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Prognostic Markers in pT3 Bladder Cancer: A Study from the International Bladder Cancer Tissue Microarray Project

Gang Wang^{1,#}, Peter C. Black^{2,#,*}, Peter J. Goebell³, Lingyun Ji⁴, Carlos Cordon-Cardo⁵, Bernd Schmitz-Dräger^{3,6}, Debra Hawes⁷, Bogdan Czerniak⁸, Sarah Minner⁹, Guido Sauter⁹, Frederic Waldman¹⁰, Susan Groshen⁴, Richard J. Cote¹¹, Colin P. Dinney¹², For the International Bladder Cancer Tissue Microarray Project

¹Department of Pathology, University of British Columbia, Canada

²Department of Urologic Sciences, University of British Columbia, Canada

³Department of Urology, Friedrich-Alexander-University of Erlangen-Nürnberg, Germany

⁴Department of Preventive Medicine, Keck School of Medicine, University of Southern California, USA

⁵Department of Pathology, Icahn School of Medicine at Mount Sinai, USA

⁶Urologie 24, Nürnberg, Germany

⁷Department of Pathology and Laboratory Medicine, Keck School of Medicine, University of Southern California, USA

⁸Department of Pathology, The University of Texas MD Anderson Cancer Center, USA

* **Correspondence:** Peter C. Black, MD, FACS, FRCSC, Department of Urologic Sciences, University of British Columbia, Level 6, 2775 Laurel St, Vancouver, BC V6N 2W6, Phone: 604-875-4301, Fax: 604-875-5604, pblack@mail.ubc.ca.

#Co-first authors

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Steven Shen, Baylor College of Medicine, Houston, USA

Peter Clark, Wake Forest University, USA

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Charles Rosser, University of Florida Shands – Jacksonville, USA

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Carlos Cordon-Cardo, Memorial Sloan Kettering Cancer Center, USA

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Martin Gleave, University of British Columbia, Canada

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Arndt Hartmann, University of Regensburg, Germany

Peter Goebell, University of Essen, Germany

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⁹Institute of Pathology, University Medical Center Hamburg-Eppendorf, Germany

¹⁰Department of Laboratory Medicine and Urology, University of California San Francisco

¹¹Department of Pathology and Immunology, Washington University School of Medicine, USA

¹²Department of Urology, The University of Texas MD Anderson Cancer Center, USA

Abstract

Purpose: We evaluated the prognostic value of 10 putative tumor markers by immunohistochemistry in a large multi-institutional cohort of patients with locally advanced urothelial cancer of the bladder (UCB) with the aim to validate their clinical value and to harmonize protocols for their evaluation.

Materials and Methods: Primary tumor specimens from 576 patients with pathologic (p)T3 UCB were collected from 24 institutions in North America and Europe. Three replicate 0.6-mm core diameter samples were collected for the construction of a tissue microarray (TMA). Immunohistochemistry (IHC) for 10 previously described tumor markers was performed and scored at three laboratories independently according to a standardized protocol. Associations between marker positivity and freedom from recurrence (FFR) or overall survival (OS) were analyzed separately for each individual laboratory using Cox regression analysis.

Results: The overall agreement of the IHC scoring among laboratories was poor. Correlation among the three laboratories varied across the 10 markers. There was generally a lack of association between the individual markers and FFR or OS. The number of altered cell cycle regulators (p53, Rb, and p21) was associated with increased risk of cancer recurrence ($p < 0.032$). There was no clear pattern in the relationship between the percentage of markers altered in an 8-marker panel and FFR or OS.

Conclusions: This large international TMA of locally advanced (pT3) UCB suggests that altered expression of p53, Rb, and p21 is associated with worse outcome. However this study also highlights limitations in the reproducibility of IHC even in the most expert hands.

Keywords

Prognostic Markers; Bladder Cancer; Tissue Microarray

INTRODUCTION

Urothelial carcinoma of the bladder (UCB) is a heterogeneous disease characterized by multiple molecular alterations and a variable clinical course.¹ Beginning with the finding that point mutations in the p53 cell cycle regulatory gene correlated strongly with increased expression of the non-functional protein due to increased half-life², Esrig et al showed that overexpression of p53 was strongly associated with UCB recurrence and death, especially in early invasive disease (pT1–2)³. Subsequently, Cote et al showed that alterations in p53 made UCB more susceptible to cisplatin-based therapy⁴, which led to the first clinical trial in UCB that used the alteration of a molecular marker (p53) to stratify patients for adjuvant treatment⁵.

Subsequent studies demonstrated that other cell cycle regulatory genes and proteins, including p21 and pRB, are associated with UCB clinical outcome in patients undergoing cystectomy.⁶⁻¹⁴ Chatterjee first reported that a combination of alterations in cell cycle regulatory proteins (p53, p21, pRb) provided more information regarding recurrence and death from UCB than any single marker alone¹⁵. Lotan et al, in a multicenter analysis, validated and extended these findings using a panel of five immunohistochemical markers related to the cell cycle and proliferation (p53, p21, p27, cyclin E1, and Ki-67) that correlated with recurrence and cause-specific mortality after radical cystectomy for muscle invasive UCB. However, many marker studies have been limited by small sample size, by the use of univariate statistical analysis to identify the putative marker of interest, by not analyzing coexpression of other potential biomarkers, and by lack of independent validation.

The primary goal of this study was to evaluate the prognostic value of 10 predefined putative tumor markers in a large multi-institutional cohort of patients with pT3 UCB. Stage pT3 was selected because these patients are often considered for adjuvant therapy, and we aimed to test if established biomarkers could be used to stratify risk in patients being considered for adjuvant therapy. To address this aim, we created tissue microarrays (TMA) by using submitted tumor specimens from all participating institutions, taking advantage of the collaboration between the Bladder Cancer SPORE Project and the International Bladder Cancer Network.¹⁶ A tissue microarray (TMA) is extremely useful to analyze many molecular markers in a large number of samples concurrently.¹⁷ We also aimed to evaluate the reproducibility of immunohistochemistry (IHC) for the 10 selected markers by comparing results across three independent, experienced laboratories.

