Fatty Acid Binding Protein 6 Is Overexpressed in Colorectal Cancer

Takahiro Ohmachi,1,2,3 Hiroshi Inoue,1,2 Koshi Mimori,1,2 Fumiaki Tanaka,1,2 Atsushi Sasaki,1,2 Tatsuo Kanda,4 Hiroshi Fujii,4 Katsuhiko Yanaga,5 and Masaki Mori1,2

Abstract

Purpose: Fatty acid binding protein 6 (FABP6) is a cancer-related protein that acts as an intracellular transporter of bile acid in the ileal epithelium. Because bile acids are implicated in the carcinogenesis of colorectal cancer, we evaluated FABP6 expression in colorectal cancer.

Experimental Design: The expression of FABP6 mRNA was evaluated in 78 paired samples of cancer/normal tissue representing colorectal cancer cases, plus 16 adenomas, and 16 metastatic lymph nodes. An immunohistochemical study was conducted with paraffin sections. In vitro transfection was done to determine FABP6’s biological roles.

Results: The expression of FABP6 mRNA was significantly higher in cancer (75 of 78, 96.2%) than in normal tissue ($P < 0.001$). The expression of mRNA was increased in cancer compared with adenoma, but was dramatically decreased in node metastases. Tumors with high FABP6 expression were smaller in size ($P < 0.01$), more often in the left colon ($P < 0.05$), and had shallower invasion into the bowel wall ($P < 0.05$) compared with those with low expression. There was no significant difference between high- and low-expression tumors regarding clinicopathologic variables such as histologic type, lymph node, or liver metastasis, Dukes’ classification, and prognosis. Immunohistochemical study revealed that FABP6 expression was primarily observed in cancer cells. In vitro transfection revealed that transfectants showed weaker invasiveness ($P < 0.05$), more dominant proliferation ($P < 0.001$), and less apoptosis than mock cells.

Conclusions: The expression of FABP6 was higher in primary colorectal cancers and adenomas than in normal epithelium, but was dramatically decreased in lymph node metastases, suggesting that FABP6 may play an important role in early carcinogenesis.

Materials and Methods

Tissue sampling. A total of 186 tissue samples obtained from 104 patients were used in this study. Tumors and adjacent normal tissues were obtained surgically from 78 patients in Kyushu University Hospital in Beppu, Japan, and immediately embedded in Tissue Tek optimum cutting temperature compound medium (Sakura, Tokyo, Japan), samples were kept frozen at $-80^\circ$C until used. Sixteen (4 samples from the aforementioned 78 patients, and an additional 12 samples) metastatic lymph nodes were also obtained and prepared for analysis. Finally, an adenoma obtained by endoscopic mucosal resection from an additional 16 patients was also used.

To further our understanding of cancer biology, it is important to discern cancer-related genes. To identify such genes, we evaluated differences in the gene expression profiles between colon cancer cells and normal colonic epithelial cells using a combination of laser microdissection and cDNA microarray techniques. Consequently, we identified 84 cancer-related genes that were overexpressed in cancer cells.5 One example is fatty acid binding protein 6 (FABP6), which acts as an intracellular transporter of bile acids in ileal epithelial cells, helping to catalyze and metabolize cholesterol. It is also known that bile acids induce colon carcinogenesis and cancer development in experimental models (1–3). Bile acids induce apoptosis and inflammation in colonic epithelial cells (4) and indirectly cause oxidative DNA damage that leads to genetic modulation during epithelial restoration (5). However, the significance of the expression of FABP6 in colorectal cancer has not been studied to date. In this study, we clarified, through clinical and experimental studies, that the relative expression level of FABP6 is associated with colorectal cancer.
written informed consent was obtained from all patients. The study design was approved by the Institutional Review Board in Kyushu University prior to implementation.

