TITLE:

IMMUNOHISTOCHEMICAL EXPRESSION OF CORTACTIN AND FOCAL ADHESION KINASE PREDICTS RECURRENCE RISK AND LARYNGEAL CANCER RISK BEYOND HISTOLOGICAL GRADING

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

RUNNING TITLE: CTTN and FAK: cancer risk biomarkers

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Key Words: laryngeal cancer risk, CTTN, FAK, histological grading, immunohistochemistry.

ABSTRACT

Background: Cortactin (CTTN) and the Focal Adhesion Kinase (FAK) are two major candidate genes to respectively drive 11q13- and 8q24-associated aggressive behavior in various cancers. Recent evidence uncovered their clinical relevance in early stages of tumorigenesis as promising biomarkers for cancer risk assessment.

Methods: Using a multicenter validation study CTTN and FAK expression was evaluated by immunohistochemistry in a cohort of 109 patients with laryngeal precancerous lesions, and correlated with clinicopathologic parameters and laryngeal cancer risk. The pathophysiological role of CTTN and FAK was further investigated using functional studies in cellular models.

Results: Positive CTTN and FAK expression (scores 2 and 3) was detected in 49 (41%) and 35 (32%) laryngeal dysplasias, respectively. Univariate Cox analysis showed that CTTN and FAK expression but not histological grading were significantly associated with both recurrence risk and laryngeal cancer risk. Patients carrying strong CTTN- or FAK-expressing lesions (score 3) experienced the highest laryngeal cancer incidence (log-rank $P<0.001$). In multivariate stepwise analysis, FAK expression (HR=13.91, 95% CI 4.82-40.15; $P<0.001$) and alcohol consumption (HR=2.22, 95% CI 1.17-4.20; $P=0.014$) were significant independent predictors of laryngeal cancer development. Targeting FAK by either RNAi or pharmacological inhibitors effectively blocked cell growth, colony formation and invasion into 3D collagen matrices.

Conclusions: CTTN and FAK emerge as powerful predictors of laryngeal cancer risk and recurrence risk beyond histological grading.

Impact: Our work supports the applicability of immunohistochemical CTTN and FAK as complementary markers for risk-stratification in patients with laryngeal precancerous lesions.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1]. Despite the latest advances using combined modality therapies (surgery, radio/chemotherapy), the survival rates have not improved significantly over the last years [2]. HNSCC is a very complex and heterogeneous disease involving multiple genetic alterations and highly variable biological behavior and disease outcome [3,4]. This highlights the urgent need of novel methods to enable more accurate and effective cancer detection, prognostication and also the development of personalized therapeutic strategies.

Amplification of the chromosomal regions 8q23-24 and 11q13 are two of the most recurrent genetic alterations in HNSCC, which have been associated with recurrent and metastatic disease and poor disease outcome [5]. The genes *FAK/PTK2* and *CTTN* (formerly *EMS1*) that encode the focal adhesion kinase and the actin-binding protein Cortactin have emerged as major candidate genes to respectively drive 8q24- and 11q13-associated aggressive behavior and tumor spreading [5].

We and others have consistently demonstrated the association of both *CTTN* gene amplification and protein overexpression in tumor progression, found to correlate significantly with poor prognosis and reduced survival in HNSCC patients [6-8] and other carcinomas [9-11]. FAK overexpression has also been linked to increased invasive and metastatic potential in various cancers [12-15].

This prompted us to investigate the role of CTTN and FAK in early stages of HNSCC tumorigenesis and their contribution to tumor initiation and

acquisition of an invasive phenotype. Our findings uncovered the clinical and biological relevance of CTTN and FAK in HNSCC tumorigenesis and, more importantly, their potential utility as biomarkers for cancer risk assessment [16,17].

Using a multicenter validation study, we herein demonstrate that immunohistochemical determination of CTTN and FAK robustly predicts recurrence risk and laryngeal cancer risk beyond histological grading, therefore recommending their clinical application as complementary markers for risk-stratification. Functional analysis in HNSCC-derived cell lines further contributed to delineate the pathobiological role of CTTN and FAK. Our results provide strong evidence supporting the involvement of FAK in laryngeal tumorigenesis and malignant transformation. Effective inhibition of cell growth, colony formation and 3D invasion unveils that pharmacological targeting of FAK could constitute a promising therapeutic strategy for HNSCC prevention and treatment.

MATERIALS AND METHODS

Patients and tissue specimens

Surgical tissue specimens from patients who were diagnosed of laryngeal dysplasia between 2004 and 2010 were retrospectively collected, in accordance to approved institutional review board guidelines. Patients must meet the following criteria to be included in the study: *i*) pathological 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; *v)* a minimum follow-up of five years (or until progression to malignancy occurred); and *vi)* patients with a diagnosis of laryngeal dysplasia who developed cancer

within the next six months were excluded from the study. One-hundred and nine patients who met these criteria were included in this study. This series included patients treated at the Hospital Universitario Central de Asturias (n = 23) and two other collaborating institutions in Barcelona, the Hospital de la Santa Creu i Sant Pau (n = 46) and the Hospital Clínic (n = 40). There was no overlap with the patients enrolled in our previous study [16]. All experimental procedures were conducted in accordance to the Declaration of Helsinki and approved by Institutional Ethics Committees of the Hospital Universitario Central de Asturias, Hospital Sant Pau and Hospital Clínic, and by the Regional CEIC from Principado de Asturias (date of approval 18th of July 2013; approval number: 81/2013) for the project PI13/00259. Informed consent was obtained from all patients. All the patients were treated with macroscopically complete excisional biopsy of the lesion, either with CO_2 laser or with cold instruments. Microscopically surgical margins were not assessed. No other treatments were administered. Patients were followed up every two months in the first six months after completing the treatment, every three months until the second year, and every six months thereafter.

