Short Communication

Genoproteomic Mining of Urothelial Cancer Suggests γ -Glutamyl Hydrolase and Diazepam-Binding Inhibitor as Putative Urinary Markers of Outcome after Chemotherapy

Courtney Pollard,* Matt Nitz,* Alex Baras,[†]
Paul Williams,[‡] Christopher Moskaluk,[†]
and Dan Theodorescu*[§]

From the Departments of Molecular Physiology,* Pathology,[†] and
Public Health Services,[‡] and the Mellon Urologic Cancer
Institute,[§] University of Virginia, Charlottesville, Virginia

Urinary biomarkers for the detection of bladder cancer have been developed, but no similar markers exist for prediction of clinical outcomes after receiving chemotherapy. Here we evaluate an approach that combines genomic, proteomic, and therapeutic outcome datasets to identify novel putative urinary biomarkers of clinical outcome after neoadjuvant methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC). Using this method, we identified γ -glutamyl hydrolase (GGH), emmprin, survivin, and diazepam-binding inhibitor (DBI). Interestingly, GGH is a protein associated with methotrexate resistance, whereas emmprin, survivin, and DBI had been previously identified as predictors of outcome after platinum-containing chemotherapeutic regimens when assessed on tumor tissue. Using disease-free survival as a marker for clinical outcome, we evaluated the ability of GGH, emmprin, survivin, and DBI expression in tumor tissue to stratify 27 patients treated with neoadjuvant MVAC. DBI ($P = 0.046$) but not GGH ($P = 0.190$), emmprin ($P = 0.066$), or survivin ($P = 0.393$) successfully stratified patients. When GGH was used with DBI the significance of stratification improved ($P = 0.024$), whereas the addition of survivin or emmprin to this latter two-gene model reduced its significance ($P = 0.036$ and $P = 0.040$, respectively). Although these predictive results were obtained on tumor tissues, the presence of GGH and DBI in urine serves as a rationale for developing them as urinary markers of clinical outcomes for patients treated with neoadjuvant MVAC. (*Am J Pathol* 2009, 175:1824–1830; DOI: 10.2353/ajpath.2009.090155)

In 2008 approximately 69,000 individuals in the United States were diagnosed with bladder cancer, and 14,000 will ultimately die as a result of the disease.¹ Most of the patients who die initially present with muscle-invasive disease that later metastasizes.² Randomized studies have shown that neoadjuvant chemotherapy with MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin) in patients with muscle-invasive disease improves median survival from 46 to 77 months. However, despite these promising results, only a subgroup of patients benefit from this approach and determining who they are is not currently possible. Hence, biomarkers that predict such outcomes would be of great practical utility, allowing only patients who are likely to derive the greatest benefit to be treated.

Despite the fact that bladder cancer is ideally suited for noninvasive molecular evaluation based on analysis of urine, no urinary biomarkers have been found to robustly predict clinical outcomes after chemotherapy. Recently, tissue expression of two genes, emmprin and survivin, was found to independently predict outcome in patients with bladder cancer treated with cisplatin-containing regimens.³ Interestingly, both emmprin and survivin have been identified in urine.^{4,5} In addition, a recent study described a 14-gene signature predictive of therapeutic response in patients with bladder cancer treated with neoadjuvant MVAC.^{6,7} One of the 14 genes in this model, diazepam-binding inhibitor (DBI), is found in urine.⁸

Taken together, these findings suggested it may be possible to develop a general method that can identify genes encoding proteins that are both predictors of clin-

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Address reprint requests to Dan Theodorescu, Department of Molecular Physiology, University of Virginia, PO Box 800422, Charlottesville, VA 22908. E-mail: dt9d@virginia.edu.

ical outcomes and are found in urine. Here we accomplish this and in the process provide a scientific rationale for the development of detection assays for two proteins in urine as noninvasive biomarkers of clinical outcomes in urothelial cancer.

