Abstract. Midkine is a heparin-binding growth factor highly expressed in various human malignant tumors. However, its role in the growth of oral squamous cell carcinoma is not well understood. In this study, we analyzed the antitumor effect of a novel midkine inhibitor (iMDK) against oral squamous cell carcinoma. Administration of iMDK induced a robust antitumor response and suppressed cluster of differentiation 31 (CD31) expression in oral squamous cell carcinoma HSC-2 cells and SAS cells xenograft models. iMDK inhibited the proliferation of these cells dose-dependently, as well as the expression of midkine and phospho-extracellular signal-regulated kinase in HSC-2 and SAS cells. Moreover, iMDK significantly inhibited vascular endothelial growth factor and induced tube growth of human umbilical vein endothelial cells in a dose-dependent fashion. These findings suggest that midkine is critically involved in oral squamous cell carcinoma and iMDK can be effectively used for the treatment of oral squamous cell carcinoma.

Although oral squamous cell carcinoma is one of the most common types of human cancer, there has been little improvement in patient morbidity in the past 50 years (1). Despite advances in early detection, diagnosis, and management of oral squamous cell carcinoma, long-term survival rates have improved only marginally over the past decade (2, 3). Therefore, novel therapeutic approaches for oral squamous cell carcinoma are needed.

Materials and Methods

Cell lines and culture conditions. Human oral squamous cell carcinoma cell lines SAS and HSC-2 obtained from the Human Science Research Resources Bank (Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. Human vascular endothelial cells (HUVECs), newly obtained from the RIKEN BioResource Center Cell Bank (Tsukuba, Japan) were cultured in EBM-2 supplemented with 10% heat-inactivated fetal bovine serum. All of these cell lines were characterized by genotyping at the cell banks. All cell lines were cultured in an atmosphere of 10% CO₂ at 37°C.
Agent. iMDK was purchased from ChemDiv (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO).

Histochemical and immunohistochemical analysis of surgically resected samples. The study was approved by the Ethical Review Committee of the Okayama University Graduate School of Medicine and Dentistry, and Pharmaceutical Sciences (protocol number: 1949). Hematoxylin-eosin-stained specimens were prepared from surgically resected oral squamous cell carcinoma samples. Sections from the deepest part of the tumor invasion were evaluated primarily by light microscopic observation. All of the patients were examined and treated at Okayama University Hospital (Okayama, Japan) between 2000 and 2014, and the diagnoses were clinicopathologically confirmed. No patient had received chemotherapy or radiation therapy before surgery. All tumor samples were obtained with the consent of the patients. The sections were sequentially deparaffinized through a series of xylene, graded ethanol, and water immersion steps. After being autoclaved in 0.2% citrate buffer for 15 min, the sections were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. A primary antibody to midkine (rabbit IgG) from Abcam (Cambridge, MA, USA) was used for the immunohistochemical analysis. The specimens were incubated with 1:200 dilution of the antibody overnight at 4˚C, followed by three washes with tris-buffered saline (TBS). The slides were then treated with a streptavidin-biotin complex, EnVision horseredox peroxidase (HRP) (Dako, Carpinteria, CA, USA) for 60 min at a dilution of 1:100. The immunoreaction was visualized using a 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System; Dako), and counterstaining was carried out with hematoxylin. Finally, the sections were immersed in an ethanol and xylene bath and mounted for examination.

Cell proliferation assay. SAS and HSC-2 cells were plated in a 96-well plate at 5×10³ cells per well in the presence of 1-500 nM iMDK. An MTS assay was performed to obtain a relative cell number after 24 hours of incubation under the experimental conditions (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA).

