

# MicroRNA Dysregulation and Non-Muscle-Invasive Bladder Cancer Prognosis

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## Abstract

**Background:** The high rate of non-muscle-invasive bladder cancer recurrence is a major challenge in patient management. miRNAs functionally regulate tumor cell proliferation and invasion, and have strong potential as biomarkers because they are robust to degradation. The objective of this project was to identify reproducible prognostic miRNAs in resected non-muscle-invasive bladder tumor tissue that are predictive of the recurrent tumor phenotype.

**Methods:** We utilized patients diagnosed with primary non-muscle-invasive bladder cancer in three independent cohorts for a biomarker discovery/validation approach. Baseline tumor tissue from patients with the clinically challenging, non-muscle-invasive primary low stage (Ta), high grade, and T1 tumors (tumors extending into the lamina propria) comprised the discovery cohort ( $n = 38$ ). We isolated the tumor tissue RNA and assessed a panel of approximately 800 miRNAs.

**Results:** miR-26b-5p was the top-ranking prognostic tumor tissue miRNA, with a time-to-recurrence HR 0.043 for levels above versus below median, ( $P_{\text{adj}} = 0.0003$ ). miR-26b-5p was related to a dose-response reduction in tumor recurrence, and levels above the median were also associated with reduced time-to-progression ( $P_{\text{adj}} = 0.02$ ). We used two independent longitudinal cohorts that included both low-grade and high-grade Ta and T1 tumors for validation and found a consistent relationship between miR-26b-5p and recurrence and progression.

**Conclusions:** Our results suggest that miR-26b-5p levels may be prognostic for non-muscle-invasive bladder cancer recurrence, and can feasibly be assessed in baseline tumor tissue from a wide variety of clinical settings.

**Impact:** Early identification of those non-muscle-invasive bladder tumor patients with refractory phenotypes would enable individualized treatment and surveillance.

## Introduction

Non-muscle-invasive bladder tumors are prevalent in the population. An estimated 500,000 patients with a history of urothelial carcinoma currently reside in the United States (1). Bladder cancer recurrence rates vary considerably (2) and tumor behavior within a single histopathologic group is highly heterogeneous (3). Of patients diagnosed with non-muscle-invasive bladder cancer, 50% to 75% experience recurrences within 6 to 12 years of diagnosis and 10% to 30% of tumors progress to muscle-invasive disease (4). The high rate of disease recurrence and progression is a major challenge in patient management (5). Because we lack reliable predictive markers to distinguish those patients who will experience recurrence, the need to screen all

patients for these events frequently (every 3–6 months by the invasive cystoscopy procedure) makes bladder cancer one of the most expensive malignancies (6, 7).

Primary tumor clinicopathologic characteristics used to predict recurrence include multiplicity, tumor size, T category (depth of invasion), presence of carcinoma *in situ*, tumor grade, and patient gender (8). Patients with low stage (Ta), low-grade (LG) tumors can remain disease free for many years, but poorly differentiated tumors with a high grade (HG) often recur within one year and frequently progress to muscle-invasive disease (5). Management of these patients with TaHG or tumors extending into the lamina propria (T1) is clinically challenging. A subset of patients experience recurrent or progressing tumors that are refractory to treatment, and bladder removal by cystectomy may need to be performed (9). Early identification of those patients with non-muscle-invasive bladder tumor with recurrent, progressing, and refractory phenotypes would enable individualized treatment and surveillance recommendations, reducing patient burden and disease mortality.

Noncoding RNAs, particularly the miRNAs have emerged as useful prognostic biomarkers in cancer in part because their small size makes them stable to degradation and thus robust to variations in sample handling (10). The miRNAs regulate their target genes by binding to specific sites, usually in the 3' untranslated region of the target gene. The miRNA then modifies the target gene via translational repression, cleavage, degradation, or sequestration (11). The objective of this project was to identify reproducible prognostic miRNAs in resected non-muscle-invasive bladder tumor tissue that are predictive

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of the recurrent tumor phenotype as potential biomarkers and molecular therapeutic targets.

## Materials and Methods

All study procedures were approved by the Committee for the Protection of Human Subjects at Dartmouth College and the Veteran's Institutional Review Board of Northern New England. We utilized patients diagnosed with primary non-muscle-invasive bladder cancer in three independent cohorts for a biomarker discovery/validation approach.

