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Original Article

Establishment of an oral squamous cell carcinoma cell line expressing vascular endothelial growth factor a and its two receptors

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KEYWORDS
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Abstract *Background/purpose:* Vascular endothelial growth factor receptor (VEGFR) expression in oral squamous cell carcinoma (OSCC) promotes tumor growth through both autocrine and paracrine signaling. VEGF-positive OSCC cases are associated with a high depth of invasion, increased metastasis, and poor prognosis. In this study we established and then molecularly and functionally analyzed an OSCC cell line that co-expresses VEGF-A, VEGFR-1, and VEGFR-2, termed HCM-SqCC010 cells.
Materials and methods: VEGF-A, VEGFR-1, and VEGFR-2 expression in HCM-SqCC010 cells were examined by immunohistochemistry and immunoblotting. Expression and inhibition of VEGF-A, VEGFR-1, and VEGFR-2 in HCM-SqCC010 cells were verified by quantitative real-time PCR.
Results: Our analysis of HCM-SqCC010 cells revealed that their proliferation depended on VEGF-A, and selective inhibition of VEGFR-1 or VEGFR-2 resulted in decreased cell growth.
Conclusion: We established an OSCC cell line, HCM-SqCC010, that expresses VEGF-A, VEGFR-1, and VEGFR-2. This triple-positive cell line showed no effect from a molecular targeted drug

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toward VEGF-A, but it did show strong cell growth inhibition in response to a VEGFR inhibitor. Thus, new therapeutic strategies against OSCC should include a VEGFR inhibitor.

Introduction

Oral cancer is a type of head and neck malignancy located in the lip, tongue, floor of mouth, buccal mucosa, gingiva, or palate.[1] More than 90% of head and neck cancers are squamous cell carcinoma (SCC), which primarily occurs in the oral cavity and oropharynx, or so-called oral squamous cell carcinoma (OSCC).[2,3] OSCC patients often present with late-stage tumors, and the 5-year survival rate is less than 50%.[4] The poor survival rate of OSCC is attributed to the high frequency of local recurrence and distant metastasis;[5] more than 50% of OSCC cases have lymph node metastasis.[6] Hence, it is important to understand the mechanisms of metastasis, as it is the leading cause of death in OSCC patients, and a major challenge in OSCC treatment are patients who present with lymph node metastases.

Tumor vascularization, which is a crucial feature in cancer development and progression, is based on angiogenesis and vasculogenesis and is driven by vascular endothelial growth factor (VEGF) signaling.[7–9] VEGF, also known as VEGF-A, is a key angiogenesis stimulator.[10,11] VEGF-A binds both VEGF receptor (VEGFR) -1 and VEGFR-2, but the tyrosine kinase activity of VEGFR-1 is very weak, approximately 10-fold lower than that of VEGFR-2.[12] Downstream signaling pathways that are activated by VEGFR include extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and phosphoinositide 3′ kinase (PI3K)/AKT.[13] Previous studies have established that OSCC cells have the capacity to express VEGF[14–16] and VEGFR.[17–20] However, a recent paper suggested that tumor-derived VEGF provides not only paracrine survival cues for endothelial cells but may also activate autocrine signaling in tumor cells expressing VEGFRs that plays a role in resistance to existing anti-angiogenesis therapies.[21–23] Studies have suggested a mean positivity rate of 77% for VEGF-positive OSCC,[24] and others have shown that the level of VEGF-expression among VEGF-positive OSCC cases increases with increasing tumor invasion depth.[25,26]

In this paper, we established an OSCC cell line with triple-positive expression of VEGF-A and the VEGFRs (VEGFR-1 and VEGFR-2) to evaluate autocrine VEGF signaling in OSCC.

Materials and methods

Patient

An 80-year-old Japanese man with swelling in his upper gingiva was referred to our department. Intraoral findings showed a diffuse granulomatous tumor with ulcer formation (Fig. 1A-). Bilateral cervical lymph nodes were painless and mobile. The clinical diagnosis was a malignant tumor of the upper gingiva. Magnetic resonance imaging (MRI) showed an irregular mass measuring 43 × 38 mm in the middle of the upper gingiva. Positron emission tomography—computed tomography (PET-CT) revealed uptake in the primary lesion (SUVmax: 13.82). A biopsy was performed, and the malignancy was pathologically diagnosed as well differentiated SCC. The patient then underwent partial resection of the hard palate. Microscopically, hematoxylin and eosin (H-E)-stained tumor tissue showed well differentiated SCC. Five months later, swelling of the bilateral Level IB and IIA lymph nodes was revealed (Figs. 1A—2, A-3), and bilateral modified radical neck dissection was performed. The final diagnosis was invasive keratinizing SCC, pT4aN2bM0 (Grade 1, YK-2, pHM0, pVM0, ly0, v0, neu0). The Level IB and IIA left cervical lymph nodes showed metastases. At 85-month follow-up, he had already died owing to dementia but had maintained a cancer-free status.