Immunoblot analysis. SAS and HSC-2 cells after treatment with 20 nM iMDK were rinsed once with ice-cold phosphate-buffered saline and lysed in an ice-cold lysis buffer (50 mmol/l Tris-HCl (pH 7.4), containing 150 mmol/l NaCl, 1% Triton X-100, 1% NP-40, 10 mmol/l NaF, 100 mmol/l leupeptin, 2 mg/ml aprotinin, and 1 mmol/l phenylmethyl sulfonyl fluoride). Cell lysates containing 25 µg of total protein in a lysis buffer were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Each membrane was incubated with primary and secondary antibodies according to the ECL chemiluminescence protocol (RP2109; Amersham Biosciences, Buckinghamshire, UK) to detect secondary antibody binding. Antibodies against phospho-protein kinase B (rAkt) (Ser473) (1:2000) and phospho-p44/42 mitogen-activated protein kinase (MAPK) (ERK1/2) (Thr202/Tyr204) 1:1000 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody to midkine (1:1000) was purchased from Abcam; antibody to pleiotrophin [a member of a family of structurally related to midkine (12)] (1:500) was purchased from Sigma Aldrich (St. Louis, MO, USA) and used at the indicated dilution. HRP-conjugated goat anti-rabbit antibodies or goat anti-mouse IgG were used as secondary antibodies at a 1:5000 dilution.

In vitro angiogenesis assay. Experiments on the angiogenesis using HUVECs were conducted in 24-well dishes with an angiogenesis kit (Kurabo, Osaka, Japan) according to the manufacturer’s instructions. Briefly, HUVECs were cultured with or without vascular endothelial growth factor (VEGF) and iMDK, and the medium was changed every 3 days. After 11 days, HUVECs were stained for CD31 with a Tubule Staining Kit (Kurabo). The luminal area was evaluated with an Angiogenesis Image Analyzer (Kurabo) in three different fields for each well and analyzed statistically.

Animal experiments. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine and Dentistry (Ethics Committee reference number: OKU-2015577). Human oral squamous cell carcinoma xenografts were established in 5-week-old female BALB/c nude mice (Clea Japan, Tokyo, Japan) by subcutaneous inoculation of 1×10⁶ SAS and HSC-2 cells into the dorsal flank as described previously (17, 18). The mice were randomly assigned into two groups. After 2 weeks, iMDK-treated group of mice was intraperitoneally injected with 100 µl solution containing iMDK (9 mg/kg/wk) every day. The tumor volume was measured from 14 days to 28 or 36 days after tumor inoculation. The tumor volume (mm³) was calculated using the equation 4πr³/3, where r₁=longitudinal radius, and r₂=transverse radius, as described previously (17, 18). All of the mice were sacrificed on day 29 or 37 after tumor inoculation, and tumors were removed and prepared for immunohistochemistry; sections were sequentially deparaffinized through a series of xylene, graded ethanol, and water immersion steps. After being autoclaved in 0.2% citrate buffer for 15 minutes, the sections were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. A primary antibody to midkine (rabbit IgG), and anti-CD31 (rabbit IgG) from Abcam were used for the immunohistochemical analysis. The specimens were incubated with 1:200 (midkine) and 1:50 (CD31) dilutions of the antibody overnight at 4˚C, followed by three washes with tris-buffered saline (TBS). The slides were then treated with a streptavidin-biotin complex, EnVision HRP (Dako) for 60 minutes at a dilution of 1:100. The immunoreaction was visualized using a 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System; Dako), and counterstaining was carried out with hematoxylin. Finally, the sections were immersed in an ethanol and xylene bath and mounted for examination.

Apoptosis assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed to detect apoptosis using the DeadEnd colormetric TUNEL system (Promega) according to the manufacturer’s protocol.

