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BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

A Common MicroRNA Signature Consisting of miR-133a, miR-139-3p, and miR-142-3p Clusters Bladder Carcinoma in Situ with Normal Umbrella Cells

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miRNAs are small noncoding RNAs with critical roles in a large variety of biological processes such as development and tumorigenesis. miRNA expression profiling has been reported to be a powerful tool to classify tissue samples, including cancers, based on their developmental lineage. In this study, we have profiled the expression of miRNAs in bladder carcinoma in situ (CIS) and distinct cell compartments of the normal bladder, namely umbrella and basal-intermediate urothelial cells, as well as the muscularis propria. We identified several miRNAs differentially expressed between umbrella and basal-intermediate cells (miR-133a, miR-139-3p, miR-142-3p, miR-199b-5p, and miR-221). In situ hybridization confirmed the expression of miR-133a and miR-139-3p in umbrella cells, and miR-142-3p in basal-intermediate cells. Strikingly, miRNA expression levels of CIS most closely resembled the miRNA profile of umbrella cells. Finally, we examined well-established umbrella and basal-intermediate cell immuno-histochemical biomarkers in an independent series of CIS samples. Again, this analysis revealed the significant expression of umbrella-specific markers in CIS when compared to non-CIS lesions. Overall, our studies represent a comprehensive and accurate description of the different miRNAs expressed in CIS tumors and three distinct histological areas of the urinary bladder. Notably, this study provides evidence of the possible origin relationship between CIS and normal umbrella cells. (Am J Pathol 2013, 182: 1171—1179; http://dx.doi.org/10.1016/j.ajpath.2013.01.006)

The urothelium is composed of specialized epithelial cells that coat the entire urinary tract, including the renal pelvis, ureter, bladder, and female proximal urethra. In humans, the urothelium of the bladder is a stratified epithelium, comprised of three different cell layers: i) basal, ii) intermediate, and iii) superficial umbrella cells. The basal membrane separates the urothelium from the stroma of the bladder, which includes the lamina propria and the muscularis propria.

Umbrella (UM) cells are distinct from basal-intermediate (B/i) cells in both morphology and expression of specific biomarkers. UM cells express high levels of uroplakin II (UPKII),[1] low molecular weight cytokeratins (CKs; eg, CK18 and CK20),[2] and Lewis X determinant,[3] however, none of these markers are expressed in B/i cells. In contrast, B/i cells stain strongly for high molecular weight cytokeratins

(eg, CK5, CK10, CK14), p63,[4] and mature A and B blood group antigens, all of which are negative in umbrella (UM) cells. Although it has been proposed that UM cells mature from the underlying B/i cells in a manner similar to epithelial development of the skin, the vast number of differences between the two cell types suggests that UM cells may originate from a precursor distinct from B/i cells.[5]

The urothelium functions as a permeability barrier between blood and urine and is constantly exposed to potential carcinogens in urine. It is not surprising, therefore, that 90% of bladder tumors originate from the urothelium.[6] Bladder tumors are classified into two groups based on phenotypic and molecular profiles. Low-grade tumors are always papillary (ie, protruding into the lumen), and usually

superficial and nonmuscle invasive. In contrast, high-grade tumors can be papillary or nonpapillary, such as carcinoma *in situ* (CIS), and are often invasive. Nonmuscle invasive bladder tumors (low-grade tumors and carcinoma stages Ta, Tis, T1) comprise 75% to 85% of total bladder cancer cases at initial presentation.[6] CIS presents as a flat lesion, found in association with 75% to 90% of high-grade papillary cases and in 45% to 65% of all invasive urothelial tumors.[7,8] Presence of CIS greatly increases the risk of disease progression to invasive urothelial carcinoma; once the tumor is metastatic, the median survival rate is approximately 7 to 20 months.[6,9] CIS is characterized by aneuploidy, and its histological abnormalities include enlarged nuclei and nucleoli and hyperchromasia.[7] Given the heterogeneity of bladder tumors, it is possible that the two distinct populations of normal urothelium (ie, UM cells and B/i cells) may have different implications in bladder tumorigenesis. A better characterization of both normal urothelium and bladder cancer cases is therefore needed to delineate the role of these cells in tumorigenesis.

miRNAs have been implicated in development and tumorigenesis, as reviewed in Zhang et al,[10] and He and Hannon,[11] and Esquela-Kerscher and Slack.[12] To date, more than 21,000 distinct mature miRNA sequences have been identified in 193 different species (miRBase 19) with more than 2000 miRNAs identified in humans.[13,14] Transcribed from independent miRNA genes or as introns of protein-coding mRNAs, miRNAs repress specific mRNA expression through 3′-UTR base pairing.[15] Furthermore, miRNA profiles can classify tumors based on their respective developmental lineage.[16] To the best of our knowledge, miRNA profiles of UM and B/i cells have not been examined. Moreover, several groups have compared miRNA expression between normal mucosa and bladder tumors,[17−25] but none has assessed miRNA expression in CIS samples relative to subpopulations of normal urothelium.