Representative tissue sections from the original biopsy material were obtained from archival, paraffin embedded blocks and the histological diagnosis and epithelial dysplasia grade was confirmed in all the cases by an experienced pathologist (A.A.). The sections selected for study also contained normal epithelia as internal controls. The premalignant lesions were classified into the categories of low-grade and high-grade dysplasia following the WHO classification (4^{th} Edition) [18].

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were cut into 3-µm 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 performed 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, San Diego, CA) at 1:200 dilution or mouse anti-FAK monoclonal antibody Clone 4.47 (Upstate Biotechnology, Lake Placid, NY) at 1:250 dilution using the Dako EnVision Flex + Visualization System (Dako Autostainer). Counterstaining with hematoxylin was the final step.

Since CTTN and FAK staining showed a homogeneous distribution, a semiquantitative scoring system based on staining intensity was applied, as previously reported [16]. Immunostaining of the dysplastic areas was scored blinded to clinical data by two independent observers as negative (absence of expression, 0), weak (staining similar to that observed in normal epithelium, 1), moderate (intermediate staining, 2) and strong protein expression (intense and homogeneous staining, 3), with a high level of inter-observer concordance (> 95%).

HNSCC-derived Cell Lines and Cell Culture Conditions

FaDu cells originated from an undifferentiated hypopharyngeal carcinoma were purchased to the American Type Culture Collection (ATCC HTB-43). The HNSCC cell line SCC42B derived from a primary laryngeal carcinoma (T4N3M0) was initiated and kindly provided by Dr. R. Grenman in 2003 (Department of Otolaryngology, University Central Hospital, Turku, Finland)

[19]. Cell line authentication was performed by DNA (STR) profiling at the SCT Core Facilities (University of Oviedo, Asturias, Spain) in 2016. All the cell lines used in this study have been passaged and kept fewer than 4 months after re-authentication or thawing. All cell lines were tested periodically for mycoplasma contamination by PCR to specifically amplify a conserved region of the mycoplasma 16S ribosomal RNA gene (Biotools Detection kit).

Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 200 mg/mL streptomycin, 2mmol/L L-glutamine, 20mmol/L HEPES (pH 7.3) and 100 mmol/L non-essential amino acids.

Transfection with siRNAs

siRNA duplexes were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). Human ON-TARGETplus SMARTpools were used to knockdown specifically CTTN and FAK expression, and siGENOME RISC-Free Control was used as a negative control siRNA.

HNSCC cells were plated into 6-well plates in antibiotic-free medium at a density of 60,000 cells/well. The next day, cells were transfected with pooled siRNAs duplexes using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

Western blotting

Cells were lysed in Laemmli sample buffer and sonicated for 15 s before centrifugation. Samples were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred subsequently to nitrocellulose membranes (Amersham Protran, GE Healthcare).

The membranes were blocked for 1h with Odyssey blocking buffer and incubated overnight with the following specific primary antibodies at 1:1,000

dilution: Cortactin (Clone 30) from BD Transduction, FAK (Clone 4.47) from Upstate Biotechnology, Phospho-FAK pTyr397 (44-624G) from Invitrogen, or GAPDH (MAB374; at 1:10,000 dilution) from Millipore. The Alexa Fluor secondary antibodies IRDye 800CW Goat anti-Rabbit IgG (H+L) and IRDye 680 RD Goat anti-Mouse IgG (H+L) were used for detection. Membranes were scanned with the Odyssey Fc Dual-Mode Imaging System (LI-COR Biosciences) using the red (700 nm) and green (800 nm) channels.

Soft agar colony formation assay

Colony formation assay was carried out using the CytoSelectTM 96-Well Cell Transformation Assay Kit (Cell Biolabs Inc, San Francisco, CA). Briefly, 50 µL of base agar matrix was added in the bottom of each well of a 96-well plate. When the agar was solid, 75 µL of cell suspension/soft agar matrix containing 2000 cells was layered on top followed by 50 µL of 2× complete medium after matrix solidification. After 7 days, the agar matrix was solubilized and the relative growth of colonies was determined in quadruplicates using a tetrazolium-based MTS test (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, Madison, WI, USA) reading the absorbance at 490 nm in a Synergy HT plate reader (BioTek, Winooski, VT)**.**

Cell growth analysis

HNSCC cells were plated into 96-well plate at a density of 2,000 cells/well, treated with the vehicle (DMSO) or different doses of PF-573228, PF-562271, VS-6063 (also known as defactinib or PF-04554878) from Selleck Chem. Cell proliferation was measured at 72 hours. For siRNA assays, cells were seeded 72h post-transfection and cell viability measured after 96h.

Quantification of cell number was determined in quadruplicates using a tetrazolium-based MTS test (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega) and reading absorbance at 490 nm with the use of a Synergy HT plate reader (BioTek, Winooski, VT). The proliferation rate for each experimental condition was quantified by dividing the absorbance data at the endpoint between the absorbance data at the initial point and these data were normalized to either vehicle-treated or siControl-transfected cells.

