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ORIGINAL ARTICLE

Focal Adhesion Kinase (FAK) Overexpression and Phosphorylation in Oral Squamous Cell Carcinoma and their Clinicopathological Significance

Aki Kato[1] · Koroku Kato[1] · Hiroki Miyazawa[1] · Hisano Kobayashi[1] · Natsuyo Noguchi[1] · Shuichi Kawashiri[1]

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Abstract
Focal adhesion kinase (FAK) is involved in progression of various cancers, and FAK overexpression has been associated with cancer invasion and metastasis. However, the involvement of FAK expression in the clinicopathological malignancy of oral squamous cell carcinoma (OSCC) remains unknown. In addition, there is no consensus regarding the role of p16 expression in OSCC. In this study, the immunohistochemically measured expression of FAK, phosphorylated FAK (FAKpY397) and p16 expressions and their associations with clinicopathological features and 5-year survival rates were examined in surgical samples from 70 patients with primary OSCC. FAK and FAKpY397 were expressed at high levels in 42 cases (60.0%) and 34 cases (48.6%), respectively, and 9 cases (12.9%) were positive for p16. FAK expression was significantly correlated with local recurrence, subsequent metastasis, and the mode of invasion. FAKpY397 expression was significantly correlated with both N classification and the mode of invasion. p16 expression was significantly correlated with clinical stage only. Patients having high expression of FAK, FAKpY397, or both showed significantly worse prognosis, but p16 expression showed no significant relation to prognosis. The results suggested that overexpression and phosphorylation of FAK in OSCC may affect cancer progression, such as local invasion and lymph node metastasis, and thereby contribute to life prognosis.

Keywords Focal adhesion kinase · FAK · FAKpY397 · p16 · Oral squamous cell carcinoma · Prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck, and its incidence continues to increase [1]. Despite great advances in treatment, its prognosis is inadequate, as OSCC leads to relapse through active local invasion and metastasis. TNM classification and the degree of histological differentiation are widely used factors for evaluating tumor properties. However, these indicators do not account for various characteristics thought to affect prognosis, such as the depth of invasion, tumor volume, number of metastatic lymph nodes, and presence of extranodal spread. Therefore, it is necessary to elucidate the molecular biological factors that reflect the clinicopathological characteristics of OSCC.

Cell adhesion is one of the important mechanisms underlying cancer progression, invasion, and metastasis. Focal adhesion kinase (FAK), a member of the protein tyrosine kinase family, plays a major role in cell adhesion. When FAK is stimulated by integrin or growth factor signals, autophosphorylation of tyrosine 397 occurs, and signals are transmitted to downstream AKT and MAPK [2, 3]. FAK is thought to plays very important roles in cancer related cellular events such as cell proliferation, differentiation, migration and apoptosis [4]. FAK is overexpressed in various malignant cancers, including thyroid, prostate, cervical, rectal, and ovarian cancers [5–9], and some studies have reported a close association between FAK expression and both cancer progression and clinical prognosis [10–12]. However, the relationships between the clinicopathological characteristics and FAK expression/activation in OSCC remain unclear.

Human papilloma virus (HPV) has been reported to be involved in head and neck cancer, especially oropharyngeal cancer, and X et al. reported that 70% of their patients with

✉ Koroku Kato
k-koroku@oral.m.kanazawa-u.ac.jp

[1] Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8641, Japan

oropharyngeal cancer were infected with HPV [13–15]. Infection with HPV is considered to be an important prognostic factor for oropharyngeal cancer, and HPV-related oropharyngeal tumors exhibiting a good therapeutic response and a good prognosis [16]. However, In OSCC, although there has been a report examining the relationship between the presence or absence of HPV infection and prognosis, the prognostic significance of HPV infection has not been clearly delineated.

In the present study, we evaluated the relationships between the expression patterns of FAK, phosphorylated tyrosine 397 (FAKpY397) and p16 in OSCC and the clinicopathological factors (including cancer progression) and life prognosis.

