Overexpression of Focal Adhesion Kinase in Head and Neck Squamous Cell Carcinoma Is Independent of *fak* Gene Copy Number

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Abstract The development of human malignancies can involve the aberrant regulation of intracellular signal transduction pathways that regulate cell-extracellular matrix interactions.

Purpose: In the current study, we aimed to evaluate focal adhesion kinase (FAK) at both genetic and protein expression levels in head and neck squamous cell carcinomas (HNSCC) and to explore the prognostic significance of FAK.

Experimental Design: A total of 211 tissue specimens, including 147 primary tumors, 56 lymph node metastases, 3 benign hyperplasias, and 5 dysplasias, were analyzed using immunohistochemistry. The *fak* gene dosage was determined in 33 tumors. Correlations among DNA, protein, and clinicopathologic variables were analyzed.

Results: FAK protein was overexpressed in HNSCCs compared with corresponding normal mucosa. High expression levels were found in 62% of the samples. Positive immunostaining was also detected in benign hyperplasias and preinvasive dysplastic lesions. All lymph node metastases examined showed FAK overexpression, with significant correlation with the expression in matched primary tumor. DNA copy number ratios for *fak* were higher in 39% of the tumors compared with normal mucosa. However, elevated FAK expression did not correlate with gains on DNA level, and not all cases with an amplification of the *fak* gene displayed protein overexpression. Similar data were obtained in five HNSCC-derived cell lines, in which FAK mRNA levels were precisely correlated with FAK protein levels. FAK protein overexpression in tumors correlated with nodal metastases.

Conclusions: These findings suggest an involvement of FAK in the onset and progression of HNSCC and provide an insight into a mechanism of FAK activation alternative to gene amplification.

Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of tumors worldwide. Despite recent advancements in diagnosis and treatment, the overall survival has undergone little improvement over the past few decades (1, 2). The major cause of the lethal progression of this type of cancer is the spreading of the malignant cells to regional lymph nodes, which represents a major prognostic indicator and serves as a guide for therapeutic strategies. Although many efforts have been devoted to better understand the molecular mechanisms involved in the progression of this type of cancer, accurate and reliable biomarkers that predict patients at highest risk for lymphatic metastases have yet to be defined.

Loss of adhesion of the epithelial cells to the extracellular matrix is one of the fundamental pathways that promote tumor cell migration, invasion, and metastasis. A key factor involved in the control of cell-extracellular matrix interactions is the focal adhesion kinase (FAK), an intracellular tyrosine kinase protein that is localized to cellular focal contact sites (3). FAK is activated and tyrosine phosphorylated upon integrins clustering (4). Evidences also suggest that FAK is a key component of growth factor receptor signaling pathways, such as those activated by platelet-derived growth factor and epidermal growth factor (5). The identification of the majority of biological processes regulated by FAK have been done in nonneoplastic cells, where it has been well documented that the activation of FAK contributes to the regulation of integrins and growth factor signals that promote cell adhesion and spreading, anchorage-independent growth, cell motility, and apoptosis (6, 7). Although less is known regarding FAK signaling in malignant cells, evidence is emerging that aberrant FAK expression, activity, and signaling can potentially contribute to the development of typical features of malignancy (8, 9). Analysis of tumor cell lines reveals that FAK controls cell survival as well as the motile, proliferative, and invasive cell

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Received 7/22/05; revised 1/11/06; accepted 1/31/06.

Grant support: Fondo de Investigación Sanitaria (M.D. Chiara); ISCIII, Red de Centros de Cáncer RTICCC grant C03/10; Obra Social CajAstur, Asturias, Spain (Instituto Universitario de Oncología del Principado de Asturias); and Spanish Ministry of Education and Science (M. Canel).

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doi:10.1158/1078-0432.CCR-05-1583

phenotypes (10–13). A recent report has also shown that FAK inhibition causes block of experimental metastasis formation (14). Additionally, FAK protein overexpression has been shown in tumor biopsy samples from sarcomas, astrocytomas, and carcinomas of the breast, colon, thyroid, prostate, oral cavity, liver, stomach, and ovary as well as in early preinvasive and metastatic lesions (see ref. 9 and references herein). Furthermore, FAK overexpression has been correlated with the invasive potential of a tumor and poor patient prognosis (15–19).

Whereas FAK overexpression has been reported in several tumor types, the mechanism responsible for its overexpression has not been determined. Elevated FAK mRNA levels have been detected in colon, breast, prostate, and hepatocellular carcinomas (20). Increased *fak* gene copy number has been reported in invasive squamous cell carcinomas and some carcinoma cell lines (21). However, the level of gene amplification in cell lines was not precisely correlated with FAK protein expression. To our knowledge, there is no available report on the correlation between copy number change in the *fak* gene and its expression at the protein level in tumor tissue samples.

The purpose of this study was to evaluate FAK protein expression and *fak* gene copy number in patients with HNSCC aimed to determine whether changes in gene dosage is the mechanism responsible for FAK overexpression. We also studied the possible relationship of FAK protein deregulation with clinicopathologic variables and prognosis.

