Identification of Nuclear Matrix Protein Alterations Associated with Human Colon Cancer

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ABSTRACT

The early diagnosis of colorectal cancer and the early detection of recurrence are central to effective treatment, as prognosis is directly related to the stage of the disease. When colorectal cancer is diagnosed at an early, localized stage, 5-year survival is 90%. With regional lymph node metastases, survival drops to 45–60%, and with distant metastases, 5-year survival is <5%. Development of tumor markers that can detect colon cancer at an early stage should have a major impact in mortality from this disease. The nuclear matrix is the structural scaffolding of the nucleus, and specific nuclear matrix proteins (NMPs) have been identified as an oncological “fingerprint” for bladder, renal, and prostate cancers. We have successfully used this approach to develop an immunoassay that detected bladder cancer early in a clinical trial with a sensitivity of 96.4% and a specificity of 100%. The objective of the present study was to identify the existence of a specific NMP fingerprint for human colon cancer, using high-resolution, two-dimensional gel electrophoresis, and thereby identify unique human colon cancer NMPs. Ten matched colon cancer and adjacent normal samples and 4 normal donor samples were analyzed. Analysis of multiple gels for each sample identified four proteins present in all tumor samples that were not present in the matched normal adjacent and normal colon tissue and six proteins present only in normal adjacent and normal colon tissue. Additionally, two proteins were found in all cancer and normal tissues, but not in the normal adjacent tissue. Data provided here demonstrate that examination of the nuclear matrix composition allows differentiation of colon cancer tissue from normal adjacent and normal colon tissue. Development of an assay to detect these specific NMPs by examining tissue, serum, and stool specimens is a promising modality for early detection of colorectal cancer. In addition, the functional characterization of these proteins and their early detection through the generation of NMP antibodies could significantly impact on the understanding of cancer progression and its diagnosis.

INTRODUCTION

Improvements in surgical procedures, adjuvant therapies, and screening programs have facilitated an overall decline in the mortality of colon cancer in the last 20 years (1). Nevertheless, colorectal cancer still accounts for 11% of all cancers in the United States, with an estimated 130,200 new cases and 48,100 deaths expected in the year 2001 (2). When colorectal cancer is diagnosed at an early, localized stage, 5-year survival is 90%, but only 37% of incident cases are diagnosed at this point. Although this disease is curable when identified at an early stage, frequently the tumor has become metastatic by the time an individual presents to his or her physician with symptoms; thus the mortality from this disease can be high. As a public health problem, it is essential that more effective screening and preventative measures for colorectal cancer be developed. This may include new molecular approaches to accurately detect, diagnose, and provide prognostic information about individuals with this disease.

The earliest detection procedures available at present for colorectal cancer involve using tests for fecal blood or endoscopic procedures. However, significant tumor size must typically exist before fecal blood is detected. The sensitivity of the guaiac-based fecal occult blood tests is ~26%, which means 74% of patients with malignant lesions will remain undetected (3). The visualization of precancerous and cancerous lesions represents the best approach to early detection, but colonoscopy is invasive with significant costs, risks, and complications (4, 5). The identification of an early tumor marker that would allow reliable early cancer detection or provide early prognostic information could lead to a diagnostic assay that would greatly aid in the management of this disease.

At present, diagnostic blood tests based on the detection of CEA, a tumor-associated glycoprotein, are available. CEA is increased in 95% of colorectal, gastric, and pancreatic cancers and in the majority of breast, lung, and head and neck carcinomas (6). Elevated CEA levels have also been reported in patients with nonmalignant disease, and many patients with colon cancer have normal CEA levels in the serum, especially during the early stage of the disease (7–9). The utility of CEA in detecting recurrences is controversial and has yet to be widely applied (10, 11). In light of the available data, serum CEA determination possesses neither sensitivity nor the specificity to enable its use as a screening test for colorectal cancer in the asymptomatic population (12).

To identify highly specific tumor markers, investigators have studied structural changes that are associated with neoplastic transformation. Alterations in the cellular and nuclear structure are hallmarks of the carcinogenic process. The identification of cancer is based on the presence of certain unique features of tumor cells. Changes in nuclear shape, size, and DNA organization, including major morphological transformation, are unique characteristics of cancer cells. The changes in nuclear structure are so prevalent in cancer cells that they are commonly used as pathological markers of transformation. The nuclear structure is determined by the nuclear matrix. Alterations in nuclear shape or structures that occur with neoplastic transformation are accompanied by changes in nuclear matrix composition and architecture (13). Additionally, changes in the framework or composition of the nuclear structural framework may alter cellular phenotype by affecting transcription, replication, and/or other nuclear processes.

