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Tyrosine 397 phosphorylation is critical for FAK-promoted Rac1 activation and invasive properties in oral squamous cell carcinoma cells

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Oral squamous cell carcinoma (OSCC) is a common cancer worldwide. Despite advances in diagnosis and therapy, treatment options for patients with metastatic OSCC are few, due in part to the limited understanding of the molecular events involved in the invasion and metastasis of OSCC. In this study, we investigated the expression of focal adhesion kinase (FAK) and its tyrosine 397 phosphorylation (pY397) in the tissue specimens of OSCC. The roles of pY397 in regulating the activities of Rac1 and cortactin and the invasive properties of OSCC cells were further determined. Results from immunohistochemical analyses in 9 benign, 19 premalignant, and 19 malignant oral tissues showed that the immunoreactivity of FAK was observed in 5 benign (56%), 19 premalignant (100%), and 18 malignant tissues (95%), whereas the immunoreactivity of pY397 was only found in 1 of 9 (11%) benign lesions but was observed in 9 premalignant (47%) and 12 malignant (63%) lesions. Compared with the low-invading SCC4 cells, the high-invading OECM-1 cells exhibited higher levels of FAK expression and pY397, correlating with higher levels of GTP-bound Rac1 and cortactin phosphorylation. Manipulation of FAK expression or Y397 phosphorylation in SCC4, FaDu, OECM-1, or HSC-3 cells regulated their Rac1 activities and invasive properties. Furthermore, treatment of NSC23766, a Rac1-specific inhibitor, in OECM-1 and HSC-3 cells led to reduced invasive properties. Nevertheless, knockdown of FAK expression or suppression of pY397 had no effect on the cortactin activity in OECM-1 cells. The data collectively suggest that pY397 plays critical roles in the FAK-promoted Rac1 activation and invasive properties in OSCC cells. Thus, the inhibition of FAK phosphorylation at Y397 or Rac1 activity can serve as a therapeutic strategy for treating patients with metastatic OSCC.
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Oral squamous cell carcinoma (OSCC) is the most common malignancy occurring in the oral cavity that usually arises from the buccal mucosa and tongue. During the tumor progression, OSCC cells gradually spread and invade to neighboring tissues, including the nasal cavity or the maxillary facial structures, and eventually metastasize to neck lymph nodes and distant organs.[1] Similar to other malignant diseases, tumor metastasis is a great challenge for the treatment of OSCC. This notion is supported by the fact that only 50% of diagnosed OSCC patients can survive for 5 years, and this is mainly because OSCC patients continue to die from tumor relapse at regional or distant sites.[2] Despite advances in diagnosis and therapy in past decades, the promising strategy for treating patients with metastatic OSCC is still underdeveloped, due in part to the limited understanding of the molecular events involved in invasion and metastasis of OSCC. The underlying molecular basis of OSCC metastasis deserves intensive study.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase and functionally involves the regulatory processes of multiple cellular events, including adhesion, migration, invasion, proliferation, and survival. Intracellular signaling elicited by integrins or growth factor receptors initiates the phosphorylation of FAK at tyrosine 397 (Y397) that is a critical for c-Src binding and FAK itself activation.[3] The activated FAK/c-Src complex can phosphorylate the downstream signaling molecules and the cytoskeleton-associated proteins, promoting cell migration and invasion.[4] In different cancer

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types, increased expression of FAK is frequently associated with tumor metastasis and poor patient prognosis.[5] In OSCC cells, the expression of FAK in the primary or invasive OSCC tumors is higher than that in benign oral tissues, and modulation of FAK expression or activity regulates the invasive properties of OSCC cells.[6–8] However, the role of Y397 phosphorylation (pY397) in the FAK-promoted invasiveness in OSCC cells and its possible mechanism is mostly unknown.

Several lines of evidence indicate that two actin cytoskeletal regulators, Rac1 and cortactin, are overexpressed in OSCC tumors.[9,10] Importantly, their activities can be modulated by FAK signaling.[11,12] Rac1 is a member of Rho GTPases that cycle between an inactive GDP-bound form and an active GTP-bound form. In response to extracellular signals, the active Rac1-GTP can interact with different effectors and activate multiple intracellular signals that regulate actin cytoskeletal reorganization, vesicle trafficking, and cell migration.[13] In OSCC, elevated expression of Rac1 is found in tumor lesions and its activity can be upregulated by Vav2, correlating with the enhanced invasive properties.[9,14] Differing from Rac1 as an enzyme, cortactin is an adaptor protein that binds and activates the Arp2/3 complex, functioning to regulate the formation of branched actin networks. Different kinases can promote cortactin activity and c-Src is the best-characterized kinase that phosphorylates cortactin at tyrosine residues 421, 466, and 482 in a sequential manner.[15]

In the present study, we examined the expression and pY397 of FAK in the clinical specimens of the oral cavity. We further determined the functional roles of FAK and its pY397 in regulating Rac1 and cortactin activities and invasive properties in OSCC cells.

MATERIALS AND METHODS

Reagents and Antibodies

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Polyclonal anti-FAK, anti-GAPDH, anti-β-actin, and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against phospho-FAK (pY397), phospho-c-Src (pY416), and phospho-cortactin (pY421) were purchased from Cell Signaling (Danvers, MA, USA). Monoclonal anti-Rac1 and anti-cortactin antibodies were obtained from Millipore (Billerica, MA, USA) and Epitomics (Burlingame, CA, USA), respectively. All chemicals were purchased from Sigma (St Louis, MO, USA).

Cell Culture

Human HNSCC cell lines including SCC4, OECM-1,[16] FaDu,[17] and HSC-3 cells[18] were purchased from the BCRC (Biosource Collection and Research Center, Taiwan) and JCRB Cell Bank (Osaka, Japan), or kindly provided by Dr KW Chang (National Yang-Ming University, Taipei, Taiwan). SCC4 and OECM-1 cells were maintained in DMEM/F12 and RPMI 1640 medium, respectively. FaDu and HSC-3 cells were cultured in DMEM medium. Each medium was supplemented with 5% FBS, 2 mM glutamine, and 0.05 mg/ml gentamicin.

