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Upregulation of Focal Adhesion Kinase by 14-3-3ε *via* NFκB Activation in Hepatocellular Carcinoma

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Abstract: Focal adhesion kinase (FAK) is implicated in cancer cell survival, proliferation and migration. Expression of FAK expression is elevated and associated with tumor progression and metastasis in various tumors, including hepatocellular carcinoma (HCC). Increased 14-3-3ε expression is shown to be a potential prognostic factor to predict higher risk of distant metastasis and worse overall survival in HCC. The aim of this study is to investigate whether FAK is associated or regulated by 14-3-3ε to modulate tumor progression in HCC. In this study, 114 primary HCC tumors including 34 matched metastatic tumors were subjected to immunohistochemistry analysis of FAK and 14-3-3ε expression. Overexpression of FAK was significantly associated with increased risk of extrahepatic metastasis ($p=0.027$) and reduced 5-year overall survival rate ($p=0.017$). A significant correlation of FAK and 14-3-3ε expression was observed in primary tumor ($p<0.001$) and also metastatic tumors. Furthermore, overexpression of 14-3-3ε induced FAK expression and promoter activity which were determined by Western blotting analysis and luciferase-reporter assay. Moreover, 14-3-3ε enhanced NFκB activation and increased nuclear translocation of NFκB. Results from chromatin immunoprecipitation assay revealed that 14-3-3ε induced NFκB binding on FAK promoter region. These findings suggest that FAK expression is correlated with and upregulated by 14-3-3ε *via* activation of NFκB. Target to suppress or inactivate FAK alone, or combine with 14-3-3ε is thus considered as the potential therapeutic strategy for preventing HCC tumor progression.

Keywords: 14-3-3ε, Focal adhesion kinase, Hepatocellular carcinoma, Metastasis, NFκB, Survival.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common and lethal malignancy around worldwide. HCC is considered a serious public health problem in endemic areas of hepatitis B or C viral infection, including Africa and Southeast Asia [1]. Despite recently improved progress in surgical and non-surgical treatment, the prognosis for HCC patients is still dismal because a substantial portion of the disease is discovered at an advanced stage including high rates of local recurrence or metastasis [2-4]. Therefore, investigating accurate prognostic biomarkers to identify patients at high risk of recurrence or metastasis is of utmost importance for developing preventive strategies to improve the outcomes of HCC patients.

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic protein tyrosine kinase localizes to cell focal adhesion sites [5-7] and plays as a crucial role in regulating cell adhesion [8], migration [9], spreading [10], and apoptosis [11]. Activation of FAK and its downstream signal pathways have been extensively implicated in the progression of variety of malignant tumors. Elevated expression of FAK has been shown in colorectal cancer [12], breast cancer [13], sarcomas [14], cervical carcinomas [15], prostate carcinoma [16], thyroid cancer [17], neuroblastoma [18], and hepatocellular carcinoma (HCC) [19-21]. FAK was indicated to be an independent prognostic factor in distinct cancer types including ovarian [22], esophageal [23] and colon cancer [13]. In addition, FAK is overexpressed in about two third of HCC and is correlated with invasiveness and aggressiveness [21, 24]. Furthermore, activation of FAK by autophosphorylation at Tyr-397 leads to tumor progression in gastric carcinomas [25] and is highly involved in invasion and metastasis of HCC [26]. Therefore, FAK plays an important role in tumor progression and is a potential molecular target for anti-cancer drug therapy. The human FAK promoter region has been cloned and characterized previously [27]. FAK promoter harbors several potential transcriptional-regulator binding domains [27, 28], including the tumor suppressor protein p53 and nuclear factor kappa B (NFκB) binding sites [27]. It has been shown that NFκB increases FAK promoter activity and protein expression *via* direct binding to the FAK promoter region. Therefore, activation of NFκB and/or defective p53 may associate with and affect FAK expression in regulating tumor growth and progression.

