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Original Article

Urinary microRNAs as potential biomarkers for differentiating the "atypical urothelial cells" category of the Paris system for reporting urine cytology

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Abstract: MicroRNA is known to be an important regulator of gene expression. The microRNAs that were found to be differentially expressed in normal urothelium and urothelial carcinomas in the literature were selected and meaningful microRNAs were validated using urothelial tumor cells lines; formalin-fixed and paraffin-embedded (FFPE) tissues; and urine samples for both the definitely benign and malignant categories. Diagnostic utility was also assessed by applying validated microRNAs to urine specimens in the "atypical urothelial cells" category of the Paris System for Reporting Urine Cytology. Among the 25 consistently directed microRNAs in 2 or more studies, 14 microRNAs were upregulated and 11 downregulated. In urothelial tumor cell lines, 3 microRNAs were upregulated and 8 microRNAs were downregulated. In the step using FFPE tissues, four microRNAs (miR-145, miR-133a, miR-125b, and miR-99a) levels were downregulated and four microRNAs (miR-96, miR-141, miR-200c, and miR-205) were upregulated in the entire spectrum of urothelial neoplasms. Similar expression profiles were observed in urine specimens of "negative for high-grade urothelial carcinoma" and "urothelial carcinoma". The expression of miR-99a revealed different trends among the atypical urothelial categories. The findings from this study show that consistently upregulated or downregulated microRNAs might be involved in tumorigenesis or progression of urothelial carcinoma and that they could be used as potential diagnostic or prognostic markers.

Keywords: MicroRNAs, urinary bladder, transitional cell carcinoma, urine, diagnosis

Introduction

Bladder cancer is the fourth most common carcinoma in males in the United States [1]. Urothelial carcinoma is the most common histologic type of bladder tumors [2], and it tends to recur and occasionally progress to a higher grade or stage. It is important to evaluate histologic grade, invasiveness to proper muscle, and the presence of carcinoma in situ lesions, as they are closely related to a patient's survival rate [3]. Cystoscopy and urine cytology or biopsy are used for the diagnosis of urinary bladder lesions. Although biopsy is the most important diagnostic tool, it is sometimes difficult to evaluate the invasive lesions. Therefore, there is a growing need to introduce new biomarkers.

MicroRNAs (miRNAs) are small non-coding RNA that has 19 to 25 nucleotides. They regulate gene expression by mRNA degradation or translational inhibition [4]. They guide the RNA-induced silencing complex to miRNA target sites that are prevalent in the 3' untranslated region of an mRNA. It has been demonstrated that miRNAs have been implicated in the regulation of a variety of biological processes, including apoptosis, proliferation, cellular differentiation, gene regulation, metabolism, and cancer development or prognosis [5]. Dysregulation of miRNAs plays an important role in many cancers [6, 7]. As altered expressions of specific miRNAs are observed in cancers, they can be used as diagnostic markers or prognostic predictors.

Profiling studies of miRNA expression in neoplastic and normal tissues have been undertaken in many cancers using the miRNA microarray or direct sequencing method. However, differentially expressed miRNAs are different or the direction of expression (increase or

decrease) is inconsistent among the studies. This inconsistency could result from differences in samples, platforms, and analysis methods. The commonly altered miRNAs may play an important role in tumorigenesis or progression, and they could be used as biomarkers. Therefore, to assess the miRNAs that show prominent differences between neoplastic and non-neoplastic tissues, a meta-analysis was carried out in this study. Literature of miRNA profiling study of urinary bladder tumors was selected, and commonly upregulated or downregulated miRNAs were listed. The validation of remarkably different miRNAs was performed in 5 urothelial tumor cell lines; the different stages of formalin-fixed and paraffin-embedded (FFPE) urothelial tumor samples; and different categories of urine samples. Validated miRNAs were also applied to the category of "atypical urothelial cells" of the Paris System for Reporting Urine Cytology and their diagnostic utility was evaluated.

Materials and methods

Literature search

The relevant literature on the miRNA expression profiling of urothelial carcinoma was retrieved from the PubMed repository. Studies written in English that used tissue samples from neoplastic and adjacent non-neoplastic urothelial tissues were selected. Review articles and studies focusing on miRNA functions were excluded.

Selection of differentially expressed miRNAs

The retrieved studies were reviewed for a focus on increased or decreased expression of miRNAs. Information on miRNA chromosomal location, pre-miRNA lengths, mature sequence, and validated targets was obtained from mirbase (http://www.mirbase.org/). As some articles showed only meaningful miRNAs with a significant fold change or logistic value, a precise comparison could not be carried out. Consistently altered miRNAs described in more than 2 articles were listed using a ranking method suggested by Griffith and Chan [8, 9].

Cell lines

Five human bladder urothelial tumor cell lines (RT4, T24, 5637, HT1376, and 253J) were purchased from the Korean Cell Line Bank (Seoul, Korea). They were maintained in the RPMI 1640 medium (for T24, 5637, HT1376, and 253J) or McCoy medium (for RT4) mixed with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. RT4 was established from urothelial papilloma (transitional cell papilloma). T24, 5637, HT1376 and 253J were established from urothelial carcinoma (transitional cell carcinoma). Normal human urothelial tissues obtained from non-neoplastic bladders were used as a control for validation of the urothelial cell lines.

