Bladder Cancer-associated Nuclear Matrix Proteins[1]

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ABSTRACT

The early diagnosis of bladder cancer is central to the effective treatment of the disease. Presently, there are no methods available to easily and specifically identify the presence of bladder cancer cells. The prevailing method for the detection of bladder cancer is the identification of bladder cancer cells by morphological examination of exfoliated cells or biopsy material by a pathologist. A hallmark of the malignant or transformed phenotype is an abnormal nuclear shape, the presence of multiple nucleoli, and altered patterns of chromatin organization. Nuclear structural alterations are so prevalent in cancer cells that they are commonly used as markers of transformation for many types of cancer. Nuclear shape is determined by the nuclear matrix, the dynamic skeleton of the nucleus. The nuclear matrix is the structural component of the nucleus that determines nuclear morphology, organizes the DNA in a three-dimensional fashion that is tissue specific, and has a central role in the regulation of a number of nuclear processes, including the regulation of DNA replication and gene expression. Previous investigations into prostate and breast cancer have revealed that nuclear matrix protein (NMP) composition undergoes alterations with transformation and that the nuclear matrix can serve as a marker for the malignant phenotype. In this study, we have identified NMPs with which it is possible to differentiate human bladder tumors from normal bladder epithelial cells. We examined the NMP composition of 17 matched tumor and normal samples from patients undergoing surgery for bladder cancer. We have identified six proteins present in all tumor samples that are not present in the corresponding normal samples and three proteins that are unique to the normal bladder tissues in comparison with the tumor samples. Five of the six bladder cancer-associated proteins were also identified in three human bladder cancer cell lines examined (253j, UMUC-2, and T24). Therefore, we have demonstrated that nuclear matrix composition is able to differentiate bladder cancer from normal bladder tissue and may provide useful tools for early detection and recurrence of the disease. Importantly, these markers may provide valuable tools for cytopathological screening for bladder carcinoma.

INTRODUCTION

The nuclear matrix is the framework of the nucleus and consists of the peripheral lamins and pore complexes, an internal ribonucleic protein network, and residual nucleoli (1). The NMPs[3] represent ~10% of all nuclear proteins and are virtually devoid of lipids, DNA, and histones (2). It has been demonstrated that the nuclear matrix plays a central role in the regulation of important cellular processes, such as DNA replication and transcription (3).

These structural components of the nucleus are known to have a central role in the specific topological organization of DNA. DNA in the nucleus is not randomly organized, and although ~10% of the

DNA actually encodes proteins, only specific genes are positioned in a manner that permits the expression of both housekeeping and cell type-specific genes. The DNA has many forms of higher-order structure, including topological organization by the nuclear matrix, which gives rise to a tissue-specific pattern of organization that results in the expression of appropriate tissue-specific genes.

A cellular hallmark of the transformed phenotype is an abnormal nuclear shape and the presence of abnormal nucleoli. Nuclear structural alterations are so prevalent in cancer cells that they are commonly used as pathological markers of transformation in many types of cancer. Nuclear shape is thought to reflect the internal nuclear structure and processes and is determined, at least in part, by the nuclear matrix (4).

Most known NMPs are common to all cell types and physiological states. Numerous NMPs, which may be unique to certain cell types or states, have been identified (reviewed in Ref. 3). It has been demonstrated that mitogenic stimulation and the induction of differentiation alter the composition of NMPs and structure (5, 6). The nuclear matrix contains a number of associated proteins that have been found to be involved in transformation. Berezney et al. (7) first showed by examining hepatoma NMPs that the nuclear matrix is altered in transformation. Fey and Penman (8, 9) demonstrated that tumor promoters induce a specific morphological signature in the nuclear matrix-intermediate filament scaffold of kidney cells, and that the pattern of NMPs differed between normal and tumorigenic cell lines.

We have previously characterized NMPs that are able to serve as markers to differentiate prostate cancer from the normal prostate in both a rat model system (10) and in human prostate samples (11). In addition, NMP composition was able to distinguish between metastatic and nonmetastatic tumors (10). NMPs that are associated with human breast cancer and are not found in normal breast tissue have also been identified (12). Recently, an antibody to a NMP, termed NM-200.4, was raised from the breast carcinoma cell line T-47D (13). This antibody reacts strongly with human breast carcinoma specimens as well as specimens from lung, thyroid, and ovarian cancers, but does not react with normal epithelial cells of similar origin, suggesting that certain anti-NMP antibodies may be developed as diagnostic tools. The strong evidence linking changes in the nuclear matrix with cancer has led to the suggestion that the nuclear matrix might serve as an excellent target for anticancer agents (14).

