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Clinical-Bladder cancer

Predictive value of phenotypic signatures of bladder cancer response to cisplatin-based neoadjuvant chemotherapy

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Abstract

Background: Cisplatin-based neoadjuvant chemotherapy (NAC) for muscle-invasive urothelial carcinoma of the bladder confers only a modest survival advantage. Patients with pathologic progression on NAC have poor outcomes related to a delay in definitive surgical management.

Objective: To characterize the value of epithelial-mesenchymal transition (EMT) effectors and other novel biomarkers to predict response to NAC.

Methods: A tissue microarray was constructed from patients with clinical stage T2 urothelial carcinoma of the bladder using transurethral resection (TUR) specimens (N = 69) and case-matched post-NAC cystectomy specimens (N = 51). Patients were stratified based on pathologic response to NAC and cancer-specific survival. Biomarker expression in TUR specimens was correlated with pathologic response to NAC and clinical outcomes. Phenotypic changes in expression induced by cisplatin-based NAC were characterized in primary TUR and post-NAC cystectomy and lymph node metastasis specimens.

Results: Increased expression of mesenchymal markers and actin-cytoskeleton regulators, as well as low apoptosis index, in TUR specimens was associated with pathologic progression on cisplatin-based NAC. Overexpression of N-cadherin and decreased apoptosis was predictive of disease-specific mortality. NAC decreased cofilin phosphorylation and induced a mesenchymal-epithelial transition phenotype.

Conclusions: The epithelial-mesenchymal transition phenotype and actin-cytoskeleton remodeling are associated with pathologic progression on NAC. Chemotherapy induced an mesenchymal-epithelial transition phenotype and decreased cofilin phosphorylation, providing new insights into therapeutic resistance mechanisms. Primary tumors with high apoptosis rates exhibited favorable response to NAC and lower mortality rates, indicating that these tumors may be sensitized to therapy. Further validation will establish these novel signatures as predictors of therapeutic response. Published by Elsevier Inc.

Keywords: Biomarker; Cisplatin; Epithelial-mesenchymal transition; Urothelial carcinoma; Neoadjuvant chemotherapy; Pathologic response

1. Introduction

Over 81,000 new cases of urothelial carcinoma of the bladder were diagnosed in 2018, accounting for over 17,000 deaths [1]. Treatment with cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy is the reference standard for muscle-invasive bladder cancer, owing to a 5% to 10% survival advantage compared to cystectomy alone [2−3]. However, 30% to 35% percent of patients with clinical stage T2 disease treated with NAC exhibit pathologic progression at the time of cystectomy [4−5]. Patients who progress during NAC may benefit from timely upfront radical cystectomy, but their prospective identification using clinical and pathologic variables has been challenging.

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Efforts have established a spectrum of molecular subtypes of bladder tumors with prognostic utility [6−10], but most are RNA-based platforms with limited commercial adoption and availability. Recent studies have established the ability of these subtypes to predict response to NAC [11−13]. Evidence from our group recently identified the value of epithelial-mesenchymal transition (EMT) and actin-cytoskeleton effectors as potential biomarkers of advanced stage and grade urothelial carcinoma of the bladder [14]. The present study investigated expression of effector proteins involved in EMT, actin-cytoskeleton organization, and apoptosis to predict response to cisplatin-based NAC towards refining sequence of definitive therapy and patient selection for NAC.

2. Materials and methods

2.1. Patient selection

Patients with clinical stage T2 urothelial carcinoma of the bladder who underwent radical cystectomy at our institution between January 1, 2010 and January 1, 2016 were included. Patients with variant histology components or pure adenocarcinoma, squamous cell carcinoma, or small cell carcinoma were excluded, as were patients who underwent partial cystectomy or with unavailable transurethral resection (TUR) specimens. Patients were treated with cisplatin-based NAC followed by radical cystectomy with pelvic lymph node dissection (N = 69); patients managed with upfront radical cystectomy served as controls (N = 21). All NAC regimens consisted of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) or gemcitabine and cisplatin. Pre-NAC TUR specimens, post-NAC radical cystectomy specimens, and tissue from lymph node metastases were included in the construction of a tissue microarray (TMA) (N = 69 case matched TURBT and cystectomy specimens; N = 11 case-matched TURBT, cystectomy, and lymph node metastasis specimens). Final pathology was stratified into organ-confined (OC) (≤ypT2N0) vs. extravesical (EV) (≥ypT3 or N+) disease or complete pathologic response (ypT0N0) vs. residual disease (>ypT0N0). Clinical outcomes data were obtained from chart review and from the Kentucky Cancer Registry.

2.2. TMA construction

Representative tumor was prospectively outlined on hematoxylin and eosin stained slides and paraffin-embedded tissue blocks were selectively cored to construct a TMA through the University of Kentucky Markey Cancer Center Biospecimen and Tissue Procurement Shared Resource. Approval for the use of human tissue was obtained from the Institutional Review Board. Two tissue cores (2 mm) from each patient's TUR, cystectomy, and lymph node metastasis were included in the TMA, if applicable and enough tissue was available.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on deidentified 4 μM sections cut from the formalin-fixed, paraffin-embedded TMA using tissue procured from surgical archives of University of Kentucky and other treating facilities. Slides were deparaffinized, hydrated, and heat-induced epitope retrieval was performed in a Biocare Medical Decloaking chamber at 100°C (20 minutes) utilizing Dako's high or low pH Target Retrieval Solution and a Dako Universal Plus Autostainer. All antibodies were detected using EnVision+-HRP, Dako EnVision Flex-HRP, or Vector Biolabs ABC-HRP kit with secondary antibody, according to manufacturer's instructions and visualized using 3,3′-diaminobenzidine chromogen (Dako, CA) and hematoxylin counterstain. Antibodies and conditions are outlined in Table 1.