Case selection

A total of 139 FFPE cases were retrieved from the files of the Departments of Pathology of Chonnam National University Hospital and Chonnam National University Hwasun Hospital (2004-2012). They consisted of 109 urothelial tumors and 30 adjacent non-neoplastic urothelium. The urothelial tumors were 16 with papillary urothelial neoplasm of low malignant potential (PUNLMP), 45 with non-invasive low-grade papillary urothelial carcinoma (NILGUC), 33 with non-invasive high-grade papillary urothelial carcinoma (NIHGUC) and 15 with invasive urothelial carcinoma (invasive UC). The histopathologic diagnosis was made according to the 2016 WHO Classification of Tumors of the Urinary System and Male Genital Organs [10]. The adjacent normal urothelial tissues were obtained from cystectomy specimens. Urine cases were selected that consisted of 28 "negative for malignancy", 47 with "atypical urothelial cells" (23 negative for malignancy and 24 with urothelial carcinoma when followed up), and 30 "urothelial carcinoma" cases. The cytologic diagnoses were made according to the Paris System for Reporting Urinary Cytology [11].

RNA extraction

Total RNA from bladder urothelial tumor cell lines, normal human urothelial tissue, and urine samples was extracted using the miRNeasy Kit (Qiagen, USA) according to manufacturer's protocol. RNA from FFPE tissue was extracted using the miRNeasy FFPE Kit (Qiagen, USA). A representative slide of each case was selected, and the sections were cut 10 μm thick. The tumor areas were scraped with a scalpel from 5 to 10 sections. FFPE tissues of invasive UC were scraped under the inverted

Table 1. Seven microRNA expression profiling studies included in the review

Reference	Platform			No. of differentially expressed miRNA	Up-regulated features	Down-regulated features	Sample number
	RNA extraction	Microarray method	RT-PCR quantification				
Catto et al. [10]	The mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA)	Human miR v1.0 (Applied Biosystems, Foster City, CA, USA)	The TaqMan® assay (Applied Biosystems, Foster City, CA, USA)	16	13	3	78
Dyrskjøt et al. [11]		Oligonucleotide probe library (mercury LNA array ready to spot v.7.1, Exiqon)	The TaqMan® assay (Applied Biosystems)	20	8	2	124
Friedman et al. [12]		LC-miRNA microarray (μParafloTM, LC Sciences, Houston, TX, USA)	The TaqMan® assay (Applied Biosystems)	12	6	6	21
Song et al. [13]	Trizol (Invitrogen, Carlsbad, CA, USA)	LC-miRNA microarray (μParafloTM)	SYBR Green kit (Qiagen, USA)	51	20	31	50
Gottardo et al. [14]	Trizol (Invitrogen)	Micro-RNA oligonucleotide microchips (self-developed)		10	10	0	27
Ichimi et al. [15]	ISOGEN (Nippon Gene, Tokyo, Japan)	-	The TaqMan® assay (Applied Biosystems)	27	8	19	138
Han et al. [16]	Trizol (Invitrogen)	Deep sequencing (Illumina Cluster Station and Genome Analyze, Illumina, CA, USA)	All-in-One TM miRNA qRT-PCR Detection Kit (GeneCopoiea, Rockville, MD, USA)	656	15	15	18

Table 2. Upregulated microRNAs reported in more than 2 expression profiling studies

miRNAs name	Chromosomal localization	Pre-miRNA length	Mature sequence	Studies (reference)	Total sample sizes	Validated target
miR-183	7q32.2	110 nt	27|5'-UAUGGCACUGGUAGAAUUCACU-3'|48	4 [3, 4, 6, 7]	227	KIF2A, ITGB1
miR-182	7q32.2	110 nt	23|5'-UUUGGCAAUGGUAGAACUCACACU-3'|46	3 [3, 4, 7]	89	FOXO1
miR-21	17q23.1	72 nt	8|5'-UAGCUUAUCAGACUGAUGUUGA-3'|29	2 [1, 2]	202	PDCD4, PTEN, RECK
miR-224	Xq28	81 nt	8|5'-CAAGUCACUAGUGGUUCCGUU-3'|28	2 [3, 6]	159	API5L1
miR-96	7q32.2	78 nt	9|5'-UUUGGCACUAGCACAUUUUUGCU-3'|31	2 [6, 7]	156	KRAS
miR-20a	13q31.3	71 nt	8|5'-UAAAGUGCUUAUAGUGCAGGUAG-3'|30	2 [2, 7]	142	TGFBR2, TSG101, ARHGAP12
miR-205	1q32.2	110 nt	34|5'-UCCUUCAUUCCACCGGAGUCUG-3'|55	2 [4, 5]	77	SHIP1, ERBB3
miR-10a	17q21.32	110 nt	22|5'-UACCCUGUAGAUCCGAAUUUGUG-3'|44	2 [3, 4]	71	HOXA1, MAP3K7
miR-141	12p13.31	95 nt	59|5'-UAACACUGUCUGGUAAAGAUGG-3'|80	2 [4, 7]	68	PTEN, BRD3, UBAP1
miR-200a	1p36.33	90 nt	54|5'-UAACACUGUCUGGUAACGAUGU-3'|75	2 [4, 7]	68	CTNNB1, SIP1, BAP1
miR-200b	1p36.33	95 nt	57|5'-UAAUACUGCCUGGUAAUGAUGA-3'|78	2 [4, 7]	68	ZEB1, BAP1
miR-200c	12p13.31	68 nt	44|5'-UAAUACUGCCGGGUAAUGAUGGA-3'|66	2 [4, 7]	68	ZEB1, BAP1
miR-429	1p36.33	83 nt	51|5'-UAAUACUGUCUGGUAAAACCGU-3'|72	2 [4, 7]	68	ZEB1, FOG2, ERBB2IP
miR-203	14q32.33	110 nt	65|5'-GUGAAAUGUUUAGGACCACUAG-3'|86	2 [3, 5]	48	TP63

