It is becoming increasingly clear that no single marker will have the sensitivity and specificity necessary to be used on its own for diagnosis/prognosis of tumors. Interpatient and intratumor heterogeneity provides overwhelming odds against the existence of such an ideal marker. With this in mind, our laboratory has been applying a long term systematic approach to identify multiple biomarkers that can be used for clinical purposes. As a result of these studies, we have identified and reported several candidate biomarker proteins that are deregulated in bladder cancer. Following the conceptual biomarker development phases proposed by the Early Detection Research Network, we have taken some of the most promising candidate proteins into post-discovery validation studies, and here we report on the characterization of one such biomarker, the bladder cancer-associated protein (BLCAP), formerly termed Bc10. To characterize BLCAP protein expression and cellular localization patterns in benign bladder urothelium and urothelial carcinomas (UCs), we used two independent sets of samples from different patient cohorts: a reference set consisting of 120 bladder specimens (formalin-fixed as well as frozen biopsies) and a validation set consisting of 2,108 retrospectively collected UCs with long term clinical follow-up. We could categorize the UCs examined into four groups based on levels of expression and subcellular localization of BLCAP protein and showed that loss of BLCAP expression is associated with tumor progression. The results indicated that increased expression of this protein confers an adverse patient outcome, suggesting that categorization of staining patterns for this protein may have prognostic value. Finally, we applied a combinatorial two-marker discriminator using BLCAP and adipocyte-type fatty acid-binding protein, another UC biomarker previously reported by us, and found that the combination of the two markers correlated more closely with grade and/or stage of disease than the individual markers. The implications of these results in biomarker discovery are discussed. 

BLCAP, a Potential Prognostic Biomarker in Bladder Cancer

may form the basis for improved diagnosis and prognosis of patients with bladder cancer as well as identify novel potential targets for therapeutics (4). Toward this aim, we have examined the protein expression profiles of more than 1,000 samples (benign as well as tumors of various histopathological grades and stages) using gel-based proteomics, and we have identified a number of potentially useful biomarkers, such as adipocyte-type fatty acid-binding protein (A-FABP), 14-3-3 protein isoform σ, psoriasin (S100A7), GST Mu, 15-hydroxyprostaglandin dehydrogenase, tropomyosin 4, disulfide isomerase precursor (ERP60), homeobox protein (HOX-1.3), keratins 8 and 13, MRP-14, CD24, and the cytochrome oxidase III subunit among others (5–12), whose expression strongly correlates with a particular step of bladder cancer progression.

Recently, we identified bladder cancer-associated protein (BLCAP) as a novel potential biomarker based on a limited study of 30 bladder UCs (13). We showed that loss of BLCAP mRNA expression correlates with the invasive potential of UCs, and subsequent studies by others in several cancer types, such as cervical (14, 15) and renal (16) as well as human tongue carcinoma (17) and osteosarcoma (18), have all shown differential expression of this protein in cancer. Moreover, overexpression of BLCAP in the human cervical cancer HeLa cell line (14, 15) and tongue carcinoma Tca8113 cell line (17) has been shown to inhibit cell growth and to induce apoptosis, suggesting a role for this protein in the regulation of these cellular processes. BLCAP is a small (87-amino acid), evolutionarily conserved protein with no homology to any known protein, and its cellular function is largely unknown. A computer-based search identified BLCAP as a target for RNA editing via adenosine to inosine (A-to-I) RNA editing catalyzed by members of the double-stranded RNA-specific adenosine deaminase acting on RNA (ADAR) family, a premise subsequently confirmed experimentally (19, 20). Selective A-to-I RNA editing is a mechanism to generate proteome variation that is carried out by the ADAR family of RNA molecules and is essential for life. Various diseases, such as epilepsy, depression, amyotrophic lateral sclerosis, and cancer, have been associated with a defective pattern of RNA editing (21–29). Although BLCAP has been shown to undergo A-to-I RNA editing (19, 20, 30–32), in bladder cancer, no correlation was found with altered BLCAP RNA editing levels and the development of transitional cell carcinoma (30). Also, the editing levels of a coding sequence target were only marginally higher in brain tumors (1.3-fold increase; p = 0.03) with lung and oral cavity samples showing no significant difference in editing levels between normal and cancer samples (26), suggesting that RNA editing of BLCAP transcript does not play a major role in cellular malignancy in these tissues either.

In the present study, we report on the detailed characterization of the BLCAP protein expression and cellular localization patterns in benign bladder urothelium and UCs in an effort to evaluate the value of BLCAP as a prospective biomarker in bladder cancer. We established novel reagents (antibodies) and characterized them extensively, and we developed and implemented a potentially useful assay, evaluated its reproducibility, and determined the appropriateness of the target for decision making in a very large number of well characterized samples (over 1,800 UCs were examined) with long term clinical follow-up. The design of the study allowed us to establish in a very large number of samples that BLCAP protein expression is lost with tumor progression. At the same time, we showed that BLCAP is overexpressed in ~20% of the cases examined and that overexpression is linked with poor survival, suggesting that BLCAP may also have prognostic value.

MATERIALS AND METHODS

Patient Tissue Specimens—One hundred and twenty bladder specimens, consisting of random biopsies judged to be histologically normal and UCs of various grades and stages, collected over a period of 6 years at Skejby Hospital, Aarhus, Denmark were analyzed. Tumors were classified by an experienced pathologist according to Bergkvist et al. (33). All tumors were evaluated according to tumor-node-metastasis (TNM) stages and morphological grades and are presented according to standard nomenclature (e.g. pTa1G1 is stage Ta, grade 1). The Scientific-Ethical Committee of Aarhus County approved the project.

