Biomarkers, Genomics, Proteomics, and Gene Regulation

miR-143, miR-222, and miR-452 Are Useful as Tumor Stratification and Noninvasive Diagnostic Biomarkers for Bladder Cancer

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**Altered microRNA (miRNA) expression may occur
early in bladder cancer and may play a role in carci-
nogenesis and tumor behavior. We evaluated whether
alterations in miRNA expression could improve dis-
ease stratification and outcome prognosis in bladder
tumors and noninvasive diagnosis in urinary sam-
ples. miR-143, miR-222, and miR-452 expression lev-
els were analyzed by quantitative RT-PCR (RT-qPCR)
in paired urinary and matching tumors and in two
independent prospective series of tumors and uri-
nary specimens. Differential expression of miR-143,
miR-222, and miR-452 in urine were verified by *in situ*
hybridization in matching tumors. Tumor miRNA ex-
pression by RT-qPCR correlated with tumor grade, size,
and presence of carcinoma *in situ* for miR-222, recur-
rence (miR-222 and miR-143), progression (miR-222
and miR-143), disease-specific survival (miR-222), and
overall survival (miR-222). Protein expression patterns
of potential miRNA targets, including vascular endothe-
lial growth factor, BCL2, v-erb-b2 erythroblastic leuke-
mia viral oncogene (ERBB) homolog 3, and ERBB4, were
evaluated by IHC in tissue arrays containing tumors for
which miRNAs were assessed by RT-qPCR. Target expres-
sion correlated with expression of their predicted regula-
tory miRNAs, recurrence (ERBB3), progression (ERBB4),
disease-specific survival (ERBB3 and ERBB4), and overall
survival (ERBB3 and ERBB4). Furthermore, RT-qPCR of
miR-452 (area under the curve, 0.848) and miR-222 (area
under the curve, 0.718) in urine provided high accuracies
for bladder cancer diagnosis. Thus, bladder tumors were
characterized by changes in miRNA expression that
could aid in tumor stratification and clinical outcome
prognosis, and miRNAs were detected in urinary speci-
mens for noninvasive diagnosis.** *(Am J Pathol 2012, 180:
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MicroRNAs (miRNAs) are small non–protein-coding
RNAs that regulate gene expression post-transcription-
ally by interacting with partially complementary target
sites in mRNAs, either inducing their degradation or im-
pairing their translation. miRNAs are implicated in several
diseases and cellular functions, including apoptosis, dif-
ferentiation, and proliferation, among others. Aberrant
miRNA expression levels are associated with tumorigen-
esis, progression, and metastases, acting as oncogenes
and/or tumor suppressors.[1–17] The tissue-specific na-
ture of miRNA expression suggests that different tu-
mors would express specific miRNA signatures. An
increasing number of studies designed to decipher
miRNAs specific to bladder cancer have attempted to
understand regulatory effects of miRNAs on bladder
tumorigenesis and progression and to search for po-
tential roles of miRNAs as diagnostic and/or prognos-
tic/predictive biomarkers.[1–17]

Following the hypothesis that altered miRNA expres-
sion occurs early in bladder carcinogenesis and contrib-
utes to tumor behavior, we tested whether miRNAs pre-
viously shown to be differentially expressed in bladder
tumors[4,17] could be detected in urinary specimens and,

thereby, useful in bladder cancer diagnosis. Specifically, miR-143, miR-222, and miR-452 were analyzed in matching urothelial tissues and independent sets of tumor specimens to determine their expression and correlation with pathological variables and outcome. Protein expression levels of potential miRNA targets by immunohistochemistry (IHC) were assessed to confirm their association with miRNA expression and clinicopathologic assessment, as shown in the experimental design (see Supplemental Figure S1, A–E, at *http://ajp.amjpathol.org*).

