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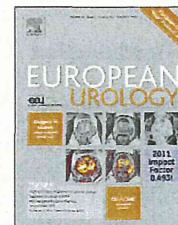
Urothelial Cancer

Methylation of a Panel of MicroRNA Genes Is a Novel Biomarker for Detection of Bladder Cancer

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Article info

Abstract

Background: Dysregulation of microRNAs (miRNAs) has been implicated in bladder cancer (BCa), although the mechanism is not fully understood.

Objective: We aimed to explore the involvement of epigenetic alteration of miRNA expression in BCa.

Design, setting, and participants: Two BCa cell lines (T24 and UM-UC-3) were treated with 5-aza-2′-deoxycytidine (5-aza-dC) and 4-phenylbutyric acid (PBA), after which their miRNA expression profiles were analyzed using a TaqMan array (Life Technologies, Carlsbad, CA, USA). Bisulfite pyrosequencing was used to assess miRNA gene methylation in 5 cancer cell lines, 83 primary tumors, and 120 preoperative and 47 postoperative urine samples.

Outcome measurements and statistical analysis: Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of the miRNA gene panel.

Results and limitations: Of 664 miRNAs examined, 146 were upregulated by 5-aza-dC plus PBA. CpG islands were identified in the proximal upstream of 23 miRNA genes, and 12 of those were hypermethylated in cell lines. Among them, miR-137, miR-124-2, miR-124-3, and miR-9-3 were frequently and tumor-specifically methylated in primary cancers (miR-137: 68.7%; miR-124-2: 50.6%; miR-124-3: 65.1%; miR-9-3: 45.8%). Methylation of the same four miRNAs in urine specimens enabled BCa detection with 81% sensitivity and 89% specificity; the area under the ROC curve was 0.916. Ectopic expression of silenced miRNAs in BCa cells suppressed growth and cell invasion.

Conclusions: Our results indicate that epigenetic silencing of miRNA genes may be involved in the development of BCa and that methylation of miRNA genes could be a useful biomarker for cancer detection.

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1. Introduction

MicroRNAs (miRNAs) are a group of small noncoding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs. In that way, they play important roles in a wide array of biologic processes, including cell proliferation, differentiation, and apoptosis [1]. Increasing evidence suggests that dysregulation of miRNA expression contributes to the initiation and progression of human cancer [2,3]. Altered miRNA expression is thought to play an important role in the pathogenesis of bladder cancer (BCa) and in certain tumor phenotypes. For instance, high-grade BCa exhibits upregulation of several miRNAs, including miR-21, which suppresses p53 function [4]. In addition, miR-21-to-miR-205 expression ratios are elevated in invasive BCa cells [5], while miR-200 family members regulate epithelial-to-mesenchymal transition by targeting transcription repressors ZEB1 and ZEB2 in BCa cells [6].

Although the mechanisms underlying miRNA dysregulation in cancer are not yet fully understood, recent studies have shown that the silencing of several miRNAs is tightly linked to epigenetic mechanisms, including histone modification and DNA methylation [7,8]. For example, treatment with a histone deacetylase (HDAC) inhibitor and a DNA methyltransferase (DNMT) inhibitor restored expression of various miRNAs in cancer cells [7,9], and the list of miRNA genes methylated in cancer is rapidly growing [10]. Studies have also shown that restoration of epigenetically silenced miRNAs may be an effective strategy for treating cancer and that aberrant methylation of miRNA genes could be a useful biomarker for cancer detection [10,11]. In addition, it was recently shown that the silencing of miRNA expression in BCa is associated with DNA methylation, often involving the CpG island (CGI) or CpG shore [12,13]. In an effort to identify novel biomarkers and treatment targets in BCa, we aimed to identify miRNAs epigenetically silenced in BCa cells by screening for miRNAs whose expression is upregulated by DNA demethylation and HDAC inhibition. We also investigated the methylation of miRNA genes in urine specimens and assessed its clinical usefulness as a biomarker for detection of BCa.

2. Materials and methods

2.1. Cell lines and tissue samples

BCa cell lines (T24, UM-UC-3, HT-1197, HT-1376, SW780, and 5637) and a normal urothelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Supplementary Table 1). A colorectal cancer cell line HCT116 harboring genetic disruptions within the DNMT1 and DNMT3B loci (DNMTs KO) have been described previously [8]. T24 and UM-UC-3 cells were treated first with 1 μM or 0.1 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich, St Louis, MO, USA) for 72 h, and then with 3 mM 4-phenylbutyric acid (PBA; an HDAC inhibitor, Sigma-Aldrich) for 72 h, replacing the drug and medium every 24 h. A total of 83 primary BCa specimens were collected from patients who underwent radical cystectomy (RC) or transurethral resection of bladder tumor (TURBT; 66 males and 17 females; median age: 72 yr; range: 34–90 yr). Of the 83 patients, 73 underwent surgical resection after initial diagnosis, 7 received chemotherapy before surgery, and 3 are recurrent cases. Samples of nontumorous bladder tissue adjacent (<2 cm) to and distant (>2 cm) from the tumors were also collected. Six samples of normal urothelial tissue from renal cell carcinoma (RCC) patients who underwent nephrectomy were also collected. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the institutional review board. Total RNA was extracted using a *mir*Vana miRNA isolation kit (Life technologies, Carlsbad, CA, USA). Genomic DNA was extracted using the standard phenol-chloroform procedure.