Materials and Methods

Patient Tissue Samples and Microarray Datasets

All patient samples were obtained at the University of Virginia School of Medicine using protocols approved by the University of Virginia Institutional Review Board (HIC 9179). Twenty-five bladder tissue samples were collected from surgical pathology specimens including 6 normal urothelial samples and 19 urothelial tumor samples.⁹

Five other bladder cancer expression microarray datasets were used in our study. The first evaluated 48 normal bladder samples and 109 bladder carcinoma samples.¹⁰ The second evaluated 14 normal bladder samples, 27 superficial transitional cell carcinoma samples, and 13 invasive transitional cell carcinomas.¹¹ Data from 30 microarray chips profiling bladder cancer downloaded from the Gene Expression Omnibus (accession number GSE3167)^{12–14} were also used. A fourth study^{6,7} on 45 patients with urothelial carcinoma of the bladder treated with neoadjuvant MVAC followed by radical cystectomy, transurethral resection of the bladder tumor, or radiotherapy was used to evaluate disease-free survival outcomes in patients. Finally, a fifth study provided disease-free survival status in 16 patients treated with gemcitabine and cisplatin (GC), another standard chemotherapy regimen for bladder cancer.³

Genoproteomic Method

We used the Affymetrix NetAffx web tools (Affymetrix, Inc, Santa Clara, CA) to match proteins identified by Adachi et al⁸ as being present in the urine proteome to transcripts queried on the human U133A Affymetrix array. Next, to determine whether any of these transcripts are up-regulated in bladder cancer compared with benign tissue, we examined our microarray data in our cohort of 25 bladder samples described above. A two-tiered approach was used in the identification of up-regulated transcripts. First, using the present, marginal, and absent calls, we screened out genes that did not exhibit at least 75% present or marginal calls in at least one group (tumor or normal). Second, we used the Kolmogorov-Smirnov test to better characterize the degree of overlap between the two distributions and the direction of change. We specifically focused on genes whose expression was increased in bladder cancer compared with that in benign urothelium. The resulting nominal *P* values from each comparison were corrected for multiple testing using a false discovery rate of <0.01. The probe sets that passed these criteria were ordered based on fold change of cancer compared with benign urothelium.

Real-Time PCR in Urothelial Tissues

Microarray gene expression data were validated by quantitative real-time PCR using the SYBR Green SuperMix protocol (Bio-Rad Laboratories, Hercules, CA) with standard curves on an iCycler (Bio-Rad Laboratories). RNA was from 7 of the bladder tumor tissue samples and 3 of the normal urothelium samples that remained from the original 25 surgical pathology specimens used in expression profiling above. RNA was converted to cDNA using the iScript Reverse Transcription kit (Bio-Rad Laboratories). The PTC-200 Peltier thermal cycler (Bio-Rad Laboratories) was used for reverse transcription-PCR reactions. Quantitative real-time PCR reactions were performed in 96-well plates with 12.5 μ l of SYBR Green, 0.5 μ l of primer, and cDNA in a total reaction volume of 25 μ l. GGH was amplified using the following primers: forward 5'-ACTGCCAATTTCCATAAG-3' and reverse 5'-TGCCATCTGTATTGTAGTA-3'.

Statistical Evaluation of Gene Expression as a Function of Tumor Stage and Clinical Outcome after Chemotherapy

We used data from 23 of the 25 aforementioned local samples, as well as 30 microarray chips from GSE3167. The robust microarray average method was used to quantile-normalize, background-adjust, and summarize the gene expression values from these arrays.^{15–17} Microarray profiles were classified according to the type of tissue hybridized to the array including 15 chips with RNA from normal urothelium, 14 chips with non-muscle-invasive bladder tumors (stage Ta or T1 tumors and grade 1 to 2), and 24 chips with muscle-invasive bladder tumors (stage T2 or greater, any grade). We measured the correlation of gene expression (HG-U133A probe set 203560_at) with invasiveness as classified in three groups: normal, non-muscle-invasive tumors, and muscle-invasive tumors. Correlation of gene expression with invasiveness was evaluated using Pearson correlation Spearman rank-based correlation approaches.

Genes discovered by the Genoproteomic method or those found in the literature as predictors of clinical response to cisplatin-containing chemotherapeutic regimens and found in urine were evaluated as predictors of clinical outcomes to MVAC^{6,7} or GC.³ For each gene candidate, we used a weighted-voting technique¹⁸ to generate response predictions in each patient. Kaplan-Meier curves were used to assess the ability of each gene or gene combinations to stratify disease-free survival. All *P* values were determined using the log-rank test.

