

*Advances in Brief***Nuclear Matrix Protein Alterations Associated with Colon Cancer Metastasis to the Liver¹****Gisela Brünagel, Robert E. Schoen,
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Abstract

Purpose: The development of colon cancer markers that can detect liver metastases early and predict which patients are at risk to develop liver metastases would have a major impact on this disease. We have previously identified G. Brünagel, *et al.*, *Cancer Research*, 62: 2437–2442, 2002, nuclear matrix proteins (NMPs), which are associated with colon cancer. The objective of this study is to identify the existence of a specific NMP “fingerprint” for human liver metastasis from colon cancer.

Experimental Design: Using high-resolution two-dimensional gel electrophoresis, we analyzed the NMP expression of 12 matched liver metastases and adjacent normal samples and three normal donor liver samples. These were compared with colon cancer NMP patterns, along with several primary cell systems and lines.

Results: Analysis of multiple gels for each sample revealed three proteins present in all liver metastases, which are not present in normal liver tissue and normal hepatocytes. These three proteins were also present in colon cancer samples.

Conclusion: Data provided here demonstrate that the NMP composition is able to differentiate liver metastases from normal liver tissue and normal hepatocytes and that these proteins are also expressed in colon cancer. These results further show that the adjacent normal liver tissue changes its NMP pattern of expression. Development of an assay to detect these specific NMPs in tissue and/or serum specimens is a promising modality for early detection of liver metastases from colon cancer or potentially as a prog-

nostic tool. In addition the functional characterization of these proteins will significantly enhance our understanding of the development of liver metastases of this disease.

Introduction

Colorectal cancer is a worldwide public health concern. In the United States, colorectal cancer accounts for 11% of all cancers with 147,300 new cases and 56,600 deaths estimated for 2002 (1). Although early detection of colon cancer is important, for many individuals it does not occur or is ineffective and almost one-half of all patients with colon cancer die of metastatic disease.

Patients with stage II (Dukes' stage B) colon cancer have a 5-year survival of 70–80% after curative surgery, but it is difficult to identify the 20–30% of patients who will have recurrence. For stage III patients (Dukes' stage C) with involvement of regional lymph nodes, the 5-year survival rate decreases to 30–50%. A significant improvement in survival in patients with stage III disease can be achieved with adjuvant chemotherapy (2). Some patients with stage II (Dukes' stage B) disease are at high risk for recurrence and receive adjuvant chemotherapy, although its benefit in such cases is less clear.

The most common site of colon cancer recurrence is the liver (3). If metastatic disease is left untreated, the median survival is 6–12 months (4). Resection of liver metastases offers the only chance for cure, although ~70% of patients who undergo initial hepatectomy will have tumor recurrence, about one-half of them having isolated hepatic recurrence (5–7). This raises the possibility of an early tumor cell spread at the time of, or before, surgical resection. Unfortunately, micrometastases are currently not detectable by conventional staging methodology.

The identification of a specific and sensitive tumor marker that would allow reliable early detection of cancer recurrence and predict the potential to develop liver metastases after attempted curative surgery would be of great benefit for those with colon cancer (8). The indication for adjuvant chemotherapy could, therefore, be tailored to individual patient risk.

The most commonly used blood test to detect recurrence of colorectal cancer is CEA³ a tumor-associated glycoprotein. Serum CEA protein is used to follow the course of therapy in the management of colorectal cancer. In postoperative follow-up, CEA appears to be a useful marker of recurrence (specificity, 77%; sensitivity, 98%), mainly for liver metastasis, but it has been shown that only one-half of colorectal cancers shed CEA levels sufficient for detection (9, 10). The utility of CEA in detecting recurrences is, therefore, controversial (11, 12).

Alterations in cellular and nuclear structure are hallmarks of the carcinogenic process. Pathological identification of can-

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³ The abbreviations used are: CEA, carcinoembryonic antigen; NMP, nuclear matrix protein.