14-3-3 proteins are 28-33-kDa acidic polypeptides with highly conserved sequences in eukaryotic organisms [29]. Seven 14-3-3 isoforms (β, ε, γ, η, σ, τ/θ and ζ) have been identified in mammalian cells [29]. In general, homo- or hetero-dimerization is required for 14-3-3 proteins to interact with and regulate the associated intracellular proteins *via* phospho-serine/threonine binding activities [30] to modulate multiple cellular functions, including contributing to oncogenesis [29, 31]. Isoform-specific expression profile of 14-3-3 proteins is associated with various types of human malignancies. For instance, increased 14-3-3ζ expression promoted anchorage-independent cell growth in lung cancer cell lines [32], and expression of 14-3-3ζ was associated with worse disease-free survival and high recurrence rate in human breast cancer [33]. Increased expression of 14-3-3 isoforms were found in human lung cancer tissues [34]. 14-3-3β and 14-3-3η were found specifically expressed in human astrocytoma [35, 36]. Moreover, results from a proteomic study reveal expression of 14-3-3ε was increased in breast cancer [37]. Overexpression of 14-3-3β, 14-3-3ε and 14-3-3γ were shown to be prognostic factors to predict a high risk of extrahepatic metastasis and worse overall survival [38-40]. In

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addition, previous studies reported that upregulation of 14-3-3ε expression protects colorectal cancer and endothelial cells against oxidative stress-induced apoptosis [41, 42]. Suppressing 14-3-3ε expression by treatment with nonsteroidal anti-inflammatory drugs induces cancer and endothelial cell death [41, 43]. Thus, 14-3-3 may be a novel prognostic biomarker and therapeutic target of distinct human tumors. In this study, we discovered that expression of 14-3-3ε is significantly associated with FAK in HCC. Elevated 14-3-3ε expression induced FAK expression and promoter activity *via* increase of NFκB binding on FAK promoter.

MATERIALS AND METHODS

Patients and Clinical Specimens

Tissue samples were obtained from 114 HCC patients who underwent surgery for tumor resection or biopsy in Tai-Chung Veterans General Hospital was retrospectively enrolled (From January 1999 to December 2001) in this study. The mean follow-up was 58.6 months. Among these 114 HCC tumors, 34 were tissue-proved to develop metastasis of 3 to 87 months after the diagnosis and surgery for primary HCC. The metastasis sites include bone, abdominal and chest wall, brain, mesentery, gall bladder, peritoneum, adrenal gland, and retroperitoneum. The paraffin-embedded surgical specimens composed of the primary and metastatic tumor and surrounding non-cancerous liver parenchyma underwent pathology examination. Pathological features, including Barcelona-Clinic Liver Cancer (BCLC) staging [44], and clinical outcomes were analyzed. This study was approved by the Institutional Review Board of Taichung Veterans General Hospital.

Immunohistochemical Analysis

For immunohistochemistry analysis, an automatic immuno-staining device and ultraView detection kit (Ventana XT Medical System, Tucson, AZ) to detect FAK and 14-3-3ε expression in paraffin-embedded tissues by use of primary antibodies against FAK and 14-3-3ε (Santa Cruz Biotechnology, Santa Cruz, CA). A negative control was prepared by the same staining procedure but not incubated with the primary antibody. The intensity of FAK and 14-3-3ε protein staining was semi-quantitatively scored by a Quick-score (Q-score) method based on intensity and heterogeneity [21, 38-40, 45, 46]. Briefly, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). For heterogeneity, the proportion of tumor cells positively stained with FAK and 14-3-3ε was scored as 0 (0%); 1 (1-25%); 2 (26-50%); 3 (51-75%) and 4 (76-100%). The Q-score of a given tissue sample was the sum of intensity and heterogeneity scores and ranged from 0 to 7. A Q-score ≥2 was considered overexpressed or positive expression, and a Q-score <2 was considered normal or negative expression. Some rare cases with <5% weakly stained specimens were considered negative expression.

Cell Culture and Transfection

SK-Hep-1 human hepatoma cells were maintained in DMEM (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone Thermo Fisher Scientific, Waltham, MA), 100 units/ml penicillin, and 100 units/ml streptomycin, in a humidified incubator with 5% CO_2 at 37°C. 14-3-3ε cDNA was amplified by PCR and then subcloned into the pcDNA3.1 vector. For 14-3-3ε overexpression, SK-Hep-1 cells were transfected with pcDNA3.1 (Control) or 14-3-3ε cDNA (14-3-3ε) by use of TurboFect™ transfection reagent (Fermentas Thermo Fisher Scientific Inc. Waltham, MA) according to the manufacturer's instruction.