microscope to minimize the contamination of stromal tissue.

cDNA synthesis and quantitative real-time polymerase chain reaction

Single-stranded cDNA was synthesized with 1 μg of total RNA using reverse transcriptase (RT Kit, Qiagen, USA). Real-time PCR quantification was conducted using the miScript PCR Kit (Qiagen, USA) in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The initial PCR step was a 15-min hold at 95°C; the cycles (n = 40) consisted of a 15-sec denaturation step at 94°C followed by 30-sec annealing at 55°C and 35-sec extension at 70°C. All reactions were performed in triplicate. Nineteen miRNAs were used for validation, namely miR-1, miR-10a, miR-29c, miR-96, miR-99a, miR-100, miR-125b, miR-133a, miR-141, miR-145, miR-182, miR-183, miR-195, miR-200a, miR-200c, miR-203, miR-205, miR-224 and miR-429. RNU6-2 and miR-222-2 were used as endogenous controls.

Proliferation assay

A proliferative assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was performed. Cells (1×10^3 per well) were seeded into 96-well culture plates in 100 μL of the medium. After overnight incubation at 37°C with 5% CO_2, the culture supernatant was removed and fresh media was added. Cell proliferation was determined at various time periods by adding 10 μL of a 5 mg/mL MTT solution (Sigma, St. Louis, MO) and incubating them further for 2 hr at 37°C. MTT reaction was terminated by adding 100 μL of 40 mM HCl in isopropanol. The MTT formazan formed was measured spectrophotometrically.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM, New York, NY). Since we used relative quantitation method, relative quantitation (RQ) with minimal and maximal value, Ct, and ΔΔCt were obtained. Non-parametric Mann-Whitney U and Kruskal-Wallis tests were used to compare relative quantitation value between groups. The relationship between miRNAs and cell proliferation was analysed using the Spearman's rank correlation coefficient. A P value <0.05 (two-tailed) was considered statistically significant.

Results

Selection of literature and differentially expressed miRNAs

A total of 122 differentially expressed miRNAs were reported in the 7 miRNA expression profiling studies (**Table 1**) [12-18]. Seventy-three miRNAs were upregulated, and 49 miRNAs were downregulated. Twenty-eight miRNAs were reported in 2 or more studies-25 with a consistent direction and 3 with an inconsistent direction. Among the consistently directed miR-

Table 3. Downregulated microRNAs reported in more than 2 expression profiling studies

miRNAs name	Chromosomal localization	Pre-miRNA length	Mature sequence	Studies (reference)	Total sample sizes	Validated target
miR-145	5q32	88 nt	16|5'-GUCCAGUUUUCCCAGGAAUCCCU-3'|38	5 [2-4, 6, 7]	351	FSCN1, SWAP70
miR-143	5q32	106 nt	61|5'-UGAGAUGAAGCACUGUAGCUC-3'|81	4 [2-4, 7]	213	ERK5, MMP13, MAPK7
miR-125b	11q24.1 or 21q21.1	88 nt or 89 nt	15|5'-UCCCUGAGACCCUAACUUGUGA-3'|36 or 17|5'-UCCCUGAGACCCUAACUUGUGA-3'|38	3 [2, 4, 6]	312	TP53, MUC1, E2F3
miR-133b	6p12.2	119 nt	66|5'-UUUGGUCCCCUUCAACCAGCUA-3'|87	3 [1, 4, 6]	266	FSCN1, MET
miR-100	11q24.1	80 nt	13|5'-AACCCGUAGAUCCGAACUUGUG-3'|34	3 [4, 6, 7]	206	PLK1
miR-133a	18q11.2 or 20q13.33	88 nt or 102 nt	53|5'-UUUGGUCCCCUUCAACCAGCUG-3'|74 or 59|5'-UUUGGUCCCCUUCAACCAGCUG-3'|80	3 [4, 6, 7]	206	FSCN1, TAGLN2, GSTP1
miR-195	17p13.1	87 nt	15|5'-UAGCAGCACAGAAAUAUUGGC-3'|35	3 [4, 6, 7]	206	CDK6, E2F3, CCND1
miR-99a	21q21.1	81 nt	13|5'-AACCCGUAGAUCCGAUCUUGUG-3'|34	3 [4, 6, 7]	206	FGFR3, MTOR
miR-1	18q11.2 or 20q13.33	85 nt or 71 nt	53|5'-UGGAAUGUAAAGAAGUAUGUAU-3'|74 or 46|5'-UGGAAUGUAAAGAAGUAUGUAU-3'|67	3 [3, 4, 7]	89	HCN2, GJA1, TAGLN2
miR-29c	1q32.2	88 nt	16|5'-UGACCGAUUUCUCCUGGUGUUC-3'|37	2 [2,3]	145	-
miR-145*	5q32	88 nt	54|5'-GGAUUCCUGGAAAUACUGUUCU-3'|75	2 [4, 7]	68	-