Immunohistochemistry

Formalin fixed tissue was stained by routine H-E staining or immunohistochemistry (IHC). The following primary antibodies were used for IHC: anti-VEGF-A antibody (Merck Millipore Japan, Tokyo, Japan), anti-VEGFR-1 (R&D Biosystems, Minneapolis, MN, USA), and anti-VEGFR-2 antibody (R&D Biosystems). All primary antibodies were incubated with tissues at a 1:100 dilution for 60 min. The secondary antibody Dako EnVision™+ Dual Link System-HRP (Dako, Tokyo, Japan) was then incubated with the tissues for 15 min at room temperature. Protein expression was visualized using a 3,3′-diaminobenzidine tablet (Dako).

Culture of patient-derived HCM-SqCC010 cells

The surgical specimen, including metastatic lymph nodes, from this patient was obtained with consent in accordance with the policies of Hyogo College of Medicine Institute and in accordance with an approved IRB protocol of Hyogo College of Medicine (No. 276). This study was also conducted in accordance with the Declaration of Helsinki. Cell culture techniques were described in our previous report.[27]

Culture of OSCC cell lines

The OSCC cell lines SCCKN, HSC-3M, Ca9-22, OSC19, SAS, and were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and incubated with Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS at 37 °C in 5% CO_2.

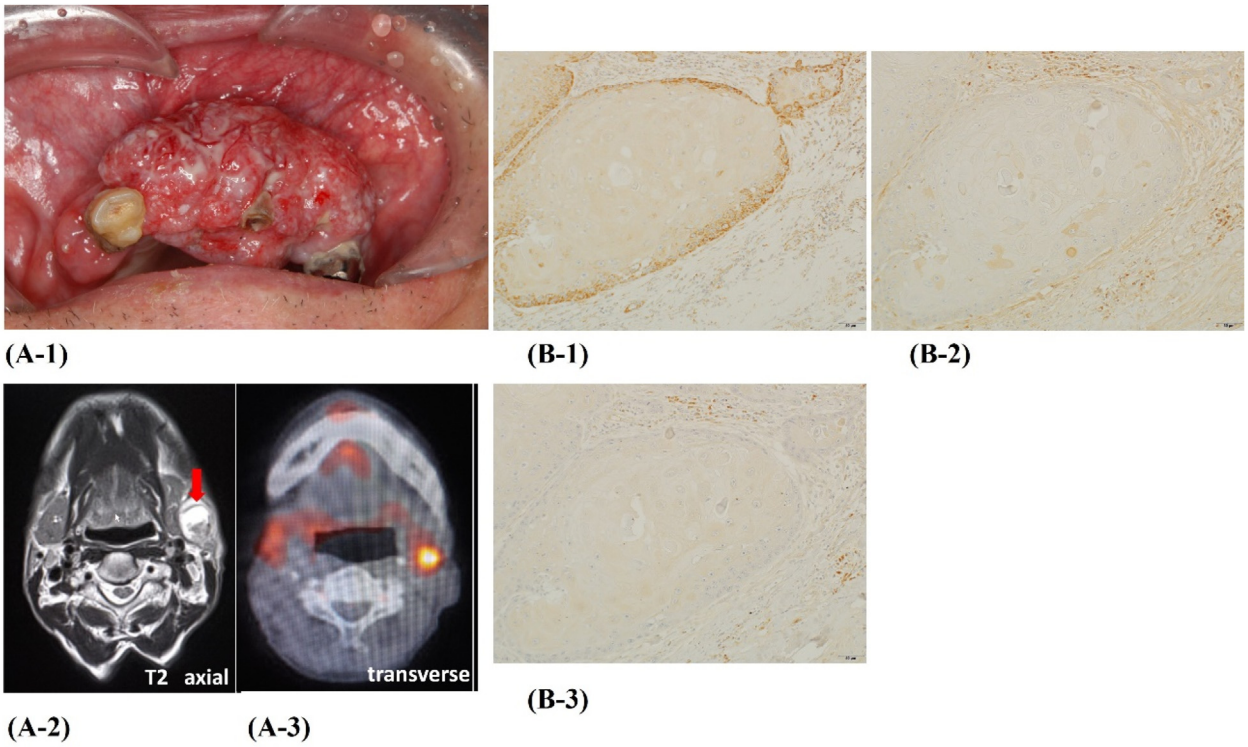

Figure 1 (A-1) Intraoral findings showed maxillary gingival squamous cell carcinoma (T4aN2bM0, Stage IVa). (A-2) Magnetic resonance imaging (MRI) showing a well-defined, high-signal contrasted mass in the lateral aspect of the left submandibular gland. Arrow indicates metastatic lymph nodes. (A-3) Positron emission tomography-computed tomography (PET-CT) revealed tracer accumulation in the left mandibular lymph node. (B) Immunohistochemical analysis of vascular endothelial growth factor (VEGF)-A, VEGF receptor (VEGFR)-1, and VEGFR-2 (magnification: 200 $\times$). VEGF-A expression was strongly positive in tumor cells, especially at edges of invasion (B-1). VEGFR-1 (B-2) and VEGFR-2 (B-3) were positive in primary tumor nests.