In 1974, Berezney and Coffey (14) first described the nuclear matrix as the structural framework scaffolding of the nucleus, consisting of the peripheral laminis, protein complexes, an internal ribonucleic protein network, and residual nucleoli. The nuclear framework contains ~10% of the nuclear proteins and is virtually devoid of lipids, DNA, and histones (15). Most of the NMPs identified to date are common to all cell types, but several identified NMPs are tissue and cell line specific (16). NMPs have been demonstrated to participate in many vital cellular functions, such as steroid hormone binding and gene transcription and translation (17–20). Given that the nuclear
matrix plays an important role in these vital cellular functions, changes in nuclear matrix structures could result in altered DNA topology and alterations in the interaction of various genes with the matrix, which could then participate in a cascade of events.

Individual cancer cell lines can be distinguished based on an aberrant pattern of expressed NMPs (21). Cell type-specific “fingerprinting” of aberrant NMPs and their appearance in cancer development has led to the analysis of NMP composition of a variety of tumors in an effort to determine whether these proteins can be developed as diagnostic and/or prognostic markers for cancer. Using high-resolution, two-dimensional electrophoresis, we have demonstrated that specific NMP alterations exist in prostate, bladder, and renal cancers (22–24). This oncological fingerprint can be used as a specific and reliable diagnostic test even when a distinction may not be made accurately on histological basis alone (25).

As an example, we have identified a specific NMP pattern that is unique to patients with bladder cancer (26). On the basis of the unique expression of a NMP, BLCA-4, in bladder cancer, we were able to develop an assay to detect its presence in urine. BLCA-4 is a sensitive (96.4%) and specific (100%) marker for the diagnosis of bladder cancer in the urine, and its appearance is an early change in the development of this disease. A large national clinical trial using this assay is under way (27).

Additionally, tumor-specific alterations in nuclear matrix composition in various cancers (prostate, renal, breast, cervix, and squamous cell carcinoma of the head and neck) have been identified (22, 23, 28–31).

The detection of NMPs in the serum of patients with various types of cancer has also been accomplished (32). Detection of NMPs in the serum and urine is possible attributable to release as tumor cells undergo degeneration and lysis. Antibodies developed to identify aberrant NMPs in colon cancer could become clinically important early markers with great specificity. The objective of this study was to investigate the NMP composition in colon cancer in an effort to identify unique cancer-associated proteins.

**MATERIALS AND METHODS**

**Tissue Processing.** Colon adenocarcinoma samples (n = 10) and matched adjacent normal tissues (n = 10) were collected through the Early Detection Research Network of the University of Pittsburgh Medical Center under Institutional Review Board approval. The patients ranged in age from 36–82 years, with a mean age of 71 years. Sixty percent of the sample population was female. Normal colon tissue was obtained from trauma victims. Two of these individuals had gunshots wound, one had been in an automobile accident, and one was an organ donor (n = 4). The patients ranged in age from 20–59 years, with a mean age of 47.2 years. These normal individuals were all male. Diagnosis was obtained from pathology reports that accompanied each specimen and was confirmed histologically. Tumors were staged according to the standard TNM system and are described in Table 1. The tissues were stored at ~80°C before processing. The colon cancer cell lines SW480 and CaCo 2 were obtained from the American Type Culture Collection. Both cell lines had been obtained from primary human colon cancer cells. The SW480 cell line was grown in Leibovitz medium with 10% fetal bovine serum at 37°C without CO2. The CaCo 2 cell line was grown in DMEM supplemented with 10% fetal bovine serum, 1% l-glutamine (200 μM), 1% penicillin/streptomycin, 1% sodium pyruvate (100 mM), 1% MEN nonessential amino acids, and 1.5% HEPES buffer (1 μM) at 37°C in a 5% CO2 atmosphere.