Western Blot Analysis

Western blotting analysis was performed as previously described [41-43]. In brief, 14-3-3ε overexpression and control cells were harvested and lysed in ice-cold RIPA buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA; Millipore, Temecula, CA) containing cocktail protease inhibitors (Roche, Indianapolis, IN). Cell lysates were clarified by centrifugation at 15,000 rpm for 20 minutes at 4°C. Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). In total, 20 μg protein of each sample was applied to a gradient SDS-PAGE gel and immunoblotted onto PVDF membranes. The membranes were blocked for 1 hour in PBST (0.1% Tween 20, 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) containing 5% nonfat dry milk. Membranes were incubated with primary antibodies overnight and washed 3 times with PBST for 5 minutes. The membranes were immersed in PBST containing horseradish peroxidase-conjugated secondary antibody for 1 hour and protein levels were determined by use of enhanced chemiluminescence reagents. Antibodies against 14-3-3ε, FAK and NFκB (p65) were purchased from Santa Cruz Biotechnology.

FAK Promoter Activity and NFκB Reporter Assay

The luciferase-based reporter plasmid with human FAK promoter and NFκB reporter plasmid were obtained as described previously [27, 28]. Cells were transfected with FAK promoter and NFκB reporter constructs, washed with PBS and lysed with 1X lysis buffer (Promega Corp., Madison, WI). Luciferase activity was measured by use of Luciferase Assay Reagent (Promega), and the emitted light was determined in a luminometer.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed with the ChIP easy™ kit (Upstate, Temecula, CA) described previously [28]. Briefly, after being transfected with 14-3-3ε overexpressing or control vectors for 48 h, cells were washed with PBS, then crosslinked with 1% formaldehyde. After sonication, DNA was immunoprecipitated by use of antibodies against p65 or control IgG (Upstate). The forward and reverse primers of ChIP assay for the protein binding sites in the FAK promoter region were 5'-CAGGAGGGAGGTTCTCCAG-3' and 5'-ACGAGGAAAGCCCTGGTC-3' for p65; 5'-GCTTCCT GGCTTGTGGTTAG-3' and 5'-GGAGGGCTTTAAGCAGGAAG-3' for control primer. PCR products were analyzed on ethidium bromide-stained agarose gel.

Subcellular Fraction of NFκB

Cells transfected with 14-3-3ε overexpressing or control vectors for 48 h and nuclear proteins were extracted by nuclear extraction kit (Chemicon) according to the manufacturer's instruction. 20 ug protein of each samples was mixed with equal volume of Laemmli sample buffer (Bio-Rad) and resolved on an 8%-SDS polyacrylamide gel. Protein levels of p65, actin and lamin A were analyzed by Western blotting as described above.

Statistical Analysis

One-way ANOVA was used to analyze differences among clinicopathological variables by FAK and 14-3-3ε expression. Student's *t* test was used to analyze differences of continuous variables among 2 groups. Kaplan-Meier curve were plotted and the log-rank test was used to analyze time-related probabilities of metastasis and overall survival.

RESULTS

Association of FAK and 14-3-3ε Expression in HCC

Previous studies demonstrated that FAK and 14-3-3ε are overexpressed and play as prognostic factors to predict a higher incidence of extrahepatic metastasis and worse survival of HCC [21, 38]. To examine whether the expression of FAK is associated with 14-3-3ε, we compared the expression and correlation of both proteins by immunohistochemistry analysis in HCC tumors. FAK and 14-3-3ε expression was examined in a cohort of 114 primary

HCC tumors with surrounding non-cancerous parenchyma and 34 matched extrahepatic metastatic tumors. Negative control slides were unstained for FAK and 14-3-3ε (Fig. (**1A** and **1B**)). FAK and 14-3-3ε was overexpressed in 65 (57.0%) and 71 (62.3%) of 114 primary HCC tumors (Fig. (**1C** and **1D**), Table (**1**)). These results indicate a significant association of 14-3-3ε with FAK expression in primary HCC ($p<0.001$, Table (**1**)). Moreover, FAK was overexpressed in 33 and 14-3-3ε was positively stained in all of 34 metastatic HCC tumors, as illustrated representatively in metastatic lesion in chest wall (Fig. (**1E** and **1F**)) and gallbladder (Fig. (**1G** and **1H**)). In addition to the correlation with 14-3-3ε, increased expression of FAK was significantly associated with age, tumor size, tumor multiplicity, capsular formation, alpha-fetoprotein level and subsequent extrahepatic metastasis (Table (**1**)). Moreover, we have confirmed that increased FAK expression in primary tumors had a significant reduction in overall survival (Fig. (**2**)) and increase in probability of extrahepatic metastasis (Fig. (**3**)).