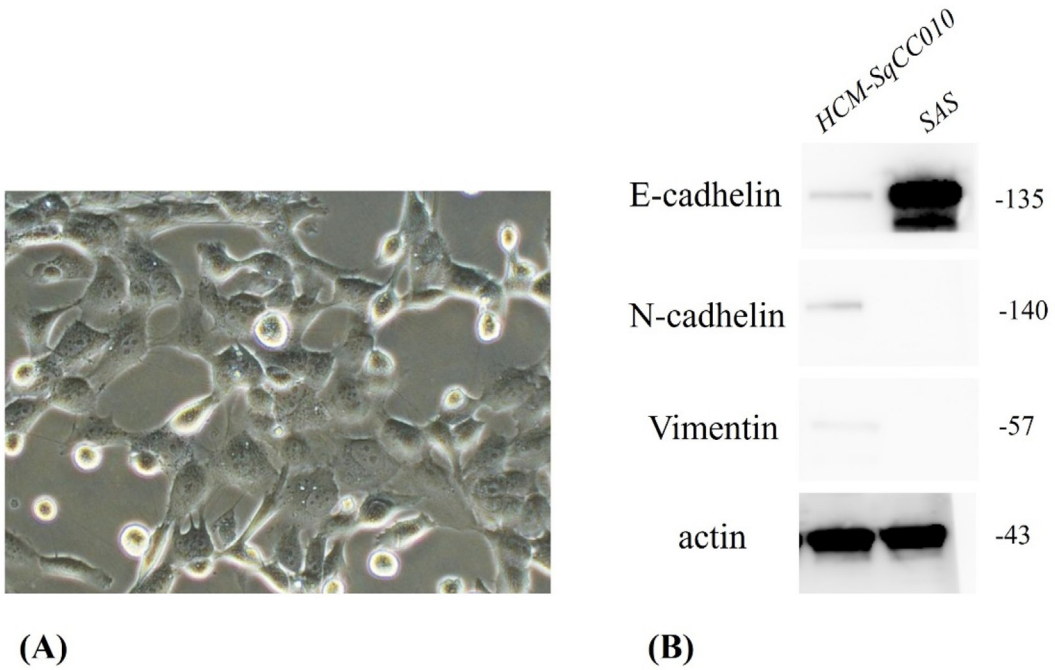

Figure 2 (A) Morphological findings showed polygonal or spindle cells. (B) Western Blotting in the HCM-SqCC010 cell line. E-cadherin was expressed. Low expression of N-cadherin and vimentin were observed.

Recombinant VEGF-A was purchased from Wako Pure Pharm. Co. (Osaka, Japan).

Short tandem repeat authentication of HCM-SqCC010 cells

To verify the identity of HCM-SqCC010 cells and to check for cross contamination with other SCC cell lines, genomic DNA was extracted from blood of the patient whose tumor sample was used to generate HCM-SqCC010 cells as well as the included cell lines using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's protocol. DNA genotyping by short tandem repeat (STR) profiling was performed using the GenePrint 10 System (Promega, Madison, WI, USA) and the Applied Biosystems 3130XL Analyzer (Applied Biosystems, Waltham, MA, USA), and the data were analyzed at BEX Co., Ltd. (Tokyo, Japan) authentication service.

3-D cell culture

HCM-SqCC010 tumor cells and their fibroblasts were mixed at 1:1 concentration in an ultralow attachment culture dish™ (Coring Co., Corning, NY, USA) and incubated for 7 d. Palettes (termed artificial organoids: AOs) were precipitated by centrifugation and fixed in 4% formaldehyde in PBS. The Specimen was used for H-E, and IHC detection of VEGF-A, VEGFR-1, and VEGFR-2.

Immunofluorescence staining

The immunofluorescence staining protocol was described in our previous report.[27] Rhodamine phalloidin (Cytoskeleton, Denver, CO, USA) was used for actin staining, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained using a confocal laser-scanning microscope LSM780 (Carl Zeiss, Oberkochen, Germany).

Reverse-transcription PCR

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and reverse-transcription PCR was performed using the Prime SCRIPT RT-PCR kit (Takara Bio, Kusatsu, Japan) in accordance with the manufacturer's instructions. The primers used are listed in Table 1. Amplification of *GAPDH* was performed as a control.

Real-time quantitative PCR

HCM-SqCC010 cells were stimulated with 100 ng/mL VEGF-A for 24 and 48 h cDNA acquired from the total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) was prepared as the template. Following PCR using the specific primer pairs listed in Table 2 and SYBR Green PCR Master Mix (7500-01; Applied Biosystems), the products were quantified in a real-time PCR machine. Relative gene expression values ($2^{-\Delta Ct}$) were normalized to *GAPDH* in the same cDNA using the ΔCt method. ΔCt values were determined by subtracting the average *GAPDH* Ct value from the average Ct value of the target gene.