Materials and Methods

Cell lines. The five established human HNSCC cell lines used in this study were kindly provided by Dr. R. Grenman (Department of Otolaryngology, University Central Hospital, Turkey, Finland; ref. 22). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 200 μg/mL streptomycin, 2 mmol/L L-glutamine, 20 mmol/L HEPES (pH 7.3), and 100 μmol/L nonessential amino acids. For immunocytochemical analysis, cells were plated on glass coverslips.

Tissue specimens. Paraffin-embedded surgical tissue specimens from 147 patients with HNSCC were obtained, following institutional review board guidelines, from the pathologic files of the Hospital Universitario Central de Asturias. To have a homogeneous population for survival analysis, 107 of these tumor samples were selected from the same location, the supraglottic larynx. In 56 cases, metastatic lymph nodes were also collected. In 33 cases, a portion of the surgical tumor tissue specimen was also obtained in the operating room: the tumor was sharply excised, placed in sterile tubes, snap-frozen in liquid nitrogen, and stored at −80°C for Western blotting/multiplex ligation-dependent probe amplification (MLPA) analysis. In these cases, clinically normal adjacent mucosa was also collected. The remaining tissue was fixed in buffered formaldehyde, dehydrated in graded alcohol solutions, and embedded in paraffin for use in histologic analysis and immunohistochemical studies. Three benign hyperplasias/hyperkeratosis and five premalignant lesions (one mild dysplasia, two moderate dysplasias, and two severe dysplasias/carcinomas *in situ*) were also obtained from archival, paraffin-embedded blocks. Representative sections from tissue were used for immunohistochemical study, and the diagnosis was confirmed for each lesion by a pathologist (A.H.). Informed consent was obtained from each patient. None of them had received radiotherapy/chemotherapy before intervention.

Clinicopathologic data are shown in Table 1. The stage of disease was determined after the surgical resection of the tumor according to the tumor-node-metastasis staging system of the International Union Against Cancer, sixth edition (23). The histologic grade was determined according to the degree of differentiation of the tumor (Broder's classification). All patients were habitual tobacco and alcohol consumers.

Table 1. Correlations of FAK protein expression and patients and tumor variables

	n	FAK expression levels in the primary tumor		
		+	++/+++	*P*
pT classification				
T_1-T_2	64	29	35	0.126*
T_3-T_4	83	27	56	
pN classification				
N_0	62	31	31	0.016*
N_+	85	25	60	
Disease stage				
I-II	36	18	18	
III	37	13	24	0.236†
IV	74	25	49	
Histopathologic grade				
Well differentiated	72	33	39	
Moderately differentiated	49	17	32	0.103†
Poorly differentiated	26	6	20	
Recurrence‡				
No recurrence	53	23	30	0.662*
Recurrence	34	13	21	

*Fisher's exact test.
†Pearson χ^2.
‡Data of recurrence association with FAK expression levels considered only the 107 cases of supraglottic localization.

Immunohistochemical studies. The formalin-fixed, paraffin-embedded tissues were cut into 4-μm sections and dried on capillary gap glass slides (DakoCytomation, Glostrup, Denmark). The sections were deparaffinized with standard xylene and hydrated through graded alcohol into water. Antigen retrieval was done by heating for 10 minutes in a pressure cooker with citrate buffer. Proteinase K (DakoCytomation) treatment during 10 minutes was used for antigen retrieval. Tissue slides were placed for 20 minutes in a 3% hydrogen peroxide blocking medium and then allowed to react with mouse IgG anti-FAK monoclonal antibody clone 4.47 (Upstate Biotechnology, Inc., Lake Placid, NY) at 1:250 dilution for 1 hour and 30 minutes. Anti-FAK pY397 clone 14 (Becton Dickinson Transduction Laboratories, Erembodegem, Belgium) was used at 1:200 dilution. Immunodetection was done at room temperature on an automatic staining workstation (TechMate 1000, BioTek Solutions, Santa Barbara, CA) using the Envision peroxidase mouse system and diaminobenzidine chromogen as substrate (Envision Plus, DakoCytomation). Counterstaining with hematoxylin for 1 minute was the final step. After staining, the slides were dehydrated through graded alcohol and mounted with a coverslip using a standard medium. Negative controls with an omission of the antiserum from the primary incubation were also included. The slides were analyzed randomly by three of the authors without knowledge of clinicopathologic data. Positive immunostaining was observed in all tumor cells with equal intensity within a tumor specimen. Differences in the intensity of staining were observed between individual tumor samples. Therefore, tumor immunoreactivity was scored by taking into account the intensity of staining in tissue samples: +, slight staining (38% of the tumors) and ++/+++, moderate to strong staining (62% of the tumors).

Confocal immunofluorescence microscopy. Cells were fixed with 3.7% paraformaldehyde in TBS, permeabilized with TBS containing 0.5% Triton X-100 and 1% bovine serum albumin for 15 minutes, and blocked with 5% fetal bovine serum in TBS for an hour. Monoclonal

anti-FAK pY397 (Biosource, Camarillo, CA) and anti-vinculin (Sigma, St. Louis, MO) antibodies were used at 1:100 dilution. Cells were washed and incubated with FITC- or Texas Red–conjugated anti-mouse or anti-rabbit IgG antibody (The Jackson Laboratory, Bar Harbor, ME) diluted 1:100 and examined using a confocal microscope (MRC600, Bio-Rad, Hercules, CA).

