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Tumorigenesis and Neoplastic Progression

Biological Significance of Focal Adhesion Kinase in Ovarian Cancer

Role in Migration and Invasion

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Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that is activated by integrin clustering. There are limited data regarding the functional role of FAK in ovarian cancer migration and invasion. In the current study, FAK expression was evaluated in ovarian cell lines (nontransformed and cancer), 12 benign ovarian samples, and in 79 invasive epithelial ovarian cancers. All three ovarian cancer cell lines overexpressed FAK compared to the nontransformed cells. The dominant-negative construct called FAK-related nonkinase (FRNK) was introduced into two ovarian cancer cell lines (SKOV3 and 222). FRNK promoted FAK dephosphorylation without changing total FAK levels in these cell lines. Furthermore, FRNK decreased the *in vitro* invasive ability of ovarian cancer cells by 56 to 85% and decreased migration by 52 to 68%. FRNK-transfected cells also displayed poor cell spreading. Immunohistochemical analysis revealed that the surface epithelium from all benign ovarian samples had weak FAK expression. In contrast, 68% of invasive ovarian cancers overexpressed FAK. FAK overexpression was significantly associated with high tumor stage, high tumor grade, positive lymph nodes and presence of distant metastasis (all *P* values <0.05). FAK overexpression was also associated with shorter overall survival (*P* = 0.008). Multivariate analysis revealed that FAK overexpression and residual disease >1 cm were independent predictors of poor survival. These data indicate that FAK is overexpressed in most invasive ovarian cancers and plays a functionally significant role in ovarian cancer migration and invasion. Thus, FAK may be an important therapeutic target in ovarian carcinoma. *(Am J Pathol 2004, 165:1087–1095)*

Ovarian cancer remains the most common cause of death from a gynecological malignancy.[1] The high mortality related to ovarian cancer is thought to be because of the advanced stage of disease at presentation. Tumor progression toward increasing metastatic potential is a complex, multistep process and requires the coordinated expression of metastasis-promoting genes and the down-regulation of metastasis-suppressing genes. Metastatic colonization requires disseminated cells to initiate context-dependent signaling cascades that allow them to survive, enter the cell cycle, and proliferate to become metastases. Cell migration is an important component of the metastatic process and requires repeated adhesion to and detachment from the extracellular matrix microenvironment. These events are mediated, in large part, by integrins, which on engagement with components of the extracellular matrix, reorganize to form adhesion complexes termed focal adhesions.[2–4] These focal adhesions orchestrate a signal transduction cascade initiated by integrin-mediated recognition of extracellular matrix components.

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Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that localizes to contact sites in focal adhesions.[5,6] This intracellular signaling protein is associated with the cytoplasmic domain of integrin receptors and on integrin clustering is activated and autophosphorylated on tyrosine. Many stimuli can induce tyrosine phosphorylation and activation of the catalytic activity of FAK including growth factors, neuropeptides, and mechanical stimuli.[6] However, the major mode of regulation is via integrin-dependent adhesion to the extracellular matrix in *in vitro* studies and FAK is an integral component of the integrin-signaling pathway. The focal adhesion complex regulates cell growth, differentiation, and fate through the promotion of tyrosine phosphorylation and subsequent regulation of downstream cell survival components such as PI3-kinase, and signaling pathways such as those associated with Grb2 and Ras. Elevated tyrosine phosphatase activity or expression of the FAK C-terminal, noncatalytic domain, termed FRNK (FAK-related nonkinase), as a dominant-negative inhibitor promotes FAK dephosphorylation and inhibits FAK function.[7] However, most of these findings were obtained with normal fibroblasts, and thus, it is unclear whether FAK functions in a similar manner in human tumor cells, especially ovarian carcinoma.

Overexpression of FAK protein has been reported in metastatic human colorectal, breast, thyroid, and prostate cancer cells.[8–12] There are limited data regarding the role of FAK in ovarian cancer, but FAK was reported to be overexpressed in most human ovarian cancers.[13] However, these studies have relied primarily on blotting methods to demonstrate FAK overexpression and it is difficult to distinguish the contributions of tumor versus stromal FAK with such an approach. Also, of special interest is the observation that amplification of 8q, where FAK is located, is one of the most frequent alterations in primary ovarian cancers and is associated with poorly differentiated tumors.[14,15] Thus, we undertook the present study with the following aims: to evaluate the clinical relevance of FAK expression in ovarian cancer and to evaluate the functional role of FAK in ovarian cancer migration and invasion.

