Research Square

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# Signatures containing miR-133a identified by large scale miRNA profiling in bladder cancer

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## Abstract

Background: Identifying bladder cancer-specific miRNA expression signatures by large scale miRNA profiling Methods：30 bladder cancer (BC) tissue samples and matched adjacent normal bladder tissue samples from patients with BC were collected and were divided into two groups： a training group and a blind testing group. Expressions of 1900 miRNAs and controls were detected in a BC miRNA pool and in a normal miRNAs pool, respectively. 380 differential expressed miRNAs between the BC miRNA pool and the normal miRNA pool were selected. The primers for detecting the 380 selected miRNAs and controls were used to generate one 384-well panel. This panel was used to profile miRNA expression of each individual sample in the training group and in the blind testing group. Data analysis was performed using a machine learning approach of a support vector machine classifier with a Student's t-test feature selection procedure. Results： We identified signatures consisting of three or four miRNAs that could distinguish BC from normal controls with an accuracy of 100% in the training model and an accuracy of over 95% in the blind test. All identified signatures contain hsa-miR-133a. We also revealed that the miRNA183-96 cluster and the miR200 cluster are both significantly up-regulated in BC. Conclusions：The identified signatures containing hsa-miR-133a could be used as biomarkers in the diagnosis and prognosis of BC.


## Background

Bladder cancer (BC) is the second most common urological malignancy globally with about 430, 000 new cases and 165,000 deaths in 2016 (1-3). In China, BC is the most common cancer in the genitourinary system (4).

The gold standard of BC diagnosis is the combination of cystoscopy and biopsy, however, the procedure is invasive, uncomfortable, and costly (5, 6). Urinary cytology is of high specificity (90%–95%), and relatively low sensitivity (~30%). Some urine-based tests for BC, such as BC antigen , nuclear matrix protein 22 (NMP22, Sysmex Corp., Kobe, Japan), and FISH (UroVysion, Abbott Molecular Inc, Des Plaines, IL, USA), have been approved in the applications of BC diagnosis, but the specificities of these tests are low (60%~80%)(7). The development of BC diagnostic biomarkers with high sensitivity and specificity is required.

Micro RNA (miRNA) regulate gene expression at the post-transcriptional level. (8-10). The accumulating data indicated that miRNAs play important roles in tumorigenesis, metastasis, and drug responsiveness in BC and in other cancers (11-14).

In this study, we have profiled miRNA expression on a large scale in a set of BC tissue samples and adjacent normal bladder tissue samples from patients with BC, and have identified some expression signatures consisting of three or four-miRNA that can distinguish BC from normal controls with high sensitivity and high specificity. All these identified signatures contain hsa-miRNA-133a thus indicating the importance of hsa-miRNA-133a in the tumorigenesis, diagnosis, and prognosis of BC.

# Methods

## Collection of BC tissue samples:

The tissue samples used in this study were collected in the Department of Urology, Zhongshan Hospital Fudan University from May to November 2016. The BC tissue samples and the matched adjacent normal bladder tissue samples (as normal controls) from patients with BC were confirmed by pathologists with microscopy.

All tissue samples were immediately frozen in liquid nitrogen after being removed from the body and stored at a -80°C freezer for long term storage. The collected tissue samples were divided into two groups: a training group, and a blind test group. The training group contains 19 BC tissue samples and 19 matched adjacent normal bladder tissue samples. The blind testing group contains 11 BC tissue samples and 10 matched adjacent normal bladder tissue samples.

A written informed consent was obtained from each patient involved in the study, and the study was approved by the Ethics Committee of Zhongshan Hospital Fudan University (No. B2016-148R). The demographics and clinical features of the patients are listed in Table 1.

## miRNA isolation and miRNA pool generation

Total RNA was isolated from < 50 mg of frozen tissue samples with the miRNeasy Mini Kit (Qiagen, 217004) according to the manufacturer's instructions.

A pool of BC miRNA and a pool of normal miRNA were generated by mixing equal amount of each miRNA isolated from the 19 BC tissue samples in the training group and by mixing equal amount of each miRNA isolated from the 19 matched adjacent normal bladder tissue samples in the training group, respectively.

## cDNA synthesis:

The purified total RNA was diluted to 125 ng/ml with 0.1x RNA storage buffer (Ambion, USA) containing 0.1% Tween-20 (Sigma, USA). miRNAs were added a poly(A) tail and reverse transcripted into cDNA using Sharpvue miRNA First Strand Kit (Biovue, Shanghai, China) per the instructions in the kit.

