Establishment and characterization of a squamous cell carcinoma cell line, designated hZK-1, derived from a metastatic lymph node tumor of the tongue

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Summary

The hZK-1 cell line was successfully established from the metastatic foci of a lymph node of an 82-year-old Japanese woman with squamous cell carcinoma of the tongue. The pathological diagnosis of the tumor was moderately to well-differentiated squamous cell carcinoma. The hZK-1 cells were angular in shape, and had neoplastic and pleomorphic features. Adjacent hZK-1 cells were joined by desmosomes and well developed microvilli, and many free ribosomes were observed in the cytoplasm. The doubling time of the hZK-1 cells was approximately 36, 33, and 29 h at the 10th, 20th and 30th passage, respectively. The cell line was shown to be triploid, with a chromosomal distribution of 75 to 80. Immunocytochemical staining of the hZK-1 cells revealed cytokeratin (CK) 17-, Ki67- and p53-positive staining, and negative staining for CK13. The hZK-1 cells were negative for human papillomavirus (HPV)-16 or-18 infection. Grafting was not successful when the hZK-1 cells were transplanted into the subcutis of SCID mice. The hZK-1 cells (2×10^6 cells/3 ml of growth medium) secreted vascular endothelial growth factor (VEGF) that reached a concentration of 2.6 ng/ml media after

3 days of culture. Hypoxia enhanced cellular HIF-1α expression and VEGF secretion in hZK-1 cells. The HIF-1α inhibitor YC-1 partially inhibited hypoxia-induced VEGF secretion in ZK-1 cells. The reverse transcription-polymerase chain reaction (RT-PCR) results revealed that the expression of CK17, Ki67 and p53 was elevated in the hZK-1 cells. hZK-1 cells were not sensitive to CDDP, TXT, 5-FU, or a mixture of these 3 anti-tumor agents.

Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent malignant tumor in the head and neck region, comprising more than 90% of all oral malignancies[1]. With the exception of distant metastases, nearly 50% of patients with OSCC present with clinical or pathological evidence of lymph node metastasis[2]. In addition, there are reports of metastasis to lung or bone[3]. There are several squamous cell carcinoma cell lines derived from tongue[4, 5, 6, 7, 8]. Patient's with lingual carcinoma are associated with excessive alcohol consumption, history of tobacco use, and human papillomavirus (HPV) infection[9].

Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis[10,11]. It has been reported that various kinds of cancer cells produce VEGF. VEGF induces endothelial cell proliferation, migration and the formation of blood vessels[12,13]. Hypoxia-inducible factor-1 (HIF-1) plays a critical role in angiogenesis by activating the transcription of genes that encode pro-angiogenic growth factors, including VEGF[14]. HIF-1α is an oxygen-sensitive subunit of HIF-1 and its expression is rapidly increased under hypoxic conditions[15].

We report herein the establishment and characterization of a new cell line, designated hZK-1, which was derived from a metastatic lymph node in squamous cell carcinoma of the tongue. Since it has been reported that 18-36% of cell lines contain a misidentified species or cell type[16], and that 18% of cell lines donated to the German Cell Bank were misidentified or contaminated[17], we checked cross-contamination of these hZK-1 cells with Nialym cells that we had previously established from tongue cancer[8]. The hZK-1 cell line may provide valuable information on the biological properties and therapeutic responses of specific cancer types, and may lead to the development of effective treatments.

Materials and Methods

Medical history

The patient was an 82-year-old Japanese woman. The histopathological diagnosis was moderately to well-differentiated squamous cell carcinoma of the tongue. She received TPF therapy, which is a combination of 150 mg of taxotere (TXT), 150 mg of cisplatin (CDDP) and 5000 mg of 5-fluorouracil (5-FU), as preoperative chemotherapy. Partial excision of the right side of the tongue was performed under general anesthesia.
After surgery, swollen lymph nodes were observed upon diagnostic imaging inspection, and the entire lymph node on the right side of the neck was then excised under general anesthesia.

Histopathological examination

The original tumor of the tongue and metastatic lymph node were fixed with a 10% buffered neutral formalin solution at 4 °C for approximately 7 days. After dehydration with graded ethanol, the tissues were dipped in xylene and embedded in paraffin. Next, 5-μm sections were prepared for hematoxylin and eosin (HE) staining or immunohistochemical staining.

Establishment of the cell line

The metastatic lymph nodes from the neck of the patient with lingual cancer were resected, cut into small pieces with razor blades and dissociated with 0.1% trypsin-0.02% ethylenediaminetetra-acetic acid (EDTA)/phosphate-buffered saline (-) at 37 °C for 30 min. After adding a little fetal bovine serum, the fragments were further dissociated into single cells by vigorous pipetting. The dissociated cells and small fragments were centrifuged at 1,500 rpm for 5 min at room temperature. The precipitate was resuspended in growth medium (GM) and cultured in 60-mm dishes in a CO_2 incubator (4.7% CO_2, 95.3% air) at 37 °C. The GM used in this study contained: DMEM/F-12 supplemented with 15% fetal bovine serum (Sigma, Lot no. 12E261), 100 μM of glutaMAX, 0.1% modified Eagle medium-non-essential amino acids, 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone (all purchased from Thermo Fisher Scientific Inc.). The number of floating cells and fibroblastic cells gradually decreased over the course of the culture, resulting in establishment of the hZK-1 cell line. After the cells reached confluency, they were split and inoculated at a ratio of 1:3 to 1:5 into fresh GM. The GM was changed twice a week.

Cell growth

The hZK-1 cells were seeded at a density of 1×10^4 cells/35-mm dish and were cultured

at 37 °C for 12 days. In order to draw the growth curve of the cell line, the number of cells in five dishes was counted every 2 days. The doubling time and saturation density were obtained from the growth curves.