Preparation of cDNA Constructs of FAK

The full-length human FAK cDNA carried by the pBluescriptR vector (pBluescriptR-FAK) was obtained from the BCRC that served as a template for PCR reaction. The cDNA was amplified using oligonucleotide primers: (forward): 5′-GCGATTTAAATCTAGCAAAATAATGGCAGCTGCTTACC-3′ (*Swa*I site is underlined), and (reverse): 5′-CGCGCGGCCGCACGTGCTCCTAGGGGAGGCTCA-3′ (*Not*I site is underlined). To construct the FAK-Y397F cDNA into a lentiviral expression vector, we performed site-directed mutagenesis. For the PCR reaction, the pBluescriptR-FAK plasmid was served as a template and the following oligonucleotides will be used as the primers: Y397F forward: 5′-CAGATGATTTTGCTGAGATTATAGATGAAGAAGATACTTACACC-3′ and reverse: 5′-ATCTCAGCAAAATC ATCTGTTTCTGACACAGAGACGGCGTGTGTC-3′ (mutated nucleotide is shown in bold). The mutagenized PCR fragments were sequenced to confirm the mutations and then were amplified using the same primers that are used for amplifying the wild-type FAK cDNA. Subsequently, the mutagenized PCR fragments will be digested by *Swa*I/*Not*I and ligated with the enzyme-cut pCDH-CMV-MCS-EF1-Puro (pCDH) lentiviral vector (SBI, Mountain View, CA, USA).

Lentiviral Infection

The preparation and infection of lentiviruses has been described previously.[19] For expressing FAK proteins in SCC4 or OECM-1 cells, 293FT cells (Invitrogen) were cotransfected with the pCDH vector containing the wild-type or Y397F FAK cDNAs plus packaging plasmids, using PolyJet Transfection Reagent (SignaGen Laboratories, Ijamsville, MD, USA). The control cells were cotransfected with the pCDH vector alone plus packaging plasmids. For gene knockdown experiments, lentiviral vectors carrying shRNAs against the *PTK2* or *CTTN/EMS1* gene were purchased from the RNAi Core at Academic Sinica (Taipei, Taiwan). For the preparation of the shRNA-containing lentiviruses, 293FT cells were cotransfected with the lentiviral expression vector, containing the gene-targeted shRNA and with packaging plasmids. The cells expressing shRNA against the gene encoding firefly luciferases or β-galactosidases served as a shCt control. After a 72-h incubation, the culture medium containing lentiviruses was harvested. For infection, cells grown in six-well plates were incubated with viruses in the presence of 8 μg/ml of polybrene and were centrifuged at 840 *g* for 1 h at 37 °C. The infected cells were then grown for 2–4 days in the absence or presence of 4 μg/ml puromycin (Sigma). The expression level of FAK or cortactin in these infected cells was detected by western blot analysis.

Rac1 Activation Assay

The activity of Rac1 (GTP-bound form) was determined according to the manufacturer's instructions (Millpore,

Billerica, MA, USA). Briefly, cells were washed twice with ice-cold PBS and then lysed with MLB lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM Na_3VO_4, and protease inhibitors). An aliquot of 0.5 mg lysates from each sample was incubated with Pak1 PBD agarose beads for 60 min at 4 °C. The beads were then pelleted by centrifugation at 14000 *g* for 10 s. After washing three times, agarose beads were suspended and boiled in with SDS-PAGE sample buffer, and western blot analysis was performed with antibody against GTP-bound Rac1. In parallel, 50 μg protein extract was used for analyzing total level of Rac1.

Migration and Invasion Assays

An aliquot of 1×10^5 cells were plated on uncoated 24-well inserts having a pore diameter of 8 μm (BD Bioscience, Bedford, MA, USA) for transwell migration assays, or placed on Matrigel-coated 24-well inserts for invasion assays. After 24 h, the cells attached to the upper surface of the membrane were wiped off with a cotton bud and the cells on the lower surface were fixed and stained using the Diff-Quik staining kit. The migrated or invaded cells were counted from randomly selected 4 fields in each transwell membrane under an optical microscope at 100-fold magnification.

Cell Lysis and Immunoblotting

Cell lysates were prepared as previously described.[20] For immunoblotting, an aliquot of total cell lysates in an SDS-PAGE sample buffer was electrophoresed and transferred to a nitrocellulose membrane. After blocking with 5% non-fat milk or BSA in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature (RT), the membrane was incubated with the primary antibodies for 3 h at RT or overnight at 4 °C. After rinsing to remove the excess antibodies, the membrane was incubated with HRP-conjugated secondary antibodies for 1 h at RT. The specific protein was detected by an ECL reagent kit (Amersham, Pittsburgh, PA, USA). The relative ratio of pY421 cortactin or Rac1-GTP was semiquantified by densitometric analysis using ImageJ (NIH image, Bethesda, MA, USA).

Tissue Microarray and Immunohistochemistry

Specimens analyzed included 11 benign, 19 premalignant, and 19 malignant lesions of the oral cavity that were collected from patients who underwent surgical resection at Mennonite Christian Hospital (Hualien, Taiwan). This procedure was approved by the institutional review board of the Buddhist Tzu Chi General Hospital (IRB101-115), and written informed consents were obtained from all participants. Tissue specimens were incorporated into a tissue microarray. The procedure of immunohistochemical staining has been described previously.[19] Briefly, the sections were deparaffinized in xylene, rehydrated with graded ethanol, and then boiled in 1 mM EDTA (pH 8.0) for 5 min in an autoclave. After three washes with 3% H_2O_2, the sections were blocked with a protein block serum (Dako Glostrup, Denmark) for 5 min. Subsequently, the sections were incubated with the mouse monoclonal anti-FAK antibody (Millpore) or the pY397-FAK polyclonal antibody (Abcam, Cambridge, MA, USA) in a 1:100 dilution for 30 min, and the signal was then amplified using an Envision kit (Dako) according to the manufacturer's protocol. All sections were counterstained with hematoxylin for 2.5 min. The immunostaining intensity of FAK or pY397 was evaluated using a light microscope, and the scores were divided into four grades: negative (0), low (1+), moderate (2+), and high (3+). Tumor cores with $>10\%$ of cells staining 1+ or greater were defined as positive.