For the Dartmouth-Hitchcock Medical Center (DHMC) cohort, we retrospectively selected a sequential set of patients with bladder cancer identified through the hospital tumor registry diagnosed in the years 2008 to 2014. We identified a subset of those patients with the clinically challenging non-muscle-invasive histologic types: primary Ta High-Grade and T1 tumors (TaHG/T1) who had archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks available for this miRNA expression project. We followed patients via retrospective review of their electronic medical record to identify recurrence and progression events. We ensured that all tissue samples utilized represented the tissue that was removed prior to the administration of any intravesical immunotherapy or chemotherapy. We reviewed the patient medical records carefully to ascertain clinical information related to recurrence and progression events. The study pathologists reviewed the hematoxylin and eosin (H&E)-stained slide and circled the noncauterized portion, containing tumor. For each tumor, we performed macrodissection on several of the matching 10- $\mu$ m unstained tissue sections to select only the circled portions to maximize the tumor content of the sample. RNA was isolated from this portion using Qiagen Deparaffinization Reagent followed by the Qiagen AllPrep FFPE Tissue Kit (Qiagen Inc).

For additional corroboration of our miRNA markers, we collected an independent cohort of patients with bladder cancer from the White River Junction Department of Veterans Affairs (VA) Medical Center (WRJ VAMC). Using administrative data from the VA National Corporate Data Warehouse, we searched for patients undergoing transurethral resection of a bladder tumor or bladder biopsy at WRJ VAMC (CPT codes 52204, 52214, 52224, 52234, 52235, 52240; ICD9 procedure codes 57.33, 57.49) between 2005 and 2011. This timeframe was chosen because of the availability of high-quality, consistently reported claims data and sufficient retrospective follow-up time to ascertain recurrence and progression outcomes. We obtained clinical information related to recurrence and progression events from the patient medical records. We utilized tissue that was removed without the administration of any intravesical immunotherapy or chemotherapy within the past 3 years. We used one or two 10- $\mu$ m sections of tumor for deparaffinization and RNA isolation using the Qiagen AllPrep FFPE tissue kit. We did not macrodissect the VA cohort specimens, nor select on the basis of tumor content.

The New Hampshire population-based cohort was comprised of patients with bladder cancer diagnosed in the state of New Hampshire (NH) between 2002 and 2004. Eligible cases were residents of the state of New Hampshire at the time of diagnosis identified using the State Cancer Registry, hospital pathology departments, and hospital cancer registries, as described previously (12). Information on bladder tumor clinicopathologic features and recurrences was obtained from medical records, or provided by the treating hospital(s) (both

inpatient and outpatient records, including any pathology reports) covering the follow-up period. The study pathologist used matching H&E-stained slides to ensure that the selected specimens contained a minimum of 75% tumor. We used an entire 10- $\mu$ m section for deparaffinization and RNA isolation using the Qiagen AllPrep FFPE tissue kit. Notably, this NH population cohort is unselected and included entire tissue sections and a large proportion of Ta low-grade tumors, (in contrast to the DHMC cohort, which we restricted to TaHG/T1 tumors and macrodissected).

### Tissue-matched bio-fluid samples

On a subset of DHMC patients with bladder cancer with tumor tissue samples, we also collected blood and urine samples. Subjects recruited into the study during their diagnostic appointment, but prior to tumor resection. Urine and peripheral blood samples were collected during that visit or at a subsequent visit, prior to tumor resection. Whole blood was collected in a Vacutainer EDTA (K2) Plastic Tube (Becton Dickinson) and fractionated by centrifugation at  $1,500 \times g$  for 20 minutes at 20°C. Plasma-enriched white blood cells (WBC) and red blood cells were aliquoted into cryogenic vials (Corning Inc.) and stored at  $-80^{\circ}\text{C}$ . Urine was collected midstream in Clikseal containers (Therapak), and subsequently transferred to 15-mL conical tubes (VWR) for centrifugation. Urine was centrifuged twice, first at  $1,200 \times \text{rpm}$  for 20 minutes at 20°C. Supernatant was transferred to fresh conical tube and centrifuged at  $2,500 \times \text{rpm}$  for 20 minutes at 20°C. Final supernatant was aliquoted into cryogenic vials and stored at  $-80^{\circ}\text{C}$ . For comparison with the biofluids, tRNA was extracted from  $3 \times 20\text{-}\mu\text{m}$  slices of FFPE-tumor tissue using Norgen FFPE RNA/DNA Purification Plus Kit (Norgen Biotek Corp.). Circulating and exosomal plasma miRNA was isolated from a 200- $\mu\text{L}$  plasma volume using Norgen Plasma/Serum Circulating and Exosomal RNA Isolation Kit. Cell-free miRNA from urine was obtained using Norgen Urine Exosome RNA Isolation Kit from a 4-mL volume of cell-free urine. WBC-enriched RNA was isolated using Norgen Total RNA Purification Kit. All protocols were performed according to the manufacturer's instructions. Plasma and urine miRNA was purified and concentrated using Amicon Ultra 0.5 columns (Millipore), as described previously (13).