Materials and Methods

Cell Culture

The ovarian cancer cell lines used in this study were SKOV3, EG, and 222. The derivation and sources of these cell lines have been reported previously.[16] These cells were maintained and propagated *in vitro* by serial passage in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). The immortalized nontransformed human ovarian surface epithelial cell lines (HI0-180 and H10-1120) were a kind gift from Dr. Andrew Godwin at the Fox Chase Cancer Center, Philadelphia, PA. The HI0-180 cells were maintained in Medium 199/MCDB 105 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate. All cell lines are routinely screened for mycoplasma species (GenProbe detection kit; Fisher, Itasca, IL). All experiments were performed with 70 to 80% confluent cultures.

Invasion Assay

The membrane invasion culture system chamber was used to measure the *in vitro* invasiveness of all cell lines used in this study.[16,17] Briefly, a polycarbonate membrane with 10-μm pores (Osmonics, Livermore, CA) was uniformly coated with a defined basement membrane matrix consisting of human laminin/type IV collagen/gelatin and used as the intervening barrier to invasion. Both upper and lower wells of the chamber were filled with serum-free RPMI containing 1× MITO+ serum supplement (Collaborative Biomedical, Bedford, MA). Single cell tumor suspensions were seeded into the upper wells at a concentration of 1×10^5 cells per well. After a 24-hour incubation in a humidified incubator at 37°C with 5% CO_2, cells that had invaded through the basement membrane were collected, stained, and counted by light microscopy.[16] For chemoinvasion assays, conditioned media from normal skin fibroblasts (kindly provided by Dr. Gregory Goldberg, Washington University, St. Louis, MO) was added to the lower wells. Invasiveness was calculated as the percentage of cells that had successfully invaded through the matrix-coated membrane to the lower wells compared to the total number of cells seeded into the upper wells and corrected for cell proliferation.

Cell Migration Assay

Unstimulated motility was determined in membrane invasion culture system chambers containing polycarbonate filter (with 10 μm pores) that had been soaked in 0.1% gelatin. Tumor cells (5×10^4) were seeded in each upper well, allowed to incubate at 37°C for 5.5 hours in Dulbecco's modified Eagle's medium containing 10% nuserum, and subsequently processed as described for the invasion assay.

Immunofluorescence Staining

The cells were plated on coverslips coated with fibronectin (BD Biosciences, San Jose, CA) and fixed in 3.7% paraformaldehyde for 10 minutes followed by a phosphate-buffered saline wash, and then treatment with Triton 0.5% for 6 minutes. Staining was performed with mouse anti-human FAK (dilution 1:50; BD Transduction, San Diego, CA), and Phalloidin (dilution 1:600; Molecular Probes, Eugene, OR).

Formalin-Fixed, Paraffin-Embedded Samples for FAK Immunohistochemical Staining

All samples were collected in compliance with requirements of the Institutional Review Board for the Protection of Human Subjects. Formalin-fixed, paraffin-embedded samples were sectioned at a thickness of 4 μm and

stained with hematoxylin and eosin (H&E) for identification. Sections adjacent to the H&E-stained sections were used for immunohistochemical staining. All slides were deparaffinized using xylene, 100% ethanol, 95% ethanol, followed by a thorough deionized water wash. A water bath antigen recovery technique, using citrate buffer, pH 6.0, was performed on all slides. The immunohistochemical staining for FAK was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using the Vectastain Universal Elite ABC peroxidase kit (Vector Laboratories, Inc., Burlingame, CA) to detect mouse anti-human FAK, clone 4.47 (dilution 1:800; Upstate Biotechnology, Waltham, MA). After deparaffinization and antigen recovery, slides were washed in Tris-buffered saline with Tween (TBST). Three blocking steps were applied: 0.03% hydrogen peroxide (DAKO) for 20 minutes followed by a TBST wash, avidin and biotin blocks were applied for 15 minutes in each solution followed by a TBST wash after each step, finally the Protein Block Serum-Free (DAKO) was applied for 15 minutes. The primary antibody was applied to the slides and incubated for 40 minutes; slides were rinsed in TBST, followed by application of the RTU Vectastain Secondary for 12 minutes. This procedure was followed by a TBST wash and then incubation with the RTU ABC reagent for 10 minutes. Color for the RTU procedure was produced by using DAB+ (brown) substrate (DAKO) for 2 to 3 minutes. Slides were counterstained with Mayer's hematoxylin for 3 minutes. Control reactions consisted of either a preimmune IgG serum (MOPC-21; Sigma Chemical Co., St. Louis, MO), which was isotype-matched for the FAK antibody at the same concentration, or incubation of sections in the absence of primary antibody.