These data provide a strong rationale for investigation of the differences in NMP composition that may be discerned in normal versus transformed bladder tissue. We have compared the protein composition of the nuclear matrix in bladder tumors with that in adjacent normal bladder tissue from individuals with these tumors as well as with the composition in normal bladder samples obtained from organ donors. In addition, we examined the NMP composition of several human bladder cancer cell lines. We identified six NMPs that are found in the tumor samples but are not present in the normal tissues and three NMPs that are identified in the normal tissue but are absent in the tumors. Five of the six cancer-specific NMPs were also found in all of the bladder cancer cell lines.

MATERIALS AND METHODS

Cell Lines and Tissue Processing. Matched normal and tumor bladder tissue samples were obtained from the same patients undergoing surgery for bladder cancer at the University of Pittsburgh Medical Center. All patients had transitional cell carcinoma. The tumor samples collected were of the following clinical stages according to the American Joint Committee on Cancer (15). Six of the tumors were T_{is}-T_1, eight were T_3, and three were T_4 (see Table 1). The TNM histopathological grade of the lesions revealed 4 G_1 tumors, 2 G_2 tumors, and 11 G_3 lesions (16, 17). In addition to TNM staging and grading, the tumors were graded according to the classification of Bergkvist *et al.* (16). None of the lesions were grade 1, seven were grade 2, nine were grade 3, and one was grade 4. Normal bladders were obtained from the Center for Organ Recovery and Education. Only samples that could be clearly identified by the pathologist as containing approximately pure populations of the stated tumor grade were used. The cell lines (253j, UMUC-2, and T24) were kindly provided by Dr. Monica Liebert (University of Texas M. D. Anderson Cancer Center, Houston, TX) and grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies).

Nuclear Matrix Preparation. The nuclear matrix is defined as the residual components of the nucleus, which are insoluble to a series of detergent and salt extractions following DNase treatment (1). The isolation procedure used in these experiments involves the release of cytoskeleton proteins by the use of a mild salt extraction with 0.25 M ammonium sulfate of detergent-treated nuclei that no longer possess their membrane lipid components. This is followed by DNase I and RNase A treatment to remove chromatin structures. This extraction causes minimal disruption of the NMPs and nuclear matrix structure (8, 18).

The NMPs were isolated from bladder tissue and tumors, according to our previously published adaptation of the method of Getzenberg *et al.* (10) and Fey *et al.* (18). Briefly, the tissue pieces were minced into small (1-mm³) pieces and homogenized with a Teflon pestle on ice with 0.5% Triton X-100 in a solution containing 2 mM vanadyl ribonucleoside (RNase inhibitor) to release the lipids and soluble proteins. Extracts were then filtered through a 350-μm nylon mesh and extracted with 0.25 M ammonium sulfate to release the soluble cytoskeletal elements. DNase treatment at 25°C was used to remove the soluble chromatin. The remaining fractions contained intermediate filaments and NMPs. This fraction was then disassembled with 8 M urea, and the insoluble components, which consist principally of carbohydrates and extracellular matrix components, were pelleted. The urea was dialyzed out, and the intermediate filaments were allowed to reassemble and were subsequently removed by centrifugation. The NMPs were then ethanol precipitated. All solutions contained freshly prepared 1 mM phenylmethylsulfonyl fluoride to inhibit serine proteases, 0.3 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin. Dr. Sheldon Penman (Massachusetts Institute of Technology, Cambridge, MA) has prepared antibodies to proteins of this fraction and demonstrated that they are localized exclusively in the nucleus and isolated nuclear matrix fraction. The protein composition was determined by resuspending the proteins and using the Coomassie Plus protein assay reagent kit (Pierce Chemical Co., Rockford, IL) with BSA as a standard. For gel electrophoresis, the ethanol-precipitated NMPs were dissolved in a sample buffer consisting of 9 M urea, 65 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 2.2% ampholytes, and 140 mM DTT (Oxford Glycosystems, Bedford, MA). The final pellet containing NMPs represented <1% of the total cellular proteins.

