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Research Report

The Prognostic Value of Cell Cycle Gene Expression Signatures in Muscle Invasive, High-Grade Bladder Cancer

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Abstract.
Background: Approximately half of patients with muscle invasive bladder cancer succumb to their disease. Previous work identified cell cycle related genes as a prognostic class of gene expression biomarkers in bladder cancer and found a specific 31-gene cell cycle proliferation (CCP) signature predicted outcome across multiple bladder cancer cohorts. However, the prognostic value of the CCP signature specifically in muscle invasive tumors was not evaluated.
Objective: To determine the prognostic value of cycle related genes in patients with muscle invasive bladder cancers.
Method: We collected all publicly available gene expression data for patients with high-grade, muscle invasive bladder cancer (8 cohorts, $N = 458$). We evaluated the CCP signature and two larger cell cycle gene sets: 1826 genes with a Gene Ontology (GO) annotation of "cell cycle" (GO-CCS) and 124 genes belonging to the "cell cycle" pathway in the KEGG pathway database (KEGG-CCS). An independently derived a sex identification gene signature (SIS) was developed as a positive control.
Results: While SIS distinguished males from females in all cohorts with information about patient sex, the CCP signature was not prognostic in any of the cohorts we analyzed, and the GO-CCS and KEGG-CCS were never prognostic in more than 2 independent cohorts. Furthermore, neither the CCP, GO-CCS, nor KEGG-CCS signatures were consistently enriched in prognostic genes while SIS was enriched with genes associated with sex in all cohorts.
Conclusions: Our findings suggest that cell cycle related genes have limited prognostic value in patients with high-grade, muscle invasive tumors. Their usefulness in predicting progression of noninvasive disease and patient response to chemotherapy remains to be determined.

Keywords: Cell cycle, gene expression, bladder neoplasms

INTRODUCTION

Bladder cancer is the ninth most common cancer in the world [1] and the fourth most common cancer in males in the United States [2]. For the 20–30% of patients that present with muscle invasive (T2–T4) tumors, approximately 57% experience recurrence within five years and the majority of these patients succumb to their disease [3]. The ability to predict which patients will succumb to their disease would allow clinicians to select patients most likely to benefit from adjuvant therapy while the identification of prognostic biomarkers could suggest possible targets for personalized treatment. For example, targeting overexpressed genes may lead to more efficacious treatment, as is the case for breast cancer patients who overexpress Her-2/Neu and who are treated by the monoclonal antibody trastuzamab [4]. Currently there are no prognostic biomarkers for bladder cancer in routine clinical use.

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Several studies have identified bladder cancer subtypes in patients with muscle invasive tumors. Using unsupervised clustering, Choi and colleagues identified basal and luminal subtypes that were associated with poor and good outcomes, respectively, and a p53-like subtype that was associated with increased resistance to chemotherapy [5]. Concurrently, Damrauer and colleagues also identified basal and luminal subtypes from high-grade, muscle invasive tumors and these subtypes were associated with outcome [6]. A separate analysis identified subtypes of high-grade, muscle invasive tumors, based on an integrated analysis of mRNA, miRNA, and protein data. These subtypes included papillary and basal/squamous subtypes. These authors carried out an integrated analysis of the mutation and copy-number data from 131 high-grade, muscle invasive bladder tumors found that cell cycle genes were altered in 93% of patients [7].

Cell cycle gene expression biomarkers are associated with outcome in a variety of cancers, including breast, prostate, and melanoma [8–10]. We have previously analyzed gene expression profiles from five bladder cancer patient cohorts ($N = 840$) and found that cell cycle genes were the only class of genes that consistently predicted outcome across multiple patient cohorts. Furthermore, we evaluated a specific cell cycle proliferation (CCP) signature and found that high CCP scores were associated with poor outcome in all five bladder cancer patient cohorts we analyzed. However, these cohorts all included patients with both muscle invasive and non-muscle invasive (Ta-T1) tumors [11].

The purpose of this study is to evaluate the prognostic value of the CCP signature and cell cycle related genes more broadly in patients with high-grade, muscle invasive tumors. We first demonstrate that the CCP signature is prognostic in cohorts containing low-grade, non-muscle invasive and high-grade muscle invasive tumors, before focusing specifically on patients with high-grade, muscle invasive tumors. As a putative "positive control", we apply the same methodology to the classification of males and females in the same independent cohorts where sex information is available.

MATERIALS AND METHODS

Patient cohorts and selection criteria

We have collected all published publicly available bladder cancer gene expression data for patients with high-grade, muscle invasive tumors having clinical outcome information (OS, DSS, or RFS). High-grade tumors were either classified as "high grade" according to the low vs. high grade classification system or classified as grade 3. With the exception of our power analysis (see below), patients were included only if they had high-grade, muscle invasive tumors, did not receive chemotherapy, and had radical cystectomy as definitive treatment. We identified eight patient cohorts (Table 1, $N = 458$), consisting of 44 patients profiled

Table 1
The eight patient cohorts ($N = 458$) used in the analysis and their clinical characteristics. A question mark (?) corresponds to patients where nodal or metastasis status were unknown or not available; a dash (–) indicates that information about the corresponding variable is not known. The *P*-value tests against the null hypothesis that all group means or proportions are the same using analysis of variance (ANOVA) or the Fisher Exact Test, respectively

		BLAVERI [12] ($N = 44$)	Choi [5] ($N = 22$)	CNUH [13] ($N = 28$)	Lindgren [15] ($N = 32$)	MSKCC [16] ($N = 60$)	MSKCC-CBIO [17] ($N = 47$)	Riester [14] ($N = 78$)	TCGA [7] ($N = 147$)	*P*-value
Availability*		S	GSE48277	GSE13507	GSE19915	S	cBioPortal	GSE31684	TCGA	
Endpoint		OS	0S	DSS	DSS	DSS	RFS	RFS	OS	
Age	Mean ± SEM	65.5 ± 1.59	65.9 ± 2.71	71.9 ± 1.53	–	66.5 ± 1.20	–	69.0 ± 1.1	68.8 ± 0.94	0.13
Gender	F	0.32	0.18	0.21	–	0.28	–	0.27	0.23	0.78
	M	0.68	0.82	0.79	–	0.72	–	0.73	0.77	
Stage	T2	0.16	0.23	0.57	0.25	0.15	0.21	0.22	0.37	<0.001
	T3	0.55	0.55	0.32	0.66	0.68	0.64	0.54	0.49	
	T4	0.30	0.23	0.11	0.09	0.17	0.15	0.24	0.14	
Nodal Status	pN0	0.45	0.45	0.75	–	0.58	0.57	0.49	0.68	<0.001
	>pN0	0.39	0.55	0.25	–	0.42	0.40	0.33	0.29	
	?	0.16	0	0	–	0	0.02	0.18	0.03	
Distant Metastasis	M0	0.07	0.95	0.93	0.62	–	–	0.58	0.51	<0.001
	M1	0	0.05	0.07	0.38	–	–	0.42	0.01	
	?	0.93	0	0	0	–	–	0	0.48	

*Gene expression data for all cohorts are publicly available from the Gene Expression Omnibus (GEO) [19] with the given Accession # (GSE ID), as Supplementary material to publication (S), from The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov), or from the cBioPortal [20].

by Blaveri and colleagues (Blaveri cohort) [12], 28 patients from Chungbuk National University Hospital (CNUH cohort) [13], 78 patients analyzed by Reister and colleagues (Reister cohort) [14], 32 patients profiled by Lindgren and colleagues [15], 22 patients profiled by Choi and colleages (Choi cohort) [5], 60 patients from the Memorial Sloan Kettering Cancer Center (MSKCC cohort) [16], 47 additional patients from the Memorial Sloan Kettering Cancer Center with profiles available on the cBioPortal (MSKCC-CBIO cohort) [17], and 147 patients profiled as part of The Cancer Genome Atlas project (TCGA cohort), a subset of whom were described previously [7]. Because there was no single endpoint common to all cohorts, we selected the endpoint as follows: DSS was always used if available (3 cohorts); otherwise, OS was used if available (3 cohorts); if neither DSS nor OS were available, we used RFS as the endpoint (2 cohorts). These endpoints are listed in Table 1.

Gene expression datasets

The sex identification signature was identified from a cohort of 80 patients profiled at l'Hôpital de l'Hôtel-Dieu at Laval University [18]. All other gene expression data used in this analysis are publicly available from the Gene Expression Omnibus [19], the Cancer Genome Atlas, the cBioPortal [20], or as supplementary material to publication (Table 1). Gene expression profiles were measured at the mRNA level using either Affymetrix microarrays (MSKCC and Reister), Illumina expression beadchip arrays (Choi, CNUH, and MSKCC-CBIO), non-commercial or customized arrays (Blaveri and Lindgren), or RNA-seq (TCGA). The specific platforms are listed in Supplementary Table S1. For all cohorts, processed data was downloaded and analyzed. In the Blaveri cohort, genes with missing values in >20% of samples were removed and expression values imputed using the *impute* package (*impute.knn* function) in *R* with default parameters. In the TCGA and Choi cohorts, low quality genes with an interquartile range of 0 were removed prior to analysis. Microarray probes were matched to genes based on current Affymetrix or Illumina annotation. When multiple probes were present for a gene, the probe with the highest mean expression was used [21].

Signature score calculation

CCP and additional signature scores were calculated by first normalizing each gene to have a mean of 0 and standard deviation of 1 across all samples within each cohort. Unweighted scores were calculated by taking the average normalized expression of all signature genes. Weighted scores were calculated by assigning a weight to each gene: a weight of +1 is assigned if the expression of the gene is either negatively associated with outcome (HR >1) or up-regulated in males (AUC >0.5); otherwise a weight of −1 is assigned. The weighted score is the weighted average expression of signature genes. For all analyses, continuous signature score is evaluated.

