Independent verification of circulating miRNA as diagnostic biomarkers for urothelial carcinoma

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Abstract
Urothelial carcinoma (UC) is an umbrella term for bladder cancers (BCa) and upper-tract urothelial carcinoma (UTUC), with BCa and UTUC sometimes detected concomitantly. The methods of detection for UC are often inaccurate or highly invasive, and, therefore, are thought to be unsatisfactory. Previously, we reported seven serum miRNAs as diagnostic markers for BCa. Here, we re- evaluated potential diagnostic miRNAs in different institutions. We prospectively analyzed serum samples obtained from 126 UC patients (BCa: 106 samples; UTUC: 14 samples; UTUC with BCa: six samples) and 50 noncancer controls by microarray analysis. We randomly assigned these samples into a training or a validation set. Biomarker candidate miRNAs were selected based on cross-validation scores in the training set of samples, with diagnostic power confirmed in the validation set. Among the diagnostic miRNAs identified in this way, miR-1343-5p and miR-6087 had been identified as potential diagnostic miRNAs in our previous study. In addition, we evaluated the association between the serum levels of identified miRNAs and the presence of UC risk conditions. The expression levels of several miRNAs correlate with the risk factors in participants without UC, which may be explained by the presence of a microscopic tumor or a precancerous lesion. In conclusion, we identified two robust miRNA diagnostic markers for UC detection. Further functional analysis is required to elucidate the mechanism by which alterations in the expression of these miRNAs occur.

KEYWORDS
diagnostic, liquid biopsy, miRNA, urothelial carcinoma

1 | INTRODUCTION

Urothelial carcinoma (UC) is the fourth most common malignancy worldwide.\textsuperscript{1} While BCa accounts for 90% of UC, UTUC is less common.\textsuperscript{2} However, the incidence of concomitant BCa at the time of primary UTUC is about 20%.\textsuperscript{3} Currently, the methods used in the detection of UC are urinary cytology, imaging, and endoscopic examinations, such as cystoscopy or ureteroscopy; however, these
methods are unsatisfactory, as they are often inaccurate and can be highly invasive.\textsuperscript{4,5} Thus, the development of effective and unintrusive diagnostic markers is urgently required.

miRNAs are noncoding, single-chain RNAs of approximately 18–24 base pairs, which act as post-transcriptional regulators of gene expression.\textsuperscript{6} Recently, differential miRNA expression has emerged as a hallmark of some human diseases, including cancers, cardiovascular disease, and DM.\textsuperscript{7} Moreover, miRNAs are stable in the blood stream and their expression has been shown to be reproducible.\textsuperscript{8} Therefore, circulating miRNAs have garnered a great deal of attention as novel targets for noninvasive diagnostic biomarkers in liquid biopsy.

In a previous study, we investigated circulating miRNAs as noninvasive biomarkers for BCa.\textsuperscript{9} We established a promising diagnostic model of circulating miRNAs with high accuracy [sensitivity, 95%; specificity, 87%; area under the curve (AUC), 0.97]; however, most of the samples were stored in the National Cancer Center biobank for several years before analysis; thus, further analysis is required.\textsuperscript{9}

In the present study, we prospectively collected serum samples from different institutions and conducted comprehensive miRNA microarray analyses, independently extracting biomarker miRNAs. We used this new dataset to investigate the consistency of our previous study.\textsuperscript{9} We additionally evaluated the association between serum level of identified miRNAs and the presence of risk conditions for UC, such as age, male, smoking history, and DM.

2 | MATERIALS AND METHODS

2.1 | Patient selection

Consecutive serum samples were obtained from 128 UC patients (BCa: 108 samples; UTUC: 14 samples; UTUC with BCa: six samples) admitted to or referred to the Kanagawa Cancer Center or National Hospital Organization Yokohama Medical Center between October 2016 and December 2018. Patients previously diagnosed with cancers other than UC were excluded, with two BCa patients excluded on this basis. Fifty control samples were collected from the National Hospital Organization Yokohama Medical Center; 20 were obtained from DM patients hospitalized for DM management education, and the remaining 30 were obtained from participants receiving a medical checkup. For inclusion as a control sample, the participant must have had no history of cancer. Serum was immediately prepared from whole blood and stored at −80°C. Briefly, whole blood was collected in sterilized vacuum tubes for serum separation, gently inverted five times, and incubated at room temperature for 30 min to allow complete coagulation. The clot was removed by centrifugation at 2500g for 10 min in a refrigerated centrifuge, and the resulting supernatant was designated serum.

