

Cortactin and Focal Adhesion Kinase as Predictors of Cancer Risk in Patients with Laryngeal Premalignancy

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Abstract

Novel markers are needed to accurately predict the risk of malignant transformation in laryngeal premalignancies. We therefore investigated the clinical significance of cortactin (CTTN) and focal adhesion kinase (FAK) during laryngeal tumorigenesis and their potential utility as cancer risk markers. CTTN and FAK protein expression and gene amplification were assessed in 82 patients with laryngeal dysplasia and correlated with clinicopathologic parameters and laryngeal cancer risk. Increased CTTN and FAK expression was found respectively in 41 (50%) and 40 (49%) of 82 laryngeal dysplasias; protein expression was maintained or further augmented in the corresponding patient-matched invasive tumors subsequently developed. *CTTN* and *FAK/PTK2* gene amplifications were respectively detected in 10 (12%) and 26 (32%) laryngeal dysplasias. Both CTTN and FAK protein expression increased with the grade of dysplasia; however, CTTN and FAK expression but not histology correlated significantly with increased laryngeal cancer risk ($P = 0.009$ and $P = 0.002$, respectively). Patients carrying strong CTTN- or FAK-expressing dysplastic lesions experienced a significantly higher cancer incidence ($P = 0.006$ and $P = 0.001$, respectively; log-rank test). Furthermore, FAK expression was an independent predictor of laryngeal cancer development (HR = 3.706, 95% CI: 1.735–7.916; $P = 0.001$) and the combination of FAK and CTTN showed superior predictive value (HR = 5.042, 95% CI: 2.255–11.274; $P < 0.001$). Taken together, our findings support the involvement of CTTN and FAK in malignant transformation and provide original evidence for their potential clinical utility as biomarkers for the risk of developing laryngeal cancer. *Cancer Prev Res*; 4(8); 1333–41. ©2011 AACR.

Introduction

Like other epithelial cancers, head and neck carcinogenesis seems to evolve through a multistep process that involves biomolecular changes caused by carcinogen exposure, ensuing premalignant lesions, and consequent invasive carcinoma (1–3). The spectrum of histologic changes occurring in this process has been cumulatively designated squamous intraepithelial lesions (SIL), ranging from squa-

mous hyperplasia to carcinoma *in situ* (CIS; refs. 4, 5). One of the most important issues of SILs is the risk of malignant transformation. In their evolution, some SILs are self-limiting and reversible, some persist, and some progress to squamous carcinoma despite careful follow-up and treatment. Although lesions with dysplastic features are thought to be at a higher cancer risk, some cancers develop from lesions lacking dysplastic changes. Hence, it is often difficult for clinicians to agree on the most appropriate therapeutic option for a particular grade of SIL diagnosed. Additional objective and reliable markers are therefore needed to identify high-risk lesions to improve the prognostic evaluation of SILs beyond current clinical and histopathologic criteria (3).

Recurrent chromosomal aberrations, such as amplifications or deletions, often harbor genes that participate in tumor development and progression. Amplification of the chromosomal regions 8q23-24 and 11q13 are two of the most frequent genetic alterations in head and neck squamous cell carcinomas (HNSCC), events that have been associated with invasive disease and poor patient prognosis (6). Amplification of the 11q13 region has been recognized as a major event driving HNSCC invasion and metastasis, occurring in 20% to 52% of tumors (7). Within the 11q13

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amplicon, *CCND1* and *CTTN* (formerly *EMS1*) have been considered to be the best candidate genes responsible for the 11q13-associated tumor aggressiveness on the basis of their biological roles and also their frequent coamplification and overexpression in various cancers (8, 9). *CTTN* encodes the actin-binding protein cortactin that regulates membrane dynamics, actin network assembly, cell-cell adhesion, invadopodia formation, and matrix degradation, thereby promoting cell motility and invasion (10–12). We and others have consistently shown that *CTTN* gene amplification and protein overexpression correlated with poor prognosis and reduced patient survival in HNSCC (13–15) and other carcinomas (16–18), thus reinforcing the central role of *CTTN* in the 11q13 amplicon and also in tumor progression. Nevertheless, the role of *CTTN*/cortactin in the early stages of tumorigenesis and its possible implication in malignant transformation and acquisition of an invasive phenotype remains to be determined.

8q23-24 amplification has been defined as an important early chromosomal event in HNSCC progression (19). *FAK*/*PTK2* is one of the most prominent genes located within this region, which encodes the focal adhesion kinase (*FAK*). *FAK* overexpression has been well documented in HNSCC and other types of tumors, corresponding to increased invasive and metastatic potential (20–23). In addition, *FAK* overexpression has also been implicated in malignant transformation of breast and cervical epithelia (24).