Power analysis

The selection criteria described above expanded to also include patients with low-grade, non-muscle invasive tumors. For this analysis, a cohort was analyzed if it had at least 10 patients with low-grade, non-muscle invasive tumors and at least 10 patients with high-grade, muscle invasive tumors. This expanded the Blaveri, CNUH, and MSKCC cohorts, yielding new control cohorts with 57, 114, and 72 patients, respectively. For a given cohort, let $n_1 =$ the number of patients in a cohort with low-grade, non-muscle invasive tumors and $n_2 =$ the number of patients in a cohort with high-grade, muscle invasive tumors. Let $n =$ the number of patients to randomly select, $s_1 = \min(n_1, n/2)$ and $s_2 = \min(n_2, n/2)$. Then randomly select patients s_1 with low-grade, non-muscle invasive tumors and s_2 patients with high-grade, muscle invasive tumors. Then if $s_1 + s_2 < n$, randomly select $n–(s_1 + s_2)$ additional patients. This approach maintains a balance between patients with low-grade, non muscle invasive tumors and high-grade, muscle invasive tumors. For each cohort n patients are randomly selected and the prognostic value of CCP score analyzed. This process is repeated 1000 times and the power for a sample of size n is estimated as the proportion of times CCP score was negatively and significantly (HR >1, $P < 0.05$) associated with outcome in the given cohort.

Statistical analyses

For sex identification, accuracy was quantified by the area under the receiver operating characteristic curve (AUC) with males coded as 1 and females coded as 0, and *P*-values calculated by the Wilcoxon Rank Sum Test. The AUC is equivalent to classification accuracy (number of patients correctly classified/total number of patients) when the number of male and number of female patients are the same.

For survival analyses, cox proportional hazard models were used to calculate hazard ratios (HR) for a

Table 2
Association of clinicopathological variables with outcome using the endpoints given in Table 1. For each cohort (column), the value in the table is the hazard ratio (HR) for the variable in the first column. For each row, the HR corresponds to the first category (e.g., male) with respect to the second category (e.g., female), with the exception of age, where the HR corresponds to a 1-year increase in age, and stage in the Lindgren cohort. In Lindgren, patients with T2 tumors who are pM0 have 100% survival (see Fig. S3). Because the HR corresponding to T4 vs. T2 is not defined in this case, the HR corresponding to T4 vs. T3 is given instead. A dash ('–') indicates insufficient sample size for analysis. *, $P < 0.05$ by Wald test. Also see Supplementary Figures S1–S3.

	Blaveri	Choi	CNUH	Lindgren	MSKCC	MSKCC-CBIO	Riester	TCGA
Male vs. Female	1.34	0.66	0.39	–	1.15	–	0.94	1.11
Age	1	1.06	1.09*	–	1.02	–	0.99	1.01
T3 vs. T2	1.04	0.61	2.58	$\gg 1$*	1.86	2.74	2.13	4.44*
T4 vs. T2	3.29*	5.06	7.60*	6.75*	2.92	7.95*	2.63	10.91*
pN1-3 vs. pN0	2.34*	0.56	4.32*	–	1.71	1.91	2.43*	3.19*
pM1 vs. pM0	–	–	8.32*	$\gg 1$*	–		23.29*	–

clinical variable or based on the continuous expression of a gene. Significance of clinical variables was assessed by logrank *P*-value or Wald *P*-value. Statistical significance for genes and gene signatures was assessed by logrank *P*-value or 95% confidence interval of the HR.

We evaluated whether a list of genes was enriched with predictive genes by calculating an enrichment score, given by

$$enrichment\ score = \frac{\#\ of\ significantly\ predictive\ genes}{total\ \#\ significantly\ predictive\ genes}$$

where a gene is significantly predictive if $P < 0.05$, based on the Wilcoxon Rank Sum Test (sex discrimination), or log rank *P*-value (survival association). The hypergeometric distribution is used to calculate a *P*-value for whether the test cohort is significantly enriched in predictive genes (i.e., whether the enrichment score significantly exceeds 1).

We evaluated whether a list of genes was enriched with genes associated with biological processes by using the Database for Visualization and Annotated Discovery (DAVID) [22], which identifies Gene Ontology (GO) [23] terms and KEGG pathways [24] overrepresented in lists of genes. Enrichment was evaluated at the probe level.

RESULTS

Patient cohorts and common clinical predictors of survival

We analyzed all publicly available cohorts that had patients with high-grade, muscle invasive tumors and patient outcome information (8 cohorts, $N = 458$). Patient cohorts have similar age and gender distributions, but differ with respect to stage (T2, T3, T4), nodal status (pN0, pN1-N3), and metastases status (M0, M1) (Table 1). However, stage, nodal status and metastases status were consistently associated with outcome, consistent with previous studies [3]. Specifically, stage was significantly associated with outcome in 6 out of 8 cohorts with stage information, while nodal status was predictive of outcome in 4 out of 8 cohorts. Metastases status was predictive in all cohorts where this information was available (Table 2 and Supplementary Figures S1–S3). These results suggest that performance of prognostic signatures can be fairly compared across these cohorts using the specified endpoints, since patients share common clinicopathological predictors of outcome. We expect that gene signatures or processes that capture this common tumor pathology will be predictive across multiple cohorts, though not necessarily all of them. We note that grade was not considered in this analysis because either all patients had the same grade within each cohort, or the specific high-grade designation (grade 3-4) was not available.

The prognostic value of a cell cycle proliferation signature in bladder cancer patients with high-grade, muscle invasive tumors

We have previously found that a continuous cell cycle proliferation (CCP) score, calculated as the average unweighted, normalized expression of 31 genes (see Methods), was significantly predictive of outcome in five bladder cancer patient cohorts [11]. However, these cohorts included patients with both low- and high-grade tumors, and non-muscle and muscle invasive tumors. Our first objective is to evaluate the prognostic value of CCP score in bladder cancer patients with high-grade, muscle invasive tumors.

A power analysis was performed in order to estimate whether or not each of our eight cohorts had sufficient sample size for prognostic gene expression signature evaluation, under the assumption that CCP score is independent of stage and grade. The Blaveri, CNUH, and MSKCC cohorts were expanded to include patients

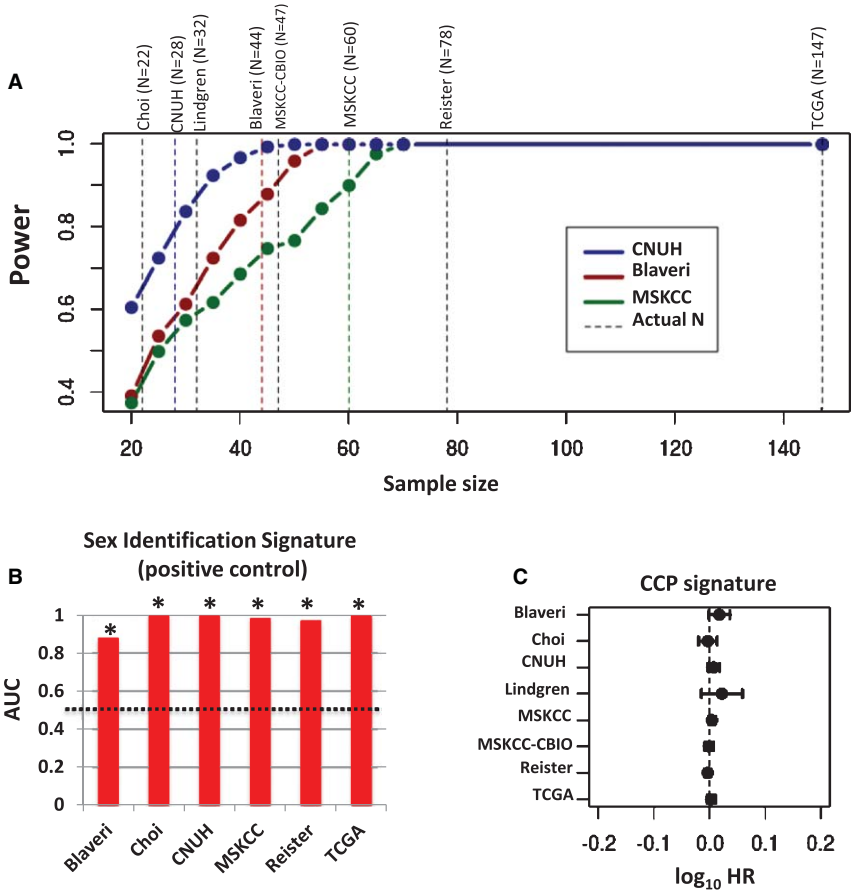

Fig. 1. Association of gene signature scores with outcome and sex. Signature scores were calculated by finding the average expression of all signature genes. A, power analysis for evaluation of CCP score in the Blaveri, CNUH, and MSKCC cohorts when patients with low-grade, non-muscle invasive tumors were included. For each sample size, the power is estimated as the proportion out of 1000 random samples where CCP score is negatively and significantly (HR >1, $P<0.05$) associated with outcome. Vertical dashed lines correspond to the sample sizes of each cohort when limited to patients with high-grade, muscle invasive tumors. B, the ability of the Sex Identification Signature (SIS) score to distinguish males from females in cohorts when limited to patients with high-grade, muscle invasive tumors. Performance is measured by AUC, which is equivalent to the probability that a randomly selected male has a higher SIS score than a randomly selected female. The dashed black line corresponds to the AUC value of an association due to random chance (i.e., AUC = 0.50). A * denotes statistical significance ($P<0.05$) of an AUC differing from 0.50 based on the Wilcoxon rank-sum test. C, the prognostic value of CCP score in each cohort. Plots show the $\log_{10}$ HR (filled circle) and 95% confidence interval for each cohort and each signature and a vertical dashed line corresponding to a $\log_{10}$ HR of no association between score and outcome (i.e., a $\log_{10}$ HR of 0).