All samples were reviewed by two board-certified pathologists (M.S.F., B,D,), who were blinded to the clinical outcome of these patients. FAK expression was determined by assessing semiquantitatively the percentage of stained tumor cells and the staining intensity, as described previously.[18] The percentage of positive cells was rated as follows: 0 points, 0 to 5%; 2 points, 6 to 50%; 3 points, >50%. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; 3 points, strong intensity. Points for expression and percentage of positive cells were added and an overall score (OS) was assigned. Tumors were categorized into four groups: negative (OS = 0), ≤5% cells stained, regardless of intensity; weak expression (OS = 1), 1 to 2 points; moderate expression (OS = 2), 3 to 4 points; and strong expression (OS = 3), 5 to 6 points.

FRNK Transfection

Cells were plated at 5×10^5 cells per well of six-well dishes. The cell lines 222 and SKOV3 were transfected with 2 μg of FRNK cDNA in pcDNA3.1 vector (Invitrogen Corp., Carlsbad, CA) using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's protocol. Forty-eight hours after the transfection, G418 containing media was added and changed every 48 to 72 hours thereafter to generate stable transfectants. Sham constructs were created by transfecting pcDNA3.1 alone into both cell lines. β-Galactosidase expression was used to determine the transfection efficiency for each cell line, which was ~35% for the SKOV3 cells and ~50% for the 222 cells.

Measurement of Cell Spreading

Cells were collected by ethylenediaminetetraacetic acid treatment and resuspended in RPMI without serum. Cells (5×10^4) were plated in triplicate in 12-well plastic dishes (coated with a defined matrix as described above) containing RPMI without serum. The cells were allowed to spread for various time intervals at 37°C. For each experiment, three random fields were counted. Unspread cells were defined as round phase-bright cells; spread cells were defined as those that had extended processes, that lacked a round morphology, and that were not phase bright.

Immunoprecipitation and Western Blot Analysis

Cells were lysed with 1× modified RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 25 μg/ml leupeptin (Sigma Chemical Co.), 10 μg/ml aprotinin (Sigma Chemical Co.), 1 mmol/L sodium orthovanadate, and 2 mmol/L ethylenediaminetetraacetic acid. Cells were removed from the dishes by cell scraping and the samples were then stored at −80°C. The protein concentration of the samples was determined using a BCA protein assay reagent kit, and whole cell lysates were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie BBR-250 (Sigma Chemical Co.) to ensure equal loading. Samples were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) membranes and blots were blocked with 5% nonfat milk for 1 hour at room temperature. Blots were incubated with the monoclonal FAK antibody (1:500 dilution; Transduction Laboratories, Lexington, KY) or FRNK antibody for 1 hour at room temperature with agitation, followed by incubation with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000; The Jackson Laboratory, Bar Harbor, ME). Blots were developed using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

For immunoprecipitation experiments, 300 μg of cell lysate were incubated with the FAK antibody for 1 hour at 4°C. Protein-antibody complexes were incubated for 1 hour at 4°C with protein A-Sepharose-conjugated beads (preincubated with rabbit anti-mouse IgG), collected by centrifugation, washed three times with the modified RIPA buffer, and boiled in Laemmli sample buffer. The proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and immunoblotting was performed as described above.

Clinicopathological Variable Analysis

All patients underwent surgical exploration and cytoreduction as the initial treatment. The treating gynecologi-

