Cell-Free Urinary MicroRNA-99a and MicroRNA-125b Are Diagnostic Markers for the Non-Invasive Screening of Bladder Cancer

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Abstract

Background: Evidence implicated the diagnostic significance of microRNAs in whole urine/urine sediments in urothelial carcinoma of the bladder (UCB). However, the contaminated blood cells in patients with haematuria significantly altered the expression profiles of urinary microRNA, influencing the test accuracy.

Methods: MicroRNA profiles of the urine supernatants of UCB patients and controls without any malignancy and profiles of malignant and corresponding normal mucosa tissues from the patients were determined by microRNA microarray and compared to identify differentially expressed microRNAs. The differential expression was verified in the tissues of an independent patient cohort by RT-qPCR. The diagnostic significance of selected microRNAs as biomarkers in the urine supernatant was investigated in the expanded cohorts.

Results: MicroRNA-99a and microRNA-125b were down-regulated in the urine supernatants of UCB patients. The degree of down-regulation was associated with the tumor grade. A diagnostic model was developed using a combined index of the levels of microRNA-99a and microRNA-125b in the urine supernatant with a sensitivity of 86.7%, a specificity of 81.1% and a positive predicted value (PPV) of 91.8%. Discriminating between high- and low-grade UCB, the model using the level of microRNA-125b alone exhibited a sensitivity of 81.4%, a specificity of 87.0% and a PPV of 93.4%.

Conclusions: The results revealed a unique microRNA expression signature in the urine supernatants of UCB patients for the development of molecular diagnostic tests. An effective cell-free urinary microRNA-based model was developed using a combined index of the levels of microRNA-99a and microRNA-125b to detect UCB with good discriminating power, high sensitivity and high specificity.

Introduction

Urothelial carcinoma of the bladder (UCB) is the second most common malignancy in the urinary system [1]. Due to its high incidence and frequent recurrence, effective diagnostic and disease monitoring tools are essential for the clinical management of patients. Cystoscopy is currently the standard clinical test for diagnosis and cancer surveillance. However, the procedure is invasive and unpleasant, and has several practical limitations. For example, it may not be able to detect small and/or flat tumors like carcinoma in situ. Therefore, an alternative non-invasive approach exhibiting high specificity and sensitivity is required. Although many blood- and urine-based biomarkers have been identified and evaluated in the literature, none have been ideal and powerful enough to replace cystoscopy [2].

MicroRNAs, which are small non-coding RNAs, have recently demonstrated significant diagnostic and prognostic value in various types of cancer [3–5]. MicroRNAs exhibit high stability and easy detectability even at low levels in various types of clinical samples [6–8]. It is considered an excellent disease biomarker for detection and monitoring. One study demonstrated that a number of microRNAs were aberrantly expressed in UCB tumor tissues and that these aberrations could be engaged in diagnosis and the staging of bladder cancer biopsies [9]. In addition, the assessment of the levels of these aberrantly expressed microRNAs that show unique signatures in whole urine or urine exfoliated cells is even more promising for the diagnosis and disease surveillance of UCB [10–11]. However, the results are sometimes unreliable when studying patients with haematuria, in whom contaminated blood cells significantly alter the urinary microRNA profiles and thus
mask the signatures. In fact, 85% of patients exhibit varying degrees of haematuria [12–13]. As a result, an alternative approach to measuring microRNAs is required. Evidence has suggested that free nucleic acids, such as DNA biomarkers in urine supernatants, provide a higher detection rate and higher sensitivity than those in sediment for UCB diagnosis [14–16]. Therefore, assessment of the levels of microRNAs in urine supernatant could improve the use of microRNAs as diagnostic biomarkers for detecting UCB, especially in patients with haematuria. In this study, the feasibility of using cell-free urinary microRNAs for diagnosing UCB was determined and models for diagnosing UCB and discriminating tumor grades were developed.

