Original Article

VEGF-C/Flt-4 axis in tumor cells contributes to the progression of oral squamous cell carcinoma via upregulating VEGF-C itself and contactin-1 in an autocrine manner

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Received August 16, 2018; Accepted September 10, 2018; Epub October 1, 2018; Published October 15, 2018

Abstract: Tumor cell-derived vascular endothelial growth factor (VEGF)-C has been primarily implicated in promoting lymphangiogenesis by activating Flt-4 (VEGFR-3) expressed on lymphatic endothelial cells via a paracrine mechanism. Flt4 has also been shown to be expressed selectively in subsets of cancer cells. However, little is known about the functional role of VEGF-C/Flt4 signaling via an autocrine mechanism, as well as the clinicopathological implication of the VEGF-C/Flt4 axis and its downstream effector molecules, in head and neck squamous cell carcinoma (HNSCC), including oral squamous cell carcinoma (OSCC). In the present study, we detected Flt-4 expression selectively in several HNSCC cell lines by quantitative PCR, and its internalization reflecting receptor activation was confirmed by immunocytochemistry in SAS and HO1U1 cells. Flt-4 stimulation upregulated the expression of contactin-1 (CNTN-1, a neural cell adhesion molecule) and VEGF-C itself in SAS cells, while Flt-4 inhibition downregulated the expression of CNTN-1 in both SAS and HO1U1 cells and that of VEGF-C itself in SAS cells. In vitro cell proliferation and migration assays using SAS cells demonstrated that both cell proliferation and migration were promoted by Flt-4 stimulation, while those were suppressed by Flt-4 inhibition. Clinicopathological factors and immunohistochemical expression of Flt-4, VEGF-C, and CNTN-1 in tumor cells were evaluated using surgical specimens from patients with tongue squamous cell carcinoma. We found a significant correlation of CNTN-1 expression with both VEGF-C and Flt-4 expression, but not between VEGF-C and Flt-4. Multivariate logistic regression analysis revealed that T classification (P = 0.003), lymphatic invasion (P = 0.024), and Flt-4 expression in tumor cells (P = 0.046) were independently predictive of neck lymph node metastasis. These results suggest that the VEGF-C/Flt-4 axis in tumor cells enhances tumor cell proliferation and migration via upregulating the expression of VEGF-C itself and CNTN-1 in an autocrine manner, thereby contributing to cancer progression of OSCC, including neck metastasis. Hence, targeting the VEGF-C/Flt-4 axis in tumor cells can be an attractive therapeutic strategy for the treatment of cancer.

Keywords: Flt-4, VEGF-C, contactin-1, tumor cell, oral squamous cell carcinoma, autocrine manner, proliferation, migration, metastasis

Introduction

As the most critical events in cancer progression, invasion and metastasis represent the aggressive phenotype of malignant tumors [1]. Metastasis, the leading cause of cancer mortality, consists of a series of sequential independent steps, each of which is regulated by a wide variety of molecular mechanisms [2, 3]. Clinical and pathological observations have shown that
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a regional lymph node is the first common site for metastasis to develop for a majority of carcinomas. Indeed, lymph node metastasis has been one of the most critical adverse prognostic factors in patients with malignant solid tumors, including head and neck squamous cell carcinoma (HNSCC) [4-6], for which the survival rate has remained unchanged over the past three decades despite various advances in combined multi-modality therapy. Thus, it is imperative to develop more effective strategies based on a better understanding of the molecular mechanisms underlying lymphatic metastasis.

During the process of lymph node metastasis, pre-existing afferent lymphatic vessels and/or newly formed lymphatic capillaries serve as an avenue for the dissemination of tumor cells [7-11]. The vascular endothelial growth factor (VEGF)/VEGF receptor (VEGF-R) system is closely involved in the growth, invasion, and metastasis of solid neoplasms by promoting angiogenesis and lymphangiogenesis [12-14]. While VEGF-A basically induces angiogenesis by activating the cognate tyrosine kinase receptors, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), VEGF-C and VEGF-D principally stimulate lymphangiogenesis by activating VEGF-R3 (Flt-4) [12-14]. During cancer progression, increased expression of VEGF-C in tumor tissue has been reported to correlate with regional lymph node metastasis in several malignancies, including HNSCC [7, 15-20]. Because Flt-4 is primarily expressed on lymphatic endothelial cells and activated by binding tumor cell-derived VEGF-C, such tumor-associated lymphangiogenesis is maintained via a paracrine mechanism in the tumor microenvironment, which has been assumed to be the principal mechanism by which VEGF-C enhances the potential for lymphatic metastasis [7-11].

However, several studies have reported that tumor lymphangiogenesis is not necessarily required for and does not affect lymph node metastasis [21-23]. Furthermore, some studies have shown that enhanced expression of VEGF-C neither correlates with increased lymphangiogenesis nor promotes the formation of functional lymphatics [22, 24, 25]. These observations suggest that VEGF-C/Flt-4 signaling may play alternative functional roles independent of lymphangiogenesis. In addition, a number of recent studies have demonstrated that Flt-4 is expressed not only in endothelial cells and Kaposi’s sarcoma, a disease with a possible lymphatic/vascular endothelial cell origin [26, 27], but also selectively in specific subsets of a variety of cancer cells such as colon cancer [28], uterine cervical malignancy [29], malignant mesothelioma [30], leukemia [31, 32], endometrial carcinoma [33], HNSCC [34, 35], prostate cancer [36], non-small cell lung carcinoma (NSCLC), lung adenocarcinoma [37], breast cancer [38-41], gastric cancer [42-44], oral squamous cell carcinoma (OSCC) [45, 46], melanoma [47], esophageal carcinoma [48], and ovarian carcinoma [49] cells, implying the existence of an autocrine stimulation mechanism of VEGF-C/Flt-4 signaling in tumor cells.

