

International Bladder Cancer Marker Network

Tissue Array Consortium

Prognostic Markers in Muscle Invasive Bladder Cancer

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Specific Aims

Transitional cell carcinoma of the bladder (TCC) is a heterogeneous disease characterized by multiple molecular alterations and a variable clinical course. Studies of TCC have identified potential phenotypic and molecular markers whose expression correlates with clinical outcome. However, many of these studies are flawed by their relatively small sample size and by their use of univariate statistical analysis to identify the putative marker of interest, without analyzing the co-expression of other potential biomarkers. These limitations can be addressed by the design of large-scale studies applying multivariate statistical analysis, which evaluate the expression of multiple biomarkers with clinical or biological significance. However, the ability of a single institution to conduct such relevant biomarker studies is limited by the relatively small number of patients available for analysis at any one institution (having known long-term outcome and stratified by stage, grade, and therapeutic intervention).

We propose to establish an international multi-institutional working group to address pertinent translational research questions that cannot be adequately addressed by a single research institution. One overlapping area of interest to researchers studying bladder cancer is the identification of biomarkers relevant to the biology and therapy of this disease. We propose to create an international multi-institutional tissue resource by sharing bladder tumor specimens and establishing tissue microarrays in order to conduct highly powered multivariate biomarker studies to improve our understanding of the relevant biology of TCC, and to allow for the development of more effective therapy.

The first step in designing such large-scale studies of TCC was to bring together representatives from institutions interested in translational bladder cancer research. An international consortium of urologists, pathologists, oncologists, statisticians, and others, met in Trento, Italy in October, 2002 to discuss possible collaborative projects. Individuals from a large number of international groups made a commitment to collaborate in multi-institutional translational research studies, and particularly to join in the development of a tissue-based bladder marker study.

As the first such study, we propose to focus on evaluating biomarkers associated with outcome in stage pT3 TCC, since patients with these tumors show a heterogeneous clinical course. We will test a set of candidate markers for clinical significance in this group of patients.

We propose:

- (1) To establish an international multi-institutional tissue resource and database to create tissue microarrays using archival paraffin blocks of stage pT3 transitional cell carcinoma of the bladder. At least 20 institutions will contribute 30 cases each for a total of 600 cases.
- (2) To exchange stained sections to evaluate the reproducibility of immunohistochemical staining and interpretation.
- (3) To evaluate the prognostic and predictive utility of multiple markers using tissue microarray blocks. Markers to be evaluated will be p53, Ki67, EGFR, erbB2, cyclinD1, Rb, VEG-F, and E-Cadherin. Outcome will be disease free survival. Data will be stratified by lymph node status and adjuvant treatment.

Background

A number of candidate immunohistochemical markers have been proposed as predictive of outcome in muscle invasive bladder cancer. In addition, recent technical approaches for high throughput analyses of molecular alterations occurring during bladder cancer development (i.e. expression arrays, CGH arrays, protein analyses) have produced enormous pools of preliminary data. These discovery techniques identify multiple candidate genes with potential diagnostic and prognostic relevance, but they require validation to define their clinical significance. Larger scale studies using tumor tissue representing the scope of bladder cancers are needed, yet the limited availability of well characterized clinical specimens and the difficulty of characterizing multiple markers in large numbers of tumors make this difficult. Using tissue microarrays facilitates such analyses of previously proposed as well as newly identified genes discovered by genomic screenings of model systems, and is a useful tool to bridge the gap between candidate discovery and candidate testing.

Patients with muscle invasive TCC show a heterogeneous clinical course. Improving our ability to predict which of these patients will die from disease will allow better choice of adjuvant therapies beyond cystectomy. Patients with higher risk of recurrence and death from disease are candidates for more aggressive adjuvant therapy, while those with little risk of recurrence and death might avoid treatment beyond cystectomy. The ability to predict response to adjuvant chemotherapy may allow the identification of patients who do not respond to standard therapy, and may be eligible for other therapies. Finally, this set of tumor samples will allow the consortium to identify future targets for gene-based therapies, which are prevalent in these tumors.