Apoptosis was detected using the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling) with PK (Dako, RTU) followed by staining with Millipore ApopTag Peroxidase in situ Apoptosis kit (Millipore S7100).

Immunoreactivity was determined based on the "Quick Score" calculated by multiplying staining intensity (on a scale of 1−3) by percentage of tumor cells with positive

Table 1
Primary antibodies utilized with manufacturer information and staining conditions.

Primary antibody	Manufacturer	Conditions
E-cadherin	Dako (IR05961-2)	High pH HIER, primary RTU for 10 min, EnVision+
N-cadherin	Abcam (ab18203)	Low pH HIER, primary at 1:100 for 1 h, EnVision Flex with Rabbit Linker
β-catenin	Cell signaling (95825)	Low pH HIER, primary at 1:50 for 1 h, EnVision+ with Rabbit Linker
Vimentin	Dako (IR63061)	High pH HIER, RTU for 10 min, EnVision Flex
α-tubulin	Abcam (6161)	Low pH HIER, primary at 1:50 for 1 h, anti-rat-bio (1:500, 30 min)
Cofilin	Sigma (C8736)	Low pH HIER, primary at 1: 5,000 for 30 min, EnVision+
Phospho-cofilin	Abcam (ab47281)	Low pH HIER, primary at 1:100 for 1 h, EnVision+
Zeb-1	Bethyl (A301-922A)	Low pH HIER, primary at 1:750 overnight at 4°C, EnVision+
BCL-2	DAKO (IR61461-2)	High pH HIER, primary RTU for 20 min, EnVision-Flex

immunoreactivity [14]. Two blinded reviewers independently reviewed 2 nonadjacent fields from each tissue core. TUNEL quantification was performed for 3 nonadjacent fields (100× magnification with 10 × 10 mm eyepiece microscope grid). A fraction was recorded with number of cells with positive staining or apoptotic bodies/number of total cells for each field.

2.4. Statistical analysis

Logistical regression models were used to study the association between expression and response to NAC. For each biomarker, Kaplan–Meier curves and log-rank tests were used to compare cancer-specific survival (CSS) between high (>median) and low (≤median) biomarker expression. The Holm's method was used to account for multiple comparisons across biomarkers and adjusted *P* values were reported. To study the difference of biomarkers between pre-NAC TUR and post-NAC cystectomy specimens/metastases, Wilcoxon signed rank tests were performed and multiple-comparison adjustments for the 3 biomarker categories (EMT, actin-cytoskeleton, apoptosis) were made using the Holm's method after normalizing to expression changes between TURBT and cystectomy specimens in patients managed with upfront cystectomy. Logistic regression using the covariate average of TUR and cystectomy specimens was used to study differential expression between TUR and cystectomy specimens in relation to pathologic response to NAC.

3. Results

Sixty-nine patients who underwent cisplatin-based NAC for clinical stage T2 urothelial carcinoma of the bladder followed by radical cystectomy were included in analysis. Baseline patient characteristics after stratification by pathologic response to NAC [OC vs. EV] are shown on Table 2. Patients in the EV group were older ($P < 0.001$) and predictably exhibited high cancer-specific mortality ($P < 0.001$) compared to the OC group. Complete pathologic response (ypT0N0) in the OC group was exhibited by 18 patients (44%).

3.1. Predictive value of EMT signature and apoptosis

EMT markers were investigated as predictors of pathologic response to NAC. TURBT specimens from patients in the EV disease cohort exhibited decreased E-cadherin, increased N-cadherin, and increased vimentin expression (Fig. 1), the latter 2 being statistically significant (P= 0.004 and $P = 0.028$, respectively). Patients in the EV group exhibited increased β-catenin (P= 0.019) and Zeb-1 expression (P= 0.396) relative to the OC group. After multiple comparisons adjustment across all EMT markers to predict response to NAC, only N-cadherin expression was significant (adjusted P= 0.027). Several EMT biomarkers predicted complete pathologic response (ypT0N0), including: low N-cadherin (P= 0.044), low vimentin (P= 0.013), and low Zeb-1 (P= 0.030) expression, suggesting that the EMT phenotype confers resistance to cisplatin-based NAC.

The ability of the apoptosis index to predict pathologic response to NAC in TURBT specimens was investigated in serial sections. Tumors from patients exhibiting poor pathologic response in the EV group had significantly lower apoptosis indices in TURBT specimens compared to the OC group ($P< 0.001$, Fig. 2). This relationship remained significant on multiple comparisons adjustment (adjusted P= 0.002). Expression of BCL-2 was similar between OC and EV groups ($P = 0.390$, data not shown).

3.2. Predictive value of the actin-cytoskeleton remodeling phenotype

We subsequently sought to identify proteins regulating the actin-cytoskeleton network serving as predictors of pathologic response to NAC. Patients in the EV group who responded poorly to NAC exhibited higher expression of Cofilin ($P = 0.148$) and P-cofilin ($P = 0.036$, Fig. 3). α-tubulin, a microtubule subunit, was also significantly up-regulated in TURBT specimens from patients with EV disease

Table 2
Patient demographics by stratification into organ-confined (OC; ≤ ypT2N0) or extravesical (EV; ≥ ypT3 or N+) disease after cisplatin-based neoadjuvant chemotherapy and radical cystectomy.