However, a limited number of those studies examined the functional role of VEGF-C/Flt-4 signaling in cellular behavior along with its downstream effectors; while a few studies showed the promotion of tumor cell proliferation without an effect on cell mobility [43, 46], others reported the enhancement of tumor cell migration but not proliferation [37] or the stimulation of both [44, 48]. Thus, the exact role of the VEGF-C/Flt-4 autocrine system and its downstream mechanism in tumor cells remain controversial. Interestingly, a study using a dominant-negative Flt-4 (the cytoplasmic domain-deleted Flt-4)-overexpressing cell model showed that the VEGF-C/Flt-4 axis functions as an autocrine loop that positively regulates the expression of VEGF-C itself to enhance the proliferation activity of OSCC cells [46]. Contactin-1 (CNTN-1), a glycosylphosphatidylinositol (GPI)-anchored neural cell adhesion molecule (NCAM) of the immunoglobulin (Ig) superfamily, was shown to be a downstream effector of the VEGF-C/Flt-4 axis that leads to increased migration of lung adenocarcinoma, gastric cancer, and esophageal cancer cells [37, 44, 48]. However, the functional role and downstream effectors of the VEGF-C/Flt-4 autocrine mechanism in HNSCC, including OSCC, have not been well studied to date. In addition, the clinical implication of the VEGF-C/Flt-4 axis and its possible downstream molecules in HNSCC, including OSCC, remain to be elucidated.

We conducted the present study to determine whether the stimulation and inhibition of the
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VEGF-C/Flt-4 axis regulate the expression of its possible effectors, CNTN-1 and VEGF-C itself, in OSCC cells in an autocrine manner, as well as their proliferation and migration activity. We also aimed to clarify whether the expression of Flt-4, VEGF-C, and CNTN-1 in tumor cells in the specimens are correlated with the clinicopathological parameters, including neck lymph node metastasis, of patients with tongue squamous cell carcinoma (TSCC).

Materials and methods

Cell culture

We used nine cell lines established from human HNSCC: HSC-2 and HO1U1 cells derived from the floor of the mouth; HSC-3, HSC-4, SAS, SCC4, and SCC25 cells from the tongue; and FaDu and Detroit562 cells from the pharynx. The human microvascular endothelial cell line HMVEC was used as the positive control for Flt-4 expression. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (for HSC-2, HSC-3, HSC-4, FaDu, and Detroit562) or a mixture of DMEM and Ham’s F-12 (for SAS, SCC4, SCC25, and HO1U1) supplemented with 10% fetal bovine serum (FBS), or EGM™-2-MV BulletKit™ (for HMVEC), in a humidified incubator (37°C, 5% CO₂).

Quantitative PCR and standard PCR

Total RNA from cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative polymerase chain reaction (PCR) was performed using the 7500 Fast Real-Time PCR system and software (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. Specific primers and probes were purchased from Applied Biosystems as TaqMan® Gene Expression Assays, with the following IDs: human Flt4/FLT4, Hs01047677_m1; CNTN-1/CNTN1, Hs00355024_m1; VEGF-C/VEGFC, Hs01099203_m1; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)/GAPDH, Hs99999905_m1. The PCR amplification conditions were as follows: 20 s at 95°C followed by 40 cycles of 3 s of denaturation at 95°C and 30 s of annealing at 60°C. We quantified the relative expression levels of the genes by the standard curve method and compared the levels after normalization using those of GAPDH as an endogenous control. For standard PCR, the primer sequences, annealing temperatures (Ta), and PCR cycles used were as follows: Flt-4 forward, GGTTCCCTCAAGATGAGAC and Flt-4 reverse, CAAGCAAGACCCAGTGTC (Flt-4 PCR product, 505 bp; Ta, 62°C; 40 cycles), and GAPDH forward, ATCATCCCTGCTCCTACTGG and GAPDH reverse, CCGTCCACCTGCTCACC (GAPDH PCR product, 188 bp; Ta, 55°C; 28 cycles) [43]. After amplification in a thermal cycler, the PCR products were electrophoresed in agarose gels, stained with ethidium bromide, and visualized under UV-light.

Immunofluorescent staining

Cells for immunofluorescent staining of Flt-4 were seeded in slide chambers (IWAKI, Tokyo, Japan). After washing the cells extensively with phosphate-buffered saline (PBS), we fixed the cells with cold methanol for 10 min at -20°C. Following another wash with PBS and blocking with 3% (v/v) normal goat serum in PBS for 1 h, the cells were incubated overnight at 4°C with primary antibodies against Flt-4 (1:50, sc-321, Santa Cruz). Subsequently, the cells were washed extensively with PBS and then incubated with the secondary antibody conjugated to FITC (Santa Cruz) for 60 min at room temperature. The nuclei were visualized by staining with Hoechst 33258 (Sigma-Aldrich). Stained cells were then mounted with Prolong Gold Antifade Reagent (Invitrogen). The fluorescent images were acquired using a confocal laser scanning microscope (FLUOVIEW FV10i, Olympus, Japan).