Materials and Methods

Patients

The samples were obtained from 70 patients who underwent surgical resection due to primary OSCC at the Department of Oral and Maxillofacial Surgery of Kanazawa University Hospital. We excluded the samples of patients who already showed distant metastasis at the first visit, those who did not undergo radical surgery because of a poor performance status, and those with a previous history of squamous cell carcinoma in the head and neck or other organs. Patient and tumor characteristics are detailed in Table 1. Tumor staging was assessed using the 7th edition of the TNM classification according to the Union International Committee on Cancer (UICC) [17]. The grade of tumor differentiation was determined according to the criteria proposed by the World Health Organization. The mode of tumor invasion was assessed according to the classification by Yamamoto et al. [18]. Subsequent metastasis was defined as metastasis to the lymph nodes in the neck observed during follow-up in cases without lymph nodes metastasis in the neck at first visit. The 5-year cumulative survival rate was defined as the percentage of patients who survived for 5 years after the first surgery.

Immunohistochemical Staining

Hematoxylin–eosin (HE)-stained specimens were prepared from surgically resected squamous cell carcinoma samples. Sections from the deepest part of the invasion were evaluated primarily by light microscopic observation. Then the site to be subjected to immunohistochemical staining was selected. Tissue specimens collected during surgery were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned to a thickness of 4-μm. Immunohistochemical staining of FAK and FAKpY397 was performed with the CSA method using an anti-FAK antibody (1:100 dilution; BD Transduction Laboratories, San Jose, CA) and an anti-FAKpY397 antibody (1:100 dilution; BD Transduction Laboratories). Staining of p16 was performed with the Envision system using an anti-CDKN2A/p16INK4a antibody (1:500 dilution; Abcam, Cambridge, MA). The sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. Then the sections were incubated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Sections were incubated with the antibodies against FAK, FAKpY397 and p16 overnight at 4 °C followed by 3 washes with Tris-buffered saline (TBS). After reacting with HRP-labeled secondary antibody at room temperature, each section was sensitized using a CSA II kit (Dako Japan, Tokyo) for FAK and FAKp397, and Dako REAL Envision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako) for p16. Color development was carried out using nickel-added 3.3′ diaminobenzidine tetrahydrochloride (DAB). After counterstaining with hematoxylin, the sections were dehydrated, penetrated and sealed, and examined. For a comparative study, normal oral epithelial tissue material was also prepared by the above method.

Evaluation of Staining

Immunohistochemical staining of FAK and FAKpY397 at the invasive front of a tumor was evaluated using the classification system of Theocharis [19], which specifies 4 groups according to the proportion of positive cells: <5% = 0, 5–24% = 1, 25–49% = 2, and 50–100% = 3. The staining intensity was also classified into 4 groups: no staining = 0, faint staining intensity = 1, weak or moderate staining intensity = 2, and strong staining intensity = 3. The scores of the positive cell proportion and staining intensity were added; a total score of 3 or lower was assigned to the low expression group and a score of 4 or more was classified into the high expression group.

In the evaluation of p16 expression, the staining intensity in the nucleus and cytoplasm of individual cells was categorized into 4 groups: none = 0, weak = 1, moderate = 2, and strong = 3. Then, the percentage of stained tumor cells were evaluated. Tumors were considered p16-positive if ≥80% of the tumor cells had moderate (2+) staining or if any percentage of cells had strong staining (3+) [20].

Statistical Analysis

JMP13 (SAS Institute, Cary, NC) was used for data analysis. The χ 2 test was used to assess the correlations between FAK, FAKpY397, p16 and clinicopathological factors. We calculated the 5-year survival rates by the Kaplan-Meier method and compared them using the log rank test. A multivariate analysis was performed using the Cox proportional hazards regression model. The significance level was set at 5% for each analysis.

Table 1 Clinicopathological parameters in relation to FAK, FAKpY397 and p16 expression ($n = 70$)