High-Resolution, Two-Dimensional Electrophoresis. High-resolution, two-dimensional gel electrophoresis was carried out using the Investigator 2-D gel system (Oxford Glycosystems; Ref. 19) as described previously (10). Briefly, one-dimensional isoelectric focusing was carried out for 18,000 V-h using 1-mm × 18-cm tube gels after 1.5 h of prefocusing. The tube gels were extruded and placed on top of 1-mm SDS Duracryl (Oxford Glycosystems) high-tensile-strength PAGE slab gels, and the gels were electrophoresed with 12°C constant temperature regulation for ~5 h. Gels were fixed with 50% methanol and 10% acetic acid. After thorough rinsing and rehydration, gels were treated with 5% glutaraldehyde and 5 mM DTT after buffering with 50 mM phosphate (pH 7.2). The gels were stained with silver stain using the method of Wray *et al.* (20; Accurate Chemical Co., Inc., Westbury, NY). Fifty μg of NMP were loaded for each gel. Protein molecular weight standards were Silver Standards from Diversified Biotechnology (Newton Centre, MA). Isoelectric points were determined using carbamylated standards [BDH (distributed by Gallard-Schlesinger, Carle Place, NY); and Sigma Chemical Co., St. Louis, MO]. Multiple gels were run for each sample, and multiple samples were run at different times. Only protein spots clearly and reproducibly observed in all the gels of a sample type were counted as actually representing the nuclear matrix components. The gels were analyzed using the BioImage 2D Electrophoresis Analysis System (BioImage, Ann Arbor, MI), which matches protein spots between gels and sorts the gels and protein spots into a data base.

RESULTS

We have obtained tumor and normal tissue from 17 matched bladder samples. NMPs have been extracted and separated by high-resolution, two-dimensional gel electrophoresis. The NMP compositions of the 17 tumors and their corresponding normal tissue were then analyzed using a computer-based gel analysis system. All tumors contain differences in their nuclear matrix compositions when compared with the nuclear matrix compositions of the matched normal tissue from the same bladders. Consistent differences were noted for all of the samples. An example of the NMP composition of a bladder tumor and normal bladder tissue is presented in Fig. 1. There are several notable differences in the nuclear matrix composition in the bladder tumor when compared with the normal tissue. We have identified six proteins (BLCA-1–BLCA-6) that are present in all of the tumors and are absent in the normal tissue (Fig. 1B) and three proteins (BLNL-1–BLNL-3) that are found in all of the normal bladder tissue samples (Fig. 1A) and are missing in the tumor samples (Fig. 1 and Table 2). These differences seem to be unique to bladder cancer in that the molecular weights and isoelectric points of the proteins do not correspond to those proteins previously reported to be different in prostate and breast cancers (Table 2). In addition, we have now examined several normal human bladders to further our investigations into the NMP composition of normal bladder tissue. We have relatively large numbers of these normal bladders, which will allow us to study, in detail, changes that occur in NMPs during the transformation from normal cells to bladder cancer. Although these comparisons are still in the preliminary stage, it seems that the nuclear matrix composition of these normal bladders contains the three normal specific proteins and does not contain the tumor specific proteins.

The tumor samples that we examined are complex mixtures of epithelial, stromal, immunological, and other cell types. To determine

Table 1 *Bladder tumor pathology*

Case	Nuclear grade	TNM stage	TNM Histopathological grade
1	III	$T_{3a}N_0M_x$	G_3
2	III	$T_{3b}N_0M_x$	G_3
3	III	$T_{4a}N_xM_x$	G_3
4	II	$T_aN_0M_x$	G_1
5	III	$T_{3b}N_2M_x$	G_3
6	III	$T_{4a}N_0M_x$	G_3
7	II	$T_4N_0M_x$	G_1
8	III	$T_1N_0M_x$	G_3
9	III	$T_{3bi}N_0M_x$	G_3
10	II	$T_{3b}N_0M_x$	G_3
11	II	$T_1N_0M_x$	G_1
12	II	$T_1N_xM_x$	G_1
13	IV	$T_{3bi}N_2M_1$	G_3
14	III	$T_{3b}N_xM_x$	G_3
15	II	$T_1N_0M_x$	G_2
16	II	$T_{is}N_xM_x$	G_2
17	III	$T_{3b}N_2M_x$	G_3