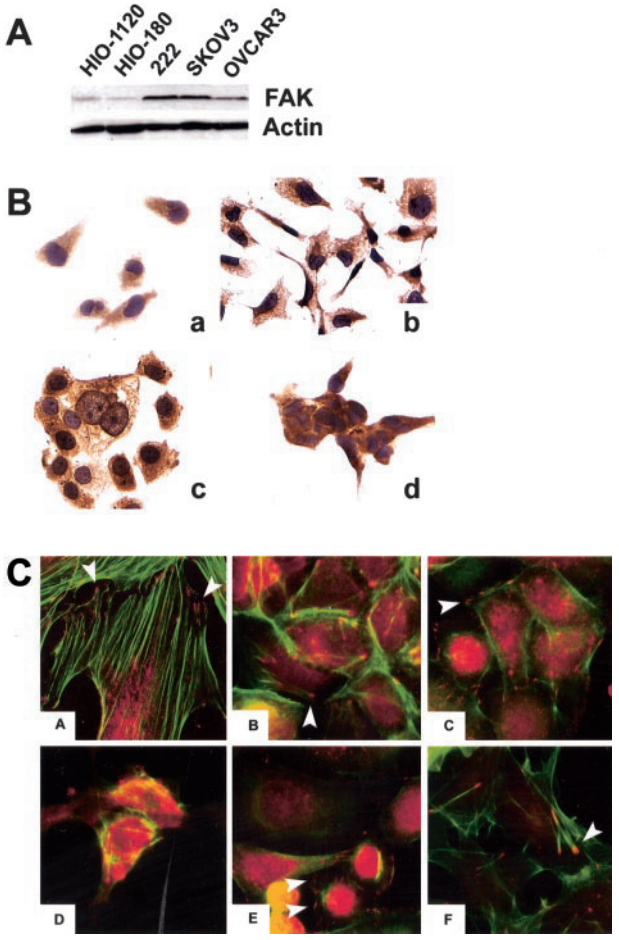

Figure 1. FAK analysis in nontransformed ovarian surface epithelial cells (HI0-180 and HI0-1120) and ovarian cancer cell lines (OVCAR3, SKOV3, and 222). FAK expression was analyzed by: **A:** Western blot of whole cell lysates; **B:** immunohistochemical peroxidase staining for FAK in HI0-180 (**a**), SKOV3 (**b**), OVCAR3 (**c**), and 222 (**d**); and **C:** immunofluorescence staining (dual) for FAK (red) and Phalloidin (green) in HI0-180 (**A**), OVCAR3 (**B**), SKOV3 (**C**), 222 (**D**), and FRNK transfectants SKOV3-FC2 (**E**) and 222-FC2 (**F**) (**arrowheads** indicate focal adhesions). Original magnifications: ×400 (**B**); ×630 (**C**).

cal oncologist determined adjuvant therapy (two patients with stage IA ovarian cancer did not receive adjuvant chemotherapy; all other patients with invasive ovarian cancer were treated with adjuvant paclitaxel and platinum chemotherapy). Diagnosis was verified by pathology review at the institutional gynecological oncology tumor board. All patients were staged according to the International Federation of Gynecology and Obstetrics surgical staging system. A gynecological pathologist reviewed the pathology for all patients.

Statistical Analysis

Chi-square or Fisher's exact test were used as appropriate to determine differences between variables using SPSS (SPSS Inc., Chicago, IL). Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. The Cox proportional hazards model was used for multivariate analysis. A P value <0.05 was considered statistically significant.

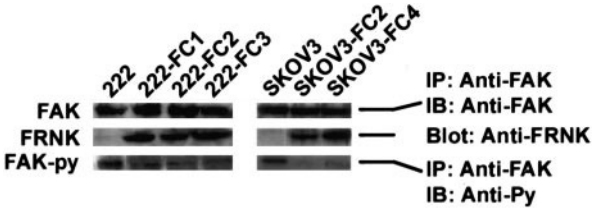

Figure 2. Western blot analysis of ovarian cancer cells (222 and SKOV3) and FRNK transfectants (222-FC1, 222-FC2, 222-FC3, SKOV3-FC2, and SKOV3-FC4). Tyrosine phosphorylation and expression of FAK was analyzed by immunoprecipitating (IP) FAK with the anti-FAK monoclonal antibody (4.47; Upstate Biotechnology, Inc.) and then immunoblotting (IB) with either anti-FAK antibody or anti-phosphotyrosine antibody. Western blot to detect FRNK was performed using a polyclonal antibody (BC4).

Results

FAK Expression in Ovarian Cell Lines Is Associated with Invasive Potential

FAK expression in ovarian cell lines was assessed by Western blot, immunohistochemistry, and immunofluorescence (Figure 1B, a). The nontransformed cell lines HI0-180 and H10-1120 are known to be poorly invasive[16] and demonstrated low FAK expression. All of the cancer cell lines had moderate to high levels of FAK by Western blot analysis (Figure 1A). Immunohistochemical peroxidase staining confirmed the low expression of FAK by the HI0-180 cells (Figure 1B, a). In contrast, all three ovarian cancer cell lines had high FAK expression (Figure 1B, b to d). Immunofluorescence labeling for both FAK and actin revealed that the HI0-180 cell line develops well-formed focal adhesions and well-developed actin stress fibers (Figure 1C, A). Both OVCAR3 and SKOV3 cell lines also demonstrate focal adhesion formation (Figure 1C, B and C). However, the highly invasive cell line 222 demonstrated decreased focal adhesion formations and had poorly organized actin staining (Figure 1C, D).