Chromosomal analysis

The cells (passage 20) were treated with 1×10^{-7} M colcemid (Thermo Fisher Scientific Inc.) for 4 h at 37 °C, placed in a hypotonic 0.075 M KCl solution for 20 min at 37 °C, and then fixed with a methanol-acetic acid (3:1) solution at 0 °C for 10 min. After fixation, the cells were stained with a 4% Giemsa solution. In addition, cells at the metaphase stage were treated with a 0.1% trypsin solution for 15 s at room temperature, stained with a 3% Giemsa solution (pH 6.8), and analyzed for G-band karyotyping. Histograms of chromosome number distribution were prepared from the values obtained from more than 50 metaphases.

Cell cross-contamination check

We carried out DNA profiling to confirm that there was no cross-contamination between the hZK-1 and the Nialym cell line[8]. Genomic DNA was isolated from these cell lines using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the genetic analysis, the AmpFlSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions, using approximately 1 ng of DNA template in a total PCR volume of 25 μL. The amplified DNA markers were: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, vWA, TPOX, D18S51, Amelogenin, D5S818 and FGA. A negative extraction and amplification control was used instead of the cell lines to check for DNA contamination. The DNA of each cell line and of the mixed cell lines was extracted and amplified in duplicate. A 1.5 μL aliquot of the amplified PCR product was combined with 24.5 μL of Hi-Di Formamide and 0.5 μL of the GeneScan 500 LIZ™ size standard. Electrophoresis was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The results were analyzed using GeneMapper ID v3.2 software (Applied Biosystems). The analytical threshold was set at 150 relative fluorescence units (RFU) in cell lines hZK-1 and Nialym. The minimum peak height threshold was set at 50 RFU in the mixed cell lines. The random match probability was calculated using the allele frequency in the Japanese population.

Electron microscopy

Cultured cells in 35-mm dishes were rinsed with Hanks' solution, and fixed with a

2.5% glutaraldehyde solution in 0.1 M phosphate buffer for 30 min. Then, the cells were rinsed with the same buffer and post-fixed with 1% osmium tetroxide. After dehydration with graded ethanol, followed by propylene oxide, the cells were embedded in Epon. Ultrathin sections were prepared using a Leica Ultracut UCT microtome (Leica.); they were stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Immunocytochemistry of hZK-1 cells

Immunocytochemical studies using antibodies against cytokeratin (CK) 17 (1:20, E3; Dako, Glostrup, Denmark), CK13 (1:400, DE-K13; Dako), Ki67 (1:100, ab15580; Abcam, Cambridge, UK) and p53 (1:100, PAb 240; Abcam) were performed. For immunocytochemistry, the hZK-1 cells were fixed with absolute methanol (Wako Pure Chemical Industries, Osaka, Japan) at 30 °C for 10 min, and then incubated in Blocking One Histo (Nacalai Tesque, Japan) for 30 min at room temperature. The cultures were incubated with the primary antibodies at 4 °C overnight. The samples were then incubated with the following secondary antibodies for 30 min in the dark at room temperature: Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (all diluted 1:1000; Invitrogen). The nuclei were stained using VECTASHIELD® HardSet™ Mounting Medium with DAPI (Vector Laboratories). Images were observed under a confocal laser scanning microscope (LSM-510; Carl Zeiss, Jena, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). Using 1 μg of total RNA, cDNA was synthesized with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems. Carlsbad. CA. USA). Amplification was performed using a PCR Supermix Platinum kit (Invitrogen, USA), a Veriti™ 96-well thermal cycler, and the primers and annealing temperatures for each gene that are shown in Supplementary Table 1[18]. The PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide.

PCR for HPV.

DNA was extracted from hZK-1 cells using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

DNA sequences encoding HPV16 and HPV18 were amplified by PCR using the Platinum PCR superMix (Life Technologies Corporation, Carlsbad, CA USA) and the

primers and PCR conditions for each gene that are shown in Supplementary Table 2. The PCR products were electrophoresed in 2% agarose gels. The HeLa and TC-YIK [19] cell lines were used as positive controls for HPV18 and HPV16, respectively.

Transplantation

The hZK-1 cells (1×10^7 cells/mouse in 0.5 ml of Hanks' solution) were subcutaneously injected into the dorsal neck skin of three SCID mice (male, 5 weeks old; Clea, Tokyo, Japan) using a 23-gauge needle. Five weeks after the transplantation, we checked a presence of formation of a tumor.

Assay of HIF-1α expression and VEGF secretion

Cellular HIF-1α expression and amount of VEGF secretion were measured using an enzyme-linked immunosorbent assay (ELISA).

Nearly confluent hZK-1 cells (1×10^6 cells, at the 10^{th}, 20^{th}, and 30^{th} passage) were incubated in GM for 4 hours under normoxic (air) or hypoxic (5% O_2) conditions at 37 °C. The cells were then fixed and processed according to the assay protocols described below. The amount of cellular HIF-1α was analyzed using the HIF-1 Alpha cell based ELISA kit (CBA-281), (Cell Biolabs, Inc. San Diego, CA, USA) and the amount of VEGF in the conditioned medium was analyzed using a VEGF ELISA kit (DVE00; R&D Systems, Minneapolis, MN, USA). Briefly, the HIF-1 Alpha cell based ELISA kit is an immunoassay that was developed for rapid detection of HIF-1α in fixed cells. Cells on a microplate are stimulated for HIF-1α stabilization, fixed, permeabilized, and then neutralized in the well. HIF-1α is then detected with an anti-HIF-1α antibody followed by an HRP conjugated secondary antibody. In addition, in order to confirm inhibition of VEGF secretion by YC-1, which is an inhibitor of HIF-1α, hZK-1 cells (1×10^6 cells, at the 10^{th}, 20^{th}, and 30^{th} passage) were treated with 80 μmol/L YC-1 in the presence of 0.5 mmol/L cobalt chloride at 37 °C for 4 hours. The amount of VEGF in the conditioned medium was then assayed using the VEGF ELISA kit.

