Reduced Expression of Focal Adhesion Kinase in Liver Metastases Compared with Matched Primary Human Colorectal Adenocarcinomas

Masako Ayaki, Keiko Komatsu, Mutsuko Mukai, Kohei Murata, Masao Kameyama, Shingo Ishiguro, Jun Miyoshi, Masaharu Tatsuta, and Hiroyuki Nakamura

Departments of Tumor Biochemistry [M. A., K. K., M. M., H. N.], Surgery [K. M., M. K.], Pathology [S. I.], Molecular Biology [J. M.], and Gastrointestinal Oncology [M. T.], Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan

ABSTRACT

The focal adhesion kinase (FAK) is implicated in integrin-mediated signal transduction pathways used in cell adhesion, cell motility, apoptosis, and anchorage-independent growth. Because cancer invasion and metastasis are thought to be associated with alterations in cellular adhesive and motile properties, we studied the expression of four focal adhesion proteins including FAK in matched samples of human normal colorectal mucosa (N), primary colorectal adenocarcinomas (T) and liver metastases (M) from 10 patients by Western blot analysis. This gave us the advantage of directly comparing levels of focal adhesion protein expression within the same genetic background. Average FAK expression level was significantly higher in T than in N and it was significantly lower in M than in T. Average paxillin expression level was also significantly higher in T than in N, but it was not significantly different between T and M. Similar results were obtained by immunohistochemical analyses of FAK and paxillin expression. Average vinculin and talin expression levels showed no significant differences among these three samples (N, T, and M). These data demonstrate that the FAK expression level increases in primary tumors compared with normal mucosa and decreases in liver metastases to the level of normal mucosa in the majority of human colorectal adenocarcinomas. Up- and down-regulation of FAK protein expression observed in this study may have a profound effect on the signal transduction.

INTRODUCTION

Invasion into surrounding tissues is a prominent phenotype of cancer cells (1, 2). Regulatory mechanisms of cell motility is undoubtedly critical in this process. We have recently shown that cooperation by a small guanosine triphosphate (GTP)-binding protein, Rho, of the generation of motile force through the organization of actin cytoskeleton and the formation of focal adhesions is necessary for the motility of rat ascites hepatoma cells (3).

The focal adhesion is an important link between the actin cytoskeleton and the plasma membrane and consists of a complex of proteins that assemble at sites of attachment of the cell to the ECM. A number of proteins are found to be preferentially associated with the focal adhesion complex, including FAK, paxillin, vinculin, and talin. Among these proteins, FAK is a critical mediator of signaling events between cells and the ECM.

FAK has been implicated in cell adhesion (5), cell motility (6, 7), apoptosis (8–10), and anchorage-independent growth (11). Because these are key processes by which a transformed cell becomes invasive and metastatic, FAK may be intimately involved in malignancies. Although overexpression of FAK protein has been reported in metastatic human colorectal (12, 13), breast (13), ovarian (14), and prostatic cancer cells (15), relatively little is known about expression levels of focal adhesion proteins in metastatic nodules compared with primary lesions. In the present study, we compared the expression levels of four focal adhesion proteins including FAK in matched samples of human normal colorectal mucosa (N), primary colorectal adenocarcinomas (T), and liver metastases (M).

MATERIALS AND METHODS

Tissue Samples. The matched tissue samples of primary colorectal adenocarcinoma, adjacent normal colorectal mucosa, and synchronous liver metastasis from the same patient were collected from 10 patients in tumor-node-metastasis stage IV undergoing surgical resection in our hospital. Adjacent normal colorectal mucosa specimens were obtained from the sites ~5–10 cm apart from the primary tumors. Tissue samples were separated from necrotic tissues by sharp dissection. Histopathological tissue confirmation was performed by a reference pathologist (S. I.). One-half of the tissues were snap-frozen in liquid nitrogen and stored at −80°C for Western blot analysis and the other one-half were formalin fixed and paraffin embedded for immunohistochemical analysis.

The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; PVDF, polyvinylidene difluoride; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

Received 1/8/01; revised 6/5/01; accepted 6/26/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan.