Statistical Analysis

Significance for group comparisons was assessed by Student's two-tailed *t*-test or a one-way ANOVA. The χ^2 test was used for comparison between categorical variables. A *P*-value of <0.05 was considered significant. All statistical analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Elevation of FAK Expression and Its pY397 in Premalignant and Malignant Lesions of the Oral Cavity

The levels of FAK expression and its pY397 were immunohistochemically analyzed in the clinical specimens of the oral cavity. As shown in Figure 1, no immunoreactivity of FAK was detected in the benign lesion, but the low and high cytoplasmic stainings were found in premalignant and malignant lesions, respectively. Similarly, no immunoreactivity of pY397 was observed in the benign lesion, whereas the premalignant tissue showed low immunoreactivities of pY397 at the region of cell–cell contacts and the malignant tissue exhibited moderate to high pY397 immunoreactivity in the cytoplasm (Figure 1). Table 1 summarizes the immunostaining intensity of total FAK and pY397 in the series of 9 benign, 19 premalignant, and 19 malignant oral tissues. The FAK expression was found in 5 of 9 cases (5/9; 56%) with a low immunoreactivity. In contrast, the immunoreactivity of FAK was detected in all premalignant tissues (19/19; 100%) and 2 of them (2/19; 10%) showed moderate to high expression. Similarly, 18 of 19 malignant lesions (18/19; 95%) displayed FAK immunoreactivity and 8 of them (8/19; 42%) exhibited a moderate to high FAK expression. The results of χ^2 tests indicated the significant difference in the intensity of FAK immunostaining among benign, premalignant, and malignant lesions of the oral cavity ($P=0.0003$). The statistical significance of FAK expression also exhibited between benign and premalignant ($P=0.006$) or malignant lesions ($P=0.011$). Similar to the pattern of FAK expression, the immunoreactivity of pY397 was only found in 1 of 9 (1/9, 11%) benign lesions

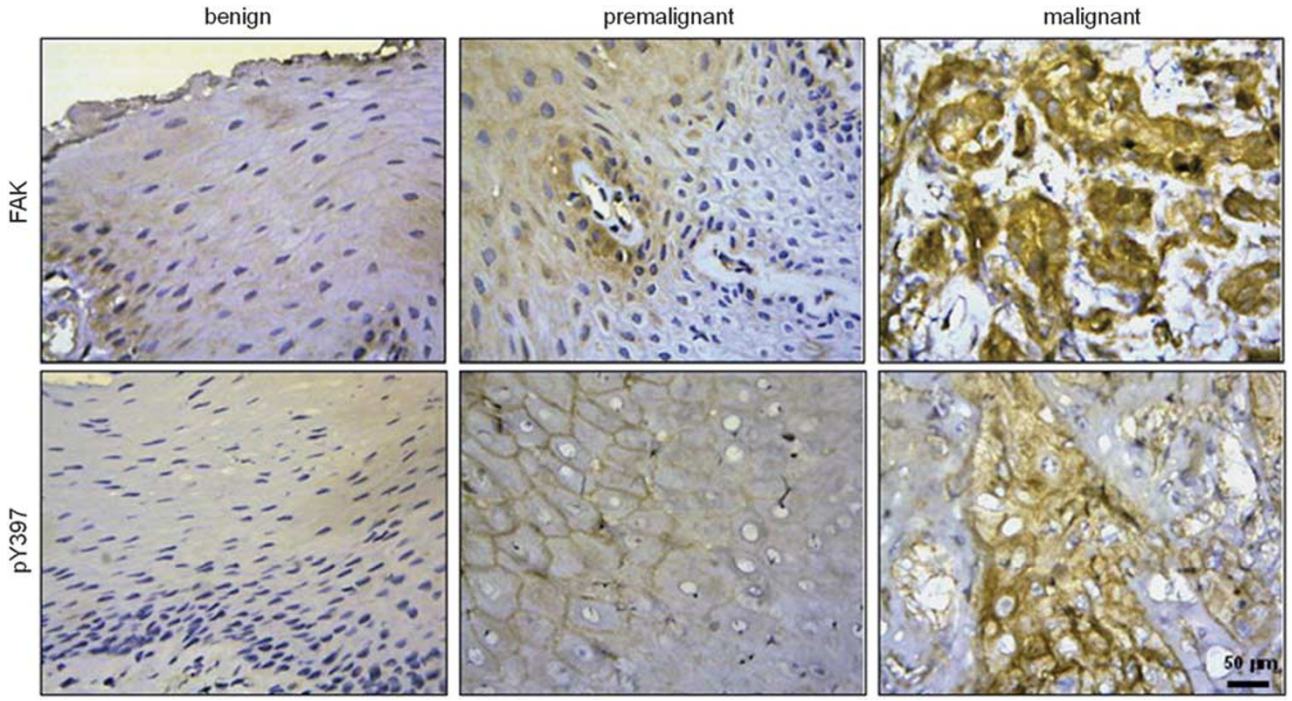

Figure 1 Immunohistochemical analyses of FAK expression and its Y397 phosphorylation in benign, premalignant, and malignant lesions of the oral cavity. Magnification ×400. The scale bar represents 50 μm.

Table 1 FAK expression and Y397 phosphorylation in different lesions of the oral cavity

	Immunoreactivity			
Tissue types	Negative (%)	Low *(%)*	Moderate to high (%)	*P*-value
Total FAK				0.0003[a]
Benign ($n=9$)	4 (44%)	5 (56%)	0 (0%)	
Premalignant ($n=19$)	0 (0%)	17 (90%)	2 (10%)	0.006[b]
Malignant ($n=19$)	1 (5%)	10 (53%)	8 (42%)	0.011[c]
pY397 FAK				0.095[a]
Benign ($n=9$)	8 (89%)	1 (11%)	0 (0%)	
Premalignant ($n=19$)	10 (53%)	7 (37%)	2 (10%)	0.16[b]
Malignant ($n=19$)	7 (37%)	7 (37%)	5 (26%)	0.032[c]

[a]*P*-value for comparison between benign, premalignant and malignant lesions.
[b]*P*-value for comparison between benign and premalignant lesions.
[c]*P*-value for comparison between benign and malignant lesions.

but was observed in 9 (9/19, 47%) of the premalignant and 12 (12/19, 63%) of the malignant lesions. Among them, 2 premalignant and 5 malignant tumors showed moderate to high expression. Although there was no significant difference in the immunostaining intensity of pY397 among lesions ($P=0.095$), the statistical significance of pY397 did exhibit between benign and malignant lesions ($P=0.032$).