Reverse transcriptase-PCR and semi-quantitative real-time reverse transcriptase-PCR. Total RNA was extracted from each sample and cDNA was synthesized from 8.0 µg total RNA as described previously (6). The purity and concentration of total RNA were determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control. Semi-quantitative real-time reverse transcriptase-PCR assay was done using 78 surgically resected paired cancer and normal tissue samples, 16 adenomas, and 16 metastatic lymph nodes. The following primers were used to amplify genes: FABP6 (sense primer, 5′-ACTATCCGGGGCCACACCAT-3′ and antisense primer, 5′-GTCTTTCTGTCACCGGCCTAGG-3′) and GAPDH (sense primer, 5′-TGTTGATCCTGGAAGACTCA-3′ and antisense primer, 5′-TGTCATCATATTTGGCAGGTTT-3′). The reaction was done in a LightCycler system (Roche Applied Science, Indianapolis, IN) using the LightCycler-FastStart DNA Master SYBR Green 1 kit (Roche Applied Science) as described previously (7). In brief, thermal cycling for all genes was initiated with a denaturation step of 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds, 64°C (60°C for GAPDH) for 10 seconds, and 72°C for each optimal length (s/25 bp). At the end of the PCR cycles, melting curve analysis was done to reconfirm the expected PCR product. We determined the levels of FABP6 and GAPDH mRNA expression by comparisons with cDNA obtained from Human Universal Reference RNA (Clontech, Palo Alto, CA). All calculated concentrations of target genes were divided by the amount of endogenous reference (GAPDH) to obtain normalized FABP6 expression values. Each assay was done in triplicate to verify the results.

Immunohistochemistry. Immunohistochemical studies for FABP6 were done on formalin-fixed, paraffin-embedded surgical sections obtained from patients with colorectal cancer. A total of 52 samples (38 colorectal cancer samples and 14 adenomas) were used for this study. After deparaffinization and blocking, the antigen-antibody reaction was incubated overnight at 4°C. LSAB+ System-HRP (Dako, Kyoto, Japan) reagents were applied to detect the signal of the antigen-antibody reaction. All sections were counterstained with hematoxylin. Purified rabbit polyclonal antibody against the purified recombinant human FABP6 protein was used at a dilution of 1:200.

Protein extraction and Western blotting. For western blotting, protein extraction was done as follows: the transfectants and mock cells were absorbed in 50 mmol/L Tris (pH 7.5) containing 154 mmol/L KCl/10 mmol/L sodium acetate/1 mmol/L phenylmethylsulfonyl fluorid, homogenized by Bioruptor (Cosmo Bio Co., Ltd., Tokyo, Japan), and centrifuged at 20,000 × g for 60 minutes, then the supernatant was used. Protein samples of 50 µg were electrophoresed in 15% concentrated SDS polyacrylamide gel and electroblotted onto GenScreen hybridization transfer membrane (Perkin-Elmer, Boston, MA) at 0.2 Å for 120 minutes at 4°C. The FABP6 protein was detected using FABP6 antibodies at a dilution of 1:1,000. Enhanced chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ) were applied to detect the signal of the antigen-antibody reaction.

DNA transfection. The open reading frame of the FABP6 gene was cloned from the human FABP6 gene–ligated plIC18 vector (8). The primers for cloning the FABP6 gene are as follows: sense primer, 5′-CACCACTGTTTCACCGCCAAGTTCGA-3′ and antisense primer, 5′-TGAAGGCACCTCCTTGTCACCGGCCTAG-3′. The FABP6 gene was ligated to pcDNA 3.1 Directional TOPO expression vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. To confirm accurate insertion into the frame of the expression vector, sequencing chemistry was done. DLD-1, the colon cancer cell line, was used for transfection assays. FUGENE6 (Roche Applied Science) transfection reagent was employed to establish permanent transfectant cells, which were then selected by neomycin treatment as described previously (9).

In vitro assays. Nine clones of transfectant cells were established by DNA transfection. Three clones which expressed a higher rank of FABP6 mRNA were used for examination to verify the results of in vitro assays. The clones from mock cells showing no difference from the parent cell line, DLD-1, were used for controls.

Invasion assays were done using the BD BioCoat Tumor Invasion System (BD Biosciences, Franklin Lakes, NJ) to evaluate invasive cells as described previously (10). Briefly, cells (5.0 × 10⁴ cells/well) were placed in the upper chamber, and the lower chamber was filled with 750 µL of DMEM with 10% FCS as a chemoattractant. After 48 hours of incubation at 37°C, membranes were labeled with Calcein-AM solutions. Invasive cells that had migrated through the membrane to the lower surface were evaluated in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm. Invasiveness was evaluated as the percentage of fluorescence of invasive cells of HT-1080, the fibrosarcoma cell line that served as a control.

