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Colon Cancer Specific Nuclear Matrix Protein Alterations in Human Colonic Adenomatous Polyps

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Abstract Most colon cancers arise within preexisting adenomatous polyps or adenomas. The slow evolution from the non-invasive premalignant lesion, the adenomatous polyp, to invasive cancer supports a strategy of early detection. Recently, we identified unique nuclear matrix proteins (NMPs) specific for colon cancer (CC2, CC3, CC4, CC5). Most of the NMPs identified are common to all cell types, but several identified NMPs are tissue and cell line specific. The objective of this study is to describe and characterize the NMP profile of premalignant adenomatous colon polyps. Specifically when in the adenoma-carcinoma sequence four specific colon cancer NMPs, previously described, appear. Using two-dimensional (2-D) gel analysis 20 colon polyps (one juvenile polyp, six tubular adenoma (TA), seven tubulovillous adenoma (TVA), six TVA with focal high-grade dysplasia (HGD), were analyzed for the presence of four (CC2, CC3, CC4, CC5) specific NMPs. CC2 was not seen in any of the premalignant polyps. CC5 was present in only two premalignant TVA with HGD and in one TA. CC3 and CC4 were present in most adenomas. None of the NMPs were seen in the juvenile polyp, which is not considered to be a precursor of colon cancer. CC2 and CC5 are NMPs expressed at the junction of an advanced adenoma and invasive colorectal cancer. CC3 and CC4 are expressed earlier in the evolution of adenomatous polyps. Development of an assay to these proteins may serve as a new method for early detection of colorectal cancer. J. Cell. Biochem. 91: 365–374, 2004.

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Colon cancer is one of the most common malignancies among populations in the United States and Western Europe, and one of the leading causes of worldwide morbidity and mortality due to cancer. The American Cancer Society predicts 105,500 new cases of colon cancer and 57,100 deaths in 2003 [Jemal et al., 2003]. The lifetime colorectal cancer risk in the general population is 6%, and the incidence rises dramatically with age. Strong circumstantial evidence supports the so-called adenoma-carcinoma hypothesis [Bond, 2000; Leslie et al., 2002]. According to this theory, human colon cancers evolve from normal to dysplastic epithelium to carcinoma over 5–10 years, in association with the accumulation of multiple clonally selected genetic alterations [Fearon and Vogelstein, 1990].

The slow evolution from the non-invasive premalignant lesion, the adenomatous polyp, to invasive cancer supports a strategy of early detection. Detection of early stage colon cancer, and finding and removal of premalignant adenomatous polyps has been shown to beneficially impact mortality and incidence of colon cancer [Winawer et al., 1993; Thiis-Evensen et al., 1999; Citarda et al., 2001].

All available screening tests for early detection of colon cancer face significant barriers to patient participation and compliance. The development of a biological biomarker that could be used to predict cancer risk is a high priority. A serum-based biomarker would be particularly

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helpful, since acceptance by the patients would be a significant improvement over what is currently available.

In order to identify highly specific tumor markers, investigators have studied structural changes that are associated with neoplastic transformation. Alterations in cellular and nuclear structure are hallmarks of the carcinogenic process [Pienta et al., 1989]. Changes in nuclear shape, size, and DNA organization including major morphological transformation are unique characteristics of cancer cells and are so prevalent in cancer that they are commonly used as pathological markers. Berezney and Coffey [1974] first described the nuclear matrix as the structural framework scaffolding of the nucleus consisting of the peripheral lamins, protein complexes, an internal ribonucleic protein network, and residual nucleoli. The nuclear framework consists of approximately 10% of the nuclear proteins and about 1% of the total cellular protein, and is virtually devoid of lipids, DNA, and histones [Fey et al., 1991]. The nuclear structure is determined by the nuclear matrix. Nuclear matrix proteins (NMPs) have been demonstrated to participate in many vital cellular functions, including steroid hormone binding, DNA replication, gene transcription and translation [Getzenberg et al., 1990; Brancolini and Schneider, 1991; Ruh et al., 1996; Martelli et al., 1997]. 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.

Cell type-specific "fingerprinting" of aberrant NMPs in cancer has led to the search for specific NMP alterations in tumors for use as diagnostic or prognostic markers for cancer [Fey and Penman, 1988; Getzenberg, 1994]. Using high-resolution two-dimensional (2-D) electrophoresis, we have demonstrated that specific NMP alterations exist in prostate, bladder, and renal cell cancers [Getzenberg et al., 1991, 1996; Konety et al., 1998].

Recently, we identified unique NMP proteins specific for colon cancer (CC2, CC3, CC4, CC5), which were not seen in normal adjacent tissue, nor in normal tissue from unaffected colon donor subjects [Brunagel et al., 2002a]. The sequence data obtained from the 2-D spots revealed some information regarding possible identities, but to date they appear to be unknown proteins. We also identified specific NMP in liver metastases of colonic origin and demonstrated that NMP changes associated with liver metastasis could be identified in liver tissue that appeared histologically normal, suggesting that NMP alteration may be an early sign of liver metastasis [Brunagel et al., 2002b]. This oncological "fingerprint" may be used as a specific and reliable diagnostic test, even when a distinction may not be made accurately on a histological basis [Hughes and Cohen, 1999].