## Quantitative real-time PCR assay:

The real-time PCR was performed with the synthesized cDNA, Sharpvue 2x Universal qPCR Master Mix (Biovue, Shanghai, China), and Sharpvue™ Human miRNA Primer Array (Biovue, Shanghai, China) according to manufacturer's protocol by using ABI 7900HT Fast Real Time PCR System (Applied Biosystems, USA). The real-time PCR reaction was incubated at 95°C for 2 minutes, followed by 3 cycles of 96°C for 5 seconds and 60°C for 1 minute, 37 cycles of 96°C for 5 seconds and 60°C for 30 seconds, and a melting curve was recorded at the end. SYBR dye and Rox dye were used as reporter and reference, respectively.

### BC miRNA panel:

Expressions of the BC miRNA pool and the normal miRNA pool were measured with primers for detecting 1900 miRNAs and controls in five 384-well plates. Each plate contains primers for detecting 380 miRNAs, 2 endogenous controls (hsa-7SL-scRNA and has-RNU48), one spiking control (cel-miR-39) and one no template control （nuclease free water）. We selected 380 differential expressed miRNAs between the BC miRNA pool and the normal miRNA pool, and used the primers for detecting the selected 380 miRNAs and controls to generate one 384-well panel. This 384-well panel was used to profile miRNA expression of each individual sample in the training group and in the blind testing group.

## Results

### Biomarker selection based on qRT-PCR

The analysis of miRNA expression data from real-time PCR was performed by using R and package e1071 with some modifications as the following: First, set Ct value as 32 to any miRNAs if their measured Ct values were greater than 32. Then, we compared the mean Ct-value of each miRNA between tumor tissue and normal control tissue for 365 microRNAs (15 miRNAs without expression were removed from analysis), 2 endogenous controls. There are many miRNAs with significant Ct value change between BC tissue and normal control tissue (data no shown). We did a T-test for each miRNA, and 70 miRNAs with P-value < 0.001 were shown in Fig. 1.

In order to prove there are tumor-related markers and to find out these markers, we trained an SVM model, and predicted 20 other samples. The model was built as the following: a). we produced 66430 new variables by computing the difference between Ct of each tow miRNAs and got 66796 total variables with the original 366 variables. b). we did a T-test on all these variables and took 2 variables with the greatest P-value and some variables with the smallest P-value. Until we had got 20 candidate miRNAs those variables included. c). for each miRNA subset with markers less than or equal to 12 of the 20 candidate miRNAs, we trained an SVM model with default parameters by function SVM and evaluated the accuracy by 50 times 10-fold cross-validation. d) Then 10 subsets with the best accuracy and AUC were selected and 10 SVMs based on these 10 marker subsets were trained by a total of 38 samples and the stat of 21

blind samples was predicted. They all showed good performances on blind samples with accuracy greater than 0.95. The best one was selected for further analysis.

### miRNA expression profiling in BC and normal controls

To identify BC-specific miRNA expression signatures as biomarkers to diagnose BC, we applied the panel to profile miRNA expression of 38 tissue samples in the training group, including 19 BC tissue samples and 19 matched adjacent normal bladder tissue samples. The 365 dysregulated microRNAs and two endogenous controls in BC tissue samples and in matched adjacent normal bladder tissue samples were presented in the supplemental Table 1. There were 121 microRNAs, including miR-182, miR-431, miR-183, miR-429, and miR-425, etc., with higher expression levels in the BC tissues than in the adjacent normal bladder tissues. In contrast, 245 microRNAs, such as miR-1, miR-133a, miR-133b, miR-125b, miR-143, and miR-145, etc, had lower expression levels in the BC tissues than in the adjacent normal bladder tissues. The aberrant expression levels of miRNAs were summarized in Table 2.

### Developing miRNAs expression signatures in the diagnosis of BC

An unpaired T-test ($p< 0.05$) with a Benjamini Hochberg FDR multiple testing correction was used to identify significantly dysregulated miRNAs that distinguish BC from normal controls. Accurate classification of BC patients from normal controls is crucial for successful BC treatment; we investigated the diagnostic value of the miRNA-expression profile in BC patients. Among the checked 70 significant miRNAs (P-value < 0.001) (these miRNAs listed in Table 2 with t-value and p-value), several expression signatures of three or four-miRNAs were developed as predictors of BC from normal controls. These signatures were selected based on a machine learning approach using support vector machine (SVM).

The 10 best groups of miRNA signatures were listed in Table 3. In the group 1, the expression of miR-133a ($log_2FC = 9.258$; $P < 0.0001$) was significantly down-regulated in BC, and the expression of miR-431 ($log_2FC = -3.268$; $P < 0.0001$) was significantly upregulated  in BC However, the expression level of miR-4251 ($P > 0.1$) in BC was slightly higher than that in normal controls. The results indicated that the use of an improved comparative Ct method may be an easily approach with potential for general clinical use and without the need for large-scale, high-throughput profiling analyses. The method could be used to develop clinically useful signatures based on tissue biomarkers (Supplemental Fig. 1).