FAK Expression and pY397 Correlates with the Activities of Rac1 and Cortactin and the Invasive Properties in OSCC Cells

To determine the involvement of FAK signaling in regulating the invasive properties of OSCC cells, we initially examined the expression and activities of FAK, Rac1, and cortactin in SCC4 and OECM-1. As shown in Figure 2a, OECM-1 cells

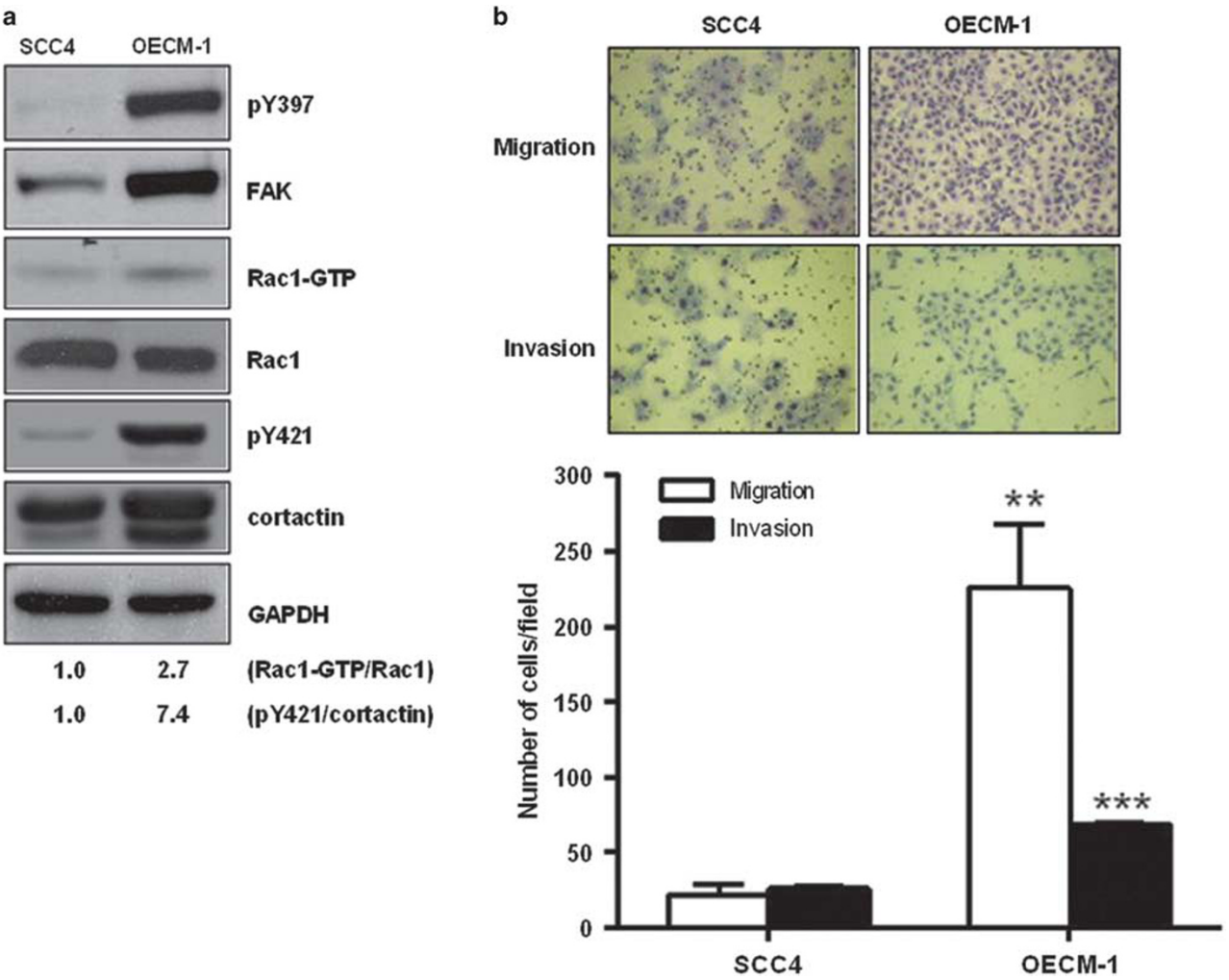

Figure 2 FAK expression and pY397 correlate with the Rac1 activity, cortactin phosphorylation, and invasive properties in SCC4 and OECM-1 cells. (**a**) Total lysates were used for immunoblotting with antibodies against the phospho-FAK at Tyr-397 (pY397), phospho-cortactin at Tyr-421 (pY421), FAK, Rac1 (including GTP-bound and total Rac1), and cortactin proteins. GAPDH served as the internal control. The relative ratio of the GTP-bound Rac1 level to the total Rac1 level or the pY421 level to the cortactin expression level in OECM-1 cells was calculated and then normalized to that of SCC4 cells. (**b**) The migration and invasion of SCC4 and OECM-1 cells. An aliquot of 1×10^5 cells were plated on the upper chamber of the transwell containing 1% FBS-supplemented medium with 2 mM hydroxyurea, whereas the 5% FBS-supplemented medium containing 2 mM hydroxyurea was added on the lower chamber. Cells were allowed to migrate or invade for 24 h and the representative images are shown at 100-fold magnification (upper panel). The numbers of migrated or invaded cells are shown as the mean ± s.d. from three independent experiments (lower panel). Each set of experiments was conducted in duplicate. $^{**}P<0.01$, $^{***}P<0.001$ *vs* the number of SCC4 cells.

expressed a higher level of FAK with a greater pY397 as compared with low levels of FAK expression and pY397 in SCC4 cells. Furthermore, OECM-1 cells exhibited high levels of GTP-bound Rac1 and cortactin phosphorylation at Y421, ~2.7- and 7.4-fold respectively, higher than those in SCC4 cells. However, the total Rac1 and cortactin expression levels were quite similar between the two cell lines (Figure 2a). We next compared the motility and invasiveness of SCC4 and OECM-1 cells. Because the growth rate of OECM-1 cells is much higher than that of SCC4 cells,[19] we performed the migration and invasion assays in the presence of hydroxyurea, a cell cycle blocker, in order to eliminate any effect caused by differential growth properties. As shown in Figure 2b, the number of migrated or invaded OECM-1 cells was ~3–10 fold higher than those in SCC4 cells ($P<0.01$). These data collectively suggested that the expression and pY397 of FAK were correlated with the activities of Rac1 and cortactin and invasive properties in OSCC cells.