The objective of this study was to examine premalignant colon polyps for the presence of the specific NMPs found to be associated with colon cancer. In particular, we wanted to describe where in the malignant process from adenoma to carcinoma the NMPs characteristic for colon cancer appear. These specific NMP could form the basis for generating an assay for an early detection marker.

MATERIALS AND METHODS

Tissue Processing

Colon polyps ($n = 20$) were collected in conjunction with the Early Detection Research Network (EDRN) of the University of Pittsburgh Medical Center under institutional IRB approval. Polyps, each measuring 1 cm or greater in size, were removed at the time of endoscopy, stored in chilled saline, and immediately transported to the pathology laboratory for sectioning. Excess tissue was stored at -80° and once clinical diagnostic issues were resolved, released for research investigation, and processing. One juvenile polyp, six tubular adenoma (TA), seven tubulovillous adenoma (TVA), and six TVA with focal high-grade dysplasia (HGD) were examined. The characteristics of the patients with colon polyps are shown in Table I. The patients ranged in age from 18 to 77 with a mean age of 58 years. Fifty-five percent of the sample population was female. Diagnosis was

TABLE I. Patients With Colon Polyps

Polyps	n	Average age	Gender
Juvenile polyp	1	18	Male 1
TA	6	58	Male 4, female 2
TVA	7	56	Male 1, female 6
With HGD	6	62	Male 3, female 3

TA, tubular adenoma; TVA, tubulovillous adenoma; HGD, focal high-grade dysplasia.

obtained from pathology reports blinded to identity, which accompanied each specimen and was confirmed histologically. The colon cancer cell line CX-1 was a kind gift from Y.J. Lee, PhD, University of Pittsburgh. The cell line has been established from primary human colon cancer cells. The cell line was grown in RPMI-1640 media with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

Nuclear Matrix Preparation

NMP were extracted from colon polyp tissues according to the method of Getzenberg et al. [1991]. In summary, the tissue was 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 dialyzing the urea 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 2-D sample buffer consisting of 9 M urea, 65 nM 3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate, 2.2% amhpolytes, and 140 mM DTT and quantitated by Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. The final pellet containing these proteins represent <1% of the total cellular proteins.

High-Resolution 2-D Electrophoresis

High-resolution two-dimensional electrophoresis was performed using the Investigator 2-D gel system (Genomic Solutions, Ann Arbor, MI) as described previously [Patton et al., 1990; Getzenberg et al., 1991]. One hundred micrograms of protein were loaded per gel onto a capillary size IEF column. One-dimensional (1-D) 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) high-tensile strength PAGE slab gels. 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% 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. [1981] (Accurate Chemical Co., Westbury, NY). Molecular weights of colon NMPs were identified using standards provided by Genomic Solutions. Isoelectric points (PI's) 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 the gels of a sample type were taken into account as those representing the described NMP's. The gels were analyzed using the BioImage 2-D Electrophoresis Analysis System (BioImage, Ann Arbor, MI), which matches protein spots between gels and sorts the gels and protein spots into a database.

RESULTS

One juvenile polyp and 19 adenomatous polyps including, six TA, seven TVA, and six TVA with HGD were examined (Table I). NMPs were extracted and separated by high-resolution, 2-D gel electrophoresis.

Using 2-D gel analysis we previously identified four NMP (CC2, CC3, CC4, CC5) that differentiate human colon cancer tissue from normal donor and normal adjacent colon tissue. Table II shows the molecular weight and isoelectic point of these four proteins. The 2-D gels obtained from the colon polyps were analysed for the presence of these four specific NMP identified in human colon cancer. Representative silver stained gels of the NMPs in colon polyps are

TABLE II. Nuclear Matrix Proteins (NMP) Specific for Colon Cancer*

Proteins associated with human colon cancer	M_r (kDa)	Isoelectric point (PI's)
CC2	56	6.22
CC3	43	6.27
CC4	43	6.22
CC5	42	6.25

*Data from Brunagel et al. [2002a].

shown in Figure 1. The distribution of their presence in the adenomas in comparison to that found in colon cancer and donor tissue is presented in Table III.

CC2 is not present in any of the premalignant polyps, 0/19 (0%), but had been present in 80% of colon cancers (8/10). CC2 is not present in normal adjacent tissue to colon cancer nor in any normal donor colon tissue. CC5 is present in only 3/19 adenomatous polyps (15.7%). CC5 is present in 2/6 (33%) TVA's with HGD and in 1/6 TA's (16.7%), but was present in all colon cancer tissues examined (10/10). In contrast, CC3 is present in 17/19 (89.5%) and CC4 in 18/19 (94.7%) of premalignant adenoma. CC3 and CC4 were present in all colon cancer tissue tested (10/10). None of the NMP are seen in the juvenile polyp, which is not considered to be a precursor of colon cancer (Table III). Representative silver stained gels areas of the NMPs CC3, CC4, and CC5 in colon polyps are shown in Figure 2.