Anticancer drug susceptibility tests of in vitro cultured hZK-1 cells

Anticancer drug (cisplatin (CDDP), docetaxel (TXT), 5-fluorouracil (5-FU) and a mixture of the 3 agents) susceptibility tests were carried out using an oxygen electrode apparatus (Aloka Co LTD., Dox-24, Tokyo, Japan) [20]. hZK-1 cell suspensions were centrifuged at 430×g for 5 min. Pellets were resuspended with 2 ml of HEPES-GM containing one of the anticancer drugs (CDDP, 0.2 μg/ ml or 1 μg/ ml; TXT, 1 μg/ ml or 5 μg/ ml; 5-FU, 1 μg/ ml or 5μg/ ml). Cell-drug suspensions were then transferred to the

oxygen electrode meter at 37 °C and dissolved oxygen in the cell-drug medium was measured for 16 h (2×10^6 cells/tube). Analysis of susceptibility to anticancer drugs was carried out using the method of Uesu and Ishikawa [21].

Results

Histopathological diagnosis

The histopathological diagnosis of the original tumor was moderately or well-differentiated squamous cell carcinoma of the tongue (Fig. 1a). Abundant blood vessels were observed in HE-stained tumor tissue (Fig. 1b). HE staining of the metastatic lymph node is shown in Fig. 1c. A blood vessel that had developed within the tumor tissue in the lymph node could be seen.

Establishment of the hZK-1 cell line

The hZK-1 cell line was established from the metastatic foci of the lymph node from the neck of the patient with lingual cancer. Epithelial cells and fibroblastic cells were intermingled in the primary culture (Fig. 2a). Over the course of serial passages, the number of fibroblastic cells gradually decreased, and the hZK-1 cell line was established.

Morphology of the hZK-1 cells

Deformity of the hZK-1 cells was common. The cells displayed a jigsaw puzzle-like arrangement. Moreover, they were observed to pile up in some places. The size of the cells varied, and the nuclear membranes were pseudomorphic. The nucleoli were clear and the cytoplasm had a bright appearance (Fig. 2b).

The growth characteristics of the hZK-1 cell line

The doubling time of the hZK-1 cells was approximately 36, 33 and 29 h, and the saturation density was 0.21×10^4, 0.23×10^4 and 0.25×10^4 cells/cm^2 for cells at the 10th, 20th and 30th passage, respectively.

Chromosomal analysis and distribution

The results of chromosomal analysis of the hZK-1 cells are shown in Supplementary Fig. 1. The cell line was shown to be triploid, with a chromosomal distribution of 75 to 80. The modal number was 76 to 78. There were many (21~27) marker chromosomes. The karyotype was determined to be 76~79(3n), -X[10], add(X)(q11)x2[10], add(1)(p11)[10], add(1)(q11)[10], del(1)(p11)[8], add(2)(q35)[7],

der(2)add(2)(p11)add(2)(q35)[3], der(2;12)(q10;q10)del(2)(q35)[8], der(2;12)(q10;q10)add(12)(q24)del(2)(q35)[9], add(3)(p21)[9], add(3)(p25~26)[10], der(3)t(3;12)(p13;q13)add(3)(q2?7)[10], -4[10],add(4)(q21)x2[10], -5[10], add(5)(p11)x2[10], -6[10], -6[9], del(6)(q13)[10], -7[10]add(7)(p11)[10], der(7)add(7)(p11)add(7)(q32)[9]-8[10], add(8)(q22)[10], add(8)(q22)[10], -9[9], del(9)(p12)x2[10]+10[5], -11[10], add(11)(p11)[9], add(11)(q13)[9], -12[10], -12[10], add(12)(q2?)[8], -13[7], add(13)(p11)[9], -14[10], add(14)(q22)[10]add(14)(q22)[10], -15[6], add(15)(p11)x2[10], add(15)[4], -16[10], -16[3], -17[10], add(17)(p11)[7]-18[9], -19[6], +20[9], +21[3], -22[4], +21~27mar.

Cell cross-contamination check

The DNA profile obtained for the hZK-1 cell line did not match that of the Nialym cell line, which proved that there was no cross contamination between these cell lines (figure not shown).

Electron micrographic analysis of the hZK-1 cells.

Electron microscopic observations revealed that the nuclear membranes were pseudomorphic, and that the cells had a clear and large nuclear body and many kinds of intracellular organelles. Although little development of rough-ER (r-ER) was seen, concentric r-ER was occasionally observed (Supplementary Fig. 2). Moreover, desmosomes and many microvilli were present (Fig. 3). There was a small number of glycogen granules (figure not shown). Lysosomes, lipid droplets, free ribosomes, and the Golgi apparatus were also observed in the hZK-1 cells. Secretory-like granules were observed around the Golgi apparatus (Supplementary Fig. 2). There were no virus-like particles. Tonofilaments, which are one of the features of epithelial cells, were poorly developed.

Immunofluorescence observations

The hZK-1 cells stained positive for CK17, Ki67 and p53, but were negative for CK13 (Fig. 4a, b, c, d).

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression of CK17, Ki67, and p53 was elevated (Supplementary Fig. 3).

PCR for Human papillomavirus (HPV)

Expression of HPV16 and HPV18 in hZK-1 cells and in the HPV-positive control cell

lines TC-YIK or HeLa, was tested using PCR with primers specific for HPV16 and HPV18 (Supplementary Table 2). This PCR analysis showed that hZK-1 cells were negative for HPV infection (Supplementary Fig. 4).