Materials and Methods

Tumor Samples

Paraffin-embedded tumors were prospectively collected following Institutional Review Board–approved protocols. Non–muscle-invasive tumors underwent transurethral resection as primary treatment.[18] Adjuvant intravesical chemotherapy was used for low-grade tumors, and maintenance *Bacillus* Calmette-Guerin was used for high-grade tumors. Radical cystectomy with pelvic lymphadenectomy was used for muscle-invasive tumors. Patients underwent surveillance stratified by their disease.[18] Inclusion criteria of primary tumors were based on histopathological characteristics, requiring high-quality RNA for miRNA analyses. A first set included tumors with available matching urinary samples ($n = 37$). Demographic information indicated 31 males and 6 females (median age, 66 years; range, 51 to 86 years). A second set included tumors ($n = 164$) for which tissue arrays were constructed. High-quality RNA was obtained from 113 tumors. Demographic information indicated 101 males and 12 females (median age, 73 years; range, 35 to 94 years).

Urinary Samples

Individuals presenting microscopic hematuria under first bladder cancer suspicion provided urine specimens prospectively collected immediately before cystoscopy, following Institutional Review Board–approved protocols. Urinary specimens for which high-quality RNA was obtained in matching bladder tumors were used to assess the correlation of miRNA expression between matching pairs of specimens ($n = 37$). An independent urine set ($n = 94$) assessed the diagnostic utility of miRNAs discriminating patients with bladder cancer ($n = 37$) from controls ($n = 57$), including healthy individuals ($n = 20$) and patients with benign urological diseases ($n = 37$). Demographic information indicated 44 males and 50 females (median age, 50.3 years; range, 16 to 87 years). The histopathological tumor stage distribution was as follows: pTa, $n = 3$; pT1, $n = 19$; and pT2+, $n = 15$. Tumor grade distribution was as follows: low grade, $n = 12$; and high grade, $n = 25$.

RNA Extraction

Total RNA from tissue and urine specimens was extracted using TRIzol (Invitrogen, Carlsbad, CA).[19] For paraffin-embedded tissues, tumor cell content was estimated to be >75% on consecutive sections after H&E staining, and corresponding pieces were digested overnight before RNA extraction using proteinase-K (Roche, Basel, Switzerland). The concentration and purity of RNA were determined with an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). RNA quality was evaluated based on 260/280 ratios of absorbances and gel electrophoresis using the 2100 Bioanalyzer (Agilent, Santa Clara, CA).

RT-qPCR Data

For quantitative determination of miR-16, miR-143, miR-222, and miR-452 expression, reverse transcription of RNA (300 ng) was performed in triplicate by quantitative RT-PCR (RT-qPCR) TaqMan miRNA assays (Applied Biosystems, Foster City, CA) using an ABI Prism 7900HT system (Applied Biosystems). miR-16 was used for internal normalization because of consistent nondifferential expression levels in tumor and nonneoplastic samples. The C_T values were calculated with SDS software version 2.1 (Applied Biosystems). Fold differences were obtained using the following equation: $2^{-\Delta C_T}$, where C_T is the threshold cycle, and ΔC_T equals the mean C_T of the sample gene minus the mean C_T of miR-16.

In situ hybridization was performed using formalin-fixed, paraffin-embedded normal urothelium biopsy specimens (5 μm thick) and bladder tumors belonging to patients with matching urinary samples analyzed by RT-qPCR. *In situ* hybridization was also performed using 10 pmol of locked nucleic acid–modified fluorescein isothiocyanate (FITC)–labeled oligonucleotide probes (Exiqon, Vedback, Denmark), complementary to miR-222, miR-143, and miR-452, overnight at 25°C lower than the predicted temperature of melting of the probe.[20,21]

