Expression and Clinical Significance of Concomitant FAK/SRC and p-Paxillin in Mobile Tongue Squamous Cell Carcinoma

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Key Words: Mobile tongue squamous cell carcinoma, FAK, SRC, paxillin, clinicopathological parameters, prognosis, immunohistochemistry.

Running title: FAK/SRC/Paxillin in Tongue Carcinoma

Clinical Study: Date of submission: 23rd December 2016

Abstract. Background/Aim: The focal adhesion kinase (FAK)/SRC phosphorylation cascade and its downstream target paxillin have been implicated in malignant transformation, tumor growth and progression, together with metastasis. The present study aimed to evaluate the clinical significance of concomitant FAK/SRC and p-paxillin expression in mobile tongue squamous cell carcinoma (SCC). Materials and Methods: FAK, SRC and phospho-paxillin expression in 48 mobile tongue SCC tissue samples was assessed immunohistochemically and analyzed with respect to clinicopathological characteristics and patient survival. Results: Concomitant high FAK/SRC expression was significantly associated with high grade of tumor differentiation (p=0.048) and longer disease-free patient survival (log-rank test, p=0.019). High p-paxillin expression was significantly associated with greater depth of invasion (p=0.002), lymph node metastasis (p=0.048) and poorer disease-free patient survival (log-rank test, p=0.021; Cox-regression analysis, p=0.031). Conclusion: The present study provides evidence that FAK/SRC and paxillin play a role in the pathophysiological aspects of mobile tongue SCC and could constitute therapeutic targets.

Focal adhesion kinase (FAK) and proto-oncogene tyrosine protein kinase SRC are ubiquitously expressed non-receptor tyrosine kinases (1, 2). They are reciprocally activated by phosphorylation (3, 4) and the FAK/SRC dual kinase complex initiates multiple phosphorylation cascades, regulating several cellular functions, including integrin-mediated cell adhesion and migration, angiogenesis, cell cycle, cell proliferation and apoptosis (5). A prominent phosphorylation substrate of the FAK/SRC kinase complex is paxillin (6, 7), which interacts with several proteins coordinating changes in the actin cytoskeleton associated with cell motility and cell adhesion.

The involvement of FAK/SRC in cellular pathways that regulate cell survival, growth, invasion and motility (8, 9) suggests that they contribute to the development of cancer and are putative drug targets for treatment (10-13). Similarly, paxillin has been implicated in tumor progression, angiogenesis and metastasis, either as a downstream target of FAK/SRC or in its own right (14-17). Clinical studies have revealed that FAK, SRC and paxillin expression are significantly correlated with clinicopathological parameters and patient survival in many cancer types (18-24).

Mobile tongue squamous cell carcinoma (SCC) is the most common malignancy diagnosed within the oral cavity (25). Despite advanced therapeutic strategies, the 5-year survival rate has not been considerably improved, mainly due to lymph node metastasis at diagnosis (26, 27). Therefore there is an urgent need to establish reliable prognostic markers in mobile tongue SCC. In light of the above considerations, the present study aimed to assess concomitant FAK/SRC and phospho (p)-paxillin expression levels immunohistochemically in 48 mobile tongue SCC samples, in association with clinicopathological parameters and patient survival.

Materials and Methods

The medical records and archival histopathological material of the 48 patients with mobile tongue SCC included in this study were described in detail previously (28). Clinical and histopathological parameters were assessed according to standard criteria as described elsewhere (28). Immunostaining for FAK, SRC and p-paxillin was performed as described elsewhere (28-30), using primary antibodies detecting FAK, c-SRC and p-paxillin (Tyr118), respectively (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Appropriate negative controls were performed by omitting the primary antibody or substituting it with an irrelevant anti-serum. As positive controls, pancreatic, thyroid and endometrial cancer tissue sections with known increased FAK and SRC immunoreactivity (29-31) and colon cancer tissue sections with known increased p-paxillin immunoreactivity (unpublished data) were used. The immunoreactivity was scored according to the percentage of FAK, SRC and p-paxillin positive tumor cells as 0: negative staining; 0-4% of cells positive; 1: 5-24% of cells positive; 2: 25-49% of cells positive; 3: 50-100% of cells positive, and its intensity as 0: negative staining; 1: mild staining; 2: intermediate staining; 3: intense staining. Expression was classified as low, if the total score was 0 or 2, and high, if the total score was ≥3 (28, 32, 33). Concomitant high FAK/SRC expression was defined as cases presenting simultaneously high FAK and SRC expression, whereas concomitant low FAK/SRC expression was defined as cases presenting low expression in either FAK or SRC or both proteins. Statistical analysis was performed as described previously (28): chi-square test was used to assess the associations of concomitant FAK/Src and p-paxillin expression with clinicopathological variables, Kaplan-Meier survival curves were compared by the log rank test and a Cox proportional-hazard regression model was developed to evaluate the association