FRNK Interferes with FAK Phosphorylation and Decreases Ovarian Cancer Invasion, Migration, and Cell Spreading

Ovarian cancer cell migration, attachment, and invasion are key steps in the metastatic process. To test the hypothesis that ectopic expression of the FAK C-terminal, noncatalytic domain, FRNK, would interfere with ovarian cancer invasion, migration, and cell spreading, FRNK cDNA was introduced into the highly invasive 222 and SKOV3 cancer cells (Figure 2). After FRNK transfection of the 222 cell line, three stably transfected clones (222-FC1, 222-FC2, and 222-FC3) were obtained and tested for their *in vitro* invasive potential. Similarly, after SKOV3 transfection, two stably transfected clones (SKOV3-FC2 and SKOV3-FC4) were obtained and tested. We determined the effect of FRNK transfection on FAK levels and phosphorylation by immunoprecipitation and immuno-

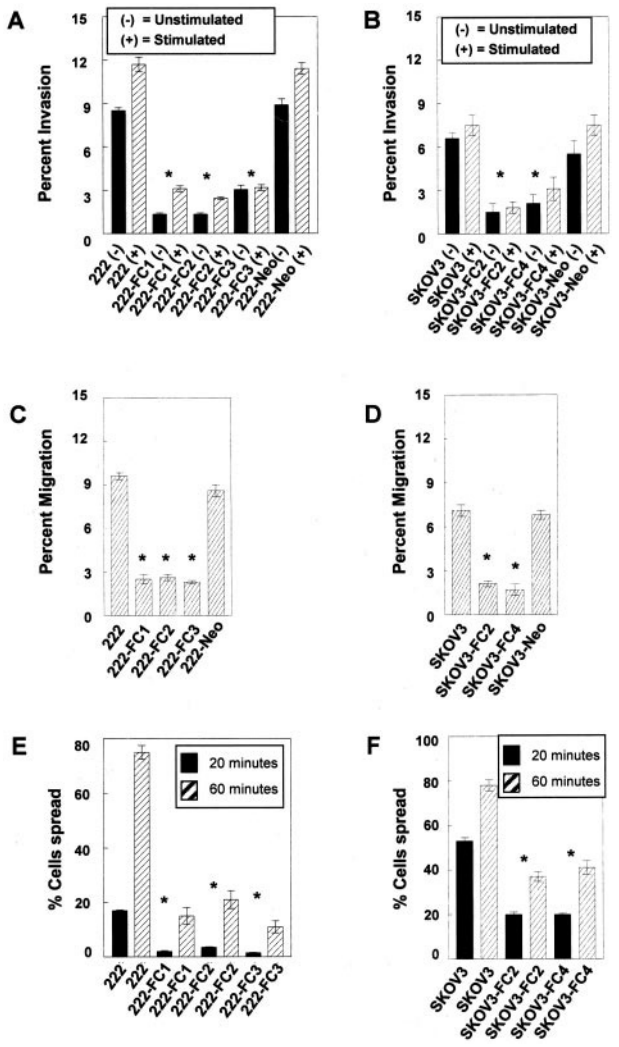

Figure 3. A and **B:** Invasion profile of ovarian cancer cell lines (222 and SKOV3) compared to FRNK transfectants (222-FC1, 222-FC2, 222-FC3, SKOV3-FC2, and SKOV3-FC4) and sham transfectants (222-neo and SKOV3-neo), based on their ability to invade a basement membrane matrix *in vitro* in the presence (**hatched bars**) or absence (**solid bars**) of a chemoattractant. **C** and **D:** Migration ability of ovarian cancer cells, FRNK transfected cells, and sham transfectants. **E** and **F:** Cell spreading of ovarian cancer cells and FRNK transfectants on fibronectin matrix at 20 and 60 minutes. Error bars represent SE.

blotting. The overall levels of FAK were not affected by FRNK transfection (Figure 2) in both cell lines, but FAK phosphorylation was markedly decreased in the FRNK-transfected cells (Figure 2). Immunofluorescence microscopy revealed that SKOV3 was still able to form focal adhesions, and FRNK-transfected 222 cells acquired the ability to form focal adhesions (Figure 1C, E and F).

The invasive potential of ovarian cancer cells and transfected cells was determined using the membrane invasion culture system assay and a defined basement membrane-coated barrier filter. The baseline invasion rates of these ovarian cancer cell lines have been reported previously.[16] The SKOV3 cell line is moderately invasive (7.5%), and the cell line 222 is highly invasive (11.7%). The dominant-negative FRNK decreased the invasive potential of the 222 cell line by 73 to 85% (all P values <0.05) in the three clones (Figure 3). The average invasive ability of the SKOV3 transfected cells decreased by 56 to 76% ($P < 0.05$) when compared with the parental SKOV3 cells.