### miRNA expression levels

Dartmouth Genomics and Microarray Core facility simultaneously assessed approximately 800 miRNA probes using the NanoString Human v3 microRNA Expression Assay (NanoString Technologies). We loaded 200 ng of tRNA into the assay. Specific tags were ligated to the 3' end of each miRNA molecule. miRNA molecules were then hybridized to a panel of miRNA: tagspecific nCounter capture and barcoded reporter probes. The nCounter Digital Analyzer counted individual fluorescent barcodes and quantified the target RNA molecules present in each sample. We used NanoString Nsolver 3.0/4.0 software to normalize the count data to the positive controls and to average geometric mean of the top 100 detected miRNAs. We estimated the background level using the counts in the negative controls (mean + 2SD), and restricted our analyses to the miRNAs expressed at counts above background in at least 1/3 of the tumors (169 miRNAs in the DHMC cohort). The miRNA levels of samples duplicated across batches were highly correlated ( $r^2 = 0.99$ ) and the coefficient of variation (% CV) for miR-26b-5p was 5.6 among these samples.

## Statistical analysis

We defined first recurrent tumor as any tumor identified following a disease-free remission period, more than 90 days after the date of initial primary bladder tumor diagnosis. These recurrent tumors include subsequent tumors of the same level of invasiveness, as well as those progressing to higher stage/grade. Persistent primary tumors that did not have a remission period were excluded from the analysis of recurrence. Time-to-recurrence was calculated as the time between the initial diagnosis date and the date of the first recurrence event. We report on overall progression, including tumors with a greater stage or grade than the initial primary bladder tumor; and report the proportion progressing to muscle invasion or metastasis (14). If no events were reported, the date the patient was last seen documented in the medical record was used for censoring.

Median times to first recurrence, or progression were calculated using the Kaplan–Meier method. Multivariate analysis of time to the first bladder tumor recurrence and progression analyses were performed using Cox-proportional hazards regression analysis with miR-26b-5p levels modeled as a continuous variable, and using median or quintile cut-off points. The standard base prognostic model included adjustment for age at diagnosis of first bladder tumor, gender, tumor size (<3, 3+cm), multiplicity (single, multiple), stage (Ta, T1)/grade (low-grade, high-grade). miRNA levels were modeled as counts, or using the median as a cut-off point. We constructed time-dependent ROC curves and area under the ROC curves (AUC) using Akita's nearest neighbor estimation of the bivariate distribution implemented in the "survival ROC" package (15). We assessed the accuracy of the multivariate models for discriminating patients at high risk of recurrence using the concordance index, which has values ranging from 0.5 to 1.0 (perfect discrimination). *P* values represent two-sided statistical tests. Analyses were performed using R 3.4.1.

## Results

As shown in Table 1, the non-muscle-invasive bladder cancer patient cohorts assessed for miRNAs included a majority of male patients (72%–100%) diagnosed with bladder cancer at mean ages between 62 and 72. Multiple tumors were present in a subset (26%–38%), and approximately half had large (3+cm) tumors.

Within the DHMC TaHG/T1 cohort, we used Cox regression analysis to assess the association between each of the miRNAs detected in tumor tissue and time-to-first recurrence. Our multivariable model was adjusted for sex, age, multiplicity, tumor size, stage, and grade. Table 2 shows the top *P* value-ranked tumor tissue miRNAs associated with recurrence. miR-424-5p,

miR-125a-5p, and miR-193b-3p showed association trends in the continuous model of miRNA counts; however, when analyzed categorically (e.g., using the median as a threshold), the relationships for miR-125a-5p, and miR-193b-3p were not statistically significant, suggesting a nonlinear relationship. Although miR-424-5p levels above the median were significantly associated with recurrence (*P* = 0.033), this marker was not statistically significantly associated with progression (*P* = 0.30).

Thus, miR-26b-5p was the miRNA most strongly associated with recurrence (continuous miR-26b-5p levels, *P* = 0.00084 adjusted for sex, age, multiplicity, tumor size, stage, grade), and with progression (*P*<sub>adj</sub> = 0.02). miR-26b-5p levels did not differ significantly by sex (*P* = 0.90), multiplicity (*P* = 0.95), tumor size (*P* = 0.62), stage/grade (*P* = 0.06), treatment with chemotherapy (*P* = 0.1), or BCG (*P* = 0.71), and were not correlated with age at diagnosis (*P* = 0.27). The tumor miR-26b-5p counts were lower than the patient-matched histologically normal adjacent tissue in 2/3 of the resected specimens assessed (Supplementary Fig. S4). Breaking the tumor tissue miRNA level at the median, the patients with miR-26b-5p above the median had longer time-to-recurrence (Fig. 1A). The recurrence HR for tumor tissue miR-26b-5p was 0.043,  $\geq$  versus <median (*P* = 0.0003, adjusted for sex, age, multiplicity, tumor size, stage, grade; Table 2), meeting the Bonferroni corrected *P* value threshold ( $\alpha$  0.05/169 miRNAs). The addition of chemotherapy and BCG treatment to the model did not materially affect the statistical significance (recurrence *P* value = 0.00025, progression *P* = 0.012). Dividing the miR-26b-5p levels into quintiles, we observe a dose-response relationship with time-to-recurrence (Fig. 1C). Using the concordance index, we assessed the accuracy of the multivariable models for discriminating patients at high risk of recurrence. The base model (sex, age, multiplicity, size, stage, grade) had a concordance index of 0.63. The concordance index improved with the addition of miR-26b-5p to 0.81 (model includes sex, age, multiplicity, size, stage, grade, and miR-26b-5p).