with low-grade, non-muscle invasive tumors. For each cohort 20 patients were randomly selected as described in Methods. The prognostic value of CCP score was then evaluated and this process repeated 1000 times each for sample sizes ranging from 20 to 147 in order to estimate the power that the CCP signature would significantly ($P<0.05$) and negatively (HR > 1) associate with outcome, which was plotted as a function of sample size (Fig. 1A). We were able to obtain power estimates for Blaveri, CNUH, and MSKCC, and these ranged from approximately 80% for CNUH ($N=28$) to 90% for MSKCC ($N=60$) (Fig. 1A). It is clear that the study is sufficiently powered for Reister and TCGA (100% power), while we estimate the power to be at least 75% for MSKCC-BIO. Our study is likely underpowered, however, for the Choi cohort ($N=22$). This analysis is an important positive control and suggests that for the majority of cohorts in Table 1, CCP score will be negatively associated ($P<0.05$) with outcome if its prognostic value was independent of stage and grade.

An additional positive control was also used. We identified a new "sex identification signature" (SIS) from a cohort of 80 bladder cancer patients with high-grade, muscle invasive tumors [18]. This cohort is not analyzed further because all patients were treated with

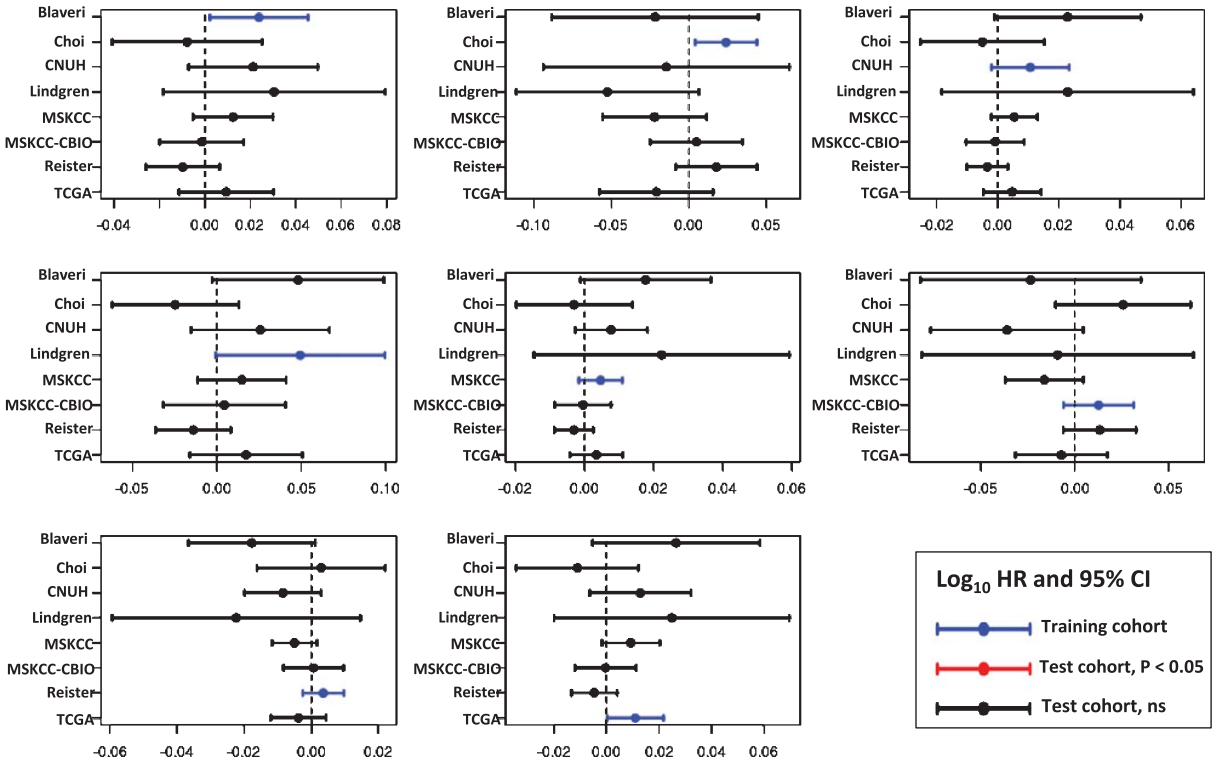

Fig. 2. Prognostic value of weighted CCP score. CCP signature genes were weighted by −1 or +1 according to whether the gene was positively or negatively associated with outcome, respectively, in each training cohort (blue lines). A weighted CCP score was then calculated and its prognostic value evaluated in the remaining cohorts (i.e., the testing cohorts). Plots show the $\log_{10}$ HR (filled circle) and 95% confidence interval for each cohort and each signature, with statistically significant results ($P < 0.05$) colored red, and a vertical dashed line corresponding to a $\log_{10}$ HR of no association between score and outcome (i.e., a $\log_{10}$ HR of 0).

adjuvant chemotherapy. The signature consists of nine genes that are up-regulated in males (Supplementary Table S2, FDR <10%).

The rest of the manuscript considers only patients with high-grade, muscle invasive tumors. We calculated SIS and CCP scores by finding the mean expression of all normalized signature genes. SIS scores accurately separated males from females in all six cohorts that had demographic information on patient sex (AUC >0.87, $P < 0.05$ in each cohort, Fig 1B). This "positive control" shows that the score from an independently derived signature, calculated as the average expression value of the signature genes, has predictive ability in the patient cohorts analyzed here. However, when we evaluated the prognostic value of CCP score in these patients, there were no cohorts for which CCP score was significantly associated with outcome (Fig. 1C).

The above calculation of CCP score assumes that each CCP gene is negatively associated with outcome. This is a reasonable assumption, since CCP genes are positively correlated with one another and this CCP score is negatively associated with outcome in prostate cancer and in bladder cancer patients when patients with low-grade and non-muscle invasive tumors are included [8, 11, 25]. However, to account for the possibility that a signature gene might be positively associated with outcome, we also analyzed the weighted average expression of all signature genes, using a training cohort to assign weights of+1 or −1 to each gene depending on whether or not the gene was negatively (HR >1) or positively (HR <1) associated with outcome, respectively. We selected one cohort as a training cohort and evaluated the weighted CCP score in the remaining testing cohorts, and this analysis was repeated with each cohort as the training cohort. In this analysis, weighted CCP score was also not significantly ($P < 0.05$) prognostic in any testing cohort (Fig. 2). These results indicate that the original and weighted CCP scores are not prognostic in patients with high-grade, muscle invasive tumors.

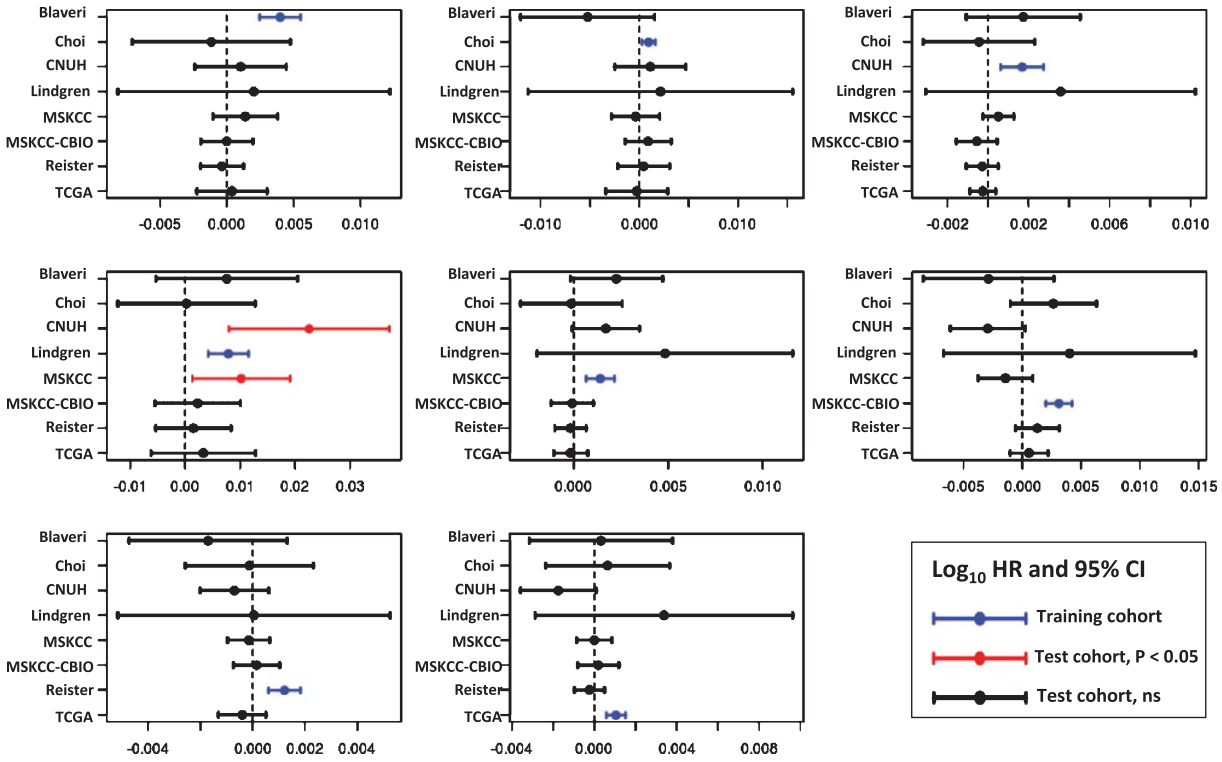

Fig. 3. Prognostic value of the Gene Ontology Cell Cycle signature (GO-CCS). Signature genes were weighted by −1 or +1 according to whether the gene was positively or negatively associated with outcome, respectively, in each training cohort (blue lines). A weighted GO-CCS score was then calculated and its prognostic value evaluated in the remaining cohorts (i.e., the testing cohorts). Plots show the $\log_{10}$ HR (filled circle) and 95% confidence interval for each cohort and each signature, with statistically significant results ($P < 0.05$) colored red, and a vertical dashed line corresponding to a $\log_{10}$ HR of no association between score and outcome (i.e., a $\log_{10}$ HR of 0).