Proliferation assays were done by cell counting for three clones each of the transfectants and mock cells. Each clone was harvested in RPMI 1640 with 10% fetal bovine serum at 37°C in a 5% humidified CO₂ atmosphere. They were plated at a density of 10⁵ cells per well in three 10-cm plates and were harvested and counted on days 3, 5, and 7. The medium was changed every 72 hours. This experiment was done in triplicate.

Apoptosis assays were done using FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody apoptosis kit 1 (BD Biosciences) and flow cytometer as described previously (11). Briefly, cells (2.0 × 10⁵) were incubated for 72 hours in serum-free medium at 37°C and fixed in 70% ethanol at −20°C. Next, the cells were washed and resuspended in the buffer with FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody. The FITC fluorescence was evaluated using an EPICS XL flow cytometer (Beckman Coulter Corp., Tokyo, Japan) until the total cell count reached 3 × 10⁴. The experiment was done in triplicate to verify the results.

Statistical analysis. Quantitative real-time reverse transcriptase-PCR data and in vitro transfection assay data were calculated with JMP 5 for Windows software (SAS Institute Inc., Cary, NC). Differences between groups were estimated using Student’s t test and χ² test. We applied Student’s t test for data in normal distribution, the nonparametric Wilcoxon/Kruskal-Wallis tests for data without normal distribution, and the ANOVA test and Tukey-Kramer test for chronological data. P < 0.05 was considered to be statistically significant.

Results

FABP6 expression in clinical samples. Reverse transcriptase-PCR analysis of six representative clinical tissue samples showed a marked level of FABP6 mRNA expression in cancer samples compared with no expression in the paired normal sample (Fig. 1A). We then submitted all 78 paired clinical samples for quantitative real-time reverse transcriptase-PCR analysis to investigate the high level of FABP6 expression in the initial cancer samples. Quantified values from the expression analysis served to calculate the FABP6/GAPDH ratio. FABP6 expression in colorectal cancer samples was significantly higher (average expression value was 52.1-fold higher, P < 0.0001) than expression in corresponding normal samples. Notably, no expression of FABP6 mRNA was observed in any of the normal samples (Fig. 1B). Figure 1C shows FABP6 expression values of different histologic cell types. Interestingly, the phased expression of FABP6 mRNA was consistent with a histologic cell type, with higher expression in cancer samples than in adenoma samples. In contrast, the expression of FABP6 mRNA in metastatic lymph nodes was markedly lower than the level obtained with adenoma samples. The significant differences in expression between cancer and normal tissue counterparts were seen across all histologic cell types, with P < 0.05 (except for the
difference in expression value between adenomas and cancer, \( P = 0.221 \). Figure 2 shows immunohistochemical results from representative clinical samples of moderately differentiated adenocarcinoma. The majority of the stain was observed in the cytoplasm of cancer cells, not in stromal cells or normal epithelium. Table 1 shows the results of staining grade of clinical samples. The difference of staining grade was not statistically significant, however, there was an inclination for stronger staining in cancer samples (\( P = 0.152 \)). The result of the immunohistochemical study was consistent with the expression of \( FABP6 \) mRNA in different histologic types, as shown in Fig. 1C.

**Fig. 1.** \( FABP6 \) mRNA and protein expression in clinical samples. A, reverse transcriptase-PCR analysis of \( FABP6 \) in colorectal cancer and paired normal samples. A marked level of \( FABP6 \) mRNA expression was observed in cancer samples, with no expression in normal samples. T, cancer sample; N, normal sample; m, marker; nc, negative control; pc, positive control. B, quantitative real-time PCR analysis of clinical samples showed marked increase in \( FABP6 \) mRNA in cancer samples (\( P < 0.001 \)). Horizontal line indicates mean. The \( P \) value was calculated by Student’s \( t \) test. C, quantitative real-time PCR analysis of different histologic cell types showed that phased expression was slightly higher in cancer samples than in adenomas. Expression in lymph node metastases was below the level observed in adenoma samples. The expression values of each sample were statistically significant (\( P < 0.05 \)) except that of cancer samples and adenoma samples. N, normal sample; Ad, adenoma; T, tumor; LN, lymph node metastasis.