Transplantation of the hZK-1 cells into SCID mice

None of the subcutaneous injections of hZK-1 cells into SCID mice resulted in a successful transplant.

Analysis of VEGF secretion and HIF-1α expression in hZK-1 cells

hZK-1 cells at the 10th, 20th and 30th passages secreted VEGF in 3-day cultures. The amounts of VEGF secreted are shown in Fig. 5a. The amount of VEGF secreted by hZK-1 cells was increased under hypoxic conditions compared to that under normoxic conditions. In the YC-1 addition group, a decrease in the amount of VEGF secretion was observed under both hypoxic and normoxic conditions in comparison with the group to which YC-1 was not added. However, the differences in the amount of VEGF secreted by the YC-1 treated groups under the different oxygen conditions were small.

The amounts of cellular HIF-1α under the different conditions are shown in Fig. 5b. The amount of HIF-1α was greatly increased under the hypoxic condition compared to that under the normoxic condition.

Anticancer drug susceptibility testing of hZK-1 cells

Susceptibility testing for anticancer drugs revealed that hZK-1 cells were not sensitive to 0.2 or 1 μg/ml CDDP, 1 or 5 μg/ml TXT, 1 or 5 μg/ml 5-FU, or a mixture of the 3 agents at low or high concentrations (Supplementary Fig. 5). Thus, hZK-1 cells were not sensitive to any of these anticancer drugs.

Discussion

The Nialym cell line[8], which we previously established from squamous carcinoma of the tongue, is composed of cells with a bright or a dark appearance under the light microscope. Of note, the dark cells have many tonofilaments. However, the hZK-1 cell line is composed of only bright cell, and tonofilaments are not well developed. Furthermore, since the hZK-1 cell line and the Nialym cell line were both derived from OSCC of the tongue, we checked the presence of cross contamination between these two cell lines by AmpFLSTR® Identifiler® PCR Amplification. Cross contamination between the two cell lines was not found.

OSCC is characterized by a high degree of local invasiveness to surrounding tissues,

which can be a cause of treatment failure. One of the difficulties in studying the metastasis of human OSCC is the lack of an appropriate animal model and OSCC cell lines[22]. Most existing OSCC cell lines have been established from metastatic lymph nodes[23, 24] as in the present study.

Since the original cancer had an abundance of blood vessels, it was assumed that the cancer cells were producing an angiogenic factor. We therefore assayed the production of VEGF (a known angiogenic factor) by the cancer cells. VEGF is a major regulator of both physiological and pathological angiogenesis[10, 11]. The mechanism of cancer cell metastasis is not yet clear. However, after the migration of cancer cells into normal tissue, the cancer cells are regarded to be in hypoxic conditions. We therefore considered that cancer cells in a metastatic focus would be in hypoxic conditions and may produce HIF1-α, which would then enhance the production of VEGF. We therefore evaluated the quantity of HIF-1α and VEGF produced by hZK-1 cells. HIF-1α plays a critical role in angiogenesis by activating the transcription of genes that encode pro-angiogenic growth factors, including VEGF, basic fibroblast growth factor (bFGF), angiopoietin-1 and -2, placental growth factor, and platelet-derived growth factor-B[14,25]. The hZK-1 cells could not be successfully transplanted into SCID mice, even though the cells secreted a moderate level of VEGF. The mechanisms behind graft rejection and angiogenesis remain unknown. It has been recently suggested that the VEGF-C/VEGFR-3 axis may be associated with lymph node metastasis of tongue squamous cell carcinoma through lymphangiogenesis[24]. High expression of VEGF-C in the primary tumor may be a good determinant for detection of occult tumor cells in the lymph nodes of OSCC cases[26]. It is therefore important to carry out research for the development of anti-tumor drugs such as VEGF-depleting and VEGF receptor-inhibiting drugs[27]. HIF-1α and CD44 immunohistochemical detection could potentially serve as a prognostic tool in therapy selection for early-stage OSCC[28]. We observed that inhibition of HIF-1α by YC-1 partially inhibited VEGF expression in hZK-1 cells. The hZK-1 cells were shown to be triploid in the karyotype analysis, and the large number of marker chromosomes suggests that the grade of malignancy of these cells was high. HPV is one of the most common sexually transmitted infections worldwide. In 2007, HPV-16 was recognized as a risk factor by the International Agency for Research on Cancer, for oropharyngeal squamous cell carcinoma (OSCC), where tonsillar and base of tongue cancer dominate. Over the past 20-40 years, the incidence of HPV-positive tonsillar and base of tongue cancer has risen in many western countries, and >70% of these cancers are in men[29]. We did not detect HPV in the hZK-1 cells, indicating that the immortalization of these cells was not caused by virus transfection. hZK-1 cells were not sensitive to any of the

anticancer drugs that we tested.

These hZK-1 cells may be a very useful cell line for the development of chemotherapies or immune therapies for HPV-independent OSCCs.

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Author Disclosure Statement

No competing financial interests exist.

Ethical approval

All procedures were approved by the ethics committee of the School of Life Dentistry, Nippon Dental University, Japan (approval number: NDU-T2013-04).

References

1. Massano J, Regateiro F, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Oral Med Oral Pathol Oral Radiol Endod (2006) 102: 67-76.

2. Som PM. Detection of metastasis in cervical lymph nodes: CT and MRI criteria and differential diagnosis. Am J Radiol (1992) 158: 961-9.

3. Yasui A, Okada Y, Mataga I, Katagiri M. An analysis of distant metastasis in oral squamous cell carcinoma. J Hard Tissue Biol (2010) 19: 27-32.