We also evaluated tumor tissue miR-26b-5p in relation to bladder cancer progression to a higher stage or grade. Stage progression to muscle-invasive disease or metastasis occurred in 33% of these events. Patients with miR-26b-5p  $\geq$  versus <median (*P*<sub>adj</sub> = 0.02), or in higher quintiles consistently had a lower risk of progression (Table 2; Fig. 1B and D). On the basis of the consistency of the association with both recurrence and progression risk, we focused on validating miR-26b-5p as a prognostic marker across other patient cohorts.

We assessed miR-26b-5p levels in an independent cohort of patient from the WRJ VAMC (*n* = 20 patients with 11 recurrence events and a single progression event). The Kaplan–Meier plot

**Table 1.** Non-muscle-invasive bladder cancer patient characteristics by cohort

		DHMC TaHG/T1 cohort		VA cohort		NH population cohort	
		<i>n</i> = 38	%	<i>n</i> = 24	%	<i>n</i> = 169	%
Age	Mean $\pm$ SD	69.84 $\pm$ 11.96		72.38 $\pm$ 10.32		62.67 $\pm$ 10.36	
Gender	Female	9	24%	0	0%	47	28%
	Male	29	76%	24	100%	122	72%
Multiplicity	Multi	10	26%	9	38%	50	30%
	Single	28	74%	14	58%	119	70%
Size	Large	17	45%	NA	—	69	58%
	Small	21	55%	NA	—	50	42%
Stage/grade	TaLG	0	0%	14	58%	131	78%
	TaHG	10	26%	8	33%	12	7%
	T1	28	74%	1	4%	26	16%

**Table 2.** Top-ranking miRNAs associated with non-muscle-invasive bladder cancer recurrence

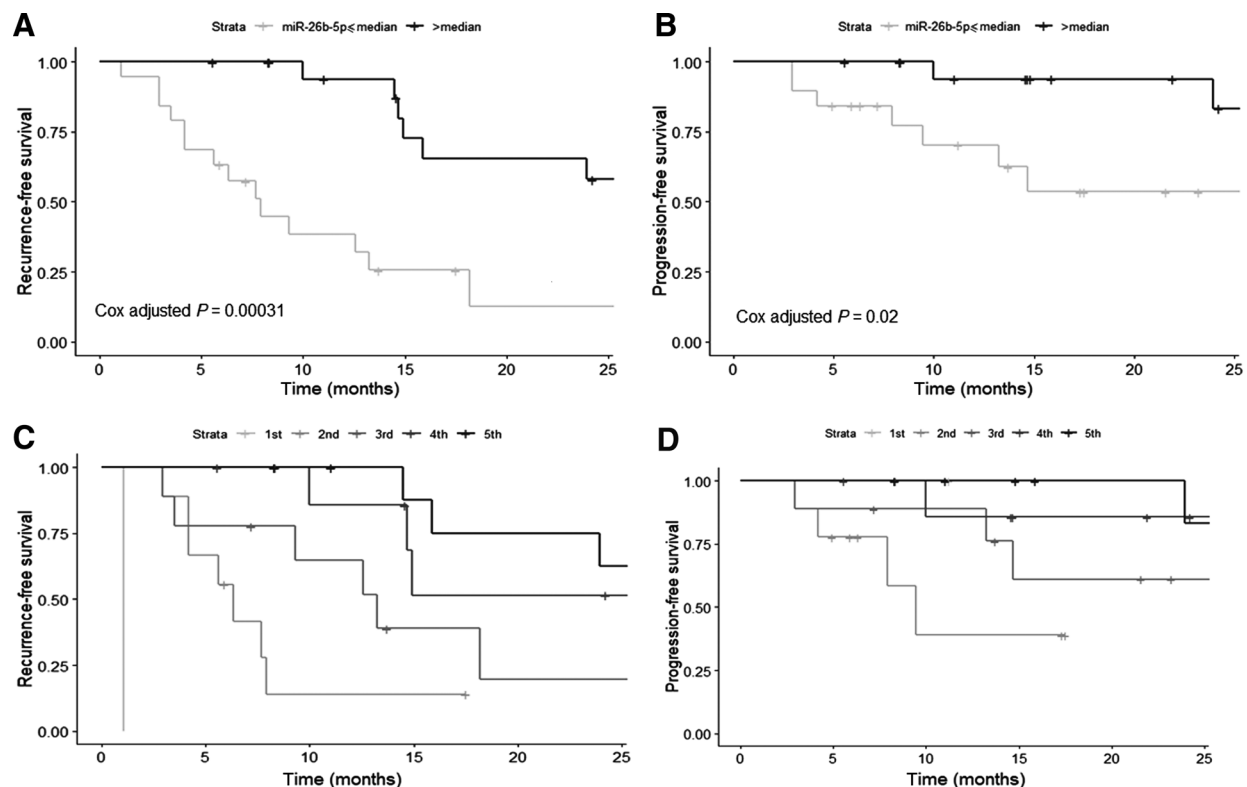
	miRNA count		miRNA levels $\geq$ versus $<$ median			miRNA levels $\geq$ versus $<$ median		
	$P^a$	Coefficient <sup>a</sup> Recurrence	HR <sup>a</sup>	(95% CI) Recurrence	$P^a$	HR <sup>a</sup>	(95% CI) Progression	$P^a$
<b>DHMC TaHG/T1 cohort</b>								
hsa-miR-26b-5p	0.00084	-0.0021	0.043	(0.0079-0.24)	0.00031	0.061	(0.0059-0.64)	0.020
hsa-miR-424-5p	0.006	0.0047	2.94	(1.09-7.93)	0.033	2.1	(0.51-8.68)	0.3
hsa-miR-125a-5p	0.0064	-0.0028	0.41	(0.13-1.33)	0.14	1.2	(0.25-5.86)	0.82
hsa-miR-193b-3p	0.0089	0.006	1.38	(0.50-3.85)	0.54	1.47	(0.30-7.23)	0.63
<b>Validation</b>								
VA cohort			Events = 20 of 38			Events = 9 of 38		
hsa-miR-26b-5p			0.66	(0.15-2.97)	0.59	NA		
NH population cohort			Events = 104 of 178			Events = 8 of 176		
hsa-miR-26b-5p			0.71	(0.47-1.05)	0.086	0.32	(0.062-1.64)	0.17