2 To whom requests for reprints should be addressed, at Department of Tumor Biochemistry, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. Phone: 81-6-6972-1181, extension 4403; Fax: 81-6-6972-7749.
Antibodies. Antibodies used in this study were mouse monoclonal anti-FAK (clone 77; Transduction Laboratories, San Diego, CA), rabbit polyclonal anti-FAK (M135, Takara Biochemicals, Kyoto, Japan), mouse monoclonal anti-paxillin (clone ZO35; Zymed Laboratories, San Francisco, CA), mouse monoclonal anti-vinculin (clone VIN-11-5; Sigma Chemical Co., St. Louis, MO), mouse monoclonal anti-talin (clone 8D4; Sigma Chemical Co.), and mouse monoclonal anti-β actin (clone AC-15, Sigma Chemical Co.). For Western blot analysis, antibodies were diluted as follows. Mouse monoclonal anti-FAK antibody (1:1000), anti-paxillin antibody (1:1000), anti-vinculin antibody (1:500), anti-talin antibody (1:200), and anti-β actin antibody (1:5000). For immunohistochemical analysis, rabbit polyclonal anti-FAK and mouse monoclonal anti-paxillin antibodies were diluted 1:500 and 1:1000, respectively.

Preparation of Tissue Extracts. Frozen surgical specimens were thawed, minced with scissors, crushed in a solution consisting of 0.05 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 6% (v/v) β-mercaptoethanol, and 10% (w/v) glycerol using polytron, and were centrifuged at 15,000 × g for 10 min. The supernatant was used immediately or stored frozen at −80°C for Western blot analysis.

Western Blot Analysis. Protein concentration was determined by Protein Assay (Bio-Rad, Richmond, CA). SDS-PAGE was performed as described by Laemmli (16). Protein samples were electrophoresed on 10% polyacrylamide gel under reducing conditions. The resolved proteins were electrophoretically transferred to PVDF membrane (17). The focal adhesion proteins and actin were detected using monoclonal antibodies listed above. Antimouse IgG (H+L) AP conjugate (Promega, Madison, WI) and BCIP/NBT Color Substrate (Promega) were used for alkaline phosphatase detection. Protein expression levels were quantitatively estimated by densitometric scanning performed using a 1200 dot per inch (dpi) flatbed scanner with NIH Image 1.55f. The focal adhesion protein concentrations were normalized to actin level and expressed as densitometric ratio.

Immunohistochemical Staining. Four-μm sections from formalin-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. They were air-dried and deparaffinized. Endogenous peroxidase activity was blocked with 0.35% hydrogen peroxide in 50% methanol for 15 min at room temperature. The sections were rehydrated and washed with PBS. After blocking nonspecific binding sites with 2% normal goat or horse serum in PBS for 30 min at room temperature, the sections were incubated overnight at 4°C with rabbit polyclonal anti-FAK antibody or mouse monoclonal anti-paxillin antibody, which was the same antibody as used for Western blot analysis. After rinsing with PBS, the sections were incubated with biotinylated goat antirabbit IgG or biotinylated horse antimouse IgG (Vector, Burlingame, CA) for 30 min at room temperature followed by washing with PBS. Immunoreactivity was detected with an avidin-biotin system (Vector) using 0.025% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen for 2.5 min. The sections were lightly stained with Mayer’s hematoxylin.

Evaluation of Degree of Antibody Reactivity. The degree of polyclonal anti-FAK or monoclonal anti-paxillin reactivity with each tissue section was scored by the percentage of stained normal or neoplastic epithelial cells in the section. In this study, normal or neoplastic epithelial tissues with more than 50% stained cells were defined as “positive.” Three persons (M. A., K. K., and H. N.) independently judged the stained cells.

Statistical Analysis. Paired Student’s t test was used for statistical analyses and P < 0.05 was considered to indicate a significant difference.

RESULTS

Western Blot Analysis of FAK, Paxillin, Vinculin, and Talin in Human Colorectal Adenocarcinomas. Human primary colorectal adenocarcinoma lysate was run on SDS-PAGE and immunoblot analysis was performed using five monoclonal antibodies. As shown in Fig. 1, only a single band locating at the corresponding molecular weight of the protein was identified in each lane.

The expression levels of four focal adhesion proteins including FAK in matched samples of human normal colorectal mucosa (N), primary colorectal adenocarcinomas (T), and liver metastases (M) were compared by Western blot analysis. Fig. 2A shows FAK and actin protein expression of matched N, T, and M from 10 cases.

In 8 of the 10 cases, FAK expression levels were higher in T than in N, and in 8 of the 10 cases, these were lower in M than in T (Fig. 2B). Average FAK expression level was significantly higher in T than in N, and it was significantly lower in M than in T (Fig. 2C).

In 8 of the 10 cases, paxillin expression levels were higher in T than in N, whereas in 6 of the 10 cases, these were lower in M than in T (Fig. 3B). Average paxillin expression level was also significantly higher in T than in N, but it was not significantly different between T and M (Fig. 3C).

In 6 of the 10 cases, vinculin expression levels were higher in N than in T, and in 6 of the 10 cases, these were lower in M than in T (Fig. 4B). However, no significant differences were observed in average vinculin expression levels between N and T and also between T and M (Fig. 4C).