Modulation of FAK Expression and Activity Regulates the Activity of Rac1 and the Invasive Properties of OSCC Cells

We next examined whether FAK can modulate Rac1 activity in OSCC cells. As shown in Figure 3a, elevation of the wild-type FAK expression in SCC4 cells led to an increased level of GTP-bound Rac1. In contrast, knockdown of FAK

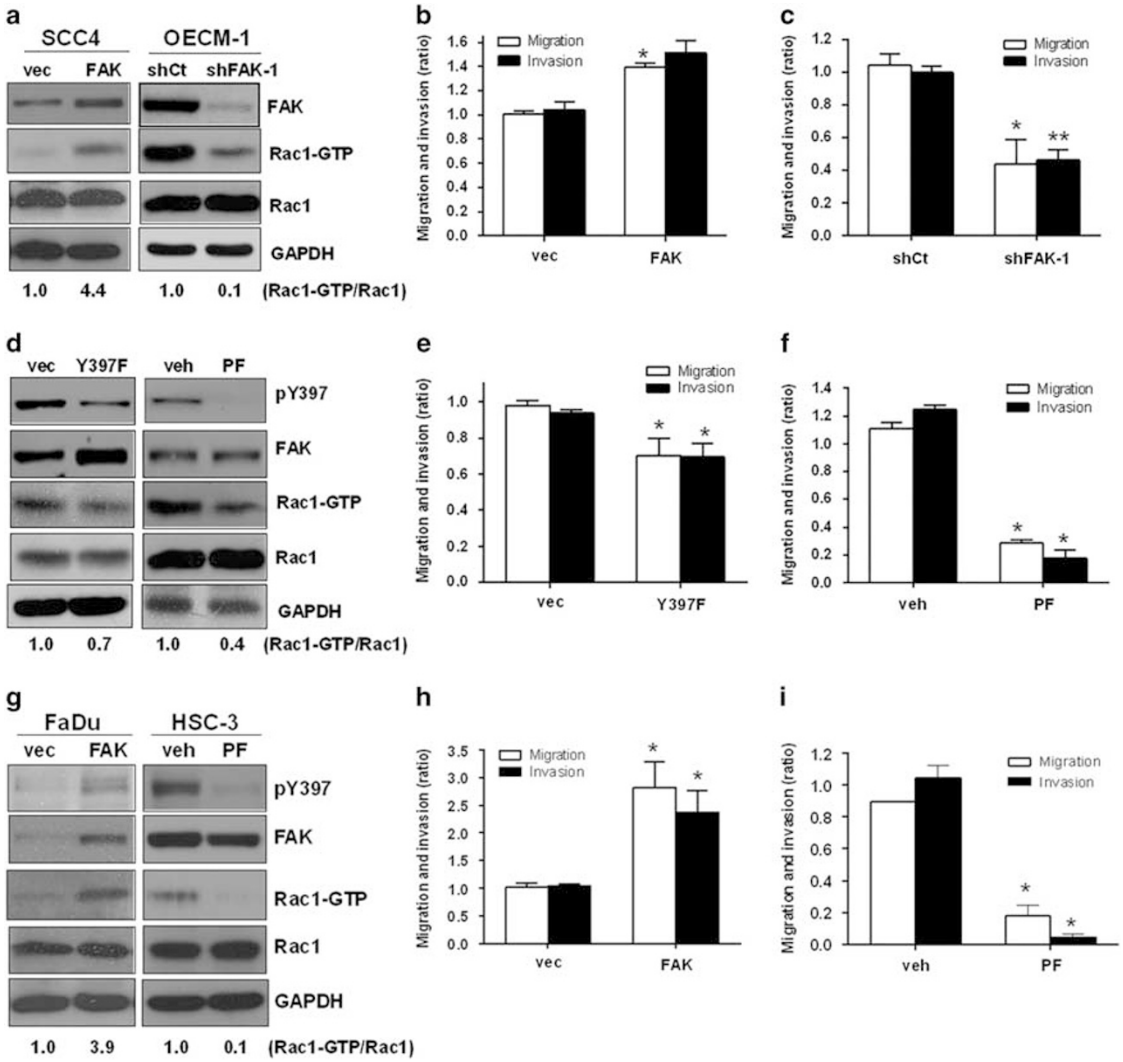

Figure 3 Modulation of FAK expression or pY397 regulates the Rac1 activity and invasive properties in OSCC cells. (**a**) SCC4 cells were infected with viruses carrying the vector control or the wild-type FAK cDNA. In addition, OECM-1 cells were infected with viruses carrying control or *PTK2*-targeted shRNA, that is, shCt or shFAK-1, respectively. After an additional 48–72 h of incubation, cells were harvested for immunoblotting with antibodies against FAK and Rac1 (including GTP-bound and total Rac1). GAPDH served as the internal control. The relative ratio of the GTP-bound Rac1 level to the total Rac1 level was calculated and then normalized to that of vector or shCt control cells. (**b** and **c**) For the migration and invasion assays, FAK-overexpressed SCC4 cells, FAK-knockdown OECM-1 cells, or the corresponding control cells were plated on the upper chamber of the transwell containing 1% FBS-supplemented medium, whereas the 5% FBS-supplemented medium was added on the lower chamber. (**d**) OECM-1 cells were infected with viruses carrying the vector control or the Y397F mutant FAK and incubated for 72 h. Alternatively, OECM-1 cells were treated with 1 μM PF-431396 (PF) or equal volume of solvent (DMSO) as the vehicle control (veh) for 24 h. Cells were then harvested and total lysates were used for immunoblotting with antibodies against the levels of pY397, FAK, GTP-bound and total Rac1, and GAPDH. (**e** and **f**) The migration and invasion assays of the Y397F-overexpressed OECM-1 cells, PF-431396-treated OECM-1 cells, and the corresponding control cells. (**g**) Total lysates from the FAK-overexpressed FaDu cells, PF-431396-treated HSC-3 cells, and their corresponding control cells were harvested and prepared for western blot analyses or (**h** and **i**) migration and invasion assays. The relative ratios of cell migration and invasion are presented as the mean ± s.d. from two to three independent experiments. Each set of experiments was conducted in duplicate. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the ratio of corresponding control cells.