2.2. Urine samples

Voided urine specimens were collected from 20 cancer-free individuals (Supplementary Table 2) and 86 BCa patients. In addition, postoperative voided urine samples were collected from 36 of the 86 patients 3–10 d after TURBT treatment. As an independent test set, preoperative urine samples were collected from 34 BCa patients, and postoperative samples were collected from 11 patients. The postoperative urine samples were collected from patients in whom tumors were successfully resected without leaving residual tumors. The urine (10 ml) was mixed with 5 ml of ThinPrep PreservCyt solution (Hologic, Bedford, MA, USA) and stored at 4 °C. Each sample was centrifuged at 3000 rpm for 10 min, and genomic DNA was extracted from the pelleted sediment using the standard phenol-chloroform procedure.

2.3. MicroRNA expression profiling

Expression of 664 miRNAs was analyzed using a TaqMan MicroRNA array v2.0 (Life Technologies). Briefly, 1 μg of total RNA was reverse-transcribed using a Megaplex Pools kit (Applied Biosystems, Foster City, CA, USA), after which miRNAs were amplified and detected using polymerase chain reaction (PCR) with specific primers and TaqMan probes. U48 snRNA (RNU48, Life Technologies) served as an endogenous control.

2.4. Quantitative real-time polymerase chain reaction of miRNA

Expression of selected miRNAs was analyzed using TaqMan microRNA assays. Briefly, 5 ng of total RNA were reverse-transcribed using specific stem-loop real-time primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. U6 snRNA (RNU6B, Life Technologies) served as an endogenous control.

2.5. Methylation analysis

Bisulfite conversion of genomic DNA, methylation-specific PCR (MSP), bisulfite sequencing, and bisulfite pyrosequencing were carried out as described previously [8]. Primer sequences and PCR product sizes are listed in Supplementary Table 3. Primer locations for methylation analysis are shown in Supplementary Figure 1.

2.6. Transfection of microRNA precursor molecules

BCa cells (1 × 10⁶ cells) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Life Technologies) or Pre-miR miRNA Molecules Negative Control #1 using a Cell Line Nucleofector kit R (Lonza, Basel, Switzerland) with a Nucleofector I electroporation device (Lonza) according to the manufacturer's instructions. The viability of the miRNA precursor transfectants was analyzed using water-soluble tetrazolium salt (WST) assays [8]. Cell invasion was assessed using Matrigel invasion assays [8].

2.7. Gene expression microarray analysis

One-color microarray-based gene expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Briefly, 100 ng of total RNA were amplified and labeled using a Low-input Quick AmpLabeling Kit One-color (Agilent Technologies), after which the synthesized cRNA was hybridized to a SurePrint G3 Human GE microarray (G4851F; Agilent Technologies). The microarray data were then analyzed using GeneSpring GX version 11 (Agilent Technologies). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE41760.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 4.0 statistical software (GraphPad Software, La Jolla, CA, USA). Quantitative variables were analyzed using a Student t test and one-way analysis of variance (ANOVA) with a post hoc Tukey test. Fisher exact test was used for analysis of categorical data. The Pearson correlation coefficient was used to evaluate correlations between continuous data. Receiver operating characteristic (ROC) curves for the diagnosis of BCa were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cut-off value for each miRNA gene was defined as the point on the ROC curve closest to the upper left corner. A diagnostic scoring system using urinary DNA methylation was constructed by analyzing the training set using the following three-step algorithm: (1) The methylation status of miRNA genes was assessed using the respective cut-off values; (2) the number of methylation-positive genes was determined, which we termed the *miR-methylation score* (M-score); and (3) the samples were classified into five groups based on the M-score. The value of $p < 0.05$ (two-sided) was regarded as significant.

3. Results

3.1. Identification of epigenetically silenced microRNA genes in bladder cancer

To identify epigenetically silenced miRNAs in BCa, we performed TaqMan array analysis using two BCa cell lines (T24 and UM-UC-3) treated with 1 μM 5-aza-dC plus 3 mM PBA. Of the 664 miRNAs examined, the drug treatment induced upregulation (more than five-fold) of 208 miRNAs in T24 cells and 200 miRNAs in UM-UC-3 cells. Of those, 146 miRNAs were upregulated in both cell lines (**Supplementary Fig. 2 and 3**; **Supplementary Table 4**). We selected 23 miRNA genes that harbored CGIs in the proximal upstream (<5 kb) of their coding regions (**Supplementary Table 5**), and subsequent MSP analysis revealed that the CGIs of 12 were hypermethylated in multiple BCa cell lines (**Fig. 1**A). These miRNAs were also induced by a low dose (0.1 μM) of 5-aza-dC plus PBA, making it unlikely that the observed induction was a secondary effect of DNA damage (**Supplementary Fig. 4**).

