Biomarker Study of Primary Nonmetastatic *versus* Metastatic Invasive Bladder Cancer[1]

Pilar Lianes, Elizabeth Charytonowicz, Carlos Cordon-Cardo,[2] Yves Fradet, H. Barton Grossman, George P. Hemstreet, Frederic M. Waldman, Karen Chew, Leon L. Wheeless, David Faraggi, and the National Cancer Institute Bladder Tumor Marker Network

Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [P. L., E. C., C. C-C.]; Department of Urology, Laval University, Laval, Quebec, Canada G1R 2J6 [Y. F.]; University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [H. B. G.]; Department of Urology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 [G. P. H.]; Division of Molecular Cytometry, University of California, San Francisco, California 94143-0808 [F. M. W., K. C.]; Department of Pathology, University of Rochester, Rochester, New York 14642 [L. L. W.]; and Division of Cancer Diagnosis and Treatment, National Cancer Institute, Bethesda, Maryland 20892 [D. F.]

ABSTRACT

A cohort of 109 patients with primary transitional cell carcinomas, stages T_2–T_3, grade 2 or higher, was identified and further divided into two groups based on lymphatic metastasis at the time of cystectomy (n = 57 cases) or absence of detectable metastatic disease over a minimum of 5 years of follow-up after cystectomy (n = 52). Blocks corresponding to the primary tumor lesions were sectioned and distributed to different laboratories to be analyzed. Immunohistochemistry on deparaffinized tissue sections was conducted for evaluation of p53 nuclear overexpression (monoclonal antibody PAb1801), assessment of proliferative index (Ki-67 antigen-monoclonal antibody MIB1), and microvascular counts (factor VIII-related antigen). DNA content/ploidy studies were performed on material obtained from thick sections. A double-blinded strategy was used for the evaluation of laboratory data *versus* clinical parameters. The cutoff value for p53 nuclear overexpression was ≥20% of tumor cells displaying nuclear staining. The median values for MIB1 (≥18% of tumor nuclear cell staining) and microvascular counts (≥40 microvessels/area screened) were used as cutoff points for these two variables. The assessment of DNA content was conducted by classifying cases as diploid, tetraploid, or aneuploid. Statistical analyses were performed using the Fisher's Exact Test (2-tailed). Results revealed that none of the markers studied had a statistically significant correlation with the end point of the study, *i.e.*, the presence of lymph node metastatic disease, in the cohort of patients studied, although an obvious trend for p53 was noted. It is concluded that alterations of p53, Ki-67 proliferative index, microvascular counts, and ploidy are not strongly associated with lymph node status in patients affected with high-stage, high-grade bladder cancer.

INTRODUCTION

Detailed molecular genetic studies of bladder tumors have led to a working hypothesis of tumorigenesis and progression (1–5). It appears that the accumulation, rather than the order, of certain genetic and phenotypic alterations acts synergistically and leads to cancer progression (6, 7). The final result is a selective growth advantage that allows cancer cells to create an uncontrollable widespread disease. A crucial concern regarding patients presenting with muscle invasive bladder carcinomas is that of their metastatic potential. We undertook this study to test the hypothesis that tumor cells that metastasize are both genotypically and phenotypically different from nonmetastatic variants and could be identified in primary tumors. A panel of markers was selected based on both preliminary data, revealing their association in bladder tumor progression, and their related biological significance to metastatic spread. These markers included altered patterns of p53 expression (8–11), Ki-67 proliferative index (12, 13), microvascular count (14, 15), and DNA content (16–18). A cohort of 109 muscle-invasive and high-grade transitional cell carcinomas was identified, consisting of 57 cases with lymphatic metastasis at the time of cystectomy and 52 cases of patients who had cystectomy and were free of metastases over a minimum of 5 years of follow-up. The collaborative effort was aimed at determining the frequency of the phenotypic alterations in these primary metastatic *versus* nonmetastatic bladder cancers. We also assessed their association with the finding of lymph node metastatic disease in high-stage, high-grade bladder cancer.