<sup>a</sup>Multivariable model adjusted for sex, age, multiplicity, tumor size, stage, grade.

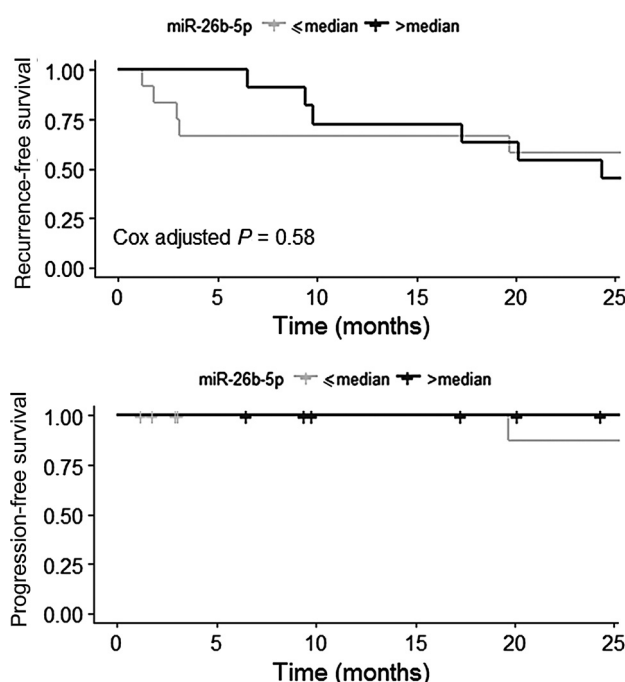
shows the probability of recurrence is lower among VA patients with miR-26b-5p levels above the median (HR 0.66), although the curves begin to intersect as time moves to 17 months (Fig. 2A).

We then assessed the value of miR-26b-5p in a broader community setting using the NH population cohort. Kaplan-Meier plots show a consistent, lower risk of recurrence and of progression with miR-26b-5p levels above the median (Fig. 3A and B). Analyses stratified into Ta low-grade and Ta high-grade/T1 (Supplementary Fig. S1), and by World Health Organization/International Society of Urological Pathology (WHO/ISUP) classification subsets show similar effects (Supplementary

Fig. S2). We constructed ROC curves to evaluate the ability of our models to discriminate patients who have recurrence from those who do not. The area under the ROC curve (AUC) at 24 months was 0.64 for the model containing miR-26b-5p, plus the base factors (sex, age, multiplicity, tumor size, stage, grade; Fig. 3C). The model containing miR-26b-5p has a consistently higher AUC (mean 0.62) compared with the base model (mean 0.59) throughout the two years of follow-up ( $P = 0.00027$ ). We also assessed the accuracy of the multivariable models for discriminating patients at high risk of recurrence. The concordance index for the model containing base factors (sex, age,

**Figure 1.**

Prognosis of DHMC discovery cohort by baseline tumor tissue miR-26b-5p levels. Kaplan-Meier plots depict recurrence (A and C) and progression (B and D) probability in patient subgroups based on the miR-26b-5p levels in their primary TaHG/T1 tumors. Patients with miR-26b-5p levels  $\geq$  median (black line) versus  $<$  median (gray line) had lower recurrence ( $P_{\text{adj}} = 0.00031$ , A) and progression probabilities ( $P_{\text{adj}} = 0.02$ , B). C and D, The shades depict the quintile, with dark lines depicting higher miR-26b-5p levels.