### Prediction of BC and control subjects by risk score analysis

To verify the accuracy and specificity of the identified miRNA signatures as BC biomarkers, we further assessed the 3 miRNAs in the former set consisting of 38 samples, including 19 BC tissue samples and

19 matched adjacent normal bladder tissue samples (Fig. 2A). The areas under the ROC curve (AUC) were 1 with 100% sensitivity and 100% specificity, respectively (Fig.2B).

### Blind test

To verify the accuracy and specificity of identified miRNA signatures as BC biomarkers, we further tested another 21 samples (including 11 BC tissue samples and 10 adjacent normal bladder tissue samples) in a blind fashion to validate the predictive ability of these miRNA-based signatures in BC diagnosis. The accuracy of the signature consisting of hsa-miR-133a, hsa-miR-431 and hsa-miR-4251 is 95.2% with 100% sensitivity and 90% specificity, respectively (Fig. 3). All 11 BC tissue samples in the blind testing group were predicted correctly. Especially, of these 11 samples, 10 samples were confirmed as stage I BC and 1 sample was confirmed as stage IV BC by pathology and imaging, thus demonstrating the power of the miRNA-based signatures in distinguishing BC from normal controls.

## Discussion

In order to find tissue-based BC-specific miRNA signatures, a comparative study was performed using a qRT-PCR array platform to profile 19 BC tissue samples and 19 matched adjacent normal bladder tissue samples from patients with BC.

The study revealed that 70 aberrant miRNAs (Table 2), hsa-miR-96, hsa-miR-182, hsa-miR-183, hsa-miR-429, hsa-miR-425, hsa-miR-431 are overexpressed in BC comparing to the normal controls. Among these miRNAs, miR-96, miR-182 and miR-183 are clustered in one locus of chromosome 7 (15). miR-429 belongs to the miR-200 family, which is clustered in the chromosomes 12, we further found that other members (200a/b/c) of miR200 family are also overexpressed in BC compared with in normal controls (Supplement Table 1). Both two miRNA clusters are well-known oncogenic miRNA clusters that have been extensively reported to be involved in tumor genesis in ovarian cancer and in other cancers including in BC (16-19).

The ten best groups of miRNAs signatures have been selected to discriminate the BC from the normal controls with 100% sensitivity and 100% specificity, suggesting their potential value for the diagnosis and prognosis of BC (Table 3, Fig. 3). These selected signatures all contain hsa-miR-133a that means hsa-miR-133a may play a key role in discriminating BC from normal controls. The number one signature contains three miRNAs, hsa-miR-133a, hsa-miR-431 and hsa-miR-4251. Hsa-miR-133a was significantly down-regulated and hsa-miR-431 was significantly up-regulated in BC. However, the expression level of hsa-miR-4251 was slightly higher in BC than that in normal controls. Furthermore, these signatures can predict BC with significantly high accuracy over 95% in a blind test. More importantly, these miRNA signatures could effectively distinguish early-stage (25 cases in stage I) BC from normal controls, suggesting their potential value in the detection of BC at an early stage.

A previous study (20) has reported the expression levels of miR-133a, miR-133b, miR-1, and miR-99a are down-regulated, however, miR-182 is up-regulated in BC. These miRNAs might be involved in the tumorigenesis and deterioration of BC. Our results confirmed the previous findings, and further demonstrated that the powers to distinguish BC from normal control of the selected signatures containing miR-133a are significantly better than that of miR-133a alone (Supplemental Fig.2). The signatures containing miR-133a identified in our study can diagnose BC even at an early stage with high sensitivity and high specificity and could be used as biomarkers in the diagnosis and prognosis of BC.

It has been shown that miR-133a-3p acts as a tumor suppressor in BC.Numerous studies demonstrated that miR-133a inhibits cell proliferation, migration, and invasion in various tumors by targeting to different signaling pathways, such as caspase signaling pathway, insulin/IGF signaling pathway, and EGFR signaling pathway (21-28). The connecting between the importance of miR-133a in tumorigenesis and significant downregulation of miR-133a in BC has shed light on the molecular mechanism of miRNA-133a in the tumorigenesis of BC.

Cystoscopy is the gold standard for diagnosis and surveillance of BC, while the procedure is invasive, uncomfortable and costly. Many potential BC biomarkers, like protein, miRNA, mRNA, DNA methylation, miRNA and miRNA signature have been reported, however, their successful applications in the diagnosis and prognosis of BC have not been demonstrated yet. As reported from other investigators (29, 30), our results also showed that miR-1, miR-133a, miR-133b, miR-143, miR-145, and miR-10b, are downregulated in BC (Supplement Table 1), suggesting a major role of these tumor suppressor miRNAs in bladder carcinoma. Of all these downregulated miRNAs, the miR-133a is the only one miRNA shown up in all ten best BC diagnosis signatures (Table 3).