The prognostic value of cell cycle gene sets in bladder cancer patients with high-grade, muscle invasive tumors

We next looked at cell cycle-related genes more broadly, rather than focusing specifically on the 31-gene CCP signature. Two cell cycle gene sets were analyzed. We identified all genes from the Gene Ontology (GO) database annotated with the biological process "cell cycle" (GO:0007049). In this database, "cell cycle" encompasses all biological processes (e.g., mitotic cell cycle, nuclear DNA replication) associated with cell division, and the set includes 1826 unique genes. The second set consists of the 124 genes belonging to the "cell cycle" pathway in the KEGG pathway database (hsa04110). We will refer to these GO and KEGG cell cycle gene signatures as GO-CCS and KEGG-CCS, respectively.

For each cell cycle signature, we calculated a weighted signature score using the method described above. One cohort was selected as the training cohort, and the remaining cohorts were used for testing. This was repeated with each cohort as the training cohort. Only one training cohort (Lindgren) yielded significantly prognostic ($P < 0.05$) GO-CCS scores in any testing cohorts, while the remaining 6 training cohorts did not produce prognostic GO-CCS scores in any testing cohorts (Fig. 3). For KEGG-CCS, no training cohort yielded significantly prognostic scores in more than one testing cohort (Fig. 4). In contrast, the SIS "positive control" produced weighted scores that significantly ($P < 0.05$) distinguished males from females in all testing cohorts regardless of which training cohort was used (Supplementary Figure S4). This latter finding demonstrates that a robust predictive signature will not be sensitive to the training cohort used. Overall, these results suggest that the expression of cell cycle associated genes have limited prognostic value in patients with high-grade, muscle invasive tumors.

Cell cycle gene lists are not enriched in genes predictive of outcome in high-grade, muscle invasive bladder cancers

Arguably, a prognostic gene signature should contain genes that are themselves individually prognostic.

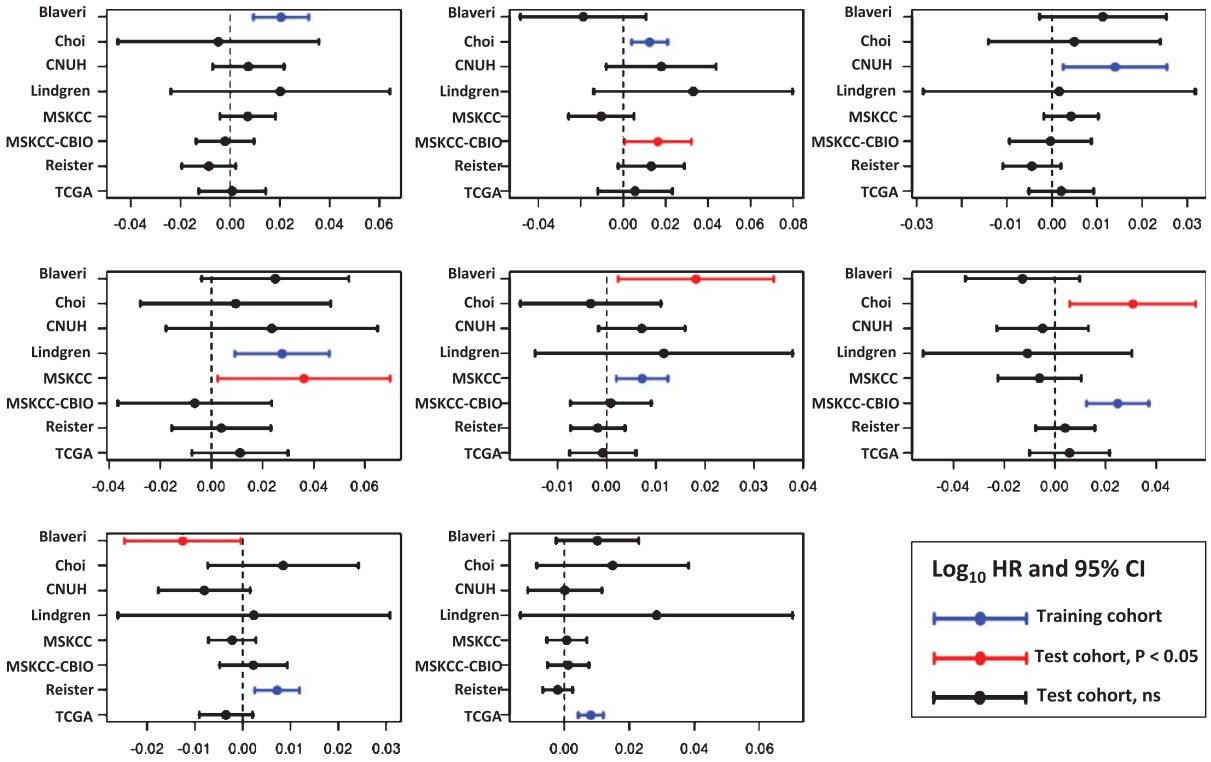

Fig. 4. Prognostic value of the KEGG Pathway Cell Cycle signature (KEGG-CCS). Signature genes were weighted by −1 or +1 according to whether the gene was positively or negatively associated with outcome, respectively, in each training cohort (blue lines). A weighted KEGG-CCS score was then calculated and its prognostic value evaluated in the remaining cohorts (i.e., the testing cohorts). Plots show the log10 HR (filled circle) and 95% confidence interval for each cohort and each signature, with statistically significant results ($P < 0.05$) colored red, and a vertical dashed line corresponding to a $\log_{10}$ HR of no association between score and outcome (i.e., a $\log_{10}$ HR of 0).

Enrichment analysis assesses whether or not a gene signature contains more significantly prognostic ($P < 0.05$) genes than what would be expected by chance. Such an analysis can be thought of as an unbiased way of assessing the prognostic value of a gene signature, since the enrichment (or lack thereof) does not depend on factors such as the specific mathematical model or gene weighting used to produce a signature score, the choice of gene normalization, or the choice of training cohort, which all can effect the performance of a gene signature.

We quantified the enrichment of the CCP, GO-CCS, and KEGG-CCS gene lists for genes that were significantly associated with outcome. For each cohort and each gene list, we calculated an enrichment score, which quantifies how much more likely the signature is to contain a prognostic gene ($P < 0.05$) than the set of all genes profiled for that cohort (see Methods for details). For example, an enrichment score of 2 indicates that the gene signature contains twice as many significantly prognostic genes than the set of all genes profiled. *P*-values assess whether an enrichment score is significantly greater than 1 (i.e., whether a signature is significantly enriched). We note that in our analysis of CCP, GO-CCS, and KEGG-CCS, we place no constraints on whether a gene is positively or negatively associated with outcome, so that a gene that is positively associated with outcome in one cohort can be negatively associated with outcome in another (or vice-versa). This is a conservative approach that may overestimate the true enrichment of a gene list, but simplifies the analysis since we do not know a *priori* whether a signature gene is positively or negatively associated with outcome. Because all SIS signature genes are up-regulated in males, however, we require that a SIS signature gene be up-regulated in males when we calculate its enrichment score.