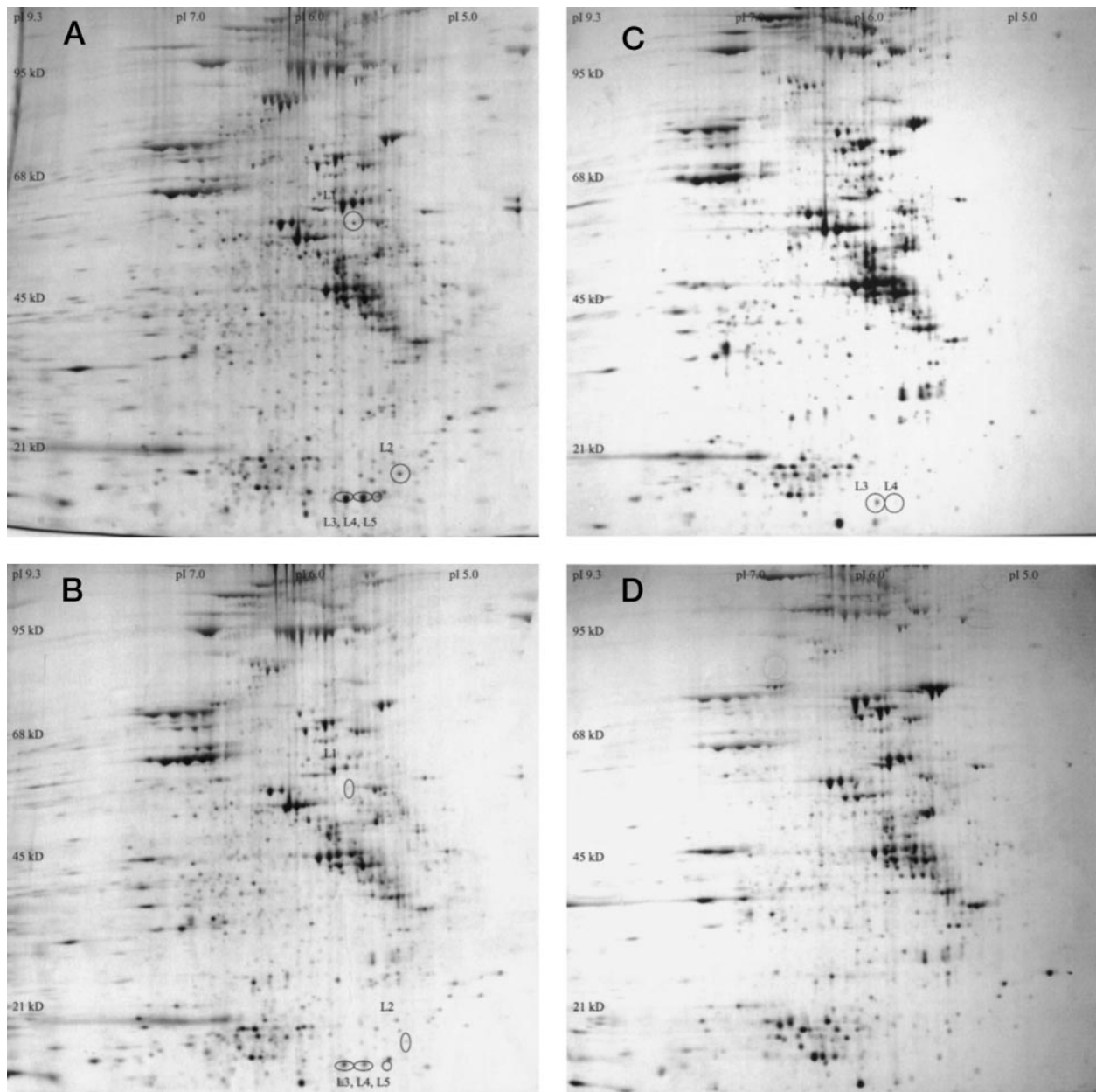


Fig. 1 Silver-stained high-resolution two-dimensional gel electrophoresis of NMPs of human liver metastasis (A) and normal adjacent (B) and normal donor (C) liver tissue and normal hepatocytes (D), representative of the nuclear matrix patterns demonstrated in these studies. *kD*, *M_r* in thousands.

cer 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. Nuclear structure is determined by the scaffolding of the nucleus, the nuclear matrix. Alterations in nuclear shape or structure that occur with neoplastic transformation are accompanied by changes in nuclear matrix composition and architecture (13). Gene expression is highly dependent on the interaction between chromatin and the

nuclear matrix (14–16). Changes in the framework of the nuclear matrix may alter gene expression by affecting DNA transcription or replication.