		FAK			FAKpY397			p16		
Variable	n	High	Low	*p* value	High	Low	p value	Positive	Negative	p value
Age				0.28			0.45			0.53
≤65	32	17	15		14	18		5	27	
65<	38 25	13			20	18		4	34	
Gender				0.48			0.59			0.36
Male	41	26	15		21	20		4	37	
Female	29	16	13		13	16		5	24	
Primary site				0.36			0.29			0.12
Tongue	31	20	11		16	15		3	28	
Gingiva	21	13	8		11	10		5	16	
Oral floor	9	3	6		2	7		0	9	
Buccal mucosa	7	4	3		3	4		0	7	
Other	2	2	0		2	0		1	1	
T category				0.28			0.14			0.74
T1	16	9	7		5	11		1	15	
T2	37	20	17		17	20		5	32	
T3	4	4	0		3	1		1	3	
T4	13	9	4		9	4		2	11	
N category				0.07			0.04			0.31
N(+)	21	16	5		14	7		4	17	
N(−)	49	26	23		20	29		5	44	
Stage				0.38			0.09			0.03
S1	15	9	6		5	10		1	14	
S2	25	12	13		10	15		1	24	
S3	14	9	5		7	7		5	9	
S4	16	12	4		12	4		2	14	
Local recurrence				0.04			0.01			0.59
+	21	18	3		13	8		2	19	
-	49	24	25		21	28		7	42	
Subsequent metastasis				0.03			0.05			0.1
+	16	12	4		10	6		0	16	
-	33	14	19		11	22		5	28	
Cell differentiation				0.55			0.51			0.56
Well	39	23	16		18	21		5	34	
Moderate	20	10	10		8	21		2	27	
Poor	11	9	2		8	3		2	9	
Mode of invasion				0.04			<0.01			0.74
1	8	3	5		0	8		0	8	
2	11	3	8		3	8		1	10	
3	22	14	8		13	9		4	18	
4C	23	17	6		13	10		3	20	
4D	6	5	1		5	1		1	5	

Results

Expression Status of FAK, FAKpY397 and p16 in OSCC

FAK and FAKpY397 were not expressed in the normal oral squamous epithelium. In OSCC, however, their expression was confirmed in all cases. In tissue specimens with OSCC, FAK and FAKpY397 were detected in the cytoplasm of tumor cells. On the other hand, p16 was detected in the nuclei or in both the nucleus and cytoplasm of tumor cells and the normal epithelium, and no cell exhibited a cytoplasmic signal alone (Fig. 1). According to the criteria adopted for the immunohistochemical evaluation, 42 cases (60.0%), 34 cases (48.6%) expressed FAK and FAKpY397 highly, respectively. Nine cases (12.9%) were judged as p16 positive.

Associations between FAK, FAKpY397 and p16 Expression and Clinicopathological Features

Table 1 shows the correlations between FAK, FAKpY397 or p16 expression and the clinicopathological factors of patients. In the clinical factors, there were significant correlations between FAK expression and either local recurrence ($p = 0.04$), or subsequent metastasis ($p = 0.03$); between FAKpY397 expression and either N classification ($p = 0.04$) or local recurrence ($p = 0.01$); and between p16 expression and clinical stage ($p = 0.03$). With respect to the pathological factors, both FAK and FAKpY397 expression levels were significantly correlated with the mode of invasion (FAK, $p = 0.04$; FAKpY397, $p < 0.01$).