miR-133a was one member of the miR-133 family, which was first experimentally characterized in mice (31). miR-133 and miR-1 are clustered in the same chromosomal locus in the human genome (18q11.2) and share the same transcriptional unit, which has shown their essential functions in controlling skeletal muscle proliferation and differentiation (32). Genes encoding miR-133 (miR-133a-1, miR-133a-2 and miR-133b) are transcribed as bicistronic transcripts together with miR-1-2 and miR-1-1. We had analyzed the potential target gene and the function of miR-133a by using www.mirnet.ca. We recognized that there is a correlation between miR-133a and miR-1 by targeting TAGLN2 and LASP1 (30, 33). In agreement with our study, miR-133a has been reported to be down-regulated in BC and in other cancers (30, 34-36). miR-133a seems to function as a tumor suppressor by inhibiting cell proliferation, invasion, migration, and apoptosis (30, 33).

# Conclusions

The BC-specific miRNA expression signatures containing miR-133a identified in our study could be used as biomarkers in the diagnosis and prognosis of BC.

# Abbreviations

BC: bladder cancer

SVM ：Support vector machine

AUC ：Areas under the ROC curve

# Declarations

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated in this study are included in this article and its supplementary documents.

### Ethics Approval and Consent to Participate

The study plan was approved by the ethics committee institute: Zhongshan Hospital Fudan University (Shanghai, China) ethics committee. Approval reference No. B2016-148R

A written informed consent was obtained from each patient involved in the study, respectively,

### Competing interests

No potential conflict of interest relevant to this article is reported

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### Authors' contributions

S.J. and J.G. study planning; S.J, M.Z., Z.X., and F.Z. experiment design and operation; S.J., M.Z., J.H., H.W., L.L. and HL. W. data analysis and result interpretation; S.J, M.Z., Z.X. and F.Z. figure preparation; S.J., M.Z., T.T., J.G., Z.L., R.T., and J.G. manuscript preparation and revision. All authors approved the final version of this manuscript.

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# Tables

**Table 1: Demographic and Clinical Characteristics of Participants.**

| | Adjacent (n=19) | | Validation | |
|---|---|---|---|---|
| Demographic features | Adjacent (n=19) | Cancer (n=19) | Adjacent (n=10) | Cancer (n=11) |
| Average age (range) | 73.0 (47-90) | | 61.4 (46-77) | 66.1 (46-88) |
| Gender | | | | |
| Male | 16 | | 9 | 10 |
| Female | 13 | | 1 | 1 |
| pT | | | | |
| Tis | 1 | | 0 | |
| Pt1 | 15 | | 10 | |
| Pt2 | 2 | | 0 | |
| Pt3 | 1 | | 0 | |
| Pt4 | 0 | | 1 | |
| pN | | | | |
| pN0 | 19 | | 11 | |
| pN+ | 0 | | 0 | |
| pM | | | | |
| pM0 | 19 | | 11 | |
| pM+ | 0 | | 0 | |
| Grade | | | | |
| High Grade | 9 | | 5 | |
| Low Grade | 10 | | 6 | |
| Multifocality | | | | |
| Yes | 9 | | 4 | |
| No | 10 | | 7 | |

Abbreviation: Tis, Tumor in situ.