Three-dimensional spheroid invasion assays

Cells were suspended in DMEM medium plus 5% Methyl cellulose (Sigma) at 80,000 cells/mL. Cell spheroids were subsequently formed by serial pipetting of 25 µL into a non-adhesive Petri dish (2,000 cells/spheroid) and incubated in an inverted position for 18h. Next day, each cell spheroid was transferred to an individual well of 96-well plate and embedded into a volume of 110 µL of 2.3 mg/mL bovine collagen type I matrix (PureCol) from Advanced Biomatrix (San Diego, CA), and filled with 100 µL of complete media. Collective cell invasion was monitored using a Zeiss Cell Observer Live Imaging microscope (Zeiss, Thornwood, NY) coupled with a CO_2 and temperature-maintenance system. Time-lapse images were acquired every 15 minutes during 24 hours using a Zeiss AxioCam MRc camera (Zeiss, Thornwood, NY). The area of each individual spheroid was measured using Image J analysis program. The invasive area was determined by calculating the difference between the final area (at each represented time) and the initial area (t = 0 h), and data were normalized to the control (untreated) cells.

Statistical analysis

χ^2 and Fisher's exact tests were used for comparison between categorical variables. 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 analysis. The hazard ratios (HR) with 95% confidence interval (CI) and *P* values were reported. The predictive potential of the studied variables was evaluated by performing receiver operating characteristic curves (ROC) analysis, and the discriminative efficacy of the individual variable was calculated by the area under the ROC curve (AUC). For quantitative variables, means were compared using Student's t-test. All tests were two-sided. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Patient characteristics

109 patients who met the above-described inclusion criteria were enrolled in the multicenter validation study. All but nine patients were men, with a mean age of 62 years (range 30-87 years). All but eleven patients were smokers, and 54 (50%) were also habitual alcohol drinkers. The mean tobacco consumption was 46 pack-year (range 3-150): 55 (51%) moderate (1–50 pack-year) and 42 (39%) heavy (> 50 pack-year) smokers. After the diagnosis, all the patients who were active smokers received smoking cessation advice; however, 30 (28%) of them continued smoking. The histological diagnosis and epithelial dysplasia grade was verified in all the cases by the same pathologist at our institution (A.A.), and after review the premalignant lesions were classified as low-grade dysplasia (27 cases, 25%), and high-grade dysplasia (82 cases, 75%).

During the follow-up period, 28 (26%) cases suffered a premalignancy recurrence, and 16 (15%) of 109 patients developed an invasive carcinoma at the biopsy site (i.e. same vocal cord). None of these patients developed laryngeal carcinomas away from the biopsy site. The mean time to cancer diagnosis in the cases that progressed was 31 months (range 10 to 60 months). No significant differences attributable to age were observed (P = 1.0) between the group of patients who developed cancer (mean, 63 years) and those who did not (mean, 62 years). The mean tobacco consumption for patients who developed an invasive carcinoma was 66 packs-year, compared to 43 packs-year for those who did not develop cancer (P = 0.09). No significant differences in laryngeal cancer risk were observed (P = 1.0) between the subgroup of patients who continued smoking (13%, 4 of 30 cases) and those who ceased smoking (16%, 12 of 77 cases).

Multicenter validation of CTTN and FAK as cancer risk markers

Immunohistochemical analysis of CTTN and FAK protein expression was carried out in a cohort of 109 patients with laryngeal dysplasia diagnosed at three different institutions in Spain: the Hospital Universitario Central de Asturias, the Hospital de la Santa Creu i Sant Pau in Barcelona and the Hospital Clínic in Barcelona.

According to our previous data [16], normal epithelia showed weak CTTN staining in the most differentiated layers and weak positive FAK expression restricted to the basal cell layer, whereas the expression of both proteins was negligible in stromal cells. Forty-nine (41%) and 35 (32%) of the 109 laryngeal lesions respectively displayed increased CTTN and FAK expression (scored as 2 and 3) in the dysplastic areas, compared with the corresponding normal

epithelia. Strong CTTN and FAK expression (score 3) was respectively detected in 14 (33%) and 10 (27%) laryngeal dysplasias (Supplementary Figure S1). 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 histopathological classification of the laryngeal lesions. We found that CTTN and FAK protein expression did not significantly correlate with the grade of dysplasia (Supplementary Table S1).

There was no statistically significant correlation between the histopathological grade and the risk of progression to laryngeal cancer in this cohort (P = 1.000; Table 1).

In marked contrast, increasing CTTN and FAK protein scores (from 0 to 3) significantly correlated with an increased laryngeal cancer risk (log-rank P < 0.001; Figure 1A-B). Consistent to our previous observations [16], strong CTTN and FAK expression (score 3) showed the highest risk of progression, and was therefore used as a cut-off point in our subsequent analyses (P = 0.016 and P < 0.001, respectively). Thus, 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 < 0.001; Figure 1C-D). Quite remarkably, five years after the patients were diagnosed, 7 (70%) of the 10 patients with strong FAK expression developed laryngeal cancer whereas only 9 (9%) of the 99 patients with weak to moderate expression of these two proteins progressed to invasive carcinoma (P < 0.001; Table 1).

