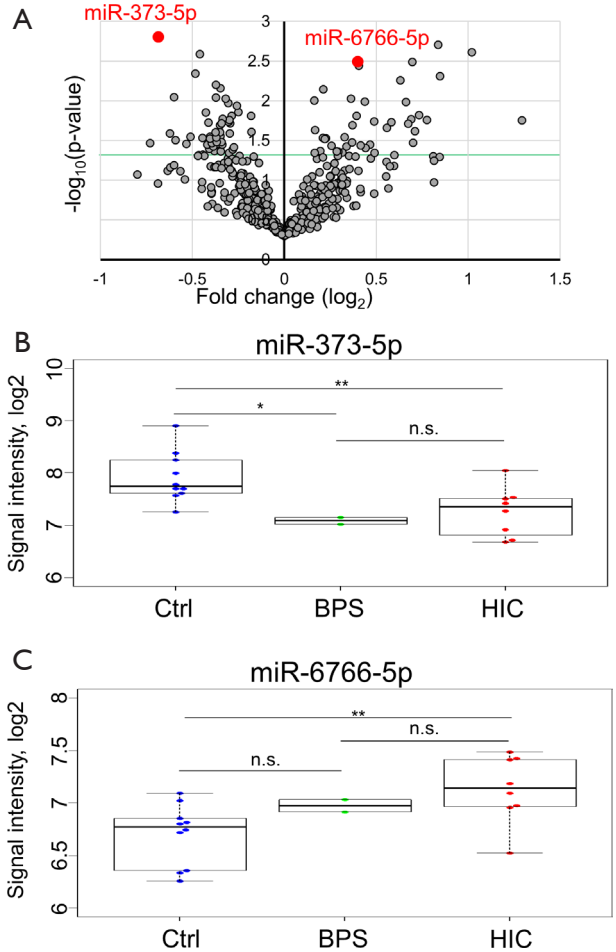

Figure S1 Evaluation of miRNA expression profiles in urine. (A) Volcano plot of the fold change of miRNAs. Green line indicates P=0.05. (B) Expression level of miR-373-5p. (C) Expression level of miR-6766-5p. Ctrl, control; BPS, bladder pain syndrome; HIC, Hunner type interstitial cystitis; miRNA, microRNA.

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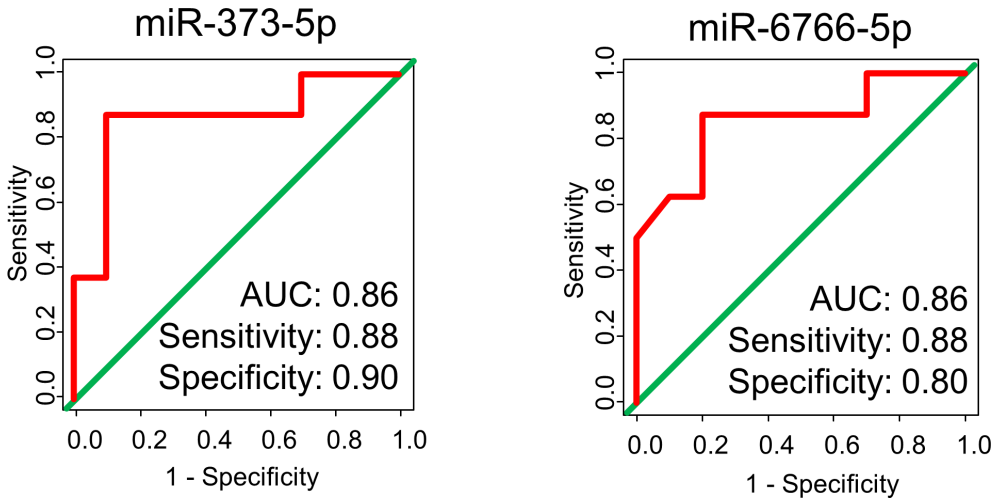

Figure S2 ROC curve analysis of miR-373-5p and miR-6766-5p in HIC patients and controls. AUC, area under the curve; ROC, receiver operating characteristic; HIC, Hunner type interstitial cystitis.