Bladder Cancer Markers

Considering the natural history of invasive bladder cancer, it is likely that accurate and reliable prognostic markers would have a significant impact on the management of this disease. An ideal prognostic marker should facilitate the identification patients who are likely to develop recurrent or progressive TCC. It could also be used to identify patients likely to respond to a specific treatment, or alternatively, it could become a target for novel therapeutic strategies. An International Bladder Cancer Network was established in 1997 with a view to improve the diagnosis, prevention and treatment of bladder cancer through the application of biologic markers (2). The group laid out the four phases for development and validation of markers for bladder cancer. Phase 1 includes the development of a reliable and reproducible assay that can be applied to clinical specimens. Markers with low prevalence in the at risk population are unlikely to be clinically useful and markers with high prevalence may be useful for diagnosis but would have limited capacity to differentiate between tumors with different behavior. Phase 2 development evaluates the utility of the marker in answering a clinical question. The marker is compared with traditional prognostic endpoints (grade and stage and outcome) to generate hypothesis regarding clinical utility. Sensitivity, specificity and appropriate cut-points are determined. In phase 3, clinical utility is tested in appropriately powered studies and in a defined clinical setting using the methodology, cut-off, and sample size determined from phase 2 studies and is usually applied at a single institutional level. In phase 4, multicenter validation of phase 3 conclusions is performed. Methodology and cut-points are validated and the assay can be tested via assessment of inter-institutional and inter-laboratory variability and quality control.

The initial phase of this study will be to evaluate a set of 8 candidate genes for prognostic significance in our patient cohort. These genes have been chosen based on a large literature evaluating a much larger set of proposed markers in a heterogeneous group of bladder cancer studies. We have chosen these 8 genes because of previous studies showing clinical significance, and because of their biologic justification. Many of these genes represent members of the retinoblastoma proliferation pathway (Ki67 as an established marker of proliferation,

Rb, p53, and cyclinD1). ErbB2 and EGFR are tyrosine receptor kinases, which are known to be overexpressed in a large number of bladder tumors, although their clinical significance is controversial. E-Cadherin, playing a role in cell-cell interactions, is frequently altered in high stage bladder tumors. Finally, VEG-F, playing a role during angiogenesis and neovascularization, is thought to play an important role during invasion and metastasis.

To date, no marker has achieved the merit of replacing the traditional indicators of prognosis – grade and stage. Many molecular marker studies have determined an arbitrary “cut-point” using a retrospective analysis of the relative presence of the marker in the original data set but few if any markers have been prospectively validated in multicenter clinical studies (phase 4). Furthermore, biomarker studies in bladder cancer should be designed with sufficient statistical power to detect differences, which are considered to be of clinical importance. Many retrospective studies contain too few cases to allow a small but significant difference to be detected.

The two markers that have received the most recent attention as potential prognostic indicators of bladder cancer are the products of the tumor-suppressor genes p53 and RB. For bladder cancer, in contrast to some other neoplasms, immunohistochemical staining for p53 correlates with mutations in the p53 gene.(3,4) In normal cells, the p53 protein is not detectable by routine immunohistochemical techniques. However, one of the difficulties in interpreting the results of p53 staining is the general lack of consistency in the technical aspects of performing the assay; another difficulty is in the interpretation of the results, since threshold values for altered p53 expression vary amongst studies. These technical considerations are the subject of a multicenter National Cancer Institute sponsored trial that is evaluating the reproducibility of the interpretation of p53 staining between different laboratories that routinely perform the assay. (5) Although the overall interlaboratory reproducibility for the interpretation of p53 staining was good for specimens with no or minimal staining or for specimens with a high number of stained tumor cells, reproducibility was not good for specimens with an intermediate range of staining. Such findings again indicate the need for caution when comparing results from different laboratories. In contradistinction to p53, the RB gene product is normally detected by immunohistochemical testing. While the loss of RB protein has long been recognized as abnormal, more recently, overexpression of RB protein by immunohistochemistry has also been shown to be associated with a poor prognosis.(6,7) p53 is an indicator of tumor progression in both superficial and locally advanced bladder cancer, (8-13) and RB protein expression also correlates with tumor progression.(14,15) Furthermore, there are data suggesting that the combination of both markers provides improved prognostic information (6,7,16).