14-3-3ε increased FAK Expression and Promoter Activity

To investigate the biological relation of 14-3-3ε and FAK, SK-Hep-1 cells were transfected with 14-3-3ε overexpression or control vectors. Transfected cells were harvested and expression of FAK was determined by Western blotting analysis. Overexpression of 14-3-3ε significantly increased FAK protein level (Fig. (**4A**)). In addition, SK-Hep-1 cells were co-transfected by FAK promoter reporter with 14-3-3ε overexpression or control vectors and FAK promoter activity was determined by luciferase reporter assay. 14-3-3ε overexpression significantly increased FAK promoter activity (Fig. (**4B**)). These results indicate that 14-3-3ε protein could activate FAK transcription and result in increased FAK expression.

14-3-3ε Induced FAK Expression *via* Activation of NFκB

NFκB has been shown to be an important transcriptional activator of FAK expression [27, 28], and 14-3-3ε was suggested to modulate NFκB activities [47, 48]. In this study, we hypothesized that 14-3-3ε may regulate FAK expression through an NFκB-mediated pathway in HCC cells. To test this hypothesis, we examined NFκB activity by co-transfection of luciferase-based NFκB-binding reporter with 14-3-3ε overexpression or control vectors, and NFκB activity was determined by luciferase reporter assay. NFκB activity was significantly increased by 14-3-3ε overexpression (Fig. (**5A**)). We next examined the subcellular location of NFκB subunit p65 in 14-3-3ε overexpressed HCC cells. Nuclear fractions of 14-3-3ε overexpression or control vectors were extracted and protein level of p65 was determined by Western blotting analysis. 14-3-3ε significantly increased p65 nuclear