Sample Preparation for Two-dimensional (2D) PAGE—Tumor samples clean of clots and contaminating tissue, as well as random biopsies diagnosed as non-malignant (clean of muscle and fat tissue) were dissected, split into small pieces with the aid of a scalpel, and subsequently labeled with [35S]methionine as previously described (6). Following labeling for 14–16 h, the medium was carefully aspirated, and the pieces were dissolved in O’Farrell lysis solution (6).

Proteomics Analysis—2D PAGE was performed as described previously (6). Gels were stained with silver nitrate and subjected to autoradiography. 2D gel autoradiographs were scanned using a Molecular Imager device (Bio-Rad) and were analyzed using PDQuest 7.1 analysis software (Bio-Rad). Only gels presenting well focused spots and showing a limited amount of protein remaining at the origin were selected for further analysis.

Immunohistochemistry and Antibodies—Immunohistochemistry assays were performed using paraffin sections of mouse and human tissue samples essentially as described (34). Tissue blocks were placed in formalin fixative and subsequently paraffin-embedded for archival use. Five-micrometer sections were mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany), baked at 60 °C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was performed by immersing the slides in Tris/EDTA pH 9.0 buffer (10 mM Tris, 1 mM EDTA) and microwaving in a 750-watt microwave oven for 10 min. Nonspecific staining was blocked using 10% normal matched serum, 0.3% H2O2 in PBS buffer for 30 min. Antigen presence was visualized by incubation for 1 h with an appropriate primary antibody followed by detection with a suitable species-matched secondary antibody conjugated to a peroxidase complex for 30 min (Envision+ poly-HRP (horseradish peroxidase) system, Dako, Glostrup, Denmark). Color development was done using DAB+ (3,3′-diaminobenzidine) chromogen (Dako). Slides were counterstained with hematoxylin. Standardization of the incubation and development times allowed accurate comparisons in all cases. The BLCAP-specific EPO21513 (raised against a mid-protein peptide) and EPO21514 (raised against a C-terminal peptide of BLCAP) and the A-FABP-specific EPO23215 rabbit polyclonal antibodies were prepared by Eurogentec (Brussels,

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RESULTS

BLCAP Protein Structure Analysis and Features: Generation and Characterization of Antibodies

BLCAP is a very small (87-amino acid; 10-kDa), evolutionary conserved protein (100% protein identity in Mammalia), and its cellular function is largely unknown. At present, there are no known human proteins with extended sequence similarity to BLCAP. We have shown previously, by hydropathy analysis (13), that the protein has two highly hydrophobic regions (positions 23–35 and 45–63). Although no matches to any known consensus were found, we demonstrated, by homology analysis of the individual hydrophobic and hydrophilic domains, that the second hydrophobic motif of 17 amino acid residues (positions 45–63) has high homology with transmembrane proteins (13, 39). Immunoblots of tissue lysates were prepared as described previously (12). Briefly, proteins were resolved by 2D gel electrophoresis, blotted onto Hybond™-C nitrocellulose membranes (Amersham Biosciences), and incubated with a BLCAP-specific rabbit polyclonal antibody (Eurogentec) followed by detection of immune complexes with a horseradish peroxidase-labeled polymer (1:200) (H2O2 detection kit, Dako) and 3,3'-diaminobenzidine (Dako) as a chromogen to detect bound antibody complexes. Slides were counterstained with hematoxylin.

Quantitative Assessment of IHC Staining and Data Analysis—An automated cellular imaging system, ACIS III (DAKO), was used to digitize and quantify the IHC staining of the TMA slides. The ACIS III system was used to derive a score of staining intensity for each core in a given TMA. Briefly, the digital images from scanned TMA sections were submitted to analysis by the TMA software analysis module that is part of the ACIS III system. A mean total intensity score was generated in this manner for each core.

Indirect Immunofluorescence Analysis—Five-micrometer sections cut from paraffin blocks of bladder tissue samples were mounted on Super Frost Plus slides (Menzel-Gläser), baked at 60 °C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was carried out as described above. Following antigen retrieval, sections were treated with Image-iT FX™ signal enhancer (Molecular Probes, Eugene, OR) to block nonspecific staining and subsequently incubated with the relevant primary antibodies at the appropriate dilution. Detection of immune complexes was done with species-specific secondary antibodies conjugated to Alexa Fluor® 488 and Alexa Fluor 594 (Molecular Probes). Nuclear material was counterstained with TO-PRO-3™. The sections were washed three times with cold PBS between incubations. Normal rabbit and mouse sera instead of primary antibody were used as a negative control. Sections were imaged using a Zeiss LSM510Meta confocal laser scanning microscope (Carl Zeiss Microlimaging GmbH, Göttingen, Germany).

2D Western Blot Analysis—The specificity of the BLCAP antibody was determined by 2D PAGE immunoblotting using protein extracts from COS1 cells overexpressing BLCAP according to published procedures (13, 39). Immunoblots of tissue lysates were prepared as described previously (12). Briefly, proteins were resolved by 2D gel electrophoresis, blotted onto Hybond™-C nitrocellulose membranes (Amersham Biosciences), and incubated with a BLCAP-specific rabbit polyclonal antibody (Eurogentec) followed by detection of immune complexes with a horseradish peroxidase-labeled polymer (1:200) (Envis+ detection kit, Dako).

