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Reviewer A

Interstitial cystitis is a urologic condition that is commonly associated with a delay to diagnosis and adequate treatment, and effective treatment is often multi-modal and driven by trial and error. Thus, effective biomarkers for diagnosis of this condition, and ultimately its response to a given treatment/treatments would be a welcome addition to the field. The authors should be commended for their efforts to do so.

I have several comments that require addressing:

Comment 1: The control group was not free from urologic pathology, but rather had a diagnosis of stress urinary incontinence and/or pelvic organ prolapse. This may confound the discriminatory capacity of miR-373-5p and miR-6766-5p. This control group should be further described. The authors note that they do not have IC, but they should also describe whether any lower urinary tract symptoms are present, or have been ruled out. They should also address this in their limitations.

Reply: Thank you for your comment. We added the following sentence "All controls did not have any lower urinary tract symptom."

Changes in the text: see Page 4, line 53 to 54.

2. The authors note that HIC and BPS were defined as per the recent Japanese guideline. The authors should briefly describe the diagnostic criteria in their text, as readership may not be familiar with this guideline terminology. Additionally, the guideline provides necessary versus optional aspects of the IC workup. The authors should specify whether all patients had a urinalysis to rule out UTI, etc., prior to diagnosis. They should also specify whether all patients (including BPS) had a cystoscopy, to rule in/rule out HLs.

Reply: Thank you for your suggestion. We added following sentence in the manuscript. "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. Additionally, IC/BPS is divided into HIC and BPS. HIC represent IC/BPS with Hunner lesion, and BPS represent IC/BPS without Hunner lesion." and "All patients had a urinary analysis to rule out urinary tract infection."

Changes in the text: see Page 3, line 47 to 51 and Page 4, line 52 to 54.

Comment 3. All patients with HIC underwent urologic instrumentation (cystoscopy) immediately prior to urine collection for this study. The authors should address this in their limitations, as a potential confounding factor.

Reply: Thank you for your suggestion. Indeed, we performed cystoscopy and urine collection in the different days. Thus, we added the following sentence in the

manuscript "Cystoscopy and urine collection were performed at least 7 days apart."
Changes in the text: see Page 4, line 55 to 56.

4. BMI is greater in the control group than in the HIC/BPS groups. Adiposity and micro-RNAs have been correlated (PMID 31611648), and thus the identified miRNAs could in part be attributed to this difference. The authors should address this in their discussion.

Reply: Thank you for your comment. We evaluated the association between miRNAs and BMI, but there was no significant association between their signal intensity and BMI. We added supplementary Figure 3 and the following sentence in the revised manuscript "As the BMI was significantly, higher in controls, we evaluated the association between the two miRNAs and BMI. There was no significant association between them (Supplementary Figure 3)."
Changes in the text: see Page 5, line 80 to 82.

5. In the final paragraph of the discussion, the authors note that there was an "omission" in functional analysis in patients with IC/BPS. However, they did perform some level of this analysis (Supplementary Figure 2). The authors should rephrase this, as it was not an omission, but rather that no significant differences were detected.

Reply: Thank you for your suggestion. miRNAs are small noncoding RNAs of 20 to 25 nucleotides in length that posttranscriptionally regulate the expression of thousands of genes. Thus, we think that functional analysis in miRNA study is to reveal the target genes of the miRNAs. From the point of the view, we used the term of "omission" in our manuscript.

Comment 6: In methodology, authors should provide the technique by which they obtained the specificity and sensitivity of their microRNAs, and provide supportive rationale for choosing their technique. For example, PMID 30633111, point 4.8, warns against choosing the top-left portion of ROC curves to generate these metrics.

Reply: Thank you for your comment, we added the following sentence in the manuscript "The cut-off levels were selected using Youden index."
Changes in the text: see Page 4, line 61 to 62.

7. Can the authors comment on whether miRNAs may also be appropriate prognostic biomarkers for treatment response? Many patients in this population require multiple lines of treatment prior to arriving at an acceptable outcome, and an accurate biomarker-driven treatment decision aid would be extremely helpful, and likely would lead to improved outcomes and reduction in associated cost burden.

Reply: Thank you for your comment. We agree with your comment. However, in the present study, we did not evaluate the kinetics of the miRNAs after treatment for interstitial cystitis. Of course, our ultimate goal of the miRNA study is establishing not only diagnostic but also prognostic biomarker, thus we will continue our research.

Reviewer B

Comment 1: This is a preliminary report focusing on the possible urinary extracellular vesicle microRNA profiling for detection in patients with interstitial cystitis. Comprehensive miRNA profiles in urine samples obtained from 10 IC/BPS (8 HIC and 2 BPS) patients and 10 controls revealed that the signal intensity of miR-6766-5p was significantly higher, and of miR-373- 67 5p was significantly lower, in HIC patients than in the control patients. However, no significant correlation of either of these two miRNAs with any of clinical parameters was demonstrated. Neither functional interpretation of these two miRNAs was evaluated.

Even though this is a preliminary investigation, the sample size is too small (8 HIC and 2 BPS) and the female/male ratio is unmatched (female percentage: HIC:75%, BPS:50%, Control:100%), which may possibly affect the results and conclusion. It is not difficult to distinguish IC/BPS patients from the other diseases by clinical symptoms and additional basic evaluations, and thus the most important point should be to find a useful marker for distinguishing HIC from BPS. However, there were only 2 BPS patients included in this study, which makes impossible to compare miRNA profiles between HIC and BPS because of too small sample size.