*Symbol is the previous official name according to the miRBase database, and now named "miR-145-3p".

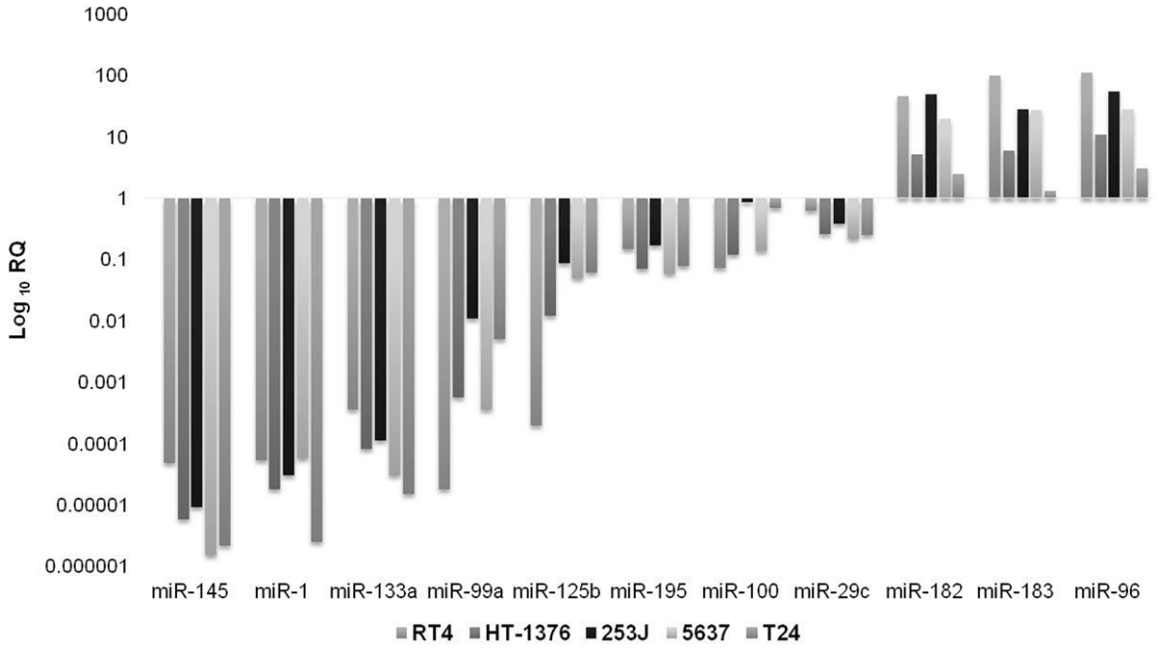

Figure 1. The expression of microRNAs in the cell lines. The expression of miR-182, miR-183, and miR-96 increased, whereas the expression of miR-145, miR-1, miR-133a, miR-99a, miR-125b, miR-195, miR-100, and miR-29c decreased in bladder tumor cell lines compared to normal human urothelial tissue. (RQ, relative quantitation).

NAs, 14 miRNAs were upregulated (**Table 2**) and 11 were downregulated (**Table 3**). The inconsistently directed miRNAs were miR-150, miR-221, and miR-223.

Validation of miRNA expression in the cell lines

Out of 25 consistently altered miRNAs, 19 miRNAs were selected for validation-11 upregulated miRNAs (miR-10a, miR-96, miR-141, miR-182, miR-183, miR-200a, miR-200c, miR-203, miR-205, miR-224, and miR-429) and 8 downregulated miRNAs (miR-1, miR-29c, miR-99a, miR-100, miR-125b, miR-133a, miR-145, and miR-195). The expression levels of the miRNAs were compared between the urothelial tumor cell lines and normal human urothelium using quantitative RT-PCR. The results showed that the levels of miR-182, miR-183, and miR-96 increased compared with the normal human urothelium (**Figure 1**). The expression of 8 miRNAs out of the 11 upregulated