Statistical Analysis—Contingency table analysis and χ2 tests were used to study the association between BLCAP expression/pattern of expression and histopathological classification (grade, stage, or grade/ stage). Survival curves were calculated using the Kaplan-Meier method with significance evaluated using the log rank test. To assess the influence of several variables (BLCAP and A-FABP expression and patient and tumor characteristics) on the survival curves, we performed multivariate analysis using the Cox proportional hazards model. Statistical analysis of data was done with NCSS version 2007 (NCSS, Kaysville, UT).
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Expression of BLCAP in Reference Set Composed of 120 Benign Bladder Biopsies and UCs

To study changes in the pattern of BLCAP expression that might take place during progression from normal bladder urothelium to bladder cancer, we analyzed a reference set of 120 bladder specimens consisting of random biopsies judged to be histologically normal and UCs of various grades and stages (24 non-malignant biopsies, 11 pTaG1, 29 pTaG2, 10 pTaG3, 13 pT1G3, 23 pT2–4G3, and 10 pT2–4G4 tumors). This sample set consisted of formalin-fixed paraffin-embedded (FFPE) tissue samples as well as matched fresh frozen biopsies. Analysis of the specimens was performed by IHC and in many cases by gel-based proteomics.

Immunohistochemistry

IHC Analysis of BLCAP Expression and Localization in Benign Specimens—IHC staining of benign bladder specimens showed that the BLCAP antigen localizes predominantly to the epithelial lining of the urinary bladder (urothelium) with weak to moderate cytoplasmic staining and interspersed moderate to strong nuclear staining of urothelial cells (Fig. 2, a and b, EPO23514, black arrow). Interestingly, nuclear staining was more frequent in the more differentiated and specialized umbrella cells that line the inner surface of the urinary bladder (Fig. 2, a and b, EPO23514, yellow arrow). Expression of BLCAP was not restricted to urothelial cells as we also detected immunostaining of vessels (Fig. 2a, EPO23514, white arrow). Moreover, sporadic nuclear staining of stromal cells was also observed. The specificity of the EPO23514 anti-BLCAP antibody for IHC analyses was further confirmed by staining consecutive bladder FFPE tissue sections with antibody preincubated with the corresponding immunizing peptide. As shown in Fig. 2c, exposure of the antibody to the immunizing peptide prior to IHC effectively blocked immunostaining of tissue sections (Fig. 2c, blocking peptide), confirming the specificity of the antibody toward the antigen. The observed expression pattern of BLCAP in non-malignant bladder tissues was further substantiated by using two additional anti-BLCAP antibodies: a second rabbit peptide-specific antibody raised against a mid-protein epitope (Fig. 2d, EPO23513) and a previously described (13) rabbit polyclonal antibody raised against the full-length recombinant BLCAP protein (Fig. 2e, antibody P1T1). The staining pattern obtained in IHC-based analyses.

Fig. 1. a, structural model for BLCAP protein based on various predictive tools. According to various prediction tools, BLCAP is potentially a transmembrane protein with three putative phosphorylation sites (circled P) in the C terminus, a signal peptide (signalP) in the N-terminal part of the protein, and a cleavage site at position 20 (black arrowhead), b, determination of antibody specificity for EPO23514 by 2D PAGE Western immunoblotting (IEF) analysis of lysates from mock-transfected COS1 cells (COS) or COS1 cells overexpressing BLCAP (COS-BLCAP); a full sized immunoblot is shown for COS1 cells overexpressing BLCAP (left-hand panel) with matched relevant sections of the immunoblots from mock-transfected COS1 cells or COS1 cells overexpressing BLCAP shown in greater magnification in the right-hand panels.

(region 1–19) with a single cleavage site (position 20) was predicted by SignalP (44) (shown in supplemental Fig. 1c). Based on these data, a working model of BLCAP was created (Fig. 1a) that was used to design two peptide-specific anti-BLCAP antibodies: EPO23514 raised against a unique C-terminal peptide of BLCAP (positions 74–87) and EPO23513 raised against a mid-protein epitope (positions 29–42). These antibodies, in addition to a previously described (13) rabbit polyclonal antibody raised against the full-length recombinant BLCAP protein, were used in this study to investigate the expression patterns of BLCAP in UCs.

Antibody specificity was determined by 2D Western blot analysis as described under “Materials and Methods,” and the results for EPO23514 are presented in Fig. 1b. Two polypeptide spots were detected in COS1 cells transiently transfected with BLCAP: a major spot corresponding to the predicted position of BLCAP (Fig. 1b, COS-BLCAP, black arrow), and one minor, more acidic spot (Fig. 1b, COS-BLCAP, white arrow). No antigen could be detected in mock-transfected COS1 cells (Fig. 1b, COS). To establish optimal IHC staining conditions and antibody performance in IHC assays, a preliminary tissue cross-reactivity screening study based on the Food and Drug Administration recommendation for antibody cross-reactivity testing was conducted using 33 different types of normal tissues. This IHC-based analysis, which will be published elsewhere, was analogous to the tissue-specific pattern of BLCAP mRNA expression (Ref. 13 and Gene Expression Omnibus GSE1133 data sets), supporting the conclusion that the antibody is specific toward the BLCAP protein in IHC-based analyses.
with these two additional anti-BLCAP antibodies was analogous to the pattern generated with the EPO23514 antibody, reiterating that BLCAP is expressed in the vasculature (Fig. 2, d and e, EPO23513 and P1T1, respectively, white arrows) and in bladder epithelial cells (Fig. 2, d and e, EPO23513 and P1T1, respectively, black arrows) with increased frequency of cells with nuclear expression in the umbrella cells (Fig. 2, d and e, EPO23513 and P1T1, yellow arrows).