We also assessed *in vitro* migration using the membrane invasion culture system assay and a gelatin-soaked filter. The migratory ability of the FRNK-transfected 222 cells decreased by 52 to 61% ($P < 0.05$) and SKOV3 cells by 65 to 68% ($P < 0.05$) (Figure 3). Next, we evaluated the ability of the parental and FRNK-transfected ovarian cancer cells to spread on fibronectin-coated dishes. At 20 minutes, 53% of the SKOV3 cells and 17% of the 222 cells had spread, and by 60 minutes, 78% and 75% of the cells, respectively, had spread (Figure 3). The cell spreading was markedly impaired by FRNK transfection in the ovarian cancer cells. At 20 minutes, only 1.5 to 3% ($P < 0.05$) of the 222 FRNK-transfected cells had spread and 20% ($P < 0.05$) of the SKOV3 FRNK-transfected cells had spread. Even at 60 minutes, only 11 to 21% ($P < 0.05$) of the 222-transfected cells and 37 to 41% ($P < 0.05$) of the SKOV3-transfected cells had spread.

Table 1. Demographic Features of Invasive Ovarian Cancer Patients

Variable	Number
Age	59.3 years (34 to 81 years)
Stage	
I	10
II	5
III	50
IV	14
Menopausal	
Yes	60
No	19
Histology	
Serous	52
Other	27
Grade	
Low (I or II)	34
High (III)	45
Ascites	
Yes	59
No	20
Cytoreduction	
Optimal	54
Suboptimal	25
Node status	
Positive	10
Negative	22
Not done	47
Status	
Alive without disease	21
Alive with disease	11
Dead of disease	43
Dead of other causes	4

FAK Expression in Human Ovarian Cancer Samples Correlates with Clinical Features

The demographic features of the patients in this study are listed in Table 1. The mean age of patients was 59.3 years. Eighty-one percent of the patients had advanced stage (III or IV) disease and 57% had high-grade (III) disease. Sixty-eight percent of the patients underwent

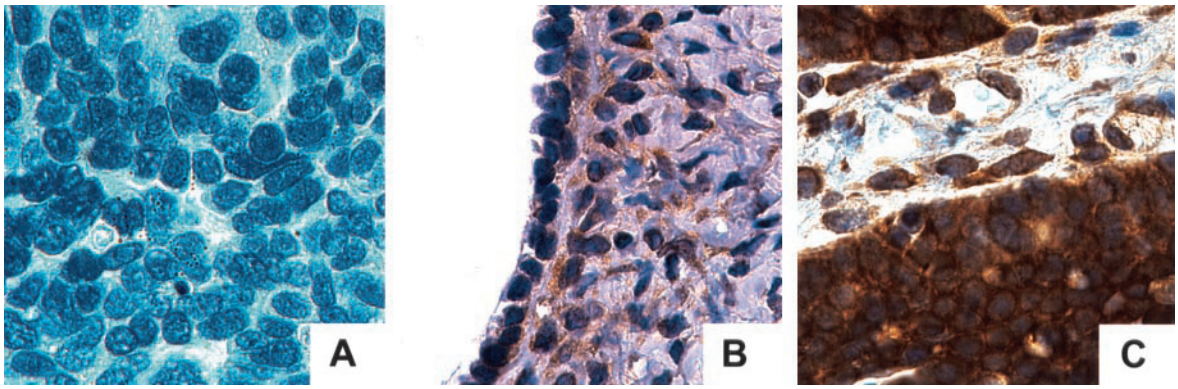

Figure 4. Representative immunohistochemical peroxidase staining for FAK in ovarian cancer-negative control (**A**), normal ovarian epithelium (**B**), and high-grade ovarian cancer (**C**) obtained from a patient. Original magnifications, ×630.

optimal surgical cytoreduction (<1 cm of residual disease at the end of surgery).

In the 12 benign ovaries all samples demonstrated weak FAK expression in the ovarian surface epithelium (Figure 4B). FAK expression was assessed using immunohistochemistry in 79 invasive ovarian cancers and representative staining results are shown in Figure 4C. In contrast to the normal ovarian surface epithelium, FAK was markedly up-regulated in the invasive ovarian cancers. FAK was detected at varying levels in all of the invasive ovarian carcinoma specimens and was overexpressed in 54 (68%) of the tumors. The correlation of FAK overexpression and various clinical variables are listed in Table 2. There was no association between FAK overexpression and histological subtypes (serous versus other), presence of ascites, and ability to achieve optimal cytoreduction. Eighty-one percent of high-stage tumors overexpressed FAK compared to only 20% of low-stage tumors ($P < 0.001$). FAK overexpression was also associated with high grade ($P = 0.01$), higher likelihood of nodal positivity ($P < 0.001$), and presence of distant metastasis ($P = 0.01$).

