Altered Expression and Localization of Creatine Kinase B, Heterogeneous Nuclear Ribonucleoprotein F, and High Mobility Group Box 1 Protein in the Nuclear Matrix Associated with Colon Cancer

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Abstract
Identification of biomarkers could lead to the development of effective screening tests for colorectal cancer. A previous study from our laboratory showed specific alterations of nuclear structure in colon cancer. In an effort to characterize these biomarkers, protein spots were selected from separations made by two-dimensional gel electrophoresis, which were analyzed by mass spectrometry. The sequences obtained from the isolated spots revealed that they have close similarity to creatine kinase B (CKB) isoforms, heterogeneous nuclear ribonucleoprotein F (hnRNP F) and high mobility group box 1 protein (HMGB1) isoforms. To determine the expression of these proteins in colon cancer, expression was studied in 9 tumor and matched adjacent normal pairs, 5 donor colons, 16 polypos, 4 metastatic liver lesions and matched adjacent normal pairs, and 3 liver donors. CKB and hnRNP F were expressed in 78% and 89% of colon tumors, respectively, and had a higher frequency of expression than CKB in premalignant polyps. With the establishment of differential expression of the proteins in colon cancer, their subcellular localization was analyzed. The subcellular fractions studied both showed high protein levels of hnRNP F in colon tumors compared with normal colon tissues. Surprisingly, subcellular levels of CKB were decreased in colon tumors, suggesting that the observed high CKB levels in nuclear matrix extracts are caused by the enhanced localization of CKB to the nuclear matrix during colon tumorigenesis. These results suggest an involvement of hnRNP F and CKB in colorectal cancer. Additionally, they suggest that hnRNP F is a potential marker for colorectal cancer progression. (Cancer Res 2006; 66(2): 763-9)

Introduction
Colorectal cancer is the third most common cancer affecting both men and women and is expected to strike >146,000 individuals and kill >56,000 in 2004 in the United States (1). Whereas screening for colorectal cancer is effective and recommended (2, 3), the process of testing with endoscopic screening or stool testing is cumbersome and difficult. Only a minority of eligible subjects have undergone colorectal cancer screening (4). There are no biomarkers that can effectively detect colorectal cancer at a localized stage (5). Developing a noninvasive, sensitive, and specific biomarker for early detection of colorectal cancer would be a welcome addition to colorectal cancer screening.

In recent years, a number of clinical assays have been successfully developed for cancer diagnosis based on observations of tumor-specific expression of nuclear matrix proteins. Some examples are the bladder cancer markers NMP-22 (88.5% sensitive) and BLCA-4 (96% sensitive, 100% specific) and the prostate cancer marker EPCA (6–8). The nuclear matrix maintains nuclear architecture by organizing the genome and supporting macromolecular assemblies that dictate DNA replication, transcription, and mRNA processing (9). Nuclear abnormalities are a common feature in cancer cells and it is hypothesized that such aberrations reflect altered nuclear matrix proteins (10). As a result, detection assays founded on nuclear matrix protein alterations are highly specific, showing promise in early detection as well as therapy of cancers. Clinical trials for cancer therapy with bizelesin were effective in patients with advanced tumors. This drug acts by selectively alkylating matrix attachment regions to nuclear matrix sites (11).

Recently, our laboratory studied tissues from sporadic colorectal cancer patients for early detection markers of colorectal cancer (12). The nuclear matrix fingerprint of 10 colon tumors and paired adjacent normal tissues as well as 4 donor normal colon tissues were examined by high-resolution two-dimensional gel electrophoresis. Four proteins (CC2, CC3, CC4, and CC5) were reported to be present in colon tumors but not in adjacent normal and donor normal colon tissues. Interestingly, two additional proteins (CC6a and CC6b) found in colon tumors, but absent in matched adjacent normal tissue, were also present in donor normal tissues. A subsequent study revealed that the nuclear matrix proteins CC3 and CC4 were also present in adenomatous polyps, suggesting an early biological role for these proteins in the course of colorectal tumor progression (13).