Results

Identification of Urinary Proteins Up-Regulated at the mRNA Level in Urothelial Cancer Tissue

To identify proteins that are present in urine and up-regulated in bladder cancer we used a multifaceted

Figure 1. Genoproteomic method. Outline of the approach used to determine that GGH is up-regulated in bladder cancer, detected in the urine proteome, and positively correlated to tumor invasiveness.

genoproteomic methodology shown in Figure 1. Adachi et al⁸ identified 1543 proteins in the urine proteome. The proteins identified as being present in the urine proteome were matched to 2184 probe sets in the human HG-U133A Affymetrix array. After specifically selecting for probe sets in our microarray data that matched identified proteins in the urine proteome, we identified 22 probe sets that had significant differential expression between normal and cancer tissue samples (Table 1). GGH, a gene associated with methotrexate resistance,^{19–23} had the highest overall fold expression in the bladder cancer samples compared with normal bladder samples at 5.5-fold.

GGH mRNA Expression Is Increased in Human Urothelial Cancer

To verify the microarray results indicating that GGH is increased in bladder cancer, quantitative real-time PCR was performed on 3 normal bladder tissue samples and 7 bladder cancer tissue samples remaining from the 25 tissue samples used in the original microarray study. The bladder cancer tissue samples showed increased expression of GGH across various tumor stages at 13.9-fold greater than normal tissue samples (Figure 2A).^{10,11} Two independent microarray data sets^{10,11} that evaluated GGH expression as a function of stage were found in the ONCOMINE database and were used to confirm our results (Figure 2B).^{10,11}

GGH mRNA Expression Is Higher in Muscle-Invasive than in Noninvasive Urothelial Cancer

To determine whether increased GGH expression was associated with muscle invasiveness in human urothelial carcinoma of the bladder, we examined GGH expression as a function of stage in microarray data from 23 of our 25 patients and 30 others (Gene Expression Omnibus accession number GSE3167) (Figure 2C).^{10,11} We measured the correlation coefficient of GGH expression compared with the invasiveness of the tumor scored as 1 for normal urothelial tissues, 2 for non-muscle-invasive tumors and 3 for muscle-invasive tumors. We found that GGH expression was significantly correlated with invasiveness (Pearson correlation = 0.572, P value = 7.47×10^{-6} , Spearman rank-based correlation = 0.614, P value = 9.87×10^{-7}).

Table 1. Genes with Up-Regulated Expression Levels in Bladder Cancer Tissue Samples for Which Protein Was Found in Urine

Probe set	Gene	Avg. normal expression	95% CI (LL)	95% CI (UL)	Avg. tumor expression	95% CI (LL)	95% CI (UL)	Fold change
203560_at	GGH	70.60	7.42	11.14	391.80	104.86	157.28	5.55
204333_s_at	AGA	35.80	9.54	14.30	106.70	23.74	35.60	2.98
202854_at	HPRT1	197.90	34.82	52.22	527.50	92.62	138.94	2.67
53071_s_at	FLJ22222	69.60	8.32	12.48	164.00	34.57	51.85	2.36
201349_at	SLC9A3R1	131.90	28.48	42.72	289.80	58.81	88.21	2.20
211725_s_at	BID	183.60	36.42	54.62	393.30	65.61	98.41	2.14
218923_at	CTBS	44.00	8.06	12.10	92.30	17.44	26.16	2.10
202118_s_at	CPNE3	231.60	49.54	74.32	482.30	80.10	120.16	2.08
217871_s_at	MIF	1354.30	297.46	446.20	2754.50	307.05	460.57	2.03
217346_at	LOC128192	106.60	17.92	26.88	213.80	24.57	36.85	2.01
219215_s_at	SLC39A4	151.10	22.53	33.79	275.00	25.46	38.20	1.82
207986_x_at	CYB561	115.50	22.66	33.98	209.30	30.03	45.05	1.81
201180_s_at	GNAI3	481.90	41.54	62.30	851.60	131.80	197.70	1.77
201098_at	COPB2	501.00	66.63	99.95	859.90	118.78	178.16	1.72
200039_s_at	PSMB2	507.20	65.35	98.03	850.20	115.46	173.20	1.68
217733_s_at	TMSB10	3242.20	291.95	437.93	5358.90	745.40	1118.10	1.65
201524_x_at	UBE2N	663.80	91.28	136.92	1083.70	143.30	214.96	1.63
208805_at	PSMA6	1145.60	82.18	123.28	1848.50	254.74	382.12	1.61
213011_s_at	TPI1	1494.50	134.62	201.92	2404.40	266.01	399.01	1.61
201268_at	NME1-NME2	1975.90	294.07	441.11	2965.70	260.61	390.91	1.50
211978_x_at	PPIA	5646.70	426.45	639.67	7513.60	634.72	952.08	1.33
214143_x_at	RPL24	4523.30	185.50	278.26	5699.70	233.53	350.29	1.26

