Identification of Nicotinamide N-Methyltransferase as a Novel Serum Tumor Marker for Colorectal Cancer

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Abstract

Purpose: The goal of this study was to identify and validate novel serum markers of human colorectal cancer as potential candidates for noninvasive detection of early colorectal neoplasm.

Experimental Design: Employing two-dimensional gel electrophoresis and mass spectrometry, we analyzed 16 matched colorectal cancer and adjacent normal tissue samples. Proteins found to be elevated in cancer tissue were further validated by generating antibodies which were used for immunoblotting of tissue samples and for the development of highly sensitive immunoassays for assessment of serum samples.

Results: In total, 735 different proteins were identified in colon tissue. Strong elevation in colorectal cancer for five proteins was confirmed by immunoblot analysis: transforming growth factor-β1 induced protein ig-h3 (igIG-H3), nicotinamide N-methyltransferase (NNMT), nucleoside diphosphate kinase A (nm23-H1), purine nucleoside phosphorylase (PNPH), and mannose-6-phosphate receptor binding protein 1 (M6P1). Elevated levels of NNMT, which is not predicted to be secreted but is known as a cytoplasmic protein, were found in serum from patients with colorectal cancer. Employing a receiver-operating characteristic curve based on the measurement of 109 patients with colorectal cancer and 317 healthy controls, we obtained an area under the curve of 0.84 for NNMT, which was superior to the established tumor marker carcinoembryogenic antigen with an area under the curve of 0.78.

Conclusions: It is proposed that NNMT serum levels may have significance in the early detection and in the management of patients with colorectal cancer.

Colorectal cancer is one of the most common cancers worldwide and continues to be a serious public health concern. It accounts for 11% of all cancers in the U.S., with an estimated 146,940 new cases and 56,730 deaths expected in 2004; the worldwide and continues to be a serious public health concern. It accounts for 11% of all cancers in the U.S., with an estimated 146,940 new cases and 56,730 deaths expected in 2004; the lifetime risk of developing the disease is close to 6% (1). The risk of recurrence and subsequent death due to colorectal cancer is closely related to the stage of the disease at the time of primary diagnosis. Indeed, recent studies have shown that shifting the detection of the disease to an earlier stage by mass screening and intervening at this stage can reduce the risk of dying from colorectal cancer (2, 3). Various screening methods for early detection of colorectal tumors are available (reviewed in ref. 4). Although the guaiac-based fecal occult blood tests lack sufficient sensitivity (5, 6), the visualization of precancerous and cancerous lesions, e.g., by colonoscopy, represents the most sensitive approach to early detection. However, colonoscopy is invasive and involves significant costs, risks, and inconvenience (7–10). Because serologic biomarkers can be analyzed relatively noninvasively and economically, they have the potential to greatly enhance mass screening programs. At present, diagnostic blood tests based on the detection of carcinoembryogenic antigen (CEA), a tumor-associated glycoprotein, are available. However, this marker possesses neither the sensitivity nor the specificity for screening an average risk population (11, 12). Therefore, new cancer biomarkers are needed that will further enhance detection of the disease and trigger a follow-up colonoscopy.

The objective of the present study was to identify cancer-associated proteins using a proteomics approach by analyzing protein expression in healthy and malignant colorectal tissues. We identified a number of proteins that were expressed at markedly higher levels in malignant tissue compared with normal colonic epithelium. To further evaluate if those proteins may constitute potential serologic cancer biomarkers, we generated antibodies to a number of proteins for immunologic
assays. We show the presence of one protein, nicotinamide N-methyltransferase (NNMT), in serum of patients with colorectal cancer. Sensitivity of this marker for colorectal cancer was higher than the established colorectal cancer tumor marker CEA, based on the measurement of 109 patients with colorectal cancer and 317 healthy controls.

Materials and Methods

Chemicals and reagents. All chemicals used were analytic grade from Merck, (Darmstadt, Germany) and Fluka (Buchs, Switzerland) if not indicated differently. Deionized water produced with a Milli-Q system (Millipore, Billerica, MA) was used for all buffers.