**Figure 2.** Prognosis of the VA replication cohort by baseline tumor tissue miR-26b-5p levels. Kaplan-Meier plots depict recurrence (11 events of 23) and progression probability (1 event of 23). Patients with miR-26b-5p levels  $\geq$  median (black line) versus  $<$  median (gray line) had lower probability of recurrence out to 10 months of follow-up.

multiplicity, tumor size, stage, grade) remained at 0.60 with the addition of miR-26b-5p for the Ta patients; however for T1 patients, the discrimination improved from 0.66 for the base model to 0.75 with the addition of miR-26b-5p.

Blood and urine can also be convenient for assessment of prognostic miRNA levels. We used a set of blood and urine samples that were patient matched to tumor tissue to assess the levels of the tumor tissue miR-26b-5p in the corresponding bio-fluid samples. Tumor tissue miR-26b-5p levels were not significantly correlated with blood plasma ( $P = 0.19$ ), white blood cell (WBC;  $P = 0.36$ ), or urine levels ( $P = 0.45$ ; Supplementary Fig. S3).

## Discussion

The objective of this project was to identify prognostic miRNAs in resected non-muscle-invasive bladder tumor tissues. We utilized non-muscle-invasive bladder cancer patient cohorts spanning three different settings: a referral hospital cohort, a Veterans Administration hospital, and a population-based study including community hospitals. We identified miR-26b-5p as consistently showing prognostic value for bladder tumor recurrence and progression. The linear nature of miR-26b's relationship with prognosis make it a better biomarker prospect than the other miRNAs identified. The utility of this miRNA was validated in unselected tissue sections from community hospital settings.

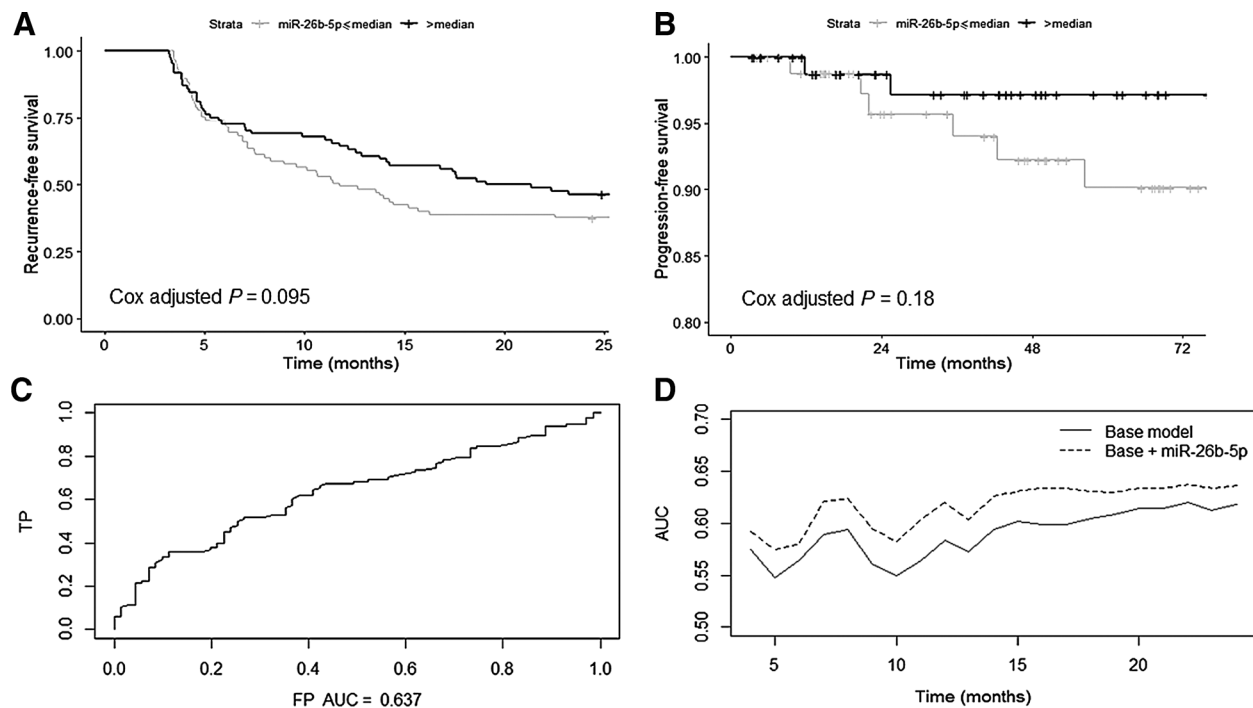
Gottardo and colleagues identified suppressed expression of miR-25b among a group of 10 miRNAs dysregulated in a cross-sectional screen of  $n = 25$  urothelial carcinoma versus  $n = 2$