4. Gioanni J, Fischel JL, Lambert JC, et al. Two new human tumor cell lines derived from squamous cell carcinomas of the tongue: establishment, characterization and response to cytotoxic treatment. Eur J Cancer Clin Oncol (1988) 24: 1445-55.

5. Chew EC, King WK, Hou HJ, Yam HF. Establishment and characterization of two new cell lines derived from squamous cell carcinoma of the tongue in Chinese patients. Anticancer Res (1992) 12: 1627-34.

6. King WWK, Lam PK, Huang DWSP, Liew CT, Li AKC. Establishment and characterization of a human cell line from a squamous carcinoma of the tongue. Clin Otolaryngol (1995) 20: 15-20.

7. Kim KM, Park EJ, Yeo YH, Cho KJ, Kim MS. Establishment of a novel HPV-negative and radiosensitive head and neck squamous cell carcinoma cell line. Head and Neck (2015) Mar 17. doi: 1002/hed.24037

8. Takahashi H, Ishikawa H, Mataga I, et al. Establishment and characterization of

human lingual squamous cell carcinoma cell lines designated Nialym derived from metastatic foci of lymph node, and Nialymx derived from transplanted tumor of Nialym cells. Human Cell (2015) 28:143-153.

9. Han J, Kioi M, Chu WS, et al. Identification of potential therapeutic targets in human head and neck aquamous cell carcinoma. Head and Neck Oncol, 2009; 1,27.

10. Koch S, Yao C, Grieb G, Prevel P, Noah EM, Steffens GCM. Enhancing angiogenesis in collagen matrices by vovalent incorporation of VEGF. J Mater Sci Mater Med 2006, 17: 735-41.

11. Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 1999;154:375-84.

12. Benjamin LE, Hemo I, KeshetE. A plasticity window for blood vessel remodeling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998; 125:1591-8.

13. Haspel HC, Scicli GM, MaMahon G, Scicli GM. Inhibition of vascular endothelial growth factor-associated tyrosine kinase activity with SU5416 blocks sprouting in the microvascular endothelial cell spheloid model of angiogenesis. Microvasc Res 2002;63: 304-15.

14. Forsythe JA, Jiang BH, Lyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mil Cell Biol 1996; 16: 4604-13.

15. Wong GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cell OXYGEN tension. Proc Natl Acad Sci 1995; 92: 5510-4.

16. Identity crisis. Nature. 2009;457 (7232):935-6

17. Chatterjee R. Cell biology. Cases of mistaken identity. Science. 2007;315 (5814):928-31

18. Patti E. Gravitt , Cheri Peyton, Cosette Wheeler, Raymond Apple, Russell Higuchi, Keerti V. Shah.Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. J Virol Methods. 2003 Sep;112(1-2):23-33

19. Ichimura H, Yamasaki M, Tamura, et al. Establishment and Characterization of a New Cell Line TC-YIK Originating From Argyrophil Small Cell Carcinoma of the Uterine Cervix Integrating HPV16 DNA. Cancer. 1991 May 1;67(9):2327-32.

20. Amano Y, Okumura C, Yoshida M et al. Measuring respiration of cultured cell with oxygen electrode as a metabolic indicator for drug screening. Hum Cell 1999; 12: 3–10.

21. Uesu K, Ishikawa H. Analysis of an in vitro susceptibility test of anticancer drugs using new yupes of oxygen electrodes. Bull Edu Res Nihon Univ Sch Dent Matsudo.

2005;7:7-21.

22. Sun R, Zhang JG, Guo CB. Establishment of cervical lymph node metastasis model of squamous cell carcinoma in the oral cavity in mice. Chinese Medical J (2008) 121: 1891-5.

23. Monose F, Araida T, Negishi A, Ichijo S, Sasaki S. Variant sublines with different metastatic potentials selected in nude mice from human oral squamous cell carcinomas. J Oral Pathol Med (1989) 18: 391-5.

24. Yokoi T, Yamaguchi A, Odajima T, Furukawa K. Establishment and characterization of a human cell line derived from a squamous cell carcinoma of the tongue. Tumor Res (1988) 23: 43-57.

25. Manalo DJ, Rowan A, Lavoie T. et al. Transcriptional regulation of vascular endothelial call responses to hypoxia. Blood 2005; 105: 659-69.

26. Dunkel J, Vaittinen S, Grénman R, et al. Prognostic markers in stage I oral cavity squamous cell carcinoma. 2013, Laryngoscope 123:2435-43.

27. Kazakydasan S, Rahman ZA, Ismail SM, et al. Prognostic significance of VEGF-C in predicting micrometastasis and isolated tumour cells in NO oral squamous cell carcinoma. J Oral Pathology & Medicine.2016.doi:10.1111/jop.12476.

28. Bozec A, Gros FX, Penault-Llorca F, et al. Vertical VEGF targeting: a combination of ligand blockade with receptor tyrosine kinase inhibition. European J Cancer 2008, 44: 1922-30.

29. Bucchi D, Stracci F, Buonoraet N, et al. Human papilloma virus and gastrointestinal cancer: a review. World J. of Gastroenterology 2016, September 7; 22(33): 7415-7430.

Figure Legends

Fig. 1

The pathological diagnosis of the original tumor is moderate-to-well differentiated squamous cell carcinoma. (a) Low magnification, and (b) High magnification of a HE-stained tumor section. Stratified squamous epithelium is indicated (arrow in a). Scale bar: 200 μm. (c) HE staining of the histopathological aspect of the metastatic focus of the lymph node. Note a blood vessel that is developing within the tumor tissue (arrows). Pleomorphic tumor cells form colonies that are scattered throughout the lymph node. Scale bar: 100 μm.