**Nuclear Matrix Preparation.** NMPs were extracted from colon cancer tissue, adjacent tissue from these individuals, and donor patients according to the method of Getzenberg et al. (24). In summary, the tissue samples described above were finely minced into small 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. The homogenized tissue was then filtered through a 350 μm nylon mesh. DNase and RNase treatments were used to remove the soluble chromatin. The remaining fraction contained intermediate filaments and NMPs. This fraction was then disassembled with 8 M urea, and the insoluble components consisting of carbohydrates and extracellular matrix were pelleted. After the urea was dialyzed out, the intermediate filaments were allowed to reassemble and were subsequently removed by centrifugation. The NMPs were then precipitated in ethanol. The protein concentration was determined by resuspending the pellet in sample buffer for the two-dimensional electrophoresis, consisting 9 M urea, 65 mM Tris-(3-cholamidopropyl)dimethyl-ammonio)-1-propanesulfonate, 2.2% ampholytes, and 140 mM DTT, and quantitated by Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. The final pellet containing these proteins represented <1% of the total cellular proteins.

**High-Resolution, Two-Dimensional Electrophoresis.** High-resolution, two-dimensional electrophoresis was performed using the Investigator 2-D gel system (Genomic Solutions, Ann Arbor, MI) as described previously (24, 33). One hundred μg of protein were loaded per gel onto a capillary-size isoelectric focusing column. One-dimensional isoelectric focusing was carried out for 18,000 V-h, using 1 mm × 18 inch tube gels after 1.5 h of prefocusing. The tube gels were extruded and placed on top of 1-mm SDS Duracryl (Genomic Solutions, Ann Arbor, MI) high-tensile strength PAGGel slabs. The gels were electrophoresed at 12°C constant temperature for 4.5–5 h. Gels were fixed with 50% methanol and 10% acetic acid. After thorough rinsing and rehydration, gels were treated with 5% glacialacetic and 5 mM DTT after buffering with 50 mM phosphate (pH 7.2). The gels were stained with silver stain according to the method of Wray et al. (Ref. 34; Accurate Chemical Co., Westbury, NY). Molecular weights of colon NMPs were determined using standards provided by Genomic Solutions. Isoelectric points (PIs) were determined using carbamylated standards (BDH, distributed by Gallard-Schlessinger, 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 identical in all of the gels of a sample type were taken into account as those representing the described NMPs. 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 database.

**Protein Sequencing.** Identification of the colon cancer-associated NMPs permitted the proteins to be isolated and sequenced. We have been successful in developing a technique for sequencing of proteins isolated from spots in two-dimensional gels. The proteins were isolated according to an adaptation of a technique developed by Gevaert et al. (35). The two-dimensional gels were negatively stained by incubation in 0.2 M imidazole for 15 min, washed several times with deionized water, and stained with warm 0.3 M zinc chloride. Deionized water was used to stop the staining, and the protein gel spots were excised and frozen at ~80°C. The spots were then thawed, pooled, and mixed with 0.25% Coomassie Blue stain (45% methanol-9% acetic acid) for 20 min. With constant agitation, the spots were destained with destaining solution (5% methanol-7.5% acetic acid) for 1 h, washed with deionized water for 1 h, and equilibrated in sample buffer [1% SDS, 10% glycerol, 50 mM DTT, 12 mM Tris-HCl (pH 7.1)] for 1 h before being loaded into the acrylamide/agarose gel. The construction of the mini-agarose gel consisted of two prewarmed (60°C) glass plates (10 × 9 cm), separated by spacers 1 cm wide and 1.5 mm thick. A strip

<table>
<thead>
<tr>
<th>Tumor stage (UICC) and grade of the 10 matched colon cancer patients</th>
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<tbody>
<tr>
<td>Colon adenocarcinoma</td>
<td>10</td>
</tr>
<tr>
<td>Matched adjacent normal tissue</td>
<td>10</td>
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<tr>
<td>Location</td>
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<tr>
<td>Right hemicolon</td>
<td>5</td>
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<td>Left hemicolon</td>
<td>5</td>
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<tr>
<td>Rectum</td>
<td>0</td>
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<tr>
<td>Tumor stage (UICC)</td>
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</tr>
<tr>
<td>I: T1N0M0</td>
<td>0</td>
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<tr>
<td>II: T2N0M0</td>
<td>5</td>
</tr>
<tr>
<td>III: T3N0M0</td>
<td>4</td>
</tr>
<tr>
<td>IV: T4N1M2</td>
<td>1</td>
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<tr>
<td>Tumor grade</td>
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<td>9</td>
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of Whatman 3-mm paper was inserted at the bottom to serve as a support for the lower agarose gel, preventing the gel from slipping during electrophoresis. The sample well was formed by a 2-cm wide \times 1.5-cm thick spacer set between two parallel spacers, each 1 cm wide \times 1.5 cm thick, inserted at the center of the glass plates and attached with adhesive tape at the top edge of the back plate. The lower gel consisted of a 2-cm deep agarose gel [1.45% agarose in 0.36 M Tris-HCl (pH 8.7), 0.1% SDS]. Once the agarose had set, it was overlayed with the polyacrylamide stacking gel [5.45% acrylamide, 0.13% bisacrylamide, 0.12 M Tris-HCl (pH 6.8), 0.1% SDS]. When the stacking gel had set, the central spacer was removed, leaving a well 2 cm high, 2 cm wide, and 1.5 mm thick. The mini-concentration gel was then mounted on a small electrophoresis tank (Bio-Rad, Hercules, CA), and the slot was filled with the equilibrated two-dimensional gel spots. The remaining volume was filled with blank gel pieces.