We next used bisulfite pyrosequencing to quantitatively analyze the methylation of the 12 miRNA genes showing CGI methylation in a series of BCa tissues ($n = 26$), a sample of normal urothelium tissue, and a normal urothelial cell line (SV-HUC-1). We found that four miRNA genes (miR-137, miR124-2, miR-124-3, and miR-9-3) were frequently methylated in primary tumors, though their methylation levels were limited in normal urothelium (**Fig. 1**B; **Supplementary Fig. 5 and 6**). In addition, we observed a marked
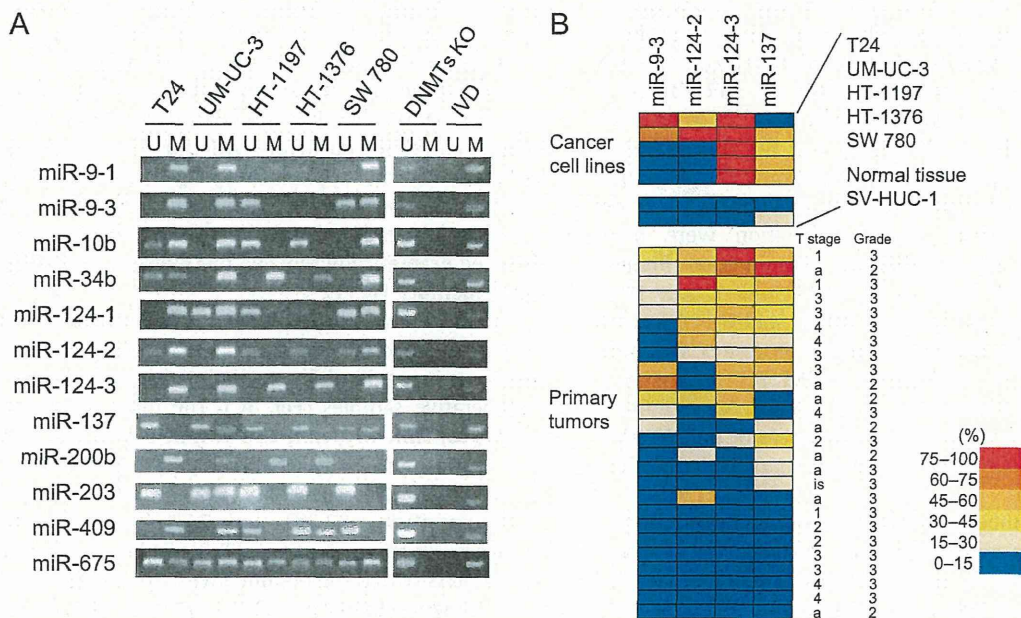

Fig. 1 – Methylation analysis of microRNA (miRNA) genes in bladder cancer (BCa). (A) Methylation-specific polymerase chain reaction (PCR) analysis of the CpG islands of 12 miRNA genes in the indicated cell lines. In vitro methylated DNA and DNA methyltransferase knockout cells served as positive and negative controls, respectively. Bands in the "M" lanes are PCR products obtained with methylation-specific primers; those in the "U" lanes are products obtained with unmethylated-specific primers. (B) Summarized results for the bisulfite pyrosequencing of miRNA genes in BCa cell lines, a sample of normal urothelial tissue, a normal urothelial cell line SV-HUC-1, and a set of primary BCa tissues ($n = 26$). Tumor stages and grades are indicated on the right.
DNMTs KO = DNA methyltransferase knockout cells; IVD = in vitro methylated DNA.

Table 1 – Correlation between microRNA gene methylation and the clinicopathologic features of bladder cancer

	(n = 83)	miR-137 met (%)			miR-124-2 met (%)			miR-124-3 met (%)			miR-9-3 met (%)		
		Mean	SD	p^*	Mean	SD	p^*	Mean	SD	p^*	Mean	SD	p^*
Age, yr:													
Median (range)	72 (34–90)	–	–	0.394	–	–	0.277	–	–	0.147	–	–	0.065
Gender:													
Male	66	26.7	19.9	–	25.1	23.9	–	32.0	24.4	–	19.8	17.4	–
Female	17	34.6	22.1	0.160	22.2	11.1	0.630	31.8	20.6	0.974	17.3	13.0	0.591
T stage:													
Ta	35	31.3	23.4	–	25.1	22.1	–	29.7	25.2	–	21.0	18.5	–
Tis	4	23.5	19.7	–	24.6	28.8	–	22.5	15.4	–	12.7	4.4	–
T1	8	33.4	19.2	–	33.9	29.9	–	42.6	25.2	–	24.9	16.2	–
≥T2	36	24.9	17.8	0.486	21.8	19.3	0.575	32.7	22.1	0.458	17.0	15.3	0.463
Grade:													
1	1	15.3	–	–	1.9	–	–	5.8	–	–	11.2	–	–
2	27	29.3	23.4	–	21.3	18.9	–	28.6	24.6	–	21.3	16.6	–
3	55	28.1	19.3	0.795	26.5	23.2	0.360	34.1	22.9	0.331	18.4	16.8	0.676
LN metastasis:													
N0	73	28.6	21.0	–	24.9	22.4	–	32.4	24.0	–	20.1	17.3	–
N1–N3	10	26.7	17.8	0.782	21.8	18.2	0.685	28.3	20.5	0.605	13.3	7.3	0.231

SD = standard deviation; LN = lymph node; ANOVA = analysis of variance.
* Pearson correlation coefficient, student t test, or ANOVA.

reduction in the methylation levels in BCa cells treated with 5-aza-dC plus PBA, which is consistent with the upregulation of miRNAs (Supplementary Fig. 7).

