Role of Expression of Focal Adhesion Kinase in Progression of Hepatocellular Carcinoma

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ABSTRACT

Purpose: Although hepatocellular carcinoma (HCC) is the most common cancer of the human liver, the mechanisms that regulate HCC development and progression remain unclear. The aim of this study was to investigate whether focal adhesion kinase (FAK) is involved in the progression of human HCC.

Experimental Design: Western blot analysis for FAK was performed on three HCC cell lines. We reviewed 64 consecutive patients who had undergone initial liver resection for HCC without preoperative treatment. Immunohistochemistry analysis for FAK was performed on paraffin-embedded tissues. FAK expression was confirmed by Western blot analysis in several clinical samples. We investigated the correlation between FAK expression and clinical outcome.

Results: FAK proteins were detected in all HCC cell lines. Hepatocytes in the normal liver and chronic hepatitis with or without cirrhosis were negative for immunohistochemical staining for FAK expression. Cytoplasmic FAK expression was observed in 18 of 64 patients (28.1%), and this positive staining was correlated with gender (P < 0.05), a lower level of serum albumin (P < 0.05), and portal venous invasion (P < 0.01). Positive staining for FAK was associated with significantly poorer survival (P < 0.05). In multivariate analysis, FAK overexpression was an independent factor in determining the prognosis of patients.

Conclusions: These data suggest that FAK plays an important role in promoting tumor progression, especially vascular invasion, in HCC. FAK could play an important role in HCC progression and would be a novel target for HCC therapeutics as well as a prognostic marker.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is a major cause of death in many countries, especially in Asia (1, 2), where there is a high prevalence of chronic hepatitis B virus and hepatitis C virus infection. In addition, the long-term prognosis remains unsatisfactory because of high recurrence rates (3–5). In particular, HCC with poorly differentiated phenotype, large type, portal venous invasion, and intrahepatic metastasis is indicative of poor prognosis. Moreover, the mechanisms underlying the development of HCC remain unclear.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is localized to focal contacts. In addition, FAK is a major mediator of signal transduction by integrin between cells and the extracellular matrix. FAK has been implicated in the regulation of cell adhesion (6), spreading (7), migration (8), and apoptosis (9–11). Activation of FAK by integrin-mediated cell adhesion to the extracellular matrix induces autophosphorylation of FAK at Tyr-397, which has been shown to be a binding site for several intracellular signaling molecules including Src family kinases (12–15), phosphatidylinositol 3’-kinase (16), phospholipase C (17), and Grb7 (18). Because these signal transductions are key processes by which a transformed cell becomes invasive and metastatic, FAK may be intimately involved in malignancies.

The overexpression of FAK mRNA in colorectal carcinoma and liver metastases has been reported previously (19). FAK overexpression at the protein level has been demonstrated in epithelial and mesenchymal tumors in the invasive and metastatic phenotype (20). Moreover, FAK is overexpressed in various tumors, including tumors derived from the head and neck (21), colon (20, 22) breast (20, 22), ovary (23), and thyroid (20). However, the role of FAK expression in HCC has not been studied.

In the present study, FAK expression in the tissue of curatively resected HCC was immunohistochemically examined, and relationships between FAK expression and clinicopathological features were investigated. The aim of this study was to investigate whether FAK is involved in the progression of human HCC.

MATERIALS AND METHODS

Cell Culture. Three established human HCC cell lines (Huh7, Hep3B, and PLC/PRF5) were used in this study. Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 500 units/ml penicillin, and 500 μg/ml streptomycin (Life Technologies, Inc.). All cells were maintained at 37°C in 5% CO2.