**Table 2: 70 aberrant miRNAs with P-value < 0.001.**

| miR name | Mean Cancer ct | Mean Normal ct | $Log_2$ FC | Fold Change | T-value | P-value |
|---|---|---|---|---|---|---|
| hsa-miR-1 | 31.11 | 26.06 | 5.05 | 33.14 | 10.0188 | 6.09E-12 |
| hsa-miR-30a | 25.79 | 22.57 | 3.21 | 9.28 | 9.7216 | 2.11E-11 |
| hsa-miR-133a | 27.60 | 24.39 | 3.21 | 9.26 | 8.9519 | 2.00E-10 |
| hsa-miR-143# | 28.65 | 25.03 | 3.62 | 12.28 | 8.4715 | 4.67E-10 |
| hsa-miR-4328 | 25.12 | 21.03 | 4.09 | 16.99 | 8.7692 | 6.55E-10 |
| hsa-miR-143 | 24.10 | 19.72 | 4.37 | 20.75 | 8.1777 | 1.27E-09 |
| hsa-miR-145 | 22.99 | 18.26 | 4.73 | 26.46 | 8.3910 | 2.74E-09 |
| hsa-miR-376c | 28.97 | 25.37 | 3.60 | 12.13 | 7.9053 | 3.84E-09 |
| hsa-miR-125b | 25.11 | 20.28 | 4.82 | 28.32 | 8.0892 | 5.55E-09 |
| hsa-miR-199b-3p | 25.03 | 20.71 | 4.32 | 19.97 | 8.4551 | 5.84E-09 |
| hsa-miR-100 | 27.55 | 22.96 | 4.59 | 24.08 | 7.9632 | 1.27E-08 |
| hsa-miR-99a | 28.15 | 23.36 | 4.79 | 27.71 | 8.1981 | 1.28E-08 |
| hsa-miR-497 | 27.11 | 24.03 | 3.08 | 8.48 | 7.5693 | 1.69E-08 |
| hsa-miR-376a | 30.03 | 26.43 | 3.61 | 12.17 | 7.7034 | 1.90E-08 |
| hsa-miR-199a-5p | 26.23 | 22.07 | 4.16 | 17.87 | 7.3058 | 2.21E-08 |
| hsa-miR-195 | 26.30 | 23.15 | 3.15 | 8.86 | 6.8696 | 5.41E-08 |
| hsa-miR-154 | 29.72 | 26.57 | 3.15 | 8.86 | 7.2751 | 9.96E-08 |
| hsa-miR-139-5p | 28.73 | 26.55 | 2.18 | 4.54 | 6.6091 | 1.13E-07 |
| hsa-miR-127-3p | 27.45 | 25.11 | 2.35 | 5.09 | 6.3966 | 2.33E-07 |
| hsa-miR-150 | 27.99 | 24.13 | 3.86 | 14.55 | 6.3678 | 2.52E-07 |
| hsa-miR-152 | 28.05 | 25.07 | 2.97 | 7.84 | 6.7012 | 2.57E-07 |
| hsa-miR-379 | 31.49 | 29.14 | 2.35 | 5.11 | 6.3446 | 2.63E-07 |
| hsa-miR-140-3p | 28.02 | 25.45 | 2.57 | 5.94 | 6.0036 | 6.91E-07 |
| hsa-miR-136# | 30.24 | 26.85 | 3.39 | 10.45 | 6.0200 | 8.19E-07 |
| hsa-miR-377 | 29.34 | 25.85 | 3.49 | 11.22 | 6.7306 | 8.48E-07 |
| hsa-miR-495 | 30.61 | 27.90 | 2.71 | 6.54 | 6.3051 | 9.28E-07 |
| hsa-miR-136 | 28.45 | 25.06 | 3.39 | 10.49 | 6.1732 | 9.73E-07 |
| hsa-miR-101 | 23.47 | 21.50 | 1.97 | 3.91 | 5.6021 | 2.47E-06 |
| hsa-miR-133b | 29.08 | 27.03 | 2.06 | 4.16 | 5.7448 | 2.57E-06 |
| hsa-miR-337-3p | 28.82 | 26.64 | 2.18 | 4.54 | 5.7657 | 2.90E-06 |
| hsa-miR-29a | 23.15 | 20.63 | 2.52 | 5.72 | 5.8648 | 2.91E-06 |
| hsa-miR-574-3p | 25.23 | 23.35 | 1.88 | 3.67 | 5.5423 | 3.96E-06 |
| hsa-miR-130a | 25.99 | 23.45 | 2.54 | 5.81 | 5.7732 | 4.45E-06 |
| hsa-miR-342-3p | 25.23 | 23.15 | 2.08 | 4.24 | 5.6557 | 5.05E-06 |
| hsa-miR-126# | 24.90 | 22.86 | 2.05 | 4.13 | 5.1135 | 1.24E-05 |
| hsa-miR-222 | 25.34 | 23.36 | 1.97 | 3.92 | 5.0994 | 1.58E-05 |
| hsa-miR-28-3p | 26.90 | 25.61 | 1.29 | 2.44 | 4.7988 | 2.87E-05 |
| hsa-miR-365b-3p | 25.38 | 24.10 | 1.29 | 2.44 | 4.8883 | 3.49E-05 |
| hsa-miR-27b | 24.19 | 22.41 | 1.79 | 3.45 | 4.7126 | 3.89E-05 |
| hsa-let-7c | 26.41 | 24.06 | 2.35 | 5.10 | 4.6752 | 4.04E-05 |
| hsa-miR-342-5p | 30.97 | 29.44 | 1.53 | 2.88 | 4.6693 | 5.39E-05 |
| hsa-miR-431 | 25.68 | 27.39 | -1.71 | -3.27 | -4.4787 | 8.06E-05 |
| hsa-miR-320c | 24.70 | 23.21 | 1.49 | 2.81 | 4.5258 | 8.33E-05 |
| hsa-miR-182 | 26.70 | 28.78 | -2.08 | -4.22 | -4.4854 | 9.35E-05 |
| hsa-miR-551b | 28.47 | 25.33 | 3.14 | 8.81 | 4.4619 | 9.38E-05 |
| hsa-miR-543 | 31.08 | 29.69 | 1.39 | 2.61 | 4.4079 | 9.56E-05 |
| hsa-miR-193b | 24.15 | 23.08 | 1.07 | 2.10 | 4.2799 | 1.39E-04 |
| hsa-miR-451 | 24.01 | 21.90 | 2.11 | 4.32 | 4.2547 | 1.45E-04 |
| hsa-miR-1247 | 23.67 | 22.36 | 1.31 | 2.48 | 4.2604 | 1.50E-04 |
| hsa-miR-140-5p | 30.30 | 28.29 | 2.01 | 4.01 | 4.2463 | 1.56E-04 |