expression in OECM-1 cells resulted in decreased Rac1 activity. Accordingly, FAK-overexpressed SCC4 cells exhibited enhanced migratory and invasive capabilities, ~40% higher than those in the vector control cells (Figure 3b), whereas FAK-knockdown OECM-1 cells showed significant inhibition in migratory and invasion capabilities (Figure 3c). To further examine whether pY397 is critical for FAK-regulated Rac1 activation and invasive properties, the Y397F mutant acting as a dominant-negative mutant of FAK was overexpressed in OECM-1 cells and the impacts on the Rac1 activity and cell invasion were analyzed. Compared with the vector control cells, the Y397F-overexpressed OECM-1 cells exhibited an elevated level of FAK proteins but had a decreased level of pY397. Importantly, expression of Y397F caused a decreased

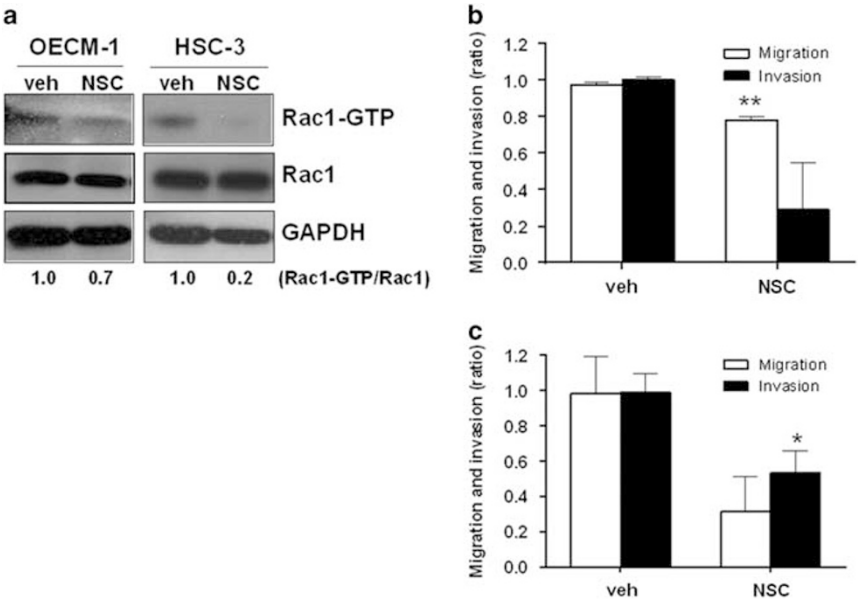

Figure 4 Inhibition of Rac1 activity suppresses the migration and invasion of OECM-1 and HSC-3 cells. (**a**) OECM-1 and HSC-3 cells were treated with 50 μM NSC23766 (NSC) or equal volume of solvent (H_2O) as the vehicle control (veh) for 24 h. Cells were then harvested and total lysates were used for immunoblotting with antibodies against the levels of GTP-bound and total Rac1. The relative ratio of the GTP-bound Rac1 level to the total Rac1 level was calculated and then normalized to that of vehicle control cells. (**b** and **c**) Effects of NSC23766 (50 μM) on the migration and invasion of OECM-1 and HSC-3 cells. The data were expressed as the mean ± s.d. from two independent experiments. Each set of experiments was conducted in duplicate. $^*P<0.05$, $^{**}P<0.01$ *vs* the ratio of vehicle control cells.

Rac1 activity, ~30% lower than that of vector control cells (Figure 3d, left panel). We further examined the impact of inhibiting pY397 on the Rac1 activity by treating OECM-1 cells with PF-431396, a highly selective pyrimidine-based inhibitor of FAK.[21] As shown in Figure 3d (right panel), treatment of 1 μM PF-431396 caused decreases in the levels of pY397 and Rac1-GTP as compared with the vehicle control. Coordinating with the changes in Rac1 activity, expression of Y397F mutant or treatment of PF-431396 led to inhibited cell migration and invasion (Figures 3e and f).

For confirming the observations above in SCC4 and OECM-1 cells, we next performed similar experiments in two other cell lines, FaDu and HSC-3. FaDu is a hypopharyngeal SCC cell line,[17] whereas HSC-3 is a tongue SCC cell line with high metastatic potential.[18] Similar to the results from SCC4 and OECM-1 cells, elevated FAK expression in FaDu cells led to increased Rac1 activity, approximately fourfold higher than that in the vector control cells (Figure 3g, left panel). Treatment of PF-431396 in HSC-3 cells caused great decreases in the levels of pY397 and Rac1-GTP (Figure 3g, right panel). Importantly, FAK-expressed FaDu cells exhibited enhanced migratory and invasive capabilities (Figure 3h), whereas treatment of PF-431396 in HSC-3 cells resulted in inhibited migration and invasion (Figure 3i). These data collectively suggested that FAK could regulate Rac1 activity in OSCC cells and that pY397 is important for FAK-modulated Rac1 activity and invasive properties.

Inhibition of Rac1 Activity Restrains the Migration and Invasion of OSCC Cells

To further examine the role of active Rac1 in FAK-promoted invasion in OSCC cells, we analyzed the effects of NSC23766, a Rac1-specific inhibitor,[22] on the migration and invasion of OECM-1 and HSC-3 cells. As shown in Figure 4a, treatment of 50 μM NSC23766 in both cells led to reduced levels of Rac1-GTP, ~30–80% lower than that of vehicle control cells. Importantly, inhibition of Rac1 activity also caused inhibited cell migration and invasion (Figures 4b and c). These data suggested the role of Rac1 activity in promoting the migration and invasion of OSCC cells.

Suppression of FAK Expression and pY397 has No Effect on Cortactin Phosphorylation in OECM-1 Cells

We next examined the role of cortactin in modulating the migration and invasion of OSCC cells. As shown in Figure 5a, cortactin-knockdown OECM-1 cells contained low levels of cortactin expression and phosphorylation compared with the control cells (shCt). After 24 h of culture, cortactin-knockdown cells exhibited lower ratios of cell migration and invasion, ~70–80% less than that of shCt control cells (Figure 5b). To further determine whether FAK can modulate cortactin activity, the expression and phosphorylation levels of cortactin were examined in FAK-knockdown OECM-1 cells. Compared with the shCt control cells, the FAK-knockdown cells showed decreased FAK expression and pY397, associating with reduced c-Src phosphorylation.