MATERIALS AND METHODS

Bladder Tumor Specimens. Paraffin-embedded blocks of primary bladder tumors (cystectomy specimens) were provided by the collaborating institutions. These paraffin tissue blocks corresponded to cases that had a minimal follow-up of 5 years. All specimens were fixed with formalin. All blocks were submitted to one center, where sections were cut for histopa-

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[2] To whom requests for reprints should be addressed, at Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-7746; Fax: (212) 794-3186; E-mail: cordon-c@mskcc.org.

thology evaluation, immunohistochemical assays, and ploidy analysis. Pertinent clinical data and the results obtained were stored in a data bank generated and maintained for the purpose of this study. All investigations were carried out blind, and the code was broken at the end of the study.

A total of 109 patients with primary transitional cell carcinomas, stages T_2–T_3, grades 2 or 3, was identified for this study. This cohort was further divided into two groups based on the presence of lymphatic metastasis at the time of cystectomy (n = 57 cases) or the absence of detectable metastatic disease (n = 52) with a minimal follow-up of 5 years after cystectomy. History of chemotherapy was the main exclusion criteria for selection of cases. Radiation therapy was not an exclusion criteria.

Immunohistochemistry and Antibodies. Evaluation of p53 nuclear overexpression was conducted using mouse monoclonal antibody PAb1801 (Ab2-IgG1; Oncogene Science, Cambridge, MA; 1:500 dilution). Evaluation of proliferative index was performed using monoclonal antibody MIB1 to Ki-67 (Immunotech SA, Marseille, France; 1:50 dilution). Microvascular counts were assessed by staining of endothelial cells using antibodies to factor VIII-related antigen (von Willebrand's factor), as reported previously (14).

The immunohistochemical method chosen for the present study was the avidin-biotin complex immunoperoxidase technique (8, 14). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and then treated for 15 min in 1% hydrogen peroxide in PBS to remove endogenous peroxidase activity. Tissue sections were washed in PBS and then incubated with 10% normal horse or goat serum (Organon Tecknika Corp, Westchester, PA) in PBS for 30 min. Blocking normal serum was drained off, and sections were incubated overnight with appropriately diluted primary antibodies at 4°C (see above). After extensive washing, sections were subsequently incubated for 30 min with biotinylated horse anti-mouse (1:500 dilution) or goat anti-rabbit immunoglobulins (1:800 dilution; Vector Laboratories, Burlingame, CA). The sections were then washed and incubated with avidin-biotin peroxidase complexes for 30 min (Vector Laboratories; 1:25 dilution). The peroxidase reaction was visualized by incubating tissue sections for 4–6 min with 5 mg of diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 100 ml of 0.5% Tris buffer containing 25 μl of 30% hydrogen peroxide. Sections were washed with distilled water, counterstained with hematoxylin, and mounted.

The cutoff value for p53 nuclear overexpression was ≥20% tumor cells displaying nuclear staining, based on studies reported previously (8, 9). The median values for MIB1 and microvascular counts were used as cutoff points for these two variables. Individual microvessel counts were conducted on the field of maximum angiogenic activity, scoring a selected ×200 field (20 × objective and 10 × ocular, 0.74 mm^2 per field; Ref. 14). Any stained endothelial cell or endothelial cluster, clearly separated from immediately adjacent microvessels, tumor cells, or other connective tissue elements, was considered a single, countable microvessel. Neither RBCs nor vessel lumens were considered necessary for a structure to be defined as a microvessel. Results were expressed as the highest number of microvessels identified and counted within any single ×200 field (ocular count; Ref. 14). Assessment of p53 nuclear overexpression, MIB1 proliferative index, and microvessel density was performed without knowledge of the lymph node status.

Paraffin-embedded tissues known to express p53 mutant proteins (an invasive bladder cancer) or a high proliferative index (a high-grade fibrosarcoma) were used for titrations and positive controls. Negative controls included substitution of the primary antibodies by irrelevant antibodies of the same species and subtype (*i.e.*, MIgS-KpI mouse monoclonal antibody; PharMingen Laboratories, San Diego, CA) or incubation with blocking serum alone.

DNA Ploidy Studies. Ploidy analyses were performed on material obtained from thick (50-μm) sections. The paraffin was dissolved with xylene, and the tissue was rehydrated. The tissue was processed by a modification of the Hedley technique (19). Sections were dissociated enzymatically with 0.5% pepsin and mechanically by repetitive syringing. The nuclei were filtered through a 105-μm mesh, and the resulting suspension was used to prepare slides for each case. Slides were fixed with 10% neutral buffered formalin and then stained with a Quantitative DNA staining kit (Becton Dickinson, Cell Imaging System, San Jose, CA) according to instructions.