For the study described herein, we used laser capture microdissection to obtain UM and B/i cell populations from normal bladder urothelium, as well as pure CIS cells. Then we profiled the expression of both miRNAs and mRNAs in CIS tumors and three distinct histological areas of the urinary bladder: i) the UM (lumen-facing cells), ii) the B/i cells, and iii) the muscularis propria (MP; stromal). We identified several miRNAs differentially expressed between UM and B/i cells. Furthermore, and for the first time, through miRNA profiling and immunohistochemical analyses, we established a correlation between CIS and UM cells.

Materials and Methods

Cell Culture

The immortalized normal urothelial cell line, Human Urothelial Cell (HUC) from ScienCell Research Laboratories, was grown in Urothelial Cell Medium (ScienCell Research Laboratories, Carlsbad, CA) at 37°C in a humidified air atmosphere at 5% CO_2. RNA was extracted from the cells using the miRVANA Kit (Ambion, Carlsbad, CA).

Laser Capture Microdissection of Normal Bladder Urothelium and CIS Biopsies

Human normal ($n = 3$) and tumor bladder (CIS, $n = 4$) tissue samples were obtained through approved protocols of the International Review Board. The three normal bladders were obtained from organ donors (males: ages 15, 25, and 28 years old) with no history of bladder cancer through the International Institute for the Advancement of Medicine. Fresh tissues were processed and frozen in optimal cutting temperature (OCT) molds, which were used for laser capture microdissection. CIS samples were obtained from patients who had undergone radical cystectomy for an invasive urothelial carcinoma and sections that did not include the invasive area were chosen for further processing. Frozen normal bladder urothelium was microdissected to obtain pure populations of UM cells, B/i cells, and MP. Frozen CIS samples were also microdissected from the bladder tissue samples to obtain pure populations of tumor cells. To rule out the possibility of normal urothelial cell contamination in CIS samples, only cases in which the CIS lesion permeated the whole thickness of the epithelium were chosen. Cases in which we could histologically identify UM or B/i cells surrounding the malignant cells by H&E stain, and cases with pagetoid spread or denuded CIS were not used. Briefly, 20-μm sections from OCT blocks were cut and mounted on MembraneSlide NF 1.0 PEN (Zeiss, Munich, Germany) and stored at −80°C until use. Slides were fixed in ethanol at −20°C for 2 minutes, washed in diethyl-pyrocarbonate-H_2O, stained with hematoxylin for 1 minute, washed in diethyl-pyrocarbonate-H_2O, and air-dried. PALM Microbeam IV Laser Capture Microscope (Zeiss) was used to perform laser microdissection. A total of 25 20-μm sections were prepared from each sample, in which the microdissected tissue was pooled per patient to extract RNA. Formalin-fixed paraffin-embedded (FFPE) CIS samples ($n = 6$) were macrodissected by scratching 40 10-μm sections in which dissected tissue was pooled per patient.

RNA Extraction and Microarray Profiling and Data Analysis

RNA was extracted from microdissected samples using RNAquesous-Micro Kit (Ambion). RNA was extracted using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion) for macrodissected samples. The quality of all RNA samples was verified using a RNA Pico Kit (Agilent, Santa Clara, CA). Only sample qualities with RNA Integrity Number >6.0 and with clean 18S and 28S peaks were used, as previously described.[26]

Agilent Human miRNA Microarray (V3, based on Sanger miRbase release 12.0) were used for measuring miRNA expression in bladder samples. Samples used for the Agilent miRNA microarray were labeled as described by the manufacturer.

Affymetrix Human U133 Plus 2.0 (Santa Clara, CA) arrays were used for measuring gene expression in bladder samples. Samples used for Affymetrix microarray were amplified using the Ovation RNA Amplification System (NuGen, San Carlos, CA) and labeled using FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's protocol. Raw intensity miRNA data were normalized and median transformed using GeneSpring GX 12 (Agilent). The raw mRNA data were log transformed and analyzed using Partek Genomics Suite 6.6 (Partek, Saint Louis, MO). Only detected probesets were used, and compromised or undetected probesets were filtered out. One-way analysis of variance and Tukey's honestly significant difference post hoc test were performed across all samples to obtain miRNAs or mRNAs differentially expressed ($P < 0.05$). Unsupervised hierarchical cluster analysis was performed on the list of differentially expressed probesets with a fold change ≥ 2 (295 miRNAs, 1113 mRNAs).

Quantitative Real-Time RT-PCR miRNA Expression Profiling

TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to convert miRNA to cDNA. Forty-four−nucleotide (nt) reverse transcription primers were designed so that the first 36 nt formed an internal stem loop and the last 8 nt were complementary to the mature miRNA sequence of interest. Qiagen Reverse Transcriptase System (Qiagen, Valencia, CA) was used to produce cDNA from mRNA. Qiagen QuantiTect PCR (Qiagen) was used to measure quantitative expression of miRNA and mRNA. PCR assays were performed as described by the manufacturer using a Stratagene MX3005P PCR system (Agilent). For normalization, we used *RNU6B* or *ACTIN* for miRNA and mRNA, respectively.