Prediction and Selection of miRNA Targets

Potential miRNA targets were predicted using four algorithms: PicTar, TargetScan, miRanda, and DIANA microT. To decrease the number of false-positive results, a putative mRNA was only considered a target if found in at least two of the four algorithms. Targets were selected based on availability of antibodies to study their protein expression: BCL2, potential target of miR-143 according to TargetScan, Pictar, and DIANA microT, and of miR-222 according to DIANA microT; vascular endothelial growth factor (VEGF), potential target of miR-452 according to TargetScan and DIANA microT, and of miR-16 according to TargetScan, miRanda, and DIANA microT; v-erb-b2 erythroblastic leukemia viral oncogene (ERBB) homolog 3, potential target of miR-143 according to TargetScan, miRanda, and DIANA microT, and of miR-222 according to miRanda and DIANA microT; and ERBB4, potential target of miR-222 according to miRanda and DIANA microT, and of miR-452 according to TargetScan.
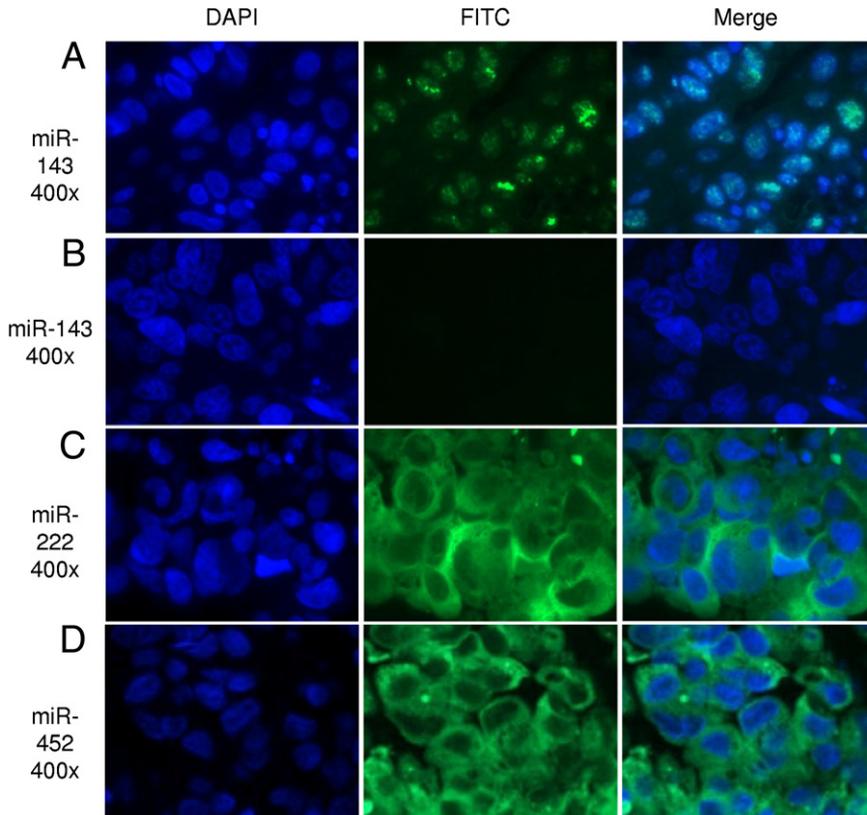
Figure 1. Verification analyses by *in situ* hybridization of the differential expression of the identified miRNAs. Representative *in situ* patterns in urothelial hyperplasia for miR-143 (**A**) and, in a high-grade pT1 bladder tumor, for miR-143 (**B**), miR-222 (**C**), and miR-452 (**D**). *In situ* hybridization was performed on tissue specimens matching the urinary samples analyzed by RT-qPCR. The miRNA locked nucleic acid probes were labeled with FITC (green) and counterstained with DAPI (blue). Original magnification, ×400.

0.005), presence of carcinoma *in situ* (*P* = 0.035), and clinical outcome end points (recurrence, *P* = 0.006; progression, *P* = 0.003; disease specific, *P* = 0.034; and overall survival, *P* = 0.023) (Figure 2, A–D). Fur