SIS, the positive control, is significantly enriched with genes that are up-regulated in males in all cohorts ($P < 0.05$), with a mean enrichment score of 22.3 (range 7.8–63.5, Fig. 5A). However, neither the CCP nor KEGG-CCS lists were significantly enriched with prognostic genes, while GO-CCS was significantly enriched with prognostic genes in only one

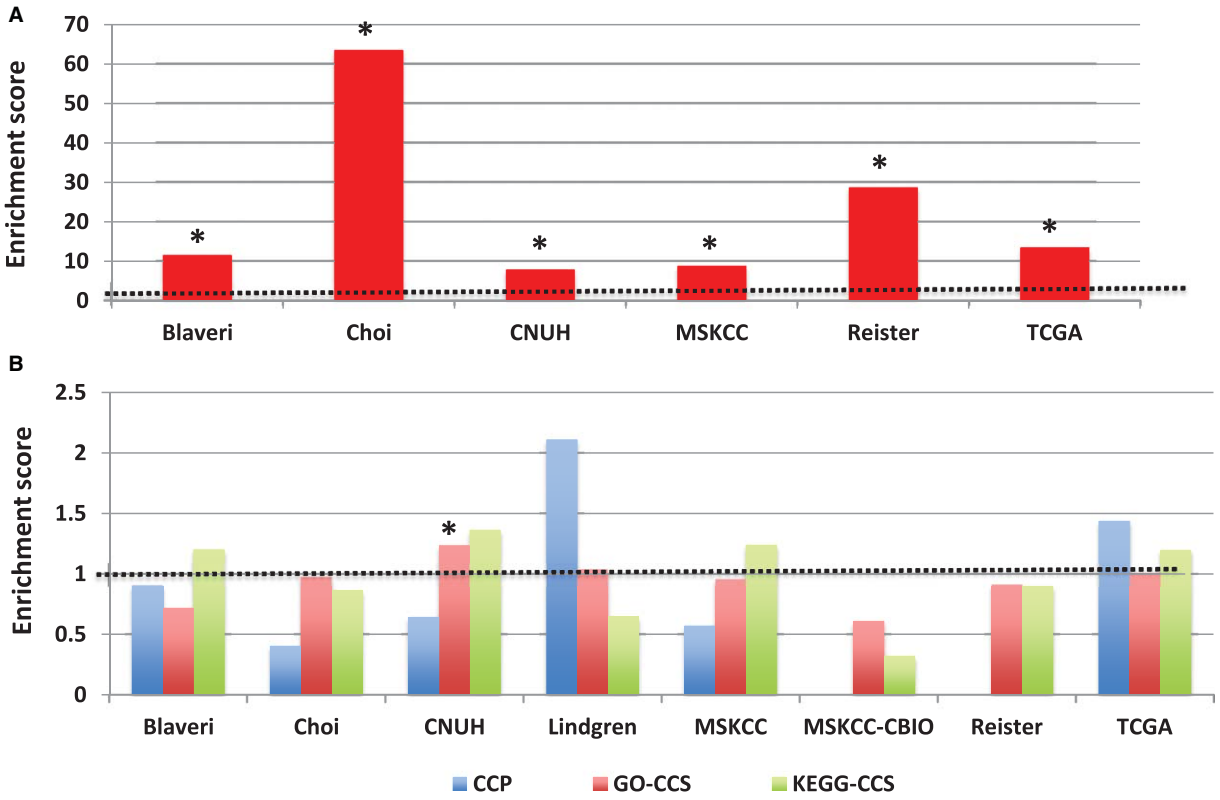

Fig. 5. Enrichment analysis of sex identification and cell cycle signatures. An enrichment analysis was carried out to test whether a gene signature was enriched in significantly predictive ($P < 0.05$) genes for sex or outcome. The enrichment score is the ratio of the number of significantly predictive genes in the signature to the number of significantly predictive genes in the dataset. A, enrichment of Sex Identification Signature (SIS; positive control) for genes that are significantly ($P < 0.05$) up-regulated in males. B, enrichment of CCP, GO-CCS, and KEGG-CCS cell cycle signatures for genes that are significantly ($P < 0.05$) prognostic. The dotted line corresponds an enrichment score of 1 (i.e., what would be expectd by chance). A * denotes statistical significance ($P < 0.05$) that a signature is enriched (i.e, the enrichment score is significantly greater than 1).

cohort (Fig. 5B). The highest enrichment score corresponded to the CCP signature in the Lindgren cohort (score = 2.11), but this was not statistically significant ($P = 0.385$), partially because only 1 out of the 11 CCP genes that were profiled was significantly prognostic. A lack of consistent enrichment in the cell cycle related gene lists for significantly prognostic genes provides strong evidence that, as a class, cell cycle associated genes are not prognostic in bladder cancer patients with high-grade, muscle invasive tumors, based on their gene expression.

Is there a functional class of genes that consistently predict outcome in bladder cancer patients with high grade, muscle invasive tumors?

A previous validation study found that bladder cancer survival signatures identified from gene expression profiling studies performed no better than chance when applied to independent cohorts containing patients with both superficial and invasive tumors [26]. However, a robust prognostic signature was later identified following the observation that cell-cycle related genes were the only class of genes consistently predictive of outcome in bladder cancer patients [11]. We therefore used an identical approach and investigated whether a class of consistently prognostic genes could be found for patients with high grade, muscle invasive tumors. The identification of a common biological process could guide the development of a consistently prognostic signature containing genes related to that process.

In each cohort, we identified all genes that were significantly associated with outcome ($P < 0.01$). We then identified GO terms and KEGG pathways that were over-represented in each list of prognostic genes, and compared these across the cohorts. We note that this analysis was identical to the enrichment analysis used previously that found that cell cycle related processes

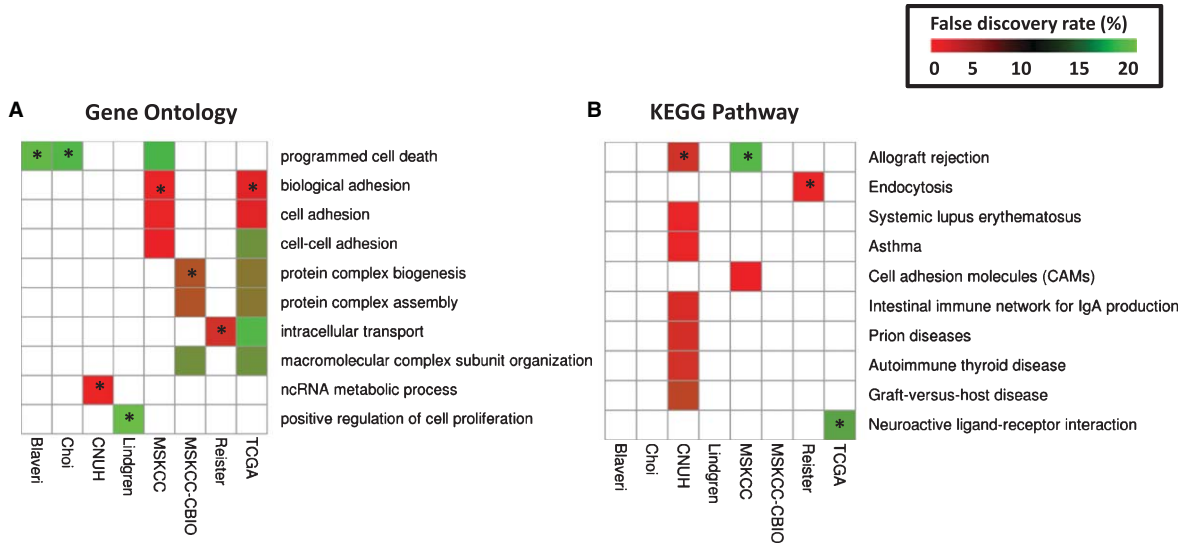

Fig. 6. Prognostic modules associated with outcome in bladder cancer patients with high-grade, muscle invasive tumors. In each cohort, (A) over-represented Gene Ontology (GO) terms and (B) KEGG pathways were identified from lists of genes significantly predictive of disease outcome ($P < 0.01$) using the DAVID gene annotation enrichment analysis toolkit. Consistently prognostic modules were identified by ranking all modules first by the number of cohorts with significant results (FDR < 20%) and then by average p-value. Each figure includes ten modules: the most consistently prognostic modules and the 'top hit' for each cohort, marked by an asterisk (*), which is defined as the module with the lowest FDR in that cohort that has an FDR < 20% in multiple cohorts, or if no such module exists, then the module with the lowest FDR.

such as "cell cycle process", as defined by GO, were the only processes consistently associated with outcome in bladder cancer patient cohorts that included patients with both low-grade, non-muscle invasive and high-grade, muscle invasive tumors [11].

Figure 6 shows the results from the gene set enrichment analysis across the 8 bladder cancer patient cohorts in our study, with all patients having muscle-invasive, high-grade tumors. The top 10 GO terms and KEGG pathways are shown. The most consistently prognostic class of genes were defined by the GO term "programmed cell death", which was associated with outcome in 3/8 cohorts (FDR <20%). Several other GO terms (such as "cell adhesion") were associated with outcome in 2 cohorts. Only one KEGG pathway ("allograft rejection") was associated with outcome in more than one cohort. For the complete set of results, see Supplementary Table S3. These results indicate that there is no single class of genes whose expression is consistently associated with outcome in bladder cancer patients with muscle-invasive, high-grade tumors.

DISCUSSION

We evaluated several cell cycle related gene signatures in bladder cancer patients with high-grade, muscle invasive tumors and found that these gene signatures had limited prognostic value in these patients. This finding was in contrast to a previous study that found that in patients with both non-muscle invasive and muscle invasive tumors, cell biomarkers robustly predict outcome in bladder cancer patients. Specifically, in a multivariate analysis of patients that included stage (muscle invasive vs. non-muscle invasive) and grade (high-grade vs. low-grade), CCP score outperformed grade and was comparable to stage when evaluated in multiple patient cohorts [11]. Our current work indicates that although cell cycle biomarkers are prognostic across patients with both non-muscle invasive and invasive tumors, these biomarkers are not prognostic in patients with high-grade, muscle invasive tumors. This may be because the prognostic value of cell cycle biomarkers is dependent on their ability to distinguish low-grade, non-muscle invasive tumors from high-grade, muscle-invasive tumors [15]. Furthermore, if nearly all high-grade, muscle invasive tumors have genomic alterations in cell cycle genes [7], then cellular proliferation may be similar across these tumors and would not distinguish between patients with good and poor prognoses.