Patients and blood donors. Clinical tissue samples were obtained from the Institute for Pathology, Klinikum Kassel, Germany, with written informed consent from all respective patients obtained before surgery and in accordance with an ethic vote of the University of Regensburg and of the Landesa¨rztekammer Hessen (State Medical Association). Malignant tissue (>80% tumor cells, as determined by pathologic assessment) from freshly isolated surgical resections was taken from the tumor, and adjacent healthy tissue, as well as stripped mucosa, were prepared from the same resection. The tissue samples were snap-frozen in liquid nitrogen immediately after resection. In total, tissue specimens from 16 patients with different stages of colorectal cancer were analyzed (Table 1). Patients who had received any chemo- and/or radiotherapeutic treatment before surgery were excluded from this study.

Forty serum samples of patients with colorectal cancer were purchased from Impath (Franklin, MA) and 69 further serum samples from patients with colorectal cancer were obtained from three clinical centers in Germany, with written informed consent from all respective patients, and in accordance with an ethic vote of the University of Regensburg and of the Landesa¨rztekammer Hessen (State Medical Association): Stadtkrankenhaus R¨usselsheim; Medizinische Klinik II, Hochtaunuskliniken, Bad Homburg; and Krankenhaus Nordwest, Frankfurt. An anonymized case report form comprising all relevant clinical data of the patient was created. The 109 cancer samples were grouped to the following stages: UICC 0, 3; UICC I, 33; UICC II, 23; UICC III, 21; UICC IV, 23; unknown, 6. Two hundred and seventy-one specimens from individuals, aged 19 to 86 years (median 37 years), in apparently normal health were donated on a voluntary basis by Roche employees. Forty-six specimens from donors aged 12 to 89 years (median 62 years) with a negative colonoscopy (i.e., no colon pathology except for hemorrhoids in some cases) collected at the three clinical sites mentioned above, were included. Informed consent from all blood donors was documented in every single case.

Venous blood samples. Approximately 9 mL of blood were drawn from the cubital vein, using standard sample tubes without any gel separator. For serum specimens, blood was collected without anticoagulant, kept at room temperature for 20 to 30 minutes, then centrifuged, separated, and frozen at −70°C.

Protein extraction. Frozen tissue samples (−1 g) were cut into small pieces, transferred to the chilled grinding jar of a mixer ball mill MM301 (Retsch, Haan, Germany) and subsequently ground to yield a fine powder. This tissue powder was transferred to a Dounce homogenizer (Wheaton, Millville, NJ), suspended in the 10-fold volume of lysis buffer (40 mmol/L Na3 citrate, 5 mmol/L MgCl2, 1% Genapol X-080, 0.02% sodium azide, Complete Protease Inhibitor; Roche Diagnostics, Mannheim, Germany) and homogenized with both loose and tight fittings (15 strokes each). The homogenate was subjected to centrifugation (10 minutes at 5,000 × g), the supernatant was transferred to another vial, followed by a second centrifugation step (15 minutes at 20,000 × g). The resulting supernatant is henceforth referred to as tissue lysate.

To prepare the samples for two-dimensional PAGE, 2 to 3 mL of tissue lysate were diluted in the 5-fold volume of denaturing buffer (7.7 mol/L urea, 2.2 mol/L thiourea, 4% chlomidopropyl(dimethylammonio)-propanesulfate, 0.4% DTT) and subsequently concentrated

Table 1. Clinical features of analyzed patients

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<th>pH 6-9</th>
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<th>NNMT*</th>
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*Protein was identified on tumor (T) and/or control (C) two-dimensional gel electrophoresis of the subject indicated.

*Subject was used for immunoblot analysis.
using Amicon Ultra-15 tubes with a cutoff of 10 kDa (Millipore) until a volume of 0.5 to 1 mL was reached. These steps were repeated twice and the protein concentration of the resulting denatured samples was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

**Isoelectric focusing and SDS-PAGE.** For all steps, chemicals and equipment from Amersham Biosciences (Uppsala, Sweden) were used. 30-100 μL of the denatured samples (corresponding to 1.5 mg of protein) were diluted in denaturing buffer containing 0.5% of ampholytes. Buffering in a final volume of 350 μL. Samples were loaded on 18 cm IcPcPore DryStrips (pH 4-7 and pH 6-9, respectively), by sample rehydration overnight. Subsequently, the strips were subjected to isoelectric focusing on a Multiphor using the following protocol: (a) step and hold, 500 V, 1 minute; (b) gradient, 3,500 V, 2 hours; (c) hold, 3,500 V, 22 hours, resulting in ~82 kVh. Subsequently, the strips were stored at ~80°C.