In this article, we investigated the role of *CTTN* and *FAK* in laryngeal tumorigenesis and established their potential utility as cancer risk markers. *CTTN* and *FAK* protein expression and gene amplification were analyzed in a large series of laryngeal dysplasias and their correlations with clinicopathologic parameters and the risk of progression to laryngeal cancer.

Materials and Methods

Patients and tissue specimens

Surgical tissue specimens from patients who were diagnosed of laryngeal dysplasia at the Hospital Universitario Central de Asturias between 1996 and 2004 were retrospectively collected, following Institutional Review Board guidelines. Patients must meet the following criteria to be included in the study: (i) pathologic diagnosis of laryngeal dysplasia, (ii) with lesions of the vocal folds, (iii) no previous history of head and neck cancer, (iv) complete excisional biopsy of the lesion, and (v) a minimum follow-up of 5 years (or until progression to malignancy occurred). A total of 95 patients who met these criteria were included in this study. Patients were followed up every 2 months in the first 6 months after completing the treatment, every 3 months until the second year, and every 6 months thereafter.

Excisional biopsy of lesions was carried out by using stripping microflap technique with cold instruments. A complete macroscopic exeresis of the lesion was done in all cases, but the microscopic margins were not addressed. Representative tissue sections were obtained from archival,

paraffin-embedded blocks and the histologic diagnosis was confirmed by an experienced pathologist (M.F.F.). The premalignant lesions were classified into the categories of mild, moderate, or severe dysplasia following the WHO classification (5). Tumor blocks were also obtained from those patients who developed an invasive carcinoma. Normal laryngeal epithelium obtained from nononcologic surgery (phonosurgery) was used as control.

Gene amplification analysis

The protocol for DNA extraction from paraffin-embedded tissue sections has been described elsewhere (25). DNA extracted from normal mucosa obtained from nononcologic patients was used as calibrator sample. Gene amplification was evaluated by real-time PCR (Q-PCR) in an ABI PRISM 7500 Sequence Detector (Applied Biosystems) by using Power SYBR Green PCR Master Mix and oligonucleotides with the following sequences: for *CTTN* gene, Fw, 5'-GATCTCATTGACCCTGATGACATC-3' and Rv, 5'-CGTACCGGCCCTTGCA-3'; for the *CCND1* gene, Fw, 5'-GGAAGATCGTCGCCACCTG-3' and Rv, 5'-GAAACGTGGGTCTGGGCAAC-3'; for *FAK* (*PTK2*) gene, Fw, 5'-AATGATGTAATCGGTGCAATTGAA-3' and Rv, 5'-TGGAGGCATTGGTAATCCTTCC-3', and for the reference genes *TH* (Tyrosine Hydroxylase, located at 11p15), Fw, 5'-TGAGATTCGGGCCTTCCG-3' and Rv, 5'-GACACGAAGTAGACTGACTGGTACGT-3' and *PLAT* (Plasminogen activator, tissue, located at 8p11), Fw, 5'-ACTGACTGCCTCCTCGTCTT-3' and Rv, 5'-CGAAACTGAGGCTGGCTGTACT-3'. The relative gene copy number for *CTTN* and *FAK* was calculated by using the $2^{-\Delta\Delta C_T}$ method. The $\Delta\Delta C_T$ represents the difference between ΔC_T of dysplasia and ΔC_T of normal mucosa, with ΔC_T being the average C_T for the target gene (*CTTN*, *CCND1* or *FAK*) minus the average C_T for the reference gene (*TH* or *PLAT*, respectively). Values higher than 2.0 were considered positive for gene amplification.

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were cut into 3- μ m sections and dried on Flex IHC microscope slides (Dako). The sections were deparaffinized with standard xylene and hydrated through graded alcohols into water. Antigen retrieval was done by using EnVision Flex Target Retrieval solution, high pH (Dako). Staining was done at room temperature on an automatic staining workstation (Dako Autostainer Plus) with mouse anti-cortactin monoclonal antibody Clone 30 (BD Biosciences Pharmingen) at 1:200 dilution, mouse anti-Cyclin D1 monoclonal antibody DCS-6 (Santa Cruz Biotechnology, Inc. sc-20044) at 1:100 dilution, or mouse anti-*FAK* monoclonal antibody Clone 4.47 (Upstate Biotechnology) at 1:250 dilution by using the Dako EnVision Flex + Visualization System (Dako Autostainer). Counterstaining with hematoxylin was the final step.