From the analysis in Figure 1, 22 genes showed up-regulation of mRNA transcripts in bladder cancer tissue versus normal bladder tissue. Genes were ranked in order of fold change in bladder cancer versus normal urothelium samples.
 CI, confidence interval; LL, lower limit; UL, upper limit.

Figure 2. Internal and external validation of increased GGH gene expression in bladder cancer. **A:** Quantitative real-time PCR (qRT-PCR) expression of GGH mRNA in normal bladder mucosa ($n = 3$) and T_a, T₂, T₃, and T₄ bladder tumors ($n = 7$). Tissue was obtained from original samples used in microarray analysis. Columns, average expression of normal and tumor tissue using quantitative real-time PCR; bars, 95% confidence interval of measured expression across each category ($P = 0.05$). Fold change from normal to tumor is 13.9. **B:** Box plots taken from the ONCOMINE database that show up-regulation of GGH in bladder cancer from two published microarray studies.^{10,11} Both datasets have been log-2 transformed with the median set to 0.0 and SD set to 1. Study 1 and study 2 have P values of 1.5E-20 and 5.9E-6 respectively. **C:** Relative microarray-derived GGH expression values obtained from 23 of our 25 patients and 30 others (Gene Expression Omnibus accession number GSE3167) as described in *Materials and Methods* were calculated among three cohorts of patients, as stratified into normal ($n = 15$), non-muscle-invasive tumors ($n = 14$), and muscle-invasive tumors ($n = 24$). Center bar is median for each cohort. Boxes are bound by first and third quartiles of GGH expression in each cohort.

GGH and DBI Stratify Clinical Outcome after Systemic Chemotherapy

In prior studies, DBI, survivin, and emmprin expression was found to predict outcomes after platinum-based

Figure 3. Algorithm used to determine whether selected genes were predictive of clinical outcomes in response to MVAC. Outline of the approach that was used to determine whether GGH, DBI, emmprin, or survivin either alone or in combination could be predictive of clinical outcomes after MVAC chemotherapy. Disease-free survival was used as the measure of clinical outcome.

bladder cancer chemotherapy and were present in human urine³⁻⁸ (Figure 3). Hence, together with GGH, which is known to be associated with methotrexate resistance, we labeled these genes as candidate urinary biomarkers of clinical outcomes after chemotherapy.

We used the gene-weighted voting technique of Golub and Slonim¹⁸ to predict whether GGH, DBI, survivin, or emmprin expression correlated with clinical outcome as assessed by tumor response and/or disease-free survival after treatment with the MVAC^{6,7} or GC³ chemotherapy regimens. In the studies of Takata et al,^{6,7} “responders” are defined as those patients who achieved tumor downstaging after receiving two courses of MVAC neoadjuvant chemotherapy and “nonresponders” are defined as those patients who did not achieve tumor downstaging after receiving two courses of MVAC neoadjuvant chemotherapy. The metric of downstaging was determined by analyzing bladder tissue specimens obtained during cystectomy and/or radiological imaging using computed tomography or magnetic resonance imaging.