Stimulation and inhibition of Flt-4 using its specific reagents

Stimulation reagents were recombinant human VEGF-C (rVEGF-C) and VEGF-C (Cys156Ser) protein, which is a selective agonist of Flt-4 and does not bind VEGFR-2 (R&D Systems, Minneapolis, MN, USA). Inhibition reagents were a recombinant Flt-4/Fc chimera that specifically neutralizes VEGF-C (R&D Systems) and MAZ51, a Flt-4-specific tyrosine kinase inhibitor (Calbiochem, Darmstadt, Germany). SAS or HO1U1 cells were seeded in six-well plates at a density of 2×10⁵ cells per well and incubated overnight.
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Figure 1. Baseline mRNA expression of Flt-4, VEGF-C, and CNTN-1 in HNSCC cells. The mRNA expression levels of each gene in the HNSCC cell lines were assessed by quantitative real-time PCR. The relative expression levels of the genes were compared after normalization using those of GAPDH. A: Flt-4. B: VEGF-C and CNTN-1, in which the relative expression levels were calibrated by dividing each value by that of HSC-2 for the sake of convenience. C: The selective expression of Flt-4 in SAS and HO1U1 cells was further confirmed by standard PCR. D: Immunofluorescent staining of SAS and HO1U1 cells, as well as HMVEC cells, revealed the cytoplasmic localization of Flt-4, indicating the receptor internalization that reflects its activation. Nuclei were stained with Hoechst 33258. Scale bar: 10 μm.

in serum-deprived medium (2% FBS medium). The cells were then treated with different selective Flt-4 stimulators: rVEGF-C (100 ng/ml) or VEGF-C (Cys156Ser) (50 ng/ml for SAS and 100 ng/ml for HO1U1), or inhibitors: recombinant Flt-4/Fc (100 ng/ml for SAS and 200 ng/ml for HO1U1) or MAZ51 (10 μM). These concentrations of the reagents were each found to be optimal with no toxic effect on cell viability up to at least 24 h based on our preliminary experiments. Treatments with only the solvent for a reagent: PBS for rVEGF-C, VEGF-C (Cys156Ser), and recombinant Flt-4/Fc, or dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) for MAZ51, were set as controls. For the evaluation of alterations in gene expression associated with Flt-4 stimulation or inhibition, total RNA was extracted after a 12-h incubation. The experiment in each condition was performed at least three times to assess for consistency of response.

In vitro cell proliferation assay

Cell proliferation rates were assessed using the Cell Counting Kit-8 (Dojindo Laboratories, Japan) according to the manufacturer’s instructions. Briefly, SAS cells were plated at 6×10³ cells per well in 96-well plates and incubated in culture medium containing 5% FBS with or without reagents for 24 h at 37°C. The water-soluble tetrazolium salt WST-8 (10 μL) was added to each well, and the plate was incubated for 2 h at 37°C. Viable cells were quantified by measuring the optical density (OD) values of the absorbance at 450 nm using a microplate reader. The experiment was conducted five times and run in sextuplicate each time.

In vitro cell migration assay

Cell migratory ability was evaluated using 24-well Transwell inserts (polycarbonate filters)
Figure 2. Effects of Flt-4 stimulation and inhibition on the mRNA expression levels ofCNTN-1 and VEGF-C in OSCC cells. Alterations in the mRNA expression ofCNTN-1 and VEGF-C in OSCC cells were examined by quantitative real-time PCR using recombinant VEGF-C or VEGF-C (Cys156Ser) (selective Flt-4 agonist) as stimulation reagents and recombinant human Flt-4/Fc chimera (specific VEGF-C neutralizer) or MAZ51 (specific Flt-4 inhibitor) as inhibition reagents. In SAS cells, Flt-4 stimulation upregulated both CNTN-1 and VEGF-C expression compared to the control (A), whereas in contrast, Flt-4 inhibition downregulated both CNTN-1 and VEGF-C expression compared to the control (B). In HO1U1 cells, Flt-4 stimulation resulted in relatively less upregulation of CNTN-1 expression and no change in VEGF-C expression (C), whereas Flt-4 inhibition led to downregulation of CNTN-1 expression but no change in VEGF-C expression (D). The values represent the mean ± standard deviation. Difference between each condition and control was statistically analyzed using a two-tailed t-test: *, P < 0.05; N.S., not significant.

with 8 μm pores (BD Biosciences). SAS cells suspended in serum-free medium were plated onto the Transwell inserts at 6×10⁴ cells per well. Medium containing 10% FBS was added to the bottom of the wells as a chemoattractant. The inserts were incubated with or without reagents for 24 h at 37°C. The filters were removed, and then, cells on the lower surface of the filters were fixed and stained with a Diff-Quick kit (Sysmex Corp., Japan) according to the manufacturer’s instructions. The migratory capacities were quantified as total cell numbers counted in five random fields for each insert under a light microscope at 200× magnification. The assay was performed five times and conducted in quintuplicate each time.

Patients and tissue samples

We reviewed the medical records of patients with histologically verified TSCC who underwent primary surgery at the Department of Otorhinolaryngology-Head and Neck Surgery, Keio University Hospital (Tokyo, Japan) between 2003 and 2012. The tumor stages were classified according to the American Joint Committee on Cancer TNM staging system (2010, 7th edition). Patients who had received chemotherapy or radiotherapy prior to primary surgery or who previously had double cancer in the head and neck region were excluded from the study. Formalin-fixed and paraffin-embedded (FFPE) surgical specimens were obtained from the 55
patients eligible for the study, among whom 17 patients had neck lymph node metastasis and 38 patients had no neck metastasis. Informed consent was obtained from all patients before the study, of which protocols were approved by the Institutional Ethics Review Board of the Ethics Committee of Keio University School of Medicine. All procedures for clinical tissues were performed in accordance with the ethical standards of the institutional research committee and with the principles of the 1964 Helsinki Declaration and its later amendments.

**Histopathological evaluation**

The FFPE TSCC specimens were sliced into 4-μm-thick serial sections. Two pathologists who were blinded to the clinical information reviewed all slides from each patient stained with hematoxylin and eosin to assess the histopathological characteristics, including differentiation (histological grade), vascular invasion, and lymphatic invasion.