MATERIALS AND METHODS

Patient Selection

Primary tumor specimens from 576 patients with muscle invasive UCB were collected from 24 institutions in North America (13 centers; 335 patients) and Europe (11 centers; 241 patients). The specimens were staged and graded according to the American Joint Committee on Cancer's *AJCC Cancer Staging Manual*, 7th Edition, and the 2004 World Health Organization classification, respectively.^{18,19} All samples were derived from radical cystectomy specimens with tumors extending into perivesical fat (pT3) without distinction of microscopic (pT3a) versus gross (pT3b) invasion. At least one lymph node had to be examined in each patient to ensure that some degree of pelvic lymph node dissection had been performed. Only patients with a minimum of 3 years of clinical follow-up were included unless a recurrence event occurred before this time. Patients receiving neoadjuvant chemotherapy and/or radiation therapy were excluded. No restrictions were made regarding adjuvant therapy. Squamous and/or glandular differentiation was allowed, but other variant histologic patterns or pure adenocarcinoma or squamous cell carcinoma were excluded. The corresponding pathologic and clinical data were provided for each patient by the contributing institutions and were approved by each institutional review board.

Tissue Microarray

The TMA was constructed by an expert bladder cancer pathologist at one center (G.S.). Histologic features, tumor grade, and disease stage were confirmed by blinded review of new hematoxylin and eosin (H&E)-stained sections cut from duplicate archival paraffin blocks for each radical cystectomy case. Three replicate samples of 0.6-mm core diameter were collected from the tumor area and placed on separate randomly arranged spaces for the purpose of constructing TMA blocks. Sections (3–4 μm) were obtained from the TMA and were stained with H&E to confirm a high proportion of tumor and to review again the tumor histologic features and other pathologic parameters of the sampled tissue. Cores from 18 normal control tissues from heart, skin, prostate, kidney, colon, lung and endometrium were included on the TMA (Figure 1).

IHC Analysis

All IHC analyses were performed independently at three laboratories (CC at Columbia University, New York City; FW at University of California, San Francisco; and RC at University of Southern California, Los Angeles). One section of the TMA was stained with H&E and one with each of the 10 antibodies listed in Table 1. Each laboratory used aliquots of each antibody from a single batch and followed standard protocols for staining, including the same antibody retrieval methods and the same antibody concentrations.

Scoring

The results of IHC analyses were scored at each laboratory separately. The staining of each core on each section was graded according to the intensity of staining (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and by the percentage of cells staining (0% to 100% in decile increments: 0%, 1%–10%, 11%–20%, etc.) in the selected cellular location (membranous, nuclear, cytoplasmic). For all but HER2/neu and Ki-67, the primary measure used for analysis was “positive” versus “negative” staining. The criteria for positive staining included staining in the appropriate subcellular location (Table 1) in more than 10% of cells with an intensity of 1+ to 3+. For HER2/neu, only 2+ and 3+ staining was considered positive. For Ki-67, the percentage of cells stained with an intensity of 1+ to 3+ was reported, and patients were categorized into lower (<33.3%), medium (33.3% to 66.6%), and upper (≥ 66.7%) tertiles on the basis of the percentage of cells stained. For Rb, we also determined differentiated altered Rb (<5% or ≥ 51% of cells stained) from wild-type Rb (5%–50% of cells stained) based on IHC criteria.

After determining the percentage of positive staining from each laboratory, we derived consensus scores: if all three laboratories scored a marker as positive or negative, or if two laboratories scored a marker as positive or negative and the third laboratory did not score it, we considered the overall score to be positive or negative, respectively. An indeterminate overall score was attributed to scores if the individual score was missing in two laboratories or if there was disagreement between the laboratories. If all three scores were missing, an overall score could not be given.

Statistical Analysis

The primary end point for this study was freedom from recurrence (FFR), and the secondary end point was overall survival (OS). Time to each endpoint was measured from the date of radical cystectomy. Associations between marker positivity and FFR or OS were analyzed separately for each individual laboratory by using Cox regression analysis.

We derived consensus scores of marker positivity by combining scoring results from the three laboratories as described above and examined the associations between the consensus scores and FFR or OS. The rules we used to combine the scoring results from the three laboratories were as follows: (1) For all markers except Ki-67, we used the rules described in Table 1. VEGF was excluded from the consensus analysis since one of the laboratories did not score this marker. (2) For Ki-67, a patient with a score of “High, High, High” or “High, High, Medium” or “High, High, Missing” across the three laboratories was considered to have high expression of Ki-67, and a patient with a score of “Low, Low, Low” or “Low, Low, Medium” or “Low, Low, Missing” across the laboratories was considered to have low expression of Ki-67. All other combinations of scores across the three laboratories were considered to be either indeterminate or missing. (3) For Rb, we calculated a consensus score for marker positivity, but also obtained a consensus score for whether Rb was altered or wild-type and assessed its association with FFR and OS.

Marker Panels

A 3-marker panel of cell cycle regulatory proteins was defined to include Rb, p21, and p53. Patients for whom any 1 of the 3 markers was not scored by a laboratory were considered missing and were excluded from the analyses. A second 8-marker panel was defined to include Rb, p21, p53, EGFR, HER2, E-cad, p16, and cyclin D1. VEGF was excluded because it was not done at one center and Ki67 was excluded because it was scored in percentage with no cut-off for positivity. Patients who had missing or indeterminate consensus results for 6 or more of these 8 markers were excluded from the consensus analyses. The proportion of markers that were altered in an individual patient was calculated as the number of altered markers according to the consensus scoring divided by the total number of markers that had a consensus score. The patients were further stratified according to the proportion of markers altered as: a) low: patients with $\leq 25\%$ of the markers altered, b) intermediate: patients with $>25\%$ and $\leq 50\%$ of the markers altered, c) high: patients with $>50\%$ of the markers altered.