In 5 of the 10 cases, talin expression levels were higher in T than in N, and in 7 of the 10 cases, these were lower in M than
However, average talin expression levels were not significantly different among N, T, and M (Fig. 5C).

In normal liver specimens (L), FAK and paxillin were expressed at minimally detectable levels (Figs. 2A and 3A), whereas vinculin and talin were expressed at approximately the same levels as in normal mucosa and in primary tumors (Figs. 4A and 5A).

**Immunohistochemical Analysis of FAK and Paxillin Expression in Human Colorectal Adenocarcinomas.** To examine the expression of FAK and paxillin at the histological level, we performed immunohistochemical analysis using the same matched samples as used in Western blot analysis and polyclonal anti-FAK or monoclonal anti-paxillin antibody.

Staining was abolished by incubating with normal rabbit serum or normal mouse IgG instead of with antibody (data not shown). When normal and neoplastic epithelial tissues with more than 50% stained cells were defined as positive, 2 (20%) of 10 normal mucosa, 10 (100%) of 10 primary adenocarcinoma, and 8 (88%) of 9 liver metastasis specimens showed FAK positive-and diffusely stained in whole cytoplasms (Fig. 6, A–C). In 8 of 10 primary adenocarcinoma specimens, the intensity of FAK expression was remarkably increased compared with matched normal mucosa specimens (Fig. 6, A and B). In contrast, the intensity of FAK expression was markedly reduced in 7 of 9 liver metastasis specimens compared with matched primary adenocarcinoma specimens (Fig. 6, B and C).
Similarly, 6 (60%) of 10 normal mucosa, 10 (100%) of 10 primary adenocarcinoma and 9 (100%) of 9 liver metastasis specimens were paxillin positive and also diffusely stained in whole cytoplasms (Fig. 6D–F). Seven of 10 primary adenocarcinoma specimens demonstrated intense paxillin immunostaining compared with matched normal mucosa specimens (Fig. 6, D and E). In contrast, seven of nine liver metastasis specimens demonstrated reduced paxillin immunostaining compared with matched primary adenocarcinoma specimens (Fig. 6, E and F).

DISCUSSION
Histopathological confirmation of surgical specimens for Western blot analysis revealed that primary colorectal adenocarcinoma specimens consisted of adenocarcinoma cells (60–70%), stromal cells (20–30%) and normal mucosal tissues (5–10%) and that liver metastasis specimens were made up of adenocarcinoma cells (70–80%) and stromal cells (20–30%). Almost no normal liver cells were observed in liver metastasis specimens. Normal mucosa specimens consisted of only nontumorous mucosal tissues.

When human primary colorectal adenocarcinoma lysate was run on SDS-PAGE, transferred to PVDF membrane, and immunoblotted, only a single band locating at the corresponding molecular weight of the protein was identified in each lane (Fig. 1). Because all of the monoclonal antibodies used in the present study were IgG1 isotype, each immunoblot became a good
control for the other. When the antibody was replaced with normal mouse IgG1, no band was observed (data not shown).

FAK is a non-receptor protein-tyrosine kinase that indirectly localizes to sites of integrin-receptor clustering. FAK functions as part of a cytoskeleton-associated network of signaling proteins (4). FAK is essential for integrin-stimulated cell migration (7). By comparing with normal human colonic mucosa, Owens et al. (13) showed that FAK protein expression was significantly elevated in 17 of 17 invasive and metastatic colonic adenocarcinomas. Cance et al. (12) developed new monoclonal antibodies that specifically detected FAK in formalin-fixed, paraffin-embedded tissue sections and assessed FAK expression using immunohistochemical techniques. They found that FAK was overexpressed at moderate-to-strong levels in 13 of 15 invasive colonic adenocarcinomas, whereas FAK was weakly expressed in the normal human colonic epithelium. By Western blot analysis, we also found that the average FAK expression level was significantly higher in T than in N (Fig. 2C).