between the potential prognostic marker and overall and disease-free survival. A *p*-value less than 0.05 was considered the limit of statistical significance.

Results

All 48 samples of mobile tongue SCC were found to stain positively for FAK, SRC and p-paxillin and representative immunostaining results are shown in Figure 1. Concomitant high FAK/SRC expression was positively associated with histological grade of tumor differentiation (Table I, p=0.048), whereas no associations with patient age and gender or any other clinicopathological parameters examined were recorded. High p-paxillin expression was significantly associated with greater depth of invasion and lymph node metastasis (Table I, p=0.002 and p=0.048, respectively).

Kaplan–Meier survival curves indicated that patients with mobile tongue SCC with concomitant high FAK/SRC expression presented non-significantly longer (Figure 2A, log-rank test, p=0.057) and those with high p-paxillin expression shorter (Figure 2B, log-rank test, p=0.226) overall survival times compared to those with low expression. Longer overall patient survival was also significantly correlated with female gender and lesser depth of invasion (Table II, p=0.010 and p=0.047, respectively), while a trend for correlation of better survival with dense stromal inflammatory reaction and well-defined tumor shape was noted (p=0.050 and p=0.069, respectively).

Univariate analysis also showed that patients with mobile tongue SCC with concomitant high FAK/SRC expression presented significantly longer disease-free survival times compared to those with low expression (Figure 2C, log-rank test, p=0.019). Moreover, longer disease-free survival was significantly correlated with female gender, well-defined tumor shape and high histological grade of tumor

differentiation (Table II, p=0.007, p=0.034 and p=0.040, respectively), while a trend for correlation between disease-free survival and dense stromal inflammatory reaction was also recorded (p=0.054). However, concomitant FAK/SRC expression, as well as patient gender, and tumor grade and shape, did not remain significant in multivariate analysis concerning disease-free survival (Table III).

In contrast, Kaplan–Meier survival curves indicated that patients with mobile tongue SCC with high p-paxillin expression presented significantly shorter disease-free survival times compared to those with low expression (Figure 2D, log-rank test, p=0.021). In multivariate analysis, patient gender and p-paxillin expression were identified as independent factors associated with disease-free patient survival (Cox-regression analysis, p=0.033 and p=0.031, respectively).

Discussion

The role of the FAK/SRC/paxillin axis in human malignancy is well established (8-24) but its role in mobile tongue SCC is still being investigated. As an extension to our previous investigations (28), the present study evaluated the associations between concomitant FAK/SRC expression and clinicopathological parameters, together with overall and disease-free patient survival. We confirmed the correlation of concomitant FAK/SRC expression with high tumor differentiation grade, observed by Theocharis *et al.* (2012) for FAK and SRC separately, and illustrated the association of concomitant FAK/SRC expression with longer disease-free patient survival, noted previously for SRC only (28). Additionally, we investigated the possibility that p-paxillin, a downstream target of the FAK/SRC phosphorylation cascade, would show similar correlations. However we demonstrated the opposite effect suggesting that these proteins do not operate together in this context. More specifically, high p-paxillin