Because *CTTN* and *FAK* staining showed a homogeneous distribution, a semiquantitative scoring system based on staining intensity was applied, as previously reported

(13, 21). Immunostaining was scored blinded to clinical data by 2 independent observers as negative (0), weak (1), moderate (2), and strong protein expression (3), with a high level of interobserver concordance (>95%). Cyclin D1 staining was evaluated as the percentage of cells with nuclear staining in the dysplastic epithelium. Cyclin D1 staining scores were classified as negative or positive staining on the basis of values below or above the median value of 10%.

Statistical analysis

The χ^2 test and Fisher's exact test were used for comparison between categorical variables and Spearman's non-parametric correlation coefficient for comparison between protein expression in premalignant lesions and the invasive tumors subsequently developed. For time-to-event analysis, Kaplan–Meier curves were plotted. Differences between survival times were analyzed by the log-rank method. Cox proportional hazards models were utilized for univariate and multivariate analyses. The HR with 95% CI and *P* values were reported. All tests were 2-sided. The values of *P* ≤ 0.05 were considered statistically significant.

Results

Patient characteristics

All patients were men, with a mean age of 64 years (range 36–83 years). All of them were active or old smokers, and 43 were also habitual alcohol drinkers. The mean tobacco consumption was 55 packs-year (range 15–110 packs-year). After the diagnosis, all the patients that were active smokers received smoking cessation advice; however, 17 of them continued smoking. Of the 95 patients included in the study, 10 patients with a diagnosis of premalignant lesion and cancer within the next 6 months were excluded from the study and 3 additional patients were also excluded because of the lack of tissue block/quality. For the remaining 82 patients, 12 (15%) lesions were classified as mild dysplasia, 26 (32%) as moderate dysplasia, and 44 (54%) as severe dysplasia/carcinoma *in situ* (CIS). During the follow-up period, 28 (34%) of 82 patients developed an invasive carcinoma at the biopsy site and 12 patients (15%) required repeat resection of laryngeal dysplasias (Supplementary Table S1). All available tissue blocks were collected for analysis.

The mean time to cancer diagnosis in the cases that progressed was 28 months (range 11–66 months). No significant differences attributable to age were observed (*P* = 0.95) between the group of patients who developed cancer (mean, 64 years) and those who did not (mean, 63.8 years). The mean tobacco consumption for patients who developed an invasive carcinoma was 56.4 packs-year, compared with 53.7 packs-year for those who did not develop cancer (*P* = 0.56). Similarly, no significant differences in laryngeal cancer risk were observed (*P* = 0.77) between the subgroup of patients who continued smoking (35%, 6 of 17 cases) and those who ceased smoking (33%, 22 of 65 cases).

CTTN and FAK protein expression in the early stages of laryngeal tumorigenesis

Immunohistochemical analysis of CTTN and FAK protein expression was done on a set of 82 laryngeal dysplasias. Sections selected for study also contained normal epithelia as internal controls. Normal epithelia showed weak CTTN staining in the most differentiated layers, whereas positive FAK expression was restricted to the basal cell layer (Fig. 1A and B). The expression of both proteins was negligible in stromal cells. Forty-one (50%) and 40 (49%) of the 82 laryngeal lesions respectively displayed increased CTTN and FAK expression (scored as 2 and 3) in the dysplastic areas, compared with the corresponding normal epithelia