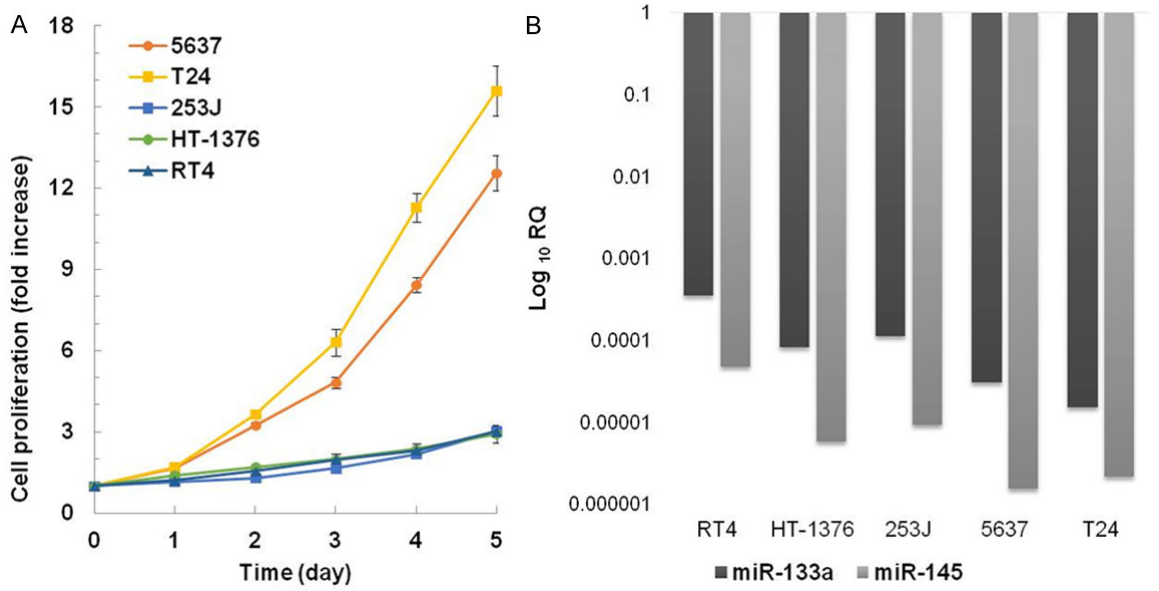

Figure 2. MicroRNAs associated with proliferative activity. A. The 5637 and T24 urothelial cancer cell lines revealed increased proliferative activity than 253J, HT1376, and RT4 cell lines in proliferation assay. B. The expression of miR-133a and miR-145 decreased as the proliferation increased. (RQ, relative quantitation).

miRNAs was inconsistent between cell lines. The levels of miR-145, miR-1, miR-133a, miR-99a, miR-125b, miR-195, miR-100, and miR-29c decreased in the urothelial tumor cell lines (**Figure 1**).

miRNAs associated with proliferative activity

To assess the miRNAs associated with cellular proliferation, proliferation assay was performed. The T24 and 5637 cell lines showed higher proliferative activity compared with 253J, HT1376, and RT4. In addition, the expression of miR-133a and miR-145 tended to decrease as the cellular proliferation activity increased (**Figure 2**).

Validation of miRNA expression in the FFPE tissues

Nineteen miRNAs were also used for validation in the FFPE tissues. They were validated in 4 different spectrums of urothelial tumors and adjacent normal urothelial tissues. The expression of miR-99a, miR-125b, miR-133a, and miR-145 decreased compared with the non-neoplastic urothelial tissues (**Figure 3**). When whole section, including stromal tissue, was used, the expression of miR-99a, miR-125b, miR-133a, and miR-145 was quite different. The expression of miR-99a, miR-125b, miR-133a, and miR-145 decreased in PUNLMP, NILGUC, and NIHGUC compared with the non-neoplastic urothelial tissues. They were upregulated in invasive UC. The expression of miR-96, miR-141, miR-200c, and miR-205 increased in the entire spectrum of urothelial tumors, which was consistent with previous studies. There was no differences of miRNA expression between the urothelial tumor groups (non-invasive versus invasive or low-grade versus high-grade).

Validation of miRNA expression in urine

Eight consistently altered miRNAs in FFPE tissues were chosen for further validation, namely miR-96, miR-99a, miR-125b, miR-133a, miR-141, miR-145, miR-200c, and miR-205. The expression of miR-200c and miR-205 increased while miR-99a, miR-125b, miR-133a, and miR-145 decreased in urine cases of "urothelial carcinoma" compared with the "negative" category urine cases (**Figure 4**). Expression of miR-96 and miR-141 was not different between the "negative" and "malignant" categories.

Application of miRNA in the urine of "atypical urothelial cells"

Five miRNAs that revealed outstanding fold changes were chosen to differentiate the "atyp-