expression was significantly associated with greater tumor depth of invasion and presence of lymph node metastasis, while patients with mobile tongue SCC with high p-paxillin expression presented significantly shorter disease-free survival times compared to those with low expression. Thus p-paxillin expression was identified as an independent factor in disease-free patient survival. The present observations support the implication of FAK/SRC and p-paxillin signalling in disease progression and recurrence. However, the opposite associations noted between concomitant FAK/SRC, and p-paxillin expression with clinicopathological parameters and patient survival suggest that FAK/SRC and paxillin mediate their effects independently. High p-paxillin expression in mobile tongue SSC is probably controlled by other signalling pathways and leads to poor prognosis in contrast to the positive prognosis associated with high FAK/SRC expression, attesting to the complexity of molecular interactions in clinical samples. It should be noted that paxillin also has an upstream regulatory role on the FAK/SRC complex by mediating its localization at focal adhesions (3, 34, 35). Interestingly, Conway *et al*. (36) reported that paxillin over expression represses FAK phosphorylation in SCC25 tongue squamous cancer cells.

In support of the above findings, Jiang *et al.* documented a significant increase in FAK protein expression levels in well-differentiated tongue tumors as compared to poorly differentiated types (37). Additionally, increased paxillin expression levels were associated with lymph node metastasis and shorter overall survival times in tongue SCC (38). Similar correlations between high paxillin expression and advanced clinicopathological parameters together with poor prognosis have been noted in salivary adenoid cystic carcinoma (39) and laryngeal SCC (40). In general, FAK and SRC constitute promising targets for anticancer therapy (11, 13). Taking into consideration the results of the present study, the FAK/SRC pathway could be

targeted indirectly in order to increase and not inhibit signalling. Although no paxillin inhibitors are available to date (41), this molecule could also have potential therapeutic utility in mobile tongue SCC.

The present study provides evidence that the FAK/SRC/paxillin axis may play a role in the pathophysiological aspects of mobile tongue SCC, being associated with crucial clinicopathological parameters for patient management and prognosis. However the current investigation only concerned a limited number of patients and relatively few clinical events, limiting our ability to draw more precise conclusions Further investigations should utilize a larger patient cohort to assess FAK and SRC activity. Both would be monitored by quantifying their activated-phosphorylated forms together with investigations of other downstream targets, apart from paxillin, in order to establish their clinical significance and therapeutic potential in mobile tongue SCC. Additionally, future research effort should focus on the assessment of the exact role of paxillin in the pathophysiological aspects that affect disease progression and prognosis in mobile tongue SCC.

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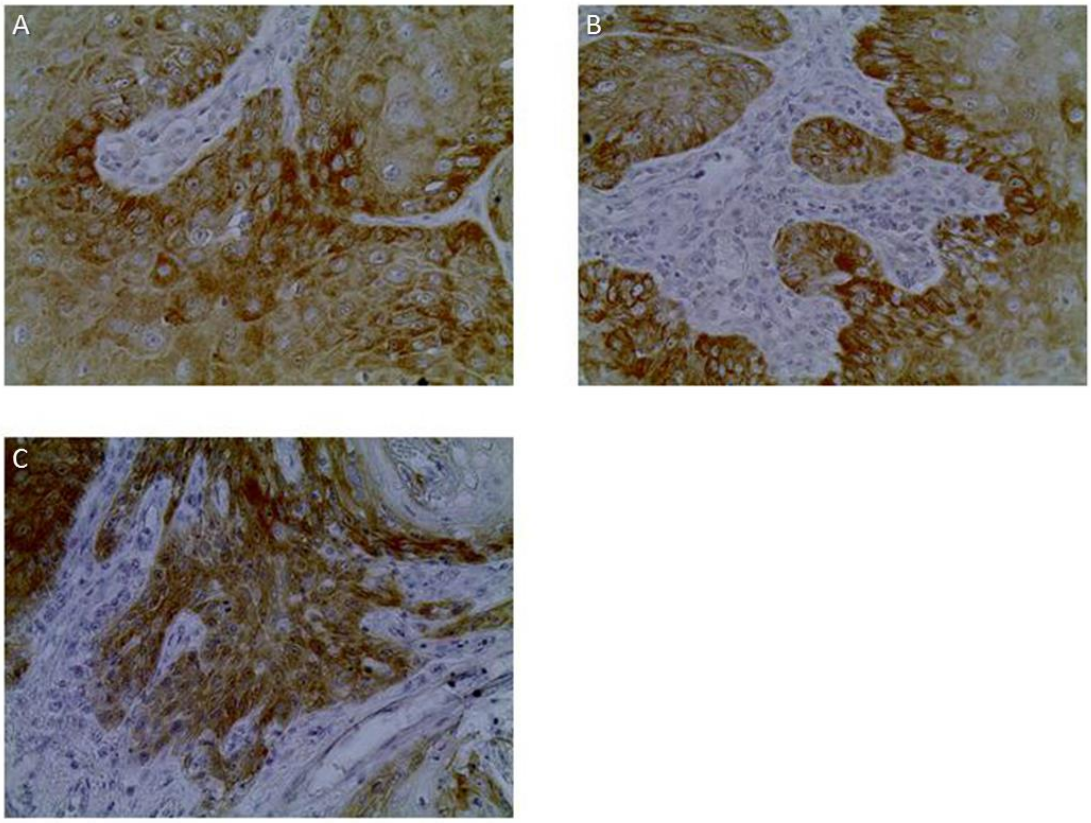