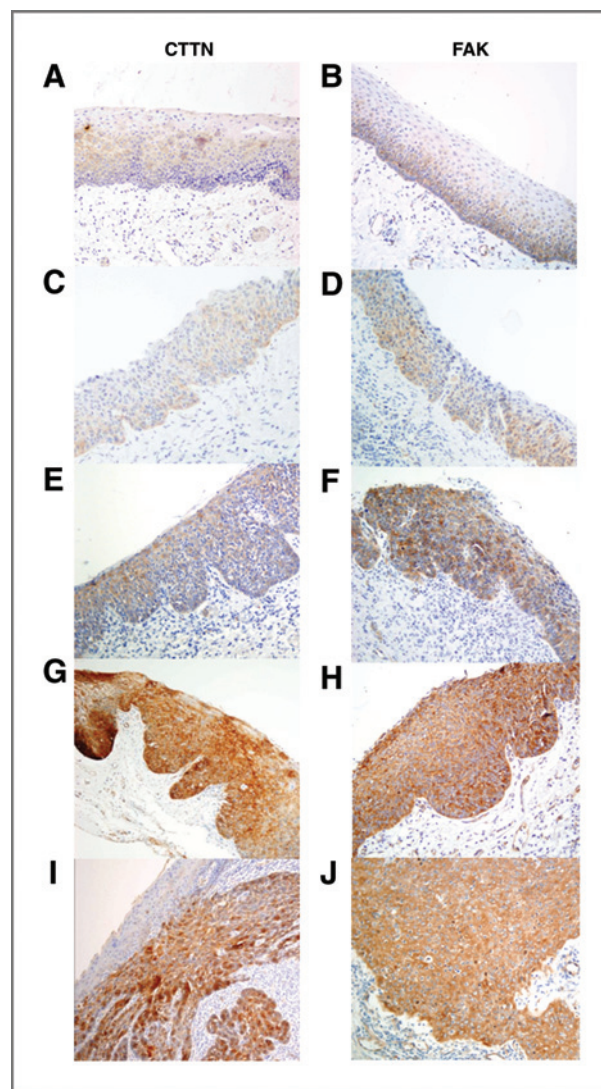


Figure 1. Immunohistochemical analysis of CTTN and FAK expression in laryngeal dysplasias. Normal adjacent epithelia showed negative staining (A and B). Representative examples of laryngeal dysplasias showing weak (C and D), moderate (E and F), and strong positive staining (G and H) and invasive tumors with strong protein expression (I and J). Original magnification $\times 200$.

(Fig. 1C–H). Strong CTTN and FAK expression was respectively detected in 21 (26%) and 23 (28%) laryngeal dysplasias (Fig. 1G and H). CTTN and FAK immunostaining preferentially yielded a cytoplasmic pattern, although some cases also exhibited protein enrichment at the cell periphery.

The expression status was analyzed in relation to the histopathologic classification of the laryngeal lesions. We found that both CTTN and FAK protein expression increased with the grade of dysplasia, although differences did not reach statistical significance. Thus, 3 (25%) of the 12 lesions with mild dysplasia, 13 (50%) of the 26 lesions with moderate dysplasia, and 25 (57%) of the 44 lesions with severe dysplasia/CIS exhibited increased CTTN protein expression ($\chi^2 P = 0.148$), whereas 3 (25%) lesions with mild dysplasia, 12 (46%) with moderate dysplasia, and 25 (57%) with severe dysplasia/CIS showed increased FAK expression ($\chi^2 P = 0.140$).

CTTN and FAK protein expression was also evaluated in 24 of the 28 invasive tumors developed in our cohort. For each patient, protein expression in the invasive tumor was compared with that of the corresponding previous premalignant lesion. Increased CTTN and FAK expression (scored as 2 and 3) was respectively observed in 19 (79%) and 21 (87%) of the 24 invasive tumors, with strong positive staining in 11 (46%) and 19 (79%) cases (Fig. 1I and J).

Statistical analysis revealed a strong positive correlation between CTTN expression in patient-matched premalignant lesions versus invasive tumors (Spearman's correlation coefficient 0.704; $P < 0.001$; Table 1). FAK expression also correlated significantly (Spearman's correlation coefficient 0.742; $P < 0.001$; Table 1). Overall, we observed that CTTN and FAK expression was maintained or further augmented in the tumor compared with the patient-matched preinvasive lesion (Table 1 and Supplementary Table S1). In addition, a high level of concordance was found when comparing CTTN and FAK protein scores in patients who developed various laryngeal lesions (Supple-

mentary Table S1). In those cases with discordant data, the highest score was assigned as final score.

CTTN and FAK gene amplification during laryngeal tumorigenesis

CTTN and FAK amplifications were both detected in early stages of laryngeal tumorigenesis, although with clear differences in both timing and frequency as summarized in Figure 2. FAK gene amplification was found in 26 (32%) of 82 laryngeal dysplasias, and its detection increased with the severity of the lesions: 2 (17%) of 12 mild dysplasia, 6 (23%) of 26 moderate dysplasias, and 18 (41%) of 44 severe dysplasia/CIS harbored FAK amplification ($P = 0.145$). CTTN amplification was detected in 10 (12%) of 82 laryngeal lesions, and although its frequency also increased with the grade of dysplasia, it was only observed in moderate (8%, 2 of 26 cases) and high-grade dysplasias (18%, 8 of 44 cases; $P = 0.163$).

In addition, we found a strong positive relationship between CTTN gene amplification and protein expression ($P = 0.007$; Supplementary Table S2). All laryngeal dysplasias harboring CTTN gene amplification showed strong CTTN expression (score 3). However, 31 amplification-negative lesions also showed increased protein expression (scores 2 and 3), indicating that gene amplification may only partially explain CTTN overexpression observed in laryngeal tumorigenesis. Even though FAK amplification correlated significantly with protein expression ($P = 0.023$; Supplementary Table S3), gene amplification did not lead to FAK protein overexpression in all cases.

Because *cyclin D1* gene (*CCND1*) is located in close proximity to CTTN within the 11q13 amplicon, we also explored its possible contribution to malignant transformation. Thus, *CCND1* amplification was detected in 13 (16%) of 80 laryngeal dysplasias (8%, 1 of 12 mild dysplasias; 4%, 1 of 25 moderate dysplasias; and 26%, 11 of 43 severe dysplasias). Eight (62%) of the 13 *CCND1*-amplified cases also showed coamplification of CTTN gene.