RESULTS

Clinical Characteristics and Outcomes

Complete clinical data were available for 504 of 576 patients. Results from all 576 patients were used to test the reproducibility of staining between the different centers. Eligibility criteria were not met in 44 and 61 were excluded due to insufficient follow-up, which left 399 patients for analysis of the association between marker status by IHC and clinical outcomes. The clinicopathologic characteristics and outcomes of these 399 patients are summarized in Table 2. The actuarial FFS probability was 75.9% at 1 year, 51.1% at 5 years, and 45.1% at 10 years (Figure S1). The actuarial OS probability was 81.0% at 1 year, 43.1%

at 5 years, and 27.1% at 10 years. Nodal metastasis, which was identified at the time of cystectomy in 99 (24.8%) patients, was strongly associated with decreased FFR (Figure 2A) and OS (Figure 2B).

Consensus scoring among the laboratories

The number of patients with successful staining results for each of the 10 protein markers is indicated in Table 3 along with the rate of marker positivity for each individual marker in each laboratory, and according to consensus scoring. Table 3 also describes the agreement between pairs of laboratories according to the percentage of cases for which both laboratories agreed that staining was positive or negative. The rate of disagreement ranged widely from 5% to 80%. The Spearman rank correlation was calculated for Ki-67 staining. These data were used to estimate the agreement between the pairs of laboratories as measured by the Kappa statistic (Table 4). This analysis suggested best agreement for p16 (72–90%) and p53 (59–78%) and worst agreement for EGFR (17–54%). Overall the agreement between laboratories was poor.

Prognostic Values of Individual Markers

FFR and OS were analyzed in univariate fashion with respect to each of the 10 markers individually according to the scoring of each laboratory separately (Figure 3) and according to the consensus determinations of positive and negative staining (Figure 4). Ki-67 was analyzed in a similar fashion by tertiles (upper and middle versus lower), and Rb was analyzed as altered versus wild-type (Figure 3). No marker showed a significant association with the primary end point of FFR except for p21 and EGFR scored by Lab C. Similarly, no association was found between marker positivity and OS except for cyclin D1 in Lab B. We also calculated the hazard ratio (HR) and 95% CI on the log scale for patients with a consensus-positive score compared with those with a consensus-negative score for each marker, as well as for Rb altered compared with Rb wild-type and for high Ki-67 compared with low Ki-67 (Table S1). All confidence intervals cross 1.0 widely, indicating again that there was a lack of association between consensus marker scores and either FFR or OS.

Prognostic Values of Marker Panels

Moving beyond single marker analysis, we also determined whether combinations of altered markers correlated to FFR or OS. VEGF and Ki-67 were excluded, and *altered* was defined as positive staining for the other markers, except for Rb, which was scored as altered or wild-type as described above.

We first investigated whether the 3-marker panel (Rb, p21, and p53) was associated with FFR or OS. When looking at data from each laboratory separately, there seemed to be a general trend toward HRs that were <1 (or log of HR <0), indicating that patients with fewer markers altered tended to have better outcomes than did those with all 3 markers altered (Figure 5A–C, Fig. S2A–C). However, none of these associations reached statistical significance ($p < 0.05$). An analysis of combined results from the three laboratories in form of consensus scores was severely limited by the small number of samples ($n=41$) for which a consensus could be determined, so that these results are essentially uninformative (Figure 5D, Figure S2D).

We then looked into the association of altered markers and FFR or OS based on an 8-marker panel including: p53, p21, p16, Rb, cyclin D1, E-cad, EGFR, and HER2. For the 8-marker panel, the proportion of altered markers was stratified as low, intermediate and high. The log of HRs and the 95% CIs were calculated for patients with a low or intermediate proportion of markers altered compared with patients with a high proportion of markers altered, for FFR and OS, respectively. There was no clear pattern in the relationship between the proportion of markers altered and FFR or OS when looking at data from each laboratory separately or when combining the scoring from the three laboratories (Figure S3).

DISCUSSION

The nidus of this project was the United States National Cancer Institute Bladder Cancer Marker Network, which aimed to evaluate the reproducibility of IHC features for measuring p53 expression in bladder tumors.²⁰ This international initiative ended up compiling IHC results from 3,570 individual patients from 26 different studies of p53 alterations in patients with UCB, which was published primarily by the International Bladder Cancer Network (IBCN).^{21,22} The IBCN subsequently launched this current international collaboration to establish this multi-center bladder cancer TMA, which was funded through the MD Anderson Cancer Center's Specialized Program of Research Excellence (SPORE) in bladder cancer. This proved to be a successful cooperation and yielded a common TMA derived from 576 patients from 24 different institutions, all with locally advanced (pT3) UCB. As far as we know, this cohort still represents the largest international and interdisciplinary TMA of UCB.

In this project we used the international TMAs to evaluate the prognostic value of 10 predefined putative tumor markers. These markers were chosen based on previous studies showing their clinical significance and based on knowledge of their biological role in UCB.^{4,23–34} Many of these proteins represent members of the retinoblastoma proliferation pathway (Ki-67, Rb, p53, p21, and cyclin D1). ErbB2 and EGFR are tyrosine receptor kinases that are overexpressed in a large number of bladder tumors, although their clinical significance is controversial.³⁵ E-cadherin, which plays a role in cell-cell interactions, is frequently altered in high-stage bladder tumors.³⁶ Finally, VEGF, which has a role in angiogenesis and neovascularization, is believed also to have an important role in cell invasion and metastasis.³⁷

The key finding, and a significant limitation, of our study is not the lack of prognostic value of the selected markers, but instead the heterogeneity of the IHC results across three highly experienced centers using standardized staining and scoring protocols, as well as identical antibodies from the same vial. The lack of association of individual markers and even panels of markers with clinical outcomes is likely due in large part to the lack of agreement in the IHC analyses across the three laboratories. Some heterogeneity may have resulted from the use of 30 sections of the TMA which was needed to perform analysis on 10 markers at 3 centers. Use of whole sections, as would usually be done in clinical practice, may also improve the results. Understanding the source of the observed discordance in IHC results will require additional analyses. We did not conduct a central review of all stained TMA slides which could have aided in determining what proportion of the interobserver variability

was due to the scoring itself. We observed the most consistent results using the markers that are most commonly used in clinical practice (e.g. p53, p16, cyclinD1 and HER2), suggesting that experience with staining methods and interpretation is important to reduce variability. Furthermore, alternative scoring methods such as the scoring systems for estrogen receptor (for markers with nuclear staining), and HER2 (for markers with membranous staining) might reduce interobserver variability.