	Organ confined (≤ypT2N0)	Extravesical (≥ypT3 or N+)	*P* value
Number of patients	41	28	
Age: Ave. y (SD)	58.3	65.8	<0.001
Noncaucasian: N (%)	1 (2.4)	2 (7.1)	0.562
Males: N (%)	35 (85.4)	20 (71.4)	0.157
Follow-up: Days from diagnosis (SD)	1,323 (731)	1,128 (815)	0.305
Cancer-specific mortality: N (%)	3 (7.3)	17 (60.7)	<0.001
Recurrence: N (%)	3 (7.3)	4 (14.3)	0.430

The *P* values were calculated based on two-sample t-tests for continuous variables or chi-squared tests for categorical variables.

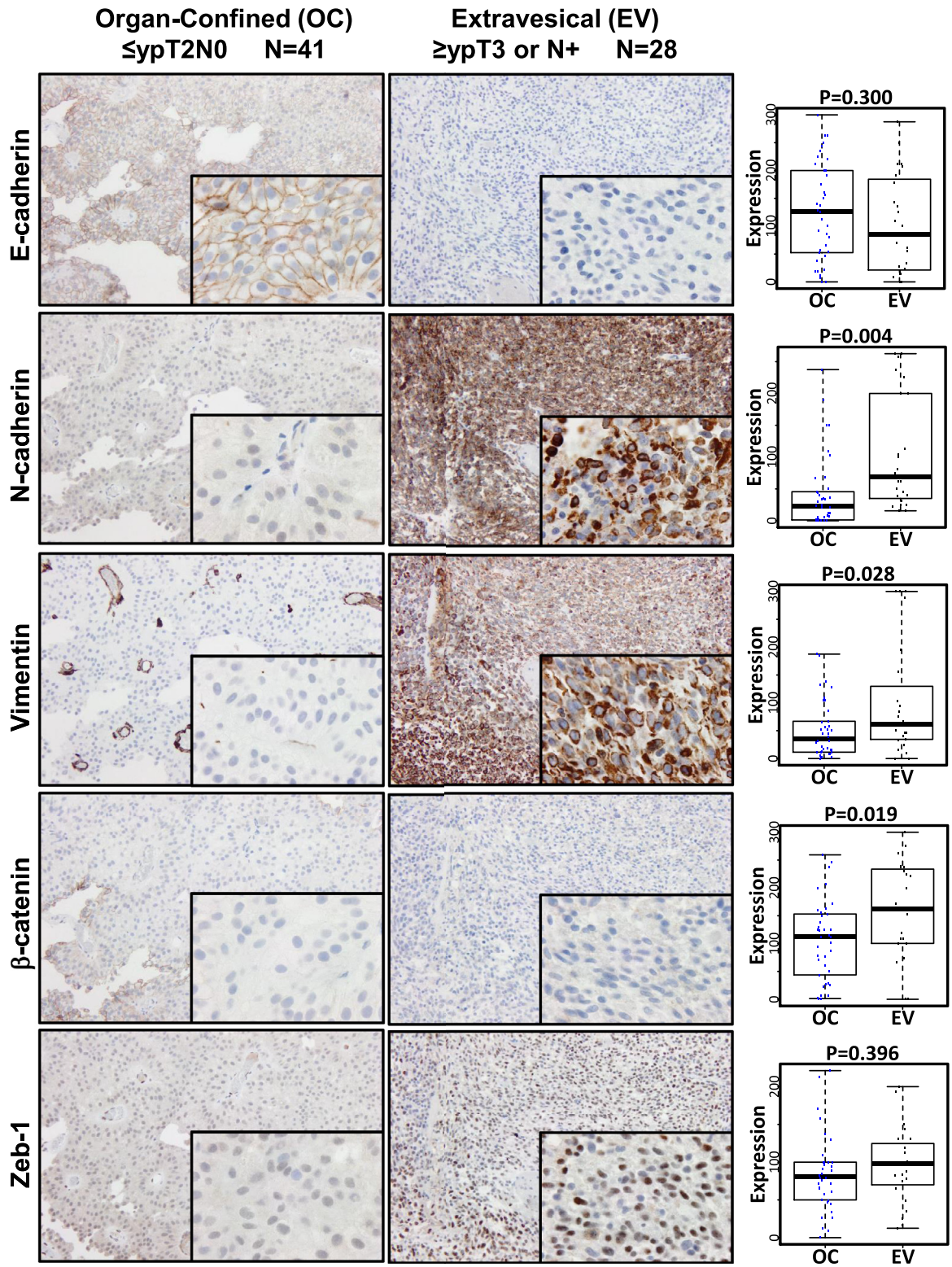


Fig. 1. EMT signature in TURBT specimens stratified by response to NAC. An EMT phenotype, characterized by decreased E-cadherin and increased N-cadherin, Vimentin and β-catenin in TUR specimens is associated with poor response to chemotherapy. Values represent the average expression score for all specimens with error bar +/− SD. All immunohistochemistry images are serial sections from a single representative patient (magnification 200× with 1,000× insert). The *P* values were calculated based on logistic regressions.