2.2 | Analysis of miRNA expression by microarray profiling

Total RNA was extracted from 300μl serum using 3D-Gene RNA extraction reagent from a liquid kit (Toray Industries) and purified with the RNeasy 96 QIAcube HT kit (Qiagen). Comprehensive miRNA expression analysis was performed using a 3D-Gene miRNA labeling kit and 3D-Gene Human miRNA Oligo Chip (Toray Industries), which was designed to detect 2565 miRNA sequences registered in miRBase release 21,\textsuperscript{10} with three miRNAs (miR-149-3p, miR-2861, and miR-4463) selected as internal normalization controls.\textsuperscript{11} Microarray analysis was performed within 5 months of sample collection, and all data are publicly available through the GEO database (GSE201359).

2.3 | Evaluation of the diagnostic power of miRNAs

Samples were randomly assigned to the training or validation set. As a quality control, we selected miRNAs with a signal >2\textsuperscript{6} in more than 50% of UC or noncancer control samples in the training set. In the training set, the best miRNAs to detect UC were investigated using Fisher linear discriminant analysis with leave-one-out cross-validation. miRNAs with a cross-validation score of >0.75 were selected as candidate biomarkers. Cutoffs to evaluate diagnostic performance in the validation set were selected using Youden index in the training set.

2.4 | Statistics

χ\textsuperscript{2} or unpaired t test was used to compare categorical or continuous variables in the two clinical groups, respectively. Linear discriminant analysis was 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.). A two-sided p value <0.05 was considered statistically significant and, where appropriate, significance at a Bonferroni adjusted p value is also indicated.

3 | RESULTS

3.1 | Patient characteristics

The discovery and validation sets consisted of 63 UC patients and 25 noncancer controls, respectively (Figure 1A). No significant difference in participant characteristics was observed between the training and validation set (Table 1). Characteristic differences between UC patients and noncancer controls are summarized in Table S1. In UC patients, the median age was 76 years (range 32–92). Ninety-six (76.2%) were male, and 25 (19.8%) were diagnosed with DM. The median Brinkman index was 663 (range 0–3060). In noncancer controls, the median age was 76 years (range 32–92). Twenty-three participants (46.0%) were male, and 21 (42.0%) were diagnosed with DM. The median Brinkman index was 0 (range 0–2280). These factors were significantly different between UC patients and noncancer controls.
3.2 | Investigation of potential diagnostic miRNAs in UC

A total of 320 miRNAs passed the quality control criteria, with principal component analysis mapping suggesting a separation between UC patients and noncancer controls (Figure 1B). Unsupervised hierarchical clustering using a heat map of the 320 selected miRNAs also suggested a segregation of UC from noncancer controls (Figure 1C). Fisher linear discriminant analysis was performed to select miRNAs for the diagnosis of UC. Twelve miRNAs were selected using cross-validation scores (Table 2). Among these, miR-1343-5p and miR-6087 had been selected as diagnostic miRNAs in our previous study.9 The diagnostic performance of each miRNA was confirmed in the validation set with high accuracy (Figure 2). The highest AUC value was obtained from miR-1273g-3p (AUC, 0.89; sensitivity, 0.81; specificity, 0.96), and miR-1343-5p and miR-6087 were validated for their performance (miR-1343-5p: AUC, 0.87; sensitivity, 0.83; specificity, 0.68; and miR-6087: AUC, 0.76; sensitivity, 0.59; specificity, 0.84, respectively). Additionally, we investigated the diagnostic performance of each miRNA for BCa and UTUC, separately. As shown in Figures S1 and S2, each miRNA had favorable accuracy for BCa and UTUC. Furthermore, the signal intensity individually plotted on the graph showed that BCa and UTUC had the same trends against controls (Figure S3).