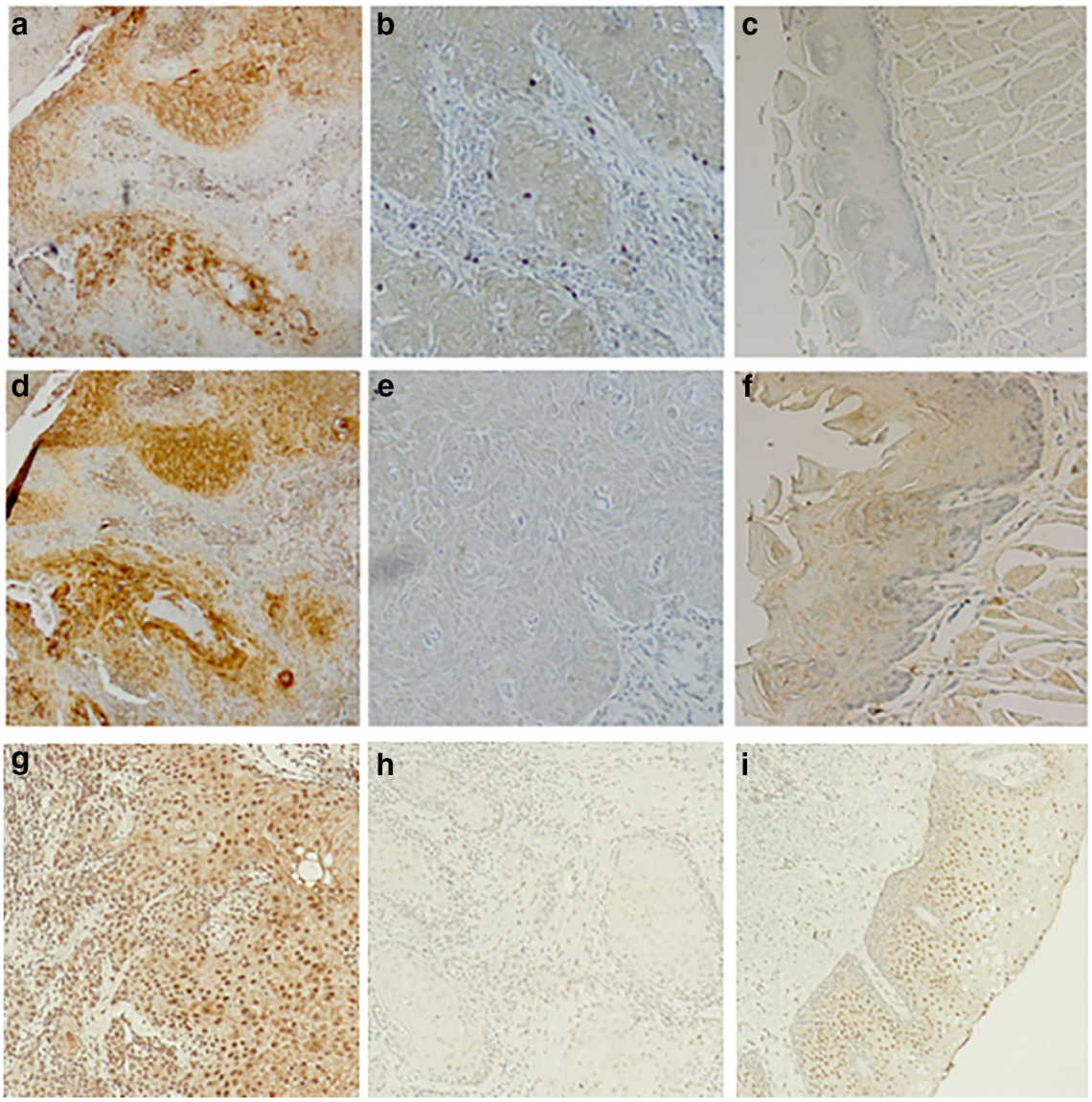

Fig. 1 Immunohistochemical staining of FAK in cancer cells of OSCC (**a** high expression; **b** low expression) and normal tissue (**c**). Immunohistochemical staining of FAKpY397 (**d** high expression; **e** low expression) and normal tissue (**f**). Immunohistochemical staining of p16 (**g** positive; **h** negative) and normal tissue (**i**). FAK and FAKpY397 immunoreactivity is observed in the cytoplasm and cell membrane of the tumor cells (original magnification ×100). p16 was detected in both the nucleus and cytoplasm of tumor cells

Correlations between 5-Year Cumulative Survival Rate and FAK, FAKpY397 and p16 Expression

The 5-year cumulative survival rate was significantly lower in cases with high FAK expression cases (53.3%) than in those with low FAK expression (75.0%) ($p = 0.046$, Fig. 2a). And the 5-year survival rate was significantly lower in cases with high expression of FAKpY397 (48.3%) than in those with low expression of FAKpY397 (75.0%) ($p = 0.015$, Fig. 2b). In the analysis of the effects of p16 expression on the 5-year cumulative survival rate, no statistically significant difference in survival was observed between the positive case (50.0%) and the negative case (64.0%) ($p = 0.130$, Fig. 2c).

Next, we examined the relationships between the FAK and FAKpY397 expression patterns and 5-year cumulative survival rates. Both FAK and FAKpY397 were expressed highly in 36 cases (51.4%). There were 6 cases with high FAK expression and low FAKpY397 expression, and 28 cases with low expression of both FAK and FAKpY397. There were no cases of low expression of FAK and high expression of FAKpY397. The cases with high expression of both FAK and FAKpY397 had the worst 5-year survival rate (48.3%) ($p = 0.052$, Fig. 3).