Immunohistochemical Detection of Biomarkers

Immunohistochemical analyses were conducted on FFPE tissue sections from 10 cases of normal human bladder and 19 CIS specimens according to the standard avidin-biotin protocol. These 10 cases of normal bladder included the three cases used for the laser microdissection (organ donors) and seven cases of histologically normal urothelium obtained from patients who had undergone a radical cystectomy for an

invasive carcinoma. From the 19 CIS specimens, 10 corresponded to biopsies of patients with primary CIS and the other nine were obtained from cystectomy specimens of patients with a diagnosis of invasive bladder cancer. Briefly, 5-μm sections were deparaffinized and submitted to antigen retrieval by steamer treatment for 15 minutes in 10 mmol/L citrate buffer at pH 6.0. Subsequently, slides were incubated in 10% normal serum for 30 minutes, followed by primary antibody incubation overnight at 4°C. Primary antibodies used were anti-UPKII goat polyclonal (Santa Cruz Biotechnology, Dallas, TX), anti-CK5 rabbit polyclonal (AF138, Covance, Princeton, NJ), anti-CK20 mouse monoclonal (clone Ks20.8, Dako, Carpinteria, CA), and anti-high molecular weight cytokeratin mouse monoclonal (clone 34βE12, Dako). Then slides were incubated with biotinylated immunoglobulins at 1:500 dilution for 30 minutes (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin peroxidase complexes at a 1:25 dilution (Vector Laboratories, Inc.) for 30 minutes. Diaminobenzidine was used as chromogen and hematoxylin as the nuclear counterstain. Slides were analyzed with a Nikon Eclipse 50i microscope (Nikon Instruments, Melville, NY) equipped with a SPOT Insight2MP Mosaic camera and SPOT Advanced software version 5.0 (Diagnostic Instruments, Sterling Heights, MI).

In Situ Detection of miRNAs

In situ detection of miRNAs was performed on 10-μm frozen tissue sections from normal human bladders obtained from healthy donor patients ($n = 3$) and B6-wt mouse bladders ($n = 15$). Sections were fixed in 4% paraformaldehyde, acetylated, and prehybridized in hybridization solution (50% formamide, 5× saline-sodium citrate, 0.5 mg/mL yeast tRNA, 1X Denhardt's solution) for 3 hours at 25°C below the predicted T_m of the probe. Five pmol probe (LNA-modified and FITC-labeled oligonucleotide; Exiqon, Woburn, MA) complementary to U6 (positive control), miR-133a, miR-139-3p, and miR-142-3p was hybridized to the sections overnight in a humidified chamber at prehybridization temperature. Posthybridization slides were washed with 5× and 0.2× saline-sodium citrate, blocked in 10% normal goat serum for 30 minutes, and incubated in anti-fluorescein isothiocyanate/alkaline-phosphatase antibody (Vector Laboratories, Inc.) at 1:400 at 4°C overnight. Five pmol sense-strand of the primary transcript of the miR-424/503 cluster (Table 1) was used as negative control. *In situ* hybridization signals were detected

Table 1   Sequence of the Sense-Strand of the Primary Transcript of miR-424/503 Cluster

Probe sequence	Hybridization temperature
5′–GGGCAGTCAACGACATTTTTCTCCATTAATCCCAAAGTCAGTGTAAAAGTTGTTTGTAAATATCATTGCC–	65°C
AAAACAAGATGGAATGTAGAGATACTTGATTGTCGCAATTTAGGGAATGGGGTTATTGTTACTAATGACTT–	
TTTTTTTTTTTACCATGACATAGTGTATTTGTTACCACTGGATCTCAGTCTGGATGTTATAAATTCGATACTT–	
AATCTTATTAAATACTCTGCACATTTTATTAACTAATAAATAGGACACTTAACATTCATTGTACTGGTTTT–	
GAAAAACCGACTTAATTTGTAAGTGTAACAAGATACTTACAGTTGGATAATTT–3′	

This probe was 3′ digoxigenin-labeled and used as a negative control in miRNA ISH stainings.

using BM purple (Roche, Indianapolis, IN). Slides were counterstained with Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO), mounted using 80% glycerol, and analyzed with a Nikon Eclipse 50i microscope (Nikon Instruments) equipped with a SPOT Insight2MP Mosaic camera and SPOT Advanced software version 5.0 (Diagnostic Instruments, Sterling Heights, MI).