To aid in the identification of some of the changes that were observed in the colon cancer nuclear matrix, selected spots were characterized by mass spectrometry (MS) and their expression patterns during colon cancer progression were elucidated. We identified creatine kinase B (CKB), heterogeneous nuclear ribonucleoprotein F (hnRNP F), and high mobility group box 1 protein (HMGB1) as nuclear matrix proteins altered in colorectal cancer. The results presented here point to the specific involvement of hnRNP F early in colorectal cancer genesis.

Materials and Methods
Tissues and Sample Processing
Colon adenocarcinoma samples and matched adjacent normal tissues (n = 9), normal colon tissue (n = 10) from donors, liver tumors from metastatic...
colorectal cancer \((n = 4)\), normal unaffected liver from the same subject, and normal livers from donors \((n = 3)\) were collected through the Early Detection Research Network of the University of Pittsburgh Medical Center under the approval of the Institutional Review Board. The patients ranged in age from 18 to 80 years with a mean age of 55 years. Normal colon and liver tissues were obtained from trauma victims. Diagnoses were obtained from pathology reports that accompanied each specimen and were confirmed histologically. Colon tumors were staged according to the standard tumor-node-metastasis system: T3-4N0M0, T2-3N0M0, T1N0M0, and T0N0M0 \((n = 7)\), T1N1M0 \((n = 4)\), and T2N1M0 \((n = 3)\).

Nuclear matrix proteins were extracted from the tissues following the method of Getzenberg et al. \(\text{(14)}\) and stored at \(-80^\circ\text{C}\) until use. For subcellular fractionation studies, cytoplasmic and nuclear proteins were prepared from colon and liver tissues using the NE-PER (Pierce, Rockford, IL) kit. Protein concentrations were determined using the Coomassie blue assay (Pierce).

**Two-dimensional Gel Electrophoresis**

High-resolution two-dimensional electrophoresis was done using the Investigator two-dimensional gel system (Genomic Solutions, Inc., Ann Arbor, MI) as previously described \(\text{(14)}\). The gels were reverse stained with the zinc sulfate-imidazole system \(\text{(15)}\). Nuclear matrix proteins, 250 and 50 \(\mu\text{g}\), were loaded for spot excision and two-dimensional immunoblot analysis, respectively.

**SDS-PAGE and Immunoblotting**

One-dimensional immunoblot analysis used 10 \(\mu\text{g}\) protein per lane. Protein samples were resolved on 10% to 16% gradient gels under reducing conditions and transferred to Immobilon-P (Millipore, Billerica, MA) using Trans-blot (Bio-Rad, Hercules, CA) for Western blot analysis. Primary antibodies used were mouse monoclonal anti-CKB (kind gift of Prof. Be Wieringa, Department of Cell Biology, University of Nijmegen, Nijmegen, the Netherlands) and mouse monoclonal anti-hnRNP F against full-length recombinant protein (3H4, Abcam, Cambridge, MA). Secondary antibodies were horseshadish peroxidase–conjugated antimmunoglobulin G (Jackson ImmunoResearch, Inc., West Grove, PA) Detection was done with the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

**Mass Spectrometry Analysis**

In-gel digestion. The reverse-stained spots were excised and destained with 2% citric acid for 5 minutes followed by washing in 100 mmol/L ammonium bicarbonate. The in-gel digestion procedure followed that of the University of California, San Francisco Mass Spectrometry facility \(\text{(16)}\). Briefly, the gel plug was chopped into smaller pieces, reduced with 10 mmol/L DTT at 56°C, alkylated with 55 mmol/L iodoacetamide, and incubated with porcine trypsin (Promega, Madison, WI) overnight at 37°C. Tryptic peptides were extracted with 5% aqueous formic acid, taken to dryness in a speed-vac, and reconstituted in 10 \(\mu\text{L}\) of 0.1% aqueous acetic acid.

