Predictive Biomarkers for the Clinical Evaluation of Bladder Cancer

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BACKGROUND

Cancer of the urinary bladder (BC) ranks second in mortality and morbidity among the genitourinary cancers causing over 16,000 deaths annually. It is the most expensive cancer to treat from diagnosis to death, due in part to its intrinsic molecular heterogeneity that makes prognosis difficult, requirement of invasive procedures such as cystoscopy, and a high incidence of recurrence. Recent efforts to improve the diagnosis for BC are rooted in identifying molecular signatures of various BC subtypes based on the Tumor Cell Genome Atlas (TCGA) database and then personalizing treatment protocols.

Urothelial Bladder Cancer arises from the urothelium, a multi-layered transitional epithelium lining the bladder lumen. Fully differentiated superficial cells overlie intermediate cells which have limited proliferative potential, and finally a basal layer composed of cuboidal cells resting on a basement membrane completes the urothelium. Thus, morphologically, BC can be divided into two molecular subtypes referred to as luminal and basal with differing clinical sensitivities to therapy. Further, these epithelial layers express distinct cytokeratin and cancer stem cell markers. Although treatment based on molecular signatures has the potential to be effective, a verification of their expression in strictly compartmentalized epithelial subtypes is not presently available. Furthermore, tumor cells with inherent genomic instability are unlikely to maintain a predictable molecular signature. We hypothesized that although the fidelity of molecular subtypes in BC may be less than optimal, expression of at least some molecules in the signature panel are likely to characterize either the basal or luminal compartment.

The future implications of this project are the formation of a bed-side toolkit that can be used following a bladder tissue biopsy to cost-effectively evaluate the subset of cancer and provide an efficient disease prognosis.

METHODS

The spectrum of UBC at presentation includes non-muscle invasive and muscle invasive disease. We analyzed genomic expression profiles in five bladder cancer cell lines (RT-4, 5637, T24, HT-1376, and 253J) ranging from a grade I cancer cell line (RT-4) to a grade IV cancer cell line (253J). Based on existing literature, we assigned these bladder cancers to one of two candidate intrinsic molecular subtypes: luminal and basal. Luminal tumors are characterized by expression profiles similar to intermediate/superficial layers of the epithelium and basal tumors correlate to the basal layer of the urothelium. Importantly, basal bladder cancers are more aggressive and lead to shorter survival times.

Through the use of RT-PCR, we analyzed genomic expression levels of eleven biomarkers (luminal: UPK, GATA-3, RAB 25, E-Cadherin; basal: CK-6, p63, CD44S, CD44V, CK-6, CyclinB1, EGFR, and CD49). Particularly, for a cost-effective analysis, the expression profiles of only two markers (GATA-3 and CD44S) could be used to identify the molecular subtype of the bladder cancer to determine a first-look prognosis at the bed-side.

To further our understanding of the differences between basal and luminal subtypes of bladder cancer, we used MTT Cell Viability Assays to test the effects of Gemcitabine and Cisplatin on five bladder cancer cell lines. Existing literature states that though basal bladder cancers are more aggressive, they are also more sensitive to chemotherapy initially compared to luminal bladder cancers. We hypothesized that the RT-4 and 5637 cell lines would be more resistant to treatment compared to the more aggressive T24, HT-1376, and 253J cell lines.

BACKGROUND FIGURES

Characteristics of the Bladder Urothelium and Bladder Cancer Cell Lines

Figure 1:

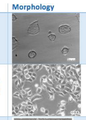
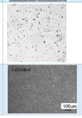
Cell Lines	Disease Classification	Morphology
RT-4	**Grade I BC:** Superficial, non-muscle-invasive BC. *Treatment:* TUR to determine extent, follow-up with another TUR and chemotherapy	
5637	**Grade II BC:** Muscle-invasive BC, but confined to the bladder. *Treatment:* partial or radical cystectomy, or TUR followed by radiation and chemotherapy	
T24	**Grade III BC:** Tumor has spread through bladder wall to surrounding muscle and tissue *Treatment:* radical cystectomy combined with chemotherapy	
HT-1376	**Grade III BC:** Tumor has spread through bladder wall to surrounding muscle and tissue *Treatment:* radical cystectomy combined with chemotherapy	
253J	**Grade IV BC:** Metastatic bladder cancer, has spread to abdomen or pelvis. *Treatment:* chemotherapy, immunotherapy	