Quantitative DNA measurements were taken on a CAS 200 instrument (Becton Dickinson). A minimum of 200 nuclei were measured on each slide. DNA histograms were generated from the integrated sum absorbance measurements. Reference cells (rat liver touch preparations) on each slide were used for calibration. The hyperdiploid fraction (the percentage of cells with DNA content values above diploid as defined by the reference population) was calculated. If the hyperdiploid fraction was ≤10%, the specimen was classified as diploid. For specimens with hyperdiploid fractions >10%, the DNA index of the nondiploid cell population was calculated relative to the reference diploid value. Specimens with a DNA index between 1.8 and 2.2 were classified tetraploid; those with DNA index values <1.8 or >2.2 were classified aneuploid.

Scoring and Statistical Considerations. The cutoff value for p53 nuclear overexpression was ≥20% tumor cell nuclei staining (8, 9). The median values for MIB1 (≥18% tumor nuclear cell staining) and microvascular counts (≥40 microvessels/area screened) were used as cutoff points for these two variables (14). The assessment of DNA content was conducted by classifying cases as diploid, tetraploid, or aneuploid. As part of the experimental design, an estimate of sample size was obtained to achieve a power of 80% when the significant level is 0.05. The cohort of 109 high-grade, muscle-invasive transitional cell carcinomas was considered to be appropriate to meet minimum sample size for the defined α and β levels. Statistical analyses were performed using the Fisher's Exact Test (2-tailed; Ref. 20).

RESULTS AND DISCUSSION

Superficial bladder tumors (T_a, TIS, or T_1) represent 70–80% of the newly diagnosed bladder cancers. Recurrences are frequent in these cases and can be as high as 80%. Tumor progression, defined as development of invasive disease or metastases, may occur in up to 30% of the TIS and T_1 lesions. On the other hand, muscle invasive carcinomas com-