The nuclear matrix consists of the peripheral lamins, protein complexes, an internal ribonucleic protein network, and residual nucleoli (17). The nuclear framework consists of ~10% of the nuclear proteins and is virtually devoid of lipids, DNA, and histones (18). Most of the NMPs identified to date are common to all cell types, but several identified NMPs are tissue- and cell line-specific and NMPs have been shown to undergo change with differentiation (19, 20).

Table 1 NMPs present in liver metastasis, adjacent tissue, donor tissue, normal hepatocytes, and human primary liver cancer cell lines (HepG 2, Huh 7); NMPs present in colon cancer, adjacent tissue, donor tissue, and human colon cancer cell lines (CaCo2 and SW480)

	L3	L4	L5	L1	L2
Liver metastases (<i>n</i> = 12)	100%	100%	83%	92%	100%
Normal adjacent liver (<i>n</i> = 12)	92%	17%	8%	42%	17%
Normal donor liver (<i>n</i> = 3)	33%	33%	0%	0%	0%
Normal hepatocytes (<i>n</i> = 1)	0%	0%	0%	0%	0%
HepG 2 (<i>n</i> = 1)	0%	0%	0%	0%	0%
Huh 7 (<i>n</i> = 1)	0%	0%	0%	100%	0%
Colon cancer (<i>n</i> = 10)	90%	60%	20%	70%	100%
Normal adjacent colon (<i>n</i> = 10)	100%	100%	0%	0%	10%
Normal donor colon (<i>n</i> = 4)	100%	75%	0%	25%	0%
CaCo 2 (<i>n</i> = 1)	100%	0%	0%	0%	100%
SW480 (<i>n</i> = 1)	0%	0%	0%	0%	100%

Individual cancer cell lines can be distinguished based on a signature patterns 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, renal, and colon cancers (22–25). The detection of NMPs in the serum of patients with various types of cancer has been accomplished (26). The detection of NMPs in the serum and urine is possible because of the release of NMPs as tumor cells undergo degeneration and lysis. The development of antibodies identifying aberrant NMPs associated with liver metastasis from colon cancer could become clinically important tumor markers.

We have recently identified a specific NMP fingerprint in colon cancer (25). Four proteins specific for colon cancer were identified that are not present in normal adjacent and donor colon tissue. These proteins are being sequenced and antibodies generated to provide reagents for additional studies. Development of an assay with which to detect these specific NMPs by examining tissue, serum, and stool specimens is a promising modality for early detection of colon cancer.

The objective of this study was to investigate the NMP composition of liver metastases from human colon cancer in an effort to identify unique liver metastasis-associated proteins. Additionally, the analysis of the function of such proteins could aid in understanding the complex biology of metastasis in human colon cancer.

Materials and Methods

Tissue Processing. All of the tissues were collected through the Early Detection Research Network (EDRN) of the University of Pittsburgh Medical Center under Institutional Review Board approval. Colon adenocarcinoma liver metastases (*n* = 12) and matched adjacent normal liver tissues (*n* = 12) were obtained. The patients ranged in age from 44–75 with a mean age of 62.4 years. Sixty % of the sample population was female. Normal liver tissue was obtained from trauma victims, including one sample from a gunshot victim and two from

automobile accidents (*n* = 3). The patients ranged in age from 36–48 with a mean age of 40.3 years. Two of these normal individuals were male and one was female. None of the patients had liver cirrhosis. Thirty % of the patients had mild steatosis. The tissues were stored at -80°C before processing. Sporadic colon adenocarcinoma samples and matched adjacent normal tissues and normal donor colon tissues were collected as described previously (25).

The colon cancer cell lines SW480 and CaCo 2 were obtained from the American Type Culture Collection. Both cell lines have been established from primary human colon cancer cells. The SW480 cell line was grown in Leibovitz medium with 10% fetal bovine serum at 37°C without CO_2 . The CaCo 2 cell line was grown in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), 1% penicillin/streptomycin, 1% sodium pyruvate (100 mM), 1% MEM nonessential amino acids, and 1.5% HEPES buffer (1 M) at 37°C in a 5% CO_2 atmosphere.