3.2. Methylation of microRNA genes in primary bladder cancer

We next examined the methylation levels of miR-137, miR-124-2, miR-124-3, and miR-9-3 in a larger set of primary tumors (n = 83), along with adjacent and distant nontumorous bladder tissues from the same patients (Table 1). Elevated levels of miRNA gene methylation (>15.0%) were frequently detected in primary BCa tissues (miR-137: 57 of 83, 68.7%; miR-124-2: 42 of 83, 50.6%; miR-124-3: 54 of 83, 65.1%; miR-9-3: 38 of 83, 45.8%), and the tumor tissues exhibited significantly higher methylation levels than their nontumorous counterparts (Fig. 2A). In addition, we found that levels of miRNA gene methylation were more frequently elevated in adjacent nontumorous bladder tissues (AN; miR-137: 26 of 74, 35.1%; miR-124-2: 19 of 74, 25.7%; miR-124-3: 15 of 74, 20.3%; miR-9-3: 12 of 74, 16.2%) than in more distant nontumorous tissues (DN; miR-137: 18 of 83, 21.7%; miR-124-2: 6 of 83, 7.2%; miR-124-3: 11 of 83, 13.3%; miR-9-3: 9 of 83, 10.8%). No significant correlation was found between the levels of miRNA gene methylation and the clinicopathologic characteristics of the patients (Table 1).

When we examined the methylation status of miR-137 in selected tissue specimens in more detail, we observed dense methylation in tumor tissues but only scattered methylation in nontumorous tissues (Fig. 2B). We then compared the levels of miR-137 expression determined in TaqMan assays with the methylation levels obtained by bisulfite pyrosequencing in selected pairs of tumors and corresponding distant nontumorous tissues (Fig. 2C). We found that there was an inverse relationship between the expression of miR-137 and its methylation, which suggests

that CGI methylation is associated with the downregulation of miR-137 in BCa tissues.

3.3. Detection of microRNA gene methylation in urine samples

To assess the usefulness of miRNA gene methylation, we collected voided urine specimens from 86 BCa patients (Table 2) and 20 cancer-free individuals. Upon performing bisulfite pyrosequencing, we observed elevated methylation of miR-137, miR-124-2, miR-124-3, and miR-9-3 in the urine samples from the cancer patients (Fig. 3A) but only limited methylation of the genes in cancer-free individuals (Fig. 3B). Moreover, the methylation levels in the urine samples correlated positively with those in the corresponding tumor tissues (Supplementary Fig. 8). Notably, when we then collected postoperative voided urine samples from 36 of the 86 patients after surgical resection of their tumors, we observed dramatically reduced methylation levels (Fig. 3A; Supplementary Fig. 9).

To further evaluate the clinical usefulness of the miRNA gene methylation in urine samples, we carried out ROC curve analysis to assess its ability to distinguish preoperative from postoperative samples (Fig. 3C). The most discriminating cut-offs for miR-137, miR-124-2, miR-124-3, and miR-9-3 were 5.2% (sensitivity, 77.9; specificity, 77.8%), 5.2% (sensitivity, 69.8; specificity, 88.9%), 12.0% (sensitivity, 65.1; specificity, 97.2%), and 7.2% (sensitivity, 69.4; specificity, 86.1%), respectively (Table 3). We next compared these results with those obtained with urine cytology. Based on the urinary cytology using Papanicolaou's classification of the 86 patients, 55 (64%) were diagnosed as class I or II, 15 (17%) were class III, and only 16 (19%) were class IV or V (strongly suggestive or conclusive of malignancy), suggesting that the sensitivity of urinary methylation for detection of BCa is significantly greater than that of conventional cytology (Supplementary Table 6).
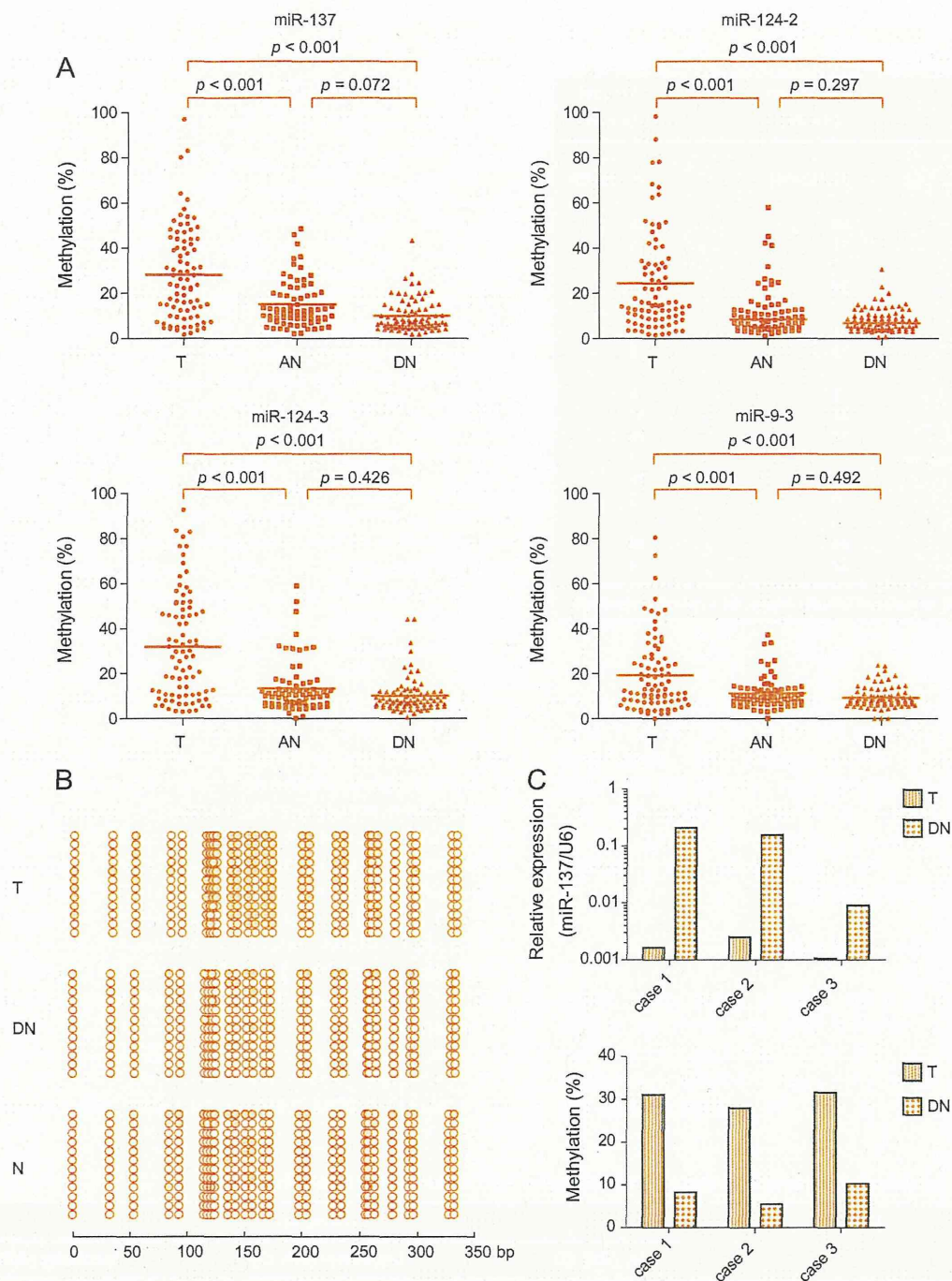
Fig. 2 – Analysis of microRNA (miRNA) gene methylation in primary bladder cancer. (A) Summarized results of bisulfite pyrosequencing of the indicated miRNA genes in primary tumors (T; *n* = 83), nontumorous bladder tissues adjacent to the tumors (AN; *n* = 74), and nontumorous bladder tissues distant from the tumors (DN; *n* = 83). *p* < 0.05. (B) Bisulfite sequencing analysis of the miR-137 CpG island (CGI) in a pair of tumor (T) and distant nontumorous tissues (DN). (C) Inverse relationship between the expression and methylation of miR-137 in three pairs of tumor (T) and distant nontumorous tissues (DN). Expression was assessed in TaqMan assays (upper panel), and methylation was determined by bisulfite pyrosequencing (lower panel).