Western blotting. HNSCC-derived cell lines at 80% to 90% confluence were harvested by scraping on ice in lysis buffer containing 50 mmol/L HEPES (pH 7.9), 250 mmol/L NaCl, 5 mmol/L EDTA, 0.2% NP40, 10% glycerol, and protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L Na_3VO_4). Tissue samples derived from cancer or normal mucosa were homogenized by three freeze-thawing cycles in identical ice-cold lysis buffer. Lysates were cleared by centrifugation (8,000 × *g*) at 4°C for 5 minutes. The protein concentrations were determined in the supernatant with Bradford protein assay reagent (Bio-Rad). Equal amount of proteins (75 μg per lane) were resolved on 6% SDS-PAGE and transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). Membranes were immunoblotted with mouse IgG anti-FAK antibody or anti-FAK (pY397) at 1:2,500 dilution. For protein load control, mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich, St. Louis, MO) was used at 1:5,000 dilution. Immunodetection was done with the enhanced chemiluminescence plus Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA analyses. Total RNA was isolated from HNSCC cell lines with Nucleospin RNA II (Macherey-Nagel, Easton, PA) following the manufacturer's instructions with the addition of an extra acid phenol/chloroform extraction followed by RNA precipitation. First-strand cDNA was synthesized from 5 μg of total RNA using the Superscript first-strand synthesis system for reverse transcriptase (Invitrogen, Carlsbad, CA) with random primers according to the manufacturer's directions.

Real-time PCR was done in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master mix (Applied Biosystems) and the thermocycler conditions recommended by the manufacturer. Each sample was analyzed for cyclophilin A to normalize for RNA input amounts and to perform relative quantification. Primers were designed using the computer program Primer Express (Applied Biosystems). Primers (forward, 5′-CTTCGGACAGCGTGAGAGAGA-3′ and reverse, 5′-GACGCATTGT-TAAGGCTTCTTGA-3′) were generated to exons 16 and 17 of the *fak* gene and used to amplify a 51-bp fragment. Primers (forward, 5′-CATCTGCACTGCCAAGACTGA-3′ and reverse, 5′-TTGCCAAACACCA-CATGCTT-3′) were generated to exons 4 and 5 of the *cyclophilin A* gene and used to amplify a 54-bp fragment. Melting curve analysis showed a single sharp peak with the expected T_m for all samples. mRNA relative quantities were obtained using the $2^{-\Delta\Delta Ct}$ method (24).

DNA analyses. Genomic DNA was isolated using the QIAmp DNA Mini kit (Qiagen, Inc., Chatsworth, CA) and subsequently treated with RNase A (1 unit/mL) at 37°C for 5 minutes. MLPA was done using a chromosome 8–specific MLPA probe mixture (Medical Research Council-Holland, Amsterdam, the Netherlands). The principles of MLPA analysis of gene copy numbers are described elsewhere (25). This technique has been previously validated for the chromosome 8 probe set by array comparative genomic hybridization (26). The MLPA mixture contained 34 probes, covering 25 genes on chromosome 8, including a probe for the *fak* gene and nine control probes detecting genes on chromosomes 3, 5, 11, 12 and 13, and 17. MLPA analysis was done as previously described (25). Experiments were carried out in duplicate. Analysis of the amounts of the MLPA PCR products per gene was done at the Oviedo University DNA Analysis Facility on an ABI PRISM 3100 Genetic Analyzer platform (Applied Biosystems) according to the manufacturer's instructions.

Analyses were done with Genescan 3.7 (Applied Biosystems). Each gene peak area was divided by the sum of the nine control peak areas for that sample. These relative areas were then compared with the corresponding average relative area obtained from six normal mucosa samples, thus creating a ratio (case/normal). The median ratio obtained from at least two independent experiments for each sample was considered the final result. For a normal mucosa, this ratio was expected to be 1. The cytogenetic interpretation for gains has been previously described (27). For statistical analyses, gene copy number change was scored by taking into account the following criteria: no gain, ratio of ≤1.3 (61% of the cases); gain, ratio > 1.3 (39% of the cases).

To validate the MLPA technique, *fak* DNA copy number was also quantified by real-time PCR in six HNSCC tumor samples, five cell lines, and two normal DNA. PCR conditions were as described above. Primers (forward, 5′-AATGATGTAATCGGTCGAATTGAA-3′ and reverse, 5′-TGGAGGCATTGGTAATCTTTCC-3′) were generated to exon 22 of the *fak* gene and used to amplify a 51-bp fragment. The *sla* gene, also located at 8q24, was used as housekeeping gene, as no changes in copy number ratios had been observed by MLPA. Primers (forward, 5′-CCTGTGCCTTCCTCGTTCC-3′ and reverse, 5′-GTTGCTAAACTGGCCG-CATAC-3′) were generated to exon 2 of the *sla* gene and used to amplify a 51-bp fragment. *fak* gene relative quantity was obtained using the $2^{-\Delta\Delta Ct}$ method (24). The relative quantity in each sample was normalized with the quantity in the normal DNA. Values higher than 1.44 were considered as gains, given that this value would be the expected fold change for an increase in one copy of the gene.