There are several technical aspects of our study that must be addressed. First, because patient cohorts were profiled on different platforms, probes for cell cycle genes may not be comparable across platforms. Second, five of the eight cohorts we analyzed have

modest sample sizes of less than 50. We addressed these potential limitations in our study through a power analysis (Fig. 1A), which shows directly that CCP score is prognostic in three cohorts (Blaveri, CNUH, MSKCC) when patients with low-grade, non-muscle invasive tumors are included, despite the fact that different platforms were used for gene expression profiling (a custom cDNA array, an Illumina bead array, and an Affymetrix microarray; Supplementary Table S1) in these cohorts. For the sample sizes we analyze (Table 1), the power of our study is at least 80% for each of these three cohorts. Despite this, CCP score was not significantly associated with outcome in any of these three cohorts when only patients with high-grade, muscle invasive tumors were analyzed (Fig. 1C). This result strongly suggests that it is the lack of patients with low-grade, non-muscle invasive tumors that diminishes the prognostic value of CCP score, rather than differences between platforms or sample sizes.

Stage, nodal status, and metastasis status are strongly associated with outcome in bladder cancer [3]. In two cohorts (Choi and MSKCC), however, none of these clinical variables were significantly associated with outcome. These cohorts are clearly not representative of typical patients and therefore the lack of prognostic signatures in these cohorts is not surprising. Nevertheless, although the remaining cohorts differed with respect to stage, nodal status, metastasis status, and endpoints, they did share common clinicopathological predictors of survival. If a signature was associated with outcome because of correlation with one of these predictors, we would expect that signature to predict outcome in all cohorts were that clinicopathological factor was predictive. Therefore, for example, we would expect a signature associated with the metastatic nature of a tumor to predict outcome in Lindgren, CNUH, and Reister, since metastasis status was associated with outcome in these three cohorts (Table 1). However, no signature we analyzed was prognostic in these three cohorts. In fact, no signature we analyzed was prognostic in Reister, despite its relatively large sample size ($N = 78$). In addition, no signature we analyzed was consistently prognostic across cohorts where either nodal status or stage was associated with outcome. For example, the GO-CCS signature, when trained on Lindgren, was prognostic in CNUH, a cohort where stage, nodal status, and metastasis status were all individually associated with outcome. However, GO-CCS was not prognostic in any other cohort where stage, nodal status, or metastasis status were prognostic.

The primary objective of our study was to determine the prognostic value of CCP score using the same weighting scheme previously found to be prognostic in both bladder and prostate cancer [8, 11, 25]. We also considered a simple weighting scheme with weights of +1 or −1 assigned to each gene, for the CCP, GO-CCS, and KEGG-CCS signatures. Arguably, a more flexible weighting scheme could result in more robust classification. However, the CCP, GO-CCS, and KEGG-CCS gene lists do not contain any more prognostic genes than are expected by chance (Fig. 5B). These results strongly suggest that these signatures would not be consistently prognostic, regardless of the weighting scheme or classification method used.

Our analysis of cell cycle biomarkers were based on their transcription profiles, rather than genomic alterations or protein expression. Mitra et al. reviews immunohistochemical cell cycle biomarkers in bladder cancer and concludes that markers of cell growth receptor signaling, the p53 and retinoblastoma pathways, and cell proliferation (i.e., KI-67) have prognostic value, and that multimarker panels have more prognostic value than individual biomarkers [27]. However, none of the studies referenced within this review explicitly evaluated KI-67 in patients with high-grade, muscle invasive tumors. One study found that KI-67 protein expression significantly associated with outcome in patients with muscle invasive tumors ($P = 0.045$), but the finding was not significant in a multivariate analysis that included stage and grade [28]. Another study found that KI-67/p27 together were prognostic in muscle invasive cancers in a multivariate analysis [29]. These findings do not contradict our conclusions. However, we note that because mRNA levels explain only about 40% of protein levels [30], investigation of both protein and mRNA biomarkers may yield contradictory results.

Finally, our gene set enrichment analysis was unable to identify any process associated with outcome in the majority (>3) of cohorts, based on GO biological processes and KEGG pathway annotations. This was surprising, since prognostic signatures are often consistently enriched in biological processes despite containing different numbers of genes [31]. Additionally, Mitra and colleagues identified a 15 gene signature with prognostic value independent of stage and grade, and this signature was enriched in GO terms related to WNT and MAPK signaling, focal adhesion, and cancer-related pathways [32]. Previous studies have also found that basal and luminal subtypes of muscle invasive tumors were associated with survival [5, 6]. However, these subtypes are not present in the GO or

KEGG pathway database. Nevertheless, our findings suggest that high-grade muscle invasive bladder cancer is a heterogenous disease and that there may be a variety of biological pathways that drive outcome, and that these pathways are independent of clinicopathological variables. The activation or repression of such pathways would define genomic subtypes that are associated with outcome. If this is the case, Fig. 6 provides insight into these potentially prognostic pathways and suggests that "programmed cell death" is altered in one subtype. Interestingly, increased apoptosis is associated with poor outcome in patients with invasive breast cancer [33] while down-regulation of caspase-9, which is required for apoptosis, is associated with poor outcome in patients with stage II colorectal cancer [34].

In summary, we find that cell cycle related biomarkers have limited prognostic value in bladder cancer patients with high-grade, muscle invasive tumors. The prognostic value of cell cycle markers in patients with basal or luminal subtypes and the value of these markers in predicting patient response to chemotherapy remains to be determined.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1
Gene expression profiling platforms

Cohort	Platform
Blaveri	Custom cDNA microarrays
Choi	Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip
CNUH	Illumina human-6 v2.0 expression beadchip
Lindgren	Swegene
MSKCC	Affymetrix Human Genome U133A Array
MSKCC-CBIO	Illumina Human HT-12 Expression BeadChip
Riester	Affymetrix Human Genome U133 Plus 2.0 Array
TCGA	Illumina HiSeq RNASeq V2

Table S2
Sex Identification Signature (SIS)

Probe	Gene	FC	*P*-value	FDR
214131_at	TXLNG2P	6.987855541	6.80E-008	0.000350503
206700_s_at	KDM5D	10.84569913	8.51E-008	0.000350941
204409_s_at	EIF1AY	3.217134489	2.74E-007	0.000942252
205000_at	DDX3Y	4.56386144	2.55E-007	0.000942252
201909_at	RPS4Y1	3.077204565	4.20E-007	0.001333226
232618_at	TXLNG2P	2.340764001	7.35E-007	0.002166596
236694_at	TXLNG2P	2.236285184	8.44E-007	0.002321415
205001_s_at	DDX3Y	1.857529079	1.04E-006	0.00267436
223646_s_at	TXLNG2P	1.706286494	1.78E-006	0.004325388
204410_at	EIF1AY	1.497917623	6.57E-006	0.015057486
211149_at	UTY	1.762116434	1.02E-005	0.022151127
230760_at	ZFY	1.695563985	1.23E-005	0.025342139
228492_at	USP9Y	1.794467909	1.39E-005	0.027300876
223645_s_at	TXLNG2P	1.806342642	2.19E-005	0.041086848
232684_at	ZNF503-AS1	1.276909382	5.45E-005	0.097688317

Table S3
Gene Ontology (GO) terms and KEGG pathways associated with prognostic genes ($P < 0.01$) in high-grade, muscle invasive bladder cancer

	Blaveri	Choi	CNUH	Lindgren	MSKCC	MSKCC-CBIO	Reister	TCGA
GO:0012501~programmed cell death	18.77	16.17			14.38			
GO:0022610~biological adhesion					0.09			2.15
GO:0007155~cell adhesion					0.09			2.18
GO:0016337~cell-cell adhesion					1.12			11.34
GO:0070271~protein complex biogenesis						6.26		9.26
GO:0006461~protein complex assembly						6.26		9.26
GO:0046907~intracellular transport							3.57	16.19
GO:0043933~macromolecular complex subunit organization						11.10		11.32
GO:0000059~protein import into nucleus, docking								0.00
GO:0007156~homophilic cell adhesion								0.00
GO:0034660~ncRNA metabolic process			0.04					
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II			0.05					
GO:0007268~synaptic transmission								0.08
GO:0019226~transmission of nerve impulse								0.16
GO:0030182~neuron differentiation								0.18
GO:0045597~positive regulation of cell differentiation					0.23			
GO:0006396~RNA processing			0.24					
GO:0007267~cell-cell signaling								0.31
GO:0007398~ectoderm development					0.32			
GO:0043623~cellular protein complex assembly								0.34
GO:0034470~ncRNA processing			0.40					
GO:0002696~positive regulation of leukocyte activation					0.59			
GO:0042592~homeostatic process		0.60						
GO:0050870~positive regulation of T cell activation					0.60			

(continued)

Table S3
(continued)