The gels were run at 100 V, allowing the proteins to elute out of the combined gel pieces and into the acrylamide. At this time, the central spacer was reinserted into the sample well until the dye front passed the two parallel 1-cm wide spacers. At that point, the central spacer was removed, and electrophoresis continued until the dye front entered the agarose and reached the filter paper.

The agarose section of the gel was fixed in fresh 50% methanol-10% acetic acid, with shaking, at room temperature for 30 min. The gel was stained with 0.05% Coomassie Blue stain (50% methanol-10% acetic acid) for 5 min and then destained in 5% methanol-7% acetic acid for 2 h with constant agitation. The protein band was then excised in a minimal volume of agarose gel, transferred into an sterile tube, and sent for peptide sequencing (Department of Biochemistry, Michigan State University, East Lansing, MI). Sequences were analyzed using the BLAST database, and sequence homologies were identified.

RESULTS

We obtained tumor and adjacent normal tissue from 10 matched adjacent normal and colon cancer samples as well as 4 normal donor
samples. NMPs were extracted and separated by high-resolution, two-dimensional gel electrophoresis. Image analysis distinguished a normal and adjacent NMP fingerprint from the colon cancer NMPs (Fig. 1 and Table 2). All colon cancer gels strongly expressed six unique NMPs (CC2 through CC6a and -b), which were undetectable in adjacent normal tissue. A seventh protein was expressed at a higher level in the carcinoma extracts compared with adjacent normal or donor tissue (CC1). Additionally, all colon cancer gels completely lost the expression of five NMPs (N2–N6), which were expressed in all adjacent normal extracts. Furthermore, a sixth NMP (N1) was expressed less in the cancer extracts compared with the normal adjacent extracts.

The four normal donor samples expressed all five NMPs, which were expressed in adjacent normal tissue and were not found in colon cancer (N2–N6). Two of the NMPs (CC6a and -b) detectable in colon cancer tissue, were also expressed in donor tissue. The electrophoretic characteristics of the identified proteins are described in Table 3. Five of the colon cancer-associated proteins detected in the present study appear to be unique in that the isoelectric points and molecular weights are completely different from those proteins detected in previous studies of other normal and cancer tissue types. Two of the identified proteins do indeed appear to be similar to those found in other cancers (CC6a and -b).

Tissue samples are complex mixtures of epithelial, stromal, immunological, and other cell types. To determine whether the nuclear matrix changes detected actually represented changes that were occurring in the neoplastic epithelial cells, as well as to identify potential models to study, the NMP compositions of two human colon cancer cell lines were examined. Although the NMP fingerprints from the pure cell lines would be expected to be distinct from the three-dimensional complex colon cancer specimens, they serve as tools for generating reagents as well as examining a single cell type. The human colon cancer cell lines CaCo 2 and SW480 were grown, and their NMPs were isolated. These NMPs were then separated and analyzed as described above. Representative silver-stained gels of NMPs from the two cell lines are shown in Fig. 2. Both cell lines expressed none of the proteins, which were expressed in the adjacent or donor gels (N1–N6). The cell line SW480 expressed the colon cancer-associated proteins CC1, CC6a, and CC6b; the CaCo 2 cell line expressed CC1, CC3, CC4, CC6a, and CC6b.