To develop a more efficient diagnostic method for detecting BCa, we constructed a scoring system using the urinary methylation of the four methylated miRNA genes (Fig. 4). Using the cut-off value for each gene (Table 3), we classified the samples into five groups based on the M-score. A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish preoperative from postoperative urine samples by plotting the sensitivity over 1-specificity at each point (Fig. 4B). We then validated the diagnostic system by analyzing an independent test set

Table 2 – Clinicopathologic characteristics of the patients in the training and test sets

	Training set (n = 86)	Test set (n = 34)
Age, yr:		
Median (range)	73 (42–90)	71 (58–93)
Gender, no.:		
Male	69	25
Female	17	9
T stage, no.:		
Ta	34	16
Tis	7	5
T1	12	8
≥T2	33	5
Grade, no.:		
1	1	0
2	28	14
3	57	20
Lymph node metastasis, no.:		
N0	81	32
N1–N3	5	2
Treatment, no.:		
TURBT	64	30
RC	22	4

TURBT = transurethral resection of bladder tumor; RC = radical cystectomy.

(**Table 2**). AUCs in both sets were high (training set: 0.916; test set: 0.910), confirming the accuracy of our system for detecting BCa using urinary miRNA gene methylation (**Fig. 4**). We also found that our scoring system could effectively detect early-stage Ta and low-grade (grades 1 and 2) BCa (sensitivity: 0.679; specificity: 0.889; AUC = 0.862), which was undetectable using urinary cytology (**Supplementary Fig. 10**).

3.4. Functional analysis of microRNAs

To test whether any of the miRNAs could act as tumor suppressors, we transfected BCa cells with an miRNA precursor molecule or a negative control, and then carried out cell viability assays. The assays showed that ectopic expression of miR-137 or miR-124 suppressed BCa cell proliferation, whereas miR-9 exerted no significant suppressive effect on growth (**Supplementary Fig. 11 and 12**). We then carried out Matrigel invasion assays to test the effect of the miRNAs on cell invasion. Although we detected no effect of miR-137 and miR-124 on cell invasion, ectopic expression of miR-9 suppressed the invasiveness of BCa cells (**Supplementary Fig. 13**).

Finally, to further clarify the effect of miRNAs, we carried out a gene expression microarray analysis of SW780 cells transfected with a miR-137 precursor or a negative control. We found that 1326 probe sets (1016 unique genes) were downregulated (more than two-fold) by ectopic miR-137 expression, including the previously reported miR-137 target genes cyclin-dependent kinase 6 (*CDK6*), cell division cycle 42 (*CDC42*), and aurora kinase A (*AURKA*) [14,15]. Among the 1016 downregulated genes, the TargetScan program predicted that 144 genes are potential targets of miR-137 (**Supplementary Table 7**). Moreover, Gene Ontology analysis revealed that genes related to the cell cycle were significantly enriched among the affected genes (**Supplementary Table 8**). Our results strongly suggest that the miRNAs in question act as tumor suppressors in BCa.