Univariate and Multivariate Analyses in Prognosis

We performed a Cox proportional hazards regression analysis to examine the significance of the predictive factors (Table 2).

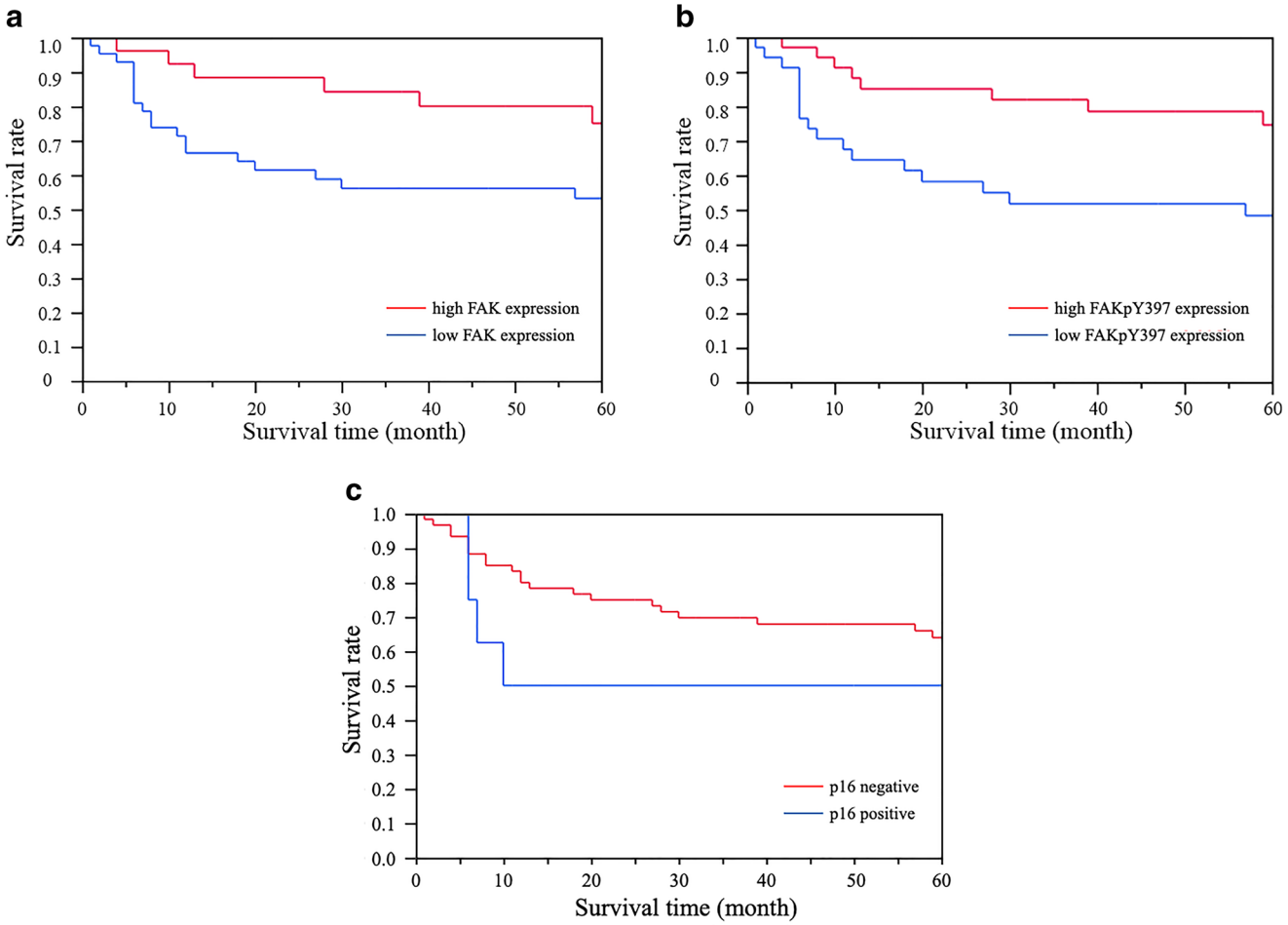

Fig. 2 Kaplan-Meier survival estimates for overall survival based on FAK (**a**), FAKpY397 (**b**) and p16 (**c**) expression

A univariate analysis revealed that the size of the primary tumor, local recurrence, mode of invasion, and high expression levels of FAK and FAKpY397 were significant prognostic indicators. A multivariate analysis showed that only T classification was an independent factor for prognosis; and the hazard ratio was 3.50.