Figure 1. Representative immunostaining results for focal adhesion kinase (A), proto-oncogene tyrosine protein kinase SRC (B) and phospho-paxillin (C) protein expression in tumor cells of mobile tongue squamous cell carcinoma. Streptavidin-biotin-peroxidase, 3,3-diaminobenzidine (DAB) chromogen, Harris hematoxylin counterstain (original magnification ×400).

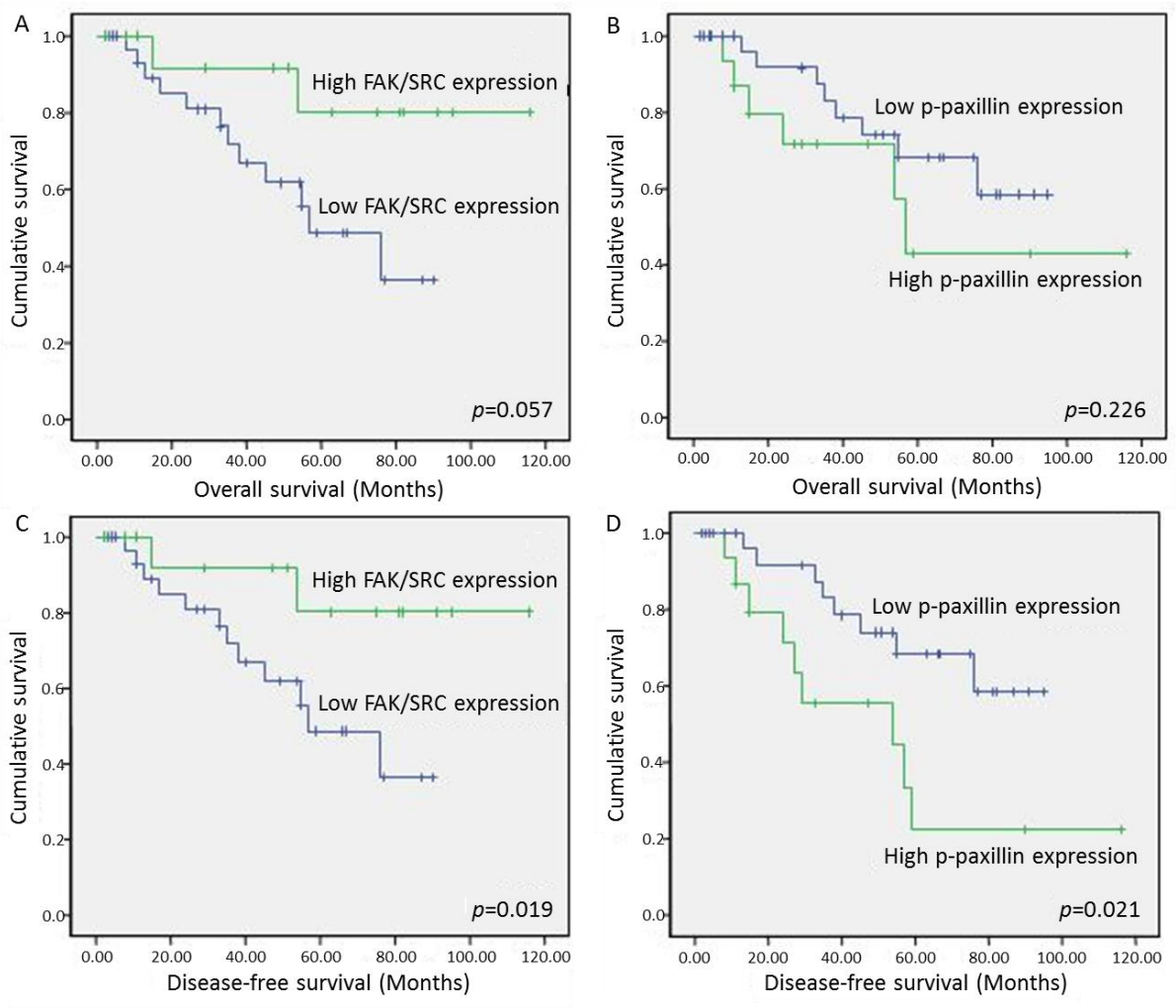

Figure 2. Kaplan–Meier survival analysis of overall (A, B) and disease-free (C, D) survival stratified according to concomitant focal adhesion kinase (FAK)/proto-oncogene tyrosine protein kinase SRC (A, C) and phospho-paxillin (B, D) protein expression in patients with mobile tongue squamous cell carcinoma.

Table I: Associations of concomitant focal adhesion kinase (FAK)/proto-oncogene tyrosine protein kinase SRC and phospho-paxillin expression with clinicopathological parameters in 48 patients with mobile tongue squamous cell carcinoma.

Clinicopathological characteristic	Concomitant FAK/SRC expression			p-Paxillin expression		
	Low, n (%)	High, n (%)	*p*-Value	Low, n (%)	High, n (%)	*p*-Value
N=48	33 (68.8)	15 (31.2)		33 (68.8)	15 (31.2)	
Age mean±SD			0.636			0.636
≤62.2±14.6 years	20 (41.7)	8 (16.7)		20 (41.7)	8 (16.7)	
>62.2±14.6 years	13 (27.1)	7 (14.6)		13 (27.1)	7 (14.6)	
Gender			0.259			0.459
Male	19 (39.6)	6 (12.5)		16 (33.3)	9 (18.8)	