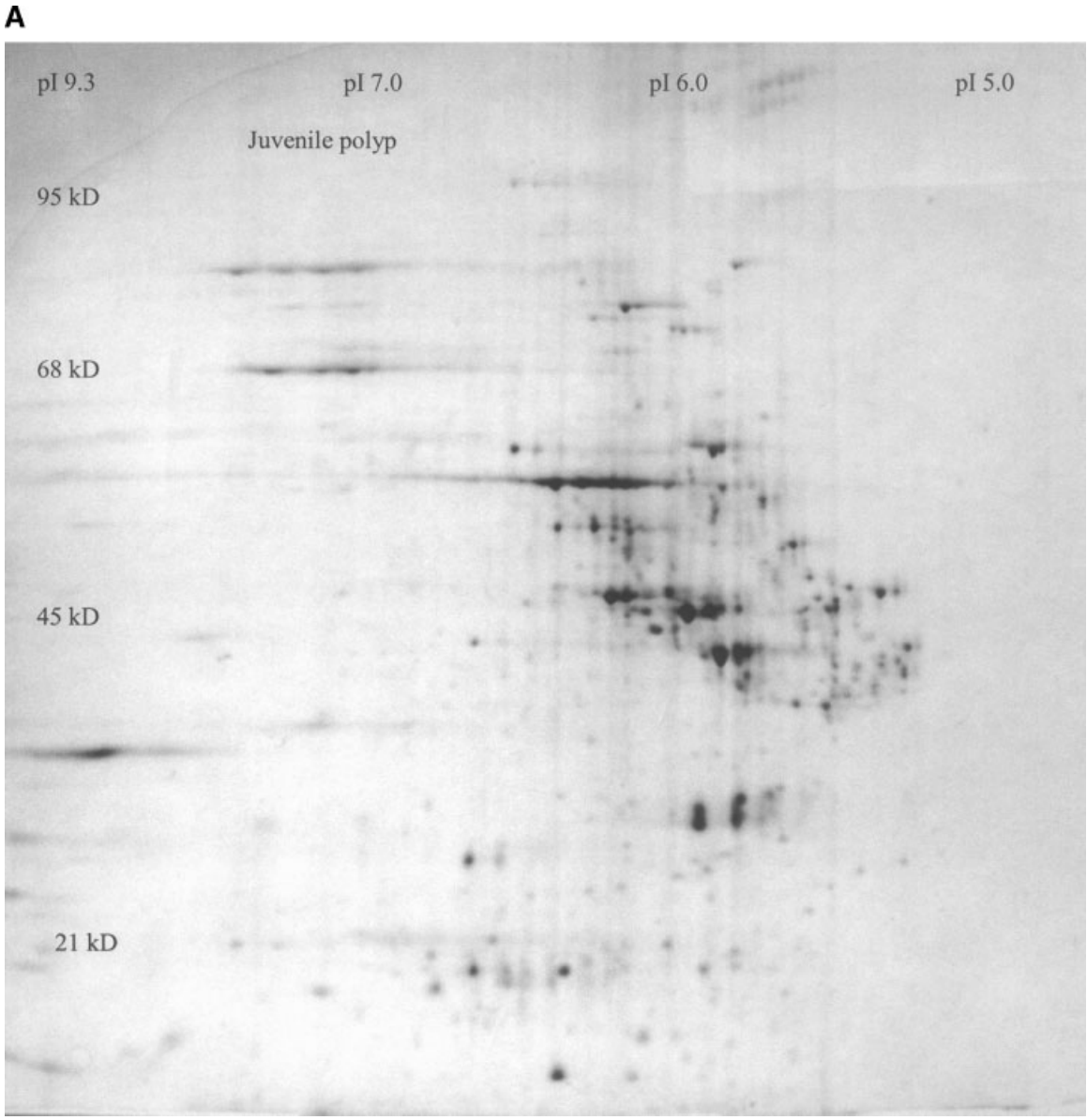

Fig. 1. Silver stained high-resolution two-dimensional (2-D) gel electrophoresis of nuclear matrix proteins (NMP) of human colon polyps: juvenil polyp (**A**) and tubular adenoma (TA, **B**) and tubulovillous adenoma (TVA, **C**) and TVA with focal high-grade dysplasia (HGD, **D**) tissue representative of the nuclear matrix patterns demonstrated in these studies.