3.3 | The influence of risk factors on miRNA expression

It has previously been suggested that DM and smoking history are associated with increased incidence and poor prognosis in UC,12,13 while age and gender are also accepted risk factors.14,15 Therefore, we investigated the relationship between miRNA expression and these risk factors.

Expression of miR-1343-5p and miR-6768-5p was significantly higher in noncancer control participants with DM than in those without DM. Conversely, miR-4433a-3p and miR-7641 expression was significantly lower in noncancer controls with DM than in those without DM. Interestingly, these trends were not observed in UC patients (Table 3). In addition, expression of miR-1343-5p and
miR-6768 increased significantly with age in noncancer controls but not in UC patients (Table 4). There was no relationship between miRNA expression and gender or smoking history (Tables 5 and 6).

Finally, as the risk factors were not matched between participants with or without UC, we performed multivariate logistic regression analysis. After adjusting for these risk factors, miRNA expression was still a significant indicator for UC, suggesting that these miRNAs are independently associated with the presence of UC (Table S2).

4 | DISCUSSION

In our previous study, we comprehensively analyzed miRNA profiles from 392 BCa patients, 100 noncancer controls, and 480 patients with other cancers. Using the Fisher linear discriminant analysis, we identified several miRNAs that can be used to discriminate BCa from noncancer and other cancers with high accuracy. However, samples were collected from different institutions and stored under different conditions, which might have had an impact on data quality. In the present study, therefore, we evaluated the association between circulating miRNA profiles and UC from samples stored under unified conditions. Samples were collected and stored at −80°C for no more than 5 months before microarray analysis. In addition, we collected detailed patient information to enable us to control for the presence of UC risk factors in our patient samples. This is the first study to evaluate circulating miRNA profiles in relation to UC risk factors. Furthermore, we investigated their diagnostic power for UC, which is composed of BCa and UTUC. Most UC, whether BCa or UTUC, originate in the transitional epithelium. Establishing diagnostic markers that can detect both BCa and UTUC is, therefore, clinically important.

miR-6087 and miR-1343-5p were identified as potential diagnostic miRNAs in both our current and previous study. Especially, miR-6087 was previously reported to be a highly sensitive and selective marker (AUC of 0.89, sensitivity of 93%, and specificity of 77%). Although the interinstitutional bias could critically influence the miRNA expression, the identification of the two suggests their stability and versatility. On the contrary, the other miRNAs were not selected in our previous study. In the present study, we managed the sample conditions more strictly than in our previous study, in

### TABLE 1 Patient characteristics

| Characteristics          | Discovery set | Validation set | p Value |
|--------------------------|--------------|----------------|---------|
| Cancer                   |              |                |         |
| BCa, N (%)               | 53 (84.1)    | 53 (84.1)      | 1.00    |
| UTUC, N (%)              | 7 (11.1)     | 7 (11.1)       |         |
| UTUC with BCa            | 3 (4.8)      | 3 (4.8)        |         |
| Age (median [range])     | 74 (32–92)   | 76 (51–91)     | 0.17    |
| Sex, N (%)               |              |                |         |
| Male                     | 51 (81.0)    | 45 (71.4)      | 0.21    |
| Female                   | 12 (19.0)    | 18 (28.6)      |         |
| DM, N (%)                | 13 (20.6)    | 12 (19.0)      |         |
| Tumor stage, N (%)       |              |                |         |
| <pT2                     | 41 (65.0)    | 48 (76.2)      | 0.12    |
| ≥pT2                     | 22 (35.0)    | 14 (23.8)      |         |
| Metastasis, N (%)        | 2 (3.2)      | 3 (4.8)        | 0.65    |
| Urothelial carcinoma, N (%) | 59 (93.7) | 60 (95.2)      | 0.68    |

Control

| Characteristics          |          |                |         |
| Age (median [range])     | 58 (46–81) | 57 (46–80)     | 0.58    |
| Sex, N (%)               |          |                |         |
| Male                     | 9 (36.0)  | 14 (56.0)      | 0.16    |
| Female                   | 16 (64.0) | 11 (44.0)      |         |
| DM, N (%)                | 11 (44.0) | 10 (40.0)      | 0.77    |

Abbreviations: BCa, bladder cancer; DM, diabetes mellitus; UTUC, upper-tract urothelial carcinoma.