Results

Isolation of Pure Populations of UM, B/i, and MP by Laser Capture Microdissection

As previously described,[5,27] UPKII and CK5 were shown to be differentially expressed in UM versus B/i cells in normal bladder urothelium (Figure 1A). Membranous expression of UPKII was observed exclusively in the UM layer, whereas cytoplasmic CK5 expression was specific for the cells in the B/i layers. Three cases of normal bladder samples and four CIS lesions were used to perform the study (Figure 1B). The urothelium was microdissected to separate UM from B/i cells as explained in *Material and Methods* (Figure 1C). The MP population was obtained by removing the urothelium and lamina propria through macrodissection. Quantitative real-time RT-PCR (RT-qPCR) showed on average an eightfold decrease in UPKII levels in B/i versus UM, and a twofold increase in CK5 in B/i versus UM (Figure 1D). These results demonstrated that the distinct urothelial subpopulations could be efficiently separated by microdissection.

miRNA Microarray Profiling Associates CIS with Umbrella Cells

Once we had isolated pure populations of urothelial subtypes and stroma, we examined the miRNA expression in these cells, along with four CIS cases and an HUC line. Of the 984 human miRNAs evaluated, there were 295 miRNAs differentially expressed (fold change ≥2) across the normal bladder, HUC line, and CIS. Interestingly, hierarchical cluster analysis of these 295 miRNAs separated samples were based on cell type of origin instead of normal versus cancer. Endoderm-derived cells, including the urothelium, CIS, and HUC, clustered into one arm, whereas mesoderm-derived MP branched into another arm (Supplemental Figure S1). Expression of individual samples was averaged into groups, with the exception of the HUC line. Notably, the level of expression of miRNAs in CIS most closely resembled the level observed in normal UM cells, whereas the HUC line shared greater similarity in miRNA expression with B/i cells. So far, an analysis of a broad sampling of miRNAs indicates a correlation between UM and CIS cells.

Then we focused our analyses on the individual miRNA and mRNA profiles of UM, B/I, and CIS, because MP is not epithelial in origin and HUC is a cell line. With the removal of MP and HUC, there were 41 miRNAs and 1113 mRNAs differentially expressed across UM, B/i, and CIS samples. A hierarchical cluster analysis of individual cases revealed distinct miRNA expression profiles between UM cells and B/i cells; interestingly, the majority of CIS samples clustered with UM cells (Figure 2A). From these 41 miRNAs,
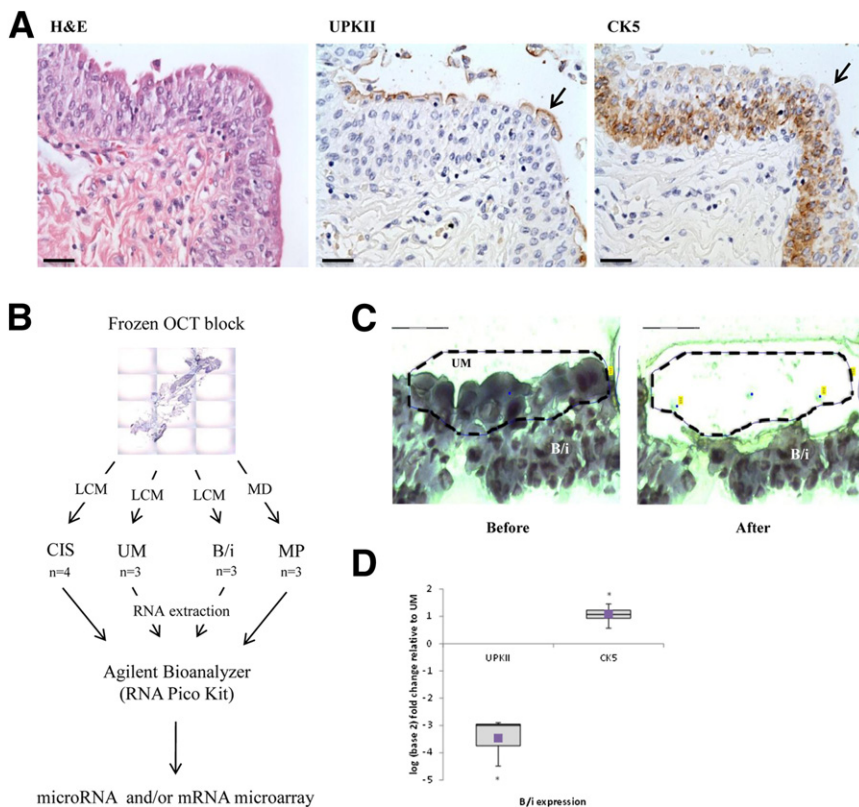