Protein identification by MS. The peptides \((0.5 \mu\text{L})\) were spotted on a stainless steel matrix-assisted laser desorption/ionization (MALDI) target and mixed with 0.5 \(\mu\text{L}\) of the matrix solution \((10 \text{mg/mL} \ \alpha\text{-cyano-4-hydroxycinnamic acid in } 50\% \text{ aqueous acetonitrile, } 0.1\% \text{ acetic acid})\) and allowed to dry. MS and tandem MS \((\text{MS/MS})\) spectra were acquired on a MALDI tandem time-of-flight \((\text{TOF/TOF})\) instrument \((4700 \text{ Proteomics Analyzer, Applied Biosystems, Foster City, CA})\). Positive-ion MS spectra were acquired averaging 2,000 laser shots. An external calibration was done using the instrument-manufacturer supplied calibration mixture to yield an accuracy of better than 30 ppm. An internal calibration using trypsin autolysis peptides was done to increase mass accuracy to better than 20 ppm. \([\text{M} + \text{H}]^+\) ions of peptides of interest were further analyzed by MS/MS. MS/MS data were acquired using the default 1 kV method of the manufacturer after updating the default calibration with the MS/MS spectrum of Glu-fibrinopeptide B in the calibration mixture.

Data were analyzed with the GPS Explorer software \((\text{Applied Biosystems})\), which uses the Mascot program \((\text{Matrix Sciences, London, United Kingdom})\) to interrogate the National Center for Biotechnology Information \((\text{NCBI})\) and Swiss-Prot databases for matches.

**Results**

**Identification of two-dimensional gel separated proteins by MS.** Nuclear matrix proteins isolated from human colorectal tumors were resolved by two-dimensional gel electrophoresis as previously described. After reverse zinc staining, the spots which appeared to be colorectal tumor specific were excised for mass spectrometric analysis. Gel pieces were destained, reduced, and alkylated, and then digested overnight with trypsin. Mass spectra were acquired for the tryptic peptides on a MALDI TOF/TOF mass spectrometer for analysis by peptide mass fingerprinting. Additionally, tandem mass spectra were acquired on the most abundant peptides for an added degree of certainty in the peptide mass fingerprinting identification. A search using combined MS and MS/MS data was done against the nonredundant NCBI database with the Mascot program. Search results were manually inspected to validate matches. Peptide mass fingerprinting identified all the spots with high confidence, with the exception of one which had insufficient ion signal for an unambiguous identification. Figures 1 and 2 represent the full MS spectrum on the left and the complete sequence of the protein it matched to, using peptide mass fingerprinting, on the right. MS and MS/MS spectra from spots 1 and 2 matched to CKB with sequence coverages of 46% and 44%, respectively \((\text{Fig. 1A-D})\). The observed mass of 42 kDa for these proteins is consistent with the calculated mass of 43 kDa for CKB. Isoelectric point \((\text{pI})\) predicted for CKB is 5.34 and this is close to the values 6.2 and 6.27 observed.

Spot 5 was identified as hnRNPF F with 33% sequence coverage \((\text{Fig. 2A and C})\). The observed and calculated mass as well as \(\text{pI}\) values were consistent with each other. Figure 2B is a representative mass spectrum of spots 3 and 4, which matched to high mobility group 1 isoforms \((\text{HMGI-1}; \text{Fig. 2D})\).

**Two-dimensional immunoblot validation.** To validate the protein identity from the MS results, we did two-dimensional immunoblot analysis on nuclear matrix proteins from colon tumors. Figure 3A shows nuclear matrix proteins from the Caco-2 cell line and the human colon tumors separated by two-dimensional gel electrophoresis and analyzed by Western blotting using a monoclonal antibody against CKB. Spots were observed with a molecular weight of 42 kDa and \(\text{pI}\) of \(~6.2\). These values are within those reported for spots 1 and 2 in silver-stained, two-dimensional gels, validating the results from peptide mass fingerprinting. Figure 4A represents a two-dimensional immunoblot from colon tumor nuclear matrix proteins probed with a monoclonal antibody for hnRNPF F. Two spots were observed and these are within the range of expected molecular weight and \(\text{pI}\) for hnRNPF F, confirming the peptide mass fingerprinting analysis.