Statistical analyses. All statistical analyses were done using the SPSS statistical software version 8.0 (SPSS, Inc., Chicago, IL). Correlations among pT classification, pN classification, histopathologic grade or recurrence, and FAK protein expression were computed using the χ^2 test. This test was also used to compare *fak* copy number and FAK protein expression. For comparison between the intensity of FAK immunostaining in the primary tumor and the corresponding metastasis, the two-tailed Spearman's nonparametric correlation was employed. Survival curve was calculated using the Kaplan-Meier product limit estimate. Deaths from causes other than the index tumor or its metastases were not considered treatment failures, and these patients were censored in all analysis involving the length of survival. Differences between survival times were analyzed by the log-rank method. $P < 0.05$ was considered statistically significant.

Results

Immunohistochemical analysis of FAK expression in human HNSCC and premalignant lesions. Immunohistochemical analysis of FAK protein expression was done on 147 primary carcinomas, 56 nodal metastases, 3 benign hyperplasias, and 5 dysplasias.

All sections of primary tumors selected for study contained both normal and malignant epithelia. FAK labeling was identified in the morphologic normal mucosa, where staining was weak and confined to the basal cell layer of the epithelium with a cytoplasmic expression pattern (Fig. 1A). Positive staining was also observed in vascular endothelial cells and the acinary cells of the seromucose glands, whereas stroma cells showed negligible FAK expression.

Most tissue sections of the primary carcinomas contained a combination of normal, hyperplastic, dysplastic, and invasive tissue, providing us the possibility to compare levels of FAK expression in the different stages of epithelial transformation within the same genetic background. Interestingly, we observed FAK immunoreactive cells in the transformed but noninvasive epithelia adjacent to the tumor tissues. In contrast to the normal mucosa, the cytoplasmic staining of those epithelia was not restricted to the basal cell layer but extended towards cells in the intermediate layer in hyperplastic epithelia (Fig. 1B) and towards all the cell layers in the dysplastic epithelia (Fig. 1C). Immunohistochemistry of benign isolated hyperplasias and