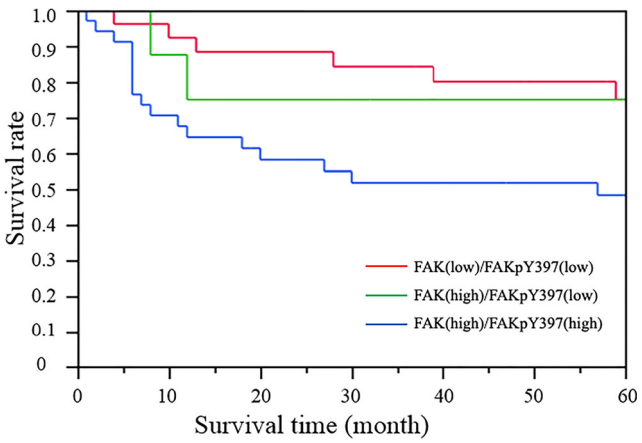

Fig. 3 Kaplan-Meier survival estimates for overall survival based on the expression pattern of FAK and FAKpY397

Discussion

There are numerous reports on the relationships between clinicopathological characteristics and prognosis in OSCC. Among the histopathological factors in OSCC, the mode of invasion is useful as an indicator of malignancy, with the prognosis deteriorating as the invasion progresses [21]. Kurokawa reported that the morphological features of tumor cells at the invasive front are the most important factors related to prognosis in tongue squamous cell carcinoma [22]. Moreover, in OSCC, although several reports have examined the relationships between clinicopathological factors and cell adhesion [23, 24], there is no unified opinion on the relationship between the degree of invasion and cell adhesion.

FAK is involved in cell migration by regulating adhesion between the extracellular matrix (ECM) and cells, and is known as an important mediator of cell motility [25, 26]. In addition, FAK has multiple binding sites, with tyrosine 397 serving as the major site of autophosphorylation, as well as a binding site for various interacting partners including Src family kinases and p85; FAK is also associated with tumor malignancy [11, 27–31]. In human epithelial malignant tumors,

Table 2 Univariate and multivariate analyses of the relation between clinicopathological parameters, FAK, FAKpY397, and p16 expression and the overall survival in 70 patients with OSCC

Variable		Univariate		Cox regression p value	Hazard ratio (95% CI)
		χ^2	p value		
Age	≤65 / 65<	1.59	0.1598		
Gender	Male / Female	0.93	0.8536		
T category	T1–2 / T3–4	4.29	0.0002	0.0097	3.49 (1.344–10.291)
N category	N- / N+	1.37	0.3524		
Stage	S1,S2 / S3.S4	2.35	0.0105	0.7848	1.14 (0.408–2.785)
Local recurrence	- / +	1.71	0.1046		
Cell differentiation	Well / Mod, Poor	1.43	0.2790		
Mode of invasion	1,2,3 / 4C,4D	1.91	0.0493	0.1537	1.64 (0.823–3.299)
FAK	High / low	2.18	0.0280	0.8260	1.16 (0.256–3.917)
FAKpY397	High / low	2.48	0.0067	0.4561	1.56 (0.520–6.771)
p16	- / +	2.30	0.1297		

there are some reports that FAK expression is associated with tumor invasive ability and prognosis [32, 33]. FAKpY397 was not detected in normal tissues and was highly expressed in highly invasive tumors [34]. However, the details of the relationship between the expression of FAK or FAKpY397 and clinicopathological characteristics in OSCC are unknown. Therefore, in the present study, in order to clarify the significance of FAK and FAKpY397 expression in OSCC, we evaluated the relationships between their expression patterns and clinicopathological factors and prognosis.

FAK and FAKpY397 were not detected in normal tissues. On the other hand, their expression in OSCC was enhanced in cancer cells at the invasive front of a tumor. Moreover, FAK and FAKpY397 were extremely highly expressed in cases with a high grade of invasion. Phosphorylation of tyrosine 397 of FAK has been reported to be important for the invasive properties of OSCC cells [35]. Together, these results suggested that the accumulation and activation of FAK occur in cancer cells at the invasive front and may affect cancer cell invasion in OSCC.