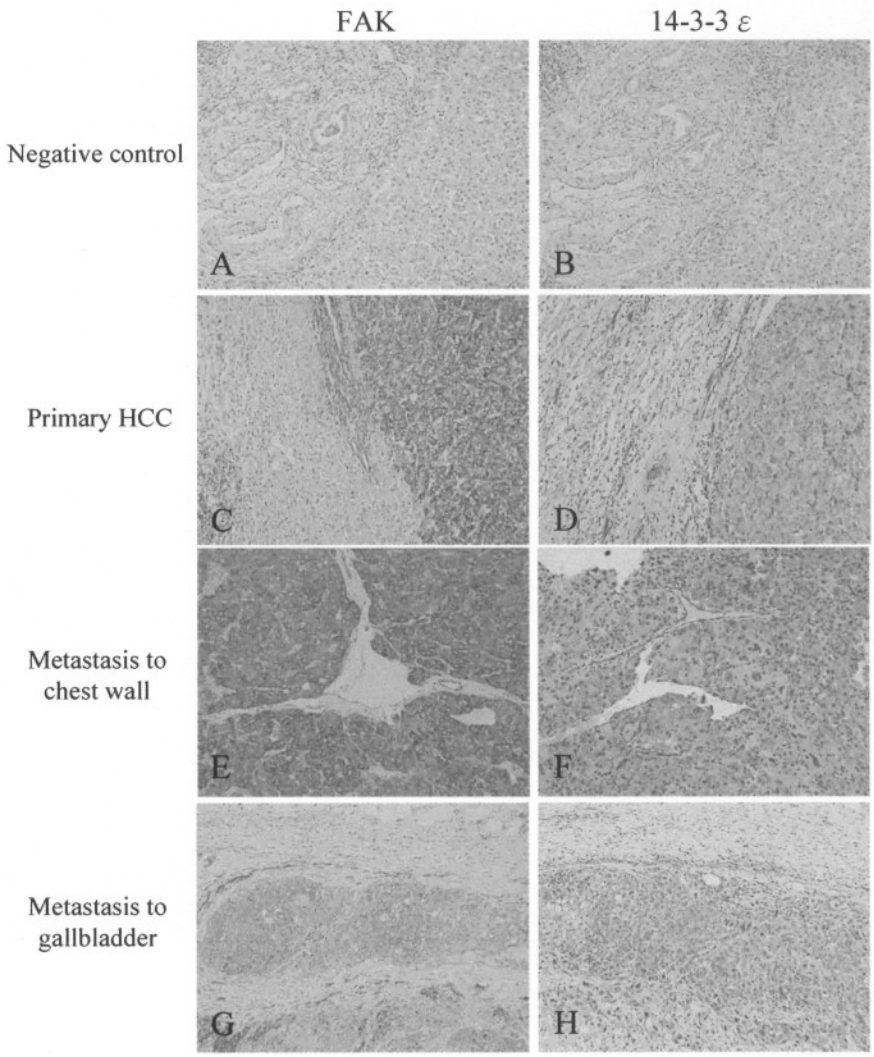

Fig. (1). Immunohistochemical analysis of FAK and 14-3-3ε in primary and metastatic HCC tumors. A and **B**) Negative control staining. **C** and **D**) Representative staining of FAK and 14-3-3ε in primary HCC tumors. **E** and **F**) Positive staining of FAK and 14-3-3ε in metastatic HCC lesions in chest wall. **G** and **H**) Positive staining of FAK and 14-3-3ε in metastatic HCC lesions in gallbladder.

Table 1. Correlation of FAK Expression with 14-3-3ε and Clinicopathological Characteristics in Primary HCC Patients

Parameters	FAK Positiveity % (n)	*p*-value
Overall (n=114)	57.0% (65)	
14-3-3ε		<0.001#
Positive (n=71)	78.9% (56)	
Negative (n=43)	20.9% (9)	
Age		0.002#
≥ 60 years (n=60)	43.3% (26)	
< 60 years (n=54)	72.2% (39)	
Gender		NS
Male (n=86)	60.5% (52)	
Female (n=28)	46.4% (13)	
Histology grade		NS
1 (n=7)	28.6% (2)	
2 (n=81)	56.8% (46)	
3 (n=26)	65.4% (17)	
Types of surgery		NS
None* (n=3)	100% (3)	
Wedge resection (n=39)	51.3% (20)	
Segmentectomy (n=54)	57.4% (31)	
Lobectomy (n=18)	61.1% (11)	
Surgical margin		NS
No operation (n=3)		
Free (n=84)	54.8% (46)	
Involved (n=27)	59.3% (16)	
BCLC staging&		NS
Not available (n=5)		
Early (n=57)	50.9% (29)	
Intermediate (n=50)	62.0% (31)	
Advanced (n=2)	100% (2)	
Tumor Size		0.036#
Not available (n=2)		
≥ 5.0 cm (n=37)	70.3% (26)	
< 5.0 cm (n=75)	49.3% (37)	
Tumor multiplicity		0.010#
Not available (n=1)		
Single (n=87)	63.2% (55)	
Multiple (n=26)	35.7% (9)	
Capsular formation		0.036#
Not available (n=11)		
Yes (n=60)	46.7% (28)	
No (n=43)	67.4% (29)	
Micro-vascular thrombi		NS
Not available (n=3)		
Yes (n=48)	62.5% (30)	
No (n=63)	50.8% (32)	
Liver cirrhosis		NS
Not available (n=6)		
Yes (n=56)	53.6% (30)	
No (n=52)	61.5% (32)	
Viral Hepatitis*		NS
Not available (n=7)		
Hepatitis B (n=58)	63.8% (37)	
Hepatitis C (n=31)	45.2% (14)	
Both (n=15)	46.7% (7)	
None (n=3)	33.3% (1)	
Alpha-fetoprotein level		0.002#
Not available (n=12)		
≧ 80ng/ml (n=37)	75.7% (28)	
< 80 ng/ml (n=65)	44.6% (29)	
Subsequent extrahepatic metastasis		0.020#
Yes (n=34)	73.5% (25)	
No (n=80)	50.0% (40)	