Table 1. Cross-tab to evaluate correlations between CTTN and FAK protein expression in premalignant lesions and patient-matched invasive tumors

CTTN expression in premalignant lesion (%)	CTTN protein expression in tumor (%)			No. of cases
	Weak	Moderate	Strong	
Weak	4 (50)	4 (50)		8
Moderate		3 (60)	2 (40)	5
Strong	1 (9)	1 (9)	9 (82)	11
FAK expression in premalignant lesion (%)	FAK protein expression in tumor (%)			No. of cases
	Weak	Moderate	Strong	
Weak	3 (50)	2 (33)	1 (17)	6
Moderate			5 (100)	5
Strong			13 (100)	13

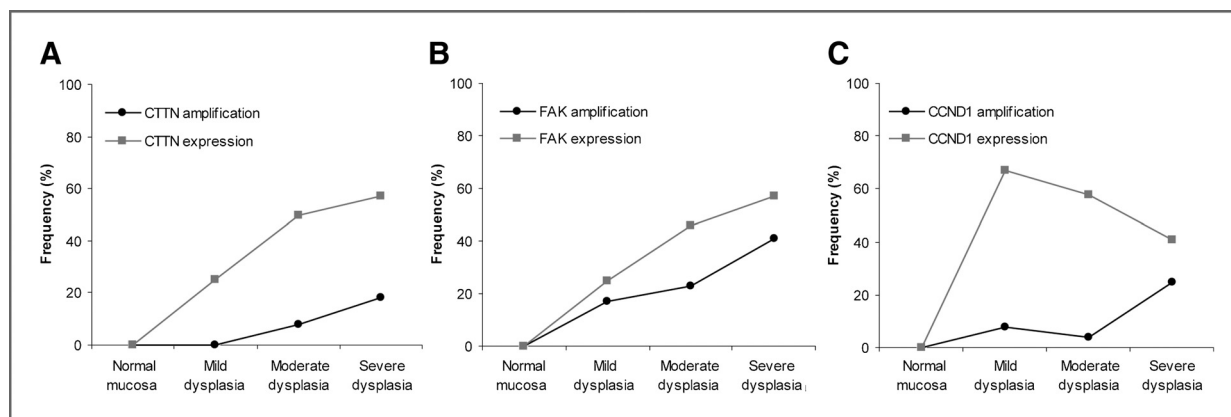


Figure 2. Frequencies of CTTN (A), FAK (B), and CCND1 (C) protein expression and gene amplification in the different stages of laryngeal tumorigenesis.

Furthermore, *CCND1* amplification significantly correlated with cyclin D1 protein expression ($P = 0.003$). However, increased cyclin D1 expression was detected at a high rate along the different stages of laryngeal tumorigenesis (67%, 8 of 12 mild dysplasias; 58%, 15 of 26 moderate dysplasias; and 42%, 18 of 43 severe dysplasias; Fig. 2C and Supplementary Data S4).

Associations with laryngeal cancer risk

There was no statistically significant correlation in this cohort between the histopathologic grade and the risk of progression to laryngeal cancer ($P = 0.378$; Table 2), although severe dysplasias showed a higher cancer risk (HR = 1.763, 95% CI: 0.519–5.989; $P = 0.364$; Table 3).

Table 2. Evolution of the premalignant lesions in relation to histopathologic diagnosis, *CTTN*, *CCND1* and *FAK* gene amplification, and protein expression

Characteristic	No of cases (%)	Progression to carcinoma (%)	P
Histopathologic diagnosis			
Mild dysplasia	12 (15)	3 (25)	0.378 ^a
Moderate dysplasia	26 (32)	7 (27)	
Severe dysplasia	44 (54)	18 (41)	
<i>CTTN</i> gene amplification			
Negative	72 (88)	22 (31)	0.083 ^b
Positive	10 (12)	6 (60)	
<i>FAK</i> gene amplification			
Negative	56 (68)	18 (32)	0.622 ^b
Positive	26 (32)	10 (38)	
<i>CCND1</i> gene amplification			
Negative	67 (88)	19 (28)	0.009 ^b
Positive	13 (12)	9 (69)	
<i>CCND1</i> protein expression			
Negative	40 (49)	12 (30)	0.485 ^b
Positive (>10% stained nuclei)	41 (51)	16 (39)	
<i>CTTN</i> protein expression			
Weak-Moderate (scores 0–2)	61 (74)	16 (26)	0.016 ^b
Strong (score 3)	21 (26)	12 (57)	
<i>FAK</i> protein expression			
Weak-Moderate (scores 0–2)	59 (72)	14 (24)	0.004 ^b
Strong (score 3)	23 (28)	14 (61)	
<i>CTTN</i> and/or <i>FAK</i> expression			
Weak-Moderate (scores 0–2)	52 (63)	9 (17)	<0.001 ^b
Strong (score 3)	30 (37)	19 (63)	

^a χ^2 and ^bFisher's exact tests.