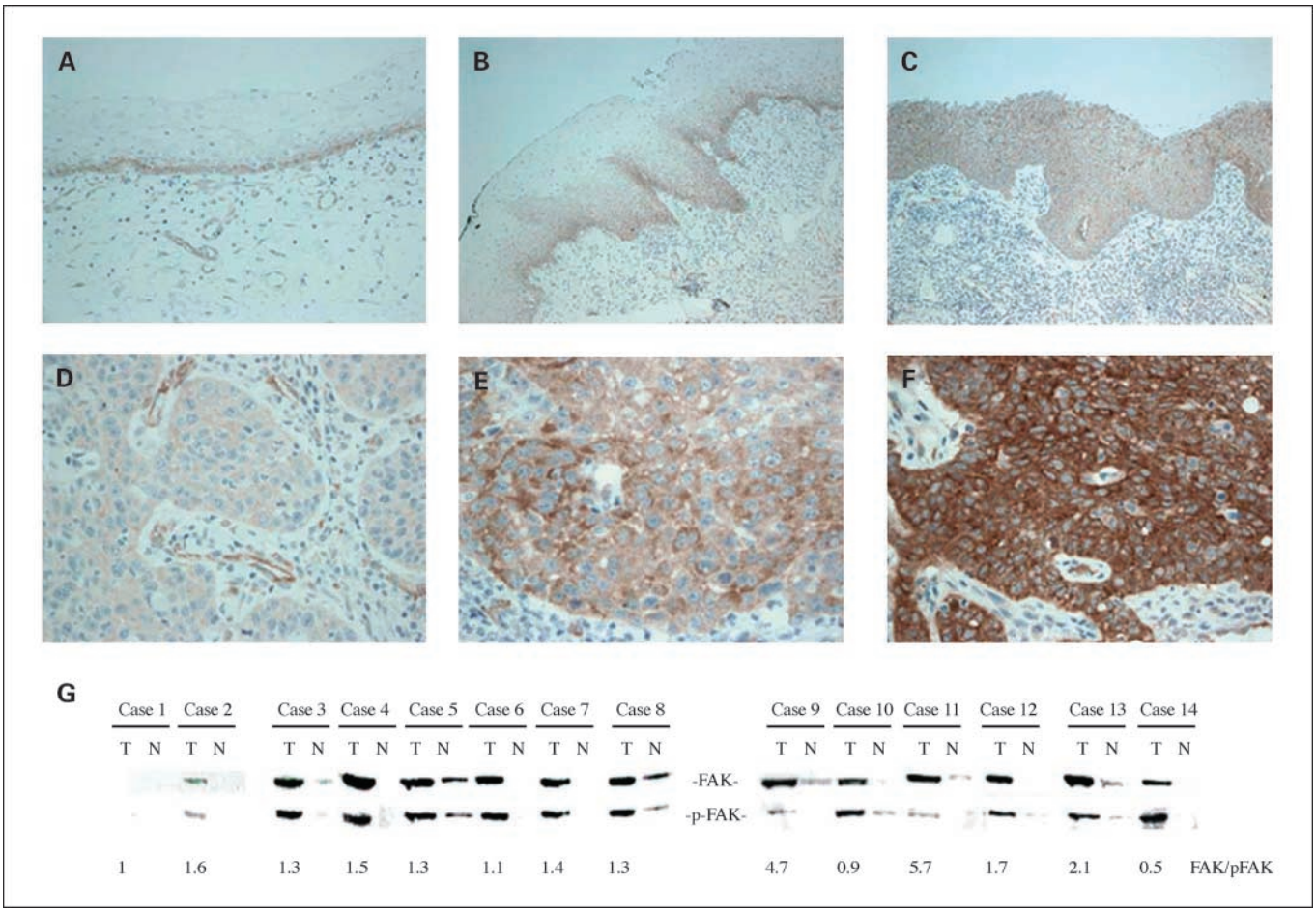

Fig. 1. FAK expression by immunohistochemistry (*A-F*) and Western blot (*G*) in normal, noninvasive transformed epithelia and HNSCC samples. *A,* immnostaining of normal mucosa. *B* and *C,* immunostaining of pathologically modified epithelia adjacent to tumor mass (*B*, hyperplasia; *C*, dysplasia). *D-F,* FAK expression in tumor tissue samples showing a weak (*D*), moderate (*E*), or strong (*F*) immunostaining. Note that the sample with weak FAK staining of the tumor cells shows intense immunostaining of blood vessels. Original magnifications, ×200 (*A-C*), ×400 (*D-F*). *G,* Western blot analysis of FAK and FAK pY397 expression in paired normal and tumor tissue samples from 14 patients with HNSCC (*T*, tumor tissue; *N*, normal tissue). The ratio FAK/FAK pY397 was estimated by densitometry.

isolated dysplastic lesions yielded similar pattern of immunostaining (data not shown).

In marked contrast to the normal epithelium, FAK expression in the tumor tissue displayed a homogeneous distribution throughout the whole tumor area. The intensity of staining varied among individual tumors: 56 (38%) of the tumors showed a weak immunostaining (Fig. 1D), whereas 91 (62%) displayed a moderate to strong FAK expression (Fig. 1E-F).

The expression of FAK in primary tumor tissues and patient-matched normal epithelium was also evaluated by Western blot analyses in 14 patients included in the above histologic studies. As shown in Fig. 1G, different levels of expression of FAK were observed in tumors, whereas little or no expression was detected in the adjacent normal epithelium. The Western blot analysis agreed with the immunohistochemical analysis in clinical samples. Cases 1 and 2 were from the group with weak FAK staining based on immunohistochemical data, whereas the other cases were from the group with moderate to strong FAK staining. Given that autophosphorylation of FAK at Y397 is a crucial event for its activation and function, expression of FAK pY397 was also analyzed in these samples. Immunoblotting with anti-FAK (pY397) antibody yielded similar data to those obtained with anti-FAK antibody in most samples (Fig. 1G).

In 56 patients, paired lymph node metastases were available for immunostaining. As in the primary tumors, the staining was detected in the cytoplasm, and the positive tumor cells were homogeneously distributed. Moderate to strong intensity of staining was observed in 73% of the samples. Interestingly, there was a significant correlation between the intensity of staining in the metastases and in the matched tumor samples (Spearman's rho correlation, $P < 0.001$).

Assessment of* fak *gene copy number ratios in HNSCC tumors. MLPA was done in 33 carcinomas and 6 normal tissues. DNA copy number ratios for *fak* were significantly higher in 39% of the tumors compared with normal mucosa. The MLPA analysis of gene copy numbers has been widely validated by different authors using the comparative genomic hybridization technique (26). Here, the findings of the MLPA analysis were confirmed by real-time PCR analysis in six tumor samples (see Supplementary Data). Comparison of the genetic and protein expression data revealed that there was not statistically significant difference in FAK expression between tumors with and without gain of the *fak* gene (Table 2, Fisher's exact test, $P = 1.000$).

Table 2. Comparison of the FAK protein and *fak* gene levels in HNSCCs

FAK expression levels in the primary tumor	*n*	*fak* gene copy number ratio		
		<1.3	>1.3	*P*
+	9	5	4	1.000
++/+++	24	15	9	

The MLPA data also showed that *fak* gene overrepresentation was frequently associated with gains of two other cancer-related genes located in close proximity to *fak* at 8q24, *ptp4a3* and *khdrbs3* (Table 3). Comparative analysis of these genetic alterations revealed that all carcinomas with *fak* gain displayed *ptp4a3* amplification, and 69% of the tumors with *fak* amplification also had an increase in *khdrbs3* DNA copy number. Regarding *myc*, which resides 13.1 Mb more centromeric than *fak*, our data revealed that 31% of the carcinomas with *fak* amplification also harbored gains of *myc*.