*Specimens from core tumor biopsy

#$p<0.05$

BCLC, Barcelona-clinic liver cancer; NS, Not significant; Q-score, quick score

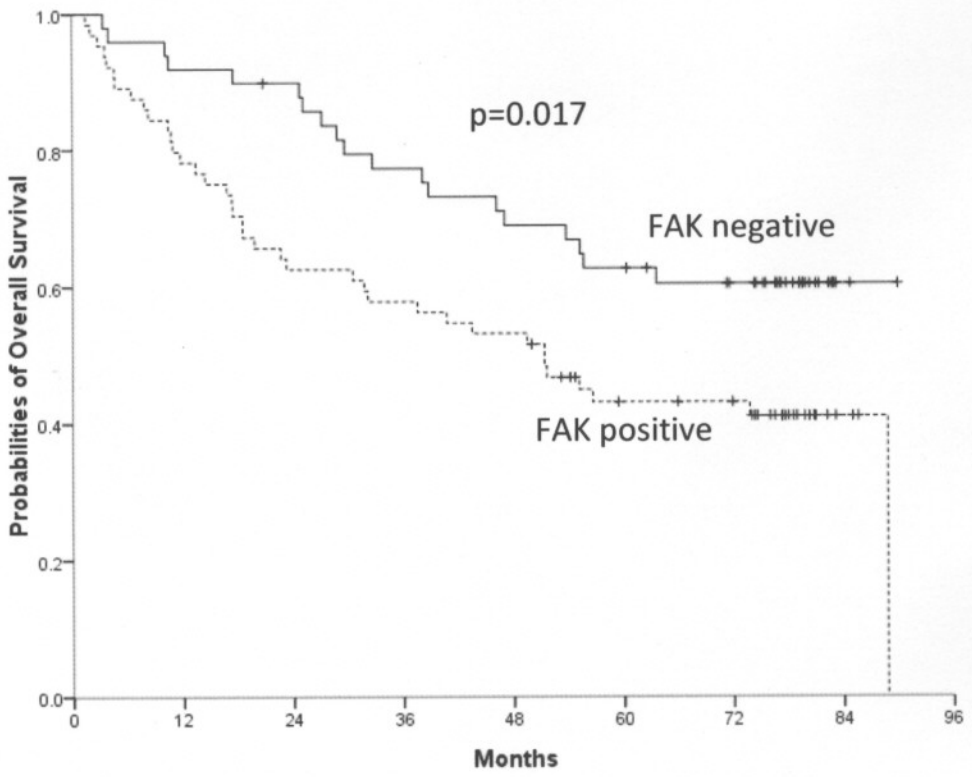

Fig. (2). Kaplan-Meier analysis of FAK expression with overall survival of HCC patients. Patients with FAK positive staining in primary HCC tumors had a significantly worse 5-year overall survival rate than negative-stained tissues.