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Table 3. Univariate Cox proportional hazards model to estimate laryngeal cancer risk

Characteristic	P	HR	95% CI
Age (above vs. below the mean)	0.919	1.039	0.494–2.185
Smoking (above vs. below the mean)	0.532	1.270	0.600–2.685
Histology (severe vs. mild to moderate dysplasia)	0.364	1.763	0.519–5.989
<i>CTTN</i> amplification (positive vs. negative)	0.032	2.695	1.089–6.670
<i>FAK</i> amplification (positive vs. negative)	0.537	1.276	0.589–2.766
<i>CCND1</i> amplification (positive vs. negative)	0.002	3.438	1.545–7.652
<i>CTTN</i> expression (score 3 vs. 0–2)	0.009	2.726	1.287–5.774
<i>FAK</i> expression (score 3 vs. 0–2)	0.002	3.248	1.544–6.835
<i>CCND1</i> expression (positive vs. negative)	0.344	1.435	0.679–3.036
<i>CTTN</i> and/or <i>FAK</i> expression (score 3 vs. 0–2)	<0.001	5.040	2.270–11.190

Interestingly, we found that increased *CTTN* and *FAK* protein scores (from 0–3) significantly correlated with an increased laryngeal cancer risk (log-rank $P = 0.048$ and $P = 0.020$, respectively; Fig. 3A and B). Because lesions with strong *CTTN* and *FAK* expression (score 3) showed the highest risk of progression, this was used as a cutoff point in our subsequent analyses ($P = 0.016$ and $P = 0.004$, respectively; Table 2). Consistent with these results, patients carrying strong *CTTN*-expressing lesions and strong *FAK*-expressing lesions experienced a significantly higher laryngeal cancer incidence than those with weak to moderate expression (log-rank $P = 0.006$ and $P = 0.001$, respectively; Fig. 3C and D). The simultaneous analysis of *CTTN* and *FAK* as predictive markers revealed that lesions with strong expression of either one (group 1) or both proteins (group 2) reflected a significantly higher cancer risk than those with weak to moderate expression (group 0; log-rank $P = 0.008$, group 2 vs. 0; $P < 0.001$, group 1 vs. 0; Fig. 3E); however, strong expression of both proteins together did not correspond to a higher cancer risk compared with a single protein (log-rank $P = 0.195$, group 2 vs. 1; Fig. 3E). Strong expression of *CTTN* and/or *FAK* showed the most robust association with laryngeal cancer risk (log-rank $P < 0.001$; Fig. 3F). Five years after the patients were diagnosed, 19 (63%) of the 30 patients with strong expression of *CTTN* and/or *FAK* developed laryngeal cancer, whereas only 9 (17%) of the 52 patients with weak to moderate expression of these 2 proteins progressed to invasive carcinoma ($P < 0.001$; Table 2). In addition, we also found that *CCND1* gene amplification, but not protein expression, significantly associated with a higher risk of progression ($P = 0.009$; Table 2).

Univariate Cox analysis showed that *CTTN* and *CCND1* gene amplification and *CTTN* and *FAK* expression, but not histologic grading, were significantly associated with laryngeal cancer risk (Table 3). In multivariate stepwise analysis including histology, *CTTN* and *FAK* expression, and *CTTN* and *CCND1* gene amplification, only *FAK* expression (HR = 3.517, 95% CI: 1.656–7.472; $P = 0.001$) and *CCND1* gene amplification

(HR = 3.909, 95% CI: 1.733–8.817; $P = 0.001$) were significant independent predictors of laryngeal cancer development. When *CTTN* and/or *FAK* expression was included in the analysis, this combined factor (HR = 4.472, 95% CI: 1.995–10.023; $P < 0.001$) and *CCND1* gene amplification (HR = 2.763, 95% CI: 1.223–6.240; $P = 0.014$) were the only significant independent predictors of cancer development.

Discussion

This study is the first to investigate *CTTN* and *FAK* protein expression and gene status in laryngeal tumorigenesis to ascertain their role in malignant transformation. Our findings show that *CTTN* and *FAK* are both frequently abnormally expressed in the early stages of laryngeal tumorigenesis and that patients carrying strong *CTTN*- or *FAK*-expressing dysplastic lesions exhibit a significantly higher cancer incidence than those with weak to moderate expression. In the light of our data, *CTTN* and, more strongly, *FAK* seem to be both biologically relevant features that contribute to laryngeal cancer development and although the expression of both proteins does not seem to confer an additional advantage to tumor formation, the combination of *CTTN* and *FAK* evaluation was statistically significantly superior in terms of predictive value and also sensitivity, therefore recommending their use as complementary markers.