There is no real consensus of what “good agreement” is. Nonetheless, magnitude guidelines have appeared in the literature. Landis and Koch³⁸ characterized values of <0% as indicating no agreement, 0%–20% as slight, 21%–40% as fair, 41%–60% as moderate, 61%–80% as substantial, and 81%–100% as almost perfect agreement. This set of guidelines, however, is based on expert opinion and is not universally accepted. Equally arbitrary guidelines by Fleiss characterize kappa values of >75% as excellent, 40% to 75% as fair to good, and <40% as poor.³⁹

A previous study showed considerable inter-laboratory differences in the intensity and percentage of Ki67 nuclear staining, suggesting that antigen retrieval or staining techniques are predominantly responsible for the inter-laboratory variability, and the cut-off levels for distinguishing prognostic subgroups appear to have limited reproducibility in a multi-center approach.⁴⁰ Regardless of the nuances of cut-offs, the level of agreement between laboratories suggests that there is further work to be done to determine the “true” status of an IHC marker. One approach to this, utilized by Esrig et al for p53, is to demonstrate the association of the scoring of a molecular marker with gene status².

IHC remains a frequently utilized and reliable tool in tissue diagnosis. Many independent single institution and multi-institutional studies have clearly shown that alterations in cell cycle regulatory proteins are independent predictors of UCB clinical outcome^{2–4,7–10,12–15,21–29,33}. However, our results clearly demonstrate that routine use of prognostic markers where the IHC results have not been independently correlated with gene status and outcome remains problematic, with poor reproducibility. Another potential problem of the present study is that it focused on locally advanced (pT3) UCB, while the primary impact on prognosis, especially for cell cycle regulatory proteins, is in early stage invasive (pT1–2) disease^{3,12,14,15}. Thus, the full potential of prognostic/predictive markers in UCB has yet to be realized, and is deserving of further study. This may gain renewed interest in the current era of molecular subtyping, where several groups have suggested IHC staining as an inexpensive and pragmatic substitute for transcriptomic analysis.^{41,42}

There is no question that IHC for specific markers is firmly established in the clinical management of defined patient populations with cancer in different organ sites. Efforts to enhance the performance and reproducibility of IHC include automated quantitative analysis and machine learning with application of artificial intelligence algorithms⁴³. Furthermore, multiplex assays measuring multiple markers on one tissue section may overcome the heterogeneity between sections and allow for co-localization studies, which are particularly important for immune markers⁴⁴. Advanced technologies, including especially next generation sequencing, are entering clinical practice, for example for FGFR testing in advanced bladder cancer or for measuring total mutational burden. Next generation

sequencing is critical for evaluating some of the same markers we have assessed by IHC, including especially p53, Rb and HER2 (ErbB2). These assays are able to move from tissue to circulating tumor DNA and circulating tumor cells, which allow longitudinal assessment of the molecular landscape of the tumor in response to therapy, and may help overcome limitations of intra- and intertumor heterogeneity⁴⁵.

CONCLUSION

A panel of 10 established oncogenes and tumor suppressor genes in UCB failed to provide prognostic information in a large international cohort of patients with locally advanced (pT3) UCB, either alone or in combination. The analysis did, however, provide valuable insight into interlaboratory differences in IHC analysis of common prognostic markers. This highlights the need to standardize the IHC staining of these and other biomarkers for UCB. This remains particularly pertinent with ongoing studies of PD-L1 IHC as a predictive marker for treatment with immune checkpoint inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Expression p53, Rb and p21 was associated with outcome after cystectomy
- The fraction of markers altered in an 8-marker panel did not correlate with outcome
- IHC score agreement between 3 laboratories for 10 cell cycle markers was poor