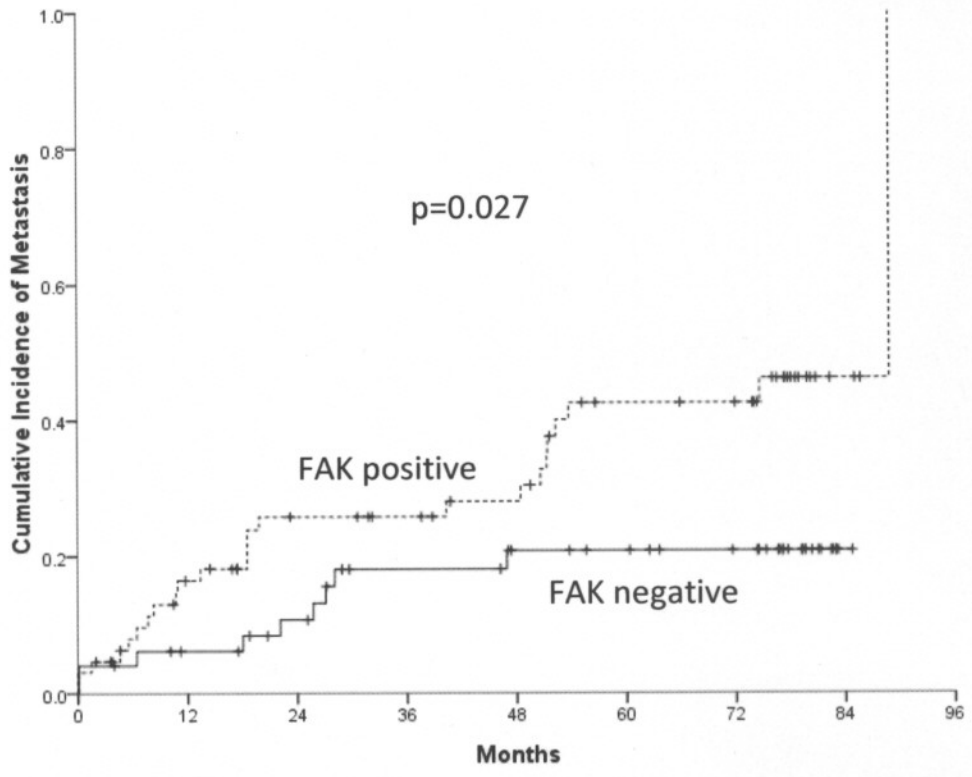

Fig. (3). Kaplan-Meier analysis of FAK expression with metastatic risk of HCC patients. The 5-year cumulative probability of metastasis for FAK negative was significantly lower than FAK positive patients.

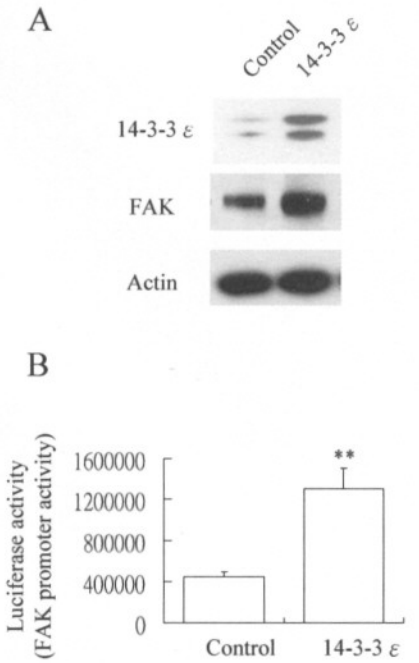

Fig. (4). Overexpression of 14-3-3ε induces FAK expression and increases promoter activity. A) 14-3-3ε-transfected SK-Hep-1 cells were harvested and protein level of FAK was determined by Western blotting analysis. Actin was used as loading control. **B**) SK-Hep-1 cells were transfected with 14-3-3ε overexpression vectors and FAK promoter activity was determined by luciferase activity. Luciferase activity assay was measured and expressed as relative light unit (RUL)/μg protein.

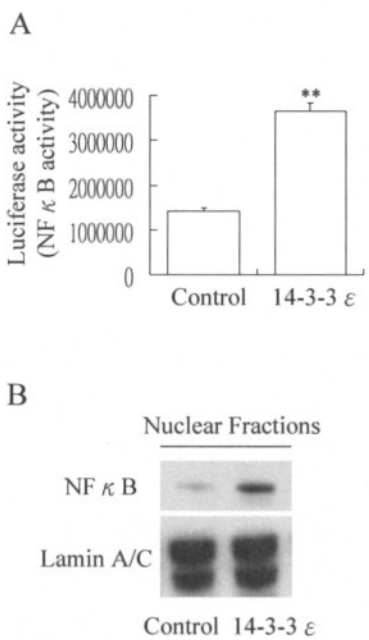

Fig. (5). 14-3-3ε overexpression induces NFκB activation. A) 14-3-3 overexpression increased NFκB binding activity was measured by luciferase activity. Luciferase activity assay was measured and expressed as relative light unit (RUL)/μg protein. **B**) 14-3-3ε overexpression induced nuclear translocation of NFκB was determined by extraction of nuclear fraction followed by Western blotting analysis. Lamin A/C was used as the loading control.

translocation (Fig. (**5B**)). To ascertain 14-3-3ε enhances the NFκB binding ability to the FAK promoter region, we performed the ChIP assay and results revealed that the binding of p65 to the FAK promoter was significantly increased by 14-3-3ε overexpression (Fig. (**6**)). Therefore, 14-3-3ε could increase FAK transcription *via* increasing NFκB activity.

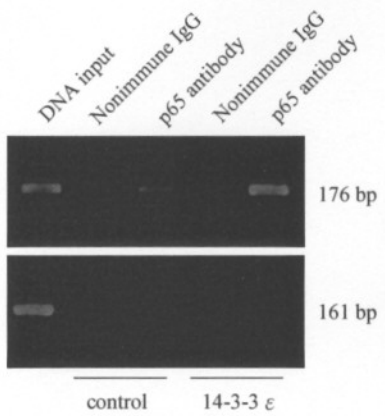

Fig. (6). 14-3-3ε enhances p65 binding on the FAK promoter region. Chromatin Immunoprecipitation (ChIP) assay of p65 binding in 14-3-3ε transfected SK-Hep-1 cells (upper panel). The nonimmune IgG and a DNA sequence located at the proximal region of FAK promoter (lower panel) were used as negative control.