Nuclear grade	I	II	III	IV
n	0	7	9	1

T stage	1	2	3	4
n	6	0	8	3

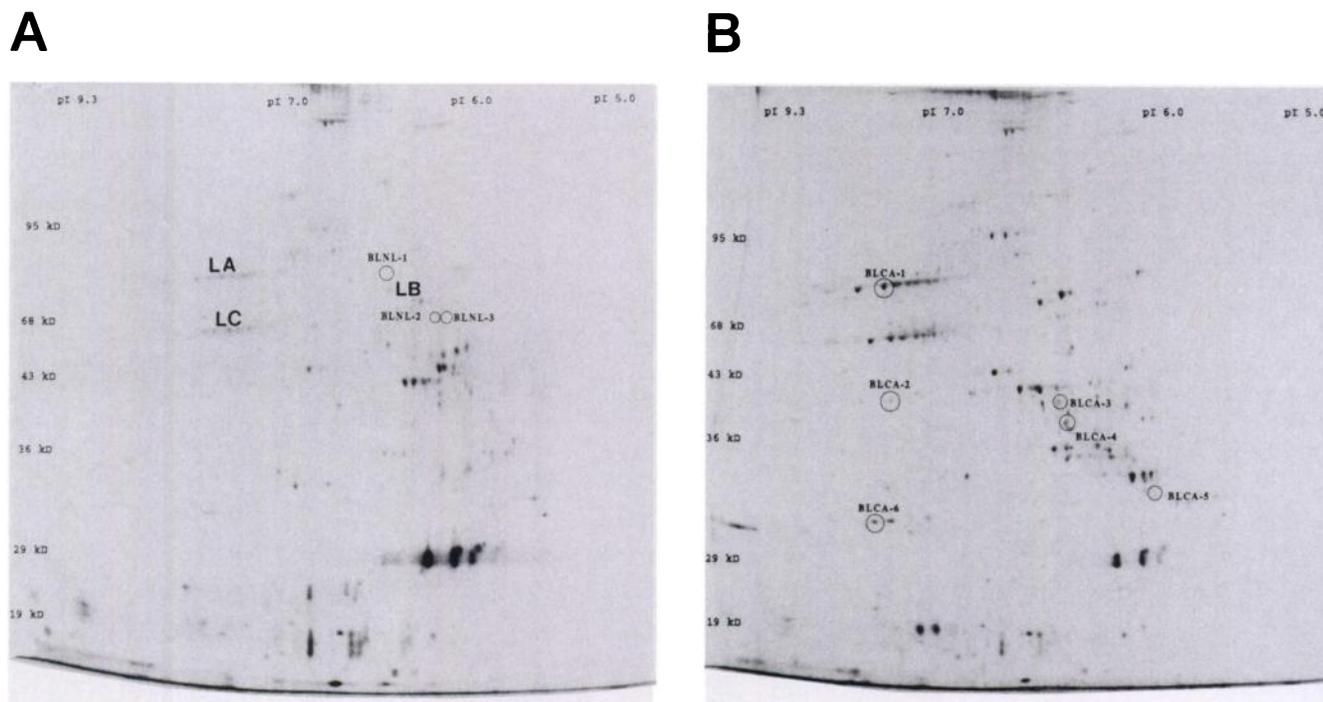| TNM histopathological grade	G_1	G_2	G_3
n	4	2	11

A

B

Fig. 1. Comparison of NMPs of human bladder cancer and normal bladder tissue. Silver-stained, high-resolution, two-dimensional gel electrophoresis of NMPs of normal bladder tissue (A) and bladder cancer (B) representative of the nuclear matrix patterns demonstrated in these studies. Several of the nuclear lamins, components of the nuclear matrix, are identified for orientation: *LA*, lamin A; *LB*, lamin B; and *LC*, lamin C.

whether the nuclear matrix changes that we were detecting actually represented changes that were occurring in the neoplastic transitional cell, we examined the NMP compositions of several bladder cancer cell lines. The human bladder cancer lines 253j, T24, and UMUC-2 were grown, and their NMPs were isolated. These NMPs were then separated by two-dimensional electrophoresis and silver stained. As demonstrated in Fig. 2, of the six NMPs determined to be found only in the bladder tumor samples, five of these proteins were identified in the three cell lines. Only the BLCA-5 protein was not identified in the three cell lines. None of the proteins found only in normal bladder samples (BLNL-1–BLNL-3) was found in these lines.

DISCUSSION

The nuclear matrix is the binding site for tumor-associated proteins, including the *myc* oncogene product, adenovirus E1A-transforming protein, polyoma large T antigen, and Tat protein from HIV-1 (21–24). Changes in the expression of a nuclear matrix-associated protein in NIH3T3 cells have been found in oncogene-transformed cells (25).