Fig. 2

(a) Phase contrast micrograph of the primary culture. In addition to epithelial cells, skeletal muscle-like cells (arrows), fibroblastic cells and multi-nucleated cells (arrow heads) can also be seen. (b) Phase contrast micrograph of hZK-1 cells at the 30th passage. Cells in a jigsaw puzzle-like arrangement are observed. Some cells have long projections and touch other cells with these projections. Many mitotic figures can be seen. Scale bar: 100 μm.

Fig. 3

Transmission electron micrographs of hZK-1 cells.

Many nuclear membranes were almost smooth in appearance. Interdigitation via microvilli was observed between the cells. Many free ribosomes were observed. Moreover, a small number of desmosomes (arrows) were seen. High magnification of the desmosomes is shown in the upper right corner. Scale bar: 2 μm

Fig. 4

Immunocytochemical analysis of hZK-1 cells. Fluorescence immunostaining of hZK-1 cells for CK-17 (a), CK-13 (b), Ki67 (c) and p53 (d). Nuclei were co-stained with DAPI (blue). hZK-1 cells were positive for CK-17, Ki67 and p53, but were negative for CK-13. Scale bar: 50 μm.

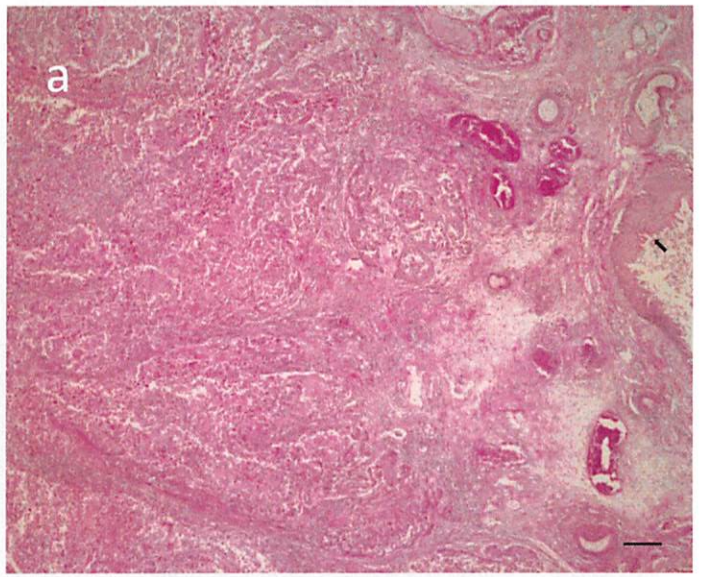
Fig. 5

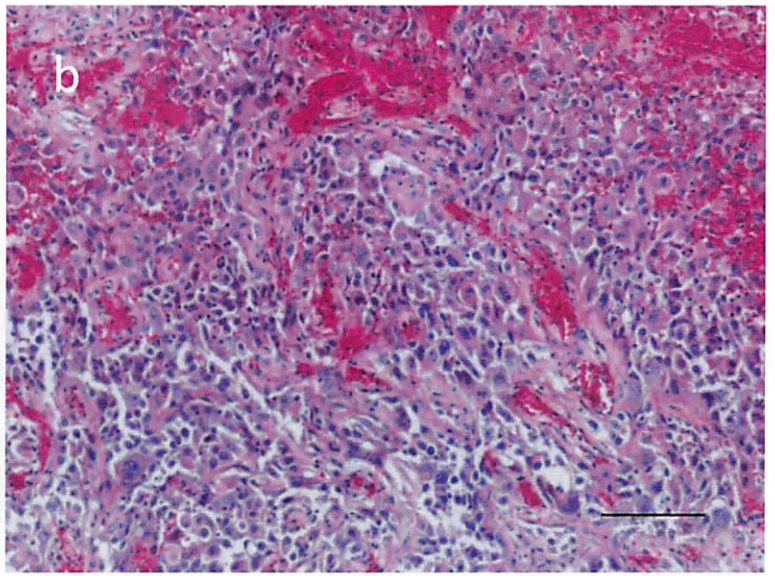
(a) Amounts of VEGF in the conditioned media of hZK-1 cells under different oxygen conditions and in the presence and absence of YC-1. Data are presented as the mean ± S.D. (b) Amounts of HIF-1α in the hZK-1 cells under different oxygen conditions. Data

are presented as the mean ± S.D.