**Immunohistochemical analysis**

For each case, specimens serially sliced to a 4-μm thickness at the central or maximum cross-section were selected. After deparaffinization and rehydration, the tissue sections were processed with antigen retrieval by boiling the slides in sodium citrate buffer (10 mmol/L, pH 6.0) for 10 min, followed by immersion in 0.3% H₂O₂ in methanol for 20 min to quench endogenous peroxidase activity. Non-specific immunoglobulin-binding sites were then blocked with normal serum (Vectastain Elite ABC kit; Vector Laboratories) for 30 min. The sections were incubated with each of the following primary antibodies overnight at 4°C: rabbit anti-FLT-4 (1:200 dilution, sc-321, Santa Cruz), goat anti-VEGF-C (1:300, sc-7133, Santa Cruz), or mouse anti-CNTN-1 (1:20, MAB904, R&D systems). After washing with PBS, the sections were incubated with biotinylated anti-rabbit, goat, or mouse secondary antibody for 40 min, followed by washing and incubation with avidin-biotin complexes (Vector Laboratories) for 30 min. After a further PBS wash, peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and counterstained with hematoxylin. The sections were mounted with a cover glass and evaluated under a microscope. Immunostaining was defined as positive if staining was observed in more than 25% of the tumor cells.

**Statistical analysis**

The data repeatedly obtained in the in vitro assays are presented as the mean ± standard deviation of three or more independent experiments. Difference in data between each condition and control was analyzed using a two-tailed Student’s t-test. The relationship of immunohistochemical expression among Flt-4, VEGF-C, and CNTN-1, and the correlation between these immunohistochemical expressions and clinicopathological variables were examined using Fisher’s exact test. Risk factors affecting neck lymph node metastasis were also examined using Fisher’s exact test for the univariate analysis. The independent significance of the variables considered significant in univariate analysis was further determined by multivariate analysis using a multiple logistic regression model with the backward stepwise selection method. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Version 20.0.

**Results**

**Baseline mRNA expression of Flt-4, VEGF-C, and CNTN-1 in HNSCC cells**

We evaluated the mRNA expression levels of Flt-4, VEGF-C, and CNTN-1 in HNSCC cell lines using quantitative real-time PCR. As shown in Figure 1A, other than positive control HMVEC, Flt-4 mRNA expression was found in several HNSCC cells (HSC-3, SAS, SCC-25, H01U1, FaDu, and Detroit562), whereas no Flt-4 mRNA expression was found in the other cells. Figure 1B shows the relative gene expression levels of VEGF-C and CNTN-1, which were normalized by dividing each value by that of HSC-2 cells as a calibrator for the sake of convenience. Based on these baseline mRNA expression levels, we selected the following Flt-4-expressing oral carcinoma cells for the in vitro experiments: SAS cells, which express a relatively high level of VEGF-C and a low level of CNTN-1, and H01U1 cells, which express the lowest level of VEGF-C and the highest level of CNTN-1. The selective Flt-4 gene expression in SAS and H01U1 cells was visualized by standard PCR (Figure 1C). Using immunofluorescent staining, protein expression was further confirmed to be mostly
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localized in the cytoplasm, seemingly as a result of receptor internalization (Figure 1D).

Effects of Flt-4 stimulation and inhibition on the mRNA expression of CNTN-1 and VEGF-C in OSCC cells

We examined the effect of Flt-4 stimulation and inhibition on the mRNA expression levels of CNTN-1 and VEGF-C in the SAS and H01U1 cell lines using rVEGF-C and VEGF-C (Cys156Ser) as stimulation reagents and recombinant Flt-4/Fc and MAZ51 as inhibition reagents. In SAS cells, Flt-4 stimulation upregulated both CNTN-1 and VEGF-C expression compared to the control, with increases of 1.5- and 2.3-fold in CNTN-1 and 1.4- and 1.8-fold in VEGF-C with rVEGF-C and VEGF-C (Cys156Ser), respectively (Figure 2A). In contrast, Flt-4 inhibition downregulated both CNTN-1 and VEGF-C expression compared to the control, with decreases of 0.4- and 0.6-fold in CNTN-1 and 0.4- and 0.5-fold in VEGF-C with recombinant Flt-4/Fc and MAZ51, respectively (Figure 2B). In H01U1 cells, Flt-4 stimulation resulted in relatively less upregulation of CNTN-1 expression, with increases of 1.3- and 1.2-fold with rVEGF-C and VEGF-C (Cys156Ser), respectively, whereas no increase
VEGF-C/Flt-4 axis in oral squamous cell carcinoma cells

![Image](90x478 to 522x720)

Figure 4. Immunohistochemistry of Flt-4, VEGF-C, and CNTN-1 in TSCC specimens. In the representative cases with positive staining shown in the top panels, staining of Flt-4 (A), VEGF-C (C), and CNTN-1 (E) was observed in the cytoplasm of the tumor cells. The bottom panels show cases with negative staining of Flt-4 (B), VEGF-C (D), and CNTN-1 (F). Original magnification: ×200. Scale bar: 100 μm.

was observed in VEGF-C expression (Figure 2C). Similarly, Flt-4 inhibition led to the downregulation of CNTN-1 expression, with decreases of 0.6- and 0.4-fold with recombinant Flt-4/Fc and MAZ51, respectively, whereas no decrease was found in VEGF-C expression (Figure 2D). These results suggest that the extent of the effect of Flt-4 stimulation and inhibition may vary depending on the cancer cell type but not necessarily on the baseline expression levels of CNTN-1 and VEGF-C in each cell line.