Western Blot Analysis. To perform SDS-PAGE and Western blot analysis for FAK expression, cells were plated onto 10-cm dishes until they reached 80–90% confluence. Cells were harvested by scraping on ice into a lysis buffer (50 mM
Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were cleared by centrifugation (14,000 rpm) at 4°C for 10 min. The tissue samples of homogenates derived from cancer or noncancerous tissue were treated with detergent buffer (50 mM Tris, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mM MgCl₂) containing containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The supernatant protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Normalized lysates were boiled in electrophoresis SDS sample buffer, run on a 10% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 h in Tris-buffered saline containing 5% skim milk with 0.05% Tween 20. The membrane was probed with FAK antibody (1:1000 dilution; mouse monoclonal antibody; clone 4.47; Upstate Biotechnology, Inc.), and rehydrated in descending dilutions of ethanol. The endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxide for 30 min. To retrieve the antigen, pretreatment with citrate buffer (0.01 M citric acid [pH 6.0]) for 30 min at 99°C in a microwave oven was performed for FAK. After sections were exposed to 10% nonimmunized rabbit serum for 10 min, they were incubated overnight at 4°C with primary antibodies diluted 1:100. The sections were subsequently incubated with a second stage biotinylated antibody for 20 min, followed by incubation with horseradish peroxidase-labeled streptavidin for 20 min at room temperature. After rinsing in PBS, the reaction products were visualized by immersing the section in diaminobenzidine tetrahydrochloride as chromogen. No significant staining was observed in the negative controls, which were prepared using the mouse immunoglobulin

Invasion Assay. According to previously described methods (24, 25), the invasive potential of HCC cell lines was determined by a Matrigel invasion assay using polycarbonate membranes (8.0-µm pore size) in the upper chamber of 24-well Transwell culture chambers coated with Matrigel (Becton Dickinson San Jose, CA). HCC cell lines (5.0 × 10⁴ cells/well) were placed in the upper chambers, and the lower chamber was filled with 750 μl of DMEM with 20% fetal bovine serum as a chemoattractant. After 36 h of incubation at 37°C, the membranes were stained with May-Grünwald and Giemsa solutions. The invasive cells that had migrated through the membrane to the lower surface were counted in three different fields under a light microscope at ×200. Each experiment was performed in triplicate wells and repeated three times.

Clinical Samples. We reviewed 64 consecutive patients who had undergone initial liver resection for HCC without preoperative treatment at the Department of Surgery and Science, Kyushu University Hospital between January 1991 and February 1999. The ages of the patients ranged from 42 to 78 years, with an average age of 61.2 years. The male:female ratio was 47:17. Forty patients (63%) had the hepatitis C virus antibody alone, 13 patients (20%) had the hepatitis B virus antigen alone, 8 patients (13%) had neither the antibody nor the antigen, and 3 patients (4%) had both the antibody and the antigen. The diagnosis was confirmed histologically in all cases, based mainly on examination of sections stained with H&E. Clinico-pathological variables were defined according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer of the Liver Cancer Study Group of Japan (26). All tumors were histologically diagnosed as well-differentiated HCC (n = 7), moderately differentiated HCC (n = 30), or poorly differentiated HCC (n = 27). Fibrous capsule formation was seen in 52 patients (81%), portal venous invasion was observed in 26 patients (41%), hepatic venous invasion was found in 6 patients (9%), and intrahepatic metastasis was exhibited in 15 patients (23%). After surgical resection, HCC specimens from each patient were fixed in 10% buffered formalin, embedded in paraffin, stained with H&E, and examined microscopically.

Immunohistochemistry. Immunohistochemical observations were performed on adjacent deparaffinized sections using the peroxidase-labeled streptavidin-biotin technique with the Histofine SAB-PO kit (Nichirei). The primary antibody used in this study was FAK mouse monoclonal antibody; clone 4.47; Upstate Biotechnology, Inc.). Three-µm-thick histological sections of 10% formalin-fixed, paraffin-embedded materials were cut, mounted on glass slides coated with 3,3'-aminoxytyliethoxyslane, and air dried overnight at room temperature. The sections were removed from paraffin with xylene and rehydrated in descending dilutions of ethanol. The endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxide for 30 min. To retrieve the antigen, pretreatment with citrate buffer (0.01 M citric acid [pH 6.0]) for 30 min at 99°C in a microwave oven was performed for FAK. After sections were exposed to 10% nonimmunized rabbit serum for 10 min, they were incubated overnight at 4°C with primary antibodies diluted 1:100. The sections were subsequently incubated with a second stage biotinylated antibody for 20 min, followed by incubation with horseradish peroxidase-labeled streptavidin for 20 min at room temperature. After rinsing in PBS, the reaction products were visualized by immersing the section in diaminobenzidine tetrahydrochloride as chromogen. No significant staining was observed in the negative controls, which were prepared using the mouse immunoglobulin.