Examination of highly vascularized areas showed that BLCAP localization to vessels was generally limited to the

![Image](image_url)
endothelial cells (ECs) lining the vessels (illustrated in Fig. 2f, black arrow). In some instances, however, we also observed immunoreactivity for the BLCAP antigen within vessels (Fig. 2f, inset). To verify that BLCAP was expressed in vascular ECs, we performed double immunofluorescence analysis of bladder FFPE tissue sections using antibodies against VEGFR-3 (Fig. 2g) and LYVE-1 (data not shown), two lymphatic vessel markers (45, 46), and von Willebrand factor (vWF; Fig. 2h), an endothelial cell marker (47, 48). As illustrated in Fig. 2h, BLCAP was expressed in vascular ECs, primarily in blood vessels (Fig. 2h, compare BLCAP expression in upper panel with vWF expression in lower panel). Although most lymphatic vessel ECs did not stain for BLCAP (Fig. 2h, BLCAP upper panel, black arrow), expression of this protein was not restricted to blood vessel ECs as we were able to detect some rare VEGFR-3-positive lymphatic ECs that also expressed the protein (Fig. 2g). This observation was corroborated by double immunofluorescence staining of bladder tissue sections with BLCAP and LYVE-1 (data not shown).

**Table I**

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<tr>
<th>Pathology classification</th>
<th>BLCAP staining pattern</th>
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<sup>a</sup> Moderate/strong cytoplasmic staining with strong nuclear staining (>80% of urothelial cells).

<sup>b</sup> Weak cytoplasmic staining with moderate/strong interspersed nuclear staining (>80% of urothelial cells).

<sup>c</sup> Weak cytoplasmic staining with weak or no nuclear staining.

<sup>d</sup> No staining.

<sup>e</sup> Carcinoma in situ.

**Fig. 3.** Immunohistochemistry analysis of BLCAP expression in bladder tissue specimens. Patterns of expression of BLCAP were categorized into four staining types. These are illustrated with an invasive UC (specimen MOB 154-2) showing type A immunoreactivity for BLCAP (a), a low grade UC lesion scored by IHC as type B (specimen MOB 763-2) (b), a malignant lesion showing low level homogenous expression of BLCAP classified as type C staining (c), and a lesion with no immunoreactivity for BLCAP (type D staining) (d). e and f, levels of protein expression in lysates from the same specimens assessed by 2D Western blot analysis (antibody EPO21514). For comparison purposes, in the Western blots, a section of the corresponding silver-stained gels is shown with the position for actin indicated.
included, with one single exception, all of the benign samples (exemplified in Fig. 2b). Type C was defined as weak cytoplasmic staining with weak/absent nuclear staining (illustrated in Fig. 3c). Type D included all samples with no detectable immunoreactivity (exemplified in Fig. 3d).

A specimen was assigned to a given staining type when more than 90% of it matched the parameters established for that category. In a few cases, because of tumor heterogeneity, a specimen was judged to consist of two or more types. All slides were independently reviewed by two of the authors, and in the few discrepant cases, a consensus was reached after joint review.

Weak immunoreactivity for BLCAP with interspersed nuclear staining (type B staining) was predominant in benign lesions (24 of 25 samples), and it was more frequent in non-invasive (17 of 48 pTa tumors; 35.4%) than in invasive (8 of 41 pT2–4 tumors; 19.5%) UCs. Equally, invasive tumors tended to lose expression of BLCAP with 21 of 41 pT2–4 tumors (51.2%) showing no expression of BLCAP (type D staining) compared with 13 of 48 pTa tumors (27.1%). Increased levels of nuclear BLCAP expression (type A staining) were observed in one single benign sample and in a median of 19.2 ± 2.5% of all the examined tumor specimens, independently of histological grade and stage. Also, type C staining patterns were rather ubiquitous, accounting for 21.2 ± 6.6% of all specimens, and also without a clear correlation to grade of atypia or stage of invasion.

In all cases, we found a good correlation between immunoreactivity of samples analyzed by IHC and protein expression levels ascertained by Western immunoblotting as shown in Fig. 3 (compare a and b with e and f, respectively). In general, samples showing strong IHC reactivity for BLCAP (Fig. 3a, type A tumor) exhibited high expression levels of this protein in 2D immunoblots (Fig. 3e), whereas specimens showing low/moderate or no IHC reactivity (Fig. 3b) displayed correspondingly lower levels or absence of the protein (Fig. 3f). Furthermore, the relative levels of post-translationally modified BLCAP (Fig. 3, e and f, white arrows) also increased in a proportional manner in samples showing higher immunoreactivity, indicating that the increased reactivity seen in some samples correlated with increased overall expression and not with a preferential enhancement in the expression of a modified form of BLCAP.

In addition to establishing a correlation between BLCAP expression and progression of UCs, our IHC analysis of BLCAP expression allowed us to make an interesting observation. Down-regulation of BLCAP has been demonstrated in bladder (13), cervical (14, 15), and renal cancer (16) as well as in human tongue carcinoma (17) and osteosarcoma (18). In addition, overexpression of this protein in the human cervical cancer HeLa cell line (14, 15) and tongue carcinoma Tca8113 cell line (17) inhibited cell growth and induced apoptosis, indicating a role for BLCAP in the regulation of cell proliferation and apoptosis. Together, these observations have led others to suggest a role for BLCAP in epithelial cell malignancy. However, careful analysis of the 72 urothelial carcinomas (constituting 60% of the 120 specimens that comprise the reference sample set) with no detectable expression of BLCAP, ascertained by lack of immunoreactivity in IHC analysis (type D staining), showed that in all cases loss of BLCAP expression was a collective event as both epithelial and endothelial cells lacked BLCAP immunoreactivity. This was not due to a technical artifact because in one specimen in particular (Fig. 4, a and b, MOB 1742-1) we could observe a gradient of BLCAP expression in malignant cells within the same sample (Fig. 4a) with the staining ranging from low/moderate expression to loss of expression with both vascular (Fig. 4a, compare black arrow with white arrow) and endothelial cells following this pattern (Fig. 4a, compare yellow arrow with red arrow). A consecutive section of the same specimen incubated with an antibody against A-FABP (6) is presented in Fig. 4b to illustrate that the gradual loss of expression observed for BLCAP was not displayed by other markers (Fig. 4, cf. a with b, yellow arrows). In all cases, staining of tandem sections with antibodies against cytokeratins 5, 8, and 13 was used as additional staining controls to rule out possible artifacts (data not shown).