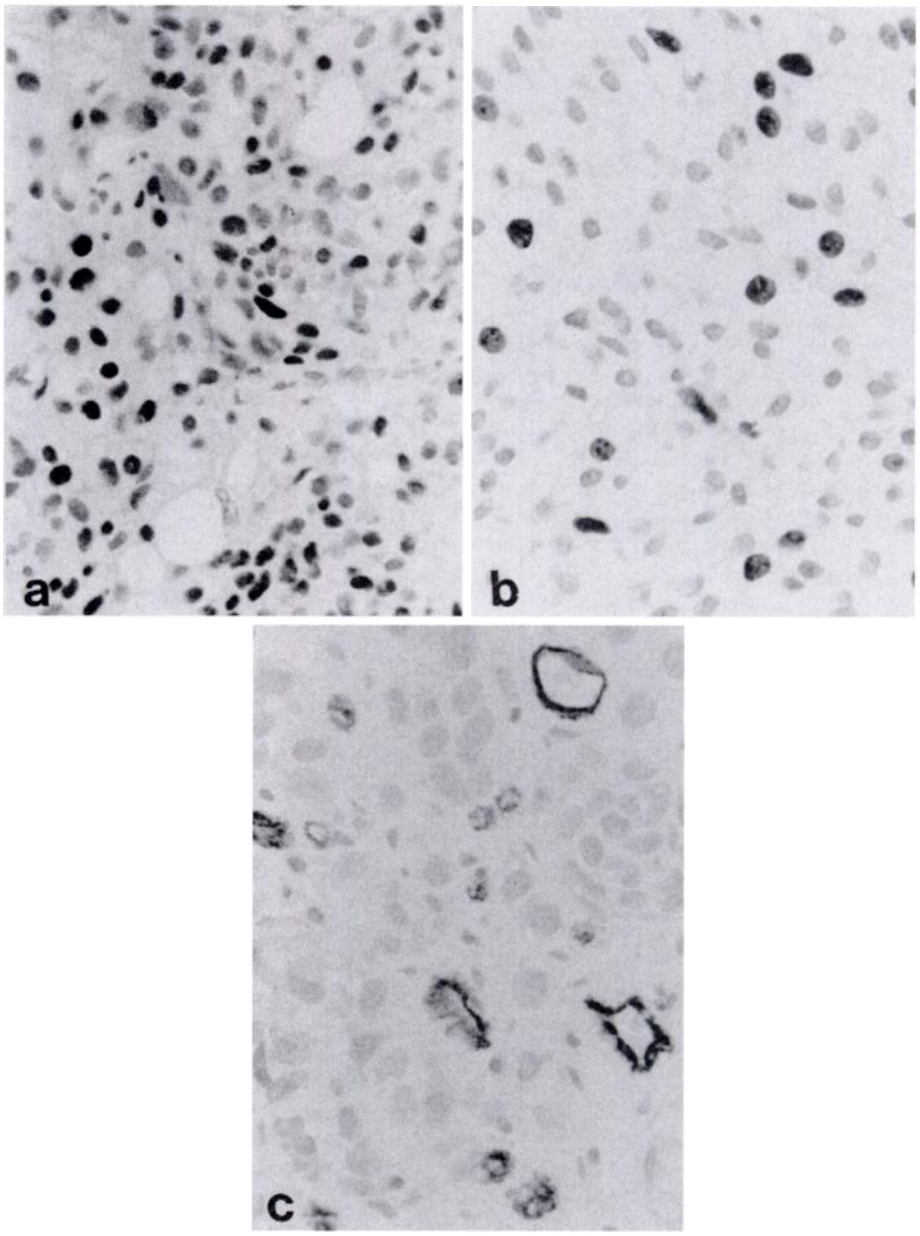

Fig. 1 Photomicrographs of selected cases of high-stage, high-grade transitional cell carcinomas of the urinary bladder using immunohistochemical staining with antibodies PAb1801 to p53 (*a*), MIB1 to Ki-67 (*b*), and anti-factor VIII-related antigen (*c*). *a* illustrates the intense nuclear staining of PAb1801 to p53 in tumor cells. *b* shows the high proliferative index phenotype, as determined by the expression of Ki-67 in the majority of tumor cells of this invasive bladder carcinoma. *c* depicts the high microvessel density identified in an invasive transitional cell carcinoma analyzed. *a*, ×200; *b* and *c*, ×250.

prise up to 30% of newly diagnosed bladder cancers. Crucial concerns regarding these cases are their metastatic potential and response to neoadjuvant regimens. We set out to address the dilemma of identifying the subset of advanced, high-grade bladder carcinomas that harbor the metastatic potential. The working hypothesis was that tumor cells that metastasize are both genotypically and phenotypically different from nonmetastatic variants and could be already identified in primary tumor samples. A cohort of well-characterized patients with metastatic and nonmetastatic muscle invasive bladder cancer was identified, and a panel of tumor markers was assessed to investigate their predictability on the development of lymph node metastatic disease.

In the nonmetastatic group, p53 nuclear overexpression was identified in 15 of 52 (29%) cases, whereas the remaining 37 (71%) cases showed a p53-negative phenotype. Overexpression of p53 was detected in 25 of 57 (44%) metastatic cases (Fig. 1*A*), whereas 32 (56%) cases had a p53-negative phenotype. There was a difference between these groups approaching but not reaching statistical significance ($P = 0.1$), which could be due to the relatively small sample size. Altered patterns of p53 expression, mainly nuclear overexpression in tumor cells identified by immunohistochemistry, have been reported to stratify bladder cancer patients in good *versus* poor risk categories (8–11). However, several previous studies have shown that the p53 phenotype is independent of lymph node status in predicting recurrence and survival (10, 21, 22). Data from this study further support the concept that p53 nuclear overexpres-

sion is not highly associated with stage of disease in bladder cancer, and that its effects on tumor progression are independent of stage.