normal bladder tissue samples (16) a finding replicated by Miyamoto and colleagues comparing a Japanese cohort of  $n = 69$  bladder tumors,  $n = 23$  normal epithelia ( $P = 0.0006$ ). Our finding that miR-26b-5p is a predictor of recurrence and progression across several longitudinally followed cohorts of non-muscle-invasive bladder cancer patients is unique, and strongly supports the *in vitro* work demonstrating a tumor-suppressive role for miR-26b-5p (17). While focused on the role of procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) as a potential miR-26a-5p/miR-26b-5p target, these cell culture transfection studies also demonstrated a functional role for miR-26b. miR-26b-5p transfection inhibited proliferation, migration, and matrigel invasion of bladder tumor cell lines, compared with miR-control (17).

miR-26b is contained within an intron of the phosphatase gene carboxy-terminal domain RNA polymerase II polypeptide (*CTDSP*). miR-26b is expressed as part of the *CTDSP2* transcript, and together they block the G<sub>1</sub>-S phase cell-cycle transition by cooperatively activating the checkpoint protein pRb (18). c-Myc is overexpressed in approximately half of non-muscle-invasive bladder tumors, with levels that are not correlated with tumor stage or grade (19). c-Myc is capable of transcriptionally repressing both *CTDSP* and miR-26b (18), possibly explaining the low miR-26b levels observed in some tumors. The levels of the mature form of miR-26b can also be controlled by inhibiting the processing of the precursor pre-miR-26b (20). Because Myc has been challenging and intractable as a direct therapeutic target, targeting miR-26b is a potential alternative intervention strategy to reduce risk of recurrence.

miR-26b levels were significantly lower in serum samples of patients with prostate cancer relative to noncancer controls  $P < 0.001$  (21). However, our study within patients with bladder cancer showed that tumor tissue miR-26b levels did not correlate with the patient-matched biofluid, suggesting that the baseline-resected tumor will likely be the more useful specimen. The tissue miR-26b levels were associated with recurrence risk even without macrodissecting the tissue specimens, increasing the logistical feasibility of this potential biomarker.

Our study provides unique longitudinal assessment of the relationship between miRNAs in non-muscle-invasive bladder cancer patients' baseline tumor tissue and their future recurrence and progression outcomes. The addition of miR-26b-5p to our multivariable models improved the accuracy for discrimination of patients at elevated risk for recurrence. The validation of this model in an external population with community hospital patients suggests potential clinical utility. Limitations of this study include a small number of progression events in the replication datasets, hampering our ability to draw definitive conclusions regarding the reproducibility of this endpoint. We deliberately restricted the DHMC cohort to patients with the more clinically challenging non-muscle-invasive tumors (26% Ta high-grade, 74% T1), to ensure we identified markers addressing the needs of this subgroup. In contrast, the majority of the patients in the replication phase from the WRJ VAMC and New Hampshire (NH) population cohorts had low stage (Ta) low-grade tumors (58 and 78%, respectively). The VA patients included some index tumors that were actually recurrences; in contrast, the DHMC and NH cohorts only assayed miRNAs in a patient's incident (first) tumor. miR-26b levels were related to recurrence risk across all three cohorts, increasing the validity of our finding.



**Figure 3.**

Prognosis of the NH population-based replication cohort by baseline tumor tissue miR-26b-5p levels. Kaplan-Meier plots depict recurrence (98 events of 169) and progression probability (8 events of 167). Patients with miR-26b-5p levels  $\geq$  median (black line) versus  $<$  median (gray line) had lower recurrence ( $P_{\text{adj}} = 0.095$ , panel **A**) and progression probabilities ( $P_{\text{adj}} = 0.18$ , panel **B**). **C**, Area under the ROC curve (AUC) is 0.64 for discrimination of patients with recurrence at 24 months in the model containing age, gender, grade, stage, multiplicity, and miR-26b-5p level in the baseline tumor. **D**, The addition of miR-26b-5p level significantly increases the AUC compared with the base model throughout the follow-up period ( $P = 0.00027$ ).

We identified miR-26b levels in baseline tumor tissue as a potential biomarker for non-muscle-invasive bladder cancer recurrence and progression. Our results demonstrate that prognostic miR-26b levels can feasibly be assessed in baseline tumor tissue from a wide variety of clinical settings. Further validation of the prognostic value of this marker in the baseline tumor tissue of additional populations is warranted.

#### Disclosure of Potential Conflicts of Interest

J.D. Seigne has ownership interest (including stocks and patents) in Johnson & Johnson. No potential conflicts of interest were disclosed by the other authors.