4. Discussion

We identified four miRNA genes (miR-137, miR-124-2, miR-124-3, and miR-9-3) that were frequently methylated in both cultured and primary BCa cells. Earlier studies have shown that these miRNAs are tumor-suppressive or tumor-related and that they are epigenetically silenced in cancers of various origins. Hypermethylation of miR-137 was first discovered in oral cancer [16] and has since been noted in other malignancies, including cancers of the colon [14] and stomach [3]. Within cancer cells, miR-137 targets *CDK6*, *CDC42*, and *AURKA*, which is indicative of its tumor-suppressive properties [14–16], whereas in normal cells, miR-137 regulates neuronal differentiation through targeting enhancer of zeste homolog 2 (*EZH2*) and mindbomb E3 ubiquitin protein ligase 1 (*MIB1*) [17,18]. Methylation of miR-124 family genes (miR-124-1, miR-124-2, and miR-124-3) was identified in colorectal cancer [19] and was also

Table 3 – Receiver operating characteristic analysis of microRNA gene methylation to detect bladder cancer

Gene name	Cut-off, %	Training set		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.782 (0.701–0.862)	77.91 (67.67–86.14)	77.78 (60.85–89.88)
miR-124-2	5.2	0.769 (0.686–0.851)	69.77 (58.92–79.21)	88.89 (73.94–96.89)
miR-124-3	12.0	0.805 (0.730–0.880)	65.12 (54.08–75.08)	97.22 (85.47–99.93)
miR-9-3	7.2	0.778 (0.697–0.860)	69.41 (58.47–78.95)	86.11 (70.50–95.33)
Gene name	Cut-off, %	Test set		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.816 (0.693–0.938)	79.41 (62.10–91.30)	63.64 (30.79–89.07)
miR-124-2	5.2	0.866 (0.758–0.975)	79.41 (62.10–91.30)	90.91 (58.72–99.77)
miR-124-3	12.0	0.901 (0.807–0.995)	58.82 (40.70–75.35)	100.0 (71.51–100.0)
miR-9-3	7.2	0.797 (0.660–0.934)	76.47 (58.83–89.25)	72.73 (39.03–93.98)

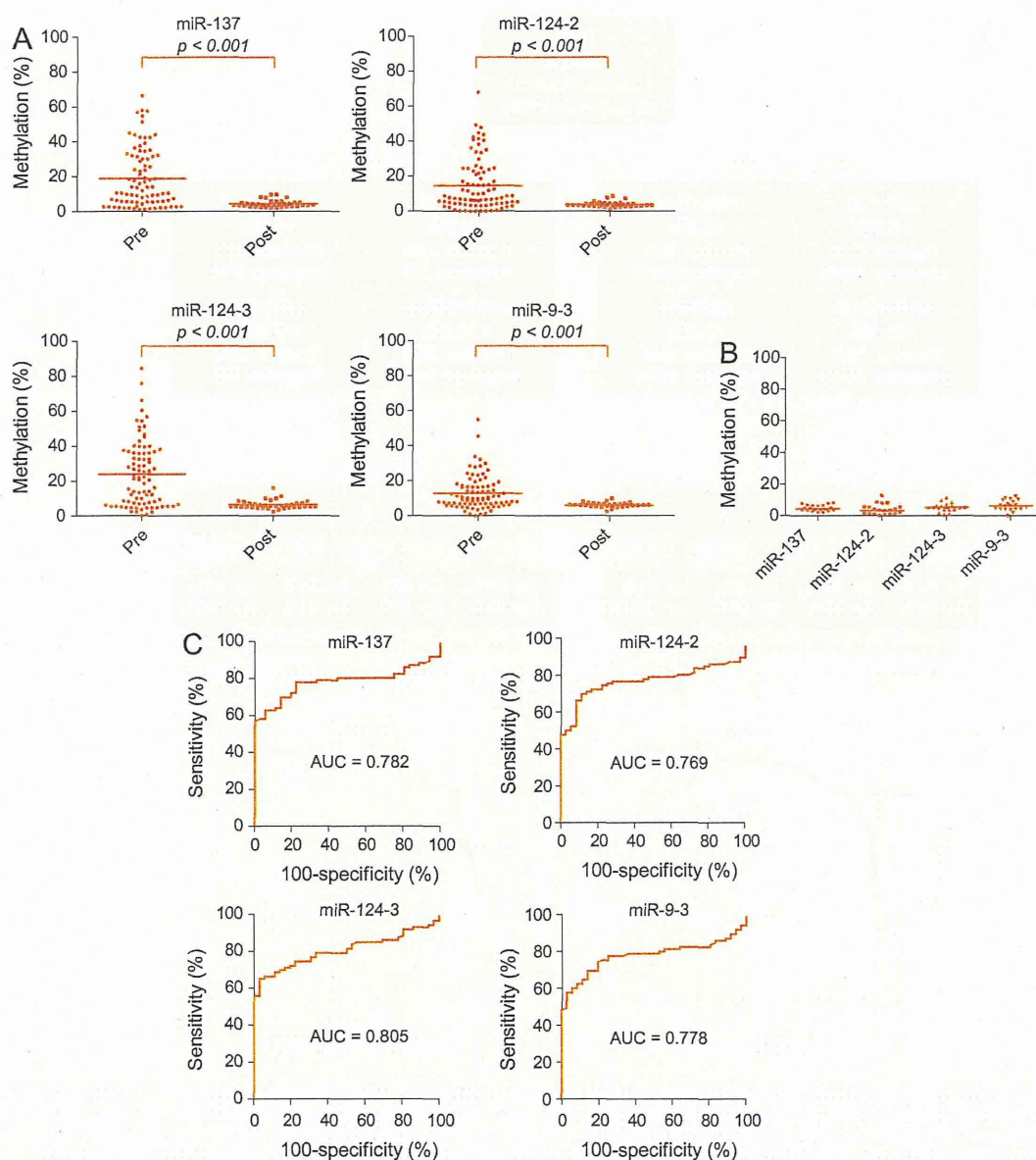AUC = area under the curve; CI = confidence interval.