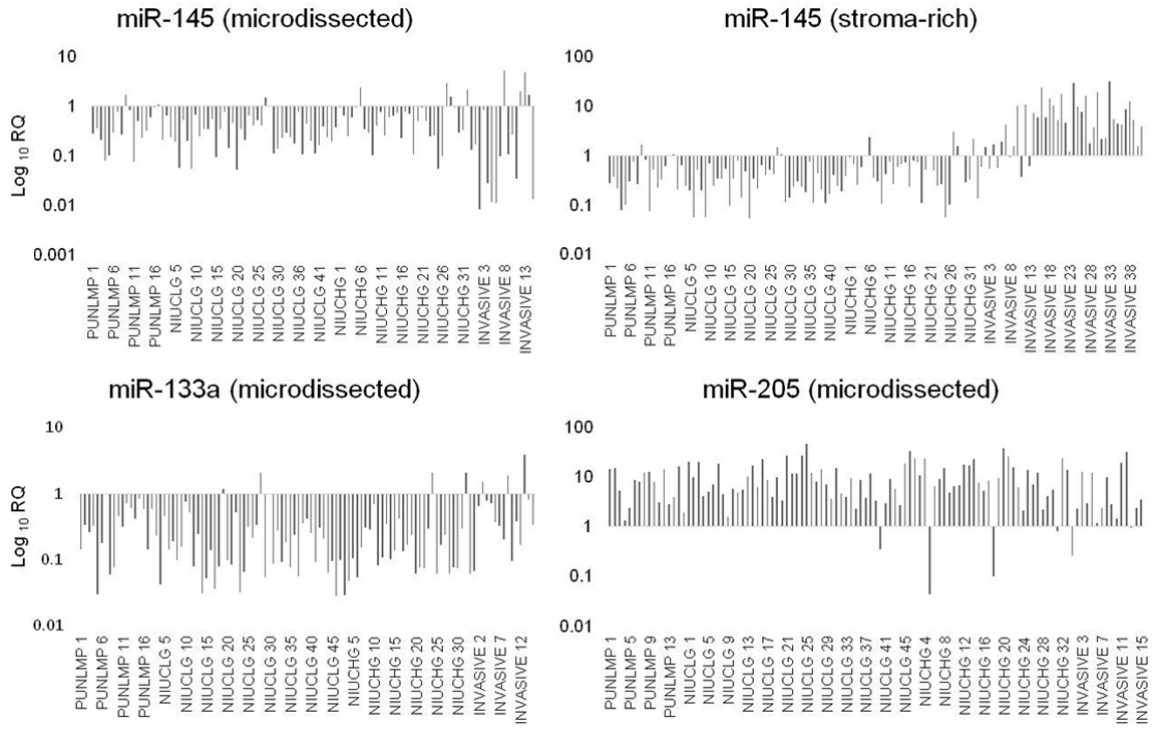

Figure 3. MicroRNAs expression in formalin-fixed and paraffin-embedded tissue of urothelial neoplasms. The expression of miR-145, miR-133a and miR-205 in formalin-fixed paraffin-embedded tissue. When whole section including stromal tissue was used (stroma-rich), the expression of miR-145 increased in invasive urothelial carcinoma. When microdissected specimens were used, the expression of miR-145 and miR-133a as what was expected. The expression of miR-205 showed similar trends as the cell lines. (RQ, relative quantitation; PUNLMP, papillary urothelial neoplasm of low malignant potential; NIUCLG, non-invasive low-grade urothelial carcinoma; NIUCHG, non-invasive high-grade urothelial carcinoma; INVASIVE, invasive urothelial carcinoma).

ical urothelial cells" category-miR-96, miR-99a, miR-125b, miR-133a, and miR-145. The expression of miR-99a was different between the atypical urothelial categories. When "atypical urothelial cells" was proven to be benign when followed up, the expression of miR-99a increased as in the "negative" category urine. When "atypical urothelial cells" was proven malignant when followed up, the expression of miR-99a decreased as in the "malignant" category urine (**Figure 5**).

Discussion

The expression of consistently upregulated and downregulated miRNAs has revealed similar trends in studies that have used cell lines, FFPE, and urine samples. These miRNAs can be used as diagnostic, prognostic, or therapeutic targets, and some studies have even shown miRNA profiles or groups to differentiate invasive carcinoma or to detect recurrent tumor during follow-up. In the present study, miRNAs applied to "atypical urothelial cells" category were considered as potential diagnostic markers. As miR-99a differentiated the "atypical urothelial cells" category into two groups with different follow-up results, it could potentially be used as a diagnostic marker.

Some studies have revealed that miR-99a inhibits cell growth or tumorigenesis in bladder cancer, renal cell carcinoma, and breast cancer [19-21]. They have therefore suggested that miR-99a is an important tumor suppressor. In bladder tumorigenesis, fibroblast growth factor receptor 3 (FGFR3) mutation is an important factor in non-invasive low-grade urothelial neoplasm, whereas the mutation of tumor suppressor genes such as TP53, phosphatase and tensin homolog (PTEN), and retinoblastoma 1 (RB1) is more prominent in high-grade urothelial neoplasm. miR-99a is known to suppress cell proliferation by targeting FGFR3 and to be downregulated in bladder cancer, as the current study shows. Xu Z. et al.