Figure 1

Fig.1





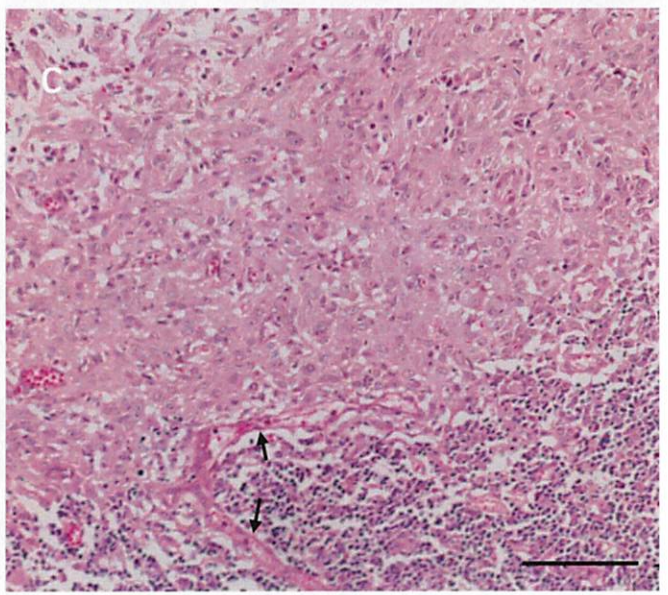


Figure 2

Fig.2 a

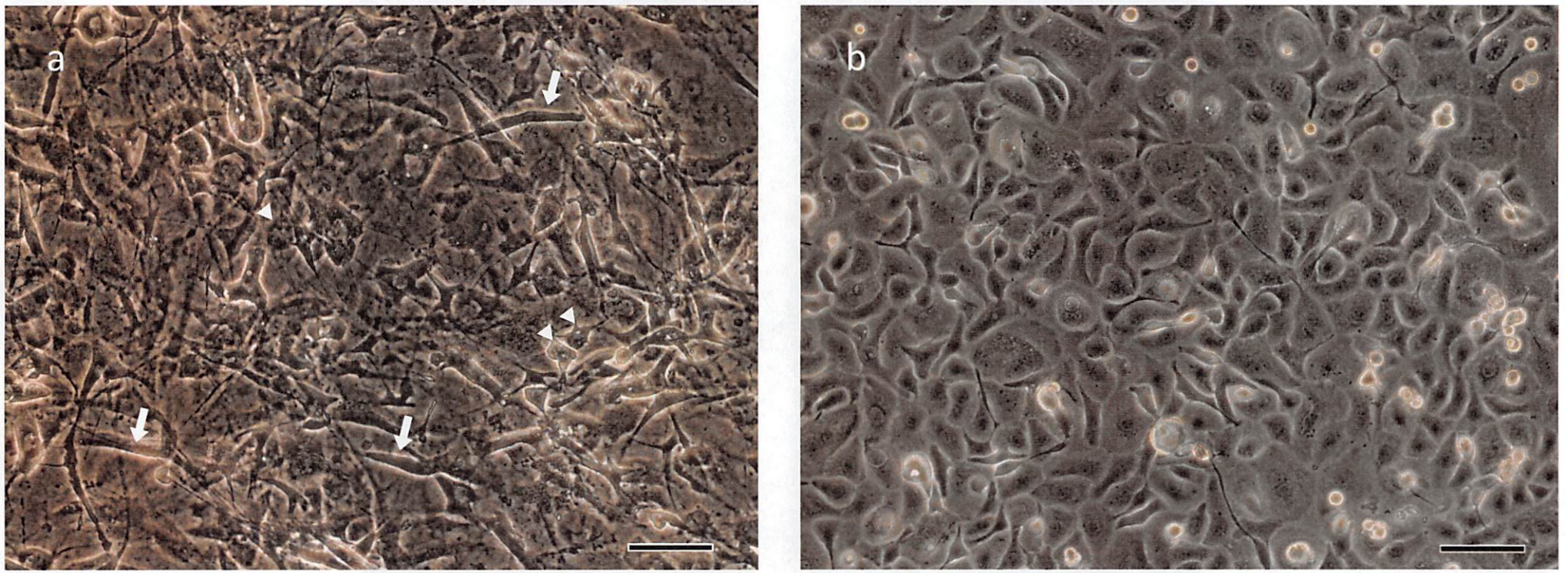


Figure 3

Fig.3