The 50×10^6 normal human hepatocytes were obtained from a 63-year-old female organ donor and were a kind gift from Dr. Stephen Strom of the University of Pittsburgh, Pittsburgh, PA. Both of the human primary liver cancer cell lines (Huh 7 and HepG 2) were a kind gift from Dr. George Michalopoulos, University of Pittsburgh. Both of these cell lines were grown in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

Nuclear Matrix Preparation. NMPs were extracted from liver metastases tissue, adjacent liver tissue from these individuals, and donor patients according to the method of Getzenberg *et al.* (24). In summary, tissue was 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

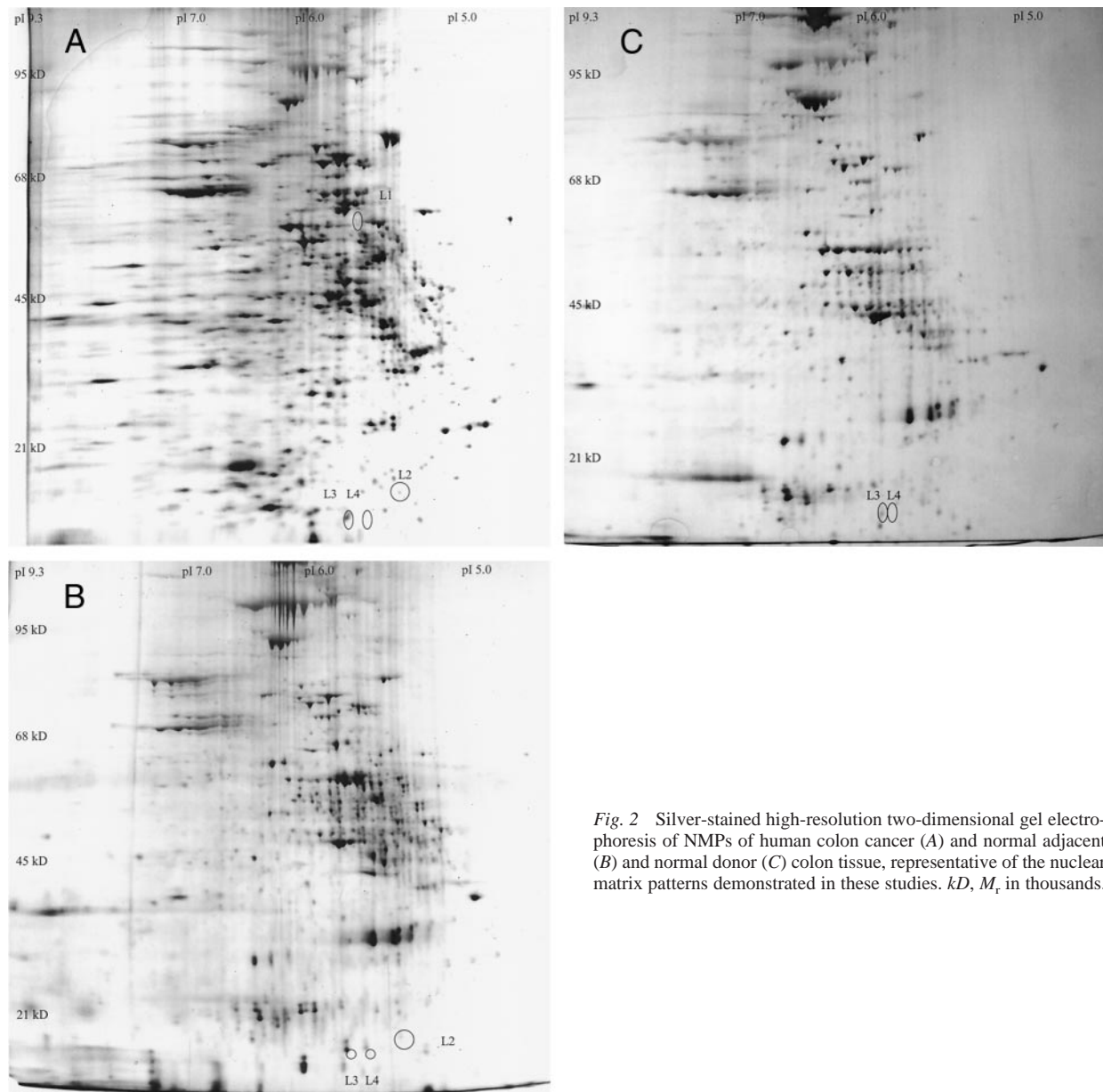


Fig. 2 Silver-stained high-resolution two-dimensional gel electrophoresis of NMPs of human colon cancer (A) and normal adjacent (B) and normal donor (C) colon tissue, representative of the nuclear matrix patterns demonstrated in these studies. *kD*, *M_r* in thousands.

protein concentration was determined by resuspending the pellet in a two-dimensional sample buffer consisting of 9 M urea, 65 mM 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate, 2.2% ampholytes, and 140 mM DTT, and was quantitated by Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. The final pellet containing these proteins represent <1% of the total cellular proteins.