| | | | | | | |
|---|---|---|---|---|---|---|
| hsa-miR-142-5p | 27.09 | 24.54 | 2.55 | 5.87 | 4.2429 | 1.56E-04 |
| hsa-miR-126 | 23.02 | 21.17 | 1.85 | 3.61 | 4.1344 | 2.10E-04 |
| hsa-miR-10b | 26.22 | 24.68 | 1.54 | 2.90 | 4.1934 | 2.28E-04 |
| hsa-miR-369-3p | 30.14 | 28.35 | 1.80 | 3.47 | 4.1931 | 2.57E-04 |
| hsa-miR-328 | 26.89 | 25.82 | 1.07 | 2.10 | 4.0529 | 2.64E-04 |
| hsa-miR-338-3p | 28.04 | 25.79 | 2.25 | 4.75 | 4.0157 | 2.89E-04 |
| hsa-miR-183 | 26.64 | 28.48 | -1.84 | -3.57 | -3.9608 | 3.49E-04 |
| hsa-miR-532-5p | 28.10 | 27.06 | 1.04 | 2.06 | 3.8482 | 4.68E-04 |
| hsa-miR-409-3p | 29.55 | 27.97 | 1.58 | 2.99 | 3.8801 | 5.41E-04 |
| hsa-miR-24 | 23.50 | 21.96 | 1.54 | 2.90 | 3.7752 | 5.87E-04 |
| hsa-miR-132 | 28.07 | 26.45 | 1.63 | 3.09 | 3.8190 | 5.97E-04 |
| hsa-miR-30e# | 26.88 | 25.28 | 1.60 | 3.02 | 3.7311 | 6.74E-04 |
| hsa-miR-429 | 23.52 | 25.04 | -1.51 | -2.86 | -3.7180 | 6.81E-04 |
| hsa-let-7i# | 26.96 | 25.52 | 1.44 | 2.70 | 3.7037 | 7.65E-04 |
| hsa-miR-142-3p | 25.15 | 22.34 | 2.81 | 7.01 | 3.6692 | 7.95E-04 |
| hsa-miR-221 | 27.09 | 25.53 | 1.56 | 2.96 | 3.7036 | 7.97E-04 |
| hsa-miR-4457 | 27.52 | 25.90 | 1.62 | 3.07 | 3.6927 | 8.31E-04 |
| hsa-miR-186 | 25.53 | 24.57 | 0.96 | 1.95 | 3.6399 | 8.80E-04 |
| hsa-miR-425 | 26.33 | 27.34 | -1.01 | -2.02 | -3.6236 | 9.07E-04 |
| hsa-miR-155 | 29.47 | 27.46 | 2.01 | 4.03 | 3.6076 | 9.51E-04 |

Mean Normal Ct = qPCR Ct value of miRNA in adjacent normal bladder tissues; Mean Cancer Ct = qPCR Ct value of miRNA in bladder cancer tissues; FC = fold change; $Log_2FC > 0$ means the expression level is decreased in cancer tissues, $Log_2FC < 0$ means the expression level is increased in cancer tissues.

**Table 3: The 10 best selected groups of miRNAs signatures to discriminate BC from normal controls.**

| miRNA signatures groups | marker1 | marker2 | marker3 | marker4 | Accuracy | AUC |
|---|---|---|---|---|---|---|
| 1 | hsa-miR-133a | hsa-miR-431 | hsa-miR-4251 | | 1 | 1 |
| 2 | hsa-miR-133a | hsa-miR-296-3p | hsa-miR-4251 | | 1 | 1 |
| 3 | hsa-miR-133a | hsa-miR-10b | hsa-miR-4251 | | 1 | 1 |
| 4 | hsa-miR-133a | hsa-miR-449b | hsa-miR-484 | | 1 | 1 |
| 5 | hsa-let-133a | hsa-miR-7a# | hsa-miR-431 | hsa-miR-4251 | 1 | 1 |
| 6 | hsa-let-133a | hsa-miR-449b | hsa-miR-7a# | hsa-miR-4251 | 1 | 1 |
| 7 | hsa-miR-133a | hsa-miR-449b | hsa-miR-1260 | hsa-miR-4251 | 1 | 1 |
| 8 | hsa-miR-133a | hsa-miR-449b | hsa-miR-296-3p | hsa-miR-4251 | 1 | 1 |
| 9 | hsa-miR-133a | hsa-miR-449b | hsa-miR-484 | hsa-miR-4251 | 1 | 1 |
| 10 | hsa-miR-133a | hsa-miR-181c# | hsa-miR-326 | hsa-miR-4251 | 1 | 1 |