Western blotting

Western blotting was performed as previously described.[28] To identify exosome markers from HCM-SqCC010 cells, the membranes were incubated overnight with anti-CD9 and anti-CD63 primary antibodies (System Bioscience, CA, USA).

Cell proliferation assay

HCM-SqCC010 cells (5×10^3 cells/well) were plated and treated with varying doses of the selective VEGFR-1 inhibitor ZM306416 (S2897) (Selleck Biotech, Tokyo, Japan) and the selective VEGFR-2 inhibitor SU5408 (S6514) (Selleck Biotech). We analyzed cell numbers using Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) after 72 h of treatment. After incubation with reagent at 37 °C, optical densities were read at 450 nm/570 nm using a microplate reader.

Exosome isolation and characterization

Exosomes were isolated from culture media using the ExoQuick™ Exosome Precipitation kit (System Bioscience)

Table 1 RT-PCR primers for VEGF-A, VEGFR-1, VEGFR-2, and GAPDH genes.

Gene		Forward primer(5′ to 3′)	Reverse primer(5′ to 3′)
VEGF-A	737	TCGGGCCTCCGAAACCATGAACTTTCT	GGTTTCTGGATTAAGGACTGTTCTGTC
VEGFR-1	545	GCTGCAAATATCTAGCTGTACCT	GGAATTGTTTGGTCAATTCGTC
VEGFR-2	312	AGACTTTGAGCATGGAAG	CCATTCCACCAAAAGATG
GAPDH	623	ACCATCTTCCAGGAGCGAGA	ACCACCTGGTGCTCAGTGTA

Table 2 RealtimePCR primer for VEGF-A, VEGFR-1and VEGFR-2 genes.

Gene	Forward primer(5′ to 3′)	Reverse primer(5′ to 3′)
VEGF-A	TGAGGAGTCCAACATCACCA	CCTCGGCTTGTCACATTTT
VEGFR-1	GGCTCTGTGGAAAGTTCAGC	GCTCACACTGCTCATCCAAA
VEGFR-2	TGCTTCACAGAAGACCATGC	GTGACCAACATGGAGTCGTG

in accordance with the manufacturer's instructions. Briefly, exosomes were isolated from HCM-SqCC010 conditioned medium by ultracentrifugation 48 h after replenishing the medium with fresh medium containing exosome-depleted FBS, and the supernatants were centrifuged at room temperature for 10 min at 2000 × *g*. The exosome-containing pellets were analyzed by Western blot to identify the exosome markers CD9 and CD63. RNA extraction from exosomes was performed using TRIzol (Invitrogen). PCR and transmission electron microscopy (TEM) were also performed.

Statistical analysis

All datasets were assessed for significant differences using the Mann–Whitney U test, with a *P*-value <0.05 considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$).

Results

Histopathological findings of the surgical specimen

The surgical specimen from the patient appeared as typical well-differentiated SCC. VEGF-A expression was strongly positive in tumor cells, and its expression was coincident with the edge of invasion (Fig. 1B-). VEGFR-1 (Figs. 1B–2) and VEGFR-2 (Figs. 1B–3) were also positive in primary tumor cells. Thus, we hypothesized that this tumor was triple-positive for VEGF-A, VEGFR-1, and VEGFR-2 and could be stimulated in an autocrine or paracrine manner.

Establishment of an OSCC cell line with VEGF-A, VEGFR-1, and VEGFR-2 expression

Histopathological examination indicated that this tumor expressed VEGF-A, VEGFR-1, and VEGFR-2. We suspected that this tumor could use autocrine and/or paracrine signing to signal for increased proliferation and tumor growth. Therefore we attempted to culture OSCC cells from this patient using outgrowth methods. In primary culture, OSCC cell colonies grew aggressively and predominantly consisted of closely packed polygonal cells with epithelial morphology (Fig. 2A). Moreover, fibroblastic cells in tumor tissue were also established. Both cell lines, termed HCM-SqCC010 and HCM-SqCC010-fibro, respectively, have been stably cultured for 4-years. HCM-SqCC010 passage has been stopped at 73 passages. Recovery from cryopreservation is not a problem, and the doubling growth time is 27.3 h. The appropriate dilution ratio for passaging is 1:4–5, but it is still possible to passage at 1:8. Furthermore, we evaluated the protein expression in the epithelial and mesenchymal markers in the HCM-SqCC010 cell line using Western Blotting. At the result, E-cadherin were expressed in the HCM-SqCC010 cells, while Vimentin expression was mildly detected (Fig. 2B).