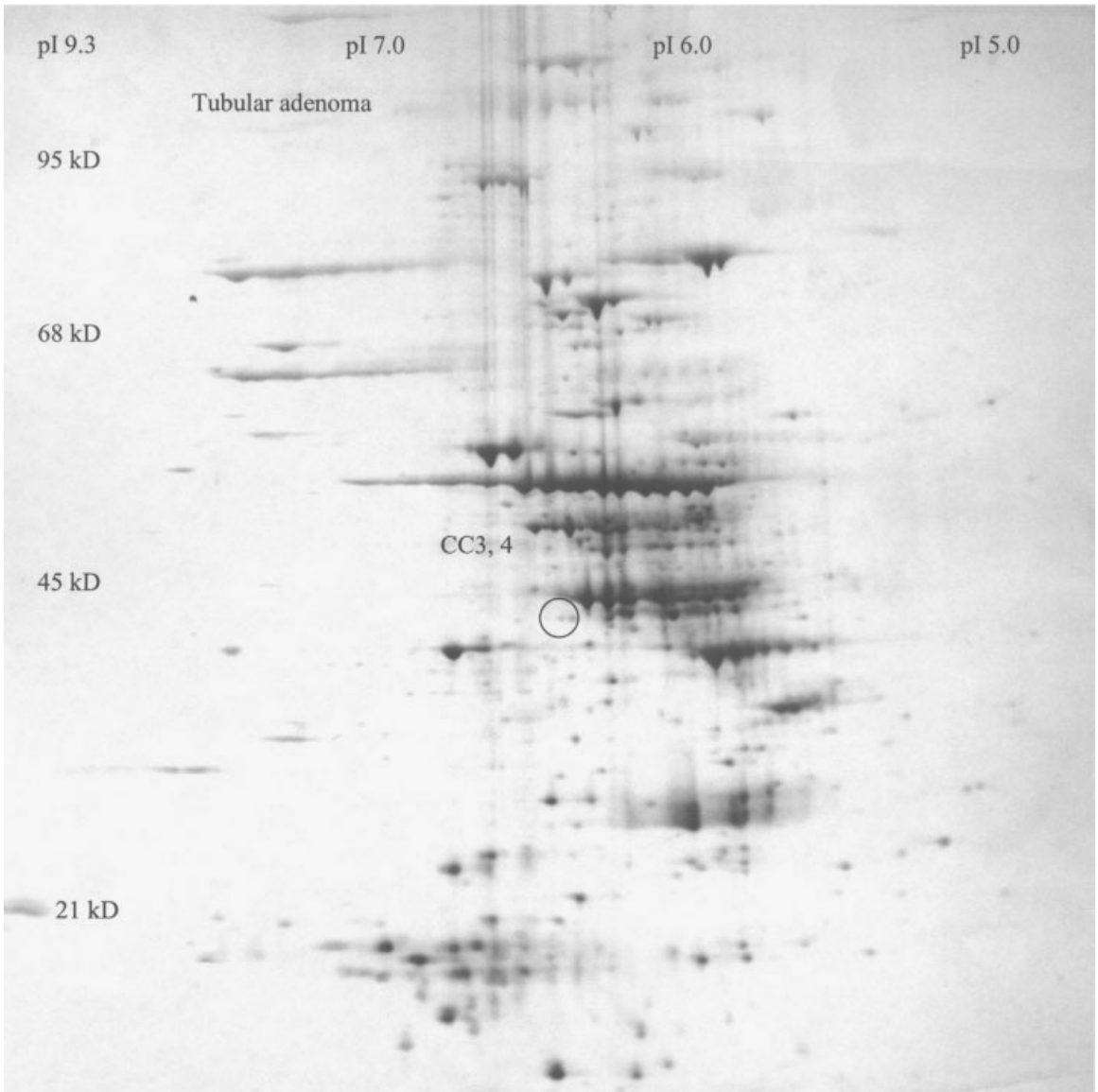

Fig. 1. (*Continued*)

Human 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 colon epithelial cells, as well as to identify potential models to study, the NMP compositions of the colon cancer cell lines $CaCo_2$ was previously examined shown to express CC3 and CC4. Additionally to find a colon cancer cell line, which expressed CC2 and/or CC5, we examined another colon cancer cell line CX-1. The human colon cancer cell line CX-1 expressed CC2, CC3, and CC4, but not CC5 (Fig. 3).

