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Oral Squamous Cell Carcinoma-derived Sonic Hedgehog Promotes Angiogenesis

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Abstract. *Background: Sonic hedgehog (SHH) signaling is related to the pathogenesis of oral squamous cell carcinoma (OSCC), but its role in OSCC is not yet well understood. In this study, we analyzed the role of SHH signaling in OSCC. Materials and Methods: We examined the expression pattern of SHH and its signal proteins in clinically resected OSCC samples by immunohistochemistry. We also evaluated the function of SHH signaling using the hedgehog signaling inhibitor cyclopamine in vivo and in vitro by proliferation, migration and angiogenesis analyses. Results: We found that SHH was highly expressed in human tongue OSCC, whereas patched (PTCH1), glioma-associated oncogene 1 (GLI1) and GLI2 proteins were expressed in the microvascular cells in the tumor invasive front. Administration of cyclopamine to mice suppressed the growth and angiogenesis of OSCC xenografts in vivo. Moreover, cyclopamine inhibited endothelial cell proliferation and migration, and reduced aorta vascular length in the rat. Conclusion: These findings suggest that OSCC-derived SHH stimulates angiogenesis at the tumor invasive front.*

Oral squamous cell carcinoma (OSCC) is the sixth most-common cancer worldwide. Despite extensive medical research, the 5-year survival rates of patients with OSCC have remained unchanged. As high mortality from OSCC is attributed to regional and distant metastasis, a more detailed analysis of the molecular events involved is necessary for the development of novel treatment strategies. The hedgehog signaling pathway governs complex developmental processes, and uncontrolled activation of hedgehog signaling drives tumor initiation and maintenance (1).

The binding of sonic hedgehog (SHH) to the patched receptor relieves the inhibition of smoothened receptor, which then activates an intercellular signaling cascade resulting in increased transcription of downstream genes including glioma-associated oncogene-1 (GLI1) and GLI2. Blocking this pathway may be a novel therapeutic avenue for the treatment of hedgehog-related cancer progression (2). Importantly, SHH signal activation has been found to be associated with disease progression and worse prognosis in OSCC (3-5), and has been suggested as a new therapeutic target in advanced OSCC (6-8). However, the molecular mechanisms leading to the activation of SHH in OSCC are not well-known.

In the present study, we investigated SHH signaling using surgically-resected OSCC samples, and *in vivo* and *in vitro* models.

Materials and Methods

Immunohistochemical analysis of surgically resected samples. Surgically resected tongue squamous cell carcinoma samples were collected as part of routine care by the Authors at Okayama University Hospital (Okayama, Japan) in the years 2000-2010. No patient had received chemotherapy or radiation therapy before surgery. The retrospective study was approved by the Ethical Review Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (protocol number: 1949). Primary antibodies anti-SHH (rabbit IgG), anti-GLI1 (rabbit IgG), anti-GLI2 (rabbit IgG), anti-CD31 (rabbit IgG) (Cell Signaling Technology, Danvers, MA, USA), and anti-patched1 (PTCH1; rabbit IgG; Proteintech, Chicago IL, USA) were used for immunohistochemical analysis as described previously (7).

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Cell lines and culture conditions. Human OSCC cell lines SAS, HSC-2, HSC-3, and HSC-4, obtained from the Human Science Research Resources Bank (Osaka, Japan), and normal human umbilical vein endothelial cells (HUVECs), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Bioscience, Lenexa, KS, USA). Human vascular endothelial cells (HUVECs; Lonza, Basel, Switzerland) were maintained as described previously (9). Hedgehog signaling inhibitor Cyclopamine (LC Laboratories, Woburn, MA, USA) was used in some experiments; it was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and added to culture medium at 0-100 μM.

Cell proliferation assay. Cells were seeded on a 6-well or 96-well culture plate and cultured with cyclopamine (0, 5, and 20 μM). The cell number was determined by TC 10™ automated cell counter (Bio-Rad, Hercules, CA, USA) or by MTS assay as described previously (10).

Trypan blue exclusion assay. Subconfluent cells were treated with different amounts of cyclopamine for 48 hours. After treatment, cells were harvested with trypsin, stained with trypan blue, and counted manually with a hemocytometer. The concentration of cyclopamine resulting in 50% inhibition (IC_{50}) was determined from a dose–response curve (10). IC_{50} values were calculated by linear regression analysis of the percentage inhibition.