Figure 1 Experimental design and isolation of UM and B/i cells from normal bladder urothelium. **A:** Representative immunohistochemical analyses of UPKII and CK5 in normal urothelium; prominent UM cells (**arrows**). Scale bar = 50 μm. **B:** Microphotograph of frozen tissue section was taken with PALM Microbeam IV Laser Capture Microscope (LCM; Zeiss). UM, B/i, and CIS samples were microdissected and analyzed with both miRNA and mRNA microarrays. MP samples were macrodissected (MD) and analyzed only in the miRNA microarray. **C:** Delineation of UM cells, before and after laser capture. Scale bar = 75 μm. **D:** RT-qPCR on UM and B/i cells for UPKII and CK5. Graph shows B/i log$_2$ fold expression; B/i expression normalized to UM. **Horizontal line** indicates the sample median. **Square** indicates the sample mean. *$P < 0.05$ by Student's *t*-test.
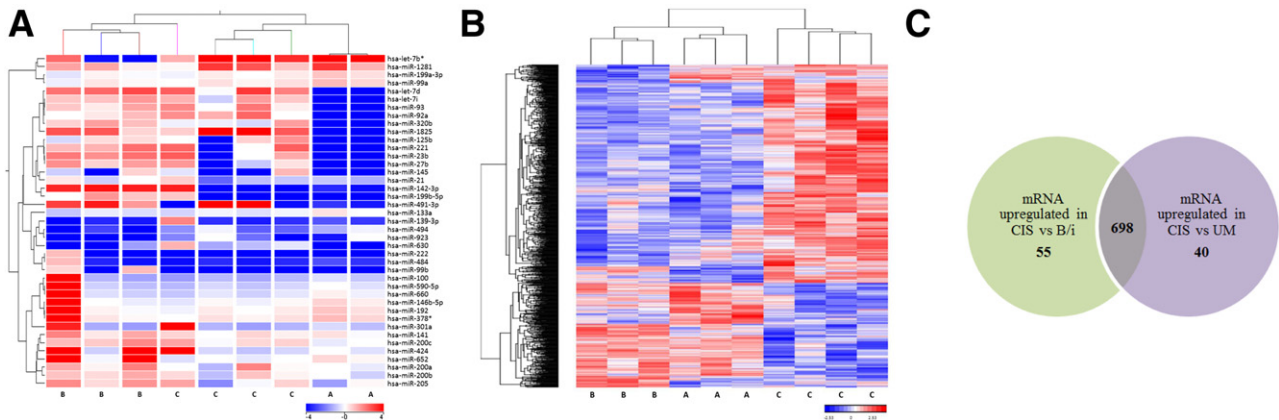
Figure 2 Hierarchical clustering of normal urothelium and CIS. **A:** Samples are in columns and miRNAs are in rows. Individual samples of normal urothelium and CIS were clustered according to the expression signature of 41 differentially expressed miRNAs ($P \leq 0.05$). **B:** Samples are in columns, mRNAs are in rows. Normal urothelium and CIS samples were clustered according to the expression signature of 1113 differentially expressed mRNAs ($P \leq 0.05$). **C:** Venn diagram illustrating 698 mRNAs upregulated in common between CIS compared to normal UM and B/i cells. There were 753 mRNAs upregulated in CIS compared to B/i cells, and 738 mRNAs upregulated in CIS compared to UM cells. Arbitrary assignment of cluster groups is as follows: A, UM cells; B, B/i cells; C, CIS.

four (ie, let-7b*, miR-1281, miR-133a, and miR-139-3p) exhibited higher levels of expression in both CIS and UM cells when compared to B/i cells. Conversely, nine miRNAs (ie, miR-142-3p, miR-199b-5p, miR-200a, miR-200b, miR-205, miR-21, miR-27b, miR-424, and miR-491-3p) displayed higher levels of expression in B/i cells than CIS and UM cells. Importantly, only five of 41 miRNAs were significantly altered between UM and B/i cells (Table 2).

In contrast, based on mRNA expression levels, normal urothelium (UM and B/i) and CIS formed two separate arms (Figure 2B). From the 1113 mRNAs differently expressed, 23 were commonly down-regulated in UM cells and CIS in comparison to B/i cells, whereas another 23 followed the opposite trend (Supplemental Table S1). Notably, *TP63* (specific to B/i cells, but absent in UM cells) was down-regulated in UM cells and CIS compared to B/i cells. Interestingly, among the genes that were upregulated in UM cells and CIS compared to B/i cells, we found *CD24*, a proposed marker of tumor-initiating cells (reviewed by Gires[28]), and genes involved in metabolism, such as *GFOD2*, *NDUFB2*, and *NQO1*.

In fact, more than half of the differentially expressed mRNAs (698) were upregulated in CIS compared to UM and B/i samples (Figure 2C). Gene ontology enrichment was performed on the 698 mRNAs to investigate biologically driven effects. This analysis revealed 74 biological processes

with enrichment scores >3 ($P < 0.05$), and as expected, the top seven ($P < 0.005$) are involved in genomic alterations, transcription regulation, and cell cycle (Supplemental Table S2). Furthermore, we examined differentially expressed pathways in the group of 698 mRNAs upregulated in CIS using Pathway analysis of variance (Partek Genomics Suite 6.6). Statistically significant pathways with more than 10 genes involved include metabolic pathways and pathways in cancer (Supplemental Table S3). Not surprisingly, in this list (Supplemental Table S4), the bladder cancer pathway was enriched (enrichment score, 3.2). As supported by gene ontology analysis, hierarchical clustering by global gene expression separated samples based on normal (UM and B/i) versus cancer (CIS).