## Supplemental File Legends

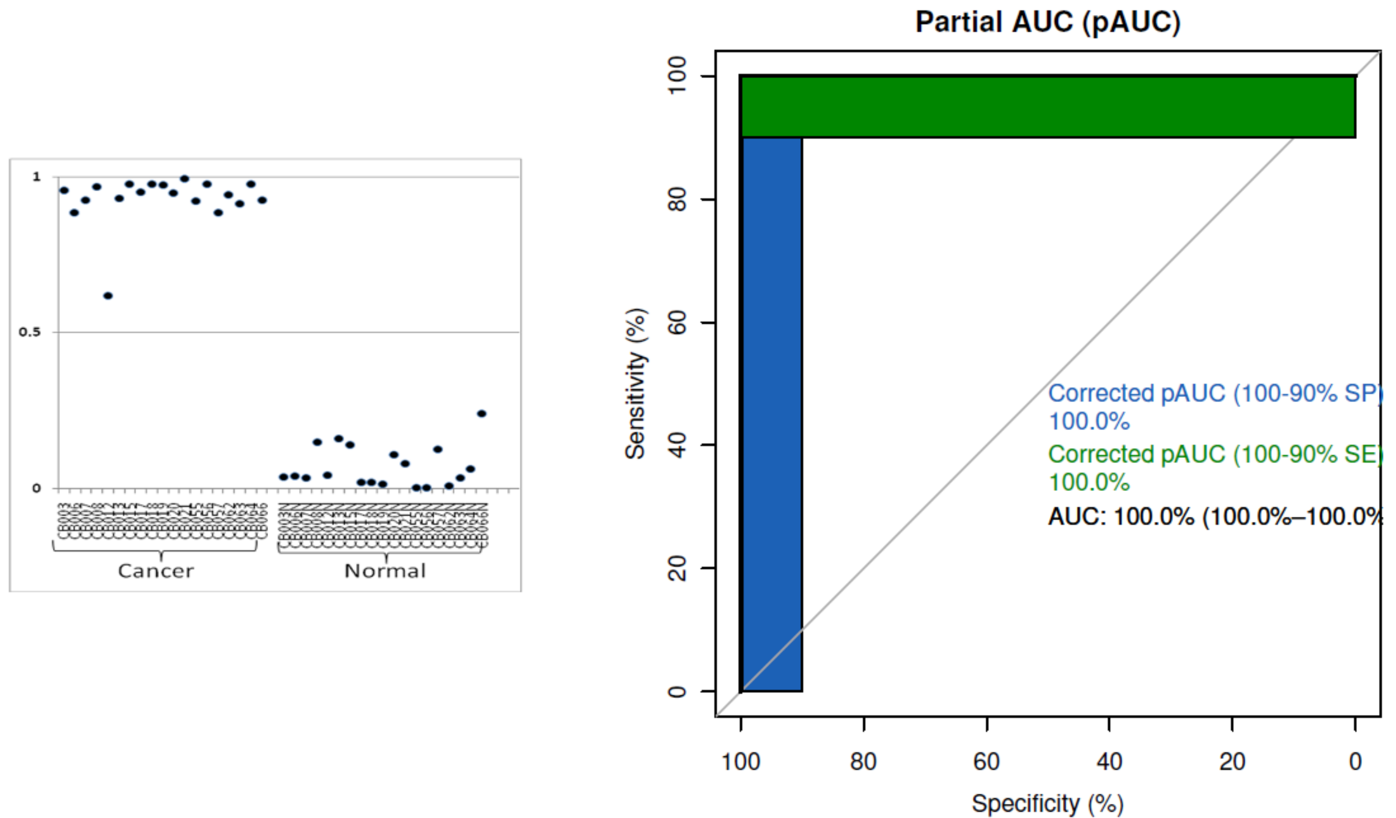
Additional information in the supporting documents of this article may be found online:

**Supplemental Table 1**. The expression levels of 365 dysregulated miRNAs and two endogenous controls in the BC tissues compared with those in adjacent normal bladder tissues. Mean Normal Ct = qPCR Ct value of miRNA in adjacent normal bladder tissues; FC = fold change; FC > 0 means expression level is decreased in BC tissues compared with adjacent normal bladder tissues; FC < 0 means expression level is increased in BC tissues compared with in adjacent normal bladder tissues.

**Supplemental Fig. 1**. Top 24 Expression Profiling of Select each 2 miRNAs ratio between BC (n= 19, red) and adjacent normal bladder tissues (n= 19, blue). The scale at the y-axis represents Ct values normalized to the global mean. Line inside the box: median. The expression levels of miR-182, miR-183, miR-431, miR-429 were significantly up-regulated in BC, and the expression levels of miR-133a, miR-125b, miR-1 were significantly down regulated in BC.