Simultaneous analysis of CTTN and FAK as predictive markers showed 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 of both proteins (group 0) (log-rank $P = 0.003$, group 2 *versus* 0; $P < 0.001$, group 1 *versus* 0; Figure 1E); however, strong expression of both proteins together did not correspond to a higher cancer risk compared to a single protein (log-rank $P = 0.045$, group 2 *versus* 1; Figure 1E). Consequently, strong expression of CTTN and/or FAK showed a robust association with laryngeal cancer risk (log-rank $P < 0.001$; Figure 1F).

Univariate Cox analysis showed that CTTN expression and FAK expression but not histological grading were significantly associated with laryngeal cancer risk (Table 2). In addition, alcohol consumption significantly correlated with laryngeal cancer risk, and tobacco showed a nearly significant correlation (Table 2).

In multivariate stepwise analysis including tobacco (above the mean of packs-year vs. below the mean) and alcohol (yes vs. no) consumption, histology (low-grade *vs*. high-grade dysplasia), CTTN and FAK expression, only FAK expression (HR = 13.91, 95% CI 4.82 to 40.15; $P < 0.001$) and alcohol consumption (HR = 2.22, 95% CI 1.17 to 4.20; $P = 0.014$) were significant independent predictors of laryngeal cancer development (Table 3).

The clinical relevance of age, tobacco and alcohol consumption, histological classification, CTTN expression, FAK expression, and the combined CTTN/FAK expression to predict laryngeal cancer risk and recurrence risk was estimated by ROC curves. The results of the area under the curve (AUC) clearly indicate that the combination of CTTN and FAK expression has the highest

predictive value for both laryngeal cancer development and recurrence (Supplementary Table S2).

Impact of CTTN and FAK inhibition on colony formation and cell growth in HNSCC-derived cell lines

To investigate the pathophysiological role of CTTN and/or FAK and their possible contribution to malignant transformation, transfections with specific siRNA pools were carried out in HNSCC-derived cells. Soft agar colony formation was used to monitor anchorage-independent growth, which is one of the hallmarks of cell transformation. Transfection with siCTTN and siFAK in FaDu cells specifically and efficiently inhibited endogenous expression of CTTN and FAK (Figure 2A), compared to siControl-transfected cells. We found that FAK depletion in FaDu cells reduced both colony formation (Figure 2B) and cell growth (Figure 2C).

Pharmacological blockade of FAK effectively prevents colony formation, cell growth and invasion in HNSCC cell lines

We next evaluated the effect of targeting FAK using different small molecule inhibitors available (PF-573228, PF-562271 and VS-6063). All three FAK inhibitors efficiently reduced the phosphorylation levels of FAK at tyrosine 397 (pY397) in a dose-dependent manner in both FaDu and SCC42B cells (Figure 3A).

In addition, the effect on cell viability was assessed using MTS assay in both HNSCC-derived cell lines. Although the three compounds significantly diminished cell growth in a dose-dependent manner in FaDu and SCC42B cells, PF-562271 and VS-6063 showed a more robust inhibitory effect than PF-573228 (Figure 3B). Nevertheless, all three FAK inhibitors were highly effective

blocking colony formation in both HNSCC cell lines (Figure 3C), thus confirming the relevant role of FAK in HNSCC tumorigenesis.

Similarly, all FAK inhibitors consistently diminished the invasive potential of both SCC42B and FaDu cells in a dose-dependent manner (Figure 4A-B and Supplementary Videos 1-4).

DISCUSSION

Advances in deep sequencing have uncovered the great complexity and heterogeneity of the HNSCC oncogenome [20, 21]. Despite the high diversity of genetic alterations underlying each individual tumor, most molecular alterations converge into few major driver pathways [22]. It has been reported that over 80% of patients harbor alterations in actionable genes [23], thus representing an excellent opportunity to develop more personalized therapeutic strategies. Nevertheless, the identification of clinically and biologically relevant features in HNSCC development and progression is fundamental to define the central nodes that may be exploited therapeutically.

CTTN and FAK have emerged as major candidates to respectively drive 11q13- and 8q24-associated aggressive behavior and tumor spreading in various cancers, including HNSCC. We and others have contributed to demonstrate extensively the impact of CTTN and FAK on the prognosis of HNSCC patients and their implication in disease progression and dissemination [6-8, 13-15]. However, very few studies have investigated, to date, the role of CTTN and FAK in early stages of HNSCC tumorigenesis and malignant transformation. We provided the first evidence demonstrating the early occurrence of CTTN and FAK expression and gene amplification in patients with laryngeal and oral premalignant lesions [16, 17], and more importantly,

uncovered their potential utility as biomarkers for cancer risk assessment. Nevertheless, these promising observations require further confirmation in large independent cohorts.

To this purpose, we performed a multicenter validation using a cohort of 109 patients with laryngeal precancerous lesions. Consistent to our previous data [16, 17], CTTN and FAK were both found to be 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. Our *in vitro* functional analyses further contributed to delineate the pathobiological role of CTTN and FAK. Results consistently showed that targeting FAK by either RNAi or pharmacological inhibitors effectively blocked cell growth, *in vitro* transformation and 3D invasion.

In the light of all these data, CTTN and more strongly FAK seem to be clinically and 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.