Cell migration assay. Cyclopamine (0, 5, and 20 μM)-treated cell mixture was added to the upper transwell chamber. As a chemoattractant, SAS cell-conditioned medium was prepared after 24-h culturing of confluent SAS cells with 4 ml Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Invitrogen) containing 10% fetal calf serum (JRH Bioscience) in a 10-cm dish. The migrated cells were examined as described previously (10).

Immunoblot analysis. Subconfluent human OSCC cells (48-h culture) were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 100 mM leupeptin, 2 mg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride]. Cell lysates containing 10 μg of total protein in lysis buffer were electrophoresed in 12% sodium dodecyl sulfate–poly acrylamide gel electrophoresis gels and the proteins were transferred to Immobilon Transfer Membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 2% nonfat dry milk in tris-buffered saline overnight at 4˚C and then incubated with a 1:1000 dilution of anti-SHH, anti-GLI1, anti-GLI2 (Cell Signaling) and anti-PTCH1 (Proteintech). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, MA, USA) was used as a secondary antibody at a 1:1,000 dilution. Bands were visualized using the ECL chemiluminescence detection method (RPN2109; Amersham Biosciences, Buckinghamshire, UK). As antibodies, were used.

Animal study. A mouse model with a xenograft at an extraskeletal site was prepared by inoculating BALB/c-nu/nu 4-week-old female mice (Clea Japan, Inc., Tokyo, Japan) with tumor cell suspensions of SAS cells (1×10^6 cells/100 μl of PBS) *via* subcutaneous injection in the right dorsal flank, as described previously (10). The mice were randomly assigned into two groups (n=6/group). Seven days after tumor cell inoculation, mice were orally administered cyclopamine (50 mg/kg) or DMSO (vehicle) from days 1 to 12. The tumor growth was determined with a caliper. The tumor volume (cubic mm) was calculated as $4\pi/3 \times (r1/2 + r2/2)^3$, where r1=longitudinal radius, and r2=transverse radius, as described previously (10). On day 12, all of the mice were sacrificed, and tumors were removed and weighed. The protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (2013108).

Rat aorta ring assay. An *ex vivo* angiogenesis assay was performed according to the slightly modified method described earlier (11). Briefly, a male Wistar rat (6 weeks old; Clea Japan, Inc., Tokyo, Japan) was sacrificed by bleeding from the right femoral artery under anesthesia. The thoracic aorta was removed and washed with RPMI-1640 medium to avoid contamination with blood. It was then turned inside out, and cut into segments about 1-1.5 mm in length. Collagen gel (gel matrix solution) was made with 8 volumes of porcine tendon collagen solution (3 mg/ml) (Cellmatrix Ia; Nitta Gelatin Co., Osaka, Japan), 1 volume of 10 × Eagle's minimum essential medium (Gibco, New York, NY, USA), and 1 volume of reconstitution buffer (0.08 M NaOH and 200 mM HEPES). These solutions were mixed gently at 4˚C. Each aortic segment was placed at the center of a well of a 12-well culture plate and covered with 0.3 ml of gel matrix solution reconstituted as described above. The solution was allowed to gel at 37˚C for 20 min, overlaid with 0.8 ml of RPMI-1640 medium (Gibco) containing 1% ITS+ (Becton Dickinson Labware, Franklin Lakes, NJ, USA), then 2 μg/ml SHH, 20 μM cyclopamine or vehicle was added. Incubation was carried out for 5 days in a fully humidified system of 5% CO_2 in air at 37˚C. The medium was changed on day 1 and 3 of the culture period. An estimation of the length of the capillaries was made under phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the end of each capillary. Microscopic fields were photographed with a digital camera. The length and area of the capillaries was measured using NIH Image (National Institute of Health, Bethesda, MD, USA). Each reported value is presented as the average of three culture samples.

cDNA expression array. HUVECs were treated with 2 μg/ml SHH for 8 h. Total RNA was isolated by using TRIZOL reagent (Life Technologies, Grand Island NY, USA) from HUVEC. Total RNA was incubated with DNase I (1 U/ml) at 37 C for 30 min. HUVEC mRNA was isolated from total RNA by using Streptavidin MagneSphere Paramagnetic Particles and Magnetic Separation system (Promega Co., Madison, WI, USA). The Atlas Human Cancer cDNA expression array (7742-1) was purchased from Clontech Laboratories Inc (Palo Alto, CA, USA). Total RNA isolation from HUVECs, and cDNA synthesis and hybridization were performed following the manufacturers' instructions. The array was exposed to an imaging plate at –80˚C for 36 h and scanned using BAS 2000 (Fuji Film Corp. Tokyo, Japan). The results were quantified using Image Gauge software (Fuji Film Corp.)