Methods

Patients and samples

An overview of this study is shown in Figure S1. With the written consent of the donors and the approval of the Joint Chinese University of Hong Kong - New Territories East Cluster Clinical Research Ethics Committee, tissue and urine samples were collected in the Urology Unit, Department of Surgery, Prince of Wales Hospital, Hong Kong. Mid-stream urine was collected from bladder cancer patients before surgery. Both tumor tissue and normal bladder mucosa located >3 cm away from the tumor edge were obtained by cystoscopy. The 1973 WHO diagnosis and grading system for bladder cancer [17] was used for diagnosis. For the normal controls, urine samples were collected from patients who had normal cystoscopic findings, absence of malignancies with a >6 months follow-up and haematuria. All of the urine samples were centrifuged at 2,500 r.c.f. for 20 minutes and the urine supernatants were collected.

MicroRNA microarray

The total RNA of the urine supernatants and frozen tissue was extracted using the MirVanaPARIS Kit (Ambion) in accordance with the manufacturer’s recommended protocols. The Agilent Human miRNA Microarray Chip (Release 13.0, Agilent Technologies, Santa Clara, CA, U.S.A.), which encompasses 866 human microRNAs and 89 viral microRNAs, was used to profile the expression of these microRNAs in the urine supernatants and cancer and control tissues. The dataset was deposited to ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) and the accession number is E-MTAB-2573. The signal intensity of each spot was median-normalized and further normalized by linear regression.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

First strand cDNA was synthesized from the total RNA of the urine supernatants and tissue samples using a universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) in accordance with the manufacturer’s recommended protocol. The resultant cDNA was subjected to quantitative polymerase chain reaction (qPCR) with SYBR Green master mix and microRNA LNA PCR primer (Exiqon) that specifically recognized the targeted miRNA in an ABI 7900HT fast RT-qPCR machine (Applied Biosystem/Life Technologies, Grand Island, NY, U.S.A.). RNU6B was used as the reference control. All of the samples were tested in duplicate. The relative level of microRNA was determined using the ∆∆Ct method.

Statistical methods

The microarray data were analyzed using the Gene Spring 11.0 software (Agilent) to normalize and identify the differentially expressed microRNAs. ABI SDS 2.3 software (ABI/Life Technologies) was used to analyze the RT-qPCR data. The quantitative data were subjected to a Mann-Whitney U test and a Wilcoxon signed-rank test using the SPSS 16.0 software (IBM Co, Armonk, NY, U.S.A.). In subsequent post-hoc analysis, a bivariate logistic regression analysis was performed to determine the Combined Index (CI) for the combination of two microRNAs expression levels. A receiver operating characteristic (ROC) curve analysis was carried out using the SPSS 16.0 software. The sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios were calculated.

Results

Identification of differentially expressed microRNAs between the urine supernatants of UCB patients and controls and between cancer and normal tissues by microRNA microarray

Urine supernatants from six UCB patients and three normal controls together with four pairs of UCB tissues and their corresponding normal bladder mucosal tissues were subjected to microRNA microarray analysis. The patient characteristics are shown in Table 1. To determine the presence and absence of microRNAs in the samples, a threshold signal was defined as the average background plus three standard deviations. Signals above this threshold were considered a “presence” and signals below the threshold were considered an “absence.” After global normalization, 117±63.8 out of 866 human microRNAs were detected in the urine supernatants and 314±83.4 microRNAs were detected in the bladder tissues. There was no significant difference in the total number of microRNAs detected between the urine supernatants from cancer patients and controls and between the cancer and control tissues. Comparing the profiles in the urine supernatants of the UCB patients with those of the normal controls, 39 microRNAs showed differential expression (Mann-Whitney test, p<0.05; fold differences >1.60). There were 78 differentially expressed microRNAs between the cancer and normal tissues (paired Mann-Whitney test, p<0.05; fold differences >2.0). Ten microRNAs were commonly dysregulated in both the urine supernatants and tissue samples (Figure 1 and Figure S1). Of these, the levels of microRNA-1, microRNA-99a, microRNA-125b, microRNA-133a, microRNA-133b, microRNA-143 and microRNA-1207-5p significantly decreased and those of microRNA-16, microRNA-96 and microRNA-103 increased in the UCB samples (Figure 1).