Effects of Flt-4 stimulation and inhibition on the cell proliferation activity of SAS cells

Alterations in cell proliferation activity induced by Flt-4 stimulation and inhibition were assessed by conducting in vitro cell proliferation assays using SAS cells. The data are presented as the fold increase in the OD values compared with the respective controls. Flt-4 stimulation moderately promoted proliferation activity in a dose-dependent manner, with increases of 1.2-1.3-fold and 1.1-1.2-fold with rVEGF-C and VEGF-C (Cys156Ser), respectively (Figure 3A). In contrast, Flt-4 inhibition mildly suppressed proliferation activity in a dose-dependent manner, with decreases of 0.8-0.85-fold with recombinant Flt-4/Fc or MAZ51 (Figure 3B).

Effects of Flt-4 stimulation and inhibition on the cell migration activity of SAS cells

An in vitro migration assay was performed using SAS cells. The results are presented as the fold increase in the number of migrated cells compared with the respective controls. To eliminate the possible influence of differences in proliferation activity, each value was normalized according to the corresponding ratio of the proliferation rate shown in the same condition. Flt-4 stimulation enhanced migration activity in a dose-dependent manner, with increases of 1.2-1.3-fold with rVEGF-C or VEGF-C (Cys156Ser) (Figure 3C). In contrast, Flt-4 inhibition attenuated migration activity in a dose-dependent manner, with decreases of 0.75-0.9-fold and 0.65-0.7-fold with recombinant Flt-4/Fc and MAZ51, respectively (Figure 3D).

Relationship of immunohistochemical expression among Flt-4, VEGF-C, and CNTN-1

Expression of Flt-4, VEGF-C, and CNTN-1 in tumor specimens from 55 patients with TSCC
were evaluated by immunohistochemistry. Positive staining of Flt-4 was demonstrated in 23 cases (41.8%), VEGF-C in 23 cases (41.8%), and CNTN-1 in 29 cases (52.7%). Figure 4 displays representative cases with the positive expression of each protein, all of which were localized to the cytoplasm of the tumor cells. As shown in Table 1, no significant correlation was found between Flt-4 and VEGF-C. However, CNTN-1, a downstream effector of the VEGF-C/Flt-4 axis, was significantly correlated with both Flt-4 (P = 0.032) and VEGF-C (P = 0.032).

**Correlation between immunohistochemical expression of Flt-4, VEGF-C, and CNTN-1 and clinicopathological variables**

We examined the correlations between the expression of each of the three molecules in tumor cells of the TSCC specimens and clinicopathological variables of the 55 patients with TSCC. As shown in Table 2, neck metastasis was significantly correlated with the expression of all three molecules: Flt-4 (P = 0.007), VEGF-C (P = 0.001), and CNTN-1 (P = 0.022). In addition, Flt-4 expression was correlated with sex (P = 0.006); VEGF-C was correlated with T-classification (P = 0.015); and CNTN-1 was correlated with vascular invasion (P = 0.016) and lymphatic invasion (P = 0.022).

**Univariate analysis of risk factors affecting neck metastasis**

To determine the risk factors affecting neck metastasis, the association of neck metastasis with other clinicopathological variables was examined. As summarized in Table 3, univariate analysis revealed that T classification (P < 0.001) and lymphatic invasion (P = 0.005) were significantly correlated with neck metastasis. All other variables, such as age, sex, differentiation, and vascular invasion, showed no significant correlation with neck metastasis.

**Multivariate analysis of risk factors affecting neck metastasis**

A multiple logistic regression model was applied to analyze the independent significance of the variables that were significantly correlated with neck metastasis in the above-mentioned univariate analyses. As shown in Table 4, T classification (odds ratio [OR] = 25.741, P = 0.003), lymphatic invasion (OR = 27.141, P = 0.024), and the expression of Flt-4 (OR = 48.136, P = 0.046) were found to be the independent risk factors affecting neck metastasis in this cohort.

**Discussion**

Our in vitro experiments revealed that Flt-4 is expressed not only in endothelial cells but also selectively in certain subsets of HNSCC cells, including OSCC cells, using real-time PCR, standard PCR, and immunofluorescent staining, and these results are partly consistent with previous reports [34, 35, 46]. Moreover, the co-expression of VEGF-C confirmed in those cells, although the extent of its expression differed in each cell line, strongly suggests the existence of an autocrine mechanism of the VEGF-C/Flt-4 signaling in these tumor cells. Regarding the cellular localization of Flt-4, its cytoplasmic localization is considered to be an indicator of receptor activation because VEGF-C was shown to induce Flt-4 internalization, to which the full downstream signaling of Flt-4 was coupled in cultured lymphatic endothelial cells [50]. Accordingly, our findings indicating Flt-4 expression mostly in the cytoplasm of tumor cells, in both in vitro and TSCC specimens, further support the likely activation of VEGF-C/Flt-4 signaling in an autocrine manner. Similar observations have been reported in several different types of cancer [29, 30, 34, 36, 45, 47, 49, 51].
Table 2. Correlation between immunohistochemical expression of Flt-4, VEGF-C, CNTN-1 and clinicopathological variables

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<td>2 (18.2)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>43</td>
<td>17 (39.5)</td>
<td>26 (60.5)</td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>43</td>
<td>17 (39.5)</td>
<td>26 (60.5)</td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
</tr>
</tbody>
</table>

*Statistically significant according to Fisher’s exact test (two-tailed).
VEGF-C/Flt-4 axis in oral squamous cell carcinoma cells