DISCUSSION

Over the past two decades our understanding of colorectal tumorigenesis has advanced rapidly. The theory of the adenoma-carcinoma sequence is now accepted, strongly supported by the demonstration of a progressive accumulation in molecular mutations, which accompany the evolution from adenoma to carcinoma. The clinical practice of polypectomy and post-polypectomy surveillance is routine. However, the utilization rate for the available screening modalities for colorectal cancer remains low [Ries et al., 2000]. The development of non-

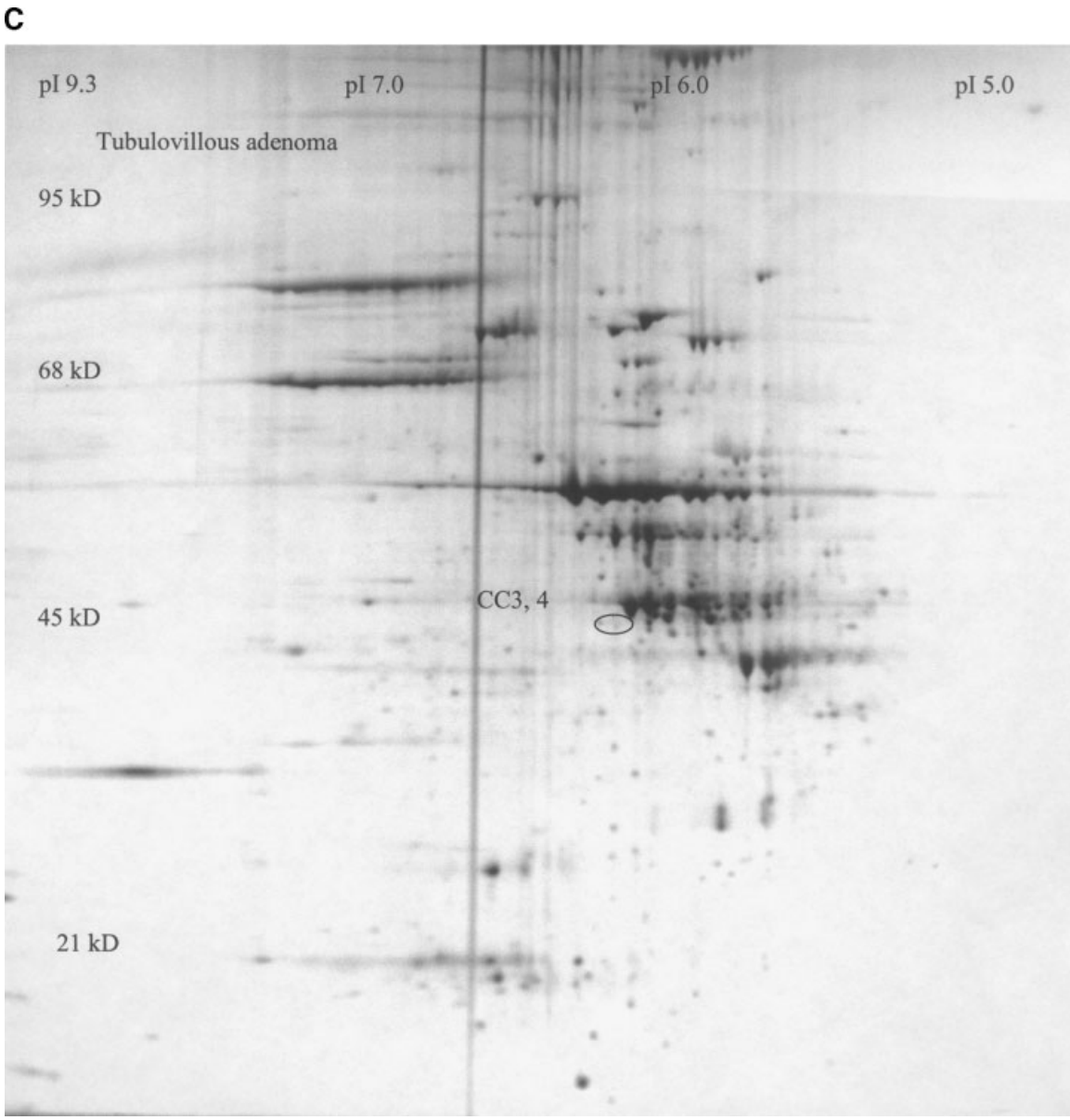

Fig. 1. (*Continued*)

invasive assays for early detection of colon cancer or adenomatous polyps, and especially a blood test for colon cancer, is the next frontier in colorectal cancer screening.

The significant disparity in prevalence of adenomatous polyps (around 30%) [Lieberman et al., 2000] and lifetime risk of colorectal cancer (around 6%) [Imperiale et al., 2000, 2002], point to the well-understood fact that only a small proportion of adenomas progress to invasive cancer. Natural history studies demonstrate that certain histologic characteristics, such as size 1 cm, villous histology, and the presence of HGD are associated with increased risk of colorectal cancer [Atkin et al., 1992]. However, although these characteristics are used to guide recommendations on the need and timing of subsequent surveillance, their accuracy in defining risk is only approximate. Markers are needed that can detect early colon cancer, that can better predict which patients with colon polyps are at increased risk for subsequent colon cancer and need closer surveillance, and that can detect premalignant lesions in the colon.