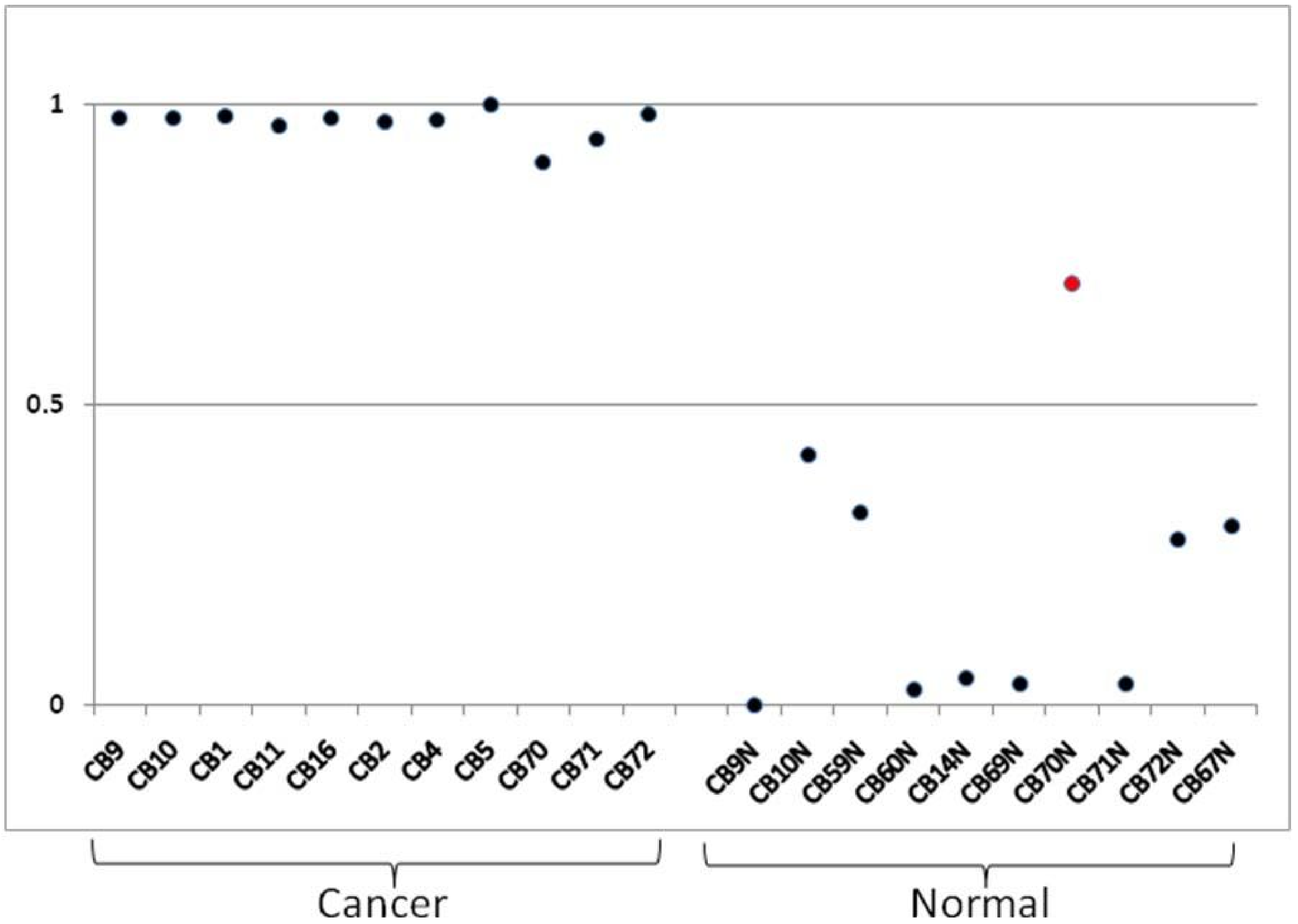
**Supplemental Fig.2.** The distributions of predicted scores of miR-133a alone and the 10 best-selected signatures. Column 0 represents the distribution of predicted score of miR-133a alone, columns 1~10 represent the distributions of predicted scores of the 10 best-selected signatures respectively.

# Figures




### Figure 1

A) The performances of the selected signature group one in classifying 19 of BC tissue samples from the 19 matched adjacent normal bladder tissue samples. SVM prediction probability for 38 samples with an error of 0. BC tissue samples and the matched adjacent normal bladder tissue samples were separated by SVM score, Cancer > = 0.5, Normal < 0.5. B) The receiver operating characteristic curve (area under the curve = 1) estimation for the miRNA panel in the BC and the normal controls.




## Figure 2

Validation of BC miRNA Expression Signature Model 1 in another 21 samples. 11 bladder cancer tissues and 10 matched adjacent controls were separated by SVM score, Cancer > = 0.5, Normal < 0.5. SVM prediction probability for 21 samples with 1 error (red).