It is also worth mentioning that CTTN and FAK showed robust associations with laryngeal cancer risk and superior predictive power than the histological grading. Quite remarkably, histology even using the new WHO classification did not show a significant role in assessing laryngeal cancer risk in this cohort nor previous cohorts studied [16, 17]. In marked contrast, expression of FAK alone or CTTN and/or FAK were independent predictors in multivariate

analysis in two independent cohorts of laryngeal precancerous lesions. Similar findings have been obtained in patients diagnosed with oral leukoplakias [17], thus extending extraordinarily the clinical applicability of these molecular markers to these highly common oral lesions. FAK expression has also been described as a marker for malignant transformation in CIS lesions of the cervix and breast [24]. These data highlight the limited value of histopathological classification in predicting outcome, despite the new WHO grading criteria recently established, while strongly and consistently suggest that CTTN and FAK protein evaluation may provide additional predictive power beyond histological features.

Histopathological diagnosis of squamous intraepithelial lesions remains the current gold standard in clinical practice for cancer risk assessment and decision-making [25]. Although high-grade lesions are thought to be at a higher cancer risk, some cancers develop from low-grade dysplasias. Histological grading is also affected by inter- and intra-observer variability. Additional objective and reliable markers are therefore needed to identify more accurately high-risk lesions beyond current clinical and histopathological criteria [26], which will subsequently help the clinicians to choose the most adequate therapeutic option. Since immunohistochemical analysis of CTTN and FAK is relatively simple and easy to interpret, it seems reasonable to recommend this molecular test to be included as complementary markers for cancer risk assessment and decision-making.

Together, these data strongly support the clinical relevance of CTTN and FAK expression in laryngeal tumorigenesis and their application as biomarkers for cancer risk assessment. Furthermore, our results suggest pharmacologic FAK

targeting with currently available inhibitors to be tested for HNSCC treatment. Laryngeal cancer treatment may have severe functional consequences and a major impact on patients' quality of life. Hence, novel cancer risk markers and/or molecular-targeted therapies will undoubtedly contribute to improve local control, overall survival, reduction of morbidity, and preservation of organ function.

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TABLES

Table 1. Evolution of the premalignant lesions in relation to histopathological diagnosis, CTTN and FAK protein expression.

Characteristic	Total No. (%)	Premalignancy Recurrence		Progression to Carcinoma	
		Cases (%)	Cases (%)	Cases (%)	*P* †
Histopathological diagnosis					
- Low-grade dysplasia	27 (25)	7 (25)	1.000	4 (15)	1.000
- High-grade dysplasia	82 (75)	21 (26)		12 (15)	
CTTN protein expression					
- Negative to Moderate (score 0-2)	95 (87)	20 (21)	0.008	9 (9)	0.001
- Strong (score 3)	14 (33)	8 (57)		7 (50)	
FAK protein expression					
- Negative to Moderate (score 0-2)	99 (73)	20 (20)	<0.001	9 (9)	<0.001
- Strong (score 3)	10 (27)	8 (80)		7 (70)	
CTTN and/or FAK expression					
- Negative to Moderate (score 0-2)	91 (83)	17 (19)	0.001	7 (8)	<0.001
- Strong (score 3)	18 (17)	11 (61)		9 (50)	

† Fisher's exact test

Table 2. Univariate Cox Proportional Hazards Model to Estimate Laryngeal Cancer Risk

Characteristic	*P*	Hazard Ratio	95% CI
Age (above *vs* below the mean)	0.89	1.072	0.402 to 2.857
Smoking (above *vs* below the mean)	0.062	2.785	0.952 to 8.149
Drinking (yes *vs* no)	0.040	1.814	1.026 to 3.205
Histology (High-grade *vs* low-grade dysplasia)	0.989	0.992	0.320 to 3.077
CTTN expression (Score 3 *vs* 0-2)	<0.001	6.810	2.533 to 18.308
FAK expression (Score 3 *vs* 0-2)	<0.001	13.296	4.891 to 36.142
CTTN and/or FAK (0 *vs* 1 or 2)	<0.001	8.864	3.293 to 23.859

Table 3. Multivariate Cox Proportional Hazards Model to Estimate Laryngeal Cancer Risk

Characteristic	***P***	**Hazard Ratio**	**95% CI**
Histology (High-grade)	0.22	0.44	0.12 to 1.63
Tobacco (Above the mean)	0.46	1.62	0.41 to 5.79
Alcohol (Yes)	0.014	2.22	1.17 to 4.20
CTTN expression (Strong)	0.37	2.02	0.44 to 9.30
FAK expression (Strong)	<0.001	13.91	4.82 to 40.15

FIGURE LEGENDS

Figure 1. 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 0-2 (negative to 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 expression *versus* negative to moderate expression. *P* values were estimated using the log-rank test.

Figure 2. Effect of CTTN and FAK knockdown on cell transformation and growth. (A) FaDu cells were transfected with either CTTN or FAK siRNAs, or siControl (as indicated), and protein expression levels analyzed by Western blot 72 h after transfection. (B) Soft agar Colony formation assay was performed for 7 days, and (C) tetrazolium-based MTS proliferation assay for 96 h in FaDu cells transfected with the indicated siRNAs. Data were normalized to the absorbance at day 0 (72 h after transfection) and relative to siControl-transfected cells. All data were expressed as the mean ± SD of at least two independent experiments performed in quadruplicate.