Our approach to developing non-invasive markers has been through study of nuclear structural elements. We previously identified

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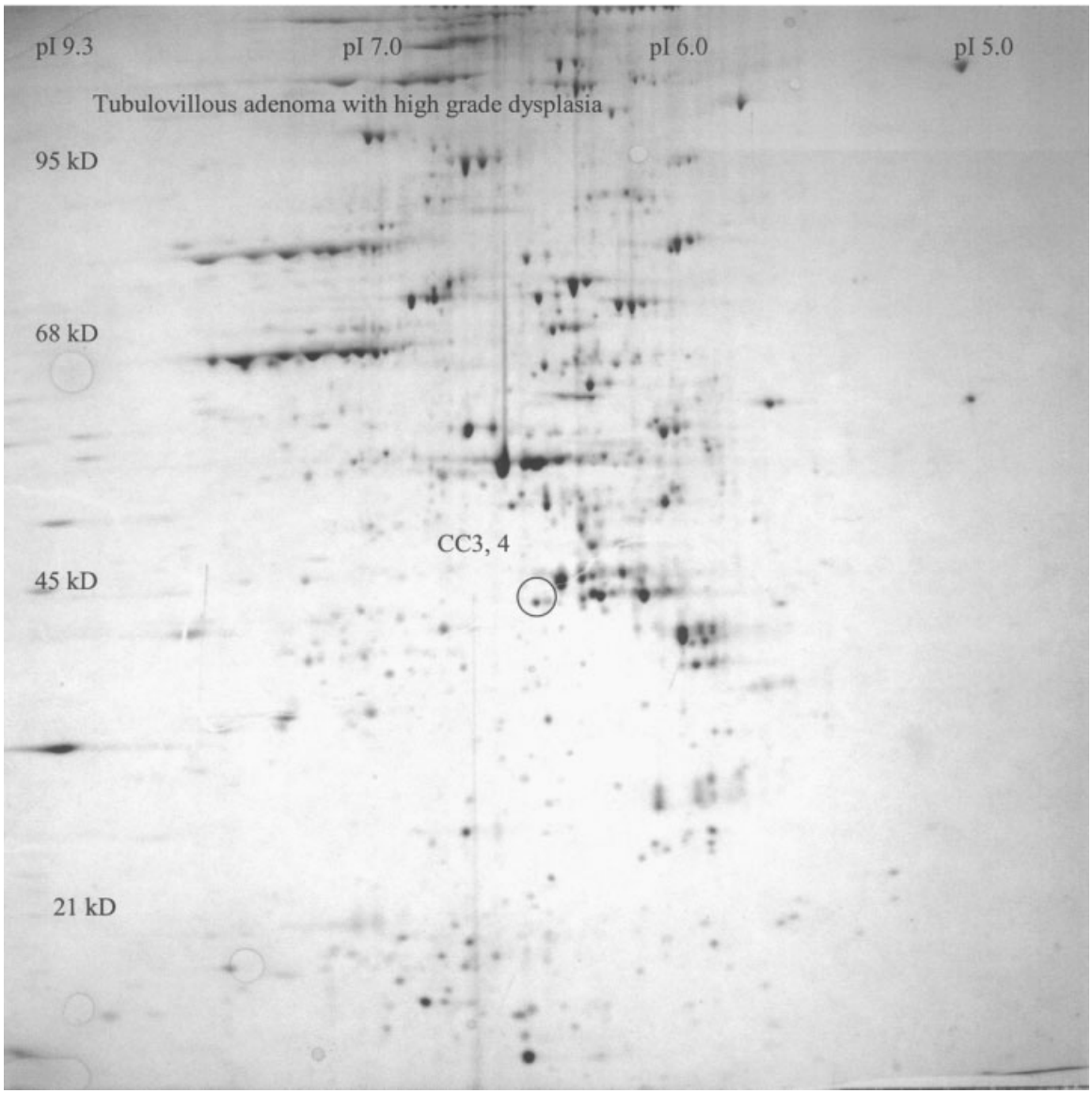

Fig. 1. (*Continued*)

TABLE III. NMPs Specific for Colon Cancer and Present in Colon Polyps and in Colon Cancer Cell Line CX-1

	CC2 (%)	CC3 (%)	CC4 (%)	CC5 (%)
TVA with HGD (n = 6)	0	83	100	33
TVA (n = 7)	0	86	86	0
TA (n = 6)	0	83	100	17
Juvenile polyps (n = 1)	0	0	0	0
Human colon cancer cell line CX-1 (n = 1)	100	100	100	0
Colon cancer (n = 10)[a]	80	100	100	100
Colon cancer normal adjacent (n = 10)[a]	0	0	0	0
Colon normal donor (n = 4)[a]	0	0	0	0
Human colon cancer cell line $CaCo_2$ (n = 1)[a]	0	100	100	0

[a]Data from Brunagel et al. [2002a].