**FABP6 expression correlates with clinicopathologic variables.** To investigate the clinical significance of \( FABP6 \) expression, samples that underwent quantitative real-time PCR were divided into two groups to examine possible correlations with clinicopathologic variables (Table 2). Tumors with high expression of \( FABP6 \) were significantly smaller in size (25 of 39, 64.1%; \( P = 0.003 \)) than those with low expression (12 of 39, 30.8%), and they were more likely (\( P = 0.024 \)) to be located in the left colon (31 of 39, 82.1%) than tumors with low expression (16 of 39, 41.0%). The incidence of serosal invasion was significantly lower (\( P = 0.028 \)) in tumors with high expression (8 of 39, 20.5%) than in tumors with low expression (22 of 39, 56.4%). Regarding other clinicopathologic variables, there was no significant correlation observed in histologic cell type (\( P = 0.240 \)), lymph node metastasis (\( P = 0.817 \)), lymphatic permeation (\( P = 0.492 \)), venous permeation (\( P = 0.185 \)), liver metastasis (\( P = 0.360 \)), peritoneal dissemination (\( P = 0.237 \)), Duke’s classification (\( P = 0.494 \)), or cancer-related death (\( P = 0.384 \)).

**Establishment of \( FABP6 \) transfectants.** To confirm that the increase in level of \( FABP6 \) mRNA was the result of DNA transfection, quantitative real-time reverse transcriptase-PCR was done to detect \( FABP6 \) mRNA expression in transfectants and mock cells. Quantitative real-time reverse transcriptase-PCR showed that a markedly higher level of \( FABP6 \) mRNA was observed (average expression, 116.7-fold higher, \( P < 0.0001 \)) in the transfectants compared with the mock cells (Fig. 3A). Figure 3B shows the results of Western blotting for the detection of \( FABP6 \) protein by chemiluminescence. \( FABP6 \)
protein was detected only in the transfectants, with no expression in the mock cells (Fig. 3B, top). The protein β-actin was used as an internal control (Fig. 3B, bottom).

In vitro invasion assay. To estimate whether high FABP6 levels affect cellular invasiveness, we did an invasion assay; results are shown in Fig. 3C. Transfectants showed weaker invasiveness than mock cells (P = 0.0119). The result is compatible with the expression-invasiveness correlation found in the clinical study.

To evaluate the significance of the FABP6 gene, we investigated the proliferation assay and apoptosis assay; the results of which are shown in Fig. 3D and E, respectively. In Fig. 3D, the transfectant showed a more dominant proliferation (P < 0.001), and in Fig. 3E, the peak of FITC fluorescence of mock cells shifted to the right side as a result of more apoptosis compared with transfectants (~20-fold). These results suggest that the overexpression of FABP6 in cancer cells contributes to the protumorigenic events as dominant proliferation and antiapoptotic activity.

**Discussion**

Several reports have shown the association between FABP gene families and cancers. Keler et al. found that liver-type fatty acid binding protein (L-FABP) promotes DNA synthesis and cell growth and preserves cell morphology in hepatocellular carcinoma (12). Hashimoto et al. clarified that L-FABP is expressed in a subset of gastric adenocarcinomas (13). Jing et al. (14) and Adamson et al. (15) revealed that cutaneous fatty acid binding protein (C-FABP) promotes invasion and metastasis in prostate cancer through up-regulation of expression of the vascular endothelial growth factor gene. Godbout et al. showed that brain fatty acid binding protein (B-FABP) mRNA is expressed in human malignant gliomas (16). In work with epidermal fatty acid binding protein (E-FABP), Sinha et al. revealed that E-FABP was overexpressed in chemoresistant pancreatic cancer cell lines (17). In work with heart-type fatty acid binding protein (H-FABP), Hashimoto et al. revealed that high H-FABP expression was associated with tumor aggressiveness and poor survival (18). As shown above, some studies with FABP families have shown that they are associated with cancer behavior as the result of various mechanisms including altered lipid metabolism (12, 19) and induction of physiologically active substances (14, 15).