Statistical analysis. Data were analyzed using unpaired Student’s t-test for the analysis of two groups, one-way ANOVA, post hoc Bonferroni and Dunnett’s test for the analysis of multiple group comparisons, and a log-rank test for Kaplan–Meier analysis using SPSS statistical software (version 10; Chicago, IL, USA). Results are expressed as means±SDs. A value of p<0.05 was considered to indicate statistical significance.
Figure 1. Expression of midkine (MDK) in mandibular oral squamous cell carcinoma and the effect of midkine inhibitor (iMDK) on oral squamous cell carcinoma xenografts in mice. A: Immunohistochemical staining for MDK in a normal epithelium and mandibular oral squamous cell carcinoma. The insets shown in the left panel are magnified in middle and right panels respectively. Scale bar=50 μm. B and C: Macroscopic appearance, volume and immunohistochemical analysis of HSC-2 (B) and SAS (C) xenograft tumors in mice with after treatment with iMDK. Left panel: Tumor volumes and images of xenograft tumors derived from HSC-2 and SAS cells, dissected from mice treated with iMDK (scale bar=10 mm). The volumes of tumors derived from HSC-2 or SAS cells were reduced after treatment with iMDK (9 mg/kg) in a xenograft mouse model. Dimethyl sulfoxide was used for the control group. Tumor growth is expressed as mean tumor volume; bars represent the SDs. Statistically significantly different at *p<0.05 and **p<0.01. Right panel: Representative histology of MDK and CD31 expression in xenograft tumors from mice treated with iMDK. Scale bar=50 μm.
Figure 2. The effect of midkine inhibitor (iMDK) on oral squamous cell carcinoma cell growth and signaling and Human vascular endothelial cell (HUVEC) tube formation. A: Growth-inhibitory effects of iMDK on HSC-2 and SAS cells. B: Detection of midkine (MDK), phospho-extracellular signal-regulated kinase (pERK), phospho-protein kinase B (pAKT), pleiotrophin (PTN) in HSC-2 and SAS cells by immunoblotting after treatment with 20 nM iMDK. C: Relative capillary length of CD31-positive HUVECs on type 1 collagen with or without 10 ng/ml VEGF in the presence of the indicated amount of iMDK. *Statistically significantly different at p<0.05.
Results

Expression of midkine in oral squamous cell carcinoma. Figure 1A illustrates a representative histologic pattern of midkine in the human gingival epithelium. Midkine was not detected in the normal gingival epithelium (Figure 1A, middle panel). The expression of midkine was strongly observed throughout the squamous cell carcinoma (Figure 1A, right panel). This result suggested that midkine might be related to the proliferation and survival of oral squamous cell carcinoma cells.

Administration of iMDK suppresses tumor growth in vivo. To analyze the antitumor effect of iMDK in vivo, we established an oral squamous cell carcinoma xenograft tumor derived from HSC-2 and SAS cells in nude mice. The animals were treated with an intraperitoneal administration of iMDK (9 mg/kg). Administration five times a week started 14 days after tumor inoculation for 14 days (HSC-2 cells) or 22 days (SAS cells), and tumor volume was measured each day during the treatment. As shown in Figure 1, both HSC-2 (Figure 1B) and SAS (Figure 1C) xenograft volumes significantly decreased in iMDK-treated mice compared with controls. No severe effects occurred on day 14 (HSC-2 cells) or day 22 (SAS cells) during the treatment. At sacrifice, after the treatment with iMDK, the tumors were excised and examined histologically. Immunohistochemical analysis showed a significant decrease of midkine expression and a reduction in CD31-positive endothelial cells in HSC-2 and SAS tumor sections from iMDK-administered mice (Figure 1B and C, right panel). These results suggested that iMDK might suppress tumor growth by the regulation of angiogenesis.

Inhibition of midkine by iMDK suppressed cell growth of oral squamous cell carcinoma in vitro. An MTS assay was performed to determine the iMDK inhibitory effect on cell growth. As shown in Figure 2A, iMDK significantly reduced the proliferation of both HSC-2 (10 nM) and SAS (20 nM) cells, and the observed effects were dose-dependent (p<0.05, Figure 2A). Next, in order to investigate which signaling pathway is involved in iMDK reduction of cell proliferation, we analyzed expression of midkine and of the molecules downstream of midkine by immunoblot analysis. As shown in Figure 2B, 20 nM iMDK inhibited midkine expression in both HSC-2 and SAS cells, indicating that iMDK has effective and specific action in midkine-positive oral squamous cell carcinoma cells. Since the activation of ERK MAPK and AKT is involved in tumorigenesis (19, 20), we sought to determine whether ERK or AKT is suppressed by iMDK. iMDK at 20 nM suppressed phosphorylation of ERK from 30 minutes in both HSC-2 and SAS cells and the suppression was continued for 12 h (Figure 2B). Moreover, 20 nM iMDK transiently up-regulated the phosphorylation of AKT for 30 min in both HSC-2 and SAS cells and then down-regulated it time-dependently compared to controls (Figure 2B). However, 20 nM iMDK administration did not change the expression of pleiotrophin.