We also examined the relationships between clinicopathological factors and the expression of FAK and FAKpY397. The results showed that the expression of FAK and local recurrence, subsequent metastasis, and the mode of invasion were significantly related. There were significant correlations between the expression of FAKpY397 on one hand and, on the other, N classification, local recurrence, and the mode of invasion. In lung squamous cell carcinoma, FAK overexpression and increased FAK phosphorylation play important roles in invasion and metastasis [36]. It is also reported that high expression of FAKpY397 is related to distant metastasis and lymph node metastasis in serous ovarian cancer [37]. Moreover, the expression of FAKpY397 is a significant prognostic factor for the recurrence of gastric cancer [38]. These results suggested that the accumulation and activation of FAK affect local invasion and cervical progression in OSCC.

The investigation of the relationship between the 5-year cumulative survival rate and clinicopathological factors showed that the overexpression of FAK and overexpression of FAKpY397 were significant prognostic factors when present concurrently, but not independent prognostic factors. Moreover, cases with co-expression of FAK and FAKpY397 had poorer prognoses than cases with other expression patterns, although the differences were not significant. It has been reported that nuclear expression of FAKpY397 correlates with prognosis in colorectal cancer [39] There is also a report showing that co-expression of FAK and FAKpY397 affects the survival periods of patients with human gliomas and is an independent prognostic indicator [40]. Finally, there are reports that the expression level of FAK protein affects the susceptibility of tumor cells to various chemotherapies [11, 41, 42]. These results suggest that examining the expression of FAK and FAKp397 is important not only for clarifying the biological characteristics of cancer cells but also for estimating life prognosis in OSCC.

The human papillomavirus (HPV) is a high-risk factor for carcinogenesis, especially for oropharyngeal cancer and cervical cancer [13, 43]. In cervical lesions, overexpression of p16, an alternative marker for HPV, is observed and it is thought to result from increases in the level of the E2F transcription factor, which is released from pRB after binding to the HPV E7 oncoprotein [44]. It is also known that the HPV E6 oncoprotein is bound to FAK via the adhesion factor paxillin [45]. Attenuation of p16 expression has been observed in many cancers, suggesting this marker is a possible prognostic factor [46, 47]. In keeping with these findings, it has been reported that the attenuation or deletion of p16 expression is associated with prognosis in head and neck squamous cell carcinomas [48]. With respect to OSCC, there have been several reports on the relation between p16 expression and this cancer, but those reports concluded that there are few cases in which p16 is expressed and p16 expression does not

affect the prognosis of OSCC [49–51]. In addition, these studies reported that p16-positive cases accounted for as little as 12.9% of OSCC cases, and there was no association between p16 and clinicopathological factors, except for clinical stage, or prognosis. Therefore, it was suggested that there was not much clinical significance of p16 expression in OSCC.

We evaluated the expression of FAK, FAKpY397 and p16 in OSCC tissue. The results suggested that determining the expression of FAK and FAKpY397 is useful not only for examining tumor characteristics but also for assessing the risk of recurrence and subsequent metastasis and viability prognosis. FAK has been established as a key component of the signal transduction pathways triggered by integrins, and we have demonstrated that integrin expression in cancer cells is related to the prognosis in OSCC [52]. Based on these results, we believe that therapy targeting these factors can be expected to improve the prognosis of OSCC. There has been a single report evaluating the clinical efficacy of the integrin inhibitor cilengitide for head and neck cancer [53], but there have been no experimental investigations into therapies targeting integrin for OSCC. On the other hand, for FAK inhibitors, beneficial results have been obtained in glioblastoma [54], melanoma [55] and osteosarcoma [56]. The anti-tumor effects achieved by the combination of a FAK inhibitor (defactinib) and an immunotherapeutic agent for various solid cancers, including pancreatic cancer, ovarian cancer, non-small-cell lung cancer (NSCLC), and mesothelioma, are being assessed in clinical collaborative research studies currently under way [57]. In vitro studies have demonstrated an antitumor effect of FAK inhibitors on OSCC [58]. On the basis of these and other results, we suggest that future treatment strategies targeting FAK will be effective in OSCC as well.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest to declare.

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