For our evaluation and model comparisons we used 27 of the 45 patients in the Takata et al MVAC study.^{6,7} We did not use all the patients because the first 18 of the 45 patients were used to train the original 14-gene prediction model. In comparison with the original 14-gene prediction model that was generated by Takata et al neither GGH, DBI, emmprin, or survivin alone or in combination was able to better predict a response to MVAC neoadjuvant chemotherapy than the 14-gene prediction model (Supplemental Table 1 see <http://ajp.amjpathol.org>).^{6,7,25,26} In addition, the remaining genes that are up-regulated in bladder cancer and secreted in urine (Table 1) were found to be weaker at predicting response to neoadjuvant MVAC compared with the Takata et al 14-gene model whether alone or in combination with GGH (Supplemental Table 2, see <http://ajp.amjpathol.org>).^{6,7,18,25-27}

When looking at stratification of disease-free survival after MVAC, we found that the GGH + DBI prediction model ($P = 0.024$) has greater statistical significance than the DBI model ($P = 0.046$) alone and had visually better separation of the survival curves (Figure 4, A and B).^{6,7,18,27} Importantly, the GGH model ($P = 0.190$),

Figure 4. Kaplan-Meier analysis of gene models as predictors of disease-free survival for 27 of the 45 patients in the MVAC study of Takata et al.^{6,7} The first 18 of the 45 patients^{6,7} were used to train their original 14-gene prediction model and thus were excluded from the current analysis. The gene-weighted voting technique as described by Golub et al¹⁸ was used to predict response to treatment for several different gene prediction models. Kaplan-Meier estimators for disease-free survival were generated for the respective gene prediction models: red curves describe predicted responders to treatment, blue curves describe predicted nonresponders to treatment. **A:** Gene-weighted voting technique applied for DBI alone. **B:** Gene-weighted voting technique applied for GGH and DBI in combination. P values were determined using the log-rank test and describe the statistical significance of stratification of disease-free survival based on predicted response.²⁷

Takata et al 14-gene model ($P = 0.077$), survivin model ($P = 0.393$), or the emmprin model ($P = 0.066$) did not provide any statistically significant stratification of disease-free survival (Supplemental Figure 1, A–D, see <http://ajp.amjpathol.org>). In addition, when survivin alone, emmprin alone, or survivin and emmprin together are added to the GGH/DBI prediction model, the significance of the two-gene model in stratifying disease-free survival after MVAC therapy is decreased ($P = 0.036$, $P = 0.040$, and $P = 0.025$, respectively) (Supplemental Figure 2, A–C, see <http://ajp.amjpathol.org>). The combination of survivin and emmprin together ($P = 0.205$) provided no statistical significance in disease-free survival (data not shown). The remaining genes from Table 1 that are up-regulated in bladder cancer and secreted in urine were also found to be weaker at stratifying disease-free survival than the GGH/DBI prediction model whether alone or in combination with GGH (Supplemental Table 2, see <http://ajp.amjpathol.org>).^{6,7,18,25–27} The study of Als et al³ only offered us the ability to evaluate the stratification of disease-free survival in patients treated with neoadjuvant GC; the GGH prediction models showed poor significance (data not shown).

Discussion

Our data implicate GGH as a novel biomarker for bladder cancer. When evaluated alone on tumor tissue, it does not significantly predict disease-free survival after MVAC chemotherapy, but its use in conjunction with DBI provides stratification superior to that of DBI alone. Interestingly, this two-gene combination predicts disease-free survival better than a previously described 14-gene prediction model^{6,7} of therapeutic response after treatment with the MVAC regimen. GGH is an intracellular lysosomal glycoprotein that hydrolyzes folyl- and anti-folypolyglutamates.²⁸ It is known to hydrolyze the polyglutamate derivatives of folates and anti-folates using both endopeptidase and exopeptidase activity.²⁹ GGH requires reduced thiols for its activity.³⁰ Secretion of GGH in cancer cells was initially established by experiments that showed increased secretion of GGH in H35 hepatoma cells compared with rat hepatocytes.³¹