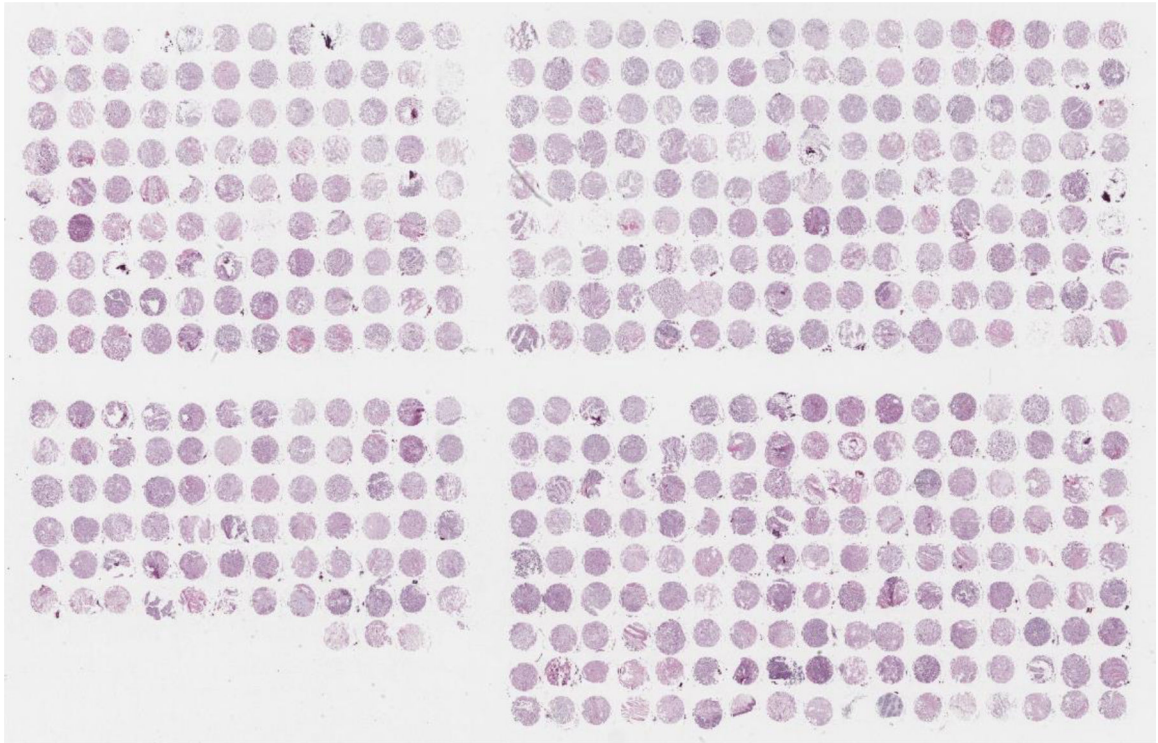


Figure 1:
Hematoxylin & eosin-stained slide of the tissue microarray.

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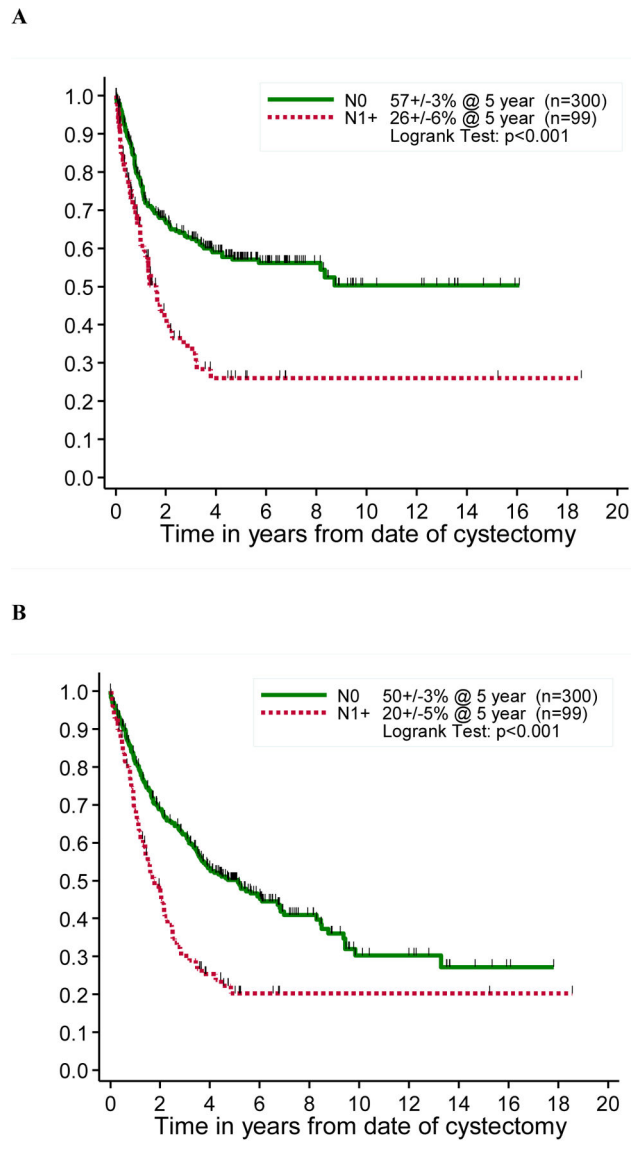
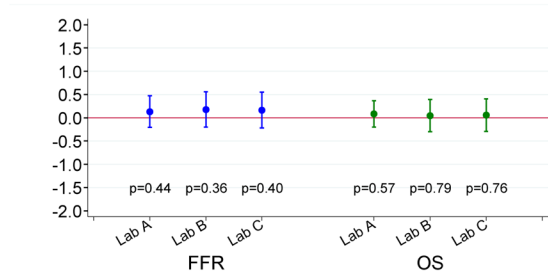
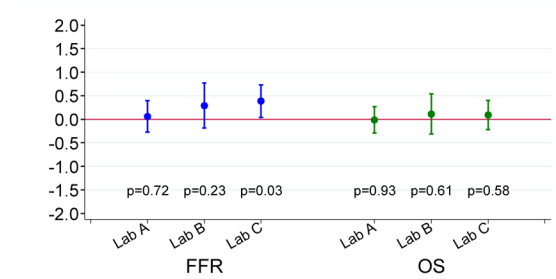


Figure 2: Freedom from recurrence (A) and overall survival (B) stratified by pathologic N-Stage. Kaplan-Meier curve depicting freedom from recurrence (FFR) and overall survival (OS), by N-stage of the disease.

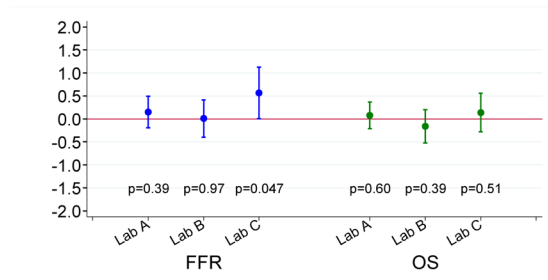
A. Marker p53 (Positive vs. Negative)



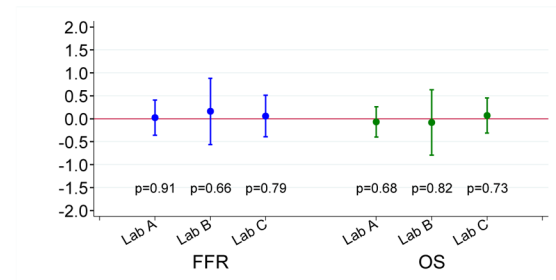
B. Marker p21 (Positive vs. Negative)