We analyzed VEGF-A, VEGFR-1, and VEGFR-2 expression in the established HCM-SqCC010 cells by immunofluorescence (Fig. 3A). The results demonstrated that HCM-SqCC010 cells expressed VEGF-A (Figs. 3A–1), VEGFR-1 (Figs. 3A–2), and VEGFR-2 (Fig. 3A-). Moreover, we developed HCM-SqCC010 AOs, which showed a well-differentiated SCC status (Figs. 3B–1) and also expressed VEGF-A (Figs. 3B–2), VEGFR-1 (Fig. 3B-), and VEGFR-2 (Figs. 3B–4). Therefore, HCM-

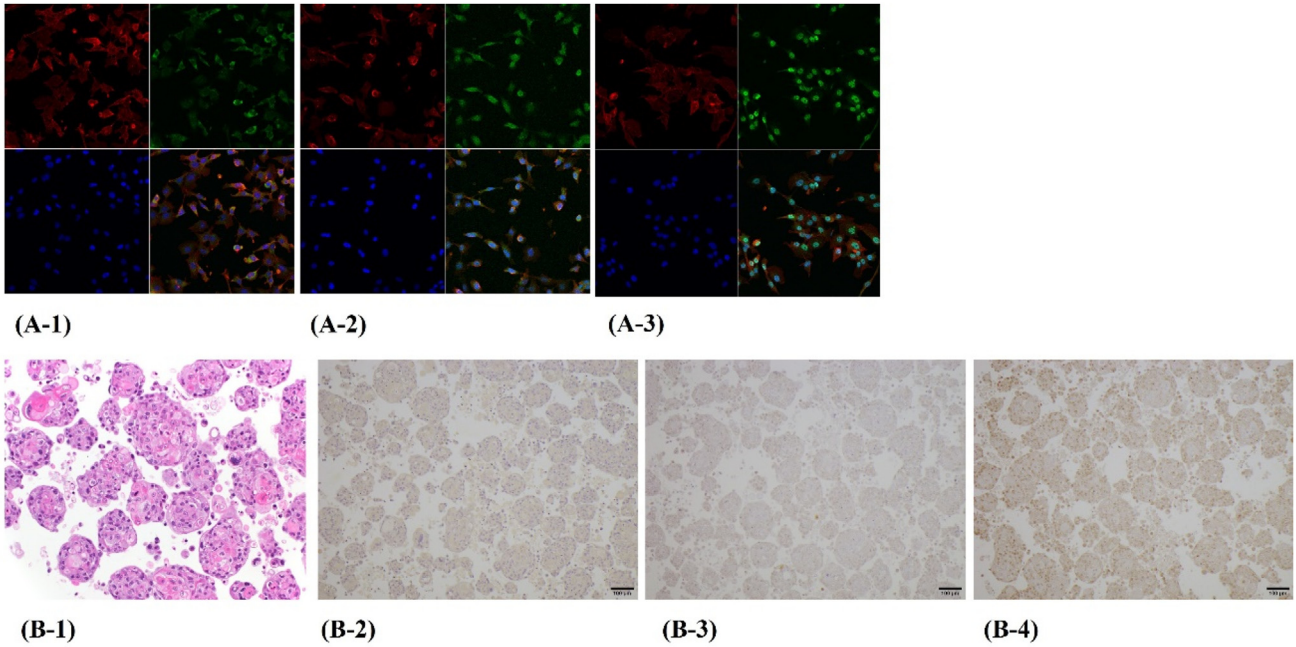

Figure 3 (A) Immunofluorescence staining. Blue, nuclei; red, actin; and green, (A-1) VEGF-A, (A-2) VEGFR-1, and (A-3) VEGFR-2 (magnification: 200 ×). (B-1) Artificial organoid (AO) of HCM-SqCC010 cells suggested well-differentiated SCC by H-E staining (HCM-SCC001 + CAF = 1:1). (B-2, 3, 4) Immunohistochemistry of spheroids showed that (B-2) VEGF-A, (B-3) VEGFR-1, and (B-4) VEGFR-2 were all positive; VEGF-A was expressed in the cytoplasm, while VEGFR-2 was mainly expressed in the nucleus (magnification: 100 ×). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