Given that *CCND1* gene is located close to *CTTN* and both genes are commonly coamplified in HNSCC (13, 14), we also compared their contribution to malignant transformation by using the same series of laryngeal dysplasias. We found that *CCND1* amplification strongly correlated with increased laryngeal cancer risk; however, it is still difficult to dissect the impact of each gene when 8 (62%) of 13 positive cases showed *CTTN* coamplification. Nevertheless, *CTTN* protein expression but not *CCND1* expression increased with the histologic grade and correlated significantly with the risk of progression, thus supporting a role for *CTTN* in malignant

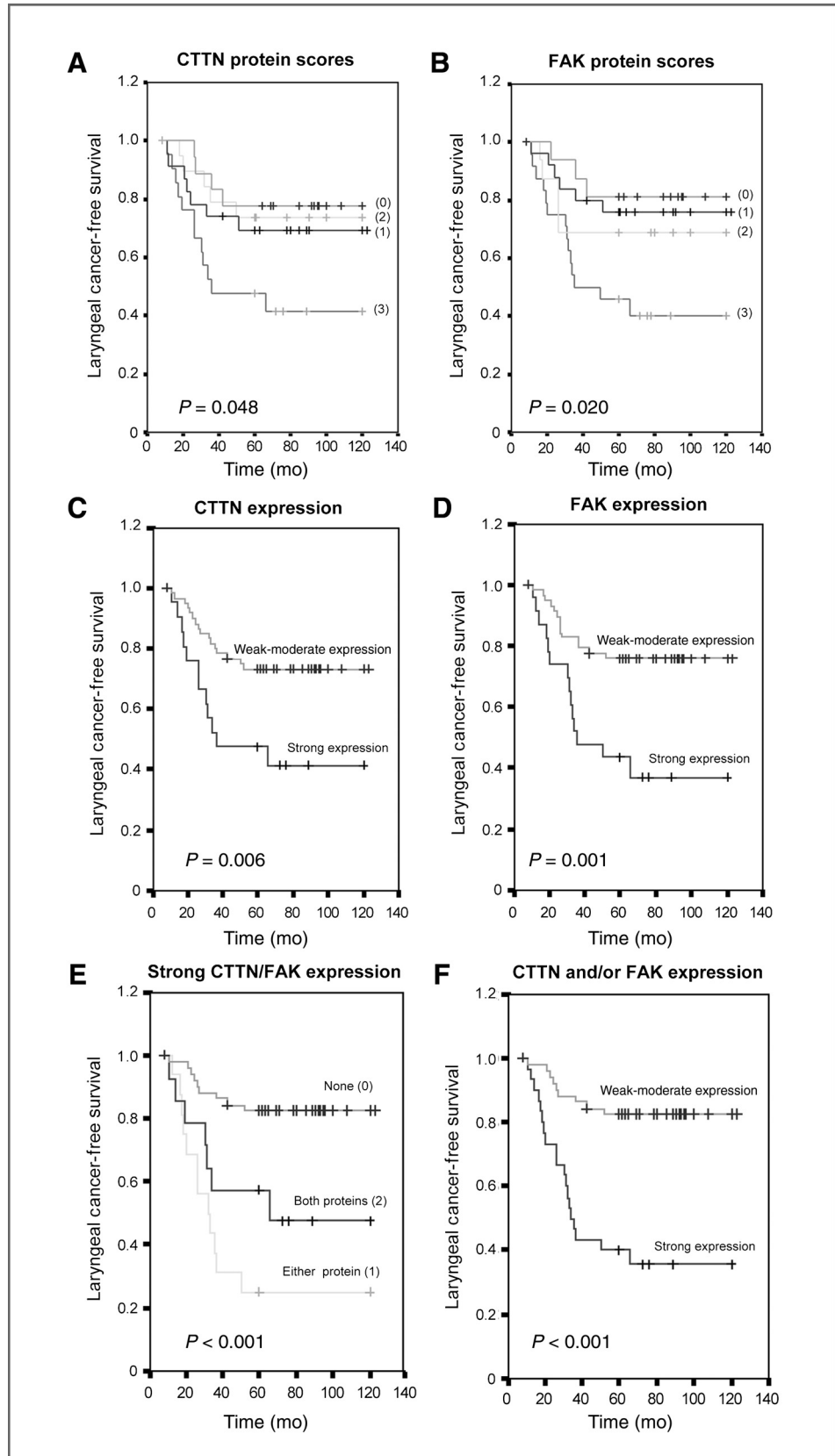


Figure 3. Kaplan-Meier cancer-free survival curves in patients with laryngeal dysplasias categorized by CTTN (A) and FAK (B) protein scores; CTTN (C) and FAK (D) protein expression dichotomized as score 3 (strong) versus scores 0 to 2 (weak-moderate); (E) CTTN and FAK expression grouped as strong expression of either one protein (1), both proteins (2), or none (0); and (F) CTTN and/or FAK protein expression dichotomized as strong versus weak to moderate. *P* values were estimated by using the log-rank test.

transformation. In addition, the early changes observed in CCND1 expression could also reflect functional differences between the 2 genes during laryngeal tumorigenesis, according to our previous findings during HNSCC progression (14). Thus, it has been reported that CCND1 overexpression during tumorigenesis precedes and probably enables gene amplification by dysregulation of cell proliferation that results in genomic instability (26).

Despite the fact that patients with severe dysplasia were at a higher risk of malignant progression (HR = 1.763), histology did not show a significant role in assessing laryngeal cancer risk in this cohort. In contrast, strong expression of CTTN and/or FAK showed robust associations with an increased laryngeal cancer risk and, more importantly, expression of FAK alone or CTTN and/or FAK were independent predictors in multivariate analysis. These data underscore the limited value of histopathologic classification in predicting outcome and suggest that CTTN and FAK protein evaluation may provide additional information beyond histologic features. Because histopathologic diagnosis of SILs is the gold standard in clinical practice for cancer risk assessment and decision making and immunohistochemical analysis of CTTN and FAK is relatively simple and easy to interpret, it seems reasonable to recommend this molecular test to be included. Nevertheless, routine implementation of CTTN and FAK expression as biomarkers for cancer risk assessment will require further confirmation in large prospective studies. It would also be of interest to delineate whether these findings could extend to other sites in the head and neck area, such as oral cavity, a location with a high rate of premalignancies (oral leukoplakias) diagnosed.