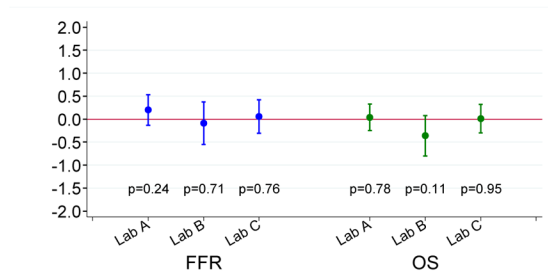
C. Marker EGFR (Positive vs. Negative)



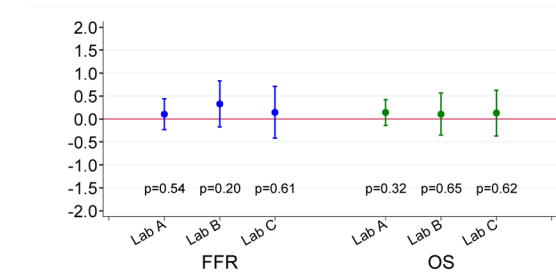
D. Marker Her2 (Positive vs. Negative)



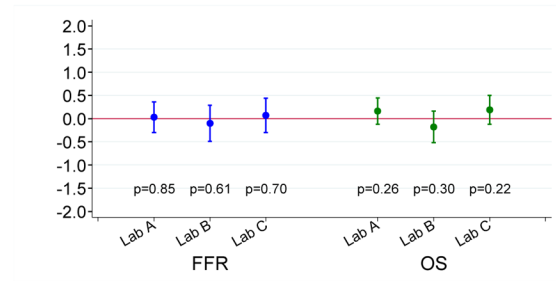
E. Marker Rb (Positive vs. Negative)



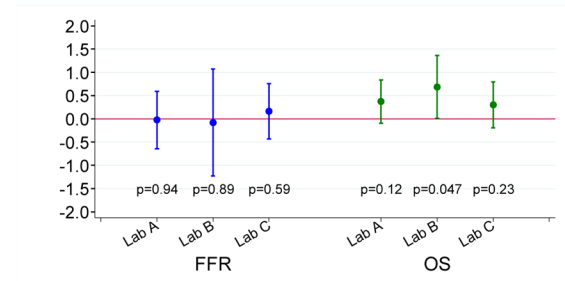
F. Marker Ecadherin (Positive vs. Negative)



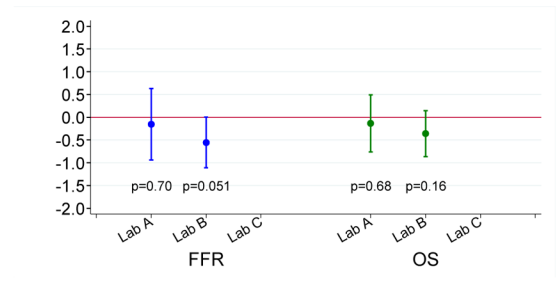
G. Marker p16 (Positive vs. Negative)



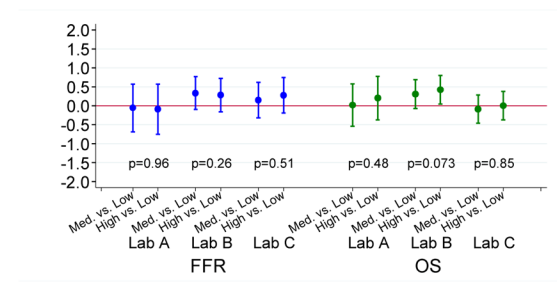
H. Marker cyclin-D (Positive vs. Negative)



I. Marker VEGF (Positive vs. Negative)



J. Marker Ki-67 (Medium/Upper Tertiles vs. Lower Tertile)



K. Marker Rb (Altered vs. Wild Type)

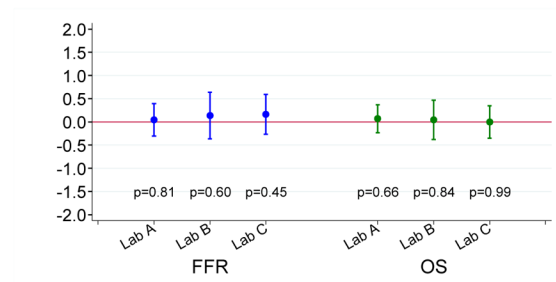


Figure 3: Patient outcome according to marker status.

Log of hazard ratios (HR) and 95% confidence intervals for patients scored as marker positive compared to those scored as marker negative. A HR of 0 on the log scale corresponds to a HR of 1, indicating a lack of association. If HR on the log scale is >0 (i.e., HR>1), it indicates that the marker positivity is associated with an unfavorable outcome.