The encouraging data regarding the use of p53 and RB are now being critically evaluated in several prognostic studies. This is particularly important because other retrospective investigations have not found these markers to be predictive(17). At Memorial Sloan Kettering Cancer Center, patients who have locally advanced bladder cancer with normal p53 expression are considered potential candidates for bladder salvage therapy. In an international trial organized through the University of Southern California, patients who have had cystectomy and have abnormal p53 expression are randomized to adjuvant chemotherapy or routine follow-up.

The fraction of proliferating cells (growth fraction) of a tumor is another important prognostic factor that defines the tumor’s biological potential. Ki-67 is a murine monoclonal antibody that reacts with a nuclear antigen expressed in proliferating cells. Increased Ki-67 expression is associated with increased grade and stage (18-20) and correlates with tumor recurrence.(21-24) Normal cell proliferation is closely regulated by cyclins such as cyclin D1, which in combination with its corresponding cyclin dependent kinase (cdk) is essential for G1/S phase transition.(25-27) A limited number of studies indicate that cyclin D1 expression is inversely proportional to stage and grade, but its role as a prognostic marker for patients with invasive bladder cancer is less clear(26,27).

Many human tumors express high levels of growth factors and their receptors that can be used as potential therapeutic targets. Tyrosine-kinase receptors, including many growth factor receptors such the receptors for epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and Her2/neu, have been found overexpressed in urothelial tumors. For many of these growth factor receptors, the degree of expression has

been associated with the progression of cancer and a poor prognosis. Among the best studied growth factor receptors are the two members of EGF receptor family EGFR(ErbB-1), and Her2/neu (ErbB-2).

Clinical studies evaluating the significance of EGFR expression in human TCC have shown that greater than 50% of human bladder cancers overexpress EGFR, and that the level of expression directly correlates with tumor grade, stage and survival.(28-35) In patients with superficial bladder cancer, EGFR expression correlates with multiplicity, time to disease recurrence and overall recurrence rate (33) Izawa et al evaluated EGFR gene expression in a series of bladder carcinomas and reported that EGFR expression was involved in bladder cancer progression as cells expressing EGFR acquired an invasive phenotype. However, the role for EGFR expression as a prognostic indicator for patients with advanced TCC remains controversial.(36)

Overexpression and amplification of Her2/neu was first identified in a human breast carcinoma cell line (37), and subsequently in approximately 30% of breast adenocarcinomas (38). It has been repeatedly shown to be a prognostic marker in breast cancer, particularly in lymph node-positive patients.(39-41) The gene amplification and/or the protein overexpression have been associated with worse outcome in breast and ovarian carcinoma (42,43). Its prognostic significance in other epithelial neoplasms, however, is less clear. Similarly, the prognostic significance of Her2/Neu overexpression in bladder urothelial carcinoma is largely unknown, mainly due to conflicting data rendered by published reports (44-50). Most studies have included both superficial and muscle-invasive disease in their study cohorts, so data concerning exclusively muscle invasive disease is for the most part lacking.

The cadherins are a family of transmembrane glycoproteins that play an important role in establishing and maintaining intercellular connections. E-cadherin, a molecule associated with epithelial cell adhesion, is lost in many types of cancers (51). The resulting decrease in intercellular adhesion can lead to a more invasive state. Restoring E-cadherin expression has been shown to abrogate the invasive phenotype.(52) A decrease in E-cadherin expression is more frequent with increasing bladder cancer stage(53-55) and is associated with decreased survival.(53,55)

Angiogenesis is the process of development of new blood vessels.(56) Blood vessels are required for tumor growth. Furthermore, the neovascularity associated with tumors may facilitate the transition of cells from the local tumor bed into the circulation resulting in metastasis.(56,57) A commonly used histological measure of angiogenesis is microvessel density (MVD).

High MVD in invasive bladder cancers, as indicated by immunohistochemical staining for Factor VIII or CD34, was reported to be a strong predictor of lymph node metastasis in one study (58) and an independent prognostic indicator for survival in two other reports conveying, a 2.5-fold greater risk of dying of bladder cancer for those patients with high microvessel counts.(59,60) Inoue et al have demonstrated that the expression levels of VEGF and bFGF as indicated by in situ hybridization, and MVD as indicated by immunohistochemistry, identify patients with muscle-invasive TCC who are at high risk of developing metastasis after aggressive therapy with systemic M-VAC chemotherapy and radical cystectomy.(61) Evidence thus supports MVD as a prognostic factor for invasive bladder cancer.