Figure 3. Effect of FAK inhibitors on cell transformation and growth in HNSCC-derived cell lines. (A) Western blot analysis of phosphorylated FAK Y397 levels in SCC42B and FaDu cells treated with increasing concentrations of the FAK inhibitors PF-573228, PF-562271 and VS-6063 for 1 h. GAPDH levels were used as loading control. (B) SCC42B and FaDu cells were treated for 72 h with the indicated concentrations of FAK inhibitors and cell viability measured by tetrazolium-based MTS assay. (C) Colony formation in soft agar was measured

at 7 days in SCC42B and FaDu cells treated with FAK inhibitors (1μM), and cell number quantified by MTS. Data were normalized to the absorbance at day 0 and relative to control (vehicle-treated) cells. All data were expressed as the mean ± SD of at least two independent experiments performed in quadruplicate.

Figure 4. Effect of FAK inhibitors on the invasive potential of HNSCC-derived cell lines. (A) Representative images from the 3D invasion assays of SCC42B spheroids embedded into a collagen matrix at initial (t = 0) and final time (t = 24 h) for the different treatments. Scale bars = 200 μm. (B) Analysis of the invasive properties of SCC42B and FaDu cells treated for 24 h with the indicated concentrations of the FAK inhibitors. Graphs represent the quantification of the invasive area at the indicated times. The invasive area was determined by calculating the difference between the final area and the initial area using Image J analysis program. Data were normalized to vehicle-treated cells and expressed as the mean ± SD of at least two independent experiments performed in quadruplicate.