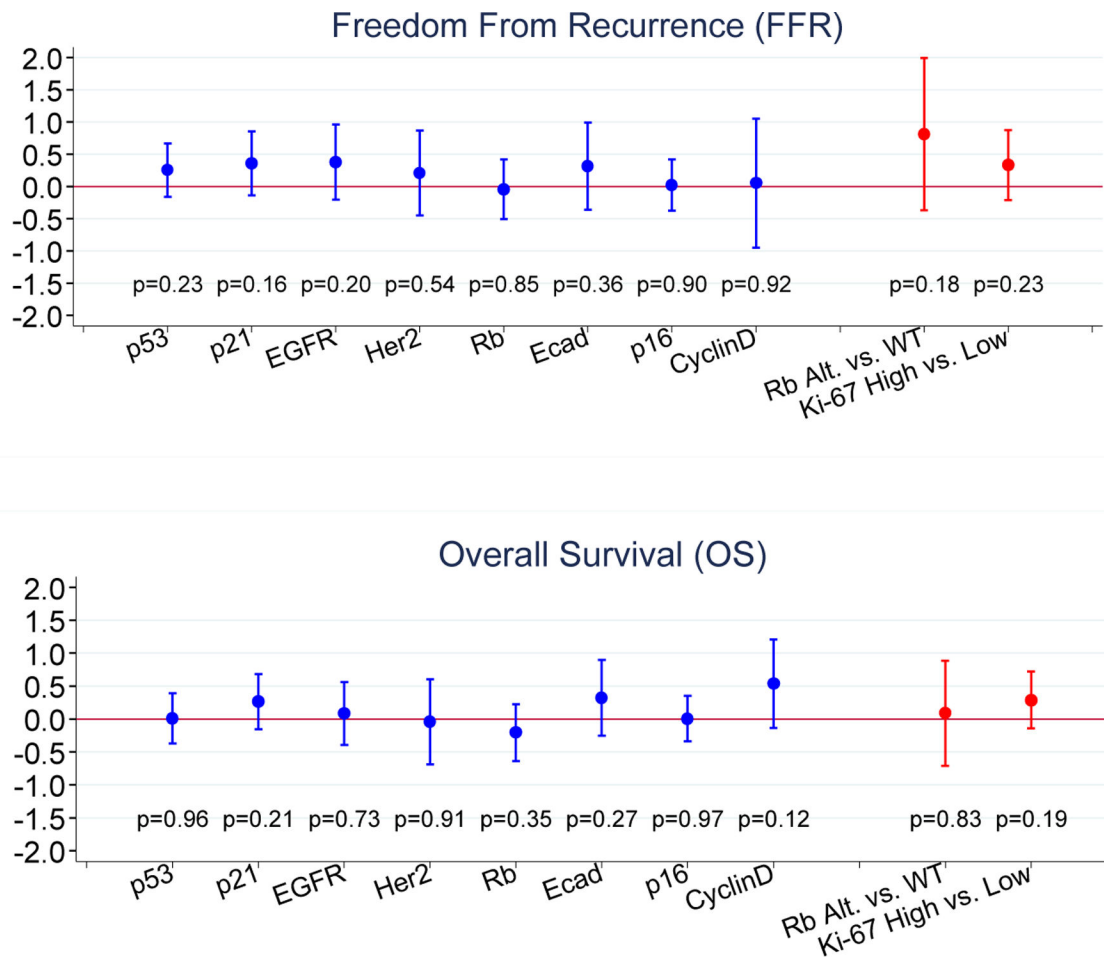
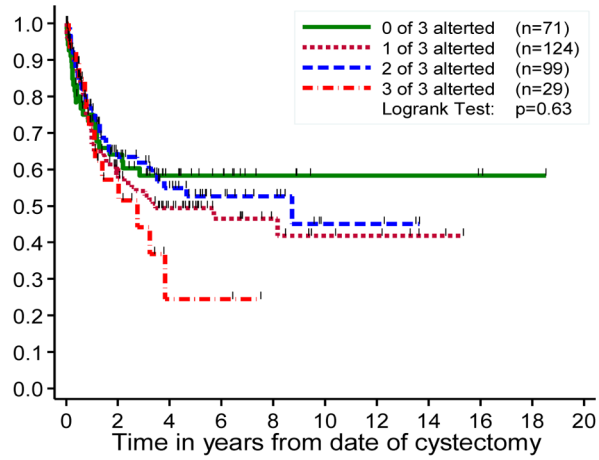


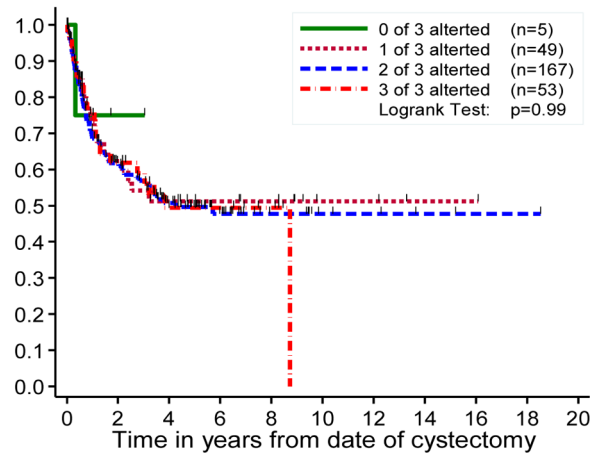
Figure 4: Consensus Analyses

-- Log of hazard ratios and 95% confidence intervals for patients scored as marker positive compared to those scored as marker negative.

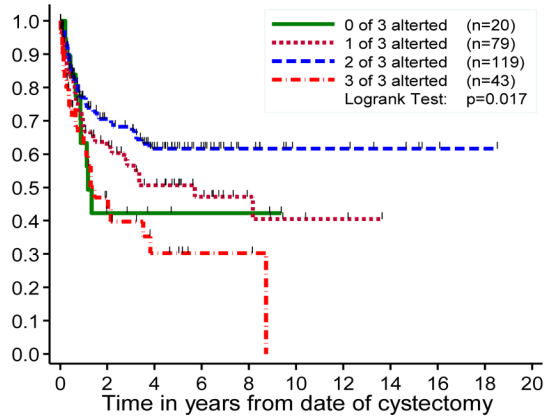
A



B



C



D

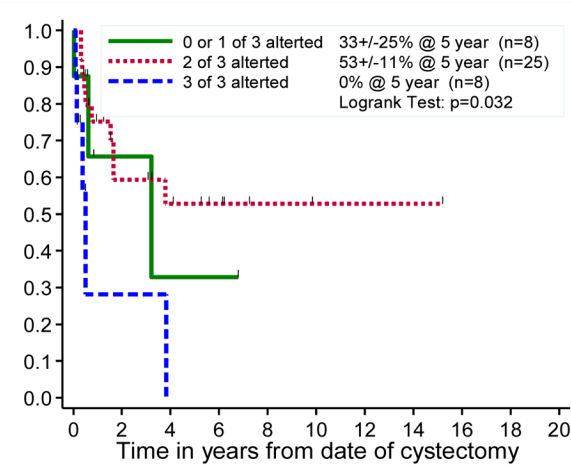


Figure 5: Freedom from recurrence stratified by the number of altered markers in a panel of three cell cycle regulators (Rb, p21 and p53).

Kaplan-Meier curve depicting freedom from recurrence (FFR) by the number of altered markers (Rb, p21 and p53), in Lab A (A), Lab B (B), Lab C (C) and consensus analysis (D).