High-Resolution, Two-Dimensional Electrophoresis.

High-resolution, two-dimensional electrophoresis was performed using the Investigator two-dimensional gel system (Genomic Solution, Ann Arbor, MI) as described previously (24, 27). One hundred μ g of protein were loaded per gel onto a capillary-size isoelectric focusing column. One-dimensional isoelectric focusing was carried out for 18,000 V-hours using

1-mm \times 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 Solution, Ann Arbor, MI) 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, the 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 an adaptation of the method of Wray *et al.* (Accurate Chemical Co., Westbury, NY; Ref. 28). Molecular weights of colon NMPs were identified 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

Table 2 NMPs that are able to differentiate human normal and donor liver tissue from liver metastases

Proteins associated with human liver metastasis	M_r	Isoelectric point
L1	50,000	6.01
L2	20,000	5.73
L3	18,000	6.09
L4	18,000	6.00
L5	19,000	5.88

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 Two-Dimensional Electrophoresis Analysis System (BioImage, Ann Arbor, MI), which matches protein spots between gels and sorts the gels and protein spots into a database.

Results

NMPs from liver metastasis, adjacent normal liver, normal donor liver, and normal hepatocytes were extracted and separated by high-resolution, two-dimensional gel electrophoresis. Image analysis distinguished a characteristic NMP fingerprint from the liver metastasis and the adjacent liver NMPs (Fig. 1 and Table 1). Between 83 and 100% of the liver metastases strongly expressed five unique NMPs (L1–L5), which were undetectable in normal hepatocytes (Fig. 1, A and D). Three (L1, L2, L5) of these proteins were undetectable in normal liver tissue, and two proteins (L3, L4) had expressed significantly lower expression in normal liver tissue compared with the liver metastasis and the normal adjacent liver tissue (Fig. 1, A, B, and C). Four of these proteins were expressed under 42% of the adjacent normal liver tissue samples (Fig. 1B; Table 1). The fifth protein (L3) was expressed in 92% of the adjacent liver tissues but was obviously stronger in the carcinoma extracts compared with adjacent tissue.

Comparing the expression of these proteins with that found in colon cancer tissue, we identified four of these proteins (L1–L4) in more than 60% of the colon cancer samples (Fig. 2A; Table 1). One protein (L5) was expressed in only 20% of the colon cancer samples.

Two proteins (L3, L4) were expressed in 100% of the normal adjacent and donor colon tissue, but much more weakly compared with the liver metastasis samples (Fig. 1A and 2, B and C). One of these five proteins (L2) was weakly expressed in 10% of the adjacent normal colon tissue. One protein (L1) was not found in the adjacent normal colon but in one of the four donor colon samples. Electrophoretic characteristics of the identified proteins are described in Table 2.

Tissue samples are complex mixtures of epithelial, stromal, immunological, and other cell types. To determine whether the nuclear matrix changes that were detected actually represented changes that were occurring in the neoplastic cells, as well as to identify potential models to study, the NMP composition of two human primary liver cancer cell lines were examined. Whereas the NMP fingerprints from the pure cell lines would be expected