Meanwhile, there have been few studies of FABP6 expression in cancers. We identified 84 genes that were overexpressed in colorectal cancer cells compared with the corresponding normal cells using a combined technique of laser microdissection and cDNA microarray analysis. Of these genes, we focused on FABP6 because it had one of the largest differences in expression between colon cancer and normal tissue. In the current study, quantitative real-time reverse transcriptase-PCR found that FABP6 mRNA expression was significantly higher (75 of 78, 96.2%) in cancerous tumors compared with corresponding normal tissue. Immunohistochemistry revealed that FABP6 protein expression was mainly in cancer cells themselves.

Why is FABP6 expression much higher in colorectal cancer than in normal colonic tissue? Previous studies of bile acids and carcinogenesis of colon cancer provide clues to understanding the underlying mechanism. The concentration of fecal bile acids, especially secondary bile acids, is higher in patients with colonic adenoma or frank colon cancer (20–23). Furthermore, Fujii et al. discovered that bile acid exposure induced FABP6 expression in the Caco-2 colon cancer cell line (8). These studies suggested to us that high concentrations of bile acids cause increased FABP6 expression in colorectal cancer. Excess bile acids, especially secondary bile acids, which infiltrate epithelial cells depending on the concentration, induce apoptosis and indirectly cause DNA damage (5), which leads to genetic modulation in the colonic epithelium.

The present study showed that FABP6 expression status was associated with tumor location, tumor size, and depth of tumor invasion. Tumors with high FABP6 expression were...
more frequently located in the left colon than the right colon. This result might be explained by the relatively longer stasis of feces in the left colon, causing prolonged exposure of epithelial cells to bile acids, with the result that more cytotoxic secondary bile acids are produced by bacterial modulation compared with conditions in the right colon. Tumors with high FABP6 expression were smaller in size than those with low expression. The expression of FABP6 mRNA was markedly increased in the sequence of normal tissue to adenoma and colorectal cancer. We speculate that FABP6 overexpression relative to normal cells might be associated with early-phase carcinogenesis but that it might not be necessary for late-stage cancer progression. In fact, the transfectants contributed the more dominant proliferation (Fig. 3D) and antiapoptotic activity (Fig. 3E) as the protumorigenic events compared with mock cells. Furthermore, the expression of FABP6 mRNA was dramatically decreased in metastatic cells derived from lymph nodes, as shown in Fig. 1C. With regard to the depth of invasion, tumors with shallow invasion showed higher FABP6 expression than tumors with deep invasion. This result is consistent with in vitro invasion assays which found that FABP6 transfectants showed weaker invasiveness than mock cells (Fig. 3C). These results in assays of invasiveness, cell proliferation, and apoptosis as the protumorigenic events support the speculation that FABP6 overexpression might be associated with early-phase carcinogenesis.

We have hypothesized the possible clinical ramifications of the high expression of FABP6 found in primary colon cancer cells. Fatty acid binding proteins are a family of small proteins that bind long-chain fatty acids and show tissue specificity. There are nine known FABP’s that exhibit tissue specificity, for example, FABP1 to liver, FABP2 to intestine, FABP3 to skeletal muscle and heart, FABP4 to adipocytes, FABP5 to epidermis, FABP6 to ileum, FABP7 to brain, FABP8 to peripheral myelin, and FABP9 to testis. Kleine et al. reported that H-FABP flowed into the blood after myocardial damage occurred, and thus, H-FABP was available for the detection and assessment of myocardial infarction (24). Rauch et al. found that E-FABP was overexpressed in head and neck cancer and was a candidate as a serum marker (25). FABP6 is one of the FABP gene families.
with a low molecular weight, ~15 kDa. Thus, FABP6 might be released into the blood during the development of colorectal cancer tumors. In other words, FABP6 is a possible molecular marker for the diagnosis of relatively early-stage colorectal cancer and/or assessment of anticancer treatment. Further studies are required to clarify this hypothesis.

In conclusion, we identified overexpression of the FABP6 gene and evaluated its biological role in colorectal cancers. There is a pressing need to investigate related genes during the cancer progression process and assess whether FABP6 is a potential biomarker for colorectal cancer. In the near future, we anticipate that FABP6 may be useful clinically in cancer diagnosis and for the assessment of cancer therapy.

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References

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