thermore, miR-143 expression significantly correlated with the following clinical outcome end points: recurrence, *P* = 0.011; and progression, *P* = 0.039 (Figure 2, E and F).
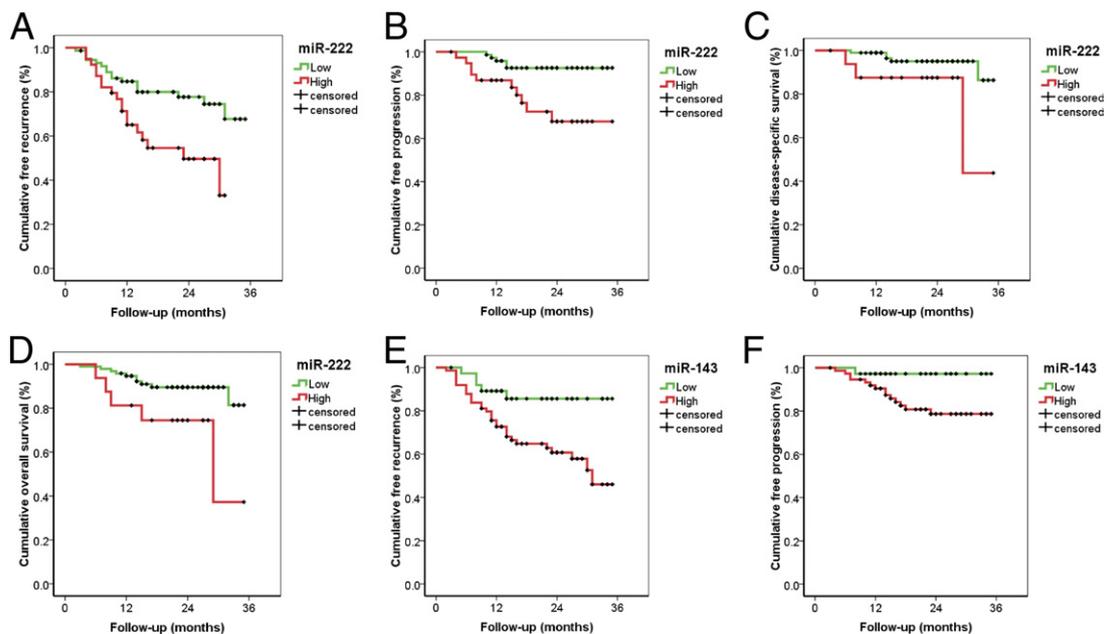

Figure 2. miRNA expression levels by RT-qPCR in tumor specimens are associated with clinical outcome end points in patients with bladder cancer (*n* = 113). miR-222 expression was associated with a high recurrence rate (log-rank *P* = 0.006) (**A**), progression into muscle-invasive disease (log-rank *P* = 0.003) (**B**), disease-specific survival (log-rank *P* = 0.034) (**C**), and overall survival (log-rank *P* = 0.023) (**D**). miR-143 expression was associated with a high recurrence rate (log-rank *P* = 0.011) (**E**) and progression into muscle-invasive disease (log-rank *P* = 0.039) (**F**).
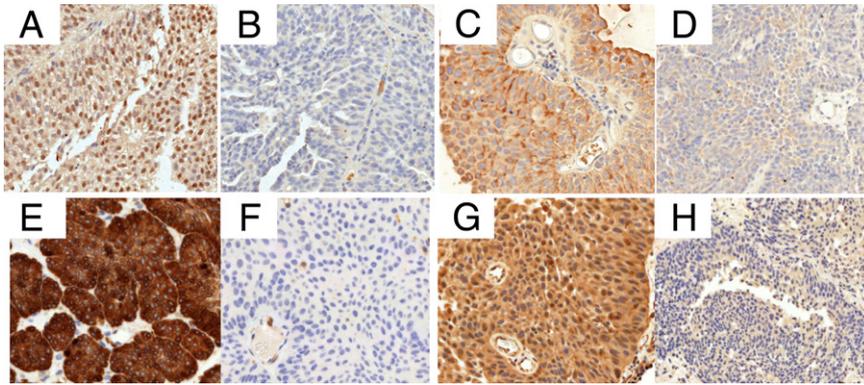
Figure 3. Verification analyses by IHC of the differential expression of the identified proteins in bladder tumors ($n = 164$). Representative protein expression patterns of the following proteins in bladder tumors contained in tissue arrays. For ERBB4 (**A** and **B**), ERBB3 (**C** and **D**), and BCL2 (**E** and **F**), protein expression was significantly different between noninvasive lesions (**A**, **C**, and **E**) and invasive tumors (**B**, **D**, and **F**). For VEGF, lower protein expression was observed in low-grade tumors (**G**) compared with high-grade tumors (**H**). Original magnification, ×400.