Table 3. Univariate analysis of risk factors affecting neck metastasis

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Positive (n = 17)</th>
<th>Negative (n = 38)</th>
<th>P value</th>
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<tr>
<td></td>
<td>n</td>
<td>(%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>26</td>
<td>6 (23.1)</td>
<td>20 (76.9)</td>
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<tr>
<td>&gt; 60</td>
<td>29</td>
<td>11 (37.9)</td>
<td>18 (62.1)</td>
<td></td>
</tr>
<tr>
<td>≤ 60</td>
<td>13</td>
<td>2 (25.0)</td>
<td>12 (75.0)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
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<td>1 (25.0)</td>
<td>12 (75.0)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>26</td>
<td>6 (23.1)</td>
<td>20 (76.9)</td>
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</tr>
<tr>
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<td>36</td>
<td>5 (13.9)</td>
<td>31 (86.1)</td>
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</tr>
<tr>
<td>T classification</td>
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<td>2 (18.2)</td>
<td>9 (81.8)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>1</td>
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<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>11</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
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<td>Differentiation</td>
<td>44</td>
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<td>32 (72.7)</td>
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</tr>
<tr>
<td>Well</td>
<td>26</td>
<td>6 (23.1)</td>
<td>20 (76.9)</td>
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</tr>
<tr>
<td>Moderately or poorly</td>
<td>18</td>
<td>6 (33.3)</td>
<td>12 (66.7)</td>
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</tr>
<tr>
<td>Vascular invasion</td>
<td>43</td>
<td>11 (25.6)</td>
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<td>Negative</td>
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<td>6 (50.0)</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>5 (16.1)</td>
<td>26 (83.9)</td>
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</tr>
<tr>
<td>Lymphatic invasion</td>
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<td>9 (20.9)</td>
<td>34 (79.1)</td>
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</tr>
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<td>4 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>5 (16.1)</td>
<td>26 (83.9)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant according to Fisher’s exact test (two-tailed).

Table 4. Multivariate analysis of risk factors affecting neck metastasis

<table>
<thead>
<tr>
<th>Step</th>
<th>Variable</th>
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<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
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<td>2.410-423.574</td>
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<td>Lymphatic invasion</td>
<td>24.52</td>
<td>1.004-598.535</td>
<td>0.050*</td>
</tr>
<tr>
<td></td>
<td>Flt-4</td>
<td>68.05</td>
<td>0.775-5976.283</td>
<td>0.065</td>
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<tr>
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<td>VEGF-C</td>
<td>7.77</td>
<td>0.45-133.979</td>
<td>0.158</td>
</tr>
<tr>
<td></td>
<td>CNTN-1</td>
<td>1.36</td>
<td>0.031-59.483</td>
<td>0.874</td>
</tr>
<tr>
<td>Step 2</td>
<td>T classification</td>
<td>31.43</td>
<td>2.474-399.297</td>
<td>0.008*</td>
</tr>
<tr>
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<td>Lymphatic invasion</td>
<td>25.43</td>
<td>1.081-598.532</td>
<td>0.045*</td>
</tr>
<tr>
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<td>Flt-4</td>
<td>64.59</td>
<td>0.838-4980.629</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>VEGF-C</td>
<td>8.29</td>
<td>0.528-130.214</td>
<td>0.132</td>
</tr>
<tr>
<td>Step 3</td>
<td>T classification</td>
<td>25.74</td>
<td>3.112-212.947</td>
<td>0.003*</td>
</tr>
<tr>
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<td>Lymphatic invasion</td>
<td>27.14</td>
<td>1.543-477.394</td>
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</tr>
<tr>
<td></td>
<td>Flt-4</td>
<td>48.14</td>
<td>1.078-2150.297</td>
<td>0.046*</td>
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</tbody>
</table>

OR, odds ratio; CI, confidence interval. *Statistically significant according to multiple logistic regression analysis.

whereas a few studies showed the membrane localization of Flt-4 in tumor cells [29, 37, 47], which can be considered to reflect its expression before internalization.

We observed that CNTN-1 expression is upregulated or downregulated by Flt-4 stimulation or inhibition, respectively, in both SAS and HO1U1 cells, indicating that CNTN-1 serves as one of the downstream effectors of the VEGF-C/Flt-4 autocrine mechanism in tumor cells. Intriguingly, the expression of endogenous VEGF-C itself is also upregulated or downregulated by Flt-4 stimulation or inhibition, respectively, in SAS but not in HO1U1 cells, suggesting that the VEGF-C/Flt-4 axis can work as an autocrine stimulation loop in which endogenous VEGF-C regulates the expression of VEGF-C itself as another downstream effector of this axis in selected tumor cells. In assays using SAS cells with Flt-4 stimulation or inhibition, we found that proliferation activity was modulated moderately, while migration activity was regulated more noticeably; both were in a dose-dependent manner. Taken together, our results suggest that VEGF-C/Flt-4 axis-induced signaling in a subset of tumor cells may promote tumor cell proliferation and migration via upregulating the expression levels of VEGF-C itself and CNTN-1 in an autocrine manner, thereby contributing to cancer progression of OSCC (Figure 5).

Our finding concerning the enhancement of cell proliferation by autocrine VEGF-C/Flt-4 signaling in tumor cells is in accordance with several
VEGF-C/Flt-4 axis in oral squamous cell carcinoma cells

Figure 5. A schematic diagram demonstrating the VEGF-C/Flt-4 autocrine system in OSCC cells. Tumor cell-derived VEGF-C binds to Flt-4 expressed on the tumor cells, and its activation upregulates the expression levels of VEGF-C itself and CNTN-1 in an autocrine manner, thereby enhancing tumor cell proliferation and migration. This autocrine mechanism of the VEGF-C/Flt-4 axis in tumor cells likely contributes to cancer progression, including the development of lymphatic metastasis, along with the primary well-known paracrine mechanism involving lymphatic endothelial cells that leads to lymphangiogenesis.