**Gel-based Proteomics**

In an effort to validate the IHC studies, we carried out a systematic 2D PAGE analysis of most of the bladder biopsies of both non-malignant and tumor origin that reacted positively with the antibodies in IHC. Silver staining of the 2D gels (Fig. 5, a, c, and d) as well as in some cases metabolic labeling with [35S]methionine for increased sensitivity (Fig. 5b) revealed that the BLCAP protein in all cases was expressed at levels that were below the detection limits of the assays and that could only be detected by 2D PAGE Western immunoblotting (cf. Figs. 4f and 5b). This was true both for whole lysates from
samples showing normal-like (IHC staining type B) patterns of expression (Fig. 5, a and b) and for samples showing overexpression of BLCAP (type A IHC staining; Fig. 5c). In addition, we could not detect BLCAP in 2D gels of the interstitial fluid that bathes the bladder tumor microenvironment (illustrated in Fig. 5d), indicating that our inability to detect BLCAP in whole tissue lysates was not due to externalization of this protein to the extracellular space but rather to the overall low levels of expression of this protein.

Analysis of BLCAP Expression Patterns in Sample Set Consisting of 2,108 UCs: Correlation with Clinicopathologic Status of Bladder Tumors

To ensure good statistical validity and considering that having an independent test sample set is an important feature in the validation of biomarkers, we proceeded to evaluate the potential relevance of BLCAP as a tumor marker by IHC analysis using a large TMA containing 2,317 samples from 1,849 bladder cancer patients (36). In 1,730 of the 2,108 UC cases (82%), the IHC yielded interpretable results (illustrated in Fig. 6a), and these are summarized in Table II. As in the case of the reference sample set, we found that the BLCAP protein expression patterns correlated with grade of atypia ($p < 0.0001$) and stage of invasion ($p < 0.0001$). Type B staining of BLCAP antigen, which was predominant in benign lesions as shown above, tended to be more prevalent in G1 (18.9%) and pTa non-invasive tumors (10.8%) than in G3 (2.6%) and pT2–4 (2.3%) invasive tumors, respectively. Loss of BLCAP expression (type D staining) was also associated with progression with 14.2% of G1 and 19.6% of pTa tumors showing no expression of BLCAP as opposed to 26.7% of G3 and 34.5% of pT2–4 tumors, respectively. Types C and A staining were ubiquitously present, accounting for about 45 and 25% of all tumors, respectively, independently of grade of atypia or stage of disease.

The data also showed that loss of expression of BLCAP is significantly correlated with grade and stage of disease (Table II; $p < 0.0001$). Thus, whereas only 14.1% of pTaG1 specimens showed no immunoreactivity for BLCAP, this number was 34.7% in pT2–4G3 tumors. Concomitantly, the number of specimens showing a type B staining pattern decreased from 18.9% in pTaG1 tumors to 2.0% in pT2–4G3 UCs.

Absolute Cellular Levels of BLCAP Staining as Means of Categorizing Tumors

Although statistically, and presumably clinically, significant, our categorization of samples according to patterns of staining is an intrinsically subjective approach requiring estimation of variable protein staining intensities as well as of the distribution pattern of the staining. Because we found a good correlation between protein expression as determined by Western immunoblotting and IHC staining, we evaluated the expression of BLCAP in the specimens present in the validation TMA using a computer-assisted image analysis system, ACIS III, with a TMA-specific application (49, 50). This application overlays a user-defined grid onto an image of the TMA, thus identifying the cores to be quantified and generating quantitative data regarding intensity of brown staining for each identified core with a linear relationship (illustrated in Fig. 5a, right-hand panels). It should be noted that this approach is not able to discriminate between the relative contribution of cancer cells and endothelial cells. It is conceivable that samples containing highly vascularized areas can present skewed score values representing a possible confusion factor in any analysis based on averaging methodologies.

In agreement with the studies described in the previous section, we found that the overall expression of BLCAP decreased with increasing grade of atypia (Fig. 6b; $p < 0.0001$). Thus, not only did the number of specimens showing no immunoreactivity for BLCAP increase with progression, but also a progressive down-expression of this protein was found.
with increasing grade of atypia (Fig. 6b; G1 versus G2 versus G3, Kruskal-Wallis analysis of variance test, \( p < 0.0001 \)). Correlation of BLCAP staining scores with tumor invasion (pT1 and pT2–4) showed that expression of BLCAP followed a similar behavior; overall expression of this protein decreased with invasion (pT1 versus pT2–4, Mann-Whitney analysis of variance test, \( p < 0.0001 \)).

**Prognostic Relevance of BLCAP Expression in UCs**

Next, we examined BLCAP expression for its prognostic relevance using the definition of prognostic factor proposed by G. M. Clark (51): “a prognostic factor is a measurement that is associated with clinical outcome in the absence of therapy or with the application of a standard therapy that patients are likely to receive.” We identified a total of 1,081 patients, with specimens present in the bladder TMA used in this study, for whom clinical data and interpretable BLCAP stainings were concurrently available. We found that the pattern of BLCAP expression, as determined by our IHC classification, had significant prognostic value with survival being correlated to the type of staining (\( p = 0.0002 \); Fig. 7a, all tumors). Thus, patients with samples with type A staining (\( n = 243 \)) had a significantly shorter disease-specific survival than patients with tumors with type B (\( n = 80 \)), type D (\( n = 276 \)), or type C staining (\( n = 482 \)).
Survival analysis performed by the Cox proportional hazards model using the ACIS III intensity score as a continuous variable showed no prognostic value of the overall expression of BLCAP (hazard ratio (HR), 0.9992; 95% CI, 0.9943–1.0042). Dichotomization of intensity scores by use of the score median (intensity score, 121) and the 25th (intensity score, 112) and 75th (intensity score, 131) percentiles showed that none of these cutoff points were statistically significant (illustrated for score median in Fig. 7b; HR, 1.001; 95% CI, 0.7536–1.330), confirming that there was no significant association between overall expression of BLCAP and disease-specific survival.