The median value for Ki-67 (MIB1 antibody) in the cohort studied was found to be 18% of tumor cells displaying nuclear immunoreactivities. High proliferative index (≥18% positive tumor cells) was found in 12 of 26 (46%) evaluable nonmetastatic tumors and 27 of 52 (52%) evaluable metastatic tumors studied (Fig. 1*B*). These data did not approach significance. As an alternative, we also conducted this analysis by using ≥20% positive tumor cells as a cutoff point, because it has been reported in several studies as a conventional figure for assessment of high proliferative index (12, 13). Using this score, 42% of the nonmetastatic lesions were found to have marked proliferation, whereas 46% of the metastatic cases showed a high Ki-67 index. Once again, these data did not have statistical significance. Similar data have been reported in other studies dealing with Ki-67 and bladder cancer (23, 24), where high Ki-67 index was found to be a reliable assay to assess cellular proliferation in transitional cell carcinomas, correlating mainly with tumor grade but not with tumor stage or lymph node status.

The median value for microvascular counts was established at 40 microvessels/area screened. This parameter segregated 15 of 26 (57%) evaluable nonmetastatic and 26 of 54 (48%) evaluable metastatic cases as having high counts (Fig. 1*C*), respectively. No statistical association was found between high microvascular counts and the presence or absence of lymph node metastases in this cohort of patients. Several studies have revealed the association between high vascular counts and poor survival in patients with high-stage bladder tumors (25, 26). However, this was not the end point of the present study. Furthermore, these studies and several other reports have shown no relationship between high vascular counts and either stage, grade, or lymph node status (14, 25–27). An important consideration in the analysis of data reported in studies dealing with characterization of tumor angiogenesis is the primary antibody used. Several authors favor the use of anti-CD31 and/or anti-CD34 monoclonal antibodies, based on their ability to identify neovascular capillary beds. Another important variant is the cutoff point used in different studies to define high angiogenic activity. Usually the mean count of several preselected areas rich in neovasculature is used as the cutoff value; however, the reported mean value for invasive bladder cancer varies from 24 counts (25), to 79 counts (27), to 138 mean counts (14). The present study was conducted using an antiserum to factor VIII-related antigen and a mean count value of 40 microvessels/area screened.

TSP[3]-1 is a potent inhibitor of angiogenesis, the expression of which is under the control of p53 (28). Functionally altered p53 products down-regulate TSP-1, enhancing tumor angiogenesis. In a recent study, Grossfeld *et al.* (28) reported the association between altered expression of TSP-1 and high microvessel density counts. However, this study also revealed the lack of association between low levels of TSP-1 and lymph node status. Nevertheless, low TSP-1 expression was significantly associated with disease recurrence and overall survival (28). It appears that alterations of p53 and angiogenesis are necessary but not sufficient for the generation of metastases.

Ploidy studies revealed that the majority of tumors analyzed were either aneuploid or tetraploid, with 20 of 28 (71%) and 30 of 50 (60%) of nonmetastatic and metastatic cases presenting with this status, respectively. No statistical associations were found between DNA content and metastatic potential. In a consensus conference on the clinical utility of DNA cytometry in bladder cancer, data concerning the role of DNA cytometry in muscle invasive tumors was inconclusive (18). The maximum utility of DNA cytometry is in stratifying grade 2 superficial tumors (18). Data from this study further support the consensus report, revealing the lack of association between DNA content and lymph node status.

The proper diagnosis and overall control of bladder cancer is a challenge in clinical oncology. Because the modality of therapy primarily depends on morphological evaluation and clinical staging, the diagnosis carries significant consequences. However, it is well known that morphologically similar tumors presenting in any assigned stage may behave in radically different fashions, a fact that seriously hampers the ability to accurately predict clinical behavior in a given case. During the past years there has been a tremendous amount of information generated dealing with the principles that govern cellular proliferation and the clinical significance of angiogenesis in certain neoplastic processes (29, 30). Biological markers, such as alterations of p53 and high microvessel density, have been found to correlate with an aggressive tumor behavior (8–11, 25, 26). Based on results summarized above, this study indicates that alterations of p53, proliferative index, microvascular counts, and ploidy status are not strongly associated with lymph node status in patients affected with high-stage, high-grade bladder cancer. This finding may further indicate that the association of these markers with the development of systemic metastases and death is independent of stage of disease. It is important to emphasize that the present study did not attempt to further evaluate the prognostic significance of the markers analyzed in bladder cancer but rather attempted to assess their potential association with the presence of lymph node metastasis at the time of primary surgery.

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[3] The abbreviation used is: TSP, thrombospondin.

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