Expression of FAK in SCC-derived cell lines. The expression of FAK at the protein level was also investigated by Western blot in five HNSCC-derived cell lines. FAK was expressed at high levels in SCC38 and SCC40, at moderate levels in SCC42B, and at low levels in SCC2 and SCC29 (Fig. 2A). In agreement with these data, FAK pY397 was detected in SCC40 cells at higher levels than in SCC29 cells (Fig. 2B). Focal contact sites, detected by anti-vinculin staining, were detected in SCC40 cells but not in SCC29 cells (Fig. 2C). Accordingly, FAK pY397 localized to vinculin-containing focal adhesions in SCC40 cells, whereas it was weakly detected in the cytoplasm in SCC29 cells.

The *fak* gene dosage in cell lines was quantified by both MLPA and real-time PCR techniques. As observed in tumors, both techniques yielded similar results. Gains in gene copy number were detected in three of the five cell lines compared with normal mucosa (Fig. 2A). The SCC38 and SCC42B cell lines, in which FAK was expressed at high/moderate levels, also exhibited increases in *fak* DNA. Similarly, the SCC29 cell line expressed low levels of FAK, and the *fak* gene dosage was not altered. However, increased *fak* DNA was detected in SCC2 cells, which expressed low levels of the protein; in addition, gene dosage was not altered in SCC40 cells, although this cell line expressed high levels of the FAK protein.

We also evaluated FAK mRNA expression levels by real-time reverse transcription-PCR analyses (Fig. 2A). The SCC38, SCC40, and SCC42B cell lines with high levels of FAK displayed associated overexpression at the mRNA levels compared with the SCC2 and SCC29 cell lines that expressed lower levels of the protein and also the mRNA. Comparison of the DNA, mRNA, and protein data in HNSCC-derived cell lines revealed that the protein expression levels strongly correlated with the mRNA levels (Pearson χ^2 test, $P < 0.001$) but not with the *fak* DNA dosage (Pearson χ^2 test, $P = 0.553$).

FAK protein expression and clinicopathologic variables. Table 1 presents the association of FAK protein expression with clinicopathologic variables. No relationship was observed between FAK protein expression levels and pT stage, disease stage, or histopathologic differentiation. There were significant differences between those tumors with and without metastases. Higher expression levels of FAK were associated with nodal metastases (Fisher's exact test, $P = 0.016$).

FAK protein expression and disease course. Given that the location of HNSCC notably influences disease course and prognosis, we have selected a homogeneous group of patients with tumors from the same localization (supraglottic larynx) for assessment of the putative role of FAK protein expression on clinical evolution of the disease. This group of patients was well balanced by tumor stage and grade, treated in the same way, and with complete follow-up.

Twenty patients that died from causes not related to the index tumor were excluded of the recurrence analysis. Of the remaining 87 cases, 6 cases developed local recurrence, 24 cases regional recurrence, and 3 cases distant metastasis. FAK protein overexpression was not associated with tumor recurrence (Table 1). On the other hand, although patients with high levels of FAK expression had a poorer disease-specific survival, it did not reach statistical significance (log-rank test, $P = 0.554$; Fig. 3).

Discussion

Our data provide evidence for the overexpression of FAK protein in HNSCC progression. Given the established role of FAK in the regulation of integrin and growth factor signaling, our data suggest that the deregulation of FAK during head and neck tumor cell progression may have an active role in the development, maintenance and, perhaps, invasion process of HNSCC. Our data also show that overexpression of FAK in HNSCC can occur without alteration in the *fak* copy number, suggesting that FAK overexpression due to mechanisms other than structural amplification is probably significant in the pathogenesis of HNSCC.

To our knowledge, there are only two published reports on FAK protein expression in tumors of the upper aerodigestive tract (28, 29). None of these studies examined the relation between the expression of FAK and clinicopathologic findings or survival rates. In addition to these studies on protein expression levels, increased *fak* gene copy number and gene amplification have been previously identified in several tumor

Table 3. Comparison of genetic gains of *fak* and three cancer-related genes located at 8q24

Locus	Gene	Mb to 8p telomer	*fak* gene*		
			n	No gain	Gain
8q24.12	*myc*				
	No gain	128,40	24	15	9
	Gain		5	1	4
	Loss		4	4	0
8q24.2	*khdrbs3*				
	No gain	136,30	21	17	4
	Gain		12	3	9
8q24.3	*ptp4a3*				
	No gain	142,10	4	4	0
	Gain		29	16	13

*Location is 141,50 Mb to 8p telomer.