Given that loss of BLCAP expression is associated with progression, itself a poor prognosis factor, we determined correlation of BLCAP staining with prognosis among patients bearing highly invasive tumors only (pT2–4). We found that expression of BLCAP correlated with disease-specific survival in these patients in an inverse fashion (\( \rho = 0.0035 \); Fig. 7c, pT2–4 tumors). In these cases, patients with tumors lacking BLCAP (type D; \( n = 77 \)) had a better prognosis than patients bearing tumors with type A (\( n = 107 \)), type C (\( n = 99 \)), or type B staining (\( n = 7 \)). Dichotomization of BLCAP to the cutoff point for staining intensity scores of BLCAP corresponding to lack of immunoreactivity (intensity score, 105; determined from no-antibody control stainings) was prognostic of disease-specific survival (HR, 1.455; 95% CI, 1.113–1.900) (Fig. 7d). The interesting additional implication of these results is that expression of BLCAP in tumors may worsen disease outcome for patients.

We also found that there was an increased likelihood of recurrence and shorter time to progression in samples showing type A staining (median, 13-month disease-free survival, 35 months to progression) as compared with type D staining (median, 19-month disease-free survival, 43 months to progression). However, none of these associations reached statistical significance.

**Addressing Molecular Mechanisms Involved in BLCAP Deregulation**

The interspersed character of the nuclear expression of BLCAP in benign samples was suggestive of an intermittent event such as cell cycle progression or a signaling event. Furthermore, the staining of serial sections with antibodies against BLCAP and cell cycle-regulated proteins (Ki67, cyclin D1, and proliferating cell nuclear antigen) showed no correlation (illustrated in Fig. 8a for Ki67 and data not shown). The one benign specimen that showed type A BLCAP staining displayed overall strong nuclear expression (data not shown), indicating that cellular differentiation state is not a major determinant of BLCAP overexpression and nuclear localization. Also, staining of serial sections with BLCAP and known differentiation state-specific proteins (uroplakin and 15-prostaglandin dehydrogenase) and oncoproteins (p53, c-Jun, and ErbB2) did not show any significant correlation (data not shown), suggesting that nuclear localization of BLCAP may instead occur in response to stimuli from the microenvironment.
Our studies showed that about 20% of the lesions examined displayed strong nuclear expression of BLCAP (type A staining) and that this phenotype was associated with overall poor disease outcome. We addressed the possible nuclear function(s) of BLCAP by performing indirect immunofluorescence analysis of some of the FFPE tissue samples that showed type A or type B staining. We found that BLCAP was diffusely distributed throughout the nucleoplasm and showed a strong, irregular, punctate fluorescent pattern that did not include the nucleoli (Fig. 8b, BLCAP). This nuclear staining pattern is compatible with localization of this protein to a nuclear body/domain such as Cajal bodies, nuclear speckles, or promyelocytic leukemia bodies (52, 53). To address this possibility, we performed indirect double immunofluorescence analysis using antibodies against BLCAP and proteins that are archetypal markers of these different nuclear bodies (coilin, SF2/ASF, and promyelocytic leukemia protein) (53). We found that BLCAP co-localized with SF2/ASF (Fig. 8b), demonstrating its association with nuclear speckles and suggesting an involvement of BLCAP in RNA metabolism, an interesting possibility given that BLCAP mRNA undergoes ADAR-mediated selective A-to-I RNA editing (19, 20).

**Multiprotein Pattern Analysis of UCs**

The old paradigm of one protein-one biomarker-one clinical decision is currently shifting with single biomarker analysis being replaced by multiparametric analysis of genes or proteins, and protein patterns are currently considered to offer better diagnostic possibilities than a single biomarker (54). Earlier we presented evidence that loss of A-FABP expression correlates with both tumor stage and tumor grade (11), and therefore it seemed relevant to ascertain whether the combination of BLCAP and A-FABP would be advantageous as compared with a single marker setup. To address this possibility, we stained the reference set of 120 bladder specimens with an antibody specific for A-FABP (11). As illustrated in Fig. 9, staining of non-malignant specimens showed that both the cellular expression and the localization of BLCAP and A-FABP were very different (Fig. 9, a and b, respectively) with A-FABP staining being stronger in the basal and suprabasal layers of the transitional epithelia and weak, but detectable, in the intermediate and more differentiated cell layers (Fig. 9b). Nuclear expression of BLCAP was not correlated with that of A-FABP as basal cells expressing A-FABP at high levels did (Fig. 9c, white arrow) or did not (Fig. 9c, yellow arrow) display a nuclear presence of BLCAP.