Association of Protein Expression Profiles of Potential Targets of miR-143, miR-222, and miR-452 in Bladder Tumors by IHC with miRNA Expression Profiles and Clinicopathologic Correlates

Differential expression for BCL2, VEGF, ERBB3, and ERBB4 was observed among the bladder tumors tested (Figure 3). Significant associations were found with tumor stage for ERBB4 ($P = 0.013$), ERBB3 ($P = 0.003$), and BCL2 ($P = 0.030$); tumor grade for ERBB4 ($P = 0.027$), ERBB3 ($P = 0.006$), VEGF ($P = 0.024$), and BCL2 ($P = 0.030$); tumor size for ERBB4 ($P = 0.025$) and ERBB3 ($P = 0.005$); and pattern of growth for ERBB3 ($P = 0.007$), VEGF ($P = 0.041$), and BCL2 ($P = 0.013$). Moreover, ERBB4 and ERBB3 protein expression patterns correlated with clinical outcome (Figure 4). Specifically, ERBB4 nuclear expression corre-lated with progression ($P = 0.001$), disease-specific survival ($P < 0.0005$), and overall survival ($P = 0.002$). ERBB3 cytoplasmic expression correlated with recurrence ($P = 0.044$), disease-specific survival ($P = 0.034$), and overall survival ($P = 0.012$). Inverse correlations between miRNA expression and protein expression patterns were observed for the following pairs: VEGF–miR-452 (τ-$\beta = -0.191$, $P = 0.010$), VEGF–miR-16 (τ-$\beta = -0.296$, $P < 0.0005$), BCL2–miR-222 (τ-$\beta = -0.216$, $P = 0.009$), BCL2–miR-143 (τ-$\beta = -0.195$, $P = 0.009$), and ERBB3–miR-222 (τ-$\beta = -0.214$, $P = 0.046$).

Diagnostic Properties of miRNAs in Urinary Specimens

The miRNA profiles by RT-qPCR were measured in an independent series of urinary specimens belonging to
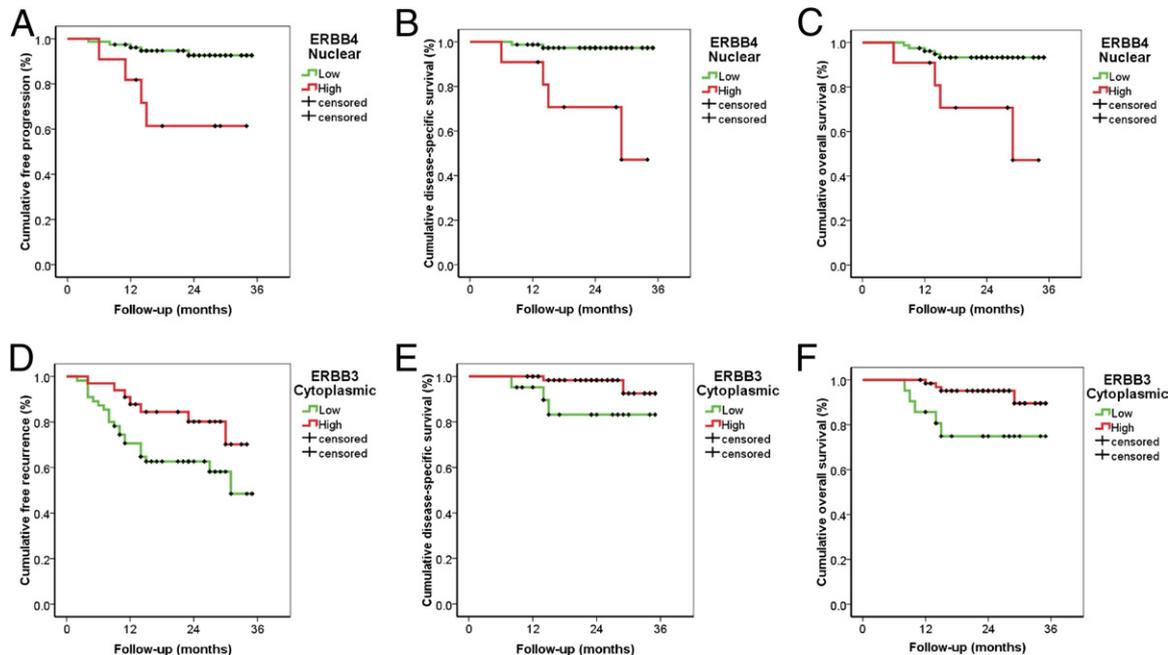