**Sequence Data and Protein Homologies.** To begin to elucidate the identities of these proteins, we collected spots from the two-dimensional gels for sequencing. Sequence data were obtained from the human spots corresponding to CC1, CC2, CC6a, and CC6b. Table 4 shows the peptides obtained for the proteins and the homologies of

![Fig. 2. Silver-stained high-resolution, two-dimensional gel electrophoresis of NMPs of the human colon cancer cell lines CaCo 2 (A) and SW480 (B).](image_url)
DISCUSSION

The early diagnosis of colorectal cancer and the early detection of recurrence are central to the effective treatment of this disease. Colon cancer has a long asymptomatic period during which it may be diagnosed at an early stage that may be curative. At present, our earliest detection procedures for colorectal cancer involve using tests for fecal blood or endoscopy. A disadvantage of the available tests for fecal occult blood is that they often fail to detect many polyps and cancers. Of particular importance is the fact that many colon cancers do not cause blood to be released into the stool in the earliest, most curable stage. In addition, the test is not highly specific. Many people who test positive will undergo the discomfort and risk of full bowel examination only to find nothing.

Colonoscopy is considered the gold standard for colon cancer diagnosis and offers the potential to both find and remove premalignant lesions, but it is associated with high cost, patient discomfort, complications, and variable sensitivity due at least in part to the experience of the endoscopist (36).

In this study we identified four unique NMPs expressed in all tumor samples but in none of the adjacent and donor tissue samples. Two (CC3 and CC4) of the four proteins for colon cancer are expressed in the CaCo 2 human colon cancer cell line, raising the potential that these proteins are present in the colonic epithelium. These four unique proteins were not found in other tissues or cancer types. The sequence data we obtained from CC2 and CC3 are being used to help raise antibodies against them. These antibodies may be useful in developing early detection assays for the stool or blood.

The sequence data obtained from collected and concentrated two-dimensional spots revealed some information regarding possible identities of these proteins. The amino acid composition of CC1 identified this spot potentially as calreticulin. The multifunctional protein calreticulin is normally found within in the lumen of the endoplasmic reticulum. Yoon et al. (37) reported the identification of calreticulin in the nuclear matrix of hepatocellular carcinoma. Paschal et al. (38) reported calreticulin as a novel nuclear export factor. Further investigations are necessary to confirm that CC1 is actually calreticulin as well as to evaluate the functions of calreticulin in the nuclear matrix of colon cancer.

Unexpectedly, two of the identified proteins (CC6a and -b) were found in the cancer and normal donor tissue but not in the adjacent normal tissue. Both proteins are also expressed very strongly in the two human colon cancer cell lines studied. One possible explanation for this finding is that the adjacent tissue is undergoing an early form of instability that is distinct from either the donor or tumor tissues. The sequence data of the human protein (CC6a) showed homologies with hemoglobin β chain and β globin. Additionally these sequences showed similarity with proteins related to the SWI/SNF complex. The identification of these proteins could aid in our understanding of the function of the proteins and perhaps give us an explanation for their absence in the normal adjacent colon tissue. Although this would be an important finding, clearly other studies are necessary to establish this.

A limited colon cancer study has previously been performed. In this study, six aberrant NMPs were expressed in 18 of 18 colon tumors and 0 of 10 normal donor tissues and adjacent tissue (31). The proteins identified in these studies appear distinct from the proteins identified here in molecular weight and isoelectric point. There are several...
possible explanations for these differences. First, the nuclear matrix isolation and electrophoresis procedures were not the same as those used by Keesee et al. (31) and could result in a different protein pattern. Second, the report by Keesee et al. only briefly described the tumors that they used without detail to the tumor stage. In addition, they described the separation of the mucosa from the muscularis propria before extraction of the nuclear matrix. It is not clear how one would separate just the mucosa from a tumor that has already invaded the muscularis propria. The study presented here along with previous works from our group demonstrate that normal adjacent tissue is different from normal donor tissue and that they therefore cannot be combined as control tissues, as has been done by others (23, 31).

The five proteins specific for the normal donor and adjacent tissue (N2–N6) were not detected in the colon cancer cell lines. Potential explanations for this observation could include that the proteins are specific for nonmalignant tissue, that the source of the proteins is not from the epithelium, or that the cell lines, which are highly dedifferentiated, have lost expression of these proteins.

These studies demonstrate that NMPs isolated from human colon cancer are distinct from normal adjacent and donor tissue, indicating both loss and gain from specific proteins. The presence or absence of unique NMPs in cancer cells could provide novel information about their function in carcinogenesis.

The functional identification of these proteins and their early detection through the generation of NMP antibodies could be used to develop tests for colon cancer diagnosis and/or prognosis. The long-term goal of these studies is to generate antibodies to detect specific NMPs in the circulation, colonic aspirates, or tissue samples. Development of assays with these antibodies potentially could serve as early detection markers with high sensitivity and specificity.

REFERENCES

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