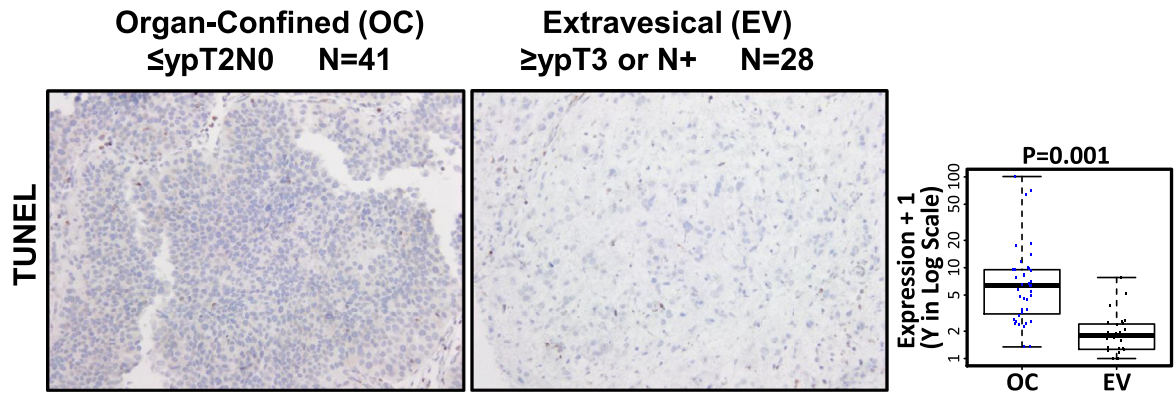


Fig. 2. Apoptosis indices in TURBT specimens stratified by response to NAC. There were significantly higher rates of apoptosis (TUNEL staining) in TURBT specimens from patients with organ-confined disease at the time of cystectomy. Values represent the average expression score for all specimens with error bar +/− S.D. Immunohistochemistry images are serial sections from the same representative patients depicted in Fig. 1 (magnification 200× with 1,000× insert). The *P* values were calculated based on logistic regressions.

at the time of cystectomy ($P = 0.007$). After multiple comparisons adjustment across all actin-cytoskeleton markers to predict response to NAC, only α-tubulin expression remained significant (adjusted $P = 0.037$). Low P-cofilin expression was predictive of complete pathologic response ($P = 0.037$). Thus, upregulation of effectors of the actin-cytoskeleton is associated with pathologic progression on cisplatin-based NAC.

The value of biomarker expression in TURBT specimens to predict clinical outcomes was investigated through Kaplan−Meier survival curves (Fig. 4). Expression levels for each biomarker was dichotomized (high/low) based on median expression level. While increased expression of the EMT effectors N-cadherin and vimentin were associated with pathologic progression on NAC, only N-cadherin expression significantly predicted disease-specific mortality ($P = 0.016$). The remaining significant predictor of disease-specific mortality was high apoptosis index ($P = 0.003$).

3.3. NAC induces phenotypic changes in primary and metastatic bladder tumors

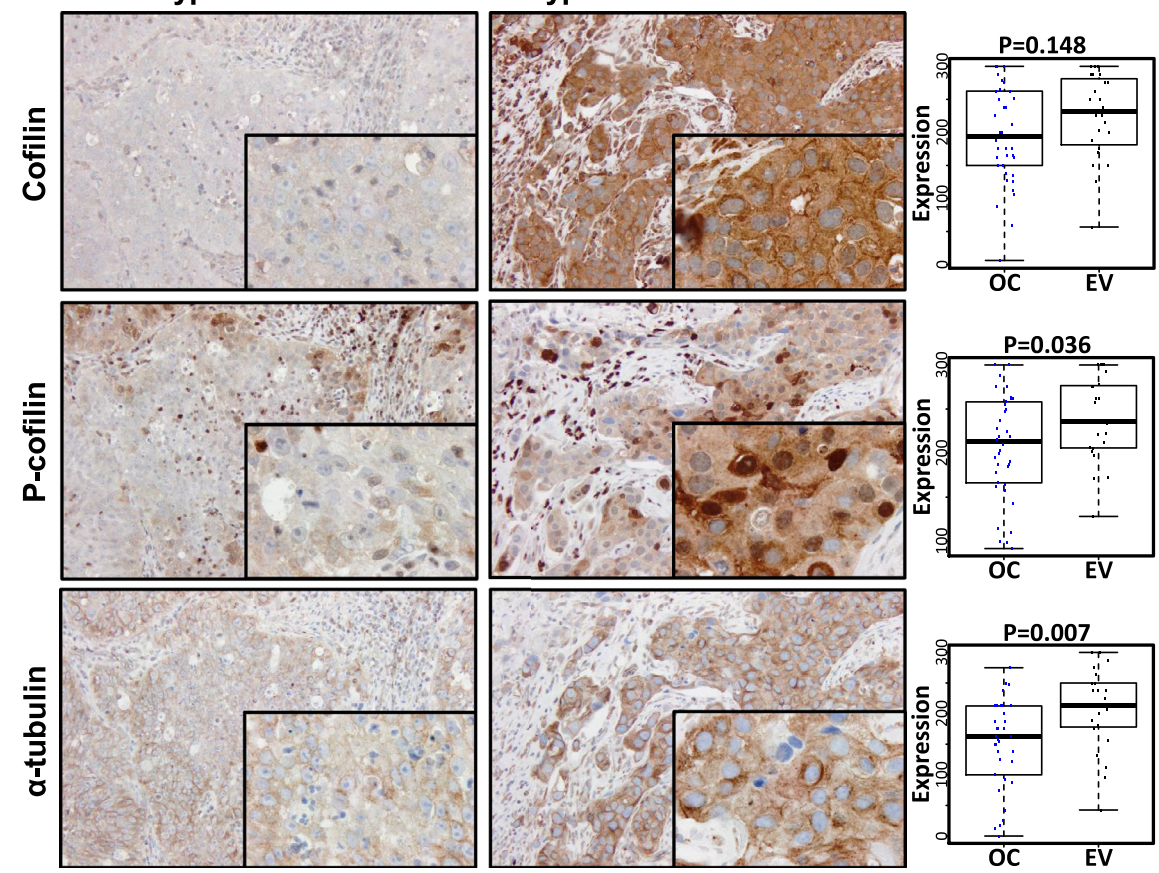
To study phenotypic changes induced by cisplatin-based chemotherapy, we compared biomarker expression in pre-NAC TUR specimens and case-matched post-NAC radical cystectomy, and lymph node metastasis specimens. Expression was normalized to changes in TUR and cystectomy specimens from control patients who were managed with upfront cystectomy. NAC induced significant decreases in the actin-cytoskeleton markers P-cofilin and tubulin. Notably, post-NAC cystectomy specimens exhibited increased total cofilin expression, suggesting the ability of cisplatin-based chemotherapy to abrogate cofilin phosphorylation (Fig. 5, Panels A and B). While NAC induced a mixed phenotype, reversal of the EMT phenotype to MET (mesenchymal-epithelial transition) was largely exhibited, characterized by decreased N-cadherin and β-catenin in cystectomy relative to TUR specimens (Fig. 5, Panel B). All biomarkers remained statistically significant on multiple comparisons adjustment. We subsequently assessed whether changes between pre-NAC TUR and post-NAC cystectomy specimens could predict pathologic response to chemotherapy. Patients with down-regulation of tubulin following NAC exhibited a favorable response to chemotherapy ($P = 0.041$), but this was not significant on multiple comparisons analysis ($P = 0.122$).