Validation of the 10 selected microRNAs in independent cohorts of tissue and urine supernatant samples

To validate the microarray results, the levels of the 10 selected microRNAs were quantified by RT-qPCR in another 18 pairs of bladder cancer and corresponding normal mucosa tissues (Table 1). Of these, the differential expression of six microRNAs between cancer and normal tissues was validated (Wilcoxon signed-rank two-related-samples test, p<0.05) (Figure 2). MicroRNA-1 was down-regulated in all 18 pairs of cancer tissues (p<0.01) and 17 out of 18 pairs showed down-regulation (p<0.01) in the remaining five microRNAs (microRNA-99a, microRNA-125b, microRNA-133a, microRNA-133b and microRNA-143). MicroRNA-1, microRNA-99a, microRNA-125b, microRNA-133a, microRNA-133b and microRNA-143 were down-regulated 266.87±2.06, 130.69±2.30, 49.52±3.39, 263.20±1.99, 261.38±2.11 and 67.18±1.83 times over in the cancer tissues, respectively.
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(A)

Down-regulated microRNAs
- miR-1
- miR-99a
- miR-125b
- miR-133a
- miR-133b
- miR-143
- miR-1207-5p

Up-regulated microRNAs
- miR-16
- miR-96
- miR-183

(B)

Down-regulated microRNAs
- miR-1
- miR-99a
- miR-125b
- miR-133a
- miR-133b
- miR-143
- miR-1207-5p

Up-regulated microRNAs
- miR-16
- miR-96
- miR-183
The discrimination power of the six validated microRNAs in the urine supernatants was then assessed using 71 urine supernatant samples, including 50 samples from bladder cancer patients (15 cases of low-grade cancer and 35 cases of high-grade cancer) and 21 samples from controls (Table 1). In this expanded set of samples, the urine supernatants of the UCB patients possessed lower levels of microRNA-99a ($p<0.01$), microRNA-125b ($p<0.01$), microRNA-133b ($p<0.05$) and microRNA-143 ($p<0.01$) than that of the controls (Figure 3A), but not of microRNA-133a and microRNA-1.

Development of cell-free urinary microRNA models for the detection of UCB and the discrimination of tumor grades

The ROC curve was determined to evaluate the diagnostic strength of these microRNAs in detecting UCB. High area under the curve (AUC) values were found for microRNA-99a (0.800, ranging from 0.715 to 0.886) and microRNA-125b (0.813, ranging from 0.729 to 0.897) (Figure 3B), whereas microRNA-133b and microRNA-143 exhibited low diagnostic strength with AUC values around 0.6. To further determine the diagnostic significance of microRNA-99a and microRNA-125b, related cut-off points were determined from the ROC curves based on Youden’s Index rule. The cut-off points for the normalized expression levels of microRNA-99a and microRNA-125b were $2^{17.76}$ and $2^{0.91}$, respectively. A patient with a urine supernatant whose microRNA level was lower than or equal to the cut-off point was considered a cancer case, and considered normal if the level was higher than the cut-off point. According to this system, the sensitivity, specificity, positive prediction value (PPV), negative prediction value (NPV), positive likelihood (LR+ ) and negative likelihood (LR-) for microRNA-99a were 78.0%, 85.7%, 92.7%, 61.0%, 5.46 and 0.26, respectively, and for microRNA-125b were 84.8%, 76.2%, 89.3%, 68.1%, 3.57 and 0.20, respectively (Table 2). To achieve better diagnostic performance, a combination of the expressions of microRNA-99a and microRNA-125b was subjected to a bivariate logistic regression. The CI performed better than the use of each microRNA alone. The AUC value increased to 0.876. The CI formula was $CI=1/[1+exp\{-(2.332+0.425 xAC_{\text{microRNA-125b}}+0.069 xAC_{\text{microRNA-99a}})}]\}$. When the cut-off point was set at 0.6244 based on Youden’s Index rule, the prediction system improved with an increased sensitivity (86.7%) and NPV (71.4%) and a decreased LR- (0.16), with comparable values for the specificity, PPV and LR+ (Table 2).