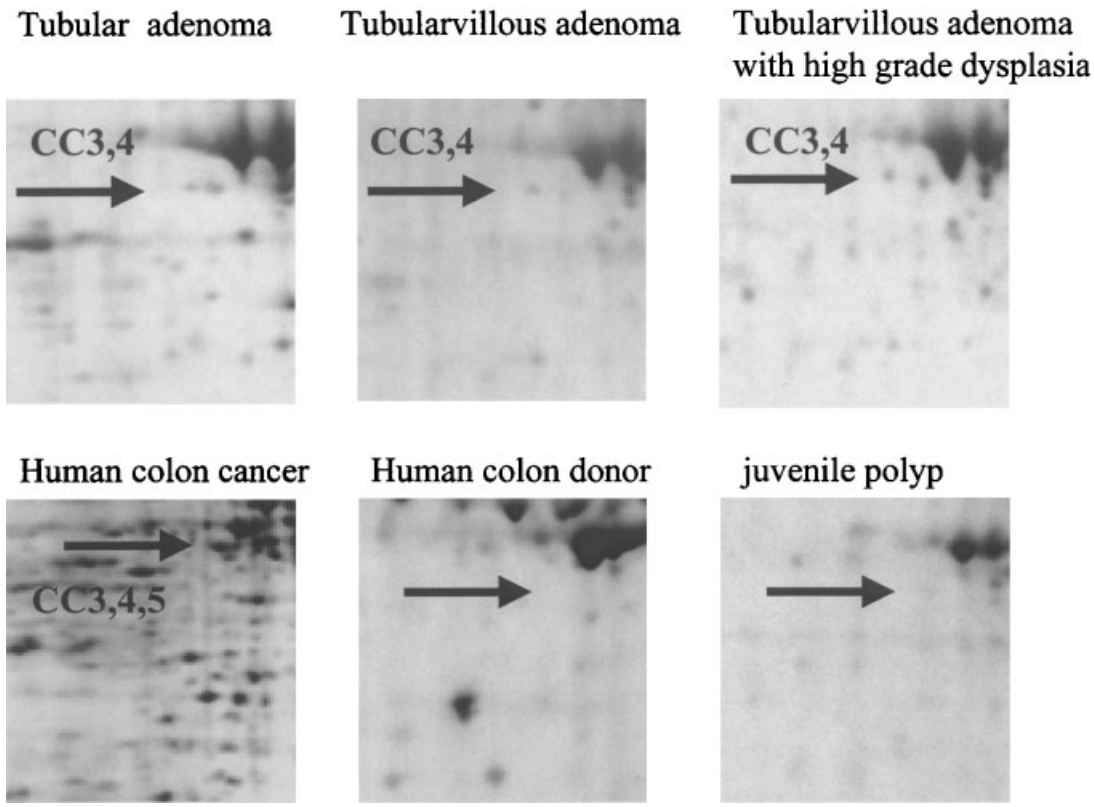

Fig. 2. Silver stained high-resolution 2-D gel electrophoresis of tissue representative areas of NMP CC3, CC4, and CC5 in human colon polyps: juvenil polyp and TA and TVA and TVA with HGD and human colon cancer and human colon donor demonstrated in these studies.

four specific, unique NMPs (CC2, CC3, CC4, CC5) that were expressed in colon cancer samples but not in the histological normal tissue adjacent to the cancer and not in normal, donor colon tissue samples. In this study, we delved further back in the adenoma-carcinoma sequence to determine where in the malignant process these proteins are expressed.

The NMP CC2 was not seen in any of the premalignant polyps we examined, but was present in 80% of colon cancers (Table III). One potential explanation for this finding is that CC2 is not expressed in the epithelium from colon cancer and therefore, cannot be found in the epithelium present in colon polyps, because we did not find CC2 in the colon cancer cell lines we examined. For this reason, we examined another epithelial colon cancer cell line, CX-1, and found the expression of CC2 in this cell line, indicating the potential that the protein is expressed in the colonic epithelium.

One open question is whether the polyps we examined will develop into colon cancer and if so, in what time frame. Even polyps adenomas with severe dysplasia could take years to progress to cancer [Kozuka et al., 1975]. However, in view of the fact that CC2 was not expressed in the advanced adenomas but was present in 80% of invasive carcinomas, suggests that CC2 is expressed at the point of invasion. Similarly, CC5 was present in only 15.7% of the adenomas, but was present in all colon cancer tissues (Table III).