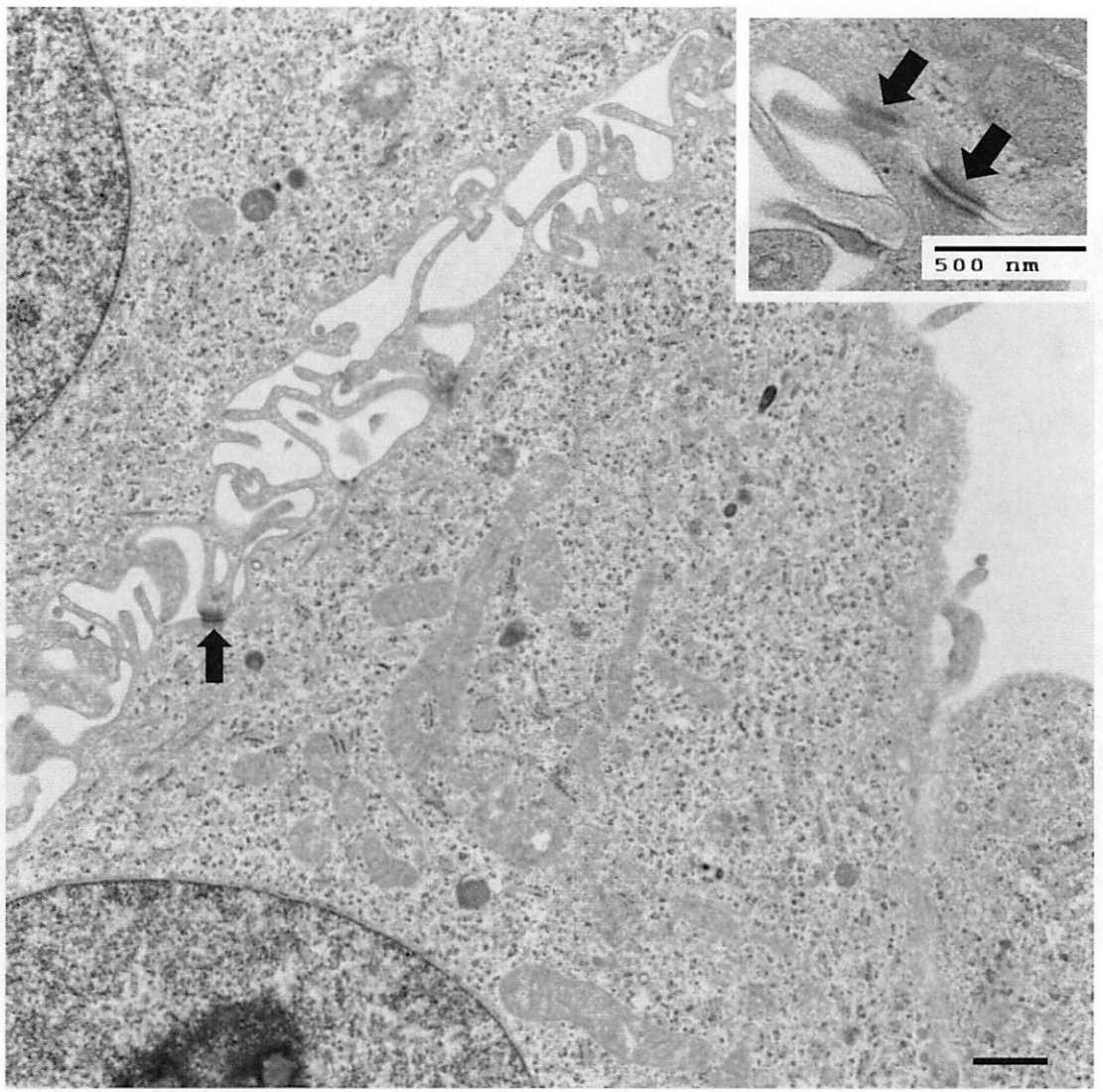


Figure 4

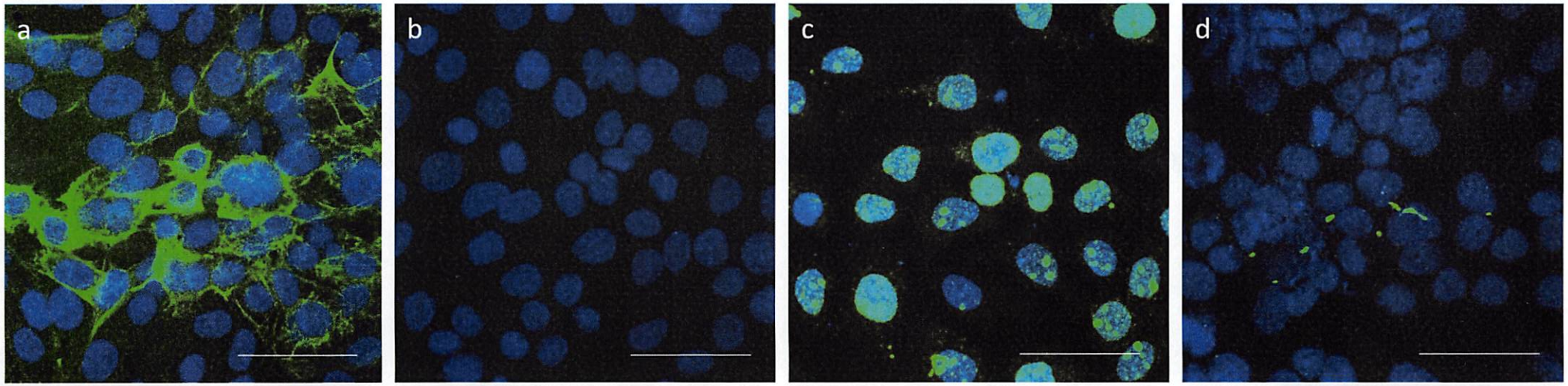


Fig.4

Figure 5

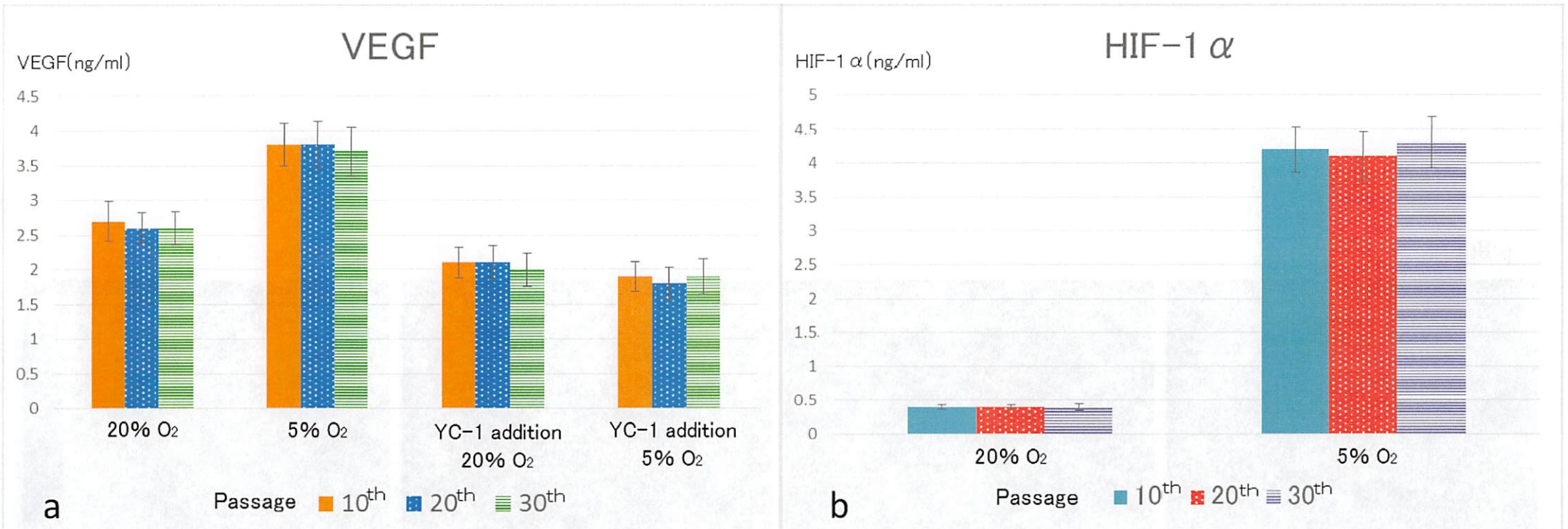


Fig.5