Validation of miRNA Arrays in UM and B/i Cells

Having examined miRNA and mRNA profiles globally in both normal urothelium and in CIS cases, we decided to further explore the five miRNAs that were statistically differentially expressed between UM cells and B/i cells (Table 2), according to the microarray data. We performed RT-qPCR to determine the expression of the mature form of these five miRNAs (Figure 3A). RT-qPCR results showed an average Pearson correlation to the microarray data of 0.95. The miR-133a and miR-139-3p were upregulated in

Table 2 Statistical Results and Location of a Set of miRNAs Significantly Differentially Expressed Between UM and B/i Cells in the Normal Urothelium

miRNA	Chromosome	Fold change (UM vs B/i)	*P* value	RT-qPCR verification	ISH verification
hsa-miR-139-3p	11q13.4	2.30	0.006	Yes	Yes
hsa-miR-199b-5p	9q34.11	−4.03	0.024	Yes	ND
hsa-miR-142-3p	2q35	−3.12	0.030	Yes	Yes
hsa-miR-133a	18q11.2	2.26	0.037	Yes	Yes
hsa-miR-221	Xp11.3	−5.04	0.048	Yes	ND

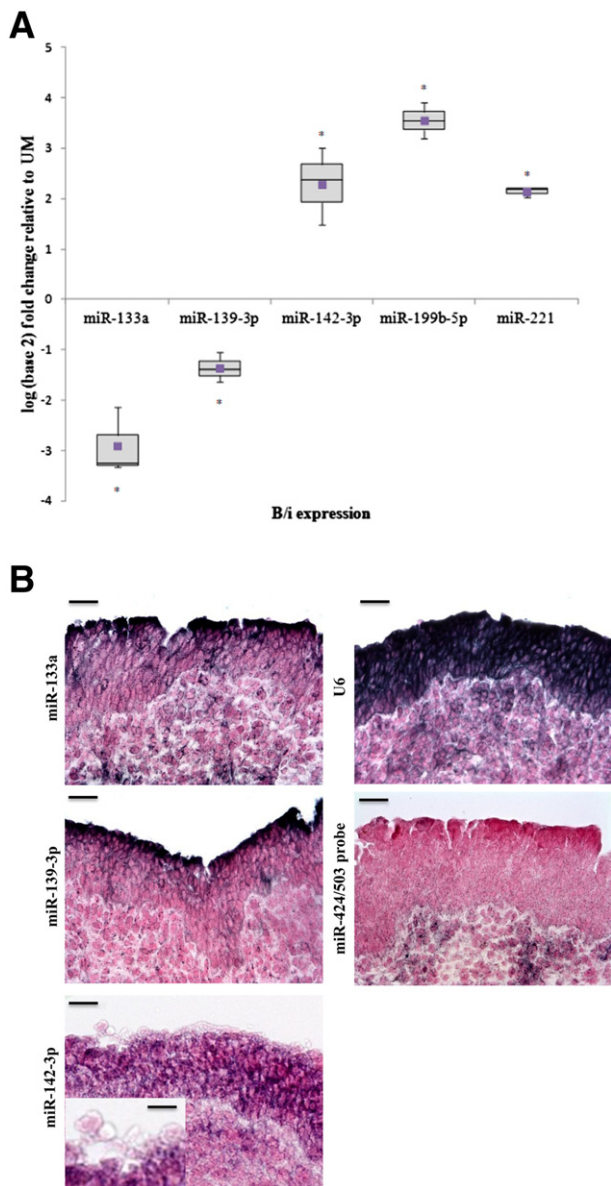ISH, *in situ* hybridization; ND, not detectable.

Figure 3 Validation of differentially expressed miRNAs from microarray data. **A:** RT-qPCR on UM and B/i cells for various miRNAs. Graph shows B/i log$_2$ fold expression; B/i expression is normalized to UM. **Horizontal line** indicates the sample median. **Square** indicates the sample mean. *$P < 0.05$ by Student's *t*-test. **B:** Localization of miR-133a, miR-139-3p, and miR-142-3p in human bladder samples by *in situ* hybridization. Scale bar = 100 μm. **Inset:** miR-142-3p shows UM cells. Scale bar = 25 μm. No staining was observed in a negative control *in situ* hybridization using the sense strand of miR-424/503, whereas a strong signal was observed in a positive control *in situ* hybridization using U6 in representative cases of normal bladder. Scale bar = 100 μm.

UM cells compared to B/i cells, whereas miR-142-3p, miR-199b-5p, and miR-221 were down-regulated in UM cells compared to B/i cells. Taken together, the RT-qPCR results confirmed that these five miRNAs were differentially expressed between UM and B/i cell populations.