VEGF, which is produced by a variety of normal and neoplastic cells, is a potent endothelial cell-specific mitogen that inhibits apoptosis of endothelial cells.(62) It promotes invasion and migration of endothelial cells, and by increasing vascular permeability it facilitates the entry of tumor cells into the circulation allowing them to metastasize to distant sites. VEGF is produced in response to hypoxia and is regulated at a transcriptional level by hypoxia-inducible factor 1.(63) Some reports have suggested that VEGF is differentially expressed in bladder cancer, with high expression in superficial tumors (stage pTa and pT1) contrasting with low expression in muscle-invasive tumors (stage > pT1).(64) Others have evaluated the prognostic significance of VEGF expression in tumor tissue and reported that VEGF expression did not correlate with risk of tumor recurrence or patient survival.(65) In contrast to these findings, Izawa et al determined that VEGF overexpression was more

common in advanced TCC, while Crew et al (66) measured VEGF expression in tumor specimens from 55 patients with superficial bladder cancer and reported that high expression was associated with recurrence and progression. They speculate that the relationship between VEGF and early tumor recurrence suggests that seeding via angiogenesis may be a major mechanism in the pathogenesis of recurrence. Inoue et al reported that VEGF expression in pre-cystectomy biopsies was an independent prognostic predictor for survival in patients with invasive TCC treated by MVAC chemotherapy and radical cystectomy.(61)

Understanding the molecular pathogenesis of bladder cancer remains an area of ongoing research, which is facilitated by the recent development of new molecular biological analytical techniques and it is intrinsically linked to the development of novel therapeutic agents. As a greater knowledge of the precise mechanisms involved in the malignant transformation of normal urothelium and the progression of superficial to invasive and metastatic TCC develops, the potential for interventions in these pathways will increase the therapeutic repertoire available to the urologist and oncologist to treat bladder cancer.

General Information on the Tissue Microarrays

The tissue microarray (TMA) technology was developed to allow efficient immunohistochemical and in situ hybridization analysis of large numbers of tumors. Tissue microarrays are constructed by collecting a cylindrical core tissue biopsy from one or more representative regions of a regular formalin-fixed, paraffin-embedded tumor block. This core (diameter 0.6 mm, height 3-4 mm) taken from the "donor" is now precisely arrayed into a new "recipient" paraffin block using a custom-built instrument (Beecher Instruments). As many as 1000 cylindrical tissue biopsy specimens from individual tumors can be distributed in a single tissue microarray, allowing analysis of a thousand tumors at the RNA, DNA, or protein level. Up to 200 consecutive sections of 4-5 μm thickness can be cut from each tumor array block. This technique allows parallel analyses of a large number of molecular markers.

Tissue Array Proposal

Tissue array blocks will be constructed from samples provided by multiple institutions. We expect that 500 node negative pT3 archival tumor blocks will be used from 20 institutions to construct a set of tissue microarrays. Arrays containing these samples will be constructed at University of Basel (Dr. G. Sauter). These arrays will be tested for expression of p53, Ki67, EGFR, erbB2, cyclinD1, Rb, VEGF and E-Cadherin using standard immunohistochemistry assays. Those assays, done in replicate sections by multiple institutions, will use agreed upon protocols for staining and established criteria for interpretation. The sections will also be exchanged to allow other institutions to score identical sections.

Expression of these 8 antigens will be tested for their ability to predict outcome in this set of 500 pT3 bladder transitional cell carcinomas. Requirements for inclusion in these arrays will be pT3 tumors, node negative (with at least one lymph node examined), having at least 3 years of follow-up (known disease status) following cystectomy. Cases will be stratified for grade and adjuvant chemotherapy. An additional 100 tumors will be requested (5/site) with known node positivity to compare prevalence of expression changes. Patients receiving Neoadjuvant and/or radiation therapy will be excluded. Pure adenocarcinoma, squamous cell carcinoma, and small cell neuroendocrine carcinoma are excluded. Presence of squamous or glandular differentiation is acceptable.