Previous studies; while the earlier reports did not refer to its specific downstream effector [26, 30, 31], a few recent studies identified several molecules as the downstream effectors of the VEGF-C/Flt-4 axis [43, 46, 48]. In a previous study using the same OSCC cell line (SAS) with Flt-4 inhibition via a dominant-negative Flt-4-overexpressing cell model or MAZ51 treatment, the expression of VEGF-C was shown to be positively regulated by autocrine activation of Flt-4 by VEGF-C itself [46]. This result implying the existence of the VEGF-C/Flt-4 autocrine loop was corroborated by our study using both Flt-4 stimulation and inhibition. The abovementioned study further demonstrated that this upregulation of VEGF-C in this autocrine loop is mediated through Flt-4-associated intracellular signal transductions, mainly via the protein kinase C (PKC), p42/44 mitogen-activated protein kinase (MAPK), and p38 MAPK pathways but not via the phosphatidylinositol-3 kinase (PI3K)-Akt pathway, although their resulting effects on cell migration activity were not assessed [46]. However, considering that in lymphatic endothelial cells the PKC-dependent p42/44 MAPK pathway was shown to mediate VEGF-C/Flt-4 activation-induced signal transductions leading to cell growth, survival, and migration [52], endogenously upregulated VEGF-C through the VEGF-C/Flt-4 autocrine loop in tumor cells is assumed to promote not only tumor cell proliferation but also tumor cell migration, as demonstrated in the present study. Besides a study using HNSCC cells, a study using VEGFR-3-expressing KKLS cells derived from gastric carcinoma showed that treatment with rVEGF-C resulted in the phosphorylation of Akt, stimulation of cell proliferation, and increased expression of cyclin D1, placental growth factor, autocrine motility factor (AMF), and autocrine motility factor receptor (AMFR) [43]. Because AMF and AMFR are known to regulate tumor cell motility, these results suggest that the migration activity of those cells could also be enhanced by VEGF-C, although it was not evaluated.

Regarding cell migration, its enhancement by the autocrine VEGF-C/Flt-4 signaling found in the present study is also in agreement with several previous studies; while some of them did not specify its specific downstream molecules [26, 39, 40, 49], a few studies demonstrated that CNTN-1 was the downstream effector of the VEGF-C/Flt-4 axis [37, 44, 48]. CNTN-1, initially found to be expressed on the external membrane surface of central and peripheral neurons [53, 54], is of vital importance for the development and maintenance of the nervous system by playing roles in axon elongation in the cerebellum [55], assembly of septate-like junctions between axons and myelinating glial cells [56], and formation of the neuromuscular junction [57] and synapses [58, 59]. Concerning its molecular function, CNTN-1 has been demonstrated to interact with a variety of cell surface proteins, including the L1 family of the Ig superfamily (L1, NrCAM, and neurofascin) [60, 61], receptor protein tyrosine phosphatase α (RPTPα) [62], RPTPβ [61, 63], tenascin-R [64], tenascin-C [65], a soluble form of RPTP zeta [66], and the Notch receptor [67].
In the field of basic cancer research, Su et al. first revealed that CNTN-1 plays a critical role in invasion and metastasis in a study using lung adenocarcinoma cells; they found that the VEGF-C/Flt-4 axis induced the upregulation of CNTN-1 through the activation of an Src-p38 MAPK-mediated C/EBP-dependent signaling pathway, leading to the promotion of cancer cell mobility and invasiveness but not cell proliferation [37]. Subsequently, the VEGF-C/Flt-4 signaling-induced upregulation of CNTN1 resulting in enhanced cell motility was reported in a few studies that used esophageal cancer cells overexpressing VEGF-C and shRNA vectors targeting VEGF-C [48] or gastric cancer cells with shRNA vectors targeting Flt-4 [44], in addition to the present study that used OSCC cells. Intriguingly, whereas tumor cell proliferation was also found to be regulated by the VEGF-C/Flt-4 axis in these last three studies [44, 48, present study], it was not the case in the study that used lung adenocarcinoma cells [37]. Considering the possible variations in the Flt-4-associated intracellular signal transductions observed among different cells, this inconsistency could be attributed to differences in the existence of downstream effectors other than CNTN-1, such as VEGF-C itself found in our study, although the other studies did not refer to any other effector molecule known to stimulate cell proliferation [44, 48]. 

Regarding the specific molecular mechanism through which CNTN-1 promotes tumor cell migration, VEGF-C/Flt-4-induced CNTN-1 expression was first demonstrated to enhance cell invasion via rearrangement of filamentous-actin (F-actin, a member of the actin cytoskeleton), which is continuously polymerized and depolymerized in migratory cells and is essential to cell motility [37, 68]. Furthermore, this CNTN1-induced F-actin polymerization was shown to be mediated by the small GTP-binding protein RhoA, which interacts with CNTN-1 at the cell membrane [69]. In addition, interestingly, a few recent studies showed that CNTN-1 induces epithelial-mesenchymal transition (EMT) by downregulating E-cadherin expression via activation of AKT and/or upregulation of Slug, thereby promoting cell migration and invasiveness [70-72]. Therefore, CNTN-1 is presumed to enhance tumor cell motility and invasiveness through a combination of the above-described mechanisms.

In addition to tumor cell proliferation and motility, VEGF-C/Flt-4 signaling has also been shown to promote cell survival and chemoresistance in leukemic cells [31], increase focus formation (anchorage-independent growth) in esophageal cancer cells [48], decrease apoptosis in gastric cancer cells [44], maintain cell survival and focal adhesion contact in breast cancer cells [41], regardless of each specific effector molecule. Intriguingly, even in the absence of VEGF-C expression, Flt-4 overexpression in breast cancer cells were shown to promote tumorigenicity and tumor growth in vivo, although neither its alternative ligand nor specific downstream effector molecule were referred to [40]. Thus, the cellular function of the VEGF-C/Flt-4 autocrine system, as well as its downstream intracellular signal and effector molecule, are assumed to depend on each organ and cancer histology.