Fig. 3 – Detection of microRNA (miRNA) gene methylation in urine specimens from bladder cancer (BCa) patients. (A) Summary of bisulfite pyrosequencing analysis of the indicated miRNA genes in voided urine samples collected from BCa patients before (Pre: n = 86) and after surgical treatment (Post: n = 36). $p < 0.001$. (B) Bisulfite pyrosequencing results for miR-137, miR-124-2, miR-124-3, and miR-9-3 in voided urine samples from cancer-free individuals (n = 20). (C) Receiver operating characteristics curve analysis of the ability of miRNA gene methylation to distinguish preoperative and postoperative urine samples.
AUC = area under the curve.

found in gastric cancer [20], hematologic malignancies [21], and hepatocellular carcinoma [22]. In addition, screening for methylated miRNA genes in metastatic cancer cell lines also identified miR-9 family genes (miR-9-1, miR-9-2, and miR-9-3) [23].

Cumulative evidence suggests that miRNAs play important roles in the pathogenesis of BCa, and previous studies demonstrated their epigenetic silencing in the disease. For example, miR-34a, which is a direct target of p53 and a candidate tumor suppressor gene, is frequently methylated and silenced in many types of cancer, including BCa [24]. In

addition, Wiklund et al. found that the silencing of miR-200 family genes and miR-205 is associated with DNA methylation in invasive BCa [12]. They also showed that reduced expression of miR-200c is associated with disease progression and poor outcome, suggesting that epigenetic silencing of miR-200 family genes could be a prognostic marker in BCa. Recently, Dudziec et al. carried out an miRNA microarray analysis after treating normal urothelium and urothelial cancer cell lines with 5-azacytidine. They identified 4 mirtrons and 16 miRNAs whose silencing was associated with DNA methylation [13]. Some of those
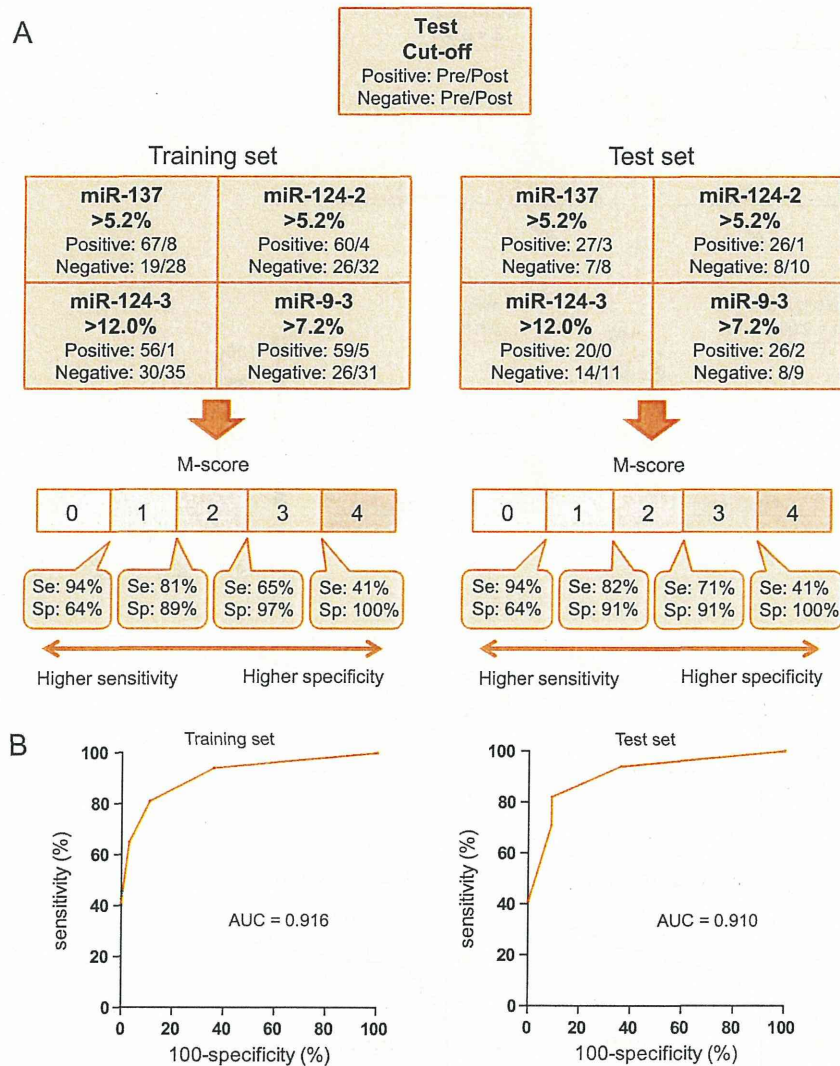
A

Test
Cut-off
Positive: Pre/Post
Negative: Pre/Post

Training set

miR-137 >5.2%	miR-124-2 >5.2%
Positive: 67/8 Negative: 19/28	Positive: 60/4 Negative: 26/32
miR-124-3 >12.0%	miR-9-3 >7.2%
Positive: 56/1 Negative: 30/35	Positive: 59/5 Negative: 26/31