Changes in the expression of biomarkers induced by NAC were comparatively analyzed between pre-NAC TUR specimens and case matched lymph node metastases. Similar to the aforementioned findings in the primary tumor, the expression of N-cadherin was decreased in lymph node metastases relative to pre-NAC TUR specimens (Fig. 5, Panel C). This is reflective of a MET phenotype as tumor cells regain cohesive capacity to establish metastatic sites. No markers remained significant in metastatic lesions after multiple comparisons adjustment.

4. Discussion

Patient selection for NAC can be challenging and portends significant consequences in clinical outcomes [15]. Recent evidence suggests that patients with residual muscle-invasive disease after NAC exhibited significantly worse survival compared with stage-matched patients managed with upfront radical cystectomy [16]. The ability to predict patients who both tolerate and respond to NAC would spare nonresponders the morbidity associated with cytotoxic therapy and delay time to definitive management. The present findings implicate the EMT phenotype, overexpression of actin-cytoskeleton regulatory effectors, and low apoptosis rates as markers of chemoresistance, in an effort to improve sequencing of definitive therapy. Previous studies have identified clinicopathologic variables associated with favorable pathologic response to NAC [4,5,16,17,18,19]. However, these studies are limited to

A



B

	OR	Lower 95% CI	Upper 95% CI	P-value
E-cadherin	0.58	0.21	1.62	0.300
N-cadherin	5.77	1.84	18.06	0.004
Vimentin	2.29	1.50	6.53	0.028
β-catenin	1.73	1.15	4.86	0.019
Zeb-1	1.73	0.62	4.86	0.396
TUNEL	0.03	0.01	0.13	0.001
Cofilin	2.29	0.80	6.53	0.148
P-cofilin	2.29	1.92	6.53	0.036
α-tubulin	5.23	1.66	16.51	0.007

Fig. 3. Profile of actin-cytoskeleton remodeling proteins in TURBT specimens stratified by response to NAC. (A) Patients with poor pathologic response to chemotherapy (EV group) exhibited increased phosphorylated cofilin and α-tubulin. Values represent the average expression score for all specimens with error bar +/− SD. Immunohistochemistry images are serial sections from the same representative patient depicted in Fig. 1 and 2 (magnification 200× with 1,000× insert). The *P* values were calculated based on logistic regressions. (B) Chart depicting odds ratio (OR) with upper and lower 95% confidence intervals (CI) for each of the studied biomarkers.

single institution, small, retrospective series with variable NAC regimens and definitions of pathologic response. Identification of patient-specific inherent tumor characteristics predicting biological behavior and response to therapy is of potentially higher clinical value.

The present study established the relationship between the EMT phenotype in pretreatment specimens with poor response to NAC and adverse clinical outcomes. The association of the EMT phenotype with aggressive disease and metastasis has been well-described in a number of solid