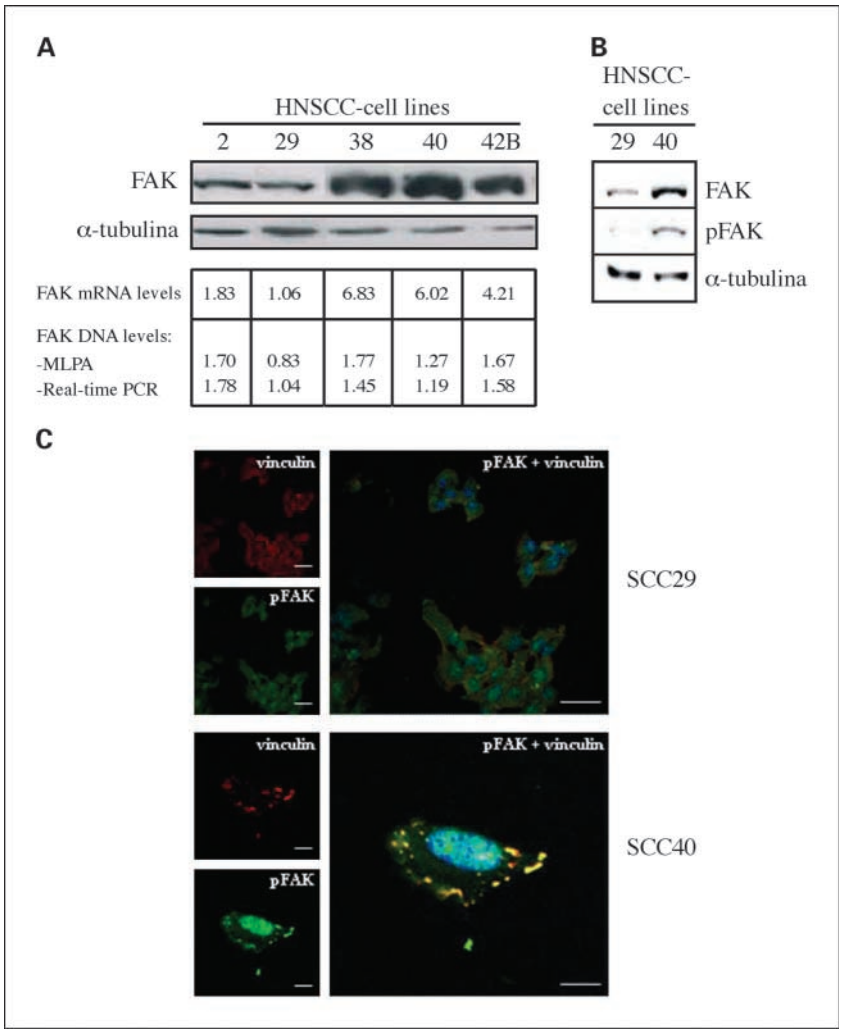

Fig. 2. FAK expression in HNSCC-derived cell lines. *A,* Western blot showing different FAK protein expression levels in HNSCC cell lines. α-Tubulin was analyzed as a control for gel protein loading. FAK mRNA and DNA relative quantities are indicated for each cell line. *B,* Western blot showing different FAK pY397 protein expression levels in SCC29 and SCC40 cells. *C,* immunocytochemistry showing that vinculin-containing focal contact sites are detected in SCC40 cells but not in SCC29 cells. FAK pY397 colocalize with vinculin in SCC40 cells whereas detected in the cytoplasm in SCC29 cells.

cell lines including head and neck (21). However, there is no available report on the correlation between copy number change in the *fak* gene and its expression at the protein level in tumor tissues.

We show here for the first time that FAK overexpression occurs in all stages of head and neck epithelial tumorigenesis. FAK up-regulation in premalignant lesions of HNSCC was detected not only in noninvasive transformed epithelia adjacent to the tumor tissues but also in isolated benign hyperplasias and in dysplastic lesions of the larynx that had not yet acquired the fully malignant and invasive phenotypes. Previous immunohistochemical studies revealed FAK overexpression in the preinvasive forms of breast, colon, and oral cancers (16, 28). These data suggest that up-regulation of FAK could be a functionally significant event in epithelial transformation and have biological significance in the early development of HNSCC and other types of cancer. Indeed, McLean et al. recently showed that loss of FAK expression from the hyperproliferative epidermal region of papillomas is incompatible with malignant progression (30). Our finding of FAK overexpression in the premalignant lesions of head and neck provides additional support for the involvement of this protein in malignant conversion. Regarding primary carcinomas, moderate to high FAK expression levels were detected in 62% of the tissues, in agreement with previous reports on laryngeal and oral squamous carcinomas (28, 29). Interestingly, the immunohistochemical data also showed that the level of FAK overexpression was significantly maintained in the progression of the tumor from its primary site to the lymph node

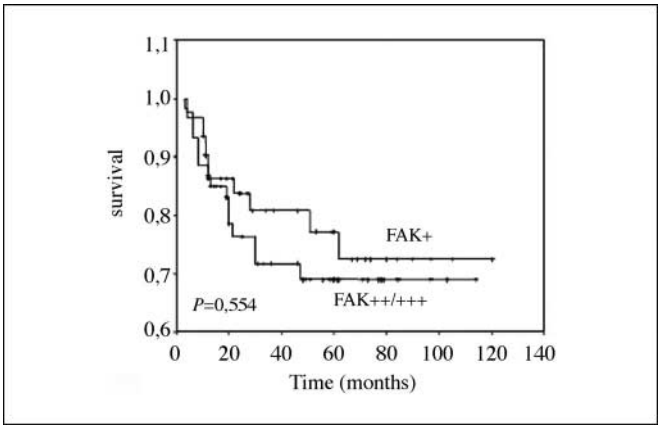

Fig. 3. Kaplan-Meier analysis showing disease-free survival stratifying patients according to FAK immunostaining levels.

metastases, which suggest a role of FAK in the invasion process of HNSCC. FAK overexpression in metastasis has been previously reported on other cancer types (15, 16).