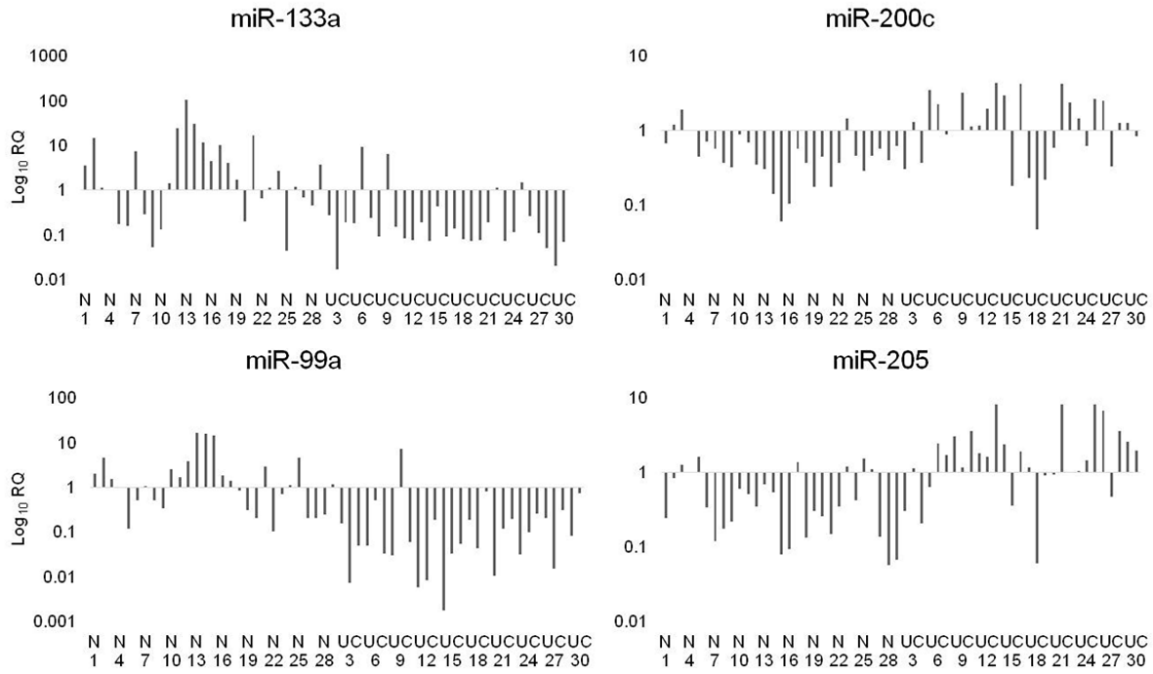

Figure 4. The expression of microRNAs in the urine samples. The expression of miR-99a and miR-133a decreased, whereas miR-200c and miR-205 increased in "malignant" urine samples. (N, "negative" category urine; UC, "malignant" urine).

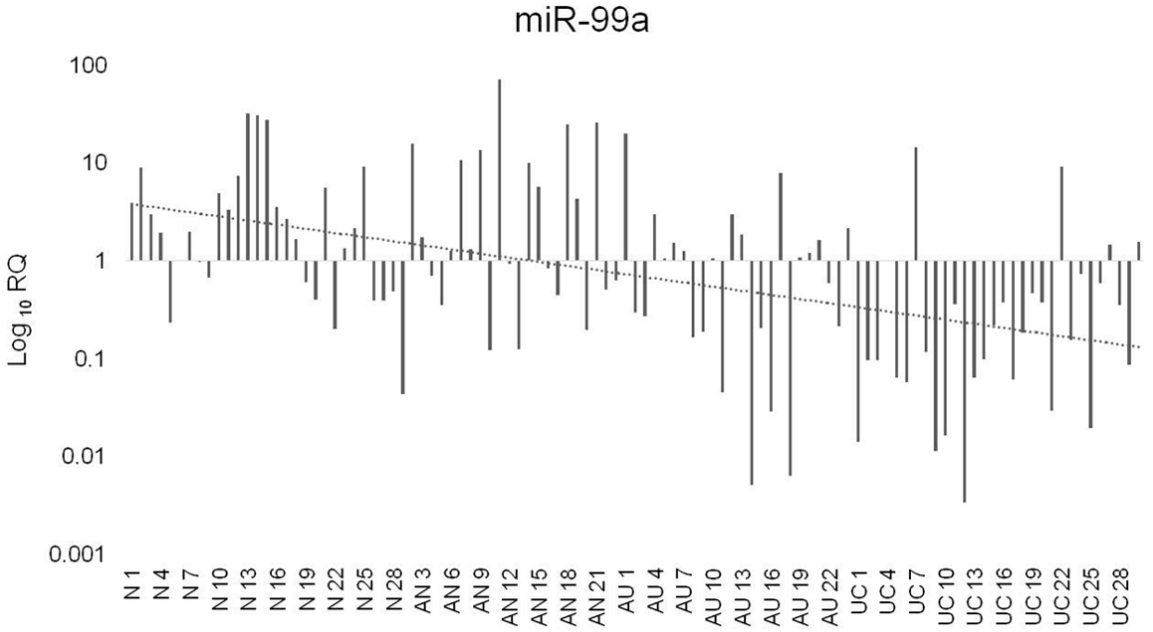

Figure 5. MicroRNA expression in the "atypical urothelial cells" category of the Paris System for Reporting Urine Cytology. When "atypical urothelial cells" was proven to be benign when followed up (abbreviated as AN), the expression of miR-99a increased as in the "negative" category urine (abbreviated N). When "atypical urothelial cells" was proven malignant when followed up (abbreviated as AU), the expression of miR-99a decreased as in the "malignant" category urine (abbreviated as UC).

revealed miR-99a expression is also associated with patient survival [22].