Figure 1

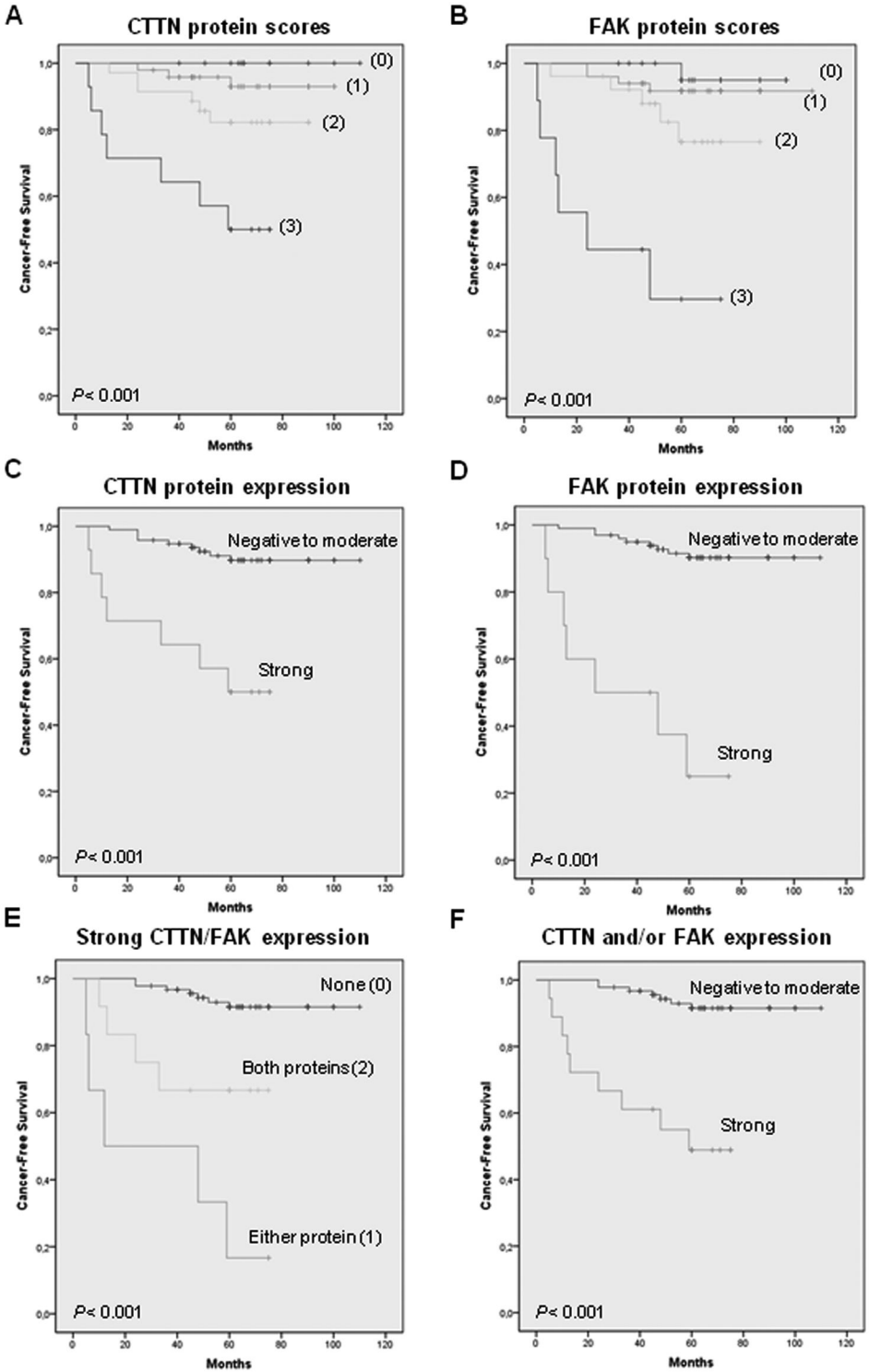


Figure 2

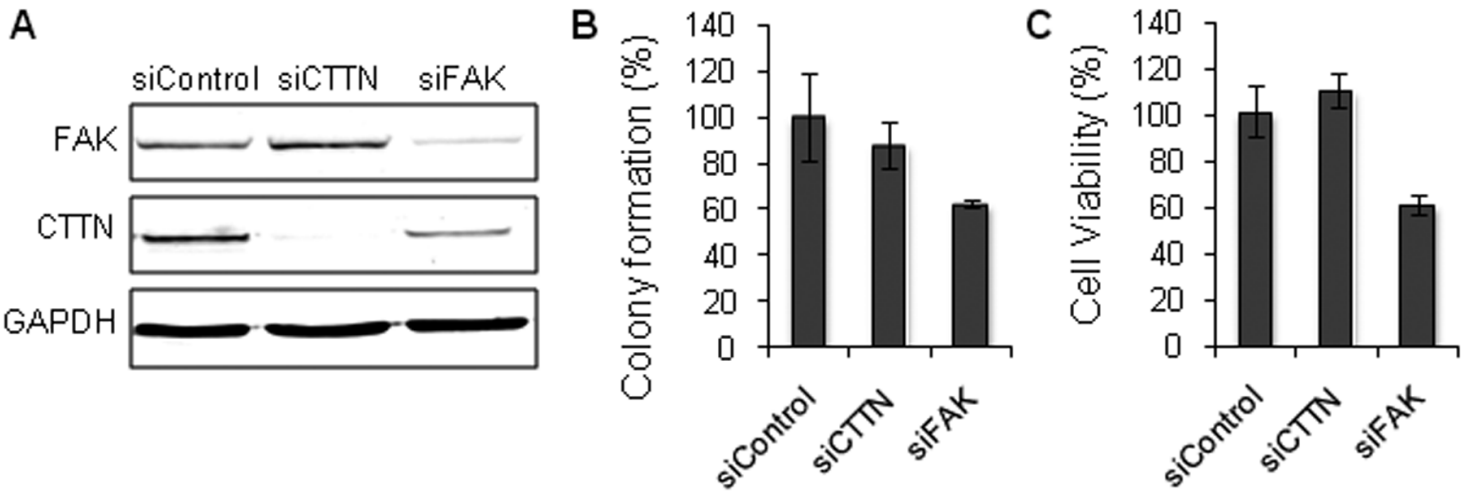


Figure 3

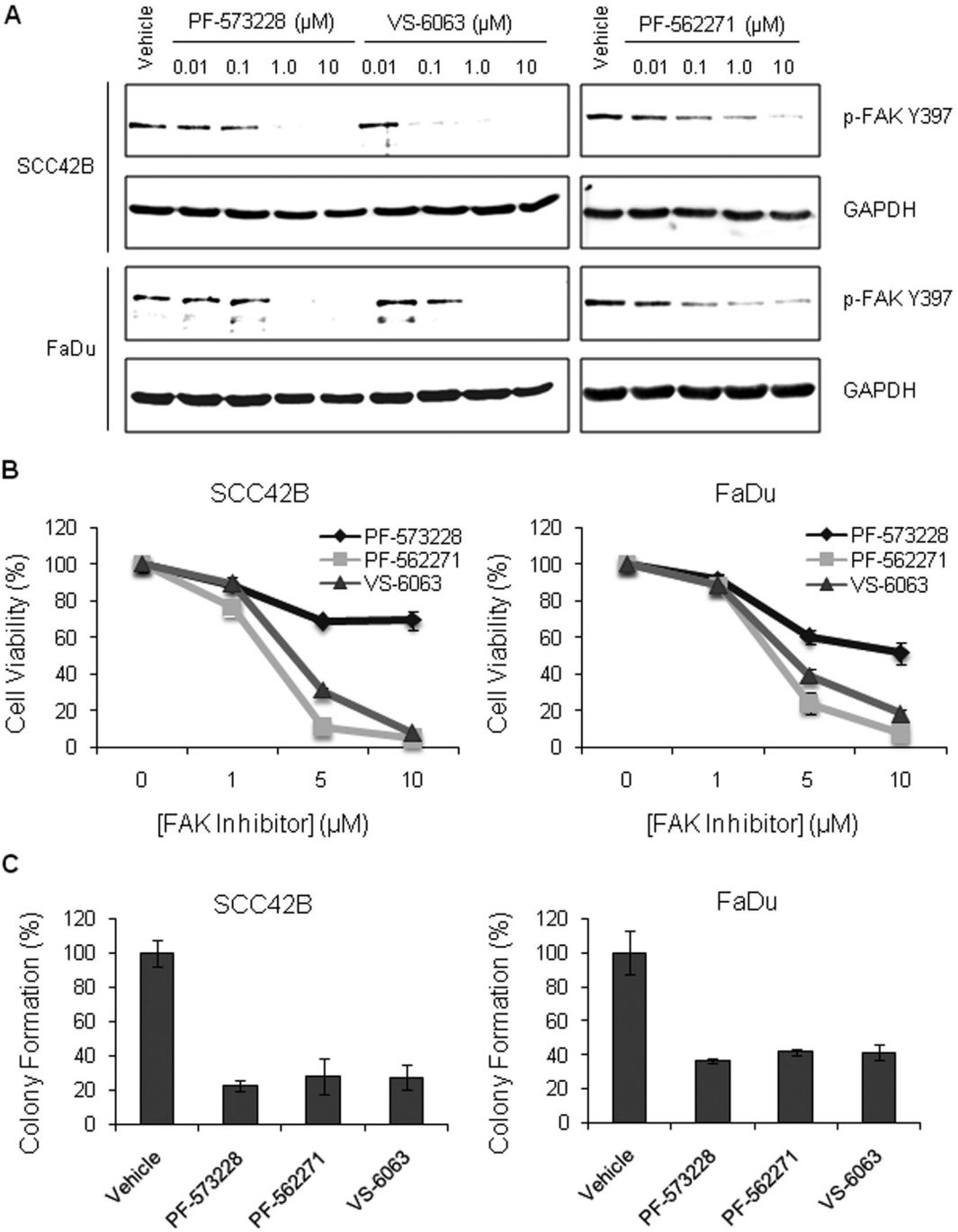
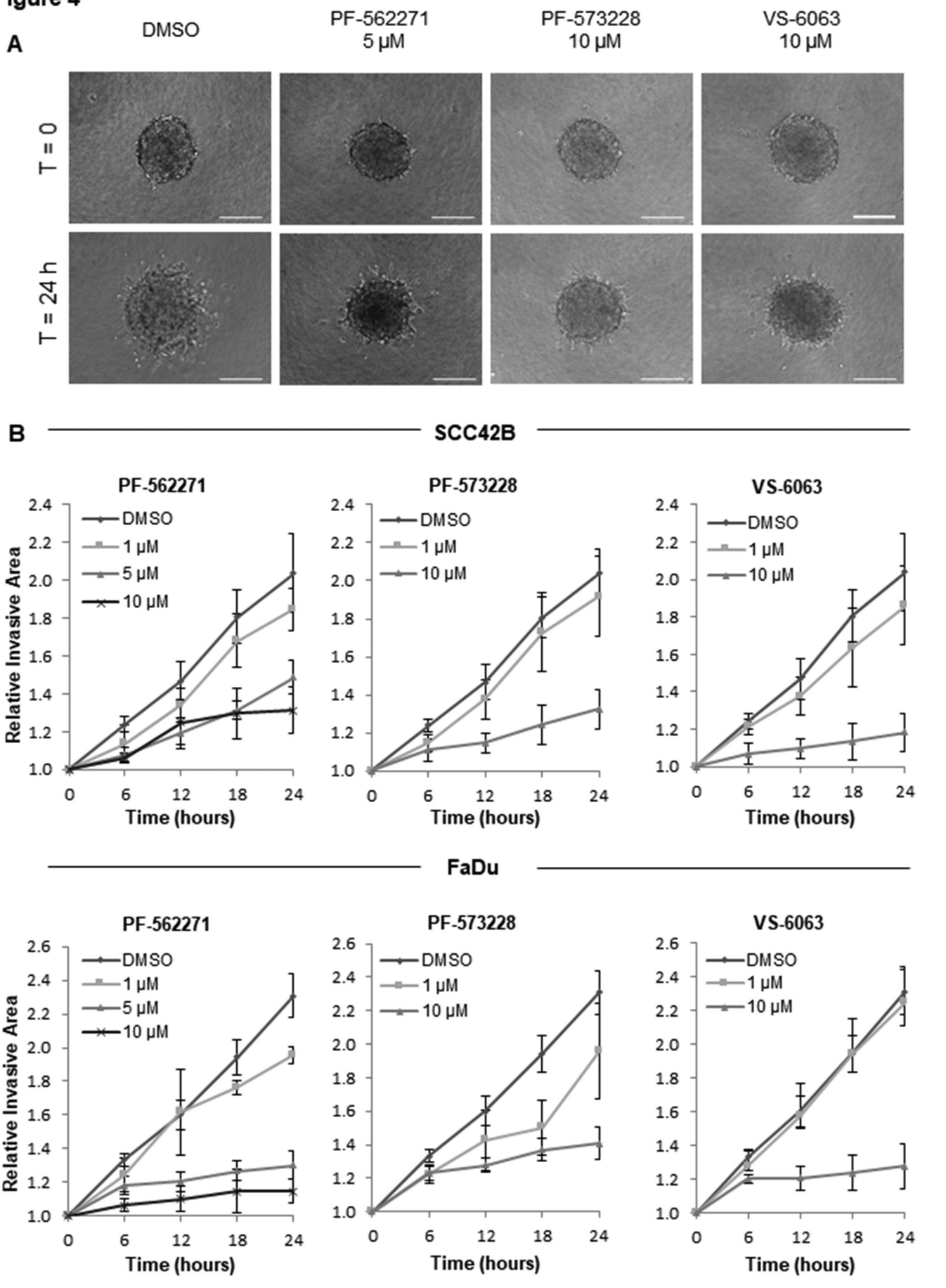


Figure 4



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IMMUNOHISTOCHEMICAL EXPRESSION OF CORTACTIN AND FOCAL ADHESION KINASE PREDICTS RECURRENCE RISK AND LARYNGEAL CANCER RISK BEYOND HISTOLOGICAL GRADING

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