	Blaveri	Choi	CNUH	Lindgren	MSKCC	MSKCC-CBIO	Reister	TCGA
GO:0007214~gamma-aminobutyric acid signaling pathway								0.61
GO:0045580~regulation of T cell differentiation					0.69			
GO:0043065~positive regulation of apoptosis		0.74						
GO:0050867~positive regulation of cell activation					0.78			
GO:0008544~epidermis development					0.81			
GO:0043068~positive regulation of programmed cell death		0.85						
GO:0010942~positive regulation of cell death		0.94						
GO:0034621~cellular macromolecular complex subunit organization								1.04
GO:0045582~positive regulation of T cell differentiation					1.06			
GO:0050863~regulation of T cell activation					1.07			
GO:0045165~cell fate commitment								1.12
GO:0051094~positive regulation of developmental process					1.19			
GO:0009952~anterior/posterior pattern formation								1.22
GO:0002708~positive regulation of lymphocyte mediated immunity					1.34			
GO:0002705~positive regulation of leukocyte mediated immunity					1.34			
GO:0045621~positive regulation of lymphocyte differentiation					1.49			
GO:0007389~pattern specification process								1.82
GO:0045619~regulation of lymphocyte differentiation					1.83			
GO:0008104~protein localization							2.05	
GO:0051251~positive regulation of lymphocyte activation					2.18			
GO:0006399~tRNA metabolic process			2.84					
GO:0060284~regulation of cell development								2.86
GO:0051960~regulation of nervous system development								2.99
GO:0042127~regulation of cell proliferation					3.02			
GO:0002699~positive regulation of immune effector process					3.52			
GO:0031349~positive regulation of defense response					3.52			
GO:0001912~positive regulation of leukocyte mediated cytotoxicity					3.53			
GO:0048666~neuron development								3.55
GO:0050778~positive regulation of immune response					3.68			
GO:0045586~regulation of gamma-delta T cell differentiation					3.70			
GO:0046645~positive regulation of gamma-delta T cell activation					3.70			
GO:0045588~positive regulation of gamma-delta T cell differentiation					3.70			
GO:0046643~regulation of gamma-delta T cell activation					3.70			
GO:0010875~positive regulation of cholesterol efflux					3.70			
GO:0003002~regionalization								4.04
GO:0051249~regulation of lymphocyte activation					4.13			
GO:0015031~protein transport							4.23	
GO:0031175~neuron projection development								4.56
GO:0045184~establishment of protein localization							4.57	
GO:0002684~positive regulation of immune system process					4.82			
GO:0032373~positive regulation of sterol transport					5.10			
GO:0045059~positive thymic T cell selection					5.10			
GO:0010874~regulation of cholesterol efflux					5.10			
GO:0032376~positive regulation of cholesterol transport					5.10			
GO:0043112~receptor metabolic process		5.11						
GO:0031343~positive regulation of cell killing					5.18			
GO:0034622~cellular macromolecular complex assembly								5.25
GO:0051056~regulation of small GTPase mediated signal transduction		5.36						
GO:0001910~regulation of leukocyte mediated cytotoxicity					5.82			
GO:0016064~immunoglobulin mediated immune response			6.22					
GO:0019725~cellular homeostasis		6.44						
GO:0010889~regulation of sequestering of triglyceride								6.64
GO:0000910~cytokinesis		6.85						

(continued)

Table S3
(continued)

	Blaveri	Choi	CNUH	Lindgren	MSKCC	MSKCC-CBIO	Reister	TCGA
GO:0035023~regulation of Rho protein signal transduction		7.10						
GO:0046578~regulation of Ras protein signal transduction		7.13						
GO:0033077~T cell differentiation in the thymus					7.22			
GO:0002706~regulation of lymphocyte mediated immunity					7.31			
GO:0019724~B cell mediated immunity			7.42					
GO:0050767~regulation of neurogenesis								7.48
GO:0022613~ribonucleoprotein complex biogenesis			7.57					
GO:0002694~regulation of leukocyte activation					7.63			
GO:0065003~macromolecular complex assembly						7.68		
GO:0031341~regulation of cell killing					7.98			
GO:0043368~positive T cell selection					8.45			
GO:0008033~tRNA processing			8.48					
GO:0008624~induction of apoptosis by extracellular signals		8.76						
GO:0045665~negative regulation of neuron differentiation								9.49
GO:0032318~regulation of Ras GTPase activity							9.54	
GO:0006820~anion transport								9.76
GO:0050865~regulation of cell activation					10.02			
GO:0007242~intracellular signaling cascade					10.45			
GO:0002703~regulation of leukocyte mediated immunity					11.06			
GO:0016192~vesicle-mediated transport							11.31	
GO:0042254~ribosome biogenesis			11.41					
GO:0030855~epithelial cell differentiation					11.56			
GO:0048598~embryonic morphogenesis								11.64
GO:0016197~endosome transport							11.91	
GO:0045664~regulation of neuron differentiation								12.17
GO:0032870~cellular response to hormone stimulus								12.17
GO:0032370~positive regulation of lipid transport					12.45			
GO:0002714~positive regulation of B cell mediated immunity					12.45			
GO:0002891~positive regulation of immunoglobulin mediated immune response					12.45			
GO:0060041~retina development in camera-type eye								12.56
GO:0034504~protein localization in nucleus								12.77
GO:0034613~cellular protein localization							12.90	
GO:0051223~regulation of protein transport							12.90	
GO:0019882~antigen processing and presentation			13.31					
GO:0070727~cellular macromolecule localization							13.41	
GO:0006917~induction of apoptosis		13.54						
GO:0030217~T cell differentiation					13.63			
GO:0012502~induction of programmed cell death		14.11						
GO:0030098~lymphocyte differentiation					14.42			
GO:0010745~negative regulation of foam cell differentiation					14.65			
GO:0060538~skeletal muscle organ development		14.72						
GO:0007519~skeletal muscle tissue development		14.72						
GO:0048584~positive regulation of response to stimulus					14.82			
GO:0070201~regulation of establishment of protein localization							15.59	
GO:0043087~regulation of GTPase activity							16.41	
GO:0006909~phagocytosis			16.45					
GO:0009451~RNA modification			16.45					
GO:0032321~positive regulation of Rho GTPase activity		16.50						
GO:0008542~visual learning								16.91
GO:0045061~thymic T cell selection					16.95			
GO:0002700~regulation of production of molecular mediator of immune response						17.08		
GO:0032990~cell part morphogenesis								17.15
GO:0006790~sulfur metabolic process	17.21							
GO:0045637~regulation of myeloid cell differentiation					17.26			
GO:0006915~apoptosis	17.53							
GO:0006606~protein import into nucleus								18.17
GO:0030534~adult behavior								18.17

(continued)

Table S3
(continued)

	Blaveri	Choi	CNUH	Lindgren	MSKCC	MSKCC-CBIO	Reister	TCGA
GO:0007166~cell surface receptor linked signal transduction					18.40			
GO:0045667~regulation of osteoblast differentiation						18.56		
GO:0046649~lymphocyte activation					18.66			
GO:0030030~cell projection organization								18.87
GO:0042102~positive regulation of T cell proliferation					19.01			
GO:0006357~regulation of transcription from RNA polymerase II promoter		19.78						
GO:0008284~positive regulation of cell proliferation				19.88				
GO:0006913~nucleocytoplasmic transport								19.99
hsa05330:Allograft rejection			3.75		17.40			
hsa04144:Endocytosis							0.11	
hsa05322:Systemic lupus erythematosus			0.12					
hsa05310:Asthma			0.16					
hsa04514:Cell adhesion molecules (CAMs)					0.93			
hsa04672:Intestinal immune network for IgA production			2.85					
hsa05020:Prion diseases			3.31					
hsa05320:Autoimmune thyroid disease			3.50					
hsa05332:Graft-versus-host disease			5.30					
hsa04940:Type I diabetes mellitus			7.25					
hsa04080:Neuroactive ligand-receptor interaction								12.66
hsa05216:Thyroid cancer								13.13
hsa05416:Viral myocarditis			16.39					

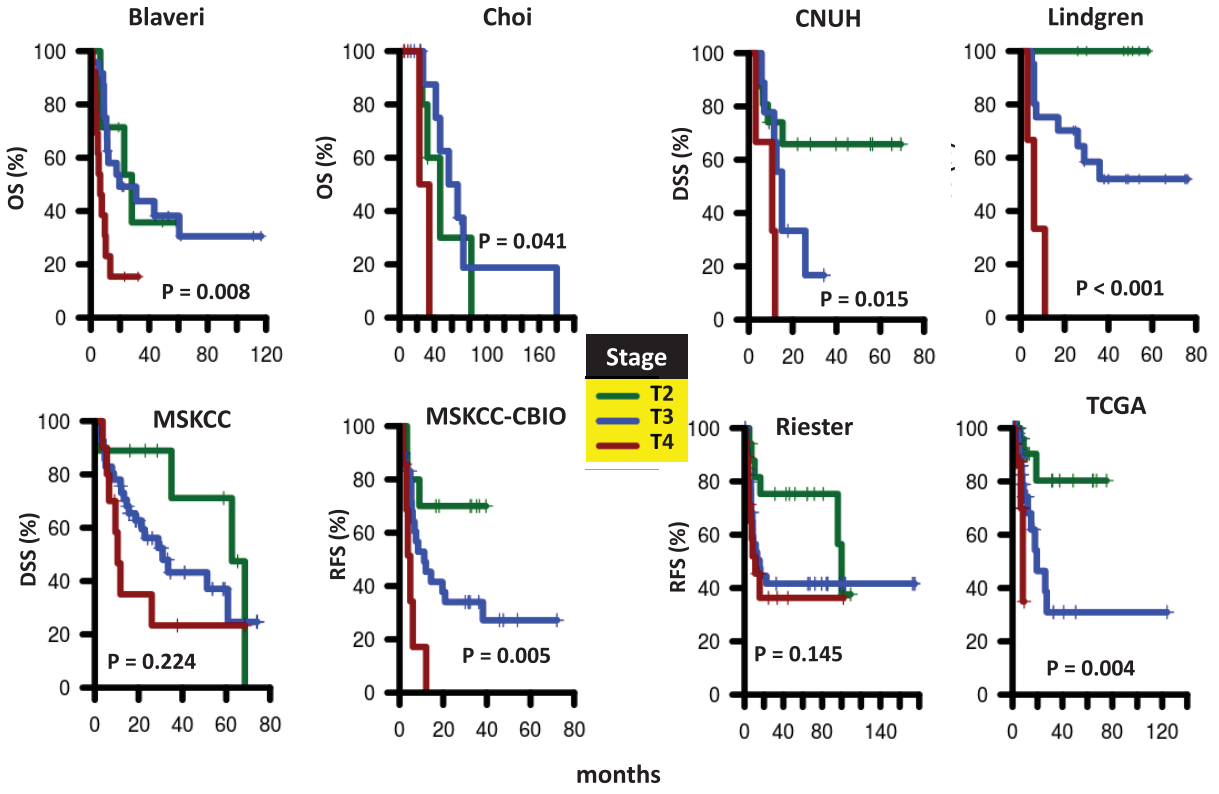

Fig. S1. Survival of patients according to tumor stage. Kaplan-Meier curves were generated for patients with T2 (green), T3 (blue), and T4 (red) tumors in Blaveri ($N = 44$), Choi ($N = 22$), CNUH ($N = 28$), Lindgren ($N = 32$), MSKCC ($N = 60$), MSKCC-CBIO ($N = 47$), Riester ($N = 78$), and TCGA ($N = 147$) cohorts. The log-rank P value is reported. Abbreviations: DSS, disease-specific survival; OS, Overall survival; RFS, recurrence-free survival.