**CKB and hnRNPF expression in the nuclear matrix during colon tumorigenesis.** We did one-dimensional immunoblotting to compare protein expression in nuclear matrix proteins extracted from human colorectal tumor and normal adjacent pairs \((n = 9)\), donor normal colon \((n = 5)\), juvenile polyps \((n = 1)\), tubular adenoma \((n = 6)\), tubulovillous adenomas \((n = 5)\), tubulovillous adenoma with high-grade dysplasia \((n = 4)\), secondary liver tumors and normal adjacent pairs \((n = 4)\), and donor normal liver tissues \((n = 3)\). Table 1 gives expression of CKB and hnRNPF F in these tissues represented as a percentage of \(n\) value. Figure 3B represents CKB expression profile in nuclear matrix proteins from premalignant and malignant tissues. We found that CKB was present at high levels in 78% of
colon tumors compared with normal colon tissues. The high frequency of CKB expression was seen in tubulovillous adenomas as compared with juvenile and tubular adenomas. Fifty percent of liver metastases showed CKB expression. hnRNP F was expressed in 89% of colon tumors and 100% of liver metastases and at a high frequency in all the polyps studied regardless of their histologic stage (Table 1 and Fig. 4B). It was also expressed in 67% (2 of 3) of liver donors.

Subcellular levels of CKB and hnRNP F in primary colon cancer. To investigate if CKB and hnRNP F had altered compartmentalization, we studied cytoplasmic and nuclear extracts from colon tumor, normal adjacent pairs (n = 6), and donor normal colon tissues (n = 5) by one-dimensional Western blot analysis. Both cytosolic and nuclear extracts revealed two bands at 50 and 42 kDa for CKB (Fig. 3C). The faster migrating band at 42 kDa corresponds to the tumor-specific spot 1 and was below detection levels in 100% of colon tumors as compared with normal adjacent and donor normal tissues regardless of cytosolic or nuclear localization. Similarly, two bands at 50 and 42 kDa were observed for HnRNP F. The 42-kDa band was similar in molecular weight to spot 5 and its level was found to be higher in colon tumors as compared with adjacent normal and donor normal tissues (Fig. 4C).

Discussion

Biomarkers for colorectal cancer are expected to be of value in disease screening, diagnosis, treatment, and surveillance. To develop biomarkers for early detection of colorectal cancer, we focused on molecular analysis of nuclear changes associated with tumor development. In the present investigation, we have identified specific changes in the proteome of colon cancer that have a high probability to be CKB isoforms and hnRNP F. Two other proteins that were present in colon tumors but absent in adjacent normal tissues are most likely HMG-1 isoforms. These findings are supported by four lines of evidence: (a) high-resolution two-dimensional gel electrophoresis; (b) MALDI MS and MS/MS mass fingerprinting; (c) two-dimensional immunoblot analysis using antibodies directed to full-length CKB and hnRNP F; and (d) one-dimensional immunoblot analysis for CKB and HnRNP F in normal and malignant colon tissues. Additionally, we evaluated the potential of CKB and HnRNP F as biomarkers for early detection of colorectal cancer by analyzing their expression pattern in premalignant polyps. CKB is an enzyme involved in energy transduction pathways, catalyzing the reversible transfer of phosphate from phosphocreatine to ADP to produce creatine and ATP. MS data identified CKB with high confidence. The two-dimensional electrophoresis data and one-dimensional immunoanalysis indicate that CKB
is associated with the nuclear matrix in colorectal cancer at significantly higher levels than in adjacent normal and donor normal colon tissues. CKB is predominantly regarded a cytoplasmic protein but several studies have shown a nuclear localization for CKB especially in embryonic olfactory neuroepithelium, ventral spinal cord, and cardiac and skeletal myoblasts (17). A nuclear localization for CKB is further supported by PSORT prediction. CKB is expressed in tissues with high fluctuating energy demands and is regarded as an energy supplier to the nucleus (17).