We originally categorized UCs into six groups based on their A-FABP IHC staining pattern (types A–F) (11); these are illustrated in Fig. 10a. Combining the different groups defined by categorization of IHC patterns of the two markers, BLCAP
and A-FABP, generates 24 possible outcomes, but most of these are only represented by a few cases in our TMA. To simplify the multimarker-based analysis of the UCs in the TMA while allowing for sufficient number of cases in each category for appropriate statistical analysis, we reclassified the IHC stainings of all samples in the TMA using a simpler categorization system we termed normal-like/up-regulated/down-regulated (NUD) score. Thus, A-FABP staining types B and D were classified as normal-like (NA-FABP); types C, E, and F were classified as down-regulated (DA-FABP); and type A was classified as up-regulated (UA-FABP). Similarly, BLCAP staining type B was reclassified as normal-like (NBLCAP), types C and D were classified as down-regulated (DBLCAP), and type A was classified as up-regulated (UBLCAP). Correlation of the combined classifiers with grade of atypia and/or stage of disease is shown in Table III. As can be seen, some classifiers such as UBLCAPA-FABP, which applies to 6.2% of pTaG1 tumors and 31.2% of pT2−4G3 tumors, respectively, and classifier DBLCAPD-FABP, which applies to 17.6% of pTaG1 tumors and 0.8% of pT2−4G3 tumors, respectively, correlate very well with grade and/or stage of disease, indicating that multimarker categorization reflects the pathological state of UCs more closely than the single marker patterns.

Single marker association analysis with disease-specific survival showed that the NUD scores for BLCAP (\(p = 0.0013\)) and A-FABP (\(p = 0.001\)) have prognostic value with up-regulation of BLCAP (UBLCAP; Fig. 10b, U) and down-regulation of A-FABP (DA-FABP; Fig. 10c, D) being associated with poor disease outcome. The combination of the two markers showed a stronger association with disease outcome (\(p < 0.0001\)). The groups that do not contain any of the poor prognostic parameters (NBLCAPNA-FABP, NBLCAPUA-FABP, DBLCAPNA-FABP, and DBLCAPUA-FABP; Fig. 8d, NN, NU, DN, and DU, respectively) had the best prognosis followed by the
groups containing a single poor prognostic parameter (\(N^{\text{BLCAP}}_{\text{DA-FABP}}\) and \(D^{\text{BLCAP}}_{\text{DA-FABP}}\) or \(U^{\text{DBLCAP}}_{\text{NA-FABP}}\) and \(U^{\text{BLCAP}}_{\text{UA-FABP}}\); Fig. 8d, ND and DD or UN and UU) with the group containing both poor prognostic parameters (\(U^{\text{ULCNP}}_{\text{DA-FABP}}\) having the worse disease outcome, suggesting that the combination of these two biomarkers in multimarker analyses appears to add prognostic information (Fig. 10d).

### DISCUSSION

Detecting a tumor at an early stage and predicting how it will behave are critical parameters in the clinical management of patients with malignancy of the urinary bladder. Current prognostic parameters such as grade and stage, multifocality of carcinomas, and lymph node status cannot predict with certainty the long term outcome of bladder cancer, and as a result, there is a pressing need to identify individual biomarkers or a group of biomarkers that may be associated with bladder cancer progression and that predict tumor behavior. The need to predict which superficial tumors will recur and/or progress has been a major driving force in our studies, and toward this aim, we have examined the protein expression profiles of more than 1,000 biopsies (both benign as well as of tumor origin of various histopathological grades and stages) using gel-based proteomics (2D gels). As a result of these studies, we have identified several potentially useful biomarkers (55). Differential protein expression of a few of these markers, including 14-3-3 protein isoform \(\sigma\) (12) and A-FABP (11), were confirmed using specific antibodies and large sample sets.

One of the main hurdles to the implementation of molecular markers into routine clinical practice is that most studies reporting the discovery of a novel cancer biomarker are largely of exploratory nature, and more often than not, promising results in the initial studies cannot be corroborated in follow-up studies. Moreover, there is often a substantial time gap between the original observation and subsequent validation studies, a crucial next step in the development of a biomarker for clinical use. The National Cancer Institute’s Early Detection Research Network has set forth a series of five phases for developing biomarkers (56). Although this proposal does not solve all problems associated with biomarker development (for a broader discussion on the subject see Ref. 57), it does provide a formalized framework to guide this process and has been adopted by the Early Detection Research Network itself as well as other biomarker research projects. As a first approximation, our laboratory is currently in the process of validating the proteins we previously reported as being deregulated in bladder cancer as potentially useful biomarkers of malignancy, alone or in combination, with the aim of subsequently testing those that show some promise of clinical usefulness. Thus, as part of this ongoing effort, we have so far examined and characterized in more detail the expression of two potential biomarkers, A-FABP and 14-3-3 protein isoform \(\sigma\) (11, 12), and here we report on a third biomarker, BLCAP. These studies are important as they represent a required key step toward the development of a novel biomarker following its discovery.

We generated and characterized antibodies that specifically recognize BLCAP and were able to show that BLCAP localizes predominantly to the epithelial lining of the urinary bladder with weak to moderate cytoplasmic staining and interspersed moderate to strong nuclear staining of urothelial cells. We found that BLCAP IHC staining pattern types B and D, which were generally associated with benign/low grade and high grade invasive lesions, respectively, may be valuable as diagnostic indicators even if the type A and C staining
patterns are not good classifiers because they appeared rather ubiquitously in all grades and stages. Interestingly, staining type A was significantly associated with poor disease-specific survival. 2D Western blot analysis of samples classified by IHC as type A or B (Fig. 3, e and f, respectively) showed that the increased immunoreactivity for BLCAP antigen observed by IHC in specimens corresponds to elevated levels of protein expression and that expression of both polypeptides (unmodified and modified forms; Fig. 3, e and f, black and white arrows, respectively) is increased. These data indicate that loss of BLCAP expression is associated with tumor progression, whereas an increased percentage of cells with high levels of nuclear BLCAP confers a poor prognosis. We showed that BLCAP is overexpressed in ~20% of the cases examined and is linked with poor survival, suggesting that BLCAP expression may be of prognostic value. In invasive tumors, expression of BLCAP confers an adverse patient outcome with patients bearing tumors that have lost expression of BLCAP faring better than those with tumors expressing BLCAP at any levels of expression (Fig. 7c, pT2–4 tumors).