Figure 2:

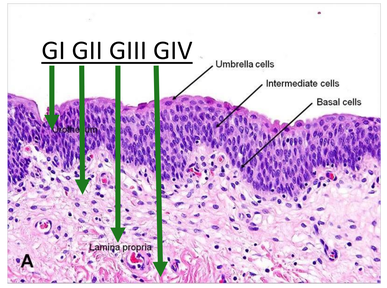


Figure 3:

	UPK	GATA-3	RAB 25	E-Cadherin	CK-6	p63	CD44S	CD44V	CD49	Cyclin B1	EGFR
RT-4	1	1	1	1	1	-1	1	-1	-1	-1	-1
5637	-1	1	1	1	-1	-1	1	-1	1	-1	-1
T24	1	1	1	1	-1	-1	1	1	1	1	1
HT-1376	1	1	-1	-1	1	1	1	1	-1	1	1
253J	1	1	1	1	-1	-1	1	1	-1	1	1
	3	5	3	3	-1	-3	5	1	-1	1	1

Figure 4:

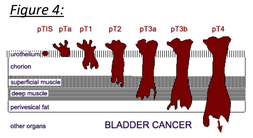


Fig. 1: Characteristics and morphology of bladder cancer cell lines used in this study. *Images: Transmission Electron microscopic images from ATCC.*
Fig. 2: Histological image of human bladder urothelial layers with general locations of different bladder cancer grades.
Fig. 3: Box-type analysis of RT-PCR results of bladder biomarkers in BC cell lines; red=positive match, blue=negative match. The green boxes indicate the two markers with the best correlations to their supposed subtype; GATA-3 as a luminal marker and CD44S as a basal marker.
Fig. 4: According to the grade and stage, pTa is of good prognosis (>90% are cured), ~20% survival at 1 year in T4 cases. Urothelial BC staging diagram, *http://atlasgeneticsoncology.org/Tumors/bladID5001.html*

RESULTS

Expression of Luminal and Basal Biomarkers in Bladder Cancer Cell Lines

Figure 5.A: *Figure 5.B:* *Figure 5.C:*

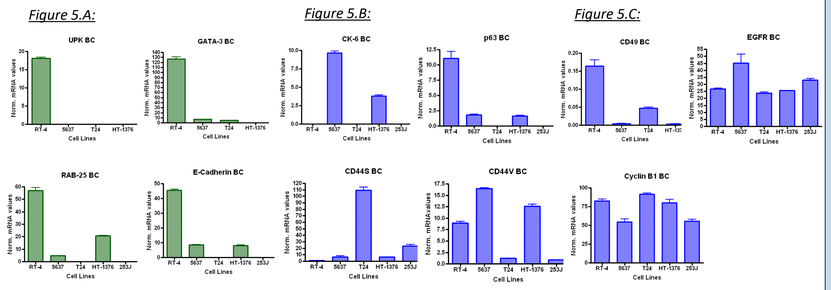


Figure 6:

Biomarker	Location	Function
UPK- Uroplakin	luminal	Protein family that associate with each other and form plaques on the apical surface of urothelium
GATA-3	luminal	Transcription factor that regulates luminal cell differentiation, upregulated in cancer cells
RAB 25	luminal	GTPase, governs cell-surface receptor recycling and activates cellular signaling pathways
E-Cadherin	luminal	Involved in epithelial cell-cell adhesion, essential for maintenance of cellular epithelium
CK-6	basal	Intermediate filament proteins that help with cell-cell adhesion and basal tissue stability
p63	basal	Transcription factor, regulates cell growth and division and differentiation
CD44S	basal	Cell surface glycoproteins involved in cell-cell interactions, adhesion, and migration
CD44V	basal	Splice variant of CD44S
CD49	basal	Adhesion and cell migration
Cyclin B1	basal	Essential for cell cycle progression through mitosis, overexpressed in many cancers
EGFR	basal	Epidermal Growth Factor Receptor, transmembrane receptor involved in downstream proliferative pathways

Fig. 5: A, B, & C: qPCR analysis for UPK, GATA-3 RAB 25, E-Cadherin, CK-6, p63, CD44S, CD44V, CD49, CyclinB1, and EGFR in BC cell lines. Data: Mean ± SD
Fig. 6: Biomarker subtype classification and function. Existing literature has categorized these biomarkers into either luminal or basal subtypes. We analyzed the validity of these pre-assigned classifications through RT-PCR experiments.