Test set

miR-137 >5.2%	miR-124-2 >5.2%
Positive: 27/3 Negative: 7/8	Positive: 26/1 Negative: 8/10
miR-124-3 >12.0%	miR-9-3 >7.2%
Positive: 20/0 Negative: 14/11	Positive: 26/2 Negative: 8/9

M-score

0	1	2	3	4

Se: 94% Sp: 64%	Se: 81% Sp: 89%	Se: 65% Sp: 97%	Se: 41% Sp: 100%

Higher sensitivity   Higher specificity

M-score

0	1	2	3	4

Se: 94% Sp: 64%	Se: 82% Sp: 91%	Se: 71% Sp: 91%	Se: 41% Sp: 100%

Higher sensitivity   Higher specificity

B

Training set

AUC = 0.916

Test set

AUC = 0.910

Fig. 4 – Diagnostic system for detecting bladder cancer (BCa) using urinary microRNA (miRNA) gene methylation. (A) Workflow of a system established based on the ability to distinguish preoperative from postoperative urine. Results of the training set are shown on the left; those of test set are on the right. The methylation status of miRNA genes in preoperative (training set: $n = 86$; test set: $n = 36$) and postoperative urine (training set: $n = 34$; test set: $n = 11$) was determined using the cut-off values in the respective boxes. A miR-methylation score (M-score) was determined from the number of methylation-positive genes, and samples were classified into five groups based on the M-score. The sensitivity (Se) and specificity (Sp) at each point are indicated below. (B) Receiver operating characteristic curve analysis of the training and test sets. Areas under the curve are shown in the graph. M-score = miR-methylation score; AUC = area under the curve.

mirtrons and miRNAs, including miR-9 family genes, more frequently exhibited CpG shore methylation than CGI methylation, suggesting that methylation in both the CpG shore and CGI is related to epigenetic silencing of miRNA in BCa. Interestingly, miR-9-1 and -9-2 were associated with both CGI and CpG shore methylation, whereas miR-9-3 showed only CGI methylation [13]. Consistent with those findings, we observed that among the miR-9 family genes, miR-9-3 most frequently showed CGI methylation.

Methylation of several miRNA genes is strongly related to the clinical characteristics of cancer, suggesting its potential usefulness as a biomarker. For instance, methylation of miR-9-1 and -9-3 is reportedly associated with metastatic recurrence of RCC, which is indicative of the possible role of miR-9 in cancer metastasis [25]. Despite this report, however, we did not find a significant difference in the levels of miR-9-3 methylation between noninvasive and invasive BCa tissues. Further study to clarify the functions of these miRNAs in BCa will be needed.

Recent studies have shown that miRNA levels in urine could serve as a molecular marker for detection of BCa. For instance, expression of miR-96 and miR-183 is reportedly upregulated in urothelial cancer, and their detection in urine strongly distinguished cancer patients from cancer-free patients [26]. Miah et al. also showed that evaluation of a panel of 10 miRNAs in urine is a highly sensitive method of detecting BCa [27]. DNA methylation is another potential molecular marker detectable in urine specimens. Several

protein-coding genes are targets of DNA methylation in BCa, and their urinary methylation appears to be a useful biomarker [28,29]. For instance, methylation of 11 protein-coding genes found in urine sediments revealed the presence of BCa with a high sensitivity and specificity [30], and in another study a panel of three genes (growth differentiation factor 15 [GDF15], transmembrane protein with EGF-like and two follistatin-like domains 2 [TMEFF2], and vimentin [VIM]) in urine could be used to accurately detect BCa [31]. In the present study, we show for the first time that methylation of miRNA genes could serve as a biomarker for detection of BCa. Methylation of miRNA genes was readily detectable in voided urine from cancer patients, and its levels were dramatically reduced after tumor resection, confirming its tumor specificity. We also showed that a combination of multiple miRNA genes could accurately distinguish between preoperative and postoperative urine samples.

Our study has several limitations. The prognostic value of miRNA gene methylation remains unclear, because the prognosis of the patients in this study is not yet available. A follow-up study in post-treatment patients will be needed to test whether urinary methylation can predict outcome or detect BCa recurrence. In addition, urinary methylation in non-BCa patients (eg, patients with other types of cancer) should be tested to evaluate the specificity of our method. Further studies to address these issues would contribute to overcoming the difficulties in translating our present findings into clinical practice.

5. Conclusions

We identified four miRNA genes that are frequent targets of epigenetic silencing in BCa. Although their specific functions in bladder carcinogenesis remain unknown, it is evident that restoration of these miRNAs may be an effective anticancer therapy. Furthermore, methylation of these miRNA genes in urine specimens could serve as a useful and noninvasive biomarker for accurate detection of BCa.

Author contributions: Hiromu Suzuki had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Suzuki, Toyota.
Acquisition of data: Shimizu, Ashida, Hatahira, Yamamoto, Maruyama, Kai.
Analysis and interpretation of data: Shimizu, Suzuki, Nojima.
Drafting of the manuscript: Shimizu, Suzuki.
Critical revision of the manuscript for important intellectual content: Taiji Tsukamoto.
Statistical analysis: Nojima.
Obtaining funding: Suzuki, Toyota, Tsukamoto.
Administrative, technical, or material support: Kitamura, Masumori, Tokino, Imai, Tsukamoto.
Supervision: Minoru Toyota, Taiji Tsukamoto.
Other (specify): None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2012.11.030.

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