Then we verified the localization of our miRNAs of interest via *in situ* hybridization. We used LNA oligonucleotide probes for detection of the miRNAs of interest in human and

mouse bladders. A positive control using U6 gave strong signals in human urothelium, whereas no signal was detected using a nonspecific probe for the miR-424/503 cluster. Robust *in situ* hybridization conditions were established for 3 of 5 miRNAs. Expression of miR-133a and miR-139-3p was observed in UM cells, whereas miR-142-3p expression was localized to B/i cells with low expression in UM cells (Figure 3B). To further validate the phenotypes described for human urothelial tissues, we extended the study to the analysis of mouse urothelium. We observed that the miRNA phenotypes of both human and murine bladder samples were consistent (Supplemental Figure S2).

CIS Samples Express UM But Not B/i Urothelium-Specific Protein Markers

To further explore whether CIS samples were more closely related to UM cells than B/i cells, as suggested by the miRNA microarray results, we examined the expression of miRNAs specific to UM cells in CIS. Compared to B/i cells, expression of the five miRNAs (Table 2) by RT-qPCR in six independent CIS biopsies were similar to that of UM cells. miR-133a and miR-139-3p were upregulated in CIS compared to B/i, whereas miR-142-3p, miR-199b-5p, and miR-221 were down-regulated in CIS compared to B/i (Figure 4A).

Because miRNA expression revealed a correlation between CIS and UM cells, we decided to assess the expression of several well-established differentiation biomarkers of the urothelium in a cohort of CIS lesions. We have previously reported B/i cells to be positive for high molecular weight cytokeratins, whereas UM cells are characterized by low molecular weight cytokeratins and UPKII.[5,27] Indeed, a high percentage of CIS lesions revealed a positive UPKII (83.3%) and CK20 (84.2%) phenotype similar to that of UM cells, as well as a negative high molecular weight cytokeratins (10.5%) phenotype (Figure 4, B and C). Table 3 summarizes the individual immuno-anatomical profiles of the 19 CIS tissue samples.

Discussion

To date, there have not been any studies examining miRNA expression within normal bladder urothelium and bladder CIS. Despite a limited number of samples, our study reveals that the phenotype of CIS most closely resembles that of UM cells, rather than B/i cells.

A novel approach undertaken in this study is the micro-anatomical isolation of the normal bladder into three compartments: i) UM cells, ii) B/i cells, and iii) MP tissue. Thus far, there is no consensus and there are several inconsistencies between different microarray studies for the majority of miRNAs reported as misexpressed. For instance, Lin et al[24] reported miR-125b and miR-199b to be significantly down-regulated in tumors compared to normal tissue, whereas Veerla et al[29] reported high expression of the same two miRNAs in invasive (T2/T3) tumors. One explanation for
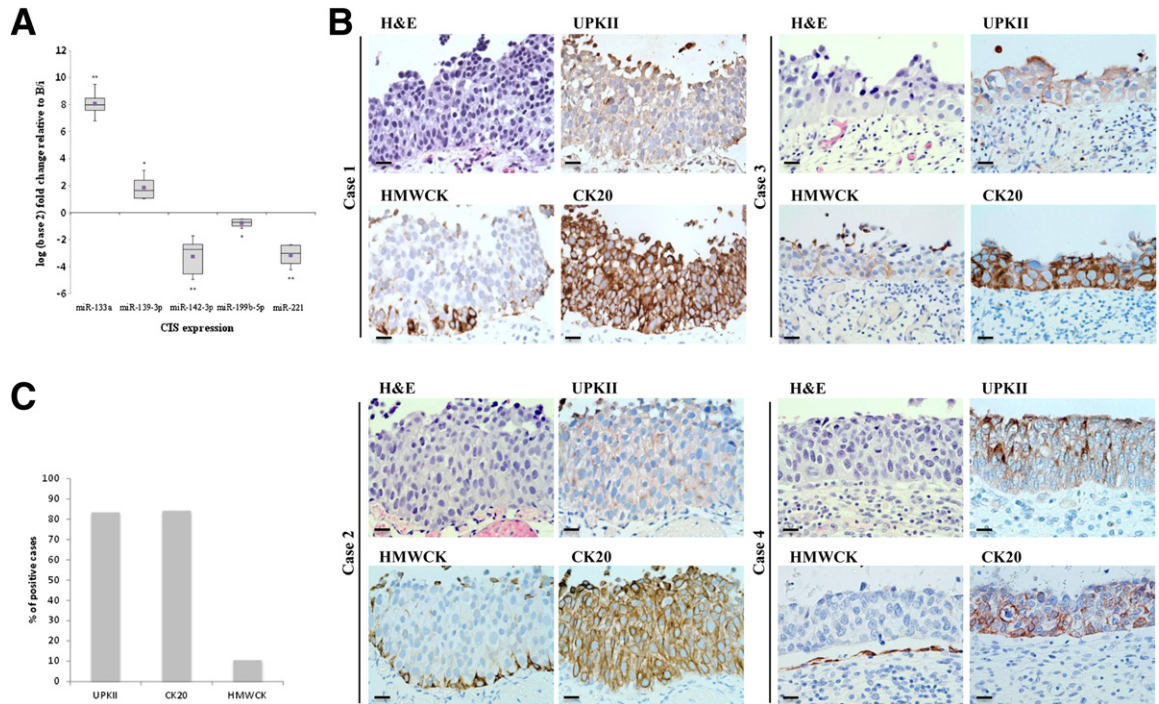
Figure 4 Phenotypic association of CIS with UM cells by RT-qPCR and immunohistochemistry. **A:** RT-qPCR results on B/i and CIS samples for various miRNAs. Graph shows CIS $\log_2$ fold expression; CIS expression is normalized to B/i. **Horizontal line** indicates the sample median. **Square** indicates the sample mean. *$P < 0.05$, **$P < 0.005$ by Student's *t*-test. **B:** Representative immunohistochemical analyses of UPKII, CK20, and high molecular weight cytokeratins (HMWCK) in four different CIS tissue samples. Scale bar = 100 μm. **C:** Quantification of the percentage of cases with expression of the respective biomarkers.