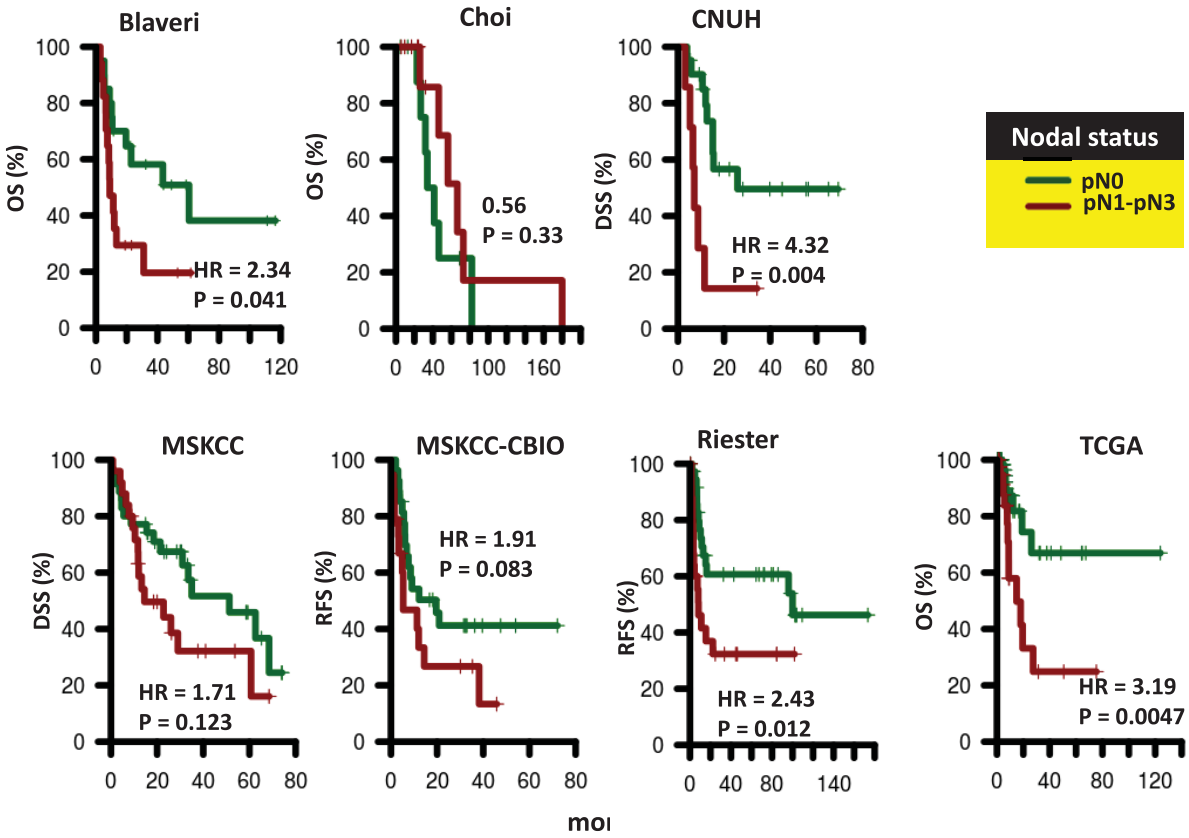

Fig. S2. Survival of patients according to nodal status at cystectomy. Kaplan-Meier curves were generated for patients with pN0 (green) or pN1-N3 (red) tumors in Blaveri ($N=44$), Choi ($N=22$), CNUH ($N=28$), MSKCC ($N=60$), MSKCC-CBIO ($N=46$), Riester ($N=64$), and TCGA ($N=143$) cohorts. The hazard ratio (HR) for patients with pN1-N3 tumors compared to patients with pN0 tumors and the corresponding log-rank P value is reported. Abbreviations: DSS, disease-specific survival; OS, Overall survival; RFS, recurrence-free survival.

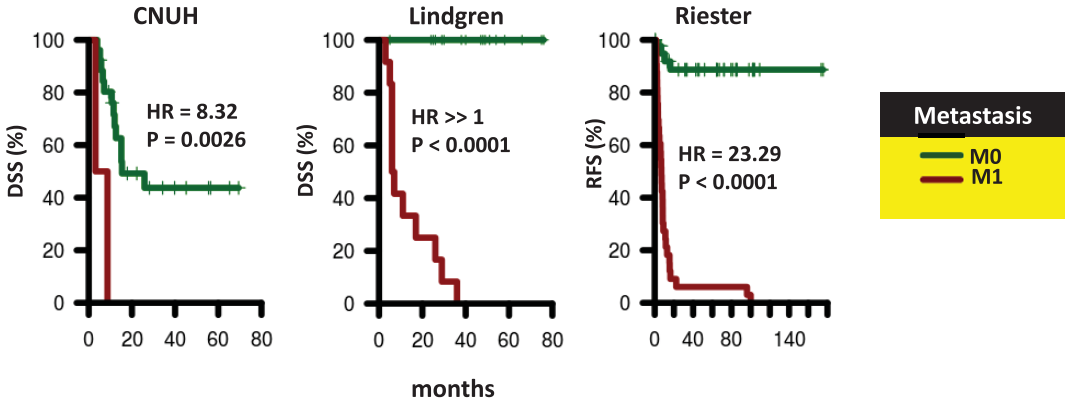

Fig. S3. Survival of patients according to presence of distant metastases. Kaplan-Meier curves were generated for patients with M0 (green) and M1 (red) tumors in CNUH ($N=28$), and Lindgren ($N=32$), and Riester ($N=78$) cohorts. The hazard ratio (HR) for patients with M1 tumors compared to patients with M0 tumors and the corresponding log-rank P value is reported. Abbreviations: DSS, disease-specific survival; RFS, recurrence-free survival.

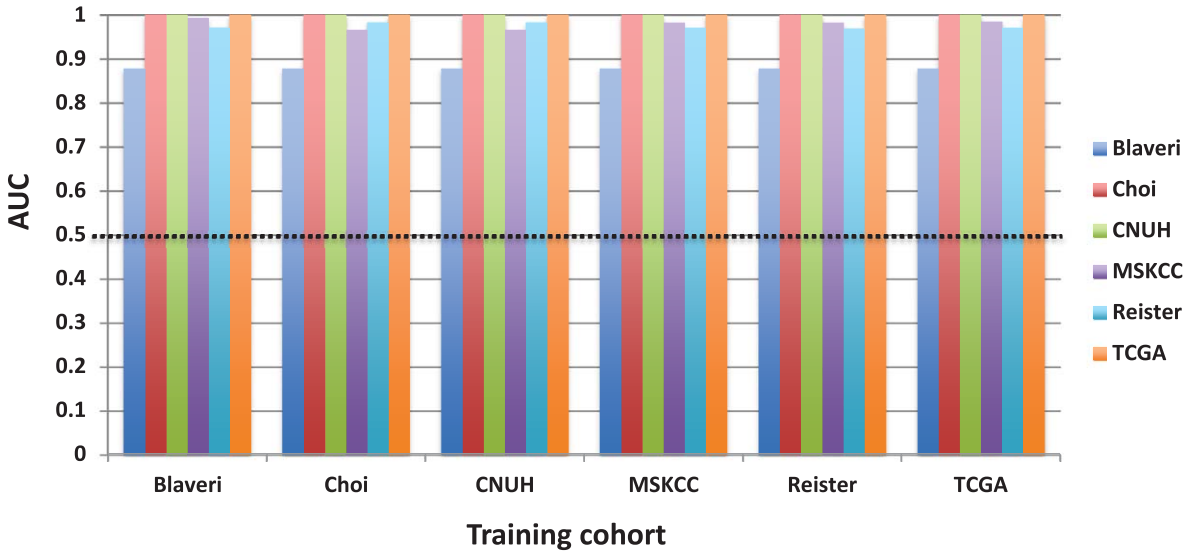

Fig. S4. Ability of the weighted Sex Identification Signature (SIS) to distinguish between males and females. SIS gene were weighted by −1 or +1 according to whether the gene was down- or up-regulated with males, respectively, in each training cohort. A weighted SIS score was then calculated and its ability to distinguish males from females value evaluated in the remaining cohorts (i.e., the testing cohorts). Performance is measured by AUC, which is equivalent to the probability that a randomly selected male has a higher weighted SIS score than a randomly selected female. The dashed black line corresponds to the AUC value of an association due to random chance (i.e., AUC = 0.50, black dotted line). All AUCs are statistically significant ($P < 0.05$) by the Wilcoxon rank-sum test.