Our analysis revealed that HNSCC frequently harbor *fak* gene gains. This was also detected in HNSCC-derived cell lines. However, in both tumors and HNSCC-derived cell lines, FAK overexpression did not correlate significantly with *fak* DNA copy number ratios. Therefore, although altered gene dosage could play a casual role in FAK overexpression in some HNSCC, a different level of regulation must exist in others. We show here that FAK expression at the mRNA level in the HNSCC cell lines showed strong correlation with the protein expression levels. Actinomycin D time course experiments revealed that the FAK mRNA half-life is similar in cell lines with different FAK mRNA expression levels.[4] Therefore, the differences of FAK mRNA levels in those cell lines do not seem to be the result of a post-transcriptional regulatory event. Based on these observations, we propose that the mechanisms of FAK up-regulation in HNSCC likely involve regulatory pathways at the level of transcription. However, these data on mRNA must await confirmation in tumor samples. Our attempts to quantify FAK mRNA levels by real-time reverse transcription-PCR in tumor samples yielded nonconclusive results as the FAK mRNA levels in normal mucosa were quite heterogeneous, probably due to variable contamination with glands and vessels, which express FAK protein at high levels. An intriguing possibility is that p53 loss or mutation in HNSCC could contribute to increased FAK transcription. Indeed, recent report showed that p53 binding to the FAK promoter was able to suppress expression of FAK (31). The role of p53 on FAK deregulation in tumors warrants further investigation.

Agochiya et al. suggested that *fak* gene gain could potentially contribute to the selection pressure for structural amplification of the 8q24 region, at least in HNSCC cell lines (21). However, our *in vivo* data revealed that the FAK DNA and protein levels were not correlated in tumor samples, which suggests that it is unlikely that FAK provides the driven force for maintaining increased dosage of the *8q24* locus in HNSCC. The most plausible explanation is that *fak* is a bystander gene that is physically located close to a gene/s critical to selective amplification of the 8q24 region. *fak* gene is flanked by two cancer-related genes, *khdrbs3* and *ptp4a3*, that were found frequently coamplified with *fak*. Protein expression studies of these candidate genes must be carried out to determine whether they are crucial and drive the selection of the chromosomal aberration of 8q24. Regarding *myc*, the paradigmatic oncogen of 8q24, our data revealed that only 30% of the tumors with *fak* gene gain harbored *myc* amplification. Therefore, amplification of the genomic region that contains *fak*, *khdrbs3*, and *ptp4a3* genes can occur independently of amplification of *myc*. Together, these data suggest that a gene or genes, other than *fak* residing close to *fak* in the 8q24 amplicon, when overrepresented may play a role in the progression of HNSCC. The putative role of *khdrbs3* and/or *ptp4a3* as "driver" gene/s of 8q24 amplification should be further explored.

Several reports have described correlations between FAK expression and reduced survival rates (17–19, 32). In contrast with those studies, our data showed that there was not a statistically significant association of FAK overexpression in supraglottic carcinomas with tumor recurrence or disease-specific survival. FAK overexpression has also been previously correlated with tumor invasiveness and lymph node metastasis in other types of cancer (15–19). We show here that FAK overexpression in HNSCC is significantly associated with the presence of lymph node metastases. To our knowledge, this is the first report showing a relationship of FAK overexpression with nodal metastasis in HNSCC. Therefore, FAK could be a critical molecular mediator of the regional aggressiveness of HNSCC, which is a major cause of mortality in patients with this type of cancer. FAK regulates key cellular properties that could mediate invasion activity, such as cell migration, invasion, adhesion, and survival. Of interest, FAK is a key convergence point of both integrins and growth factor regulated signals, which are frequently deregulated in HNSCC (33, 34). These data suggest that FAK may serve as an attractive therapeutic target as a node of integration and coordination of different proinvasive and prosurvival signals activated in HNSCC.

In conclusion, FAK overexpression is a frequent and early event in HNSCC that is maintained during tumor progression. Overexpression of FAK in these carcinomas is associated with the presence of lymph node metastasis. These data suggest that the deregulation of FAK during HNSCC progression may have an active role in the development, maintenance and, perhaps, invasion process of this type of tumors. However, FAK expression does not seem to be a good indicator of patient outcome. On the other hand, FAK expression can be independent of the copy number of *fak*. Therefore, FAK overexpression due to pathways other than structural amplification is probably significant in the pathogenesis of HNSCC, and the precise mechanisms of regulation in these tumors must be sought.

[4] P. Secades and M.D. Chiara, unpublished observations.

Acknowledgments

We thank Margaret C. Frame and members of her laboratory for their contributions to the inmunocytochemical imaging studies; Aurora Fernández, Inés Argüelles, Olivia García, and Marta Sanchez for technical assistance; and M. Hermsen for critical reading of the article.

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