Experimental Design

Tissue

Each participating institution (20 institutions total) will provide 30 formalin-fixed, paraffin-embedded tumor blocks of pT3 TCC. 25 of these should be node negative (at least one node examined) and 5 should be node positive (positive and negative nodes documented). These blocks will be combined into a single tissue microarray containing approximately 600 different tumors. Additional cores will be included in the array from 10 normal bladders, from 30 non-bladder tissues (positive and negative controls), and from 10 cell lines having known antigen status.

Cores will be taken from each block for inclusion in the array. Areas for sampling of each core will be identified from an H&E section from the surface of each block (10 thin sections will be taken from each block for control stains). Each core will be 0.6 mm in diameter. 10 cores will be taken from each block. 6 cores will be used for 6 identical arrays, and the remaining 4 cores will be used for DNA extraction.

All blocks will be returned to the originating institution after the arrays are produced.

It is expected that each array block will yield ~200 sections, each containing the full set of tumor and control cores. Sections from these blocks will be distributed to the participating institutions for further studies.

Institutional Review Board Approvals

Each participating institution will be required to receive approval from their Institutional Review Board on Human Experimentation before submitting archival blocks to the Consortium. It should be made clear to the local IRB that each sample will be coded at the originating institution and only this coded identity will be used by any of the other participants. Thus confidentiality of the patient identify will be entirely protected behind a firewall at each institution. In addition, any institution requesting sections for study must all have approval (or certification of exemption).

Clinical Data

Each institution will provide a defined set of data associated with each tumor specimen. The identification of block and corresponding data will be coded to protect confidentiality. No patient identifying information will be used. A central biostatistical core will be responsible for reviewing the data for completeness, assessing quality of data (entry consistency, etc.), organizing the information, and performing the statistical analyses of the results. The table below summarizes the minimum data requested.

Requested Information:

<i>Variable</i>	<i>Coding</i>
Final Histologic Diagnosis	Transitional Cell Carcinoma, Urothelial Carcinoma, Other (state)
Sex	m = male; f = female
Date of Birth	mm/dd/yyyy
T (pathologic stage)	PT3 yes/no/unknown
N	Lymph node status
M	Metastatic spread
Grade	Tumor grade (scale of 3)
Number of lymph nodes positive	NN
Number of lymph nodes examined	NN
Presence of CIS in specimen	Yes / No,
Was Cystectomy done?	Yes / No, -9 = do not know
Date of Cystectomy	mm/dd/yyyy
Was all primary tumor removed at the time of cystectomy?	Yes/No/Unknown
Surgical procedure by which the specimen was obtained	Cystectomy, partial cystectomy, TUR, other
Date the specimen was collected	mm/dd/yyyy
Was the bladder exposed to radiation therapy?	1 = yes, 0 = no, -9 = do not know
Did the patient receive prior intravesical therapy for bladder cancer?	1 = yes, 0 = no, -9 = do not know
Has the patient received prior systemic chemotherapy for bladder cancer?	1 = yes, 0 = no, -9 = do not know
Did the patient receive adjuvant chemotherapy?	1 = yes, 0 = no, -9 = do not know
Type of adjuvant chemotherapy for bladder cancer:	MVAC, Other (state)
Date of first recurrence	mm/dd/yyyy
Type of first recurrence	local, distant, both
Did patient receive chemotherapy following recurrence?	1 = yes, 0 = no, -9 = do not know
Date of death	mm/dd/yyyy
Death with cancer?	Yes/No/Unknown
Date of last follow-up	mm/dd/yyyy
Status at last follow-up	Alive with no evidence of recurrence, Alive following recurrence, Alive with disease status unknown*, Dead
*If patient is alive at last follow-up but the disease status is unknown (i.e. it is unknown whether the patient has recurred), then record the date that the patient is last known to be free of any recurrence	mm/dd/yyyy

Immunohistochemistry

The primary goal of this study is to evaluate the prognostic value for time to recurrence and overall survival using multivariable analysis of the 8 proposed antigens using a large multi-institutional cohort of tumors. A consequence of this analysis will be to determine whether we can identify a profile of these markers that can be used to define the probability of recurrence. In addition, reproducibility of staining and scoring will be tested as a secondary aim.

For the main study, five institutions will stain one section with H&E and one with antibodies against each of p53, Ki67, EGFR, erbB2, cyclinD1, Rb, VEG-F, and E-Cadherin. Standard protocols for staining and scoring will be distributed by the study coordinator based on prior validation with a developmental tissue array. Results will be scored at each institution separately. Sections will then be exchanged and rescored at two of the institutions. Reproducibility of staining and scoring will be evaluated based on staining and scoring at multiple institutions.