Statistical analysis. Data were analyzed using unpaired Student's *t*-test for the analysis of two groups and one-way ANOVA for the analysis of multiple group comparisons. The results are expressed as means±SD. Values of $p<0.05$ were considered to indicate statistical significance.

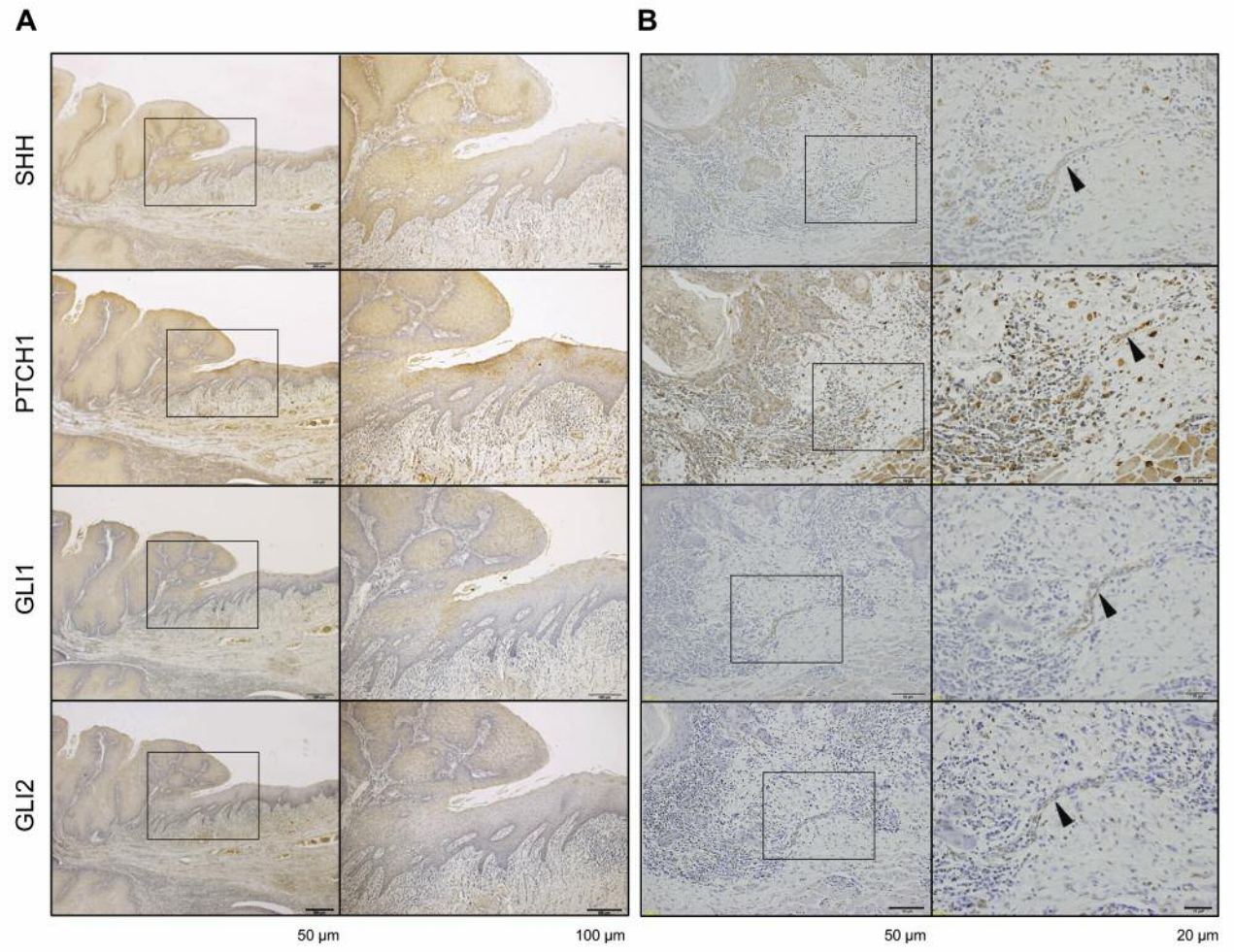

Figure 1. *Immunohistochemical staining for sonic hedgehog (SHH), patched-1 (PTCH1), glioma-associated oncogene-1 (GLI1) and GLI2 in tongue squamous cell carcinoma. A: Transition between normal mucosa and tongue squamous cell carcinoma. B: Invasive front of tongue oral squamous cell carcinoma. Each right-hand image is a magnifiied view of the corresponding inset of the left-hand image. Arrow head: Microvasculature.*