### TABLE 2 Discriminant analysis for urothelial carcinoma in the training set

| Model  | miRNA   | Sensitivity | Specificity | Accuracy | AUC | Cross-validated accuracy | Cutoff |
|--------|---------|-------------|-------------|----------|-----|--------------------------|--------|
| Model 1| miR-1273-3p | 0.87        | 0.92        | 0.89     | 0.92 | 0.83                     | 7.48   |
| Model 2| miR-1343-5p | 0.81        | 0.88        | 0.83     | 0.89 | 0.80                     | 10.21  |
| Model 3| miR-6778-5p | 0.84        | 0.84        | 0.84     | 0.89 | 0.80                     | 7.93   |
| Model 4| miR-619-5p | 0.86        | 0.84        | 0.85     | 0.90 | 0.80                     | 6.89   |
| Model 5| miR-4732-5p | 0.78        | 0.88        | 0.81     | 0.89 | 0.79                     | 5.63   |
| Model 6| miR-6087    | 0.60        | 1.00        | 0.72     | 0.85 | 0.78                     | 11.65  |
| Model 7| miR-8071    | 0.59        | 0.96        | 0.69     | 0.82 | 0.77                     | 6.75   |
| Model 8| miR-6768-5p | 0.76        | 0.92        | 0.81     | 0.85 | 0.77                     | 8.93   |
| Model 9| miR-4433a-3p | 0.56       | 0.96        | 0.67     | 0.79 | 0.75                     | 7.23   |
| Model 10| miR-7641   | 0.59        | 0.92        | 0.68     | 0.78 | 0.75                     | 7.05   |
| Model 11| miR-4454   | 0.68        | 0.84        | 0.73     | 0.81 | 0.75                     | 9.53   |
| Model 12| miR-1260a  | 0.70        | 0.84        | 0.74     | 0.80 | 0.75                     | 6.26   |
FIGURE 2 ROC curve analysis of the selected miRNAs in the validation set.
which we used preserved samples from the several biobanks. The differences of sample management methods may affect the result of the miRNA selection. Additionally, DM patients who were in hospital for the purpose of education of DM were included in the present study. The difference in characteristics of control samples may also influence the selected miRNAs.
We further examined the effect of the risk factors (DM, age, sex, and smoking history) on circulating miRNA expression in UC, which revealed that the expression of miR-1343-3p and miR-6768 is upregulated in UC patients with DM. In noncancer controls, these miRNAs were similarly upregulated in DM patients compared with non-DM participants. As DM is a risk factor for UC, this upregulation may reflect the presence of a precancerous lesion. Similar trends for these two miRNAs were observed when we focused on patient age; miR-1343-3p and miR-6768 were upregulated in older noncancer controls. By contrast, miR-4433a-3p and miR-7641 expression was downregulated in UC patients compared with noncancer controls, and upregulated in noncancer controls from DM patients compared with non-DM participants. Although the role of these miRNAs in the tumor microenvironment has not been elucidated, these trends may reflect a reactive response of tumor environment.

This study has several limitations. Although we prospectively collected samples, the sample size was relatively small; therefore, further external validation is still required.

In conclusion, we prospectively evaluated the expression of circulating miRNAs in UC patients, identifying miR-1343 and miR-6087, among others, as reliable diagnostic markers for UC. The expression of several miRNAs appears to be influenced by UC risk factors. While further study is required, these data suggest circulating miRNAs are attractive biomarkers for the diagnosis of UC.

**AUTHOR CONTRIBUTIONS**

KH and TO conceived this study. FU, JM, KH, and TO designed the methodology. FU and KH conducted data analysis with support from JM and TO. FU drafted the manuscript, conducted data interpretation with JM, FT, TK, TO, and KH. All authors have contributed to the manuscript drafting.