Table 2 *Nuclear matrix proteins that are able to differentiate normal bladder from bladder cancer samples, which were identified by high-resolution, two-dimensional gel electrophoresis*

The designation of each protein corresponds to the identified proteins in Figs. 1 and 2. Molecular weights and isoelectric points were identified as stated in "Materials and Methods."

	M_r	Isoelectric point
Proteins associated with human bladder cancer		
BLCA-1	72,000	7.70
BLCA-2	40,000	7.50
BLCA-3	39,000	6.27
BLCA-4	37,000	6.24
BLCA-5	32,000	5.80
BLCA-6	31,000	8.00
Proteins associated with normal human bladder		
BLNL-1	70,000	6.09
BLNL-2	66,000	5.84
BLNL-3	66,000	5.80

It has been demonstrated that the monoclonal antibody Ki-67, typically used to identify proliferating cells, is directed against a NMP (26). It has also been demonstrated that the nuclear matrix is the preferential binding target of the carcinogen benzo(*a*)pyrene (27).

Previous investigations from our laboratory have demonstrated that the nuclear matrix is altered in cancer cells in the Dunning rat prostate adenocarcinoma model (10). We have recently completed a study on the NMP composition in human prostate tissue (11). We compared the NMP patterns in fresh normal prostate, BPH, and prostate cancer from 21 men undergoing surgery for clinically localized prostate cancer or BPH. One protein (PC-1), a M_r 56,000 protein with an isoelectric point of 6.58, appeared in all of 14 nuclear matrix preparations from different prostate cancer specimens and was not detected in normal prostates (0 of 13) or BPH (0 of 14). Work from Khanuja *et al.* (12) has begun to determine the NMP composition of normal human breast tissue as well as breast tumors (12). A review of 10 different tumor samples revealed at least four proteins that were expressed in tumor tissue but not in normal tissue. One protein was a low-molecular-weight (M_r 18,000) protein, and the others were found at relatively low isoelectric points, between M_r 60,000 and 80,000. In addition, two proteins, of M_r 24,000 and 26,000 were found in the normal breast samples but were not detected in the tumor samples. These studies, taken as a whole, suggest that the protein composition of the human breast nuclear matrix may provide insight into the biological etiology of breast cancer and may provide biological markers of growth and gene expression in the human breast.