Fig 1. A. Western blots analyses on lysates of Huh7, Hep3B, and PLC/PRF/5 cells; 3T3/A31 cells were used as a positive control. As an internal control, the same blot was reprobed with anti-β-actin antibodies. An intense M, 125,000 band is demonstrated in all cells, and the Huh7 cell is the weakest intensity of all. B, invasive potential of hepatocellular carcinoma cell lines was determined by Matrigel invasion assay. The number of cells that invaded the lower side of the Matrigel was assessed. PLC/PRF/5 and Hep3B exhibited significantly more invasive potential than Huh7 (P < 0.01). Each experiment was performed three times.
at the same concentration. Finally, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

Bile duct cells were used as the internal positive control for FAK in the same sample. Identical reaction times allowed an accurate comparison of all samples. Compared with the internal control, positive staining of tumor cells was evaluated independently by two pathologists (T. M. and S. A.). We classified the cases as FAK-positive tumors when $>20\%$ of the carcinoma cells were stained positive for the protein.

The association between FAK expression and clinicopathological parameters was evaluated using a $\chi^2$ test for qualitative data and Fisher’s exact test for categorical data. Continuous variables were divided into two groups according to their means or medians. Adjusted survival was the time period of survival after resection to the date of the last follow-up or death by disease. The survival rate was calculated using the Kaplan-Meier method. Any differences in the survival curve were tested using the log-rank test. The Cox proportional hazards model with a stepwise procedure was used in multivariate analysis of survival data. A $P$ of $<0.05$ was considered to be statistically significant.

RESULTS

FAK Expression in HCC Cell Lines. Western blot analysis was performed to determine whether HCC cell lines express FAK. A cell lysate of 3T3/A31 cell line (Upstate Biotechnology, Inc.) was used as a positive control. FAK expression was detected in all three HCC cell lines using Western blot analysis (Fig. 1A). Huh7 expressed lower levels of FAK compared with Hep3B and PLC/PRF/5.

Invasion Potential of HCC Cell Lines. We examined the invasion potential of HCC cell lines using a Matrigel invasion assay (Fig. 1B). PLC/PRF/5 and Hep3B exhibited significantly more invasive potential than Huh7 ($P < 0.01$).

Expression of FAK Protein in Clinical Samples. We performed immunohistochemical analysis for FAK on paraffin-embedded tissues of 64 patients. Hepatocytes in normal liver or chronic hepatitis with or without cirrhosis were negative for immunohistochemical staining for FAK expression (Fig. 2A). FAK immunoreactivity was observed in normal bile duct epithelial cells (Fig. 2A). About $30\%$ of HCC (28.1%) exhibited increased cytoplasmic FAK expression (Fig. 2A). A main lesion of HCC shows FAK-positive cytoplasm (diaminobenzidine-hematoxylin; original magnification, $\times40$). T, tumor. C, a main lesion of HCC shows FAK-positive cytoplasm (diaminobenzidine-hematoxylin; original magnification, $\times200$). D, a HCC lesion with portal venous invasion. HCC lesion with portal venous invasion displays cytoplasmic positivity for FAK (diaminobenzidine-hematoxylin; original magnification, $\times100$). PVI, portal venous invasion.

Fig. 2 Immunohistochemical analysis of focal adhesion kinase (FAK) expression in hepatocellular carcinoma (HCC). A, hepatocytes in chronic hepatitis are negative for FAK. Normal bile duct cells indicate positive immunostaining for FAK, as an internal positive control. (diaminobenzidine-hematoxylin; original magnification, $\times100$). N, nontumor, BD, bile duct. B, a main lesion of HCC shows FAK-positive cytoplasm (diaminobenzidine-hematoxylin; original magnification, $\times40$). T, tumor. C, a main lesion of HCC shows FAK-positive cytoplasm (diaminobenzidine-hematoxylin; original magnification, $\times200$). D, a HCC lesion with portal venous invasion. HCC lesion with portal venous invasion displays cytoplasmic positivity for FAK (diaminobenzidine-hematoxylin; original magnification, $\times100$). PVI, portal venous invasion.