Figure 4. Protein expression patterns of miRNA targets obtained by IHC in bladder tumors spotted on tissue arrays ($n = 164$) are associated with clinical outcome end points in patients with bladder cancer. ERBB4 nuclear expression was associated with progression into muscle-invasive disease (log-rank $P = 0.001$) (**A**), disease-specific survival (log-rank $P < 0.0005$) (**B**), and overall survival (log-rank $P = 0.002$) (**C**). ERBB3 cytoplasmic expression was associated with a high recurrence rate (log-rank $P = 0.044$) (**D**), disease-specific survival (log-rank $P = 0.034$) (**E**), and overall survival (log-rank $P = 0.012$) (**F**).
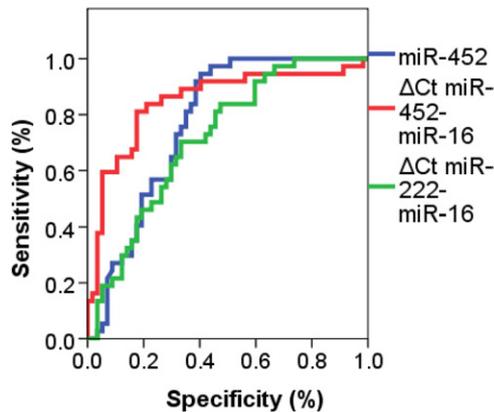
Figure 5. Utility of miRNA expression patterns for bladder cancer diagnostics in urinary specimens ($n = 94$). The receiver operating characteristic curve analyses indicated that the measurement of urinary miR-452 alone (AUC = 0.772), and those of miR-452 (AUC = 0.848) and miR-222 (AUC = 0.718), normalized by miR-16 expression, provided significant accuracies for the diagnosis of patients with bladder cancer from controls.

patients with bladder cancer ($n = 37$) and controls ($n = 57$). The receiver operating characteristic curve analyses indicated that urinary miR-452 alone (AUC = 0.772), and miR-452 (AUC = 0.848) or miR-222 (AUC = 0.718) normalized by miR-16 expression, provided significant accuracies for bladder cancer diagnosis (Figure 5).

Discussion

Both RT-qPCR correlations of matching tumor and urinary specimens and *in situ* hybridization analyses showing differential expression of candidate miRNAs in bladder tumors supported the working hypothesis by which urinary miRNA measurement could mirror the miRNA expression in bladder tumors and be potentially used for noninvasive bladder cancer diagnostics. *In situ* hybridization probes detecting premature and mature miRNAs displayed cytoplasmic or nuclear staining, such as for miR-452 and miR-143.[22,23] *In situ* localization analyses served to address the source of miRNAs in the urine, from cancer urothelial cells and/or the stroma, as shown for miR-143.

Previous miRNA array profiling reports indicated lower miR-143 expression in tumors.[16,17] Higher miR-222 expression was found in muscle-invasive tumors,[4] and high miR-452 levels were found in tumors with lymph node metastases.[4] However, the three miRNAs were not previously described as useful in bladder cancer for diagnosis (miR-452) and clinical outcome prognosis (miR-143 and miR-222). None of these miRNAs were previously detected in urinary specimens. In support of our selection of miR-16 as the reference, this candidate also previously had consistent levels of expression in normal and bladder cancer–related specimens.[6] Our results suggested that miR-452 could contribute in tumorigenesis and aid in bladder cancer diagnostics, whereas miR-143 and miR-222 could be related to tumor progression and used for clinical outcome assessment. Consistent with previous reports, our findings suggested an oncogenic role for

miR-222[4] and miR-452[4] and a tumor suppressor role for miR-143.[16,17] Whether these miRNAs are involved in carcinogenesis and/or disease progression as tumor suppressors or oncogenic events remains to be determined in functional *in vitro* and *in vivo* analyses. Such experiments are out of the scope of this study, which aims to define the prognostic and diagnostic roles of these miRNAs.