Immunoblot analysis of cytoplasmic and nuclear extracts showed that CKB levels are dramatically decreased in colon tumors as compared with adjacent normal and donor normal tissues. Proteomic analysis done by Friedman et al. (18) showed low CKB levels in colorectal cancer. Moreover, CKB levels increase with differentiation of the colon cancer cell line Caco-2 (19). Other reports from proteomic analyses of differentially expressed proteins in colon cancer found similar low levels of CKB in tumor tissues. It is not known if decreased expression of CKB is a consequence of losses in chromosome 14 in colon cancers. Alternately, high levels of CKB are reported for lung and breast cancer (20, 21). Serum levels of CKB are increased in cancer of the lung, prostate, colon, and ovary, suggesting that it is likely to be a generic marker rather than a specific marker for colorectal cancer (22–24). Nevertheless, to our knowledge, this is the first report of CKB binding with the nuclear matrix. One possible explanation for the disparate results in our study is that CKB binds to the nuclear matrix at higher than normal levels in colorectal cancer. Currently, nuclear energy pathways are poorly elucidated, making it difficult to predict the biological consequences of nuclear matrix localized CKB. Interestingly, high CKB immunoreactivities have been suggested to confer a survival advantage to cells undergoing glucose and oxygen deprivation (25). On one hand, we have shown low cellular levels of CKB in the tumor tissues and on the other hand, we have shown high levels of CKB associated with the nuclear matrix in several tumor tissues. One possible interpretation of our results is that CKB binds to and accumulates in the nuclear matrix when its cellular levels decrease. We suspect that the CKB identified in this study is posttranslationally modified because some of the high-quality fragmentation spectra did not match to the database entries. As an example, the observed precursor ions at m/z 1,244, 1,254, and 1,258 have identical tandem mass spectra. MS and MS/MS of precursor m/z 1,254 ion matched to the peptide HGGYKPSDEHK of CKB, suggesting that the precursor ions at m/z 1,244 and 1,258 correspond to homologous peptide sequences. The mass shift (+14) of m/z 1,244 to 1,258 could be due to methylation of the COOH-terminal lysine residue. However, further experiments are necessary to confirm this, and we are concentrating our efforts in this direction.

hnRNP F is a constituent of the hnRNP F splicing complex that is involved in pre-mRNA cleavage reaction within the mammalian nucleus (26). Our data show that hnRNP F protein levels are higher in primary and metastatic colorectal tumors as compared with...
adjacent normal and donor normal tissues. Similar high levels of hnRNP F were observed in adenomatous polyps, suggesting that it acts as an early switch in the adenoma-to-carcinoma sequence of colorectal cancer. Two of the liver donor tissues studied expressed hnRNP F. Pathology reports indicate that these specimens were obtained from accident victims whose livers had to be excised because of ischemia and liver failure, respectively. This may point to a role for hnRNP F in other abnormal liver pathologies. However, cross-reactivity of the antibody to shared epitopes with other members of the hnRNP family cannot be discounted. Immunofluorescence studies by Honore et al. (27) showed high expression of hnRNP F in gastric carcinoma but decreased expression in hepatocellular carcinoma. Overexpression of hnRNP F has been studied in plasma cells where it resulted in decreased expression of secreted versus membrane-bound antibodies (28). hnRNP F regulates the choice of alternative splice sites by directly binding intronic and exonic nucleic acid sequences. Early reports by Matunis et al. (29) show that hnRNP F has distinct nucleic acid binding properties, with a discrete distribution in the nucleoplasm, and is 78% homologous to hnRNP H/H'. These authors also observed that hnRNP F and H/H' are immunologically similar. This may possibly explain our observations of multiple bands in Western blots of cytosolic/nuclear proteins, which is a limitation of our study. hnRNP F is reported as predominantly cytoplasmic and its nuclear import is mediated by transportin (30). However, a cytoplasmic function for this protein is not known at present. The gene for hnRNP F is mapped to chromosome 10q11.21-q11.22. PTEN and SMAD4, two of the genes mutated in polyposis syndromes, also lie in the chromosome region 10q, which is frequently altered in colorectal cancers (31). Although it is not known if hnRNP F protein expression is regulated by its pseudogene on chromosome 10.

Figure 3. Immunoblot analysis of CKB. A, two-dimensional immunoblot validates protein identity given by MS for spots 3 and 4 represented in the Caco-2 cell line. B, expression profile of CKB in colon tumors, matched adjacent normals (Adj. Nor), colon donors, juvenile polyps (Juv), tubular adenoma (TA), tubulovillous adenoma (TVA), tubulovillous adenoma with high-grade dysplasia (TVA/HGD), metastatic liver tumors, matched adjacent normals (Adj. Nor), and donor normals. C, cytoplasmic and nuclear levels of CKB are decreased in colon tumors compared with matched adjacent normal and donor normal colon tissues.