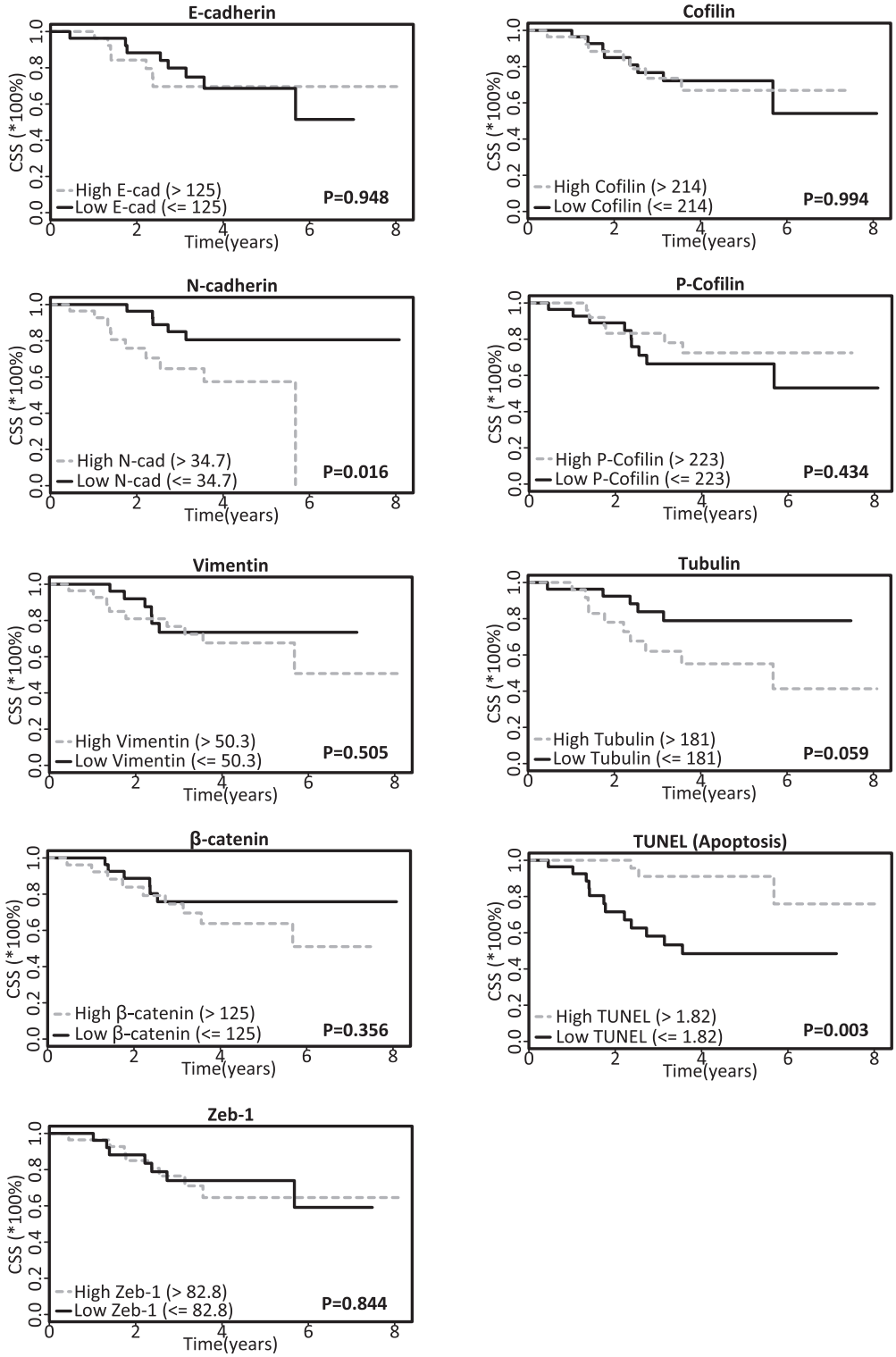


Fig. 4. Biomarker expression predicts clinical outcomes. For each biomarker, Kaplan–Meier curves and logrank tests were used to compare the cancer-specific survival (CSS) between high (> median) and low (≤ median) biomarker expression. Decreased N-cadherin expression in TURBT specimens, consistent with an EMT phenotype, was associated with decreased CSS while high apoptosis rates were associated with improved CSS.

malignancies, and implications in therapeutic resistance and molecular targeting in prostate cancer are being exploited in our lab [20–23]. Previous work from our group implicated the EMT phenotype with high stage and grade urothelial carcinoma of the bladder [14]. Work by others linking EMT signatures to response to cisplatin-based chemotherapy has been largely limited to *in vitro* and RNA-based platforms [7,8,10].