Because adenocarcinoma cells in the primary lesions overexpress FAK, we expected that all of the metastatic nodules in the liver would express FAK more strongly than would the primary lesions. To our surprise, however, we

![Image](image_url)

Fig. 6 Immunohistochemical staining patterns of FAK and paxillin in a matched sample of human colorectal adenocarcinoma, adjacent normal mucosa, and liver metastasis. A matched sample was stained with anti-FAK antibody (A, B, and C) or anti-paxillin antibody (D, E, and F). This matched sample demonstrated little detectable FAK immunoreactivity in normal colon mucosa (A), intense FAK immunostaining in primary adenocarcinoma (B), and markedly reduced FAK immunostaining in liver metastasis (C). The same matched sample demonstrated weak-to-moderate paxillin immunoreactivity in normal colon mucosa (D), intense paxillin immunostaining in primary adenocarcinoma (E), and reduced paxillin immunostaining in liver metastasis (F). ×20.
found that in 8 of the 10 cases, FAK expression levels were lower in M than in T (Fig. 2B). The average FAK expression level was significantly lower in M than in T (Fig. 2C). Immunohistochemical analysis of FAK expression confirmed these results. Adenocarcinoma cell motility may favor local dissemination from the primary cancer site and into circulation, whereas adenocarcinoma cells in the metastatic nodules may require down-regulation of FAK expression. Cajor et al. (18) showed that cultured liver metastasis-derived human colonic adenocarcinoma cells displayed a significantly lower migratory potential compared with that of their corresponding primary adenocarcinoma-derived cells and that the expression of motility-related protein (MRP-1/CD9) was down-regulated in liver metastases compared with primary lesions. Tatebe et al. (19) analyzed the relationship between apoptosis and proliferation in matched samples of primary human colorectal adenocarcinomas and liver metastases using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method and Ki-67 staining. They found that both the apoptotic indices and the Ki-67 labeling indices were significantly higher in liver metastases than in primary lesions. Both increased apoptosis and enhanced proliferation of tumor cells in the metastatic foci were also observed in animal experimental metastasis models (20, 21). We have recently reported that Ki-67 expression is significantly higher in liver metastases than in human primary colorectal adenocarcinomas (22). Attenuation of FAK expression results in decreased cell motility (23) and induces apoptosis (8, 9). Conversely, FAK overexpression results in increased cell motility (6) and suppresses apoptosis (24). These results suggest that reduced FAK expression in liver metastasis may decrease the cell motility and increase the apoptotic indices and that enhanced proliferation of tumor cells in liver metastasis may eventually overcome the apoptotic cell loss.

Paxillin is a unique focal adhesion protein with its ability to interact with certain oncogene products (25). FAK can directly interact with paxillin (26). Paxillin also functions as part of signaling proteins. Vadlamudi et al. (27) showed that heregulin stimulation of noninvasive human breast cancer MCF-7 cells resulted in the up-regulation of paxillin expression leading to the development of invasive phenotype. In contrast, Salgia et al. (28) reported that the transfection of human non-small cell lung cancer cells with paxillin cDNA consistently reduced cell motility. In the present study, the average paxillin expression level was significantly higher in T than in N, but it was not significantly different between T and M (Fig. 3C). Similar results were obtained by immunohistochemical analysis. In one of the 10 cases, paxillin expression level was extraordinarily higher in M than in T (Fig. 3B). If this case were excluded from the samples, the average paxillin expression level would still remain not significant between T and M. In this case, FAK and talin expressions were also up-regulated in M (Figs. 2B and 5B).

Vinculin and talin are actin-membrane attachment proteins that exist at the intracellular surface of the plasma membrane as adhesion plaques intervening between F-actin and integrins. Sadano et al. (29) showed by Northern blot and immunohistochemical analyses that the expression of vinculin was lower in weakly metastatic mouse B16-melanoma cells than in highly metastatic counterparts. Lifschitz-Mercer et al. (30) reported that the vinculin expression was down-regulated in human metastatic squamous cell carcinomas compared with the expression in nonmetastatic carcinoma cells. In the present study, however, average expression levels of vinculin and talin showed no significant differences among N, T, and M (Figs. 4C and 5C).

Phosphorylation of FAK and paxillin is considered to be critically involved in the signal transduction (4, 5). Up-and down-regulation of FAK and paxillin expression observed in clinical samples may also have a profound effect on this process. Increased fak gene dosage, which was associated with overexpression of FAK protein, was reported in some human colon cancer cell lines (31). Although overexpression of FAK and paxillin in primary lesions and reduced expression of FAK in liver metastases were observed in the present study, the mechanism of up- or down-regulation of FAK and paxillin expression remains to be clarified.

ACKNOWLEDGMENTS

We are grateful to Shiko Kitagaki and Youko Nagamachi for excellent technical assistance.

REFERENCES


Reduced Expression of Focal Adhesion Kinase in Liver Metastases Compared with Matched Primary Human Colorectal Adenocarcinomas

Masako Ayaki, Keiko Komatsu, Mutsuko Mukai, et al.

*Clin Cancer Res* 2001;7:3106-3112.

**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/10/3106

**Cited articles**
This article cites 31 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/10/3106.full.html#ref-list-1

**Citing articles**
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/7/10/3106.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.