The clinical relevance of the four microRNAs selected to discriminate low-grade from high-grade cancers was also assessed. The levels of urinary microRNA-99a and microRNA-125b were significantly lower in patients with high-grade cancers (G2 and G3) than in those with low-grade cancers (G1) [Mann-Whitney U test, $p<0.01$]. The levels of microRNA-133b and microRNA-143 showed no tumor grade discrimination value (Figure 3A). The AUC values differentiating between high- and low-grade cancers for microRNA-99a and microRNA-125b were 0.819 and 0.791, respectively (Figure 3B). The cut-off points of the normalized levels of microRNA-99a and microRNA-125b for tumor grading were $2^{17.76}$ and $2^{0.91}$, respectively. Based on these cut-off points, the system using the level of microRNA-99a exhibited a sensitivity of 74.2%, a specificity of 83.3%, a PPV of 91.5%, an NPV of 58.1%, an LR+ of 4.46 and an LR- of 0.31. In the case of microRNA-125b, these values were 81.4%, 87.0%, 93.4%, 67.0%, 6.11 and 0.21, respectively, for discriminating tumor grades (Table 2). When combining the levels of microRNA-99a and microRNA-125b in the system, the CI formula became $CI=1/[1+exp\{-[1.742+0.0295xAC_{\text{microRNA-125b}}+0.496xAC_{\text{microRNA-99a}}]}]\}+0.7398 as the cut-off point. The sensitivity, specificity, PPV, NPV, LR+ and LR- were 79.4%, 88.0%, 94.7%, 61.1%, 6.61 and 0.23, respectively (Table 2). All of these values were either comparable with or lower than those for the systems using either microRNA-99a or microRNA-125b alone. Taken altogether, the system using microRNA-125b alone performed better at differentiating between high- and low-grade UCB.

Correlation between expressions of microRNA-99a and microRNA-125b in urine supernatants and bladder cancer and control tissues

Via a Pearson correlation analysis, the microRNA-99a and microRNA-125b levels in both the tissues and urine supernatants of the patients and controls were highly correlated ($R=0.896$, $p<0.001$ for the tissue samples; $R=0.982$, $p<0.001$ for the urine supernatant samples) (Figure 3C), suggesting that their expression may have been co-regulated. In fact, these genes were clustered at the q21.1 region of chromosome 21 (Figure 3D).

Restoration of microRNA-99a and microRNA-125b expressions in post-operative patients

To demonstrate the crucial link between bladder cancer status and decreased microRNA-99a and microRNA-125b levels in the urine supernatants, these two microRNAs were quantified in the urine supernatants of pre-operative and post-operative patients. Twenty patients were recruited (Table 1). Post-operative urine was collected 4 weeks after a transurethral resection. After the resection, the patients were able to restore the microRNA-99a and microRNA-125b levels in the urine supernatant (Wilcoxon signed-rank two-related-samples test, $p<0.01$) (Figure 4) to levels comparable with those of the normal control samples (Figure S2).