Results

The expression of SHH in tongue squamous cell carcinoma. To investigate the expression of SHH and signaling proteins in human tongue squamous cell carcinoma, we performed immunohistochemical staining using specific antibodies. SHH, PTCH1, GLI1 and GLI2 were hardly detected in the margin of the epithelium around the tumor (Figure 1A), whereas tumor cells highly expressed SHH and its signaling proteins (Figure 1A). Importantly, more intense signals of PTCH1, GLI1 and GLI2 were detected in the microvascular cells at the tumor's invasive front (Figure 1B).

Inhibition of SHH by cyclopamine suppressed cell growth of OSCC in vitro. Firstly, in order to confirm the basal level of SHH in human OSCC, we performed immunoblot analysis. As shown in Figure 2A, SHH was highly expressed in SAS, HSC-2, HSC-3 and HSC-4 cells, whereas the SHH receptor PTCH1 and the signaling proteins GLI1 and GLI2 were highly expressed in SAS cells compared with the levels in HSC-2, HSC-3 and HSC-4 cells. Next, in order to determine the inhibitory effect on cell growth by cyclopamine and to optimize the concentration for use in further experiments, the IC_{50} was measured by the trypan blue exclusion method after treatment of cells with cyclopamine for 48 h. The IC_{50} for SAS cells was 22 μM (Figure 2B); cyclopamine significantly reduced the proliferation of SAS cells in a dose-dependent manner with 72-h treatment (Figure 2C). The results suggest that human OSCC SAS cell proliferation is enhanced by hedgehog signaling.

Hedgehog signaling is involved in subcutaneous tumor growth in vivo. In order to analyze the involvement of