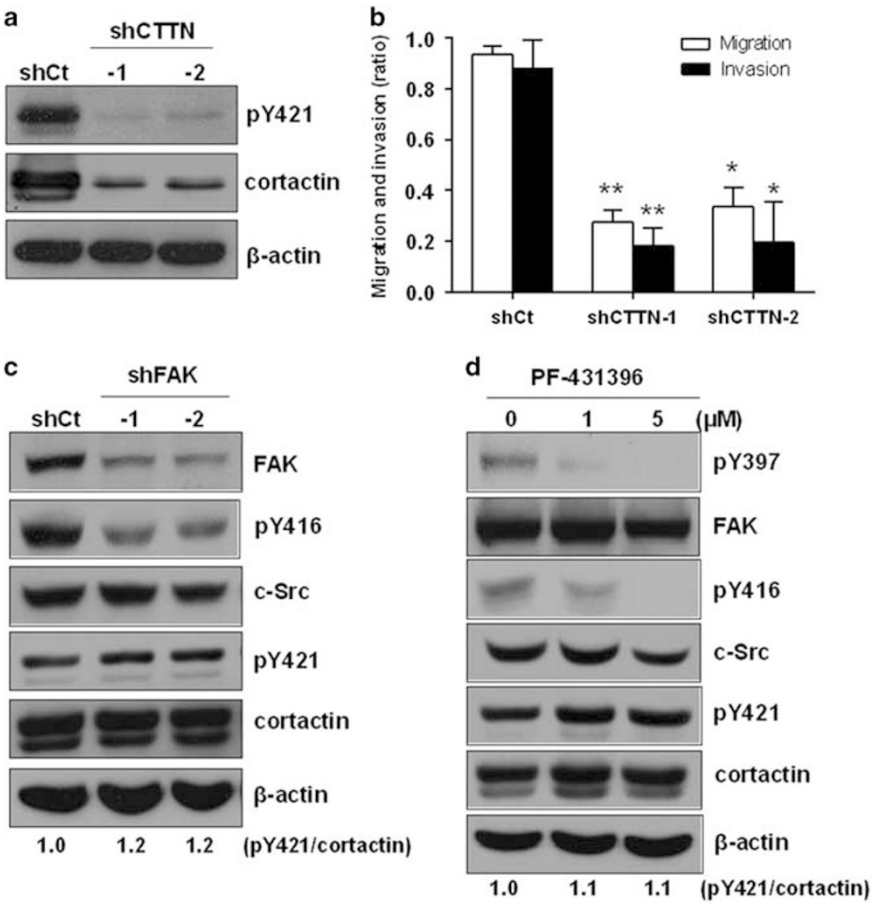

Figure 5 Cortactin activity was required for promoting the invasiveness of OECM-1 cells but was not regulated by FAK/c-Src signaling. (**a**) Cells infected with viruses carrying the control (shCt) or *CTTN*-targeted shRNAs (shCTTN-1 and shCTTN-2). After 2 days of viral infection, cells were harvested and total lysates were prepared for western blot analyses. (**b**) Cells were plated on the upper chamber of the transwell containing 1% FBS-supplemented medium with 2 mM hydroxyurea, whereas the 5% FBS-supplemented medium containing 2 mM hydroxyurea was added on the lower chamber. Cells were allowed to migrate or invade for 24 h and the ratio of cell migration and invasion are shown as the mean ± s.d. from two independent experiments. Each set of experiments was conducted in duplicate. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the ratio of shCt control cells. (**c**) Cells infected with viruses carrying the control (shCt) or *PTK2*-targeted shRNAs (shFAK-1 and shFAK-2). After 2 days of viral infection, cells were harvested and total lysates were prepared for immunoblotting with antibodies against the protein or phosphorylation levels of FAK, c-Src, and cortactin. (**d**) Cells were treated with 1 or 5 μM FAK inhibitor PF-431396 for 24 h. Cells treated with an equal volume of solvent (DMSO) served as a vehicle control. Cell lysates were prepared for western blot analyses. The relative ratio of the pY421 level to the cortactin expression level was calculated and then normalized to that of shCt or vehicle control cells.

However, no change in cortactin expression or phosphorylation was observed in these cells (Figure 5c). Similar results were also found in the cells treated with PF-431396, a FAK inhibitor. As shown in Figure 5d, treatment of 1 and 5 μM PF-431396 caused decreases in pY397 and c-Src phosphorylation levels in a dose-dependent manner but had no inhibitory effect on cortactin phosphorylation. These results indicated that cortactin could functionally regulate the migration and invasion of OECM-1 cells, but its activity was not modulated by FAK/c-Src signaling.

DISCUSSION

FAK is highly expressed in OSCC tumors and its expression is correlated with the metastatic activity. However, the functional role of Y397 phosphorylation in FAK-modulated invasive properties in OSCC cells and its underlying molecular mechanism remains unclear. In this study, we examined the clinical significance of FAK expression and its pY397 in benign, premalignant, and malignant lesions of the oral cavity. We further determined the roles of FAK and its pY397 in regulating the activities of Rac1 and cortactin and the invasive properties in OSCC cells. Our main finding was that pY397 was critical for FAK-modulated migration and invasion in OSCC cells and its underlying molecular mechanism was associated with the activation of Rac1 but not cortactin.

Results from our immunohistochemical analyses showed that the expression of FAK was detected in 95% of OSCC tumors examined and 42% of them exhibited a moderate to

high expression. Similarly, a study in 80 OSCC archival specimens showed that FAK expression was found in all tumors and 55 of them (69%) displayed a moderate to high expression.[7] A separate study also reported that FAK is expressed in 60 of 67 OSCC tumors (90%) and 38 of them (57%) exhibit a moderate to high expression.[23] Compared with the intensive studies in FAK expression in OSCC, relatively few researches examined the levels of pY397 in benign and malignant oral lesions. In this study, we showed that the ratios of pY397-positive immunostaining significantly increased from 11% (1/9) in benign, 47% (9/19) in premalignant, and 63% (12/19) in malignant lesions ($P=0.036$). Interestingly, no significant difference was found in the intensity of pY397 immunoreactivity among lesions ($P=0.095$), due mainly to the less difference in pY397 intensity between benign and premalignant lesions ($P=0.16$). Thus, our data clear indicated that the immunostaining intensity of FAK expression was significantly increased in the premalignant stage ($P=0.006$), whereas the intensity of pY397 was not elevated until OSCC was developed ($P=0.032$). We also found that FAK expression in 18 OSCC tumors was significantly correlated with tumor differentiation ($P=0.04$) but not tumor size; however, the level of pY397 was not associated with tumor differentiation or size (Supplementary Table S1).