In the clinical specimens examined using immunohistochemistry, the expression of CNTN-1 was demonstrated to correlate with that of both Flt-4 and VEGF-C, whereas no correlation was found between Flt-4 and VEGF-C. Inconsistent results have been reported on the correlation between the expression of Flt-4 and VEGF-C in tumor cells. Previous studies that examined gastric [42] and prostate cancer [73] found no correlation between the immunohistochemical expression of Flt-4 and VEGF-C, similar to our results. In contrast, other studies that examined NSCLC [74] and uterine cervical malignancy, of which the majority were pre-invasive lesions (cervical intraepithelial neoplasia) [29], found a positive correlation between the expression of these molecules, unlike our findings. This inconsistency seems attributable, at least in part, to the unequal distribution of the extent of malignancy as well as differences in the immunohistochemistry protocols and criteria for positive staining. Although little has been reported on the association between the expression of CNTN-1 and VEGF-C in tumor specimens, a recent study that investigated esophageal carcinoma specimens demonstrated that CNTN-1 expression was correlated with VEGF-C expression, both mRNA and protein levels [48], in accordance with our results. These findings are assumed to indirectly reflect the role of CNTN-1 as the downstream effector of the VEGF-C/Flt-4 axis in tumor cells.
Notably, our clinicopathological study with multivariate analysis revealed that in addition to T classification and lymphatic invasion and for the first time in HNSCC, the expression of Flt-4 in tumor cells is an independent predictive factor of neck metastasis. This result also supports the aforementioned critical role of Flt-4 expressed in OSCC cells in the development of neck metastasis, while the reliability of this predictor requires further validation with independent patient cohorts. Regarding the clinical implication of Flt-4 expression in other malignancies, in line with our observations, several studies have demonstrated a positive correlation between Flt-4 expression in tumor cells and lymph node metastasis in endometrial [33], prostate [36], and cervical cancer [51], and lung adenocarcinoma [37], although all these results were confined to univariate analysis. In contrast, a few other studies that examined colorectal [28] and gastric cancer [42] reported no correlation between Flt-4 expression and regional metastasis, suggesting differences in the importance of Flt-4 expression in tumor cells in the development of lymph node metastasis among various cancers. Concerning this implication in OSCC, Flt-4 expression in tumor cells, but not in vascular endothelial cells, was shown to have a trend towards an association with delayed neck metastasis in patients with early stage (cT1-2N0M0) OSCC, although this trend was not statistically significant [45]. The endothelial expression of Flt-4, however, was shown to positively correlate with lymph node metastasis in OSCC [75], as well as with delayed neck metastasis in stage I-II TSCC [76], although these findings were also limited to univariate analysis. Together with our results, these findings suggest that Flt-4 expression in both tumor cells and endothelial cells can be responsible for promoting lymphatic metastasis in OSCC.

A positive correlation of lymph node metastasis with VEGF-C expression has also been demonstrated in studies that analyzed prostate [36], gastric [42], cervical [51], and gallbladder cancer [77] and lung adenocarcinoma [37], as has its correlation with CNTN-1 expression in studies that assessed lung adenocarcinoma [37], OSCC [78], and gastric cancer [71], among which only the last one [71] provided confirmation using multivariate analysis. In agreement with these results, correlations of VEGF-C and CNTN-1 expression with lymph node metastasis were also found in the present study, whereas these molecules were excluded from the independent risk factors during multivariate analysis. This result is likely due to confounding among the variables in the prediction model in which both VEGF-C and CNTN-1 had a strong correlation with the other eventually independent factors; namely, VEGF-C expression was significantly correlated with T-classification (Table 2), while CNTN-1 expression was significantly associated with Flt-4 expression (Table 1) and lymphatic invasion (Table 2).

Aside from lymph node metastasis, Flt-4 expression in tumor cells has also been shown to correlate with other clinicopathological parameters such as lymphatic vessel invasion in cervical cancer [51], clinical staging and Gleason scores in prostate cancer [73], and stage in lung adenocarcinoma [37], although these results were solely indicated by univariate analysis. In addition, survival analyses have found Flt-4 expression in tumor cells to be an unfavorable prognostic factor for overall survival in colorectal cancer [28] and lung adenocarcinoma [37] and carcinoma-specific survival in gastric cancer [42], among which only the last one was qualified to be independent by multivariate analysis. Collectively, the clinical significance of Flt-4 expression in tumor cells corroborates the importance of tumor cell-produced Flt-4, together with the presumable function of the VEGF-C/Flt-4 autocrine system with its downstream effector CNTN-1, in the progression of various malignancies.

Conclusions

The present study demonstrated that the VEGF-C/Flt-4 axis in a subset of HNSCC cells provides the autocrine mechanism through which the expression levels of VEGF-C itself and CNTN-1 are upregulated, thereby enhancing tumor cell proliferation and migration. We also found the expression of Flt-4 in tumor cells to be an independent predictor of neck metastasis in patients with TSCC, which supports the critical role of tumor cell-expressed Flt-4 in promoting lymphatic metastasis. Hence, targeting the VEGF-C/Flt-4 axis in tumor cells can be an attractive therapeutic strategy for the treatment of patients with cancer, including OSCC.
References


[29] Van Trappen PO, Steele D, Lowe DG, Baithun S, Beasley N, Thiele W, Weich H, Krishnan J, Shepherd JH, Pepper MS, Jackson DG, Slee-


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