Discussion

This study developed an effective cell-free urinary microRNA-based model for detecting UCB with good discriminating power (AUC = 0.876) and high sensitivity (86.7%) and specificity (81.1%). The feasibility of using urinary microRNAs as bladder cancer diagnostic biomarkers was previously investigated. The ratio of microRNA-126 to microRNA-152 in urine was studied to detect UCB at a sensitivity of 82% and a specificity of 72%, with an AUC value of 0.768 [7]. By studying the expression levels of microRNA-96 and microRNA-183 in urine sediment, tests using these two microRNA biomarkers independently showed sensitiv-
## Table 1. Patient characteristics.

| Characteristics | microRNA microarray | qRT-PCR | qRT-PCR |
|-----------------|---------------------|---------|---------|
|                 | Tissues (TT and NT) | Urine supernatant (TT and NT) | Urine supernatant - pre- and post-operative |
|                 | TU | CU | TU | CU | |
| Total number    | 4 pairs | 18 Pairs | 50 | 21 | 20 pairs |
| Median age      | 74 | 71 | 71 | 75 | 75 |
| (years old)     | (58–81) | (61–70) | (43–85) | (32–87) | (43–85) |
| Gender          | Male | Female | Male | Female | Male | Female | Male | Female |
|                 | 3 | 1 | 5 | 1 | 2 | 1 | 17 | 10 | 13 | 14 |
| Stage           | Superficial | Invasive | New case | Recurrent case | |
|                 | 2 | 2 | 3 | 1 | |
|                 | - | 4 | - | 1 | |
| Grade           | Low grade (G1) | High grade (≥G2) | New case | Recurrent case | |
|                 | 1 | 3 | 3 | 1 | |
|                 | 1 | 5 | - | 6 | |
| Tumor status    | New case | Recurrent case | |
|                 | 3 | 1 | |
| TT: cancer tissue; NT: normal mucosa tissue; TU: urine supernatant from tumor patient; CU: urine supernatant from control. doi:10.1371/journal.pone.0100793.t001
The total RNA was extracted from 18 pairs of bladder cancer tissues and their corresponding normal adjacent mucosa tissues. The levels of these microRNAs were quantified by RT-qPCR in duplicate. The relative expression levels (2^−ΔΔCq) are presented. A paired Mann-Whitney U test was conducted to compare the tumors and their corresponding normal mucosa tissues in UCB patients. Columns, Means; Bars, S.D.; n = 18. ** denotes p < 0.001.

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Figure 3. Expression of microRNAs in the urine supernatant of UCB patients and normal controls. The total RNA was extracted from the urine supernatants of UCB patients with low-grade (n = 15) (shaded bar) or high-grade cancers (n = 35) (black bar) and normal controls (n = 21) (white bar). The levels of six microRNAs in the samples were quantified by RT-qPCR in duplicate. (A) The relative levels (2-ΔCq) of microRNA-133a, microRNA99a, microRNA-1, microRNA-143, microRNA-133b and microRNA-125b in the urine supernatant samples are presented. A Mann-Whitney U test was conducted to compare the UCB patients and normal controls. Columns, Means; Bars, S.D.; ** denotes p < 0.01; * denotes p < 0.05. (B) ROC curves of microRNA-99a alone (blue line), microRNA-125b alone (red line) and microRNA-99a and microRNA-125b in combination (black line) for the diagnosis of UCB (left) and discrimination between low- and high-grade tumors (right). The cut-off points were set and the AUC values determined based on Youden’s Index. (C) Results of a Pearson correlation analysis of the levels of microRNA-99a and microRNA-125b in the tissues and urine supernatant samples. Scatter plots of the relative levels of these two microRNAs in the samples are presented. The Pearson correlation coefficient R was calculated via a Pearson correlation analysis and the p-value was determined. (D) Chromosomal regions of the genes (hsa-miR-99a and hsa-miR-125b-2). Human Genome Reference Consortium GRCh37 Patch Release 12 assembly was used. Hsa-miR-99a is located at chromosome 21:17,909,409–17,913,489, and hsa-miR-125b-2 is located at chromosome 21:17,960,557–17,964,645.

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supernatants increased, implicating a strong association between their levels and the patients’ tumor statuses. This further supports their potential roles as diagnostic biomarkers for UCB and surveillance biomarkers for monitoring tumor recurrence. Based on these models, when a patient has a positive urine supernatant test result with a CI larger than the cut-off (0.6244), he or she probably has bladder cancer, with an accuracy rate of 91.8%. If the normalized expression level of microRNA-125b is lower than 2^{-1.71}, there is a 93.4% chance that the patient has high-grade cancer. However, because the negative predicting value was 71.4% in this study, negative results cannot be used to rule out the possibility of bladder cancer in patients.