For SDS-PAGE, the strips were equilibrated for 15 minutes in equilibration buffer (6 mol/L urea, 1.5 mol/L Tris-HCl, pH 8.8, 30% glycerol, 2% SDS) containing 32 mmol/L DTT and subsequently for 15 minutes in equilibration buffer containing 235 mmol/L iodacetamide. The equilibrated strips were transferred to 12.5% polyacrylamide gels and overlayed with 1% agarose containing traces of bromphenol blue. Afterwards, the strips were subjected to electrophoresis using an Ettan DALT II system applying constantly 1 W per gel for 1 hour, followed by 17 W per gel until the dye front reached the end of the gels (~5 hours). Gels were fixed for 1 hour (50% methanol, 10% acetic acid) and stained overnight with the Colloidal Blue Stain Kit (Invitrogen, Carlsbad, CA).

**Peptide mass fingerprinting and identification of proteins.** Peptide mass fingerprinting analysis was done as described elsewhere (13) with minor modifications. All spots on the gels were excised and placed into 96-well microtiter plates. The excised spots were destained using a Cy-Well robot (Cybio, Jena, Germany) by adding 180 μL of 100 mmol/L NH₄HCO₃ in 30% acetonitrile and incubation at room temperature for 10 minutes. The procedure was repeated twice and the gel piece was dried in a Speedvac evaporator. The dried gel piece was rehydrated with 5 μL of 20 μg/mL recombinant trypsin (proteomics grade, Roche Diagnostics) solution. After 5 hours at room temperature, 20 μL of 50% acetonitrile, containing 0.3% trifluoroacetic acid were added and the gel pieces were incubated for 15 minutes with gentle shaking.

Sample application to an Anchorchip target plate (Bruker Daltonics, Bremen, Germany) was carried out as described (13). Samples were analyzed in an Ultraflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker Daltonics). Peptide matching and protein searching was done automatically using an in-house developed software as described (14, 15). Generally, peptide mass spectra matching theoretical digest fingerprints were found solely by the calculation of the probability of a random match. A positive match was accepted if the probability of getting a random match in the protein database was <1 / (1,000 x N_proteins), where N_proteins is the number of distinct relevant proteins in the sequence database.

**Recombinant antigen production, synthesis of peptides, and generation of antibodies.** For the generation of antibodies, recombinant expression of proteins was done in order to obtain immunogens. cDNA clones were obtained from the “Deutsches Ressourcenzentrum für Genomforschung GmbH” (Berlin, Germany). Linear DNA expression fragments encoding the respective antigens in-frame to a COOH-terminal His6-tag were PCR-generated using the Linear Template Generation Set, His-Tag (Roche Diagnostics) according to the manufacturer’s instructions. The final PCR product featured all necessary T7 regulatory regions for subsequent in vitro expression using the Rapid Translation System RTS 100 Escherichia coli HY kit (Roche Diagnostics). In cases of poor expression, optimization of the N-terminal codon usage was done using the ProteoExpert software (Roche Diagnostics).

Upon successful in vitro expression, the linear PCR product was cloned into the pBAD-TOPO vector backbone (Invitrogen) for large-scale expression in E. coli BL21 (DE3; ref. 16). E. coli cells harboring the particular expression plasmid were grown at 37°C in Luria broth medium plus ampicillin (100 μg/mL) to an OD₆₀₀ of 1, and cytosolic overexpression was induced by adding isopropyl-d-thiogalactoside to a final concentration of 1 mmol/L. Four hours after induction, cells were harvested by centrifugation (20 minutes at 5,000 × g), frozen and stored at −20°C.

For cell lysis, the frozen pellet was resuspended in chilled 100 mmol/L sodium phosphate (pH 8.0), 7.0 mol/L guanidinium-HCl, 5 mmol/L imidazole, 20 mmol/L thioglycerol, and the suspension was stirred for 2 hours on ice to complete cell lysis. Insoluble material was pelleted by high-speed centrifugation. Soluble lysate was loaded onto a nickel-nitrilotriacetic acid column and washed with at least 10 to 15 column volumes of resuspension buffer. Then the column was washed in three steps of five column volumes each: (a) washing buffer [100 mmol/L sodium phosphate (pH 8.0), 10 mmol/L Tris-HCl (pH 8.0), 20 mmol/L thioglycerol] + 8 mol/L urea; (b) washing buffer + 0.5% SDS; (c) washing buffer + 0.1% SDS. Subsequently, the antigen was eluted by 100 mmol/L sodium phosphate (pH 5.0), 20 mmol/L thioglycerol, 0.1% SDS. The fractions were analyzed, pooled, and stored at 4°C in the same elution buffer.