#### Disclaimer

Opinions expressed in this article are those of the authors and do not constitute official positions of the U.S. Federal Government or the Department of Veterans Affairs.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.S. Andrew, M.R. Karagas, A.R. Schned, J.D. Seigne

**Writing, review, and/or revision of the manuscript:** A.S. Andrew, M.R. Karagas, C.J. Marsit, A.R. Schned, D.A. Armstrong, J.D. Seigne  
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**Study supervision:** M.R. Karagas

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#### References

1. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol* 2013; 63:234-41.
2. Miyata Y, Sakai H. Predictive markers for the recurrence of nonmuscle invasive bladder cancer treated with intravesical therapy. *Dis Markers* 2015;2015:857416.

3. Murta-Nascimento C, Schmitz-Drager BJ, Zeegers MP, Steineck G, Kogevinas M, Real FX, et al. Epidemiology of urinary bladder cancer: from tumor development to patient's death. *World J Urol* 2007;25: 285–95.
4. Petrovich Z, Baert L, Boyd SD, Brady LW, D'Hallewin M, Heilmann HP, et al. Management of carcinoma of the bladder. *Am J Clin Oncol* 1998;21: 217–22.
5. Honma I, Masumori N, Sato E, Takayanagi A, Takahashi A, Itoh N, et al. Local recurrence after radical cystectomy for invasive bladder cancer: an analysis of predictive factors. *Urology* 2004;64:744–8.
6. Botteman MF, Pashos CL, Redaelli A, Laskin B, Hauser R. The health economics of bladder cancer: a comprehensive review of the published literature. *Pharmacoeconomics* 2003;21:1315–30.
7. Strobe SA, Montie JE. The causal role of cigarette smoking in bladder cancer initiation and progression, and the role of urologists in smoking cessation. *J Urol* 2008;180:31–7.
8. Schmitz-Drager BJ. Identifying risk factors in patients with non-muscle-invasive bladder cancer: clinical implications. *Eur Urol* 2011;60:721–3.
9. Liakou CI, Narayanan S, Ng Tang D, Logothetis CJ, Sharma P. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. *Cancer Immun* 2007;7:10.
10. Schubert M, Junker K, Heinzlmann J. Prognostic and predictive miRNA biomarkers in bladder, kidney and prostate cancer: Where do we stand in biomarker development? *J Cancer Res Clin Oncol* 2016;142:1673–95.
11. Sempere LF, Kauppinen S. Translational implications of microRNAs in clinical diagnostics and therapeutics. In: Bradshaw RA, Dennis EA, editors. *Handbook of Cell Signaling*; Burlington, MA: Academic Press; 2009. p.2965–81.
12. Marsit CJ, Houseman EA, Christensen BC, Gagne L, Wensch MR, Nelson HH, et al. Identification of methylated genes associated with aggressive bladder cancer. *PLoS One* 2010;5:e12334.
13. Armstrong DA, Green BB, Seigne JD, Schned AR, Marsit CJ. MicroRNA molecular profiling from matched tumor and bio-fluids in bladder cancer. *Mol Cancer* 2015;14:194.
14. Kobayashi H, Kikuchi E, Mikami S, Maeda T, Tanaka N, Miyajima A, et al. Long term follow-up in patients with initially diagnosed low grade Ta non-muscle invasive bladder tumors: tumor recurrence and worsening progression. *BMC Urol* 2014;14:5.
15. Heagerty PJ, Lumley T, Pepe MS. Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics* 2000;56: 337–44.
16. Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, et al. Micro-RNA profiling in kidney and bladder cancers. *Urol Oncol* 2007; 25:387–92.
17. Miyamoto K, Seki N, Matsushita R, Yonemori M, Yoshino H, Nakagawa M, et al. Tumour-suppressive miRNA-26a-5p and miR-26b-5p inhibit cell aggressiveness by regulating PLOD2 in bladder cancer. *Br J Cancer* 2016; 115:354–63.
18. Zhu Y, Lu Y, Zhang Q, Liu JJ, Li TJ, Yang JR, et al. MicroRNA-26a/b and their host genes cooperate to inhibit the G1/S transition by activating the pRb protein. *Nucleic Acids Res* 2012;40:4615–25.
19. Christoph F, Schmidt B, Schmitz-Drager BJ, Schulz WA. Over-expression and amplification of the c-myc gene in human urothelial carcinoma. *Int J Cancer* 1999;84:169–73.
20. Zhang H, Zhang L, Sun T. Cohesive regulation of neural progenitor development by microRNA miR-26, its host gene Ctdsp and target gene Emx2 in the mouse embryonic cerebral cortex. *Front Mol Neurosci* 2018; 11:44.
21. Moltzahn F, Olshen AB, Baehner L, Peek A, Fong L, Stöppler H, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res* 2011;71:550–60.