The present study showed that the levels of microRNA-99a and microRNA-125b in the urine supernatant of UCB patients and controls were highly correlated with those in the tissues. In fact, the two genes (hsa-miR-99a and hsa-miR-125b-2) are located in intron 6 of the long intergenic non-protein coding RNA 478 (LINC00478) in chromosome 21. Their expressions are probably co-regulated by the promoter that controls the transcription of the LINC00478 gene. The underlying mechanism for the down-regulation of these two microRNAs in UCB may be related to the frequently observed loss of genomic 21q in UCB. Tzai et al. [27] reported a frequent allelic loss of 36.6% in the long arm of chromosome 21, where the two genes are located, in UCB. In addition, patients with Down syndrome exhibited a decreased risk of dying from urological neoplasms, suggesting the presence of genes in chromosome 21 suppressing urological cancer tumors [28].

MicroRNA-99a and microRNA-125b have been demonstrated to have tumor-suppressive roles in various human cancers [29-34]. In prostate cancer, microRNA-99a suppresses the expression of prostate-specific antigens and prostate cancer cell proliferation by interfering with the expression of two chromatin remodeling factors, SMARCA5 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5) and SMARCD1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1), and the growth regulatory kinase mTOR (mammalian target of rapamycin) [29]. In fact, a high expression of phosphorylated mTOR was found in 74% of muscle-invasive urothelial carcinomas, in association with increased pathological stages and decreased disease-related survival [30]. The inhibition of mTOR was found to decrease in vitro and in vivo bladder cancer cell growth [30]. As microRNA-99a targets mTOR in prostate cancer cells [29], the down-regulation of this microRNA in UCB demonstrated in the present study may abolish the tumor-promoting effects of mTOR in the cells, resulting in cancer development. However, further demonstration of the regulation of mTOR by microRNA-99a in bladder cancer cells is required. Although the data of the present study indicated a down-regulation of microRNA-99a expression in both the urine supernatants and tumor tissues of UCB patients, one report showed elevated microRNA-99a in noninvasive but not invasive lesions [31]. A down-regulation of microRNA-125b expression has been frequently observed in various tumors such as hepatocellular carcinoma HCC [32], osteosarcoma [33] and bladder cancer [34]. The ectopic expression of microRNA-125b in low-expressing cancer cells has been found to significantly decrease proliferation and promote apoptosis by targeting Bcl-2 in HCC cells [35], and to affect the proliferation and migration in osteosarcoma cell lines via the suppression of STAT3 expression in the cells [33]. In

Table 2. Diagnostic strength of microRNA-99a alone, microRNA-125b alone and microRNA-99a and microRNA-125b in combination for the detection of bladder cancer and their significance in discriminating tumor grades.

| Diagnosis of UCB | Discrimination of tumor grades |
|------------------|-------------------------------|
|                  | microRNA-99a alone | microRNA-125b alone | microRNA-99a * and microRNA-125b | microRNA-99a alone | microRNA-125b alone | microRNA-99a* and microRNA-125b |
| AUC              | 0.800 (0.715–0.886) | 0.813 (0.729–0.897) | 0.876 (0.809–0.942) | 0.791 (0.673–0.908) | 0.819 (0.712–0.926) | 0.835 (0.770–0.951) |
| Cut-off point    | REL: 2^{0.19} | REL: 2^{-1} | CI: 0.6244 | REL: 2^{-0.91} | REL: 2^{-2.71} | CI: 0.7398 |
| Sensitivity      | 78.0% | 84.8% | 86.7% | 74.2% | 81.4% | 79.4% |
| Specificity      | 85.7% | 76.2% | 81.1% | 83.3% | 87.0% | 88.0% |
| PPV              | 92.7% | 89.3% | 91.8% | 91.5% | 93.4% | 94.7% |
| NPV              | 61.0% | 68.1% | 71.4% | 58.1% | 67.0% | 61.1% |
| +LR              | 5.46 | 3.57 | 4.58 | 4.46 | 6.11 | 6.61 |
| -LR              | 0.26 | 0.20 | 0.16 | 0.31 | 0.21 | 0.23 |