Peptides were synthesized by standard solid phase technique containing an extra cysteine on the COOH terminus for maleimide coupling to hemocyanin. For generation of polyclonal antibody, recombinant antigens or synthetic peptides coupled to hemocyanin were used to immunize rabbits. Antisera were collected after 3 months.

**SDS-PAGE and Western blots.** SDS-PAGE and Western blotting were carried out using reagents and equipment from Invitrogen. For each tissue sample tested, 10 μg of tissue lysate were diluted in reducing NuPAGE SDS sample buffer and heated for 10 minutes at 95°C. Samples were run on 4% to 12% NuPAGE gels (Tris-glycine) in the MES running buffer system. Matched lysates of tumor and adjacent control tissue of each patient were always separated on adjoining lanes, to allow for visual comparison of signal intensities. The gel-separated protein mixture was blotted onto nitrocellulose membranes using the Invitrogen XCell II Blot Module and the NuPAGE transfer buffer system. The membranes were washed thrice in TBST [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] and blocked with Roti-Block blocking buffer (Carl Roth GmbH, Karlsruhe, Germany) for 2 hours. The polyclonal rabbit serum was diluted 1:10,000 in Roti-Block blocking buffer and incubated with the membrane for 1 hour. The membranes were washed six times in TBST. Subsequently, the specifically bound primary rabbit antibody was labeled with a horseradish peroxidase-conjugated polyclonal sheep anti-rabbit IgG antibody (10 μL/mL in 0.5× Roti-Block blocking buffer). After incubating for 1 hour, the membranes were washed six times in TBST. For detection of the bound peroxidase-conjugated anti-rabbit antibody, the membrane was incubated with the Lumi-LightPLUS Western Blotting Substrate and exposed to a Lumi-Film Chemiluminescent Detection Film (Roche Diagnostics).

**ELISA for βG-H3.** Polyclonal rabbit antibody was produced by immunizing animals with a recombinant βG-H3 fragment (amino acids 375-683) and rat antiserum to full-length βG-H3 was produced by genetic immunization (Genovac GmbH, Freiburg, Germany). Throughout all assay steps, 96-well plates (Nunc, Wiesbaden, Germany) were filled with a reagent volume of 100 μL/well. Wells were coated with affinity-purified polyclonal sheep anti-rabbit IgG in coating buffer (50 mmol/L NaHCO₃, pH 9.6) for 60 minutes, rinsed thrice with washing buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.05% Tween 20] and blocked with 1% bovine serum albumin in 40 mmol/L sodium phosphate (pH 7.4), containing 0.9% NaCl. After washing as before, plates were incubated for 60 minutes with a 1:1,000 dilution of polyclonal rabbit anti-βG-H3 antisemum in incubation buffer [40 mmol/L sodium phosphate (pH 7.4), containing 0.9% NaCl, 0.6% Syneronic F68, 1.025% polyethylene glycol (PEG) 40,000, 0.1% bovine IgG, 0.025% acetylated bovine-globulin (Pamela, Madison, WI), 0.01% N-methyl isothiazolone hydrochloride, and 0.1% 2-chloro-acetamide], washed and incubated again for 60 minutes with diluted...
serum samples (1:3,000 in incubation buffer + 0.6% bovine serum albumin without PEG 40,000 and bovine γ globulin). After washing as mentioned to remove unbound sample, the wells were incubated with rat anti-βG-H3 antiserum (1:2,000 in incubation buffer) for 60 minutes and washed again. The plates were incubated for 60 minutes with peroxidase-conjugated donkey anti-rat IgG (Dianova, Hamburg, Germany; 1:2,000 in incubation buffer), washed again, and incubated for 50 minutes with TMB substrate solution (Roche Diagnostics). The reaction was stopped by adding sulfuric acid and absorbance was measured at 450 nm with a correction wavelength of 620 nm using an ELISA reader. A recombinant fusion protein of full-length βG-H3 and the E. coli chaperone SlyD were used for calibration.