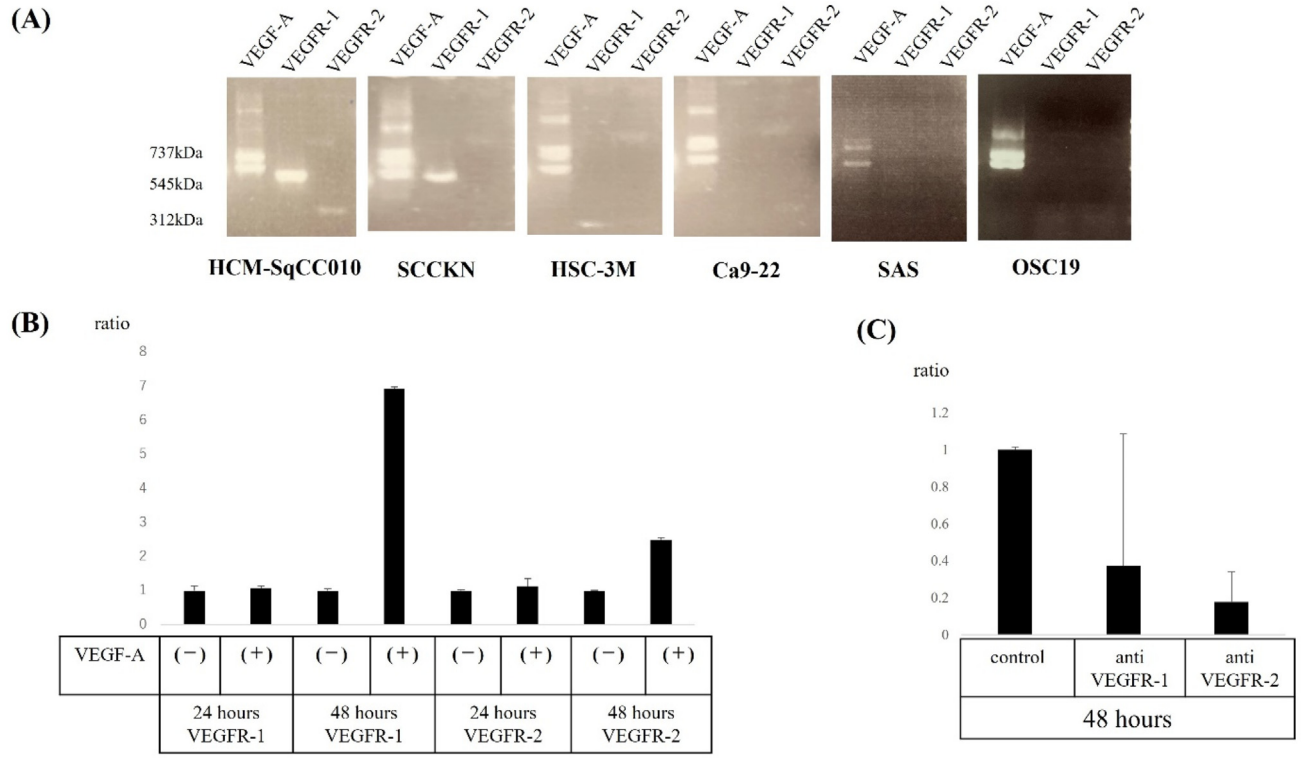

Figure 4 (A) VEGF-A, VEGFR-1, and VEGFR-2 mRNA expression in several OSCC cell lines. HCM-SqCC010 cells were found to have triple-positive expression. (B) Quantitative PCR results showed that VEGFR-1 was increased 6.92-fold at 48 h, and VEGFR-2 was increased 2.5-fold at 48 h. VEGF-A induced *VEGFR-1* and *VEGFR*-2 RNA expression in HCM-SqCC010 cells. (C) VEGFR-1 and VEGFR-2 antibodies suppressed VEGF-A expression in HCM-SqCC010 cells by 0.4-fold (VEGFR-1) and 0.2-fold (VEGFR-2), respectively, compared with controls.

SqCC010 cells sustained protein expression of VEGF-A, VEGFR-1, and VEGFR-2.

Analysis of VEGF-A, VEGFR-1, and VEGFR-2 expression in OSCC cell lines

We then examined VEGF-A expression in many OSCC cell lines by quantitative PCR, and all of the tested OSCC cell lines had VEGF-A mRNA. Double-positive expression of VEGF-A and VEGFR-2 protein in tumor cells is well documented. However, co-expression of VEGFR-1 and VEGFR-2 in solid tumors, especially OSCC cell lines, is uncommon. VEGF-A is a ligand for VEGFR-1 and -2; therefore, we also analyzed VEGFR-1 and -2 expression in six commercial OSCC cell lines. Screening by RT-PCR revealed VEGFR-1 mRNA expression in two of the six OSCC cell lines, and VEGFR-2 mRNA in two of the six cell lines. However, the only VEGFR-1 and -2 double-positive OSCC cells were the HCM-SqCC010 (Fig. 4-A).

To examine the expression levels of VEGFR-1 and -2 after stimulation with VEGF-A, VEGFR-1 and -2 transcripts were measured by quantitative PCR in HCM-SqCC010 cells treated with VEGF-A for 24 h and 48 h. The relative expression levels of VEGFR-1 to those of the control (GAPDH) after VEGF-A stimulation were 1.1 ± 0.084 for 24 h and 6.92 ± 0.059 for 48 h. Relative VEGFR-2 levels after VEGF-A stimulation were 1.1 ± 0.23 for 24 h and 2.5 ± 0.059 for 48 h (Fig. 4-B). To examine levels of mRNA activity, VEGF-A-stimulated HCM-SqCC010 cells cultured with VEGFR-1 or VEGFR-2 antibody for 48 h were collected for quantitative PCR. VEGFR-1 antibody inhibited VEGF-A mRNA by 66% at 48 h, and VEGFR-2 antibody inhibited VEGF-A mRNA by 80% at 48 h (Fig. 4-C). Therefore, we concluded that the VEGF-A/VEGFR-2 axis was the main driver of VEGFR kinase activity in HCM-SqCC010 cells.