DISCUSSION

The results from this study reveal a tightly association of FAK with 14-3-3ε expression in HCC. Positivity of FAK and 14-3-3ε expression significantly correlated with clinicopathological characteristics, including age, tumor size, alpha-fetoprotein level, subsequently extrahepatic metastasis, progression-free and overall survival. Moreover, expression of 14-3-3ε and FAK was elevated and highly associated in metastatic tumors. These findings indicate that FAK may collaborate or crosstalk with 14-3-3ε to promote HCC tumor progression.

Additionally, we provide evidences to show that increased 14-3-3ε expression activates NFκB activity, enhances NFκB binding to FAK promoter and induces FAK transcription as well as expression. To further investigate whether 14-3-3ε plays an essential role to maintain FAK expression in HCC, we have performed experiment of 14-3-3ε knockdown by transfection of specific siRNA. Surprisingly, reduced 14-3-3ε expression by siRNA did not significantly decreased FAK expression. Since the physiological function of 14-3-3 proteins is medicated by dimerization, we hypothesized that 14-3-3ε may interact with other 14-3-3 isoforms to form hetero-dimer to modulate FAK expression. Thus, knockdown single isoform of 14-3-3ε probably may not be enough to suppress FAK expression. In addition to 14-3-3ε, our recent studies indicated that expression 14-3-3β and 14-3-3γ was also increased in HCC [38-40]. Thus, combination of 14-3-3ε knockdown with other isoforms would be the way to explain how FAK expression is regulated by 14-3-3 proteins in HCC. The synergism of 14-3-3 isoforms to form hetero-dimers on regulating FAK expression in HCC needs further investigation.

14-3-3β has been reported to be interacted with integrin β1 cytoplasmic domain to promote cell spreading and migration [49]. As FAK is known to be associated and activated by integrin signaling, 14-3-3β is thus another potential regulator collaborating with FAK to promote HCC progression. In addition, 14-3-3 proteins exert the anti-apoptotic activity by binding with and sequestering the pro-apoptotic Bad proteins in the cytosol, thereby preventing Bad from mitochondrial translocation, thereby protecting cell survival [41-43]. Furthermore, it has been shown that 14-3-3 proteins interact with phosphorylated Bad is mediated

by a PI-3K/Akt-dependent mechanism [50]. Since PI-3K/Akt pathway is known to be the downstream factor activated by Integrin/FAK pathway, 14-3-3 is therefore considered to regulate and further collaborate with FAK and its downstream signal factors to enhance HCC cell survival. Taken together, these findings suggest that FAK may synergize with 14-3-3β, 14-3-3ε or other isoforms to play crucial roles in promoting cancer cell migration and HCC metastasis.

Bortezomib, a proteasome inhibitor based anti-cancer drug, repress NFκB activation by preventing the degradation and accumulation of IκB [51]. Earlier studies showed that bortezomib suppresses FAK expression by interrupting NFκB pathway [28, 52]. In addition, previous reports indicated that 14-3-3ε was upregulated to protect endothelial and colorectal cancer cell survival through a PPARδ-dependent pathway [41, 42]. Interestingly, our preliminary data indicated that expression of 14-3-3ε was also suppressed by bortezomib. However, the reduction of 14-3-3ε by bortezomib is neither regulated *via* NFκB nor PPARδ-dependent pathway (data not show). Thus, the role of bortezomib in modulating 14-3-3ε expression may be diverse and depend on cell or tissue types. In conclusion, targeting FAK, 14-3-3 proteins and their downstream mediators with the combination of bortezomib and other anti-tumor agents in the clinical application for HCC should be therefore under rigorous evaluation.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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ABBREVIATIONS

FAK	=	Focal adhesion kinase
HCC	=	hepatocellular carcinoma
NFκB	=	nuclear factor kappa B
ChIP	=	Chromatin immunoprecipitation
IκB	=	inhibitor of kappa B

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