**ELISA for NNMT.** Polyclonal rabbit antibody was produced by immunizing animals with a recombinant full-length NNMT. Wells were coated with polyclonal rabbit anti-NNMT immunoglobulin. Coating, washing and blotting was done as described for βG-H3. After blocking and washing, plates were incubated for 60 minutes with a 1:10 dilution of serum samples in incubation buffer. The plate was washed again to remove unbound sample, then wells were incubated with 10 μg/mL biotin-linked rabbit anti-NNMT immunoglobulin in incubation buffer + 2% rabbit IgG. After washing as before, wells were incubated for 60 minutes with a peroxidase-conjugated mouse antibiotin monoclonal antibody (M33), 19 μl/μl, in conjugate buffer (incubation buffer + 5 mmol/L EDTA without PEG 40,000), and washed again. Finally, bound antibody was detected using TMB substrate as described above. A lysate of HT29 tumor cells was used for calibration. The NNMT content of this material had already been estimated by Western blot, comparing the intensity of the NNMT band with known amounts of recombinant full-length NNMT.

**ELISA for nm23-H1.** For detection of nm23-H1 in human serum, a sandwich ELISA was developed using streptavidin-coated 96-well microtiter plates. Twenty microtiters of a human serum sample or a serial dilution of the recombinant nm23-H1 protein as standard antigen were incubated with 100 μl biotinylated polyclonal anti-nm23-H1-(52-65) antibody (1 μg/ml) and with digoxigenylated polyclonal antirecombinant nm23-H1 antibody (1 μg/ml) in 10 mmol/L phosphate (pH 7.4), 1% bovine serum albumin, 0.9% NaCl, and 0.1% Tween 20. After incubation overnight at room temperature, the plates were washed thrice with 0.9% NaCl, 0.1% Tween 20. For the detection of antigen-antibody complexes, 100 μl of a monoclonal antidigoxigenin peroxidase conjugate in 10 mmol/L phosphate (pH 7.4), 1% bovine serum albumin, 0.9% NaCl, and 0.1% Tween 20 were added and incubated for 2 hours. The excess of conjugate was removed by washing the plates thrice with 0.9% NaCl, 0.1% Tween 20. The amount of bound conjugate was detected using TMB substrate as described above. The concentration of nm23-H1 in serum samples was calculated from the standard curve using a serial dilution of recombinant nm23-H1.

Carcinoembryogenic antigen assay. CEA was measured by a commercially available assay (Roche Diagnostics).

**Results and Discussion**

**Identification of proteins in colon tissue.** We obtained tumor, adjacent normal tissue, and adjacent normal stripped mucosa from 16 matched normal and colon cancer samples (Table 1). Proteins were extracted and separated by high-resolution, two-dimensional gel electrophoresis (Fig. 1). For each sample, three gels were prepared. In contrast to recent previous proteomics studies on colorectal cancer (17–19), we did not apply image analysis to distinguish differentially expressed proteins in colon cancer and adjacent normal tissue, because in our opinion, image analysis may be impaired by typical features of two-dimensional gels, such as moderate reproducibility or areas with suboptimal resolution of proteins. Instead, we created a two-dimensional gel database of identified proteins for colon cancer and normal colon tissue in order to identify differentially expressed proteins.

Therefore, on each of the resulting two-dimensional gels, all visible spots were excised, and the corresponding proteins were analyzed by peptide mass fingerprinting in order to identify the protein. The number of spots detected and excised on pH 4-7 gels varied from 210 to 864; on pH 6-9 gels, 36 to 304 spots were detected and excised. In total, 58,500 spots were analyzed, out of which 30,150 led to the identification of a protein. After eliminating any redundancies, we obtained a list of 735 distinct proteins. Of these proteins, 463 (63%) were identified both in tumor samples and in control tissues; 150 (20%) and 122 (17%) proteins were found exclusively in tumor and control tissues, respectively. A complete list of all identified proteins is available as Supplementary material. This is, to our knowledge, the most comprehensive list of the colon proteome thus far.