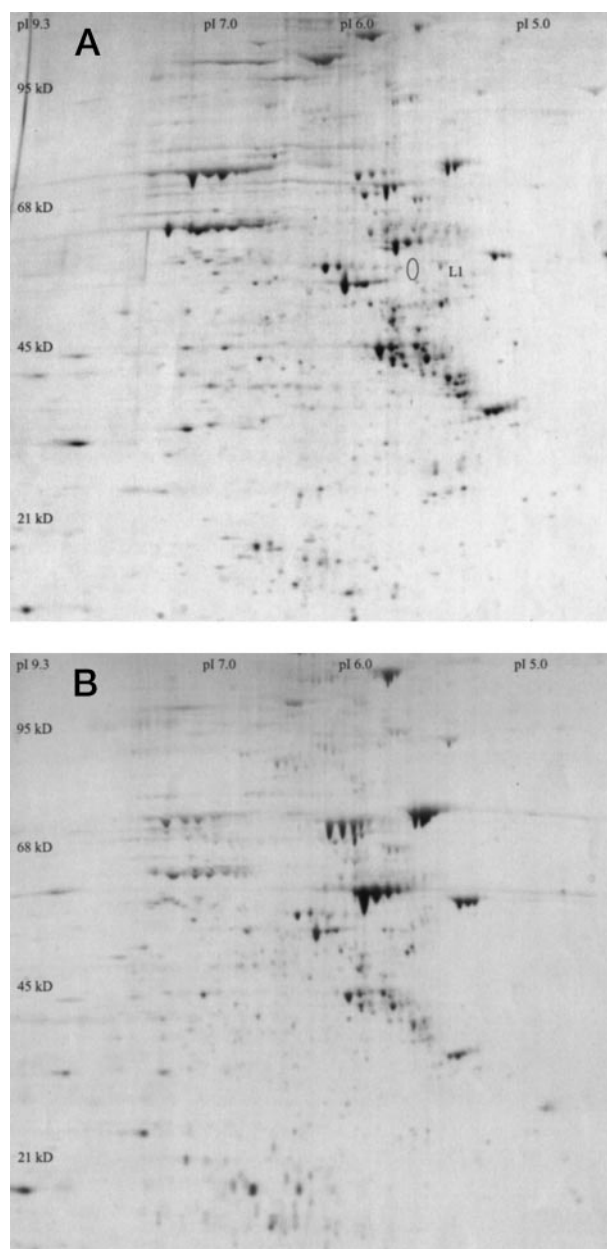


Fig. 3 Silver-stained high-resolution two-dimensional gel electrophoresis of NMPs of the human primary liver cancer cell lines Huh 7 (A) and HepG 2 (B). *kD*, M_r in thousands.

to be distinct from the three-dimensional complex of liver metastasis and colon cancer specimens, they serve as tools for generating reagents as well as for examining a single cell type. The human liver cancer cell lines Huh 7 and HepG 2 and 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 four cell lines are shown in Figs. 3 and 4. The cell line HepG 2 expressed none of the liver metastasis-associated proteins, and the Huh 7 cell line expressed one of the liver metastasis-associated proteins (L1; Fig. 3A). The cell line

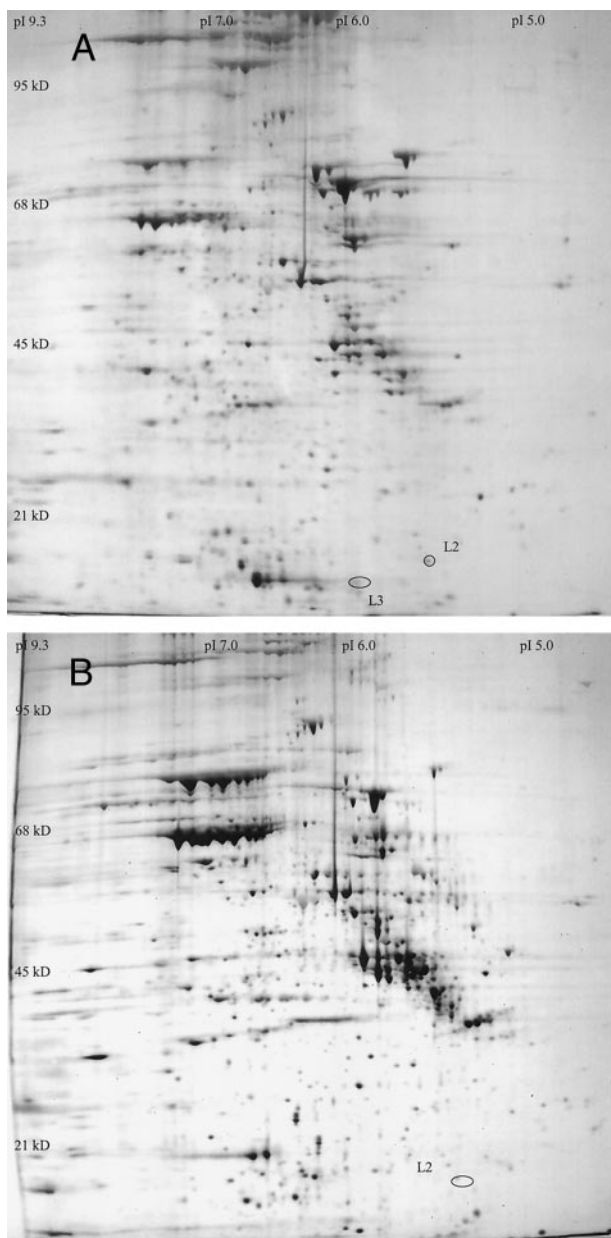


Fig. 4 Silver-stained high-resolution two-dimensional gel electrophoresis of NMPs of the human colon cancer cell lines CaCo 2 (A) and SW480 (B). *kD*, M_r in thousands.