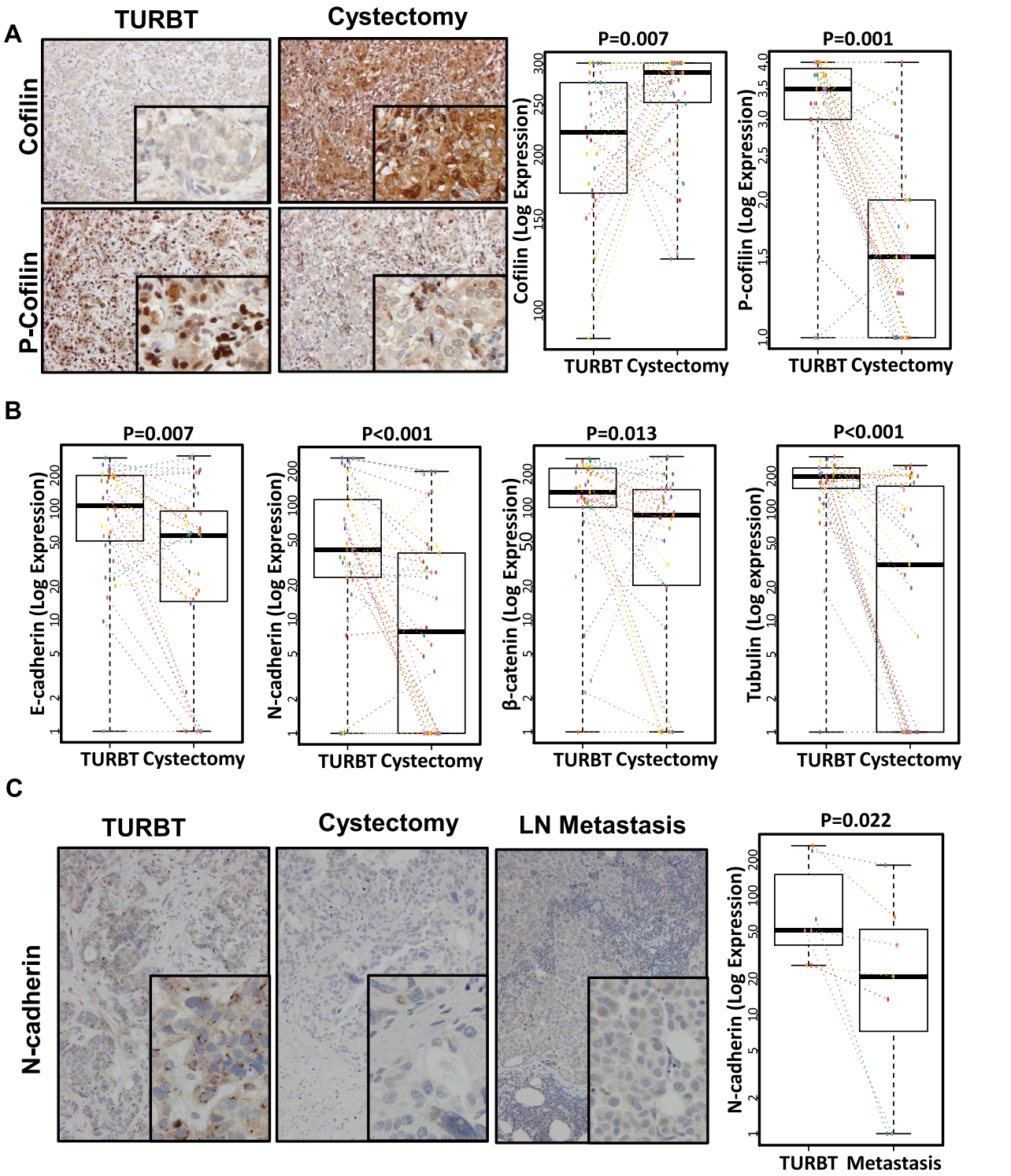


Fig. 5. Phenotypic changes in biomarker expression induced by cisplatin-based chemotherapy. (A) Case-matched serial sections from a single patient exhibiting characteristic cofilin expression profile changes in pre-NAC TUR specimens and post-NAC cystectomy specimens. In response to chemotherapy there was a marked decrease in phosphorylated cofilin and increased total cofilin. (B) Boxplots from biomarkers with statistically significant expression changes induced by NAC. Chemotherapy is associated with an MET phenotype with decreased N-cadherin and β-catenin expression in post-NAC specimens. In addition to cofilin targeting by NAC, the actin cytoskeleton regulator tubulin is down-regulated in cystectomy specimens relative to pre-NAC TUR specimens. (C) Case matched serial sections from a single patient exhibiting characteristic N-cadherin down-regulation in cystectomy and lymph node (LN) metastases relative to pretreatment TUR specimens. Boxplots represent mean expression with error bar +/− standard deviation. Immunohistochemical images are at 200× magnification (1,000× insert). The *P* values were calculated based on Wilcoxon signed rank tests.

Complete transcriptomic and somatic mutational analysis through The Cancer Genome Atlas database has been thoroughly documented in bladder cancer [6,7]. Several molecular classification schemes have been proposed and prospectively validated [6–10,27]. Markers of urothelial differentiation are highly expressed in TCGA Clusters I and II ("luminal" tumors), including epithelial markers E-cadherin and uroplakins, while cluster III ("basal/squamous-like") tumors exhibit mesenchymal properties consistent with the well-described basal-like breast cancers [7]. While basal tumors appear to be more aggressive with adverse clinical outcomes [7,12,13], patients with these tumors appear to derive the greatest benefit from NAC [11,13]. Basal tumors are characterized by poorly differentiated, mesenchymal phenotypes with overexpression of EMT effectors. While our previous data corroborate the aggressive nature of the EMT phenotype [14], in the present study we identified these tumors as being inherently chemoresistant. These contrasting conclusions about the sensitivity of tumors with mesenchymal differentiation to cisplatin-based chemotherapy could be explained by the occult incidence of proposed sub-phenotypes within the basal group, including "claudin-low" and "neuronal" phenotypes, both of which exist on a more extreme dedifferentiated spectrum and have been implicated in chemotherapeutic resistance [27]. Limitations from the TCGA analysis include lack of proteomics which limits commercial adoption. Recent efforts have focused on developing IHC signatures for this reason, including a simplified dichotomy utilizing GATA3 and KRT5/6 as surrogate luminal and basal markers, respectively [28,29]. Antibodies towards biomarkers investigated in the current study are commercially available, and several have diagnostic and prognostic clinical utility in other solid tumors.

Mitotic indices have long been studied in relation to cancer outcomes and have clinical utility as standard of care components of pathologic staging in multiple solid malignancies. Apoptosis indices have less clearly defined prognostic roles. Our findings that a high apoptotic index portends favorable response to NAC and improved CSS are in accordance with previous studies [25,26], and suggest that high apoptotic rates in TUR specimens may sensitize patients to NAC towards better pathologic response.