**Identification of up-regulated proteins in colon cancer.** Up-regulated proteins may not only be found in the group of proteins exclusively found in tumor tissue but also in the group of proteins identified both in cancer and in adjacent normal tissue. In the latter group, up-regulation may be indicated, if a given protein is identified in a significantly higher number of cancer tissues than control samples. In order to validate if our discovery approach resulted in the detection of up-regulated proteins, we selected five exemplary proteins with different features for further characterization.

Transforming growth factor-β induced protein IG-H3 (βG-H3) was identified in seven tumor tissues and was absent in all control samples. The predicted molecular weight/isoelectric point (MW/pI) for this polypeptide of unknown function is 73.1 kDa/7.37 and the position of the spot on the pH 6 to pH 9 gels corresponds nicely to these data for the full-length protein. The position of the spot on the pH 4 to pH 7 gels as well as the data from mass spectrometry rather suggest an NH₂-terminal fragment up to amino acid 420. The predicted MW/pI for this fragment is 44.0 kDa/5.86, which fits well with the position of the corresponding spot. The gene for βG-H3 was initially isolated through a differential display screen for genes induced in response to treatment with transforming growth factor-β (20). Using serial analysis of gene expression, >20-fold elevation of βG-H3 transcripts in colon adenoma and colon cancer compared with normal tissue had been shown (21).

NNMT was identified in both cancer tissue and control samples. However, it was found in all 10 cancer samples analyzed on pH 4 to pH 7 gels, and in contrast, it could only be detected in 1 control sample. Predicted MW/pI for NNMT is 17.14 kDa/6.56, which corresponds well with the position of the spot on the gels. The protein is reported to be predominantly expressed in human liver and is located in the cytoplasm (22). NNMT catalyzes the N-methylation of nicotinamide, pyridines and other structural analogues (23), thus, it is involved in the biotransformation of many drugs and xenobiotic compounds. Recently, NNMT expression was reported to be enhanced in papillary thyroid cancer (24).

Purine nucleoside phosphorylase (PNPH) was identified on pH 4 to pH 7 as well as on pH 6 to pH 9 gels. The protein was identified in 11 tumor samples, whereas being present in two control samples only, and the position of the spot fits well with the predicted MW/pI (32, KDa/6.45). The pI in the overlapping range of the two pH gradients used explains why PNPH was found both on pH 4 to pH 7 gels and pH 6 to pH 9 gels. PNPH...
catalyzes the reversible reaction of purine nucleoside + phosphate to purine + ribose-phosphate and is directly involved in a number of essential metabolic processes, such as providing constituents of nucleic acids. Enzyme activity of PNPH was reported to be increased in colorectal cancer and to be correlated with the progress of colorectal cancer as expressed by the Dukes’ stage (25, 26).

Mannose-6-phosphate receptor binding protein 1 (M6P1) was identified in seven tumor samples and in only one control sample. Predicted MW/pI (47.0 kDa/5.30) are in accordance with the corresponding spot. M6P1 is present in the cytosol and on endosomes and is required for mannose 6-phosphate receptor transport from endosomes to the trans-Golgi network in vitro and in vivo. Elevated expression of M6P1 was reported in cervical carcinoma tissues as determined by Western blot analysis (27). In the same article, the authors report that they could not find elevated expression of M6P1 in adenocarcinoma of the colon as compared with healthy tissue samples. We selected M6P1 to clarify these contradictory results.

Nucleoside diphosphate kinase A (nm23-H1) was identified on pH 4 to pH 7 gels in eight cancer tissues and in three control samples, and therefore displayed the weakest evidence with regard to up-regulation in cancer among the five proteins presented here. However, we included nm23-H1 because up-regulation of this protein was described by others using a two-dimensional gel/image analysis approach (17, 19, 28). Predicted MW/pI (17.1 kDa/5.83) for nm23-H1 fit well with the position of the corresponding spot. A number of studies addressed the biological function of nm23-H1 (29, 30) and its expression in tumors, particularly in lymphoma (31, 32), where it can be used as a prognostic factor. Its potential use as a prognostic factor in colorectal cancer is still controversially discussed (33–37). To confirm elevated expression of these proteins in colon cancer, immunoblot analysis was done. Therefore, all five proteins were expressed in E. coli as recombinant proteins and polyclonal antisera were obtained from immunized rabbits. The immunoblotting experiments verified that all five proteins were expressed at significantly higher levels in neoplastic samples, compared with matched normal colon samples. Figure 2 shows a representative compilation of immunoblot analyses for four exemplary patients. Elevation of protein levels in cancer tissue was most prominent for βIG-H3 (immunoblot analysis detected the full-length protein), NNMT, and nm23-H1, and still significant, though less pronounced, for PNPH and M6P1. These data therefore provide independent evidence that protein separation by two-dimensional gel electrophoresis combined with data-mining solely relying on mass spectrometry data, and thereby
avoiding any image analysis–based evaluation, provided an accurate indication of protein expression changes in colorectal neoplasia. Importantly, of the five up-regulated proteins identified by this two-dimensional gel electrophoresis/mass spectrometry approach, only one, nm23-H1, had been previously identified as elevated in colon neoplasia, using image analysis–based two-dimensional gel approaches (17, 19, 28).