Statistics

Evaluation of Tissue Microarray Sections

Once the TMAs are created, sections will be cut and numbered sequentially. A table of random digits will be used to select 10 sections to be sent to each of the 5 participating institutions (the current plan is to have 5 institutions participate in this phase of the project – but the methods do not depend on the exact numbers). Each institution will use one section for each of the 8 genes; one section will be used for H&E staining; 1 section will be available for an additional study or repeating a gene. The staining of each core on each section will be graded according to the intensity of staining (0=negative, 1=weak, 2=moderate, 3=strong) and by the percent of cells staining (0% to 100%). For each marker, the exact scoring will be based on the recorded intensity and percent positive, and agreed upon by all participants prior to distribution of the sections, based on published studies using these markers. Once the sections have been scored, the sections will be shipped to another participating institution for re-scoring. The current plan is to have two additional institutions rescore each section. For each TMA section, the sequence of institutions for rescore will be randomly assigned by the statistical center.

Analysis of Association of 8 Markers with Outcome

Time to recurrence will be defined as the time from diagnosis of invasive (pT₃N₀ or pT₃N₊) bladder cancer until the date of the diagnosis of the first recurrence of the disease or until last date that the patient was known to be alive and free of any recurrence. Patients who die prior to any recurrence will be censored at the time of death for time to recurrence. Overall survival will be defined as the time from the diagnosis of invasive (pT₃N₀ or pT₃N₊) bladder cancer until death or the date that the patient was last known to be alive. Death due to any cause will be used as the endpoint in overall survival. Finally, prior to formal statistical analysis of the association with outcome, a consensus status of each marker and each patient will be determined (by category and by overall negative/positive status). It will be these consensus values that will be used in the initial analyses.

The initial analyses will be undertaken to summarize the distributions of the protein expression (IHC) results, the differences between patients with and without involved lymph nodes, and the associations between the markers. For these analyses, straight-forward descriptive statistics will be used: contingency tables, percents, and Pearson's chi-square test for association. Next, univariate analyses (Kaplan-Meier plots and the logrank test), with each marker examined individually in invasive pT₃N₀ and pT₃N₊ patients separately, and for time to

recurrence and overall survival separately. In previous studies at USC (data not shown) the assumption of proportional hazards was not unreasonable for time to recurrence during the first 5 years of follow-up. Hence the current plan is to use the Cox proportional hazards model to jointly evaluate the impact of all 8 markers on time to recurrence – including main effects and two-way interactions, to the extent permitted by numbers. In addition, clinical and treatment variables will be considered in this joint model. With 8 markers there will be 28 two-way interaction terms – for a total of 36 variables. Overall conservatively, we will estimate that about 40% patients (180 to 220) with pT₃N₀ tumors will experience a recurrence within 3-5 years, and 60% of patients (i.e. 55 to 65) with pT₃N₊ tumors will experience a recurrence within 3-5 years. Using the rule of thumb that for each variable included in a model there should be 10-15 “events” or recurrences (72, 73), then for the node negative patients, we would expect approximately 15 to 18 variables and for the node positive patients, we would expect to include 5-6 variables in the final model. If the patterns in the node negative and node positive patients are similar, then consideration will be given to combining the two cohorts. Stepwise forward selection will be used to decide which interaction terms to include in the model for lymph node negative patients, and to decide which subset of markers is best associated with time to recurrence for patients with involved lymph nodes. Similar analyses will be undertaken with overall survival, using the Cox proportional hazards model if appropriate and with an alternative model (possible parametric, such as the log-logistic) if necessary.