In contrast CC3 and CC4 were present in 83–100% of the advanced adenomas we tested, and in all the colon cancer specimens. However, CC3 and CC4 were not present in normal epithelium. Thus, CC3 and CC4 are expressed earlier in the adenoma-carcinoma pathway then CC2 and CC5. Further efforts to determine whether these proteins are present in small, non-advanced adenomas, or hyperplastic polyps are needed, to determine where in the progression they appear. If they are expressed in advanced adenomas and not in non-advanced adenomas, these proteins could be an exciting target for early detection. It should be noted and emphasized that none of the four proteins were found

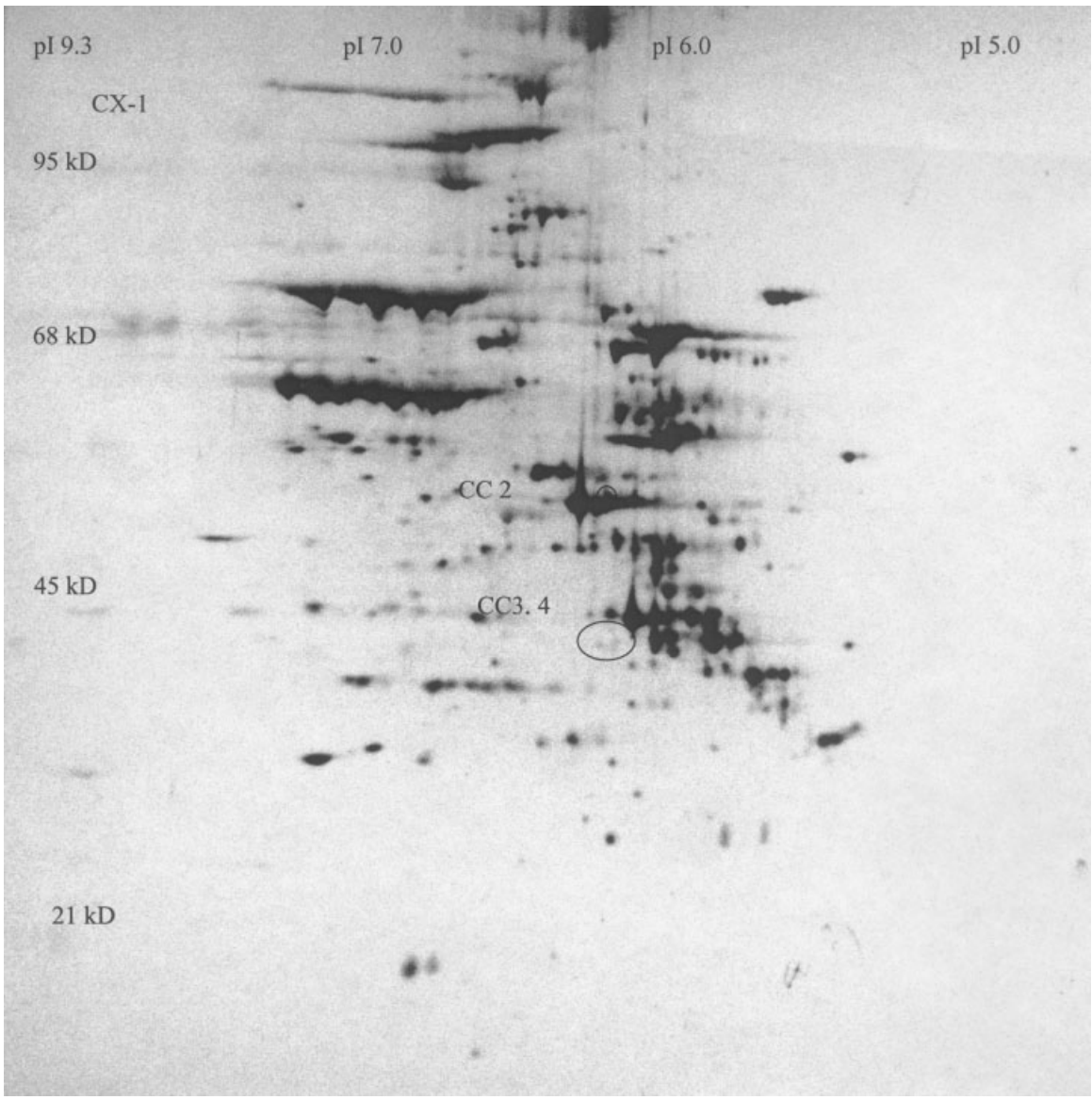

Fig. 3. Silver stained high-resolution 2-D gel electrophoresis of NMP of the human colon cancer cell lines CX-1.

in a juvenile polyp, which is not considered to be a precursor for colon cancer, further substantiating the notion that these proteins are part of the neoplastic process.

In summary, this study demonstrates that NMP found in invasive colon cancer can be identified in premalignant human adenomatous polyps. CC3 and CC4 may serve as indicators for adenomatous polyps, whereas CC2 and CC5 appear to be associated with transformation of an advanced adenomatous polyp to an invasive cancer. The combination of these proteins as markers seems to be the most promising option for early detection. The identification of these proteins and their detection through the generation of NMP antibodies could be used to develop tests for early detection of colon polyps and colon cancer.

A biologic understanding of these proteins could provide new insight into the transformation of adenomas into carcinoma. The long-term goal is to develop an assay, which can these specific proteins detect in the circulation, colonic aspirates, or tissue samples. Development of such assays could serve as early detection markers with high sensitivity and specificity.

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