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**Table 5** The difference in miRNA expression profiles from male and female patients

| miRNA     | Tumor (+) Male | Tumor (+) Female | p Value | Tumor (-) Male | Tumor (-) Female | p Value |
|-----------|----------------|------------------|---------|----------------|------------------|---------|
| miR-1273g-3p | 7.00 (0.55)    | 7.01 (0.68)      | 0.93    | 7.92 (0.40)    | 7.96 (0.49)      | 0.78    |
| miR-1343-5p | 10.43 (0.23)   | 10.43 (0.24)     | 0.91    | 10.08 (0.23)   | 9.94 (0.25)      | 0.055   |
| miR-6778-5p | 7.43 (0.61)    | 7.70 (0.62)      | 0.037   | 8.37 (0.42)    | 8.34 (0.45)      | 0.81    |
| miR-619-5p  | 6.31 (0.78)    | 6.35 (0.84)      | 0.82    | 7.38 (0.62)    | 7.44 (0.75)      | 0.77    |
| miR-4732-5p | 4.82 (1.70)    | 5.32 (1.05)      | 0.13    | 6.25 (0.43)    | 6.25 (0.56)      | 0.98    |
| miR-6087   | 11.85 (0.29)   | 11.82 (0.32)     | 0.68    | 11.55 (0.26)   | 11.52 (0.19)     | 0.69    |
| miR-8071   | 7.03 (0.64)    | 7.05 (0.63)      | 0.84    | 6.47 (0.36)    | 6.44 (0.35)      | 0.76    |
| miR-6768-5p | 9.17 (0.41)    | 9.17 (0.57)      | 1.00    | 8.77 (0.20)    | 8.65 (0.17)      | 0.026   |
| miR-4433a-3p | 7.05 (0.28)    | 7.09 (0.34)      | 0.55    | 7.36 (0.16)    | 7.37 (0.18)      | 0.93    |
| miR-7641   | 6.55 (1.33)    | 6.75 (0.82)      | 0.45    | 7.53 (0.60)    | 7.36 (0.59)      | 0.32    |
| miR-4454   | 9.03 (0.65)    | 9.28 (0.72)      | 0.073   | 9.75 (0.77)    | 10.07 (0.64)     | 0.12    |
| miR-1260a  | 5.88 (0.46)    | 6.08 (0.57)      | 0.065   | 6.42 (0.54)    | 6.64 (0.42)      | 0.11    |

Note: Mean (SD).

**Table 6** The relationship between miRNA expression profiles and Brinkman index

| Brinkman index | Tumor (+) | p Value | Tumor (-) | p Value |
|----------------|-----------|---------|-----------|---------|
| miR-1273g-3p   | 0.032     | 0.72    | -0.073    | 0.62    |
| miR-1343-5p    | 0.11      | 0.21    | 0.32      | 0.023   |
| miR-6778-5p    | -0.042    | 0.64    | -0.018    | 0.90    |
| miR-619-5p     | 0.055     | 0.54    | -0.089    | 0.54    |
| miR-4732-5p    | -0.10     | 0.27    | 0.042     | 0.77    |
| miR-6087       | -0.10     | 0.27    | 0.07      | 0.63    |
| miR-8071       | -0.071    | 0.43    | 0.17      | 0.24    |
| miR-6768-5p    | 0.056     | 0.53    | 0.22      | 0.13    |
| miR-4433a-3p   | -0.040    | 0.66    | 0.20      | 0.16    |
| miR-7641       | -0.0023   | 0.98    | 0.28      | 0.049   |
| miR-4454       | -0.22     | 0.014   | 0.018     | 0.90    |
| miR-1260a      | -0.18     | 0.049   | 0.043     | 0.77    |
DISCLOSURE
The authors declare no competing interests.

ETHICAL APPROVAL
Approval of the research protocol by an Institutional Review Board:
The present study involving human subjects was approved by the
Ethical Committee of the Independent Administrative Institution
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ical guideline of the “Declaration of Helsinki.”

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SUPPORTING INFORMATION
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