GGH has been proposed to act as a prognostic biomarker for acute leukemias in response to methotrexate therapy²¹ and also to act as a diagnostic biomarker in regard to pulmonary neuroendocrine tumors.³³ Our study focused on the interaction of GGH with MVAC chemotherapy treatment. The role of GGH as a predictor of outcome after this treatment is of particular interest because methotrexate is a component of this regimen. The ratio between GGH, which removes terminal glutamates from methotrexate, and folypolyglutamate synthetase, which adds glutamates to methotrexate metabolites, has been shown to effectively predict methotrexate accumulation in both myeloid and lymphoid blasts.^{20,21} Inhibition of GGH at the molecular level has been shown to increase methotrexate metabolites in human sarcoma cell lines as well as leukemic cell lines.^{22,23} Interestingly, methylation of the CpG1 and CpG2 regions in the GGH promoter was shown to significantly reduce GGH mRNA expression and GGH catalytic activity, which resulted in higher accumulation of methotrexate metabolites in acute lymphoblastic leukemic cells.¹⁹

Our study showed a weak correlation with marginal significance ($P = 0.190$) between GGH expression and outcome after methotrexate-containing MVAC chemotherapy treatment regimens. At first glance these data seemingly contradict previous studies that show a strong and significant reduction of methotrexate activity with increased GGH expression. We speculate that the other agents in the MVAC regimen (vinblastine, doxorubicin, and cisplatin) may have the ability to diminish the effects of GGH activity inside the cell, thus increasing the viability of methotrexate within the cell, or that they are more efficacious than methotrexate and thus drive the clinical outcome to a much greater degree.

To further study the utility of GGH in predicting outcome after MVAC activity we looked at the ability of GGH to predict MVAC treatment outcomes when paired with other proteins that are both found in urine and have been shown to predict response to MVAC. A study conducted by Takata et al^{6,7} identified a 14-gene model to predict clinical outcome after MVAC chemotherapy for bladder cancer. This model contains DBI, a gene of interest for

our study because, like GGH, it was identified in the urine proteome.⁸ DBI was initially identified as an endogenous ligand that is recognized by the β -carboline/benzodiazepine recognition site located in the GABA_A receptor.³⁵ Besides the central nervous system DBI is expressed in peripheral tissues such as the liver, adrenal gland, testis, and kidney, primarily in the mitochondrial membrane.³⁶ We show that addition of DBI to the GGH prediction model has the ability to better stratify disease-free survival than the 14-gene model of Takata et al, the DBI model, or the GGH model alone. In addition to DBI, emmprin and survivin were included in the chemotherapy response and prognostic outcome prediction model as they are both found in urine^{4,5} and have both been found to be independent prognostic markers for outcome in patients with bladder cancer treated with cisplatin-containing regimens such as MVAC and GC.³ Addition of emmprin and survivin to the GGH/DBI models weakened their ability to stratify disease-free survival after MVAC. Interestingly, the GGH/DBI prediction model has not shown an ability to predict outcomes to GC therapy. From these results, we speculate that the GGH/DBI model is specific for the three drugs used in the MVAC regimen with the exception of cisplatin and has little predictive ability for gemcitabine.

The ability of the GGH/DBI model to significantly stratify and predict disease-free survival in patients treated with MVAC has potential for clinical prognostication in patients who undergo this treatment regimen. In addition, the genoproteomic approach supports the rationale for pursuit and development of enzyme-linked immunosorbent assay; assays for urine GGH/DBI that would allow noninvasive prediction of therapeutic outcomes to MVAC based purely on urine evaluation. However, several additional steps would be required to bring this concept to clinical utility. For example, once enzyme-linked immunosorbent assay tools are developed for the leading markers, GGH and DBI, we would propose to quantitate them in urine of patients undergoing neoadjuvant chemotherapy before cystectomy for invasive bladder cancer. A receiver operating characteristic curve analysis would then be performed with the marker level and the incidence of pathological P0 stage at cystectomy. Because the presence of P0 stage at cystectomy indicates chemotherapy benefit and enhanced survival,³⁷ we would use this analysis to "calibrate" the levels of GGH and DBI to provide a reflection of this important pathological outcome. As patients are followed after cystectomy, the marker levels would be further calibrated in a second stage using actual survival outcomes, which, although predicted well by P0 stage, are the standard outcome measure.³⁷ This aim of this future study is to demonstrate "proof of principle" of a new method of defining urinary markers that may be used in combination with assays such as ELISA to predict chemotherapy response.

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