The analyses described above will evaluate the association of each marker with outcome, as well as allow us to test for interaction effects. For example, we will be able to evaluate the joint association of p53 and Rb with outcome or KI-67 and cyclin D1, or erbB2 and EGFR with outcome. Once the markers that are most strongly associated with outcome are identified, then a series of analyses will be undertaken to see if we can predict which patients are at very high risk of recurring – or at a substantially lower risk. In the first analysis, we will use the score function obtained with the Cox model and group patients by the quartiles (lowest 25%, 26th to the 50th percentiles, 51st to the 75th percentiles, and the top quarter of the patients), based on this score function. Of interest will be degree of separation between the bottom and top quarters. A second analysis will use the classification and regression tree (CART) methods with each marker considered as either positive/negative or by the percent categories (74, 75, 76). Receiver operating characteristic (ROC) curve methods will be used to evaluate whether the final models are effective in predicting which patients have a very high or very low chance of recurring within 3-5 years (77, 78, 79). Resampling methods will be used to evaluate the final models in terms of internal validity and to assess the possibility of overfitting (80). A second set of validation studies will be undertaken by using marker results from individual centers, rather than the consensus values. For each center, a sequence of 80% samples will be used to fit the final model and to “predict” the outcome in the remaining 20% of patients. The success of the resampling analyses of the center’s data will also be used to evaluate the usefulness of the final proposed model.

When the digital imaging results become available, it is entirely possible the exact percentages of cells staining will be used, rather than groupings. In this situation, we will use maximal chi-square methods to identify cut-points that produce the strongest associations between the individual marker and outcome (81). The p-values to assess the strength of the associations based on these cut-offs, will be based on bootstrap-like simulations in which the recurrence times were randomly matched to the expression levels, in order to generate a reference distribution of the maximal logrank statistic under the null hypothesis. These cut-points will be compared to the initial groupings of the results.

Justification of Numbers of Specimens

The primary goal of the proposed analyses is to verify whether each of the 8 markers is associated with outcome in patients with pT₃ N₀ transitional cell bladder cancer. With 500 such patients there will be excellent power (at least 96%) to detect such associations, as long as the association translates into a difference in the chance of progression or overall survival of at least 20% at 3 years (for example 30% chance of recurring by 3 years vs. 50% chance – or 40% chance of dying vs. a 60% chance of dying) when the IHC result for a marker is

classified as positive vs. negative and at least 20% of patients have “positive” tumors and at least 20% have “negative” tumors. When the difference in the chance of progression or overall survival is 15% or more, then the power is at least 82% (the scenario with least power occurs when 80% of patients have the unfavorable marker, 57.2% of these patients recur by 3 years, while only 42.5% of the patients with the favorable marker recur). These power calculations used methods of Rubinstein, Gail, and Santner (82) as programmed by Buckley (83), and are based on a two-sided 0.05-level logrank test and assume (for purposes of the calculations) exponential failure and uniform accrual over 2 years with 3 years of follow-up. Hence, with this series, we will be able to establish whether any of the 8 markers is associated with outcome to an extent that will be clinically meaningful. In addition, there will be at least 95% power to compare the protein expression of each of these 8 genes in patients with and without involved lymph nodes, when the difference in the percent of patients whose tumors express the positive marker is at least 20%. The power is at least 80% when the difference is at least 16%. Power calculations for the two-sided 0.05-level Fisher’s exact test are based on methods proposed by Casagrande *et al.* (84) as programmed by Buckley (83).

Evaluation of Reproducibility of IHC Staining and Interpretation

Initially each gene will be analyzed separately and the positive / negative grade from the institution performing the staining will be used to evaluate the reproducibility of staining. A logistic regression model for correlated binary data will be used analyze the overall staining positive assessments; cumulative logit proportional hazards models for correlated ordinal data will be used for the ordinal scores. To evaluate the reproducibility of the scoring, the percent agreement among the 3 scoring institutions will be calculated for each section and each “position/core” in each section.

The proposed analyses are descriptive with the goal of estimating the reproducibility of staining and scoring for each of the 8 genes. The precision of these estimates of the reproducibility of the staining is indicated by the following: (1) with 600 cores (for each gene) the percent of times that all 5 institutions agree can be estimated with a standard error of 2.0%; a 95% confidence interval will have a half-width of +/- 4%. Kappa statistics will be used to estimate the agreement between institutions.

Array Analysis by Digital Imaging

A number of groups are currently evaluating the use of imaging technologies to score tissue microarrays for immunohistochemistry and FISH (prostate, breast). These technologies allow straight-forward, efficient, and quantitative analysis of multiple cores. In addition, digital imaging allows sharing of data much more easily than does of sending the array slides to the multiple investigators. Application of these imaging and analysis procedures will be tested for web-based visualization and scoring of stained array slides.

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