Reply: Thank you for your comment. We agree with the reviewer's comment that the number of the sample is relatively small to discuss the diagnostic power of miRNAs in interstitial cystitis. However, the strong point of our study is that we comprehensively analyzed the 2,632 miRNAs, which constitute all the human miRNAs identified to date according to miRbase rel. 22. This is the first study to evaluate the profiles of miRNAs in urinary extracellular vesicles from interstitial cystitis patients. Additionally, we updated our array data in the Gene Expression Omnibus database (GSE196156), which are publicly available. Further validation study will be required, the present study is valuable as the first pilot study.

Reviewer C

This manuscript describes a small pilot study that profiled miRNA in urine from 10 IC/BPS patients (8 with HIC and 2 with BPS) and 10 non-IC/BPS patients. Although the preliminary results look interesting, they do not yet support the conclusion that the two EV miRNAs identified by the study, miR-373-5p and miR-6766-5p "will be useful in diagnosing IC/BPS patients".

Comment 1: The title of the manuscript says 'interstitial cystitis' yet the recruited patients represent IC (i.e., 8 HIC) and BPS (i.e., 2 BPS). Nearly all of the patients (8/10) are HIC, which is a very specific subgroup within IC/BPS (~10-15%) and so the miRNAs may actually be mostly related to IC with HL only, rather than a biomarker for for IC/BPS. Furthermore, although 3/10 recruited IC/BPS participants were male, (none of the controls were male), there is no mention of gender effects. For such a modest sample size it would probably have been advisable to recruit only women. This study presents some interesting results but would benefit from a larger, more targeted

(e.g., HIC vs BPS vs control; or IC/BPS vs control - with equal numbers in the groups) and/or balanced (equal numbers of M/F, or all females) sample.

Reply: Thank you for your comment. Firstly, in the present study, the definitions of HIC and BPS followed the recent Japanese guideline. In the guideline, IC/BPS is divided into HIC and BPS. HIC represent IC/BPS with Hunner lesion, and BPS represent IC/BPS without Hunner lesion. Thus, we could cover the most of the type of IC/BPS. In addition, we agree with the reviewer's comment that the sample size of our study may be critical. However, as we have replied in the above (please refer the reply for reviewer B), we believe that the present study is valuable as the first pilot study for interstitial cystitis.

Reviewer D

This manuscript reported the miRNA profiles in the urine samples of patients with Hunner's IC, bladder pain syndrome (BPS) and controls. The authors identified two miRNA in extracellular vesicles that might be useful in diagnosing Hunner's IC patients. The study is interesting and the results are novel. There are some points that the authors might comment in their revised version:

Comment 1: The urine was collected by a catheter, please report the bladder condition on urine collection, such as bladder volume, bladder sensation, and urinalysis.

Reply: Thank you for your comment. We added following sentence in the manuscript "All patients had a urinary analysis to rule out urinary tract infection."

Changes in the text: see Page 4, line 52 to 53.

Comment 2: The definition of HIC and BPS should be clearly defined.

Reply: Thank you for your comment. To clarify the definition of the HIC and BPS in Japanese guideline, we added the following sentences in the manuscript "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. Additionally, IC/BPS is divided into HIC and BPS. HIC represent IC/BPS with Hunner lesion, and BPS represent IC/BPS without Hunner lesion."

Changes in the text: see Page 3, line 47 to 51.

Comment 3: Identifying HIC from the controls is not difficult,. but differential diagnosis of HIC from patients with hypersensitive bladder might be difficult. Please comment.

Reply: Thank you for suggestion. We agree with the reviewer's comment, thus we added the following sentence in the limitation of the manuscript " 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 may be more useful."

Changes in the text: see Page 6, line 92 to 94.

Reviewer E

This is a pilot study showing what urine microRNAs may be useful as diagnostic tools in BPS/IC. The authors identify two specific microRNAs that seemed able to separate between IC patients with Hunner lesions and controls. The authors acknowledge the power limitation of the study (small sample size) and future studies should aim to increase the number in all the groups tested. Overall, this is an interesting concept study that shows the usefulness of microRNAs in the diagnosis of IC/BPS.

I have the following comments:

Comment 1: Authors state that all HIC patients had undergone cystoscopy. They do not indicate whether BPS patients also had cystoscopy. Cystoscopy is the gold standard for separating the two groups. Please clarify.

Reply: Thank you for your comment. We added the following sentence in the manuscript. "All HIC and BPS patients were clinically diagnosed by cystoscopy before urine collection."

Changes in the text: see Page 3, line 51 to Page 4, line 52.

Comment 2: Urine collection was through a catheter (even controls). If the authors want to expand their findings to a larger population size and want to make it more relevant to clinical practice they should consider using clean catch for the urines.

Reply: Thank you for your suggestion. We agree with the reviewer thus we will not use a catheter for our next study.

Comment 3: Fig 1C shows AUC for both miRNA in determining a distinction between IC/BPS patients and controls. S1B and C are more informative since they break down patients into separate subgroups (BPS and HIC). These supplementary figures show that both miRNAs levels are different in the HIC group when compared to controls. I suggest they run AUC analysis using both miRNA against their patient and control populations. The separation should be improved.

I understand the sample size is small and unbalanced (especially for BPS). Still authors should apply greater emphasis on their ability to differentiate HIC from controls using this technique.

Reply: Thank you for your comment. According to the comment, we added the Supplementary Figure 3 and the following sentences to show the diagnostic power of each miRNA in HIC and controls "In addition, we evaluated diagnostic performance of miR-375-5p and miR-6766-5p between HIC and controls, which 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) (Supplementary Figure 3)."

Changes in the text: see Page 5, line 73 to 76.

Minor comments:

Typo line 56 "was regard…" should likely be "was regarded…"

Reply: We revised the manuscript according to the comment.

Changes in the text: see Page 4, line 64.