Presence of proteins elevated in colorectal tumor tissue in serum. In order to assess the potential release of up-regulated proteins from neoplastic colon tissue into the periphery and their potential value as biomarkers for the disease, highly sensitive immunoassays were established for βIG-H3, NNMT, and nm23-H1. These three proteins were selected because they showed the strongest elevation in colon cancer in the immunoblot analysis. Many researchers have hypothesized that the best cancer biomarkers were likely to be secreted proteins (38). In addition, many classical cancer biomarkers, such as CEA, Her2/neu, and the mucins (e.g., CA 15-3) are bound to cell membranes, but their extracellular domains could be found, through shedding, in the circulatory system. Consequently, Buckhaults et al. (21) applied serial analysis of gene expression to identify transcripts encoding secreted or cell surface proteins that were expressed in tumors of the colorectum. One of the six candidate markers they identified with this approach was βIG-H3. In line with their finding, in the present report, we show strong elevation of the corresponding protein in neoplastic tissue of the colorectum. Because the protein is predicted to be secreted, its release to and detection in body fluids should be facilitated, making it an ideal biomarker candidate. However, measurement of βIG-H3 serum levels using a sandwich immunoassay revealed the presence of relatively high amounts of βIG-H3 in the plasma of healthy blood donors (low μg/mL range). βIG-H3 protein levels were not elevated in the sera of cancer patients (Fig. 3). Most classical tumor markers show very little baseline levels (low ng/mL range) in serum of healthy individuals. With this low background, release of a respective marker protein by cancer cells may lead to measurable elevation of this protein in serum. However, in the case of βIG-H3, protein levels in serum from healthy individuals seem to be two to three orders of magnitude higher (Fig. 3), rendering detection of the presumable low amounts of βIG-H3 released from the tumor impossible.

nm23 proteins are basically intracellular proteins, but several reports also indicate the presence of nm23-H1 in the plasma of lymphoma patients (32, 39). We therefore established our own immunoassay to assess nm23-H1 levels in plasma. Because nm23-H1 can be purified from erythrocytes (40), we first assessed potential contamination of serum samples by release of nm23-H1 from erythrocytes in hemolytic blood specimens. A dilution series from erythrocyte lysates was prepared and hemoglobin concentrations as marker of hemolysis were determined. Concentrations of nm23-H1 and hemoglobin correlated very well, indicating release of nm23-H1 from lysed erythrocytes (data not shown). This finding correlates well with the results of Niitsu and coworkers (39), who also assessed the release of nm23-H1 from RBC. Importantly, significant nm23-H1 release in the low ng/mL range was already detected at hemoglobin concentrations of 0.2 mg/mL. Even stronger hemolytic effects could occur upon withdrawal of blood and processing to serum or plasma in the clinical routine setting, and therefore, most immunoassays used for in vitro diagnostics are designed to tolerate hemolytic samples with levels of 10 mg/mL hemoglobin or higher. Therefore, we do not believe that release of nm23-H1 by hemolysis and corresponding disturbance of the cancer-related nm23-H1 plasma level can be sufficiently controlled during preanalytic steps in the clinical routine setting. Consequently, we stopped further assessment of nm23-H1 as serum/plasma marker for colorectal cancer.