Female	14 (29.2)	9 (18.7)		17 (35.4)	6 (12.5)	
Histopathological grade			**0.048**			0.369
I	22 (45.8)	14 (29.1)		26 (54.2)	10 (20.8)	
II	11 (22.9)	1 (2.1)		7 (14.6)	5 (10.4)	
Stromal inflammatory reaction			0.457			0.175
Mild/moderate	10 (20.8)	3 (6.3)		7 (14.6)	6 (12.5)	
Dense	23 (47.9)	12 (25.0)		26 (54.2)	9 (18.8)	
Muscular invasion			0.869			0.473
Yes	28 (58.3)	13 (27.1)		29 (60.4)	12 (25.0)	
No	5 (10.4)	2 (4.1)		4 (8.3)	3 (6.3)	
Shape			0.175			0.457
Diffuse	26 (54.3)	9 (18.7)		23 (47.9)	12 (25.0)	
Well-defined	7 (14.6)	6 (12.5)		10 (20.8)	3 (6.3)	
Vascular invasion			0.502			0.151
Yes	6 (12.5)	4 (8.3)		5 (10.4)	5 (10.4)	
No	27 (56.3)	11 (22.9)		28 (58.3)	10 (20.8)	
Perineural invasion			0.269			0.636
Yes	12 (25.0)	8 (16.6)		13 (27.1)	7 (14.6)	
No	21 (43.8)	7 (14.6)		20 (41.7)	8 (16.7)	
Depth of invasion			0.839			**0.002**
I+II	21 (43.8)	10 (20.8)		26 (54.2)	5 (10.4)	
III	12 (25.0)	5 (10.4)		7 (14.6)	10 (20.8)	
Lymph node metastasis			1.000			**0.048**
Yes	11 (22.9)	5 (10.4)		8 (16.7)	8 (16.7)	
No	22 (45.8)	10 (20.8)		25 (52.1)	7 (14.6)	

Mitotic index			0.613			0.459
≤Median	15 (31.3)	8 (16.7)		17 (35.4)	6 (12.5)	
>Median	18 (37.5)	7 (14.6)		16 (33.3)	9 (18.8)	

Table II. Association of clinicopathological parameters, concomitant focal adhesion kinase (FAK)/proto-oncogene tyrosine protein kinase SRC and phospho-paxillin expression with patient survival: Univariate analysis.