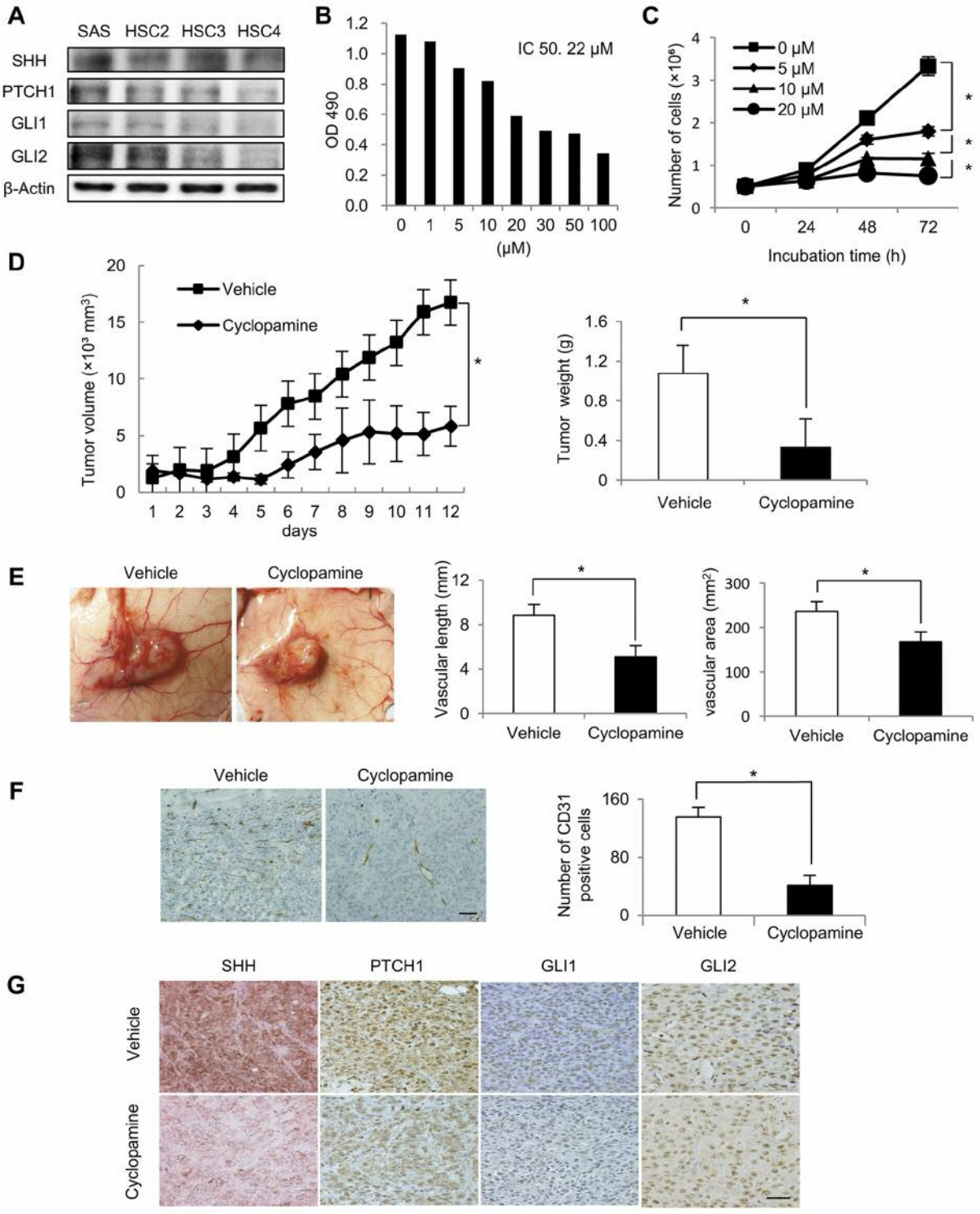

Figure 2. *Involvement of sonic hedgehog (SHH) signaling in the growth of oral squamous cell carcinoma cells in vitro and in vivo. A: Comparison of SHH signaling expression in SAS, HSC2, HSC3 and HSC4 cells. B: The half-maximal inhibitory concentration (IC_{50}) of cyclopamine in SAS cells. C: The growth inhibitory effect of cyclopamine on SAS cells. D: Tumor volume for 12 days after treatment (n=6 /group, left panel) and the tumor weight on day 12 (right panel). E: Microscopic appearance of neovascularization and vascular analysis on day 12 after treatment (n=6/group). F: Immunohistochemical staining and the analysis of CD31-positive cells. G: Immunohistochemical analysis of SHH signaling in xenograft tumors. Scale bar, 20 μm. Cell number, tumor volume and weight, vascular length and area are expressed as means, with bars representing SDs. Asterisks indicate statistically significant differences at $p<0.05$.*