In univariate analysis, survival was adversely affected by high-stage, high-grade, and residual disease >1 cm ($P < 0.05$, data not shown). FAK overexpression was associated with significantly worse survival (median, 7.6 years versus 2.98 years; $P = 0.008$; Figure 5). In multivariate analysis using the Cox proportional hazards model that involved age, tumor stage and grade, volume of residual disease, and FAK overexpression, only volume of residual disease ($P < 0.02$) and FAK overexpression ($P < 0.03$) were significant predictors of poor survival.

Table 2. Correlation of Clinicopathological Variables with FAK Overexpression in Invasive Ovarian Cancer Patients

Variable	FAK overexpression		*P*
	Yes ($n = 54$)	No ($n = 25$)	
Stage			
Low	3	12	<0.001
High	51	13	
Histology			
Serous	37	15	0.46
Other	17	10	
Grade			
Low (I or II)	4	7	0.01
High (III)	50	18	
Ascites			
No	11	9	0.14
Yes	43	16	
Cytoreduction			
Optimal	37	17	0.961
Suboptimal	17	8	
Node status			
Positive	10	0	<0.001
Negative	9	13	
Not done	35	12	
Distant metastasis			
Yes	11	0	0.01
No	43	25	

Discussion

In this study, we addressed the functional significance of FAK in ovarian cancer invasion and migration. We also evaluated the expression and clinical relevance of FAK in human ovarian cancers. Our data provide definitive evidence, at the cellular level, that the expression of FAK is

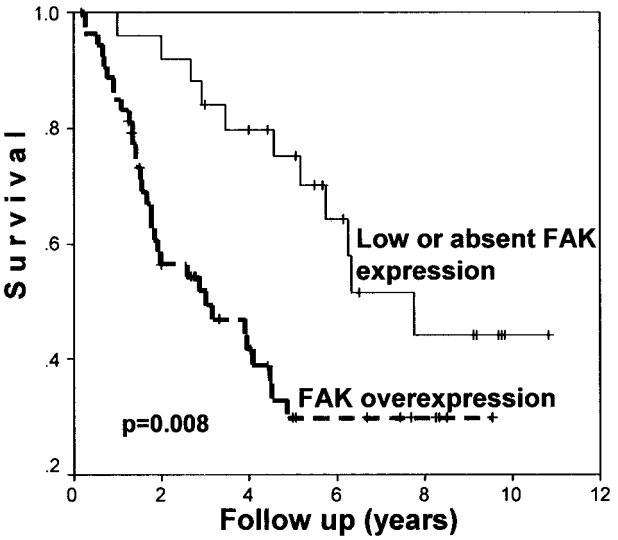

Figure 5. Kaplan-Meier survival of ovarian cancer patients based on FAK staining intensity using the log-rank statistic.

up-regulated in invasive ovarian cancer cells and is associated with aggressive tumor features and poor outcome in patients. Furthermore, inhibiting FAK phosphorylation by transfecting FRNK into highly aggressive ovarian cancer cells results in decreased invasion, migration, and cell spreading, which are key components of the metastatic process.

The ability of tumor cells to migrate from the site of the primary tumor and to invade surrounding tissues is a prerequisite for metastasis. FAK is a nonreceptor protein tyrosine kinase that is a critical mediator of signaling events between cells and their extracellular matrix, thereby facilitating invasion and migration.[19–24] FAK has been linked to integrin-signaling pathways via interactions with integrin-associated proteins such as paxillin and talin,[25–31] with resultant effects on cell migration,[31,32] cytoskeletal organization,[30,33] and apoptosis.[34–36] FAK becomes phosphorylated and activated during integrin-mediated cell adhesion. In addition to integrins, FAK can act as a transducer of certain soluble growth factors such as epidermal growth factor.[37] Cells derived from FAK−/− mouse embryos have been shown to exhibit reduced migration relative to wild-type cells.[38] Furthermore, overexpression of FAK in Chinese hamster ovary fibroblasts led to increased migration on fibronectin and a mutation in the major autophosphorylation site of FAK abolished its ability to stimulate cell migration.[32] FAK has also been shown to be a survival signal for anchorage-dependent cells[36–39] and inhibition of FAK expression in tumor cells has been shown to result in cell death.[34,35] Thus, it is likely that cancer cells up-regulate FAK to maintain survival during the metastatic process.