Clinicopathological parameters		Mean OS (95% CI), months	*p*-Value	Mean DFS (95% CI), months	*p*-Value
Age	<62.2 years	65.2 (50.9-79.5)	0.381	63.0 (48.8-77.2)	0.607
	≥62.2 years	87.2 (66.4-108.0)		78.3 (57.3-99.3)	
Gender	Male	55.9 (41.1-70.7)	**0.010**	51.7 (37.6-65.9)	**0.007**
	Female	100.5 (84.5-116.4)		95.4 (78.0-112.8)	
Histopathological grade	I	85.5 (69.4-101.7)	0.179	82.8 (66.8-98.9)	**0.040**
	II	46.2 (35.0-54.4)		41.7 (31.1-52.4)	
Stromal inflammatory reaction	Mild, moderate	53.9 (37.0-70.8)	0.050	50.4 (33.9-66.9)	0.054
	Dense	90.3 (74.2-106.4)		84.1 (67.5-100.7)	
Muscular invasion	Yes	55.0 (38.7-68.8)	0.554	49.4 (35.5-63.3)	0.995
	No	78.9 (63.4-94.3)		74.7 (59.5-89.9)	
Shape	Diffuse	63.3 (50.6-76.0)	0.069	58.3 (46.1-70.5)	**0.034**
	Well-defined	105.8 (87.6-124.0)		105.8 (87.6-124.0)	
Vascular invasion	Yes	57.4 (35.9-78.8)	0.418	57.4 (35.9-78.8)	0.645
	No	83.7 (67.7-99.7)		76.7 (60.8-92.6)	
Perineural invasion	Yes	72.3 (52.2-87.4)	0.533	72.3 (57.2-87.4)	0.185
	No	74.8 (54.2-95.4)		64.8 (45.8-83.7)	
Depth of invasion	I+II	77.8 (66.3-89.3)	**0.047**	71.1 (59.0-83.1)	0.177
	III	63.5 (39.2-87.7)		63.5 (39.2-87.7)	
Lymph node metastasis	Yes	63.2 (45.9-80.5)	0.595	59.8 (42.5-77.0)	0.631
	No	82.7 (64.7-100.6)		76.8 (59.3-94.4)	
Mitotic index	≤Median	82.2 (62.7-101.6)	0.977	73.8 (54.3-93.2)	0.831
	>Median	67.7 (53.4-81.9)		64.7 (50.1-79.3)	
Concomitant FAK/SRC expression	Low	59.5 (47.2-71.8)	0.057	54.4 (42.7-66.1)	0.019
	High	100.5 (80.9-120.0)		100.5 (80.9-120.0)	
p-Paxillin expression	Low	74.1 (62.1-86.1)	0.226	74.1 (62.1-86.1)	**0.021**
	High	69.9 (43.8-96.0)		53.4 (31.8-75.1)	

OS: Overall survival; DFS: disease-free survival; CI: confidence interval.

Table III. Association of clinicopathological parameters and concomitant focal adhesion kinase (FAK)/proto-oncogene tyrosine protein kinase SRC and phospho-paxillin expression with disease-free patient survival: Multivariate analysis.

Variable	Disease free-survival			
	Concomitant FAK/SRC		p-Paxillin	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Gender: Female *vs.* male	0.284 (0.075-1.080)	0.065	0.282 (0.088-0.902)	**0.033**
Tumor grade: II *vs.* I	1.988 (0.590-6.701)	0.268	3.014 (0.972-9.347)	0.056
Tumor shape: Well-defined *vs.* diffuse	0.203 (0.026-1.600)	0.130	0.148 (0.019-1.178)	0.071
Protein expression: High *vs.* low	0.381 (0.077-1.879)	0.236	3.148 (1.110-8.928)	**0.031**

HR: Hazard ratio; CI: confidence interval.