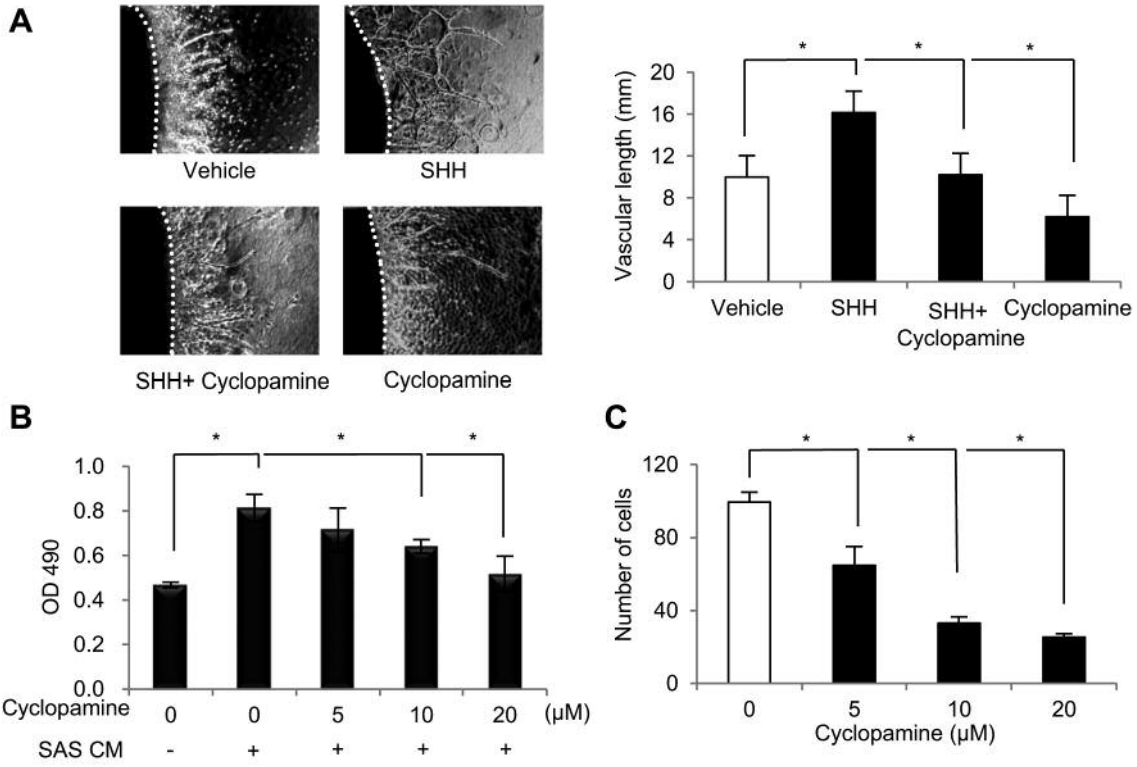

Figure 3. *Involvement of sonic hedgehog (SHH) signaling in angiogenesis. A: Representative capillary growth of rat aorta ring cultured in collagen gel (left panel) and vascular length analysis (right panel). n=3. SHH, 2 μg/ml; cyclopamine, 20 μM. B: Inhibitory effect of cyclopamine on proliferation of human umbilical vein endothelial cells (HUVECs) stimulated by SAS-conditioned media. C: Inhibitory effect of cyclopamine on HUVEC migration induced by SAS-conditioned media. Asterisks indicate statistically significant differences at p<0.05.*

hedgehog signaling in OSCC tumor growth *in vivo*, we established xenograft tumors derived from SAS cells in nude mice. The animals were treated subcutaneously with cyclopamine (50 mg/kg) or DMSO as a vehicle. Cyclopamine administration was started 7 days after tumor cell inoculation, and was carried out every day for 12 days; tumor volume was measured each day during the treatment (Figure 2D). As shown in Figure 2D, both the tumor volume (left panel) and weight (right panel) significantly decreased in the cyclopamine-treated mice compared with mice treated with the vehicle only ($p<0.05$). Figure 2E illustrates a representative macroscopic view of the subcutaneous tumors 12 days after treatment. Neovascularization around the tumors was significantly suppressed in this subcutaneous xenograft model after intraperitoneal administration of 50 mg/kg cyclopamine compared to the vehicle-treated mice (Figure 2E right panel). Immunohistochemical analysis further showed a significant reduction in CD31-positive endothelial cells in tumor sections from cyclopamine-treated mice ($p<0.05$, Figure 2F). In addition, the intensity of SHH,

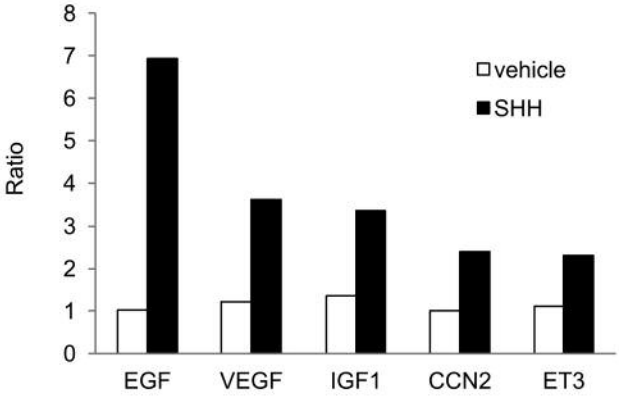

Figure 4. *Results of microarray analysis after stimulation of human umbilical vein endothelial cells (HUVECs) with sonic hedgehog (SHH). Angiogenic factors were up-regulated by 2 μg/ml SHH treatment of HUVECs for 24 h. EGF: Epithelial growth factor, VEGF: vascular endothelial growth factor, IGF1: insulin-like growth factor-1, CCN2: connective tissue growth factor, ET3: endothelin 3.*

PTCH1, GLI1 and GLI2 expression in tumor cells decreased in tumor sections from cyclopamine-treated in mice more than in the vehicle-treated group (Figure 2G). These results suggest that SHH signaling is involved in tumorigenesis by the regulation of tumor cell growth and angiogenesis.