The concomitant loss of expression of BLCAP we observed in both bladder epithelial and vascular endothelial cells suggests that this phenomenon most likely reflects a change(s) in the cellular microenvironment rather than being a causal process in epithelial carcinogenesis. Several different cancer types, such as cervical cancer (14, 15), renal cancer (16), human tongue carcinoma (17), and osteosarcoma (18), have all been shown to have differential expression of BLCAP, implying that the change(s) in the microenvironment that triggers this cellular response may be of a general character rather than tissue-specific.

Clearly, the expression of BLCAP in bladder tissue is under various regulatory constraints, and Fig. 11 depicts a working model for the regulation of BLCAP expression that takes into consideration the different influences and staining types we observed. On the one hand, interspersed nuclear expression seems to be a response to cues/stimuli from the microenvironment, whereas on the other hand, expression is also dependent on cell differentiation. Another possible degree of complexity lies in the manifestation of genetic or epigenetic alterations that lead to epithelial overexpression of BLCAP in ~20% of all tumor samples irrespective of tumor stage/grade (Table II). The latter suggest that some malignancy-associated event directly or indirectly, for example as a result of activating mutations in a transcriptional regulator, leads to increased expression of this protein. It should be noted that all examined samples showing no immunohistochemical reactivity to BLCAP had concurrent loss of expression in endothelial cells, ruling out the possibility that loss of expression can also be due to genetic or epigenetic changes. In addition, although some samples displayed augmented relative levels of BLCAP, assessed by either increased immunohistochemical reactivity or Western blot analysis, the absolute levels of this protein in all cases were never within the detection limits of 2D gel-based analyses. These data are consistent with the observation that high levels of BLCAP protein are deleterious for cells.2

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*Fig. 11. Working model for regulation of BLCAP expression. The expression of BLCAP is interspersed nuclear in benign specimens and can remain so in tumors, but changes in the microenvironment associated with tumor progression lead to loss of BLCAP expression, presumably in a progressive manner, which would generate the samples showing homogeneous low level expression of BLCAP. Genetic/epigenetic alterations and/or alterations in the tumor microenvironment can lead to overexpression of BLCAP with a strong nuclear presence.*

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2 J. M. A. Moreira, unpublished observation.
During the last two decades, a large number of molecular markers of bladder cancer with potential diagnostic and prognostic value have been identified, but when tested for their prognostic value, most markers have failed to provide any added prognostic information as compared with the clinicopathological index of the primary tumor. Presently, there is not a single marker able to predict with accuracy the biological potential of bladder cancer, and as a result, molecular prognostication is moving toward a multiplex marker setting (58–62). Multimarker phenotype analysis has been used by different groups to increase the diagnostic or prognostic value of markers. The combination of candidate markers spanning different biological pathways, in particular, has been investigated. We have explored a similar approach but using our own markers. Accordingly, we tried to determine whether the combination of two of the markers we have identified would be advantageous compared with a single marker setup. We used a two-marker discriminator that included BLCAP and A-FABP, another marker that we have previously identified (11), and found that it correlates very well with grade and/or stage of disease, suggesting that multimarker categorization using these two markers may reflect the pathological state of UCs more closely than using single markers on their own. In addition, the combination of these two markers showed a stronger association with disease outcome than single marker analysis, suggesting that combination of these markers in multimarker analyses may add prognostic information (Fig. 10d). Additional studies using more homogeneous sample cohorts (e.g. identical treatment, age-matched, and disease-matched) should establish whether the combination of these markers can contribute to a more accurate treatment of UCs. However, the acquisition of a large series of cases associated with high quality samples with clinical data is not a trivial task as it will necessitate the involvement of many laboratories in different countries in a large scale multicenter research project, a challenging prospect by all accounts (63).

In conclusion, we have been able to establish that down-regulation of BLCAP protein is associated with tumor progression presumably in response to changes in the microenvironment and that increased expression of this protein is detrimental for patients, indicating that categorization of staining patterns for this protein has prognostic value. Consequently, it is important to examine further the prognostic effects of BLCAP in particular to determine its ability to predict a differential clinical benefit from a specific treatment. The interspersed nuclear staining of BLCAP, with some cells showing the presence of this protein in the cytoplasm but not in the nucleus, suggests that localization of this protein to the nucleus is a regulated function. In this respect it is particularly interesting that protein topology prediction methods (supplemental Fig. 1, a and b) suggest BLCAP to be a transmembrane protein, whereas we observed nuclear and cytoplasmic localization. It is conceivable therefore that localization to the nucleus is a regulated process in response to stimuli from the microenvironment. Accordingly, BLCAP under certain conditions could localize transiently to the cytoplasmic membrane and in response to effectors present in the microenvironment move to the nucleus and/or cytoplasm.

Our results also suggested a relation between BLCAP nuclear expression levels and disease outcome, but the mechanism(s) underlying this phenomenon is at present unknown. We determined that BLCAP co-localizes to nuclear speckles with spliceosome factor SF2/ASF, a proto-oncogene that is up-regulated in various human tumors (64). Thus, the association we identified between nuclear patterns of staining for BLCAP and prognosis could reflect a potential role of this protein in RNA splicing. Given the nuclear localization of BLCAP, our laboratory is now investigating the possibility of BLCAP being functionally involved in RNA metabolism.

These data together with the results presented in this report underline the need for further research into a possible dual role of BLCAP in cancer cells. Dissecting the various pathways controlling BLCAP expression and localization and determining its function might provide a clearer understanding of the regulatory networks controlling expression and localization of this protein, facts that will enhance its usefulness as a bladder cancer biomarker alone or in combination with others and may provide additional insight into the molecular mechanisms underlying pathogenesis of the urinary bladder.

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§ The on-line version of this article (available at http://www.mcponline.org) contains supplemental Fig. 1.

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