Nonetheless, CTTN or FAK expression may not be sufficient to promote tumorigenesis, because 9 (17%) cancers developed from lesions with weak-moderate expression of both proteins. Alternatively, it is also plausible that these lesions were biopsied before the abnormality occurred, or that cancers were originated from lesions not clinically visible at the time of biopsy and therefore unexamined. Consistent with this notion, we found 8 patients showing strong expression of CTTN and/or FAK in the invasive tumor with weak-moderate protein expression in the corresponding premalignant lesion previously biopsied. In addition, our observation that CTTN and FAK expression is maintained or even augmented in invasive tumors further supports the role of CTTN and FAK in both the development and progression of laryngeal cancer. Various studies have shown the fundamental role of CTTN and FAK in processes of invasion and metastasis in HNSCC and other cancers (10–12, 16, 20–23, 27). Overexpression of CTTN and FAK has been associated with tumor aggressiveness and poor prognosis (9, 13–18, 22, 27). CTTN overexpression has also been linked to resistance to the epidermal growth factor receptor kinase inhibitor gefitinib (28). Therefore, in addition to the role of CTTN and FAK as prognostic and/or cancer risk markers, these data also reflect that they both may

represent promising therapeutic targets in the prevention and treatment of HNSCC. Interestingly, small molecules targeting Src kinase activity have proved to be effective inhibitors of HNSCC invasion and metastasis in preclinical settings by impairing phosphorylation of Src substrates, such as CTTN and FAK, important for invadopodia formation and ECM degradation (29–32). These compounds are currently being evaluated in clinical trials (33).

Our study also revealed important temporal and mechanistic information when comparing the timing and frequency of molecular changes along the different stages of laryngeal tumorigenesis. The overexpression of both CTTN and FAK was detected at early stages and their frequency increased with the grade of dysplasia. Furthermore, we found that CTTN overexpression precedes and occurs at a higher frequency than gene amplification during laryngeal tumorigenesis, indicating that additional mechanisms must be contributing to CTTN expression. FAK overexpression also occurred more frequently; however, in this case, FAK amplification did not perfectly match with protein expression and only showed a partial contribution. These results are in agreement with our previous observations in HNSCC progression (14, 21).

Taken together, our results indicate that CTTN and FAK are both biologically and clinically important in laryngeal tumorigenesis and in malignant transformation and provide original evidence of their potential utility as biomarkers for cancer risk assessment. Given that the treatment of laryngeal tumors presents formidable functional consequences, new molecular markers of risk will undoubtedly improve local control, overall survival, reduction of morbidity, and preservation of organ function.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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