Table 1:

Antibodies used for immunohistochemical scoring

Protein	Antibody	Manufacturer	Dilution	Preparation	Location	Threshold	Intensity
p53 (1801)	PAb1801	Calbiochem	1:100	microwave in citrate buffer	nuclear	>10%	1-3+
p21	EA10 (Ab-1)	Calbiochem	1:20	microwave in citrate buffer	nuclear	>10%	1-3+
p16	16PO7	Neomarkers	1:40	microwave in citrate buffer	nuclear	>10%	1-3+
Retinoblastoma (Rb)	3C8	QED BioSci	1:200	microwave in citrate buffer	nuclear	<5% or 51% of cells positive	1-3+
Cyclin D1	Ab-3	Calbiochem	1:20	microwave in citrate buffer	nuclear	>10%	1-3+
E-Cadherin	HECD-1	Zymed	1:1000	microwave in citrate buffer	membranous	>10%	1-3+
EGFR	2-18C9	Dako	1:200	proteinase K	membranous	>10%	1-3+
ErbB2 (HER-2/ <i>neu</i>)	A0485	Dako	undiluted	microwave in citrate buffer	membranous	>10%	2-3+
VEGF	JH121	Neomarkers	1:50	steam in EDTA buffer	membranous	>10%	1-3+
Ki-67	MIB1	Dako	1:100	trypsin+microwave	nuclear	% of cells with 1-3+ intensity	1-3+

Table 2:

Clinical and pathological characteristics of patients with corresponding clinical outcomes

Characteristics (N=399)	N	FFR			OS		
		5-year Rate±SE	Logrank p value	HR (95% CI)	5-year Rate±SE	Logrank p value	HR (95% CI)
Overall	399	51±3%	--	--	43±3%	--	--
Area							
North America	278	51±3%		Ref	46±3%		ref
Europe	121	50±5%	0.97	1.0 (0.71, 1.4)	36±5%	0.38	1.1 (0.86, 1.5)
Age at Cystectomy							
<65 years	151	57±4%		ref	50±4%		ref
>=65 years	237	49±4%	0.17	1.2 (0.91, 1.7)	38±3%	0.003	1.5 (1.2, 2.0)
Unknown	11	12±11%	(excluded)		40±15%	(excluded)	
Sex							
Male	301	50±3%		ref	42±3%		ref
Female	98	54±6%	0.38	0.85 (0.58, 1.2)	45±5%	0.48	0.90 (0.66, 1.2)
Histologic Diagnosis							
Urothelial Ca Only	326	50±3%		ref	42±3%		ref
Urothelial Ca with Other	73	53±6%	0.95	0.99 (0.67, 1.5)	46±6%	0.74	0.94 (0.67, 1.3)
N-Stage							
N0	300	57±3%		ref	50±3%		ref
N1-3	99	26±6%	<0.001	2.2 (1.6, 3.0)	20±5%	<0.001	2.0 (1.5, 2.7)
CIS Presence							
Absent	246	58±4%		ref	49±3%		ref
Present	128	39±5%	<0.001	1.7 (1.3, 2.4)	34±5%	0.002	1.5 (1.2, 2.0)
Missing	25	29±11%	(excluded)		32±10%	(excluded)	
Margin Status							
Negative	347	52±3%		ref	47±3%		ref
Positive	18	13±12%	0.008	2.4 (1.2, 4.8)	19±12%	0.006	2.2 (1.2, 4.0)
Missing	34	48±10%	(excluded)		21±7%	(excluded)	

Table 3:

Summary of pairwise agreements among 3 laboratories (n=576)

Marker	Lab B and Lab A				Lab B and Lab C				Lab A and Lab C				consensus	
	N	% of Cases			N	% of Cases			N	% of Cases			N	Positive (%)
		Both Positive	Both Negative	Disagree		Both Positive	Both Negative	Disagree		Both Positive	Both Negative	Disagree		
p53	397	29%	56%	15%	384	26%	65%	9%	388	28%	53%	19%	322	104 (32%)
p21	410	15%	50%	35%	401	13%	64%	23%	452	30%	47%	23%	283	64 (23%)
p16	400	32%	55%	13%	373	32%	56%	12%	397	41%	54%	5%	369	138 (37%)
Rb	397	18%	54%	28%	370	19%	38%	43%	412	45%	38%	17%	270	91 (34%)
Cyclin D1	385	5%	88%	7%	372	5%	90%	5%	418	7%	86%	7%	408	21 (5%)
E-cadherin	396	11%	63%	26%	370	6%	84%	10%	392	10%	62%	28%	306	29 (10%)
EGFR	386	30%	32%	38%	361	31%	18%	51%	381	66%	17%	17%	208	130 (63%)
Her-2	416	6%	77%	17%	404	6%	84%	10%	427	14%	75%	11%	367	29 (8%)
VEGF	186	10%	10%	80%	0				0				37	18 (49%)
Ki-67 (Spearman Rank Corr)	403	r=0.77			393	r=0.68			415	r=0.69				

Table 4:

Summary of pairwise agreement between 3 laboratories (n=576): Kappa estimate (95% confidence interval)*

Marker	Lab B and Lab A	Lab B and Lab C	Lab A and Lab C
p53	68% (61%, 75%)	78% (71%, 85%)	59% (51%, 67%)
p21	29% (23%, 36%)	40% (31%, 49%)	53% (45%, 60%)
p16	72% (66%, 79%)	75% (68%, 81%)	90% (85%, 94%)
Rb	41% (34%, 49%)	25% (19%, 31%)	67% (61%, 74%)
E-cadherin	36% (27%, 44%)	52% (38%, 65%)	31% (23%, 39%)
Cylin D1	55% (40%, 70%)	63% (48%, 79%)	65% (53%, 77%)
EGFR	33% (26%, 39%)	17% (12%, 22%)	54% (45%, 64%)
Her-2	35% (25%, 46%)	51% (39%, 64%)	66% (57%, 75%)
VEGF	1.40% (-1%, 4%)	-	-

* A value of 100% indicates perfect agreement and a value of 0% indicates a random relationship

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