NNMT was first identified by cDNA cloning from the liver (22) and the protein is predicted to be present in the cytosol. We established a highly sensitive sandwich immunoassay to assess serum levels of the protein in healthy controls (n = 317) and patients with colorectal cancer (n = 109). In addition, we determined the levels of the established tumor marker CEA using a commercial immunoassay. The frequency distribution of NNMT and CEA serum levels is depicted in Fig. 4. NNMT was detectable in all samples analyzed. The median NNMT level in healthy donors was 308 pg/mL. For the patients with colorectal cancer, a median level of 925 pg/mL was found. The difference between healthy donors and patients with colorectal cancer was highly significant (Wilcoxon two-sample test, P < 0.0001). Median serum levels for stage I disease were 833 pg/mL (431-1,334 pg/mL, lower and upper 95% confidence limit of the median) and 1,436 pg/mL (925 to >2625 pg/mL) for stage IV disease. Figure 5 illustrates the relationship between the specificity and the sensitivity of NNMT measurements for the detection of colorectal cancer, represented by a receiver-operating characteristic curve (41). The diagnostic accuracy of the test is expressed by the area under the curve of the ROC plot. Values may range between 1.0 (perfect separation of the two groups, i.e., apparently healthy donors and patients with colorectal cancer) and 0.5 (no difference between the two groups). When choosing 95% specificity, the sensitivity of NNMT for colorectal cancer was 50.5%, whereas CEA detected only 38.5% of cancers when 95% specificity was defined. The corresponding area under the curve was 0.84 for NNMT and 0.78 for CEA, indicating that NNMT was more suitable to discriminate between patients with colorectal cancer and apparently healthy donors than CEA.

We observed a considerable overlap of NNMT (and CEA) in apparently healthy donors and patients with colorectal cancer.

**Fig. 3.** Determination of βIG-H3 in plasma of patients with colorectal cancer and control individuals. The concentration of βIG-H3 in plasma samples from 12 patients with colorectal cancer (●) and 12 healthy blood donors (○) was determined by ELISA as previously described (see Materials and Methods).
(Fig. 4). Because NNMT is predominantly expressed in the liver (22), one possible cause for elevated NNMT serum levels may be the presence of occult liver disease in apparently healthy donors. In line with this possibility is the finding of abnormal nicotinamide metabolism and elevated levels of N-methylnicotinamide in patients with hepatic cirrhosis (42). Further studies are needed to elucidate a potential correlation of NNMT serum levels with other diseases, such as liver diseases or other cancers. In addition, the specificity of the assay could still be enhanced using more specific (monoclonal) antibodies.

The present report is the first to show a correlation of NNMT plasma levels with the presence of colorectal cancer. NNMT showed a higher sensitivity than CEA in the detection of colorectal cancer in the limited patient cohort used in this study. Therefore, NNMT could be a new, sensitive, and specific marker that aids in the detection of colorectal cancer. However, these conclusions are based on the measurement of only a limited number of patients. Further studies on larger patient cohorts including relevant disease controls are ongoing. Additionally, these studies will address the most important question of whether sensitivity for colorectal cancer could still be increased by the combination of NNMT with other markers for the disease.

**Conclusion**

The present study shows that the chosen proteomics technology is very powerful in identifying cancer-associated proteins in tissue, as verified by validation using immunoblotting. In addition, this approach may have the potential to discover new major cancer biomarkers as exemplified by the new marker NNMT. However, which of the cancer-associated proteins found in tumor tissue that eventually will be present in serum or plasma cannot be predicted a priori. Development of highly sensitive immunoassays for each candidate marker and subsequent assessment of serum/plasma samples is mandatory. Three candidate markers with comparable elevation in cancer tissue as judged by immunoblotting analysis performed quite differently when analyzed in serum. βIG-H1, a secreted protein, was the most "logical" biomarker candidate but failed due to unforeseeable high "background" levels in plasma from unknown sources. nm23-H1 failed for technical reasons because its release from erythrocytes could not be sufficiently controlled during the preanalytic steps in the clinical routine setting. In contrast, NNMT was identified as a potential new marker for colorectal cancer, although it was not a prime candidate marker due to its predicted cytoplasmic localization. The mechanism by which NNMT is released to the circulatory system is still unknown. The diagnostic accuracy of serum-NNMT for colorectal cancer seems at least comparable to that of the established marker CEA, and further studies are needed to fully assess its promising diagnostic role.
References

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