Organization and remodeling of the actin-cytoskeletal network is associated with cellular integrity as well as migratory and invasive capacities. We previously identified the actin-binding protein cofilin, and its phosphorylated isoform P-cofilin, as being up-regulated in high stage and grade urothelial carcinoma of the bladder [14]. The present study was the first to investigate the cytoskeletal regulators cofilin, phosphorylated-cofilin, and α-tubulin in response to NAC, with the overexpression of the latter 2 biomarkers being associated with chemoresistance.

While there has been tremendous effort to use genomic and immunohistochemical signatures to predict response to NAC, there exists a paucity of data regarding phenotypic changes *induced by* cisplatin-based therapy in pre- and post-treatment specimens. The identification of such phenotypic changes can have prognostic utility and provide insight into resistance mechanisms. Treatment of patients with cisplatin-based NAC induced a MET phenotype in the present study, with N-cadherin exhibiting the most prominent expression down-regulation in both cystectomy and lymph node metastasis specimens relative to the primary tumor. Choi et al. described tumors adopting a p53-like phenotype in the post-MVAC MD Anderson Cancer Center Discovery cohort, a phenotype later characterized by infiltrating stromal cells imparting chemoresistance [12]. Similar findings were reproduced in a cohort of 23 patients treated with neoadjuvant MVAC and bevacizumab [11]. The p53-like phenotype is an aggressive variant of the luminal classification, and our data contribute to evidence of a spectrum of transdifferentiation along the EMT-MET phenotype induced by chemotherapy. In relation to our findings that the EMT phenotype in the pretreatment specimen is associated with resistance to NAC, the ability of the chemotherapeutic regimen to induce transdifferentiation, with EMT reversal to MET, serves as a therapeutic mechanism of cisplatin-based therapy, and may provide synergistic with further EMT-targeted therapy currently being studied in preclinical phases. Furthermore, our data suggest that cofilin phosphorylation is inhibited by cisplatin-based NAC, evident by increased total and decreased phosphorylated isoforms in post-NAC cystectomy specimens. Recent evidence suggests that cofilin phosphorylation mediates EMT-MET interconversion [24], and these data identify this pathway as a promising target for overcoming therapeutic resistance.

Our study was limited to patients with clinical stage T2 disease as we believe this population provides the greatest room for interpretation in the value of NAC. There was intentional over representation of ypT0 patients (44% of OC patients) to study innate tumor biology as pathologic responders. The authors recognize the definition spectrum of "pathologic response" to NAC in the published literature. The pathologic stage distribution utilized in the present study (OC vs. extravesical) was based on prior evidence that patients with clinical stage T2 disease at the time of cystectomy who progress on NAC (ypT3+ or N+) exhibit worse CSS than stage-matched cohorts managed with upfront radical cystectomy [5]. Additional evidence suggests that residual noninvasive and residual organ-confined muscle-invasive disease in patients with clinical stage 2 disease at the time of cystectomy have similar 5-year OS rates (79.5% vs. 78.3%, respectively), which are inferior to the 86.2% OS of those patients who mount a complete pathologic response [30]. The authors additionally recognize the ability of aggressive TUR(s) to achieve pT0, with over 20% of patients in the aforementioned study with cT2 disease managed with upfront cystectomy exhibiting no residual disease at cystectomy. These complete resection rates of up to 40% in the published literature may confound interpretation of the ypT0 cohort in the present study.

The single-institution nature and small sample sizes in this study limit generalizability. While tumors with variant histologic differentiation were excluded to avoid this as a confounding variable, this limits interpretation to pure urothelial carcinoma. Emerging evidence suggests that there is considerable intratumoral heterogeneity and plasticity with coexistence of multiple molecular subtypes within the same tumor [31]. While our TMA was constituted of 2 discrete cores from different tissue blocks from each patient to account for this, there remained intratumoral heterogeneity which may limit decision-making about strict phenotypic categorization. Automated scoring systems may prove beneficial for standardization and clinical implementation.

5. Conclusions

Our study identified several novel biomarkers as predictors of adverse pathologic response and clinical outcomes after cisplatin-based NAC for muscle-invasive urothelial carcinoma of the bladder. The apoptosis index as well as the EMT marker N-cadherin were predictive of both chemoresistance and poor CSS. The phenotypes investigated in the present study are distinct from those previously established, and future studies are necessary to correlate them with basal and luminal bladder subtypes and possibly incorporate them into diagnostic biomarker panels to increase their predictive value. Furthermore, we identified the ability of cisplatin-based chemotherapy to impart EMT reversal and target expression of the key actin-cytoskeleton regulators cofilin and tubulin, previously implicated in cellular motility and invasive capacities. These findings provide insight into mechanisms of therapeutic targeting and resistance mechanisms.

Ongoing efforts to utilize personalized proteomic signatures, including the use of patient-derived xenografts to study *ex-vivo* response to chemotherapy, are promising strategies to develop a personalized approach to treatment sequencing [32]. Further multi-institutional studies are warranted to validate these results and reach a consensus adoption of emerging histologic and molecular subtypes towards better patient stratification, treatment sequencing, and patient outcomes. Future studies will focus on incorporating these novel biomarkers into existing molecular phenotypes, subtyping histologic variants, and identification of biomarkers predictive of response to emerging immunotherapies.

Conflict of interest

The authors report no competing interest to declare.

Acknowledgments

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