We observed inverse correlations between miRNAs and protein expression of their selected potential targets, which supported the validity of the target prediction.[22–25] Although the associations between the miRNAs and the targets evaluated in our series are novel in bladder cancer, the link between these miRNAs and the expression of these targets has been shown in other models: miR-222 and ERBB4 in embryonic stem cells,[23] miR-143 and BCL2 in human leukocytes,[24] and miR-143 and VEGF in nasopharyngeal carcinoma.[25] ERBB4 expression inversely correlated with the expression of miR-222 and miR-452, which suggested that several miRNAs could simultaneously regulate the expression of one specific protein. Conversely, miR-222 correlated with the protein expression of BCL2 and ERBB3, supporting the notion that a single miRNA could regulate the expression of more than one protein.[4,17,22]

Analyses of expression patterns of BCL2, VEGF, ERBB3, and ERBB4 served to associate the miRNA expression to their targets and were clinically relevant by being correlated with histopathological correlates of tumor progression and/or several outcome end points. ERBB4 has localized to several cellular counterparts: membrane,[26,27] cytoplasm,[26,28] and nucleus.[29] Membrane loss was related to stage, grade, size, and poor survival,[26] whereas nuclear expression correlated with advanced disease.[29] Similarly, our results revealed that increased nuclear staining was also linked to poor outcomes, suggesting a potential translocation of ERBB4 within the cell. Our studies also established novel associations of cytoplasmic ERBB3 with tumor stage, grade, size, growth pattern, and outcome (recurrence and disease-specific and overall survival). ERBB3 previously sublocalized to the membrane[30] and the cytoplasm.[27,30] Lack of associations with membranous expression was also previously observed for ERBB3[27,30] and ERBB4.[27–29] Although translocation mechanisms of ERBB3 and ERBB4 in bladder cancer progression remain to be clarified, our results highlighted the clinical relevance of the proteins' cellular sublocalization for outcome prognosis.

In the context of a prospective study, the follow-up ranged up to 3 years, as shown in the Kaplan-Meier curves. Based on the natural history of bladder cancer, many patients are expected to be alive and, thus, censored at the follow-up frame of this study. The distribution of censored patients varied depending on the miRNA and protein under analyses. To further highlight the statistical clinical outcome discrimination power of the measurements undertaken, these miRNAs and proteins served to identify patients with a poorer outcome, even in the presence of many censored alive patients. This discrimination would be expected to increase in

the presence of fewer censored patients and a longer follow-up.

In addition to the utility of miRNA expression assessment for tumor classification, our study suggested a potential diagnostic adjunct role of urinary miRNAs. The diagnostic accuracies of miR-452 (85%) and miR-222 (77%) should be considered relevant because cystoscopy as a gold standard remains invasive and relatively expensive for disease follow-up, and urinary cytological analysis provides overall diagnostic accuracies of 40% while failing in low-grade tumors. Our study suggests that, in the near future, novel miRNA-based panels might be explored for bladder cancer diagnostics. It is important to be aware that the amount of exfoliated cancer cells could be a limitation in the extraction of an adequate amount of mRNA from urinary specimens.

In conclusion, urinary miRNAs were clinically useful for noninvasive bladder cancer diagnostics (miR-452 and miR-222) and tumor stratification and outcome assessment using tumor samples (miR-222 and miR-143). Protein expression profiles of targets (ERBB3 and ERBB4) potentially regulated by these miRNAs correlated with their expression, clinicopathologic correlates of tumor progression, and clinical outcome.

Acknowledgments

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