CaCo 2 expressed two of the proteins (L3 and L2) and the cell line SW480 expressed just one protein (L2; Fig. 4, A and B).

Discussion

The early diagnosis of recurrent colon cancer is central to the effective treatment of the disease. Although more than 75% of the patients with colon cancer currently have an attempted curative resection of the primary tumor, more than one-half of them will eventually die of metastatic disease. Markers are needed to detect liver metastases early as well as to help predict which patients are more likely to develop liver metastases.

In this study, we identified one unique NMP (L5) found in liver metastasis, normal adjacent liver tissue, and in colon cancer tissue but not in the normal donor liver, hepatocytes, normal adjacent colon tissue, normal colon donor tissue, or in one of the cell lines. This protein shows a high specificity for liver metastasis and has the highest potential to serve as a biomarker of metastatic colon cancer.

Another unique NMP (L2) is expressed in liver metastases and normal adjacent liver tissue. In comparison to L5, this protein is also found in normal colon adjacent tissue. This protein is expressed in 100% of all colon cancer samples and both colon cancer cell lines. The protein L2 therefore also has potential as a biomarker for liver metastasis and differs from L5 in that it is found in colon tissue adjacent to the tumor as well.

The L1 protein, identified in 92% of the liver metastasis, is expressed in the Huh 7 human primary liver cancer cell line, raising the possibility that this protein is not specific for liver metastasis from colon cancer. The primary liver cancer cell line HepG 2 expressed none of the proteins we identified in liver metastasis from colon cancer.

Unexpectedly, all of the proteins were expressed in 8–92% in the normal adjacent liver tissue. The fact that the proteins are expressed in morphologically normal areas of liver tissue potentially supports the concept of a field effect or a global modification happening in the liver. These proteins may be altered early during the course of metastatic development and may be markers for the development of the disease. The appearance of these proteins in the adjacent liver could potentially serve to predict a risk to develop liver metastasis. In addition, we do not have any information regarding the distance at which the normal adjacent liver tissue was taken from the liver metastasis. Variation in distance of normal adjacent liver tissue from the metastatic lesion could be an explanation for the difference in expression of these proteins in the adjacent normal liver tissue.

The discovery of these proteins in morphologically normal areas of liver tissue may help to predict subsequent recurrence and may help explain the high observation of recurrence after attempted curative liver metastases resection.

All of the proteins (L1–L5) were also found in 20–100% of colon cancer tissues. Two of them (L3 and L4) were also expressed in the adjacent normal colon tissue and normal donor tissue. But in the normal adjacent and donor colon tissue, the expression was significantly less, compared with that in the liver metastatic tissue. Additionally one of these two proteins (L3) was found in the CaCo 2 cell line, which confirmed the neoplastic source of the protein already in the colon epithelia.

These studies demonstrate that NMPs isolated from human liver metastasis are distinct from normal donor tissue and hepatocytes. In addition, the normal adjacent liver tissue contains alterations in the nuclear matrix pattern similar to those found in liver metastasis. These five proteins were also found in primary colon cancer but were not found in other cancer types. The proteins currently are being sequenced and antibodies raised against them.

The functional identification of these proteins and their detection through the generation of NMP antibodies could be used to develop tests for colon cancer prognosis and early detection of metastases. The long-term goal of these studies

is to generate antibodies to detect specific NMPs in the blood or tissue samples. Assays will be developed that include the detection and combination of individual proteins identified here. The development of assays with these antibodies potentially could serve as tumor marker with high sensitivity and specificity.

Additionally the presence of unique NMPs in liver metastasis or the up-regulation in cancer cells could provide novel information about their function in the development of metastasis and perhaps provide us with additional targets for anti-cancer therapies.

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