this discrepancy is that different populations of normal bladder tissue were used. It is extremely difficult to obtain pure urothelium by macrodissection because it often leads to contamination of the urothelial sample with the lamina propria. Furthermore, although some groups use normal

Table 3 Summary of Immunohistochemical Results in Individual CIS Tissue Samples

Case No.	UPKII	CK20	HMWCK
1	−	+	−
2	+	+	−
3	+	+	−
4	+	+	+
5	+	+	−
6	+	+	−
7	+	+	−
8	+	−	−
9	−	−	−
10	+	+	−
11	+	+	−
12	+	+	−
13	−	+	−
14	NA	+	−
15	+	+	−
16	+	+	+
17	+	+	−
18	+	+	−
19	+	−	−
% Positive	83.3	84.2	10.5

NA, core not available.

bladder mucosa,[20,21,23] others use normal tissue adjacent to the tumor,[24,25] which can include nonurothelium components, such as the muscularis propria. Microdissection is necessary, therefore, to ensure clean urothelial samples, and it is the only method to dissect single-layer UM cells.

We profiled the expression of 984 human miRNA and 22,277 human mRNA genes in the subpopulations of normal bladder, CIS tumors, and a normal urothelial cell line (HUC). Surprisingly, miRNA expression profile revealed CIS tumors to share a molecular profile displayed by UM cells, although mRNA expression profile clustered B/i and UM cells together, but separate from CIS. These data suggest that gene expression analysis is able to separate normal from tumor tissues. In fact, more than half of the differentially expressed genes that were upregulated in CIS, when compared to normal UM and B/i cells, were associated with proliferative phenotypes (eg, cell cycle, metabolic pathways, and pathways in cancer). In contrast, miRNA expression has been shown to classify tissue samples based on histogenesis,[16] and our data show that CIS samples share more similarity with UM cells than B/i cells in the normal urothelium.

There were five miRNAs that were significantly altered between B/i and UM cells. RT-qPCR and *in situ* hybridization confirmed miR-133a and miR-139-3p to be specific to UM cells, whereas miR-142-3p was expressed only in B/i cells. Interestingly, the two UM cell specific miRNAs, miR-133a and miR-139-3p, are located on chromosome 18q and 11q, respectively. It is well known that deletions of

chromosomes 18 and 11 are associated with invasive bladder carcinomas,[30] which mainly arise from high-grade lesions such as CIS. Conversely, B/i specific miR-199b-5p is located on chromosome 9q, the deletion of which is associated almost exclusively with papillary, nonmuscle invasive tumors.[6,31–33] It is plausible that deletion of these miRNAs may affect downstream targets involved in bladder tumorigenesis.

It is well accepted that there are two pathways of bladder tumor progression.[6,34–36] The development of papillary, non-muscle invasive tumors is largely independent from that of CIS lesions, as demonstrated through transgenic mouse models.[37–39] Although bladder-specific expression of mutant *H-RAS* led to the growth of papillary tumors,[38] simultaneous deletion of tumor suppressors *p53* and *Pten* gave rise to CIS with progression to muscle-invasive tumors and metastasis.[37] Furthermore, the double inactivation of *p53* and *Rb* through UPKII-driven expression of SV40T also led to the development of high-grade CIS and muscle-invasive tumors.[39] A clonal origin of bladder cancer has been suggested,[40] although the study did not include CIS tumors in the analyses. It remains unclear, therefore, whether oncogenic changes that give rise to two phenotypic variants occur within one cell, or whether they arise in different progenitors (UM versus B/i). Our current study identifies a strong association between CIS and UM cells in miRNA expression and the expression of a few well-established differentiation markers of the UM cell, and provides evidence of the possible relationship between the two.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.01.006*.

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