\*Formula of CI for bladder cancer diagnosis: CI = 1/([1+exp\{-(2.332+0.425 x ΔACmicroRNA-99a-125b)+0.069 x ΔACmicroRNA-99a}\}].

\#Formula of CI for tumor grading: CI = 1/([1+exp\{-(1.742 +0.0295 x ΔACmicroRNA-99a-125b +0.496 x ΔACmicroRNA-99a}\}].

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bladder cancer, microRNA-125b inhibited colony formation in an in vitro cell model and in vivo tumor development in nude mice by targeting E2F3, which frequently showed overexpression in bladder cancer and had an expression inversely correlated with that of microRNA-125b [34]. Taken together, aberrant expressions of microRNA-99a and microRNA-125b may be involved in bladder carcinogenesis and even disease progression. Further investigations that elucidate the mechanism underlying how these two microRNAs participate in bladder cancer development and progression are required. The present study found that microRNA-99a and microRNA-125b can be used as effective biomarkers for UCB detection and disease monitoring. They can also function as therapeutic agents for UCB treatment in patients.

Conclusions

The results of this study revealed the unique microRNA expression signature in urine supernatants and tumor tissues of bladder cancer patients for the development of molecular diagnostic tests by microRNA microarray and RT-qPCR. The differential expression of microRNA-99a and microRNA-125b in an expanded patient cohort was verified. An effective cell-free urinary model using a combined index of the microRNA-99a and microRNA-125b levels for bladder cancer detection with good discriminating power, high sensitivity and high specificity was developed. The diagnostic significance of these two microRNAs was further confirmed by the reversals of the altered expression in patients after tumor resection. It was found that microRNA-99a and microRNA-125b can be used as effective biomarkers for UCB detection and disease monitoring. They can also function as therapeutic agents for UCB treatment in patients.

Supporting Information

Figure S1 Workflow of the study of microRNA profiles in urine supernatant and cancer and normal tissues of bladder cancer patients and controls. microRNA profiles were determined by microRNA microarray for identification of differentially expressed microRNAs. By RT-qPCR, the differential expression of the selected microRNAs was validated in the tissue samples of the patients and also in the urine supernatant samples of the expanded patient cohort for development of models for detection of bladder cancer. To determine the link between tumor status and the differential expression, the relative levels of the selected microRNAs in urine supernatant of pre-operative and post-operative patients was determined by RT-qPCR and compared.

Figure S2 Expression of microRNA-99a and microRNA-125b in urine supernatant of UCB patients and normal controls. Urine samples were collected from 20 UCB patients before (pre-operative) and after (post-operative) transurethral resection. Total RNA was extracted from supernatant of pre- and post-operative urine samples of UCB patients (n = 20) and also that of normal controls (n = 21). The levels of these two microRNAs in the samples were quantified by RT-qPCR in duplicate. Relative levels (2-ΔCq) of microRNA-99a and microRNA-125b in the urine supernatant samples were presented. Wilcoxon signed-rank 2 related samples test was used for the comparisons of the levels of microRNA-99a and microRNA-125b between the pre- and post-operative samples. * denotes p<0.01.

Table S1 Median sensitivity and specificity of cytology and other urine-based markers for the detection of bladder cancer.

Author Contributions

Conceived and designed the experiments: DZZ CFN GW KW RKWC. Performed the experiments: DZZ KW. Analyzed the data: DZZ KML. Contributed reagents/materials/analysis tools: CFN. Wrote the paper: DZZ KML CFN CCS ESYC.
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