Presently, the only available marker for bladder cancer identification is morphological examination of cytological samples or cystoscopic biopsies. This method is accurate for high-grade lesions; however, a significant proportion of bladder tumors (25–45%) are low grade or well differentiated and escape detection on cytological examination of exfoliated cells. In general, the diagnostic accuracy of cytological examination alone for the detection of low-grade transitional carcinoma is between 49 and 64% (if suspicious urines are
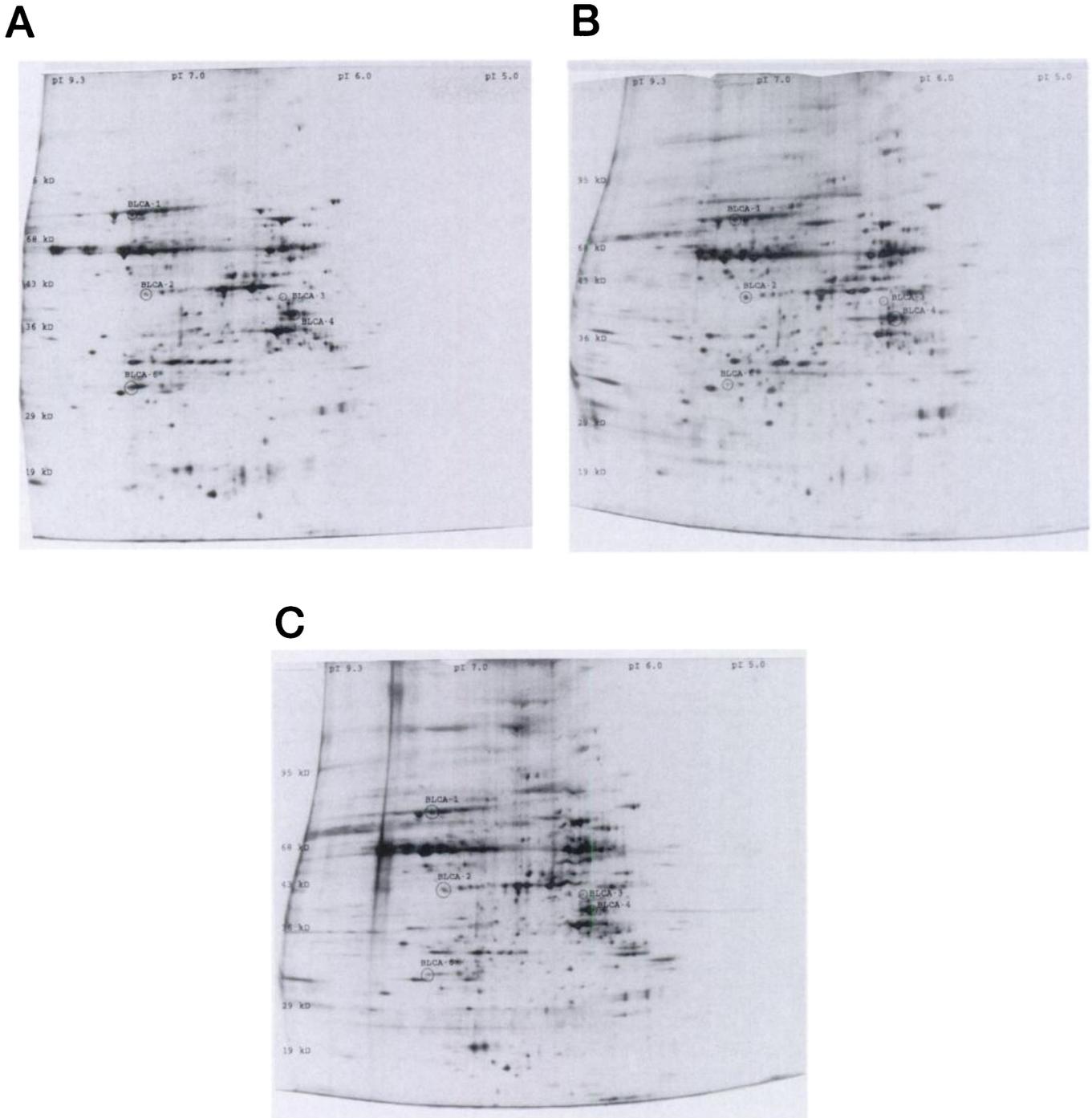
Fig. 2. NMPs associated with human bladder cancer are identified in three human bladder cancer cell lines. Silver-stained, high-resolution, two-dimensional gel electrophoresis of NMPs of the human bladder cancer cell lines 253j (A), T24 (B), and UMUC-2 (C). Identified proteins, found only in human bladder cancer samples, correspond to those in Fig. 1.

considered positive; Ref. 28). The accuracy of cytological examination can be increased by repeating the study (29); however, this is a costly and time-consuming practice for both the patient and physician. Development of a sensitive screening assay that could specifically detect bladder carcinoma would significantly facilitate patient care and the early detection of bladder carcinoma. Therefore, the identification of NMPs that indicate the presence of bladder cancer in a sample may permit the development of a diagnostic test for bladder cancer. NMPs have been shown to be shed by tumors as cells die, and it has been demonstrated that they are present in the serum and urine of cancer patients (30). Therefore, it is possible that the NMPs

described here could be used as markers for bladder cancer in serum or voided urine. These markers could aid not only in the early identification of bladder cancer but also in the detection of recurrent disease. Presently, patients who have had bladder tumors removed must undergo frequent cystoscopic examinations to rule out recurrent disease. The ability to use a blood or urine test to identify patients with disease recurrence would greatly aid in the detection of the disease and would decrease the need for these patients to have repeated cystoscopic evaluations.

We have recently obtained relatively significant quantities of the isolated NMPs BLCA-1–BLCA-6. There proteins are being se-

quenced and, in addition, are being used to raise antibodies against these proteins. This information will then be used to clone the genes encoding these proteins as well as to begin to understand their role in cancer. We have been successful in developing methods for isolating spots from two-dimensional gels in sufficient quantities for protein sequencing and antibody production. Deciphering the NMPs that are either present or absent in cancer cells will provide novel information about their role in cancer, and because the nuclear matrix plays a central role in DNA organization and the regulation of gene expression, these proteins may be crucial to the transformation process. Finally, these NMPs may serve as possible therapeutic targets by serving not only as cancer-specific markers, but also as tissue-specific addresses, thereby theoretically permitting the targeting of molecules to specific cell types within the body.

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