The activation of FAK is a multistep process and pY397 is a critical event for FAK activation and FAK-regulated functions.[24] Our data clearly showed that the levels of FAK expression and pY397 were correlated with the invasive properties in different OSCC cell lines. Changes in pY397 by manipulating FAK expression in OSCC cells can regulate their motility and invasiveness. Furthermore, inhibition of pY397 by treating PF-431396 or blockage of pY397-mediated signaling by overexpressing the Y397F mutant could modulate the migration and invasion of OSCC cells. Similarly, treatment with FAK-related nonkinase (FRNK) in HNSCC cells leads to decreased pY397 and inhibited cell invasion.[25] In addition, TAE226 that blocks the ATP binding site of FAK and suppresses pY397 greatly inhibits OSCC cell migration and invasion.[8] Alternatively, 1,2,4,5-benzenetetramine tetrahydrochloride (Y15), a molecule inhibiting specifically to the Y397 site without affecting ATP binding, can effectively suppress the adhesion and tumorigenesis of pancreatic cancer cells.[26] These results collectively support the notion that pY397 is required for FAK-promoted invasion in malignant cells. Thus, the pY397 of FAK could be an optimal target for treating patients with the metastatic malignancies.

Our data showed that manipulation of pY397 status in OSCC cells caused an impact on Rac1 activity, suggesting the important role of FAK autophosphorylation in Rac1 activation. However, the mechanism of how pY397 can regulate Rac1 activity is unclear. Because of the lack of evidence regarding Rac1 mutations in human cancers, deregulated Rac1 activity is closely associated with the elevated expression or aberrant activation of Rac1-associated guanine nucleotide exchange factors (GEFs).[27] Thus, FAK may activate Rac1 in OSCC cells by phosphorylating Rac1-associated GEFs. This notion is supported by the observations that Tiam1 and Vav2, two Rac1-associated GEFs, can be phosphorylated by c-Src and, in turn, leads to Rac1 activation and cell invasion.[14,28] Because pY397 is the critical step for FAK-mediated c-Src activation, the phosphorylation status of Y397 can greatly determine c-Src activation, and thus regulate the activities of GEFs and Rac1. Alternatively, FAK-promoted Rac1 activation can be mediated by GEF-independent mechanisms. Studies indicated that FAK can upregulate Rac1 activity through the activation of PI3K.[11,29] In addition, FAK can phosphorylate RhoGDI and cause the dissociation of RhoGDI from Rac1, thus promoting Rac1 activation.

Accumulated evidence demonstrates that *CTTN/EMS1* gene is amplified and cortactin is highly expressed in OSCC tumors that is associated with nodal metastasis of the disease.[30,31] The critical role of cortactin in modulating cell migration and invasion is further supported by the *in vitro* study that knockdown of cortactin in HNSCC cells leads to reduced motility and invasiveness.[32] Similarly, our data clearly showed that the decrease in cortactin expression led to inhibited migration and invasion in OSCC cells. However, differing from previous reports that cortactin activity is regulated by FAK or c-Src, our data indicate that knockdown of FAK expression or inhibition of pY397 in OECM-1 cells resulted in reduced c-Src activity but had no effect on their cortactin phosphorylation. Although the cause of differential observation may due to the cell-type specificity, two possible reasons may be considered. First, studies in c-Src- or FAK-regulated cortactin phosphorylation and activity were mostly done by fibroblasts or embryonic cells. It may raise a concern of whether the regulatory mechanism of cortactin phosphorylation and activity in epithelial cells is similar to that in fibroblasts or embryonic cells. Second, several tyrosine kinases other than FAK/c-Src, such as Ack1 and c-Abl, can phosphorylate cortactin.[33,34] Whether aberrant expression or activation of these kinases in OSCC cells can cause cortactin phosphorylation in a FAK/c-Src-independent manner deserves further investigation.

Although Kurio *et al*[8] first reported the immunoreactivity of pY397 in different lesions of the oral cavity, their works only provided the images of staining without the statistical data because of the small sample size with five cases. In contrast, we analyzed 47 clinical specimens and clearly showed the statistical significance of FAK expression or pY397 in benign, preliminary, or malignant tissues. Thus, our study provided statistical, quantitative results as compared with the work of Kurio *et al*[8]. Furthermore, we demonstrated the functional role of FAK and its pY397 in regulating the Rac1 activity and the migration and invasion in OSCC cells. We performed cDNA expression, RNAi-mediated gene knockdown, and FAK or Rac1 inhibitor treatment in four HNSCC cell lines. Importantly, we expressed Y397F mutant

in cells to confirm the role of pY397 in modulating Rac1 activity and invasive capability. However, in the work of Kurio *et al*,[8] only one cell line (SAS) was used for examining the effects of FAK inhibitor TAE226 on cell migration and invasion without further mechanism-related study. Thus, we took a step from the work of Kurio *et al*[8] to further demonstrate the underlying mechanism that FAK signaling via the activation of Rac1, but not cortactin, promoted OSCC cell migration and invasion.

Because of the oncogenic function of FAK in OSCC progression, small-molecule inhibitors aiming to block FAK kinase activity or inhibit Y397 phosphorylation may have therapeutic benefits for patients with OSCC. Indeed, several FAK inhibitors, including PF-00562271, PF-04554878, GSK-2256098, VS-4718, and VS-6063 (Defactinib), have undergone or completed the phase I trial (http://www.clinicaltrials.gov). Compared with FAK inhibitors, Rac1 inhibitors were under development.[35,36] Hopefully, the development of these inhibitors may provide us a new therapeutic option for both efficacious and nontoxic treatment of OSCC in the near future. In summary, we have demonstrated the elevated FAK expression and its pY397 in OSCC tumors and cell lines. Furthermore, pY397 was critical for FAK-promoted Rac1 activation and invasive properties in OSCC cells. Thus, the inhibition of FAK phosphorylation at Y397 or Rac1 activity can serve as a therapeutic strategy for treating patients with metastatic OSCC.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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