Involvement of SHH signal in tumor angiogenesis. To clarify whether SHH signaling is involved in angiogenesis, we first determined the direct effect of SHH in neovascularization using a rat aorta ring assay. SHH treatment apparently increased vascular length and tube formation ($p<0.05$, Figure 3A), whereas up-regulation of vascular length by SHH was suppressed by co-treatment with cyclopamine ($p<0.05$, Figure 3A). Next, we investigated the involvement of SHH in tumor angiogenesis by treatment with conditioned medium from SAS cells. SAS-conditioned medium significantly stimulated HUVEC proliferation whereas the up-regulation of proliferation of HUVECs was dose-dependently inhibited by treatment with cyclopamine ($p<0.05$, Figure 3B). Moreover, cyclopamine treatment apparently dose-dependently suppressed the migration of HUVECs induced by SAS cell-conditioned medium ($p<0.05$, Figure 3C). These findings suggest that SHH plays a role in regulation of tumor angiogenesis.

Hedgehog signaling is involved in activation of angiogenesis-related genes. Since SHH appears to play an important role in angiogenesis, we compared the endogenous expression levels of genes between vehicle- and SHH-treated HUVECs by microarray analysis. When compared to the vehicle-treated samples, SHH-treated samples showed increased expression of 48 factors, including nine growth factors, cytokines and chemokines, five angiogenic-related factors; four intermediate filament proteins, three intercellular kinase network members and three metalloproteinases. The up-regulated angiogenic factors were epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF1), connective tissue growth factor (CCN2) and endothelin 3 (ET3) (Figure 4). These results suggest that the SHH produced by a tumor regulates these factors produced by vascular endothelial cells and induces autocrine control of angiogenesis.

Discussion

Tongue squamous cell carcinoma is the most frequent malignant neoplasm of the oral cavity (60%) in Japan (12). Several studies have suggested the involvement of hedgehog signaling in OSCC (5, 8) although the functional significance of these findings and their application to potential treatments are not yet well understood. In our study, as tongue epithelial dysplasia progressed, the immunostained area of SHH and signaling in tumor cells were increased. Our data suggested that paracrine hedgehog signaling could increase OSCC growth through direct action on microvascular endothelial cells, suggesting the involvement of SHH in tumor-associated angiogenesis. SHH stimulates direct and indirect mobilization of endothelial progenitor cells to new vessel formation sites (13, 14). Our data show that cyclopamine significantly inhibited capillary formation and migration induced by SHH and OSCC-conditioned media. Our data indicate that paracrine SHH signaling mediated the pro-angiogenic function of SAS cells and enhanced OSCC growth.

In microarray analysis, five angiogenic factors, including EGF and VEGF, were increased by the action of SHH on HUVECs. Several recent reports have suggested the involvement of hedgehog signaling in tumor-associated angiogenesis (15, 16); for instance, tissue microarray analysis of 200 patients with triple-negative breast cancer (TNBC) showed GLI1 overexpression paired with VEGF receptor 2 (VEGFR2) expression, and showed that hedgehog signal promoted TNBC progression in an autocrine manner, resulting in a VEGF/VEGFR2 loop in cancer cells, and paracrine orchestration of tumor vascularization (15). The SHH signaling pathway promotes the expression of VEGF in the tumor environment, thereby promoting angiogenesis and tumor malignancy (16). In this study, GLI1 and GLI2 were hardly expressed in tongue carcinoma cells, but were robustly expressed in microvascular endothelial cells at the invasion front. Tumor-derived SHH can promote angiogenic processes of vascular endothelial cells in a paracrine manner, and its role in stimulating the production of secreted angiogenic growth factors could cooperatively promote tumor angiogenesis. A synergistic effect between hedgehog and EGF receptor signaling was found to account for a release of VEGF, and supported human medulloblastoma growth (17). Hedgehog and EGF signals also interact in head-and-neck OSCC; a phase 1 study on treatment with hedgehog inhibitor IPI-926 and cetuximab in a patient with recurrent OSCC showed the efficacy of this strategy (18). Clinical development of novel pharmacological combinations of hedgehog inhibitors could overcome the limitations of bevacizumab use, by acting simultaneously against tumor growth and angiogenesis (15).

In summary, this study is, to the best our knowledge, the first to show the direct and indirect mechanisms of SHH on endothelial cells. Our findings clearly show that OSCC-derived SHH is involved in endothelial cell proliferation and migration as well as tumor growth *in vitro* and *in vivo*. These results strongly suggest that targeting SHH signaling alone or in combination with other mechanisms might be a potential therapeutic approach in the future treatment of OSCC.

Conflicts of Interest

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

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