Some studies have investigated biomarkers in order to discriminate between benign lesions

and malignant tumors using urine, and a few studies have demonstrated that urinary miRNA profiles can be used to detect recurrent tumors during the follow-up period. The current study has shown that six miRNAs (miR-99a, miR-125b, miR-133a, miR-145, miR-200c, and miR-205) can differentiate between "negative" categorized and "malignant" categorized urine samples. However, there has been no study to investigate the diagnostic utility of miRNAs in the setting of "atypical urothelial cells" (borderline category) in the urine. In this study, miR-99a showed meaningful trend among the "atypical urothelial cells" category whereas five other miRNAs could not. The "atypical urothelial cells" category in urine is criticized by clinicians and pathologists because specificity or reproducibility of diagnosis is low and information for deciding what clinical actions to take is limited. The reporting rate of the "atypical urothelial cells" category ranges from 1.9% to 31%, and the risk of high-grade urothelial carcinoma in follow-up biopsies is as high as 37.4% [11]. Therefore, an ancillary test using urine could help clinicians decide.

The most consistently downregulated miRNA in this meta-analysis was miR-145, which was found in five studies. There have been some miRNA expression profiling studies that have shown that miR-145 is reduced in prostate, colorectal, breast, and pancreatic cancers and that it acts as a tumor suppressor. In the present study, miR-145 was downregulated in the urinary bladder cancer cell lines, all spectrums of urothelial tumors (PUNLMP, NIUCLG, NIUCHG, and invasive UC), and "malignant" urine cases. In addition, a few studies focusing on the function of miR-145 have shown that transfection of miR-145 in bladder cancer cell lines results in the inhibition of cell growth, migration, and invasion [23]. Transfection to a xenograft showed similar results, namely tumor growth inhibition and prolongation of survival [24]. Plasminogen activator inhibitor-1 has been found to be a target of the miR-143/145 cluster, and fascin homologue 1 (FSCN1) is a target of suppressive miR-145 [25].

miR-1 and miR-133a were found to be downregulated in three studies. miRNA expression profiling studies have revealed downregulation of miR-1 and miR-133a in urinary bladder, head and neck, and prostatic carcinomas [25, 26]. They suggest that both miR-1 and miR-133a act as tumor suppressors. miR-1 and miR-133a have been demonstrated as common regulators of prothymosin-α and purine nucleoside phosphorylase, LIM and SH3 protein 1, transgelin 2, and FSCN1 in urothelial cancer [25]. These miRNAs have been reported to be related with tumorigenesis, cell proliferation, apoptosis, migration, and invasion in functional studies.

The expression of miR-125b varies between tumors. Upregulation has been observed in colorectal cancer and non-small cell lung cancer, whereas it is downregulated in breast carcinoma, hepatocellular carcinoma, and carcinoma of the urinary bladder [27, 28]. Downregulation of miR-125b is related to tumorigenesis, cancer progression, and metastasis. In cancers of the urinary bladder, SphK1, matrix metallopeptidase 13, E2F transcription factor 3, and SIRT7 are known to be target genes of miR-125b, and miR-125b inhibits cancer development or progression by targeting them [28].

In this validation study using FFPE tissue, when the whole paraffin section including stromal tissue was used in invasive UC, the expression profiles were not what was expected. miR-99a, miR-125b, miR-133a, and miR-145 increased in invasive UC, whereas they decreased in the validation study using cell lines and urine cases. In FFPE non-invasive urothelial neoplasms, four miRNAs decreased as well as the cell lines and urine cases. It was thought that FFPE invasive UC has more abundant desmoplastic stroma than FFPE non-invasive urothelial neoplasms. Cell lines and urine samples have also much less stromal cells than invasive UC tissues. Therefore, in order to minimize the contamination of stromal tissue, we have microdissected epithelial tumor portion in invasive UC tissues. When microdissected specimens of invasive tumors were used, 4 miRNAs decreased, as in the cell lines and urine. As some studies have shown that miRNAs in the stroma could have a prognostic value, miRNA could be a crucial component in the tumor microenvironment including stroma [29, 30].

There are a few limitations in the present study. As the data described in each articles varied and were somewhat limited, there were limi-

tations in comparing the expression levels of miRNAs. Frequency was an important criterion that could cause selection bias. In addition, certain miRNAs that showed significant differences between normal and carcinoma tissue in previous studies could have been omitted from the current study because they were described in just one article.

In conclusion, this systematic review and validation study has identified 3 upregulated miRNAs (miR-96, miR-182, and miR-183) and 8 downregulated miRNAs (miR-145, miR-1, miR-133a, miR-99a, miR-125b, miR-195, miR-100, and miR-29c) that are potential diagnostic or prognostic markers for urothelial carcinoma. Validation studies have showed several meaningful miRNAs for differentiating benign and malignant lesions. The expression of miR-99a in "atypical urothelial cells" in the urine showed the possibility to predict follow-up results, indicating that it could serve as a diagnostic marker.

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Disclosure of conflict of interest

None.

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