Fig. 3 Western blot analysis of focal adhesion kinase (FAK) protein on clinical samples of hepatocellular carcinoma tissue. Lysates were derived from tumor tissues (T) and paired nontumor tissues of the liver (N). As an internal control, the same blot was reprobed with anti-β-actin antibodies. In immunohistochemical analysis, case 1 was from the FAK-negative group, and other two cases were from FAK-positive groups.
To confirm FAK expression, we performed Western blot analysis in three clinical samples (case 1 was from the FAK-negative group, and cases 2 and 3 were from the FAK-positive group based on immunohistochemical analysis). In case 1, FAK expression of tumor tissues was detected at a lower level. On the other hand, HCC tissues expressed FAK protein at significantly higher levels than nontumor tissues in cases 2 and 3 (Fig. 3). Western blot analysis in clinical samples correlated with immunohistochemical analysis.

Relationships among Immunohistochemical Results, Clinicopathological Features, and Survival. Table 1 shows a comparison of the clinicopathological factors between HCC patients with FAK-positive tumors and those with FAK-negative tumors. FAK expression correlated significantly with sex, a serum level of albumin, and portal venous invasion. Survival and disease-free survival were compared between HCC patients with FAK-positive tumors and those with FAK-negative tumors. The survival curves of the positive versus negative expressers of FAK showed a significant separation ($P = 0.035$ by log-rank test; Fig. 4).

Multivariate Analysis of Survival. We also carried out a multivariate analysis of survival using the Cox proportional hazards regression model including Edmondson-Steiner grade, histological differentiation, tumor-node-metastasis (TNM) classification, portal venous invasion, intrahepatic metastasis, and FAK expression. FAK expression was one of the independent predictors of survival ($P = 0.0071$; Table 2), as were intrahepatic metastasis ($P = 0.0421$), TNM classification ($P = 0.0130$), and Edmondson-Steiner grade 3 ($P = 0.0033$). The relative risk in patients with FAK-positive carcinomas was 3.024 times greater than that of patients with FAK-negative tumors.

DISCUSSION

Various kinds of indicators have been reported as prognostic parameters for patients with HCC after hepatic resection, including clinical factors such as liver function of patients; tumor factors such as tumor size, number, TNM classification, and serum concentration of $\alpha$-fetoprotein; and surgical factors such as surgical margin and perioperative transfusion (27–31).

![Fig. 4](image-url) The overall survival curve for positive versus negative expression of focal adhesion kinase in 64 patients with hepatocellular carcinoma showed a significant separation ($P = 0.0350$, log-rank test).
Moreover, the degree of histological differentiation (31) or vascular invasion (27, 31) is known or suspected to predict the prognosis as a pathological factor. In univariate analysis, the survival curves of the positive versus the negative expressers of FAK showed a significant separation. In this multivariate analysis, FAK expression in the main tumor for HCC, Edmondson-Steiner grade 3, and TNM classification (stage 3 or 4) were independent prognostic factors. In our study, FAK-positive staining was correlated with gender and a lower level of serum albumin. The reason for a correlation between FAK expression and gender was not known. Moreover, to our knowledge, gender is not a prognostic factor for HCC. Otherwise, we reported that the serum level of albumin was a prognostic factor for HCC is not a prognostic factor for HCC. Otherwise, we reported that a correlation between FAK expression and gender was not known. Moreover, to our knowledge, gender is not a prognostic factor for HCC. Otherwise, we reported that the serum level of albumin was a prognostic factor for HCC after hepatic resection (32), and a serum albumin level of the serum level of albumin was a prognostic factor for HCC.

In summary, we demonstrated that FAK expression is up-regulated in HCCs that have portal venous invasion. We also showed that FAK expression is a prognostic factor. Our results suggest that there may be a relationship between FAK expression in HCC and tumor survival and portal venous invasion and that FAK would be a novel target for HCC therapeutics as well as a prognostic marker.

## REFERENCES


### Table 2

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<sup>a</sup> FAK, focal adhesion kinase; Im, intrahepatic metastasis.

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