Other mechanisms by which FAK could potentially promote tumor cell invasion and migration include promotion of MMP-2 or MMP-9 secretion.[40,41] Shibata and co-workers[42] have demonstrated that FAK along with endogenous Ras is required for the activation of MMP-9 secretion by fibronectin stimulation. Similarly, Hauck and colleagues[40] have demonstrated that FAK dephosphorylation in human adenocarcinoma cells resulted in reduced epidermal growth factor-stimulated JNK activation and inhibited MMP-9 secretion, and potently blocked both random and epidermal growth factor-stimulated cell motility. Aguirre Ghiso[43] has demonstrated that active FAK is an important mediator of uPAR-regulated tumorigenicity and interruption of FAK mitogenic signaling, either through down-regulation of uPAR or by expression of FRNK, can force human carcinoma cells into dormancy. Our findings provide definitive evidence that inhibition of FAK phosphorylation results in reduced ovarian cancer cell invasion and migration.

Focal adhesions are specialized sites of cell attachment to the extracellular matrix where integrin receptors link the extracellular matrix to the actin cytoskeleton. Focal adhesions consist of several proteins that seem to serve structural roles (talin, vinculin, and paxillin) and possibly regulatory roles (protein kinase C, FAK, Src).[29–31] Transformed cells have fewer and less well-developed focal adhesions than their normal counterparts.[44] Also, the cytoskeleton is less organized and resembles that of a rounded cell about to undergo mitogenesis.[33] FAK has been shown to be a survival signal for anchorage-dependent cells,[36,39,45] and inhibition of FAK expression in tumor cells has been shown to result in cell death.[34,35] The role of FAK in focal adhesion assembly/disassembly continues to evolve.[46] It was originally thought to function in the assembly of focal adhesions; however, recent studies suggest that it might actually promote the disassembly of focal adhesions.[6,47] FAK knockout studies have demonstrated that deletion of FAK results in an early embryonic lethal mutation and the embryos died at 8.0 to 8.5 days post coitum with severe morphogenic defects.[38] Most notable defects were in the axial mesodermal tissue and the cardiovascular system. Both vasculogenesis and angiogenesis were also severely impaired.[48] Interestingly, Ilic and colleagues[38] demonstrated that FAK−/− fibroblasts exhibit larger focal adhesions than control FAK-expressing fibroblasts, but a decrease in cell migration in *in vitro* assays. The increase in focal adhesion number was somewhat surprising, but it suggests that FAK may regulate the cycles of focal adhesion assembly and disassembly rather than assembly alone. Ren and co-workers[49] have shown that FAK plays a role as a regulator of disassembly of focal adhesions. FAK may control focal adhesion disassembly by down-regulating Rho activity.[49] Our results are consistent with the role of FAK in disassembly of focal adhesions. The highly invasive cell line 222 had the highest FAK levels, but formed poor focal adhesions. The normal ovarian cell line HI0-180 had the lowest levels of FAK, but exhibited large focal adhesions. Interestingly, after FRNK transfection, the cell line 222 began to demonstrate better focal adhesion formation.

FAK overexpression has been demonstrated in other cancers including colon, breast, thyroid, prostate, and brain cancers.[8–11,44,50,51] Cance and colleagues[8] have demonstrated that FAK is only weakly expressed in most benign breast and colon epithelium; however, it is up-regulated in most of the invasive carcinomas. Interestingly, FAK expression was also up-regulated in areas of dysplastic, premalignant colon epithelium, suggesting that up-regulation occurs at an early stage in tumorigenesis. Using reverse transcriptase-polymerase chain reaction and/or Western blot analysis; FAK was shown to be overexpressed in invasive thyroid tumors[9] and invasive and metastatic prostate cancers.[11] Han and colleagues[52] have shown that FAK is overexpressed in most colorectal liver metastases, but the degree of overexpression of FAK was not a significant prognostic factor for patient survival. There are limited data regarding the role of FAK in ovarian cancer. Judson and colleagues[13] have reported that FAK levels were increased by fourfold in most invasive ovarian adenocarcinomas compared to normal ovarian tissues. However, these studies evaluated FAK expression in homogenates of total tumor tissues, and it is not possible to detect changes in FAK specifically associated with tumor cells with this technique. To the best of our knowledge, our study is the first to demonstrate that FAK overexpression is an independent predictor of poor outcome in ovarian cancer patients.

In summary, we have demonstrated that FAK expression is up-regulated in most invasive ovarian cancers. We

also showed that interfering with FAK function results in decreased invasion and migration of ovarian cancer cells. The dramatic up-regulation of FAK in ovarian cancers, combined with the relative lack of expression in normal tissues suggests that FAK may serve as an attractive therapeutic target.

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