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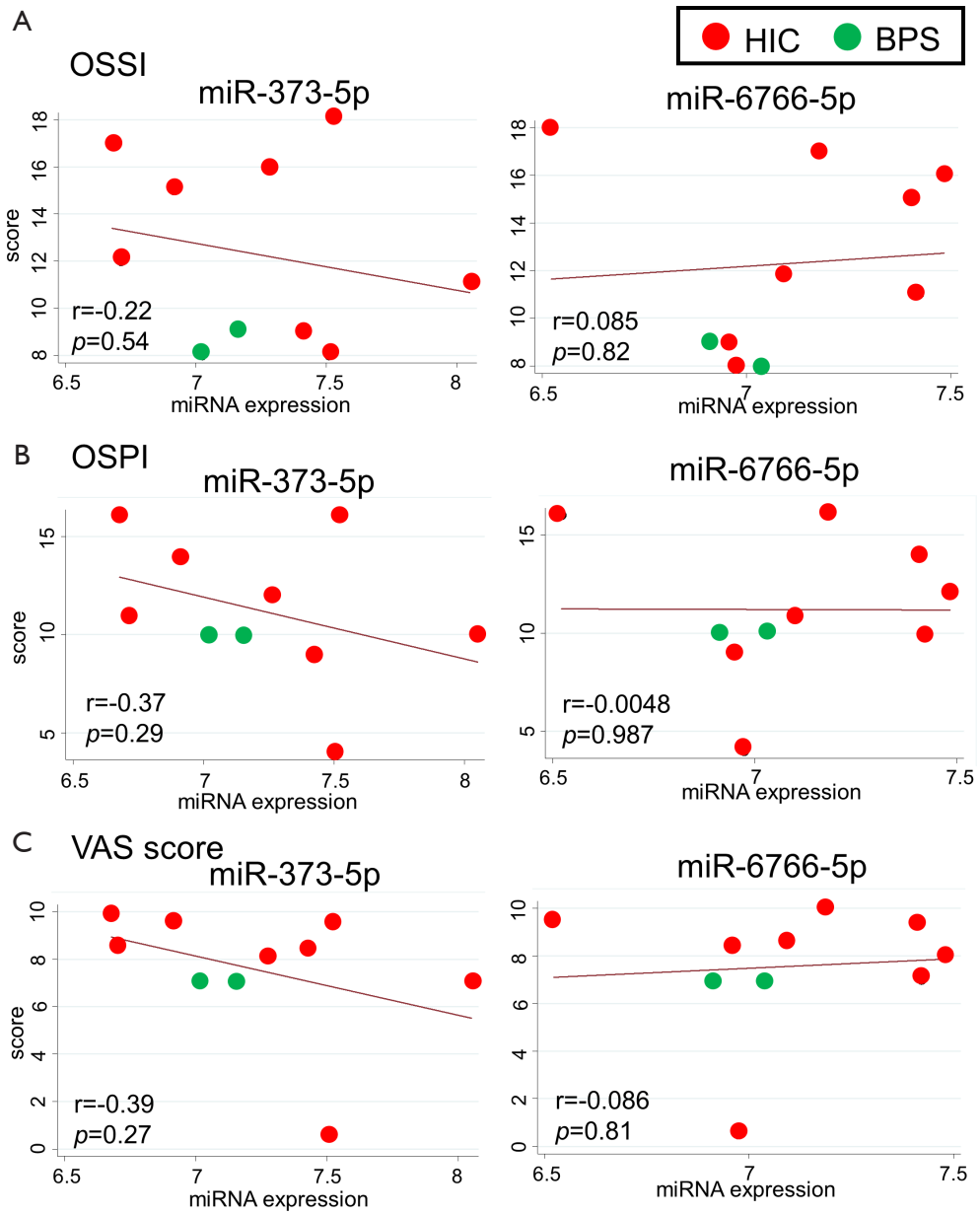

Figure S3 Scatter plots show the relationship between each miRNA expression and (A) OSSI, (B) OSPI, and (C) VAS score. HIC, Hunner type interstitial cystitis; BPS, bladder pain syndrome; OSSI, O'Leary-Sant score symptom indexes; OSPI, O'Leary-Sant score problem indexes; VAS, visual analog scale; miRNA, microRNA.

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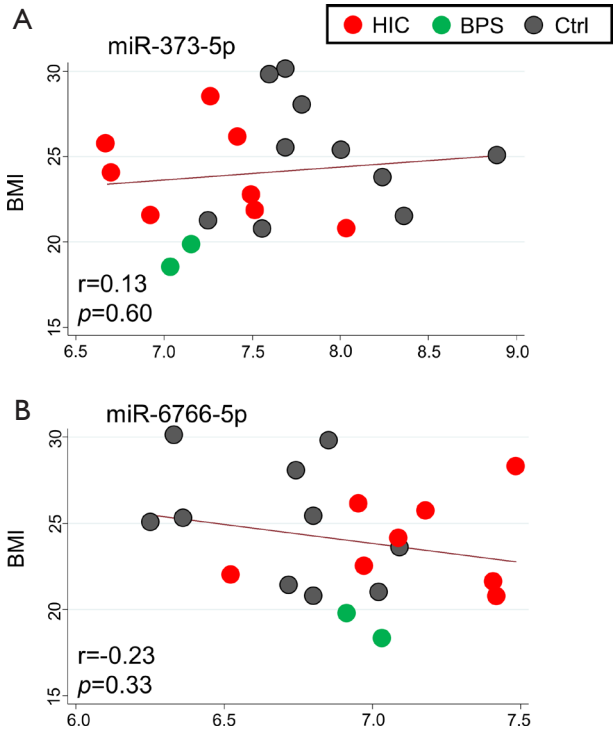

Figure S4 Scatter plots show the relationship between each miRNA expression and BMI. HIC, Hunner type interstitial cystitis; BPS, bladder pain syndrome; Ctrl, control; BMI, body mass index; miRNA, microRNA.

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