RESULTS

Sensitivity of Basal and Luminal Bladder Cancer Cells to Chemotherapy

Figure 7A: *Figure 7B:*

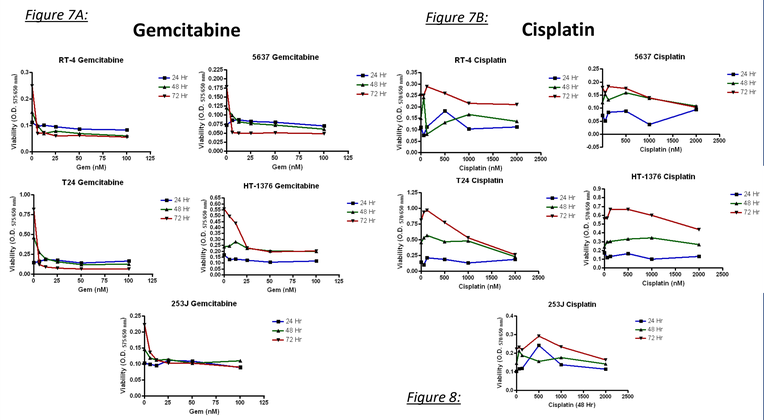
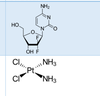
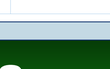


Fig. 7A & 7B: The cytotoxicity of Gemcitabine and Cisplatin was determined in BC cell lines using the MTT assay. Cells were seeded in a 48-well tissue culture plate at 10,000 cells/mL and incubated at 37ºC in a 5% CO_2 incubator for 24 hours to allow the cells to attach. Predetermined wells were treated with various concentrations of Gem and CiP. The cells were then incubated again for 24, 48, or 72 hrs. At each time point, MTT was added at a final concentration of 0.25 mg/mL, and the plates were incubated again. Non-reduced MTT and media were aspirated after 2 hours and replaced with DMSO to dissolve the MTT formazan crystals. **Fig. 8:** Structure and Function of the chemo-drugs used—Gemcitabine and Cisplatin.

Figure 8:

Chemotherapeutic Drug	Structure	Function
Gemcitabine		Pyrimidine antagonist, antimetabolite drug that inhibits DNA synthesis
Cisplatin		Interferes with DNA replication, which kills the fastest proliferating cells. Crosslinks DNA, interfering with mitosis.

CONCLUSIONS

- Less aggressive bladder cancer cell lines (RT-4, 5637) showed higher expression profiles for luminal biomarkers (UPK, GATA-3, RAB 25, E-Cadherin).
- The more invasive cell lines (T24, HT-1376, 253J) showed a slight upregulation of basal target genes (CK-6, p63, CD44S, CD44V, CD49, CyclinB1, EGFR), but these markers were also modestly present in the less invasive cancer cell lines. For a cost-effective analysis, the expression profiles of only two markers: GATA-3 and CD44S could be used to identify the molecular subtype of bladder cancer.
- MTT Assays showed the significant cytotoxic effect of Gemcitabine especially but also Cisplatin on all bladder cancer cell lines. However, contrary to our hypothesis, basal cancers (253J, HT-1376, and T24) were not *significantly* more susceptible than luminal cancers when exposed to the chemotherapeutic agents.

FUTURE EXPERIMENTS

- Protein Expression levels of the eleven biomarkers in the same five cell lines should be analyzed through the use of Western Blot Analysis to better validate the presence of luminal and basal biomarkers.
- The future implications of this project are the creation of a bedside tool-kit that can be used following a bladder tissue biopsy to cost-effectively evaluate the grade of the cancer to provide an efficient disease prognosis.

FUNDING

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