The VEGF-A/VEGFR-2 axis promotes proliferation in HCM-SqCC010 cells

VEGF-A can bind to both VEGFR1 and VEGFR2, but the tyrosine kinase activity of VEGFR-1 is very weak, approximately 10-fold lower than that of VEGFR-2.[29] Next, we analyzed cell proliferation and VEGF-A expression in HCM-SqCC010 cells following blockade with VEGFR-1 or VEGFR-2 antibody. The proliferation of HCM-SqCC010 cells was suppressed to under 30% by blocking VEGFR-2 (Fig. 5-A). Moreover, the proliferation of HCM-SqCC010 cells was suppressed in a dose-dependent manner by the selective VEGFR-1 kinase inhibitor ZM306416 and/or the selective VEGFR-2 kinase inhibitor SU5408. ZM306416 weakly inhibited the growth of HCM-SqCC010 cells, which showed only a 20% inhibition with a 10-fold increased dose (Fig. 5-B). Thus, ZM306416 may restrictively inhibit VEGFR-1 in HCM-SqCC010 cells. Conversely, SU5408 strongly inhibited the growth of HCM-SqCC010 cells (Fig. 5-C). Next, we simultaneously administered ZM306416 and SU5408 to HCM-

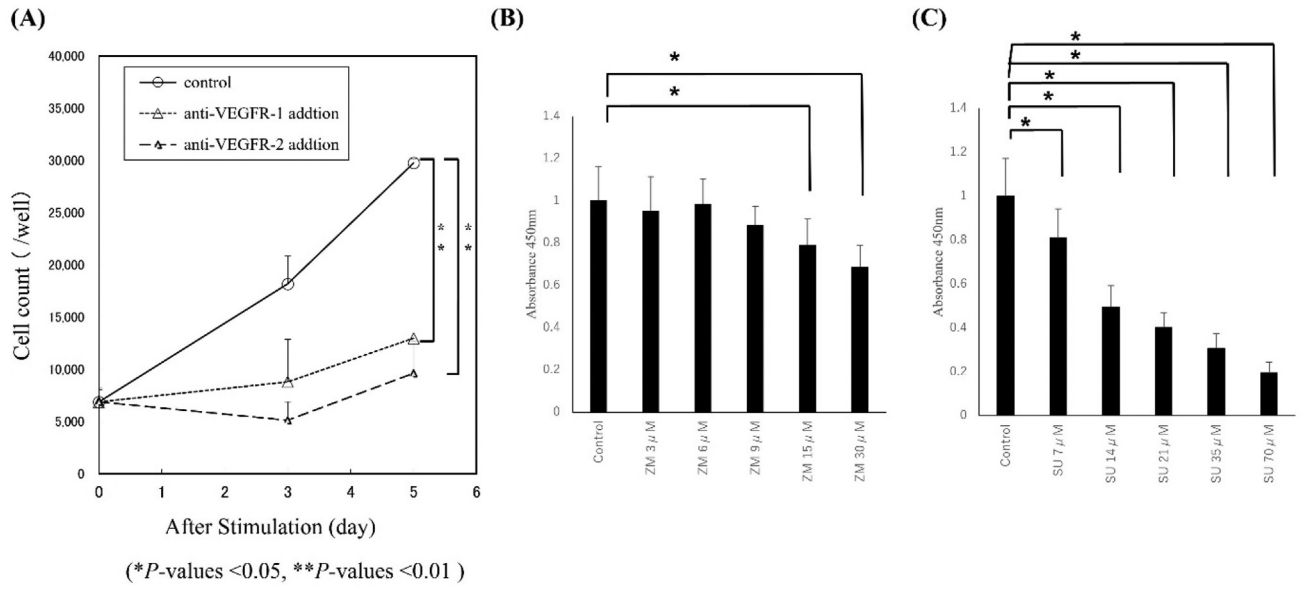

Figure 5 Proliferation assays. (A) Incubation with VEGFR-1 and VEGFR-2 antibodies inhibited cell proliferation by 0.44-fold (VEGFR-1) and 0.32-fold (VEGFR-2), respectively, compared with controls. (B) The selective VEGFR-1 inhibitor ZM306416 weakly inhibited proliferation by approximately 20%. (C) The selective VEGFR-2 inhibitor SU5408 strongly inhibited HCM-SqCC010 proliferation by approximately 80% ($P > 0.05$). ZM: ZM306416, SU: SU5408.

SqCC010 cells, but no simultaneous effect was revealed (data not shown). Therefore, we conclude that autocrine VEGF-A signaling in HCM-SqCC010 cells depends on VEGFR-2 kinase activity.