TUNEL staining was carried out to clarify whether the inhibition of midkine induces apoptosis of human oral
squamous cell carcinoma cells. iMDK treatment at 10 and 100 nM significantly increased TUNEL-positive HSC-2 cells within 48 hours compared to controls (p<0.05, Figure 3). These results suggested that inhibition of midkine by iMDK might inhibit proliferation and induce apoptosis through the ERK and AKT pathway in human oral squamous cell carcinoma cells.

**iMDK suppressed endothelial cell tube formation.** To clarify whether iMDK suppresses angiogenesis, we determined its inhibitory effect on normal HUVEC growth. In the presence of 10 ng/ml VEGF, cells exhibited efficient tube formation (Figure 2C). iMDK significantly inhibited the VEGF-induced tube growth of HUVECs in a dose-dependent fashion (p<0.05, Figure 2C).

**Discussion**

Previous studies revealed a role of midkine in oral squamous cell carcinoma (14). Because midkine is known as a key molecule for tumor cell proliferation (21), it is an ideal target for treatment of oral squamous cell carcinoma. We found that as lower gingival oral squamous cell carcinoma progressed, the midkine immunostained area of increased. Our results indicated that midkine expression can affect oral squamous cell carcinoma development and progression; furthermore, it might be a biomarker for cancer screening and predicting outcome of oral squamous cell carcinoma. In this study, we found that inhibition of midkine by iMDK might be a promising approach for the treatment of oral squamous cell carcinoma. Midkine was highly expressed in human oral squamous cell carcinoma, and iMDK treatment displayed significant inhibitory effects on the proliferation and apoptosis of oral squamous cell carcinoma cells in vitro.

Midkine is known to activate not only the AKT pathway but also the MAPK pathway in primary neuronal culture (22) and myocardium (23). In our study, iMDK inhibited the ERK and AKT pathways, and transiently activated AKT in both HSC-2 and SAS cells. The mechanisms by which iMDK inhibit the AKT pathway after transient activation are unknown. The up-regulation of the AKT pathway might be a compensation for down-regulation of the ERK pathway in HSC-2 and SAS cells. Treatment of HSC-2 and SAS cells with iMDK did not influence the expression of the pleiotrophin, indicating that iMDK specifically inhibits midkine signaling.

Angiogenesis creates a tumor microenvironment that enhances tumor progression; endothelial cells, as the major component making up blood vessels, proliferate much more rapidly during angiogenesis than under normal conditions (24). Administration of iMDK exhibited efficacy in the oral squamous cell carcinoma xenograft model. Inhibition of tumor growth was associated with clear down-regulation of midkine expression and neovascularization in mice with oral squamous cell carcinoma xenografts. Several studies have demonstrated that midkine plays an important role in tumor angiogenesis (9). It was also reported that midkine-binding peptides and novel iMDK inhibitors inhibited tumor-associated angiogenesis in hepatocarcinoma and lung cancer xenograft models in vivo (4).

In the present study, iMDK significantly inhibited capillary formation in HUVECs induced by VEGF. These data suggested that the inhibition of midkine by iMDK might suppress angiogenesis and endothelial cell survival in human oral squamous cell carcinoma.

In summary, this study is, to the best of our knowledge, the first to show that midkine should be considered a target for the treatment of oral squamous cell carcinoma. The iMDK effect was mediated through down-regulation of the midkine protein directly, which is involved in the survival and angiogenesis of cancer cells. Although further studies are needed, including identification of direct iMDK targets, and safety validation, the use of iMDK alone or in combination with other agents might be considered an attractive approach to treating oral squamous cell carcinoma.

**Conflicts of Interest**

None of the Authors have any conflict of interest with regard to this study.

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