Figure 4. Immunoblot analysis of hnRNP F. A, two-dimensional immunoblot validates protein identity given by MS for spot 5 in nuclear matrix proteins from human colon tumors. B, high levels of hnRNP F are expressed in colon tumors versus matched adjacent normals and colon donors. HnRNP F is expressed at a high frequency in juvenile polyps, tubular adenoma, tubulovillous adenoma, and tubulovillous adenoma with high-grade dysplasia, and at high levels in metastatic liver tumors versus matched adjacent normals and donor normals. C, hnRNP F levels are high in cytoplasmic and nuclear extracts from colon tumors compared with matched adjacent normal and donor normal colon tissues.
Invasive carcinoma.

HNMB1 is a nuclear protein which is reported to be involved in the cancer process as well as to participate in several other pathologies (completely reviewed in ref. 35). Surprisingly, HMGB1 is expressed in nuclear matrix proteins from tumor and donor tissues but is absent in adjacent normal tissues. Kunisuy et al. (36) reported poor prognosis for colorectal cancer patients with enhanced expression of both HMGB1 and advanced glycation end products. HMGB1 is overexpressed in several tumors although negligible expression is noted in others. Previously, HMGB1(Y), members of the HMG protein family that do not contain HMG boxes, were found localized in nuclear matrices from prostate tissues and associated with prostate cancer development (37). Because HMGB1 can bind DNA directly, thereby modulating transcription, identification of quantitative and qualitative changes in HMGB1 expression is valuable not only for use as a biomarker but also for targeting therapy. Additional studies are required to confirm HMGB1 expression and localization in colon tumors. The absence of HMGB1 in matched adjacent tissues may signify an early form of transformation. This can clarify whether HMGB1 levels are decreased in adjacent normal tissues or posttranslationally modified, signifying an early form of transformation to cancer.

In conclusion, we found that high levels of hnRNP F are present in premalignant and malignant stages of colorectal cancer, reflecting a role for this protein early in colorectal tumorigenesis. In addition, only a subgroup of tumors showed both high CKB and hnRNP F levels, suggesting that variations exist at the molecular level. Such synchronous expression of proteins with disease prognosis would be useful for predicting colorectal cancer patients at risk for hepatic spread.

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**Table 1. Expression of CKB and hnRNP F by immunoblot analysis given as a percentage of n**

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<thead>
<tr>
<th>Expression of CKB (%)</th>
<th>HnRNP F (%)</th>
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<tbody>
<tr>
<td>Nuclear matrix protein extracts</td>
<td>Donor colon, n = 5</td>
</tr>
<tr>
<td></td>
<td>Colon tumor, n = 9</td>
</tr>
<tr>
<td></td>
<td>Matched adjacent normal colon, n = 9</td>
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<tr>
<td></td>
<td>Juvenile polyp, n = 1</td>
</tr>
<tr>
<td></td>
<td>Tubular adenoma, n = 6</td>
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<tr>
<td></td>
<td>Tubulovillous adenoma, n = 5</td>
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<td></td>
<td>Tubulovillous adenoma with high-grade dysplasia, n = 4</td>
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<tr>
<td>Cytoplasmic/nuclear extracts</td>
<td>Donor colon, n = 5</td>
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<td></td>
<td>Colon tumor, n = 6</td>
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<td>Matched adjacent normal colon, n = 6</td>
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1p34.2, it is interesting to note that deletion mapping studies report 1p34.2-p35, 1p35.1-p36.3 as frequently lost in colorectal cancers (32, 33). Genetic and chromosomal instabilities are regarded as the cause for tumorigenesis and such aberrations are present in 90% of colorectal tumors. Studies by Koehler et al. (34) show that colorectal cancer progression is accompanied by very few gene expression changes and the authors concluded that alterations at the molecular level occur very early during cancer development. We show high HnRNP F protein levels beginning in the majority of adenomas studied and in all primary and metastatic tumors. It seems that a persistent “on” switch for hnRNP F may lead to sustained cell growth supporting invasive carcinoma.

**References**


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