HCM-SqCC010-derived exosomes contain VEGF-A

Because it has been reported that exosomes play essential roles in tumor metastasis, we analyzed HCM-SqCC010 exosomes. We extracted exosomes from HCM-SqCC010 culture medium and evaluated their size and purity. The isolated exosomes ranged from 50 to 120 nm in diameter (Fig. 6-A), and immunoblot analysis revealed that they expressed the exosomal surface markers CD9, CD63, and TSG101 (Fig. 6-B). Furthermore, we performed RT-PCR, and the exosome of HCM-SqCC010 contained VEGF-A (Fig. 6-C). VEGF-A is a potent tumor lymphangiogenesis factor, a potential mediator of metastasis to distant sites, and a factor that can modify a lymphovascular niche into a premetastatic niche.[29] Thus, HCM-SqCC010 cells are a good cell line to model the metastatic process of oral cancer for molecular analysis.

Discussion

The currently approved anti-angiogenic therapies for the treatment of solid tumors hamper VEGF-A, VEGFR-2, or global VEGFR activation. However, agents that interfere with VEGF-A/VEGFR-2 signaling can cause severe adverse events (e.g., bleeding, delayed wound healing, gastrointestinal perforations, hypertension, thromboembolic complications, and proteinuria) due to inhibiting physiological angiogenesis.[30] Moreover, in most clinical settings, anti-angiogenic agents have failed to provide consistent and long-lasting antitumor activity. The report by Kyzas et al.[18] demonstrated the colocalization of VEGF and VEGFR-2 in

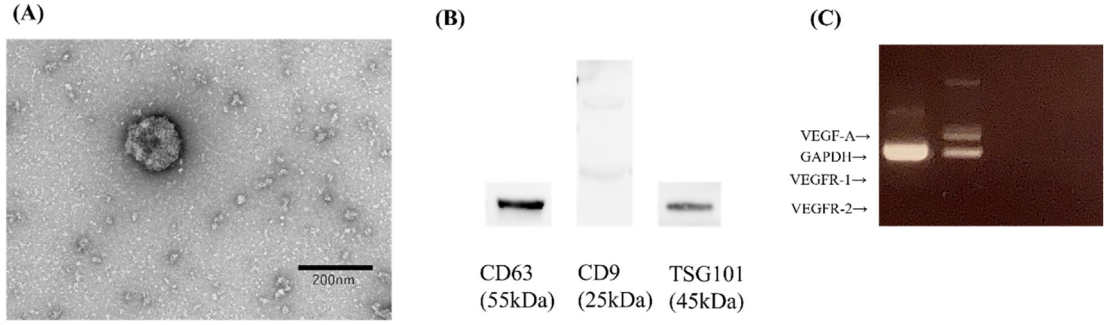

Figure 6 (A) Exosomes were extracted from HCM-SqCC010 cells. The isolated exosomes ranged from 50 to 120 nm in diameter. (B) Western blotting confirmed expression of the exosome surface markers CD9, CD63, and TSG101. (C) RT-PCR showed that exosomes from HCM-SqCC010 cells contained VEGF-A mRNA.

cancer cells and occasionally the co-expression of VEGF-A/VEGFR-2/Ki-67 in the same cell. Regarding these results, the authors suggested the existence of a VEGF autocrine loop in OSCCs but with the caveat that this might not be a general property of head and neck SCC.[18] From our data, multiple VEGFR inhibitors could decrease tumor growth. Meanwhile, VEGF antibodies such as Bevacizumab did not show an inhibition of tumor growth. Thus, multiple VEGFR inhibitors may be effective against OSCC, which express VEGFR.

Exosomes are nano-sized vesicles that are secreted from many cell types, including tumor cells. They are enclosed by a lipid bilayer and carry various biomolecules including proteins, glycans, lipids, metabolites, RNA, and DNA.[31] When exosomes are taken up by other cells, the cargoes are transferred and influence the phenotype of recipient cells. As such, exosomes are appreciated as essential mediators of cell—cell communication.[32] Tumor cells release a wide variety of tumor-derived exosomes that influence the behavior of cells in the primary tumor microenvironment.[33] Premetastatic niche formation is initiated by local changes, such as the induction of vascular leanness and remodeling of the stromatolites and extracellular matrix, followed by dramatic system effects on immune responses.[34] Recent studies have found that tumor-derived exosomes play important roles in metastasis.[35] Furthermore, tumor-derived exosomes mediate vascular leanness, which is a crucial feature of premetastatic niche formation.[36,37] VEGF-A is a potent tumor lymphangiogenesis factor, a potential mediator of tumor metastasis to distant sites, and a signaling molecule that could modify a lymphovascular niche into a premetastatic niche.[29] Therefore, future experiments will analyze the relationship between tumor-derived exosomes and premetastatic niche formation. HCM-SqCC010 cells are a good preclinical model of premetastatic niche formation with which to study metastasis. We established a new oral cancer cell line (HCM-SqCC010 cells) with VEGF-A, VEGFR1, and VEGFR2 expression. Moreover, exosomes from HCM-SqCC010 cells contained VEGF-A. Thus, HCM-SqCC010 cells are a good in vitro model for molecular analyses of metastatic processes in oral cancer.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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