FOXC2 expression is associated with tumor proliferation and invasion potential in oral tongue squamous cell carcinoma

Naomi Imayama[1], Shin-ichi Yamada[1], Souichi Yanamoto[1], Tomofumi Naruse[1], Yuki Matsushita[1], Hidenori Takahashi[1], Sachiko Seki[2], Shuichi Fujita[2], Tohru Ikeda[2], Masahiro Umeda[1]

[1]Department of Clinical Oral Oncology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences.

[2]Department of Oral Pathology and Bone Metabolism, Unit of Basic Medical Sciences, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences.

Key Words: ***FOXC2, invasion, metastasis, proliferation, oral tongue squamous cell carcinoma***

Running title: Overexpression of FOXC2 in tongue squamous cell carcinoma

Abbreviations: (OSCC) oral squamous cell carcinoma, (FOXC2) Forkhead box protein C2, (OTSCC) oral tongue squamous cell carcinoma, (VEGF) vascular endothelial growth factor.

Correspondence: Shin-ichi Yamada, DDS, PhD

Department of Clinical Oral Oncology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences.

1-7-1 Sakamoto, Nagasaki 852-8588, Japan

Email: shinshin@nagasaki-u.ac.jp

Fax: + 81 95 8197700, Tel: + 81 95 8197698

Abstract

Forkhead box protein C2 (FOXC2) is a gene encoding a transcription factor that controls the generation of mesodermal tissue including vascular and lymphatic tissues. FOXC2 has previously been associated with EMT and tumor angiogenesis in various cancers. Moreover, a relationship between the expression of FOXC2 and poor prognosis has been reported in various cancers. We herein examined the clinicopathological significance of FOXC2 in oral tongue squamous cell carcinoma (OTSCC) and attempted to clarify the function of FOXC2 in OTSCC cell lines *in vitro*. The overexpression of FOXC2 was more frequent in cancers with higher grades according to the pattern of invasion (grade 4 vs. 1-3; $p<0.05$). A correlation was observed between the expression of FOXC2 and that of VEGF-A and -C (VEGF-A; $p<0.05$, VEGF-C; $p<0.001$). The high-FOXC2 expression group had a significantly poorer prognosis than that of the low-expression group ($p<0.001$). Multivariate analysis indicated that the overexpression of FOXC2 may also be an independent prognostic factor, similar to N classification(N0 vs 1/2;$p<0.05$), stage classification(stage I/II vs III/IV; $p<0.05$), pattern of invasion(grade 1-3vs 4; $p<0.05$), local recurrence(local recurrence (+) vs (-);$p<0.01$), and the overexpression of FOXC2 (FOXC2 overexpression (-)vs.(+); $p<0.05$). In the OTSCC cell line analysis, the expression of FOXC2 was also associated with proliferation and invasion potential. These results strongly suggest that the overexpression of FOXC2 may be a potent predictor of survival in OTSCC patients.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck regions and accounts for more than 90% of cancers in the oral cavity[1]. The oral tongue is the most common site of OSCC. The primary therapeutic modality for OSCC is surgery. Although recent advances in surgical techniques and anticancer agents have improved tumor regression and survival for patients with OSCC, wide surgical resection of OSCC inevitably causes various oral dysfunctions. Therefore, new treatment strategies are urgently needed.

The presence of neck lymph node metastasis is strongly related to a poor prognosis in squamous cell carcinoma of the head and neck[2-4]. Moreover, previous studies reported that an alteration in the expression of adhesion-related molecules was associated with a poor prognosis in OSCC patients[5-8]. In addition, several tissue and biological markers have been identified as possible indicators of the tumor aggressiveness and metastatic capability of tumors[9]. Epithelial-mesenchymal transition (EMT),

angiogenesis, and lymphangiogenesis are known to be pivotal for tumor progression and metastasis in oral tongue squamous cell carcinoma (OTSCC)[10,11]. The initial steps in the sequential process of metastasis were previously found to be similar to EMT in which cells lose epithelial characteristics including cell adhesion and gain mesenchymal features including cell motility during embryogenesis and wound healing[12,13]. Acquiring the EMT, accompanied the functional loss of E-cadherin maintaining the intercellular adhesion, stimulates the dissemination of single tumor cells from primary sites through cell-to-cell contact, thereby endowing cells with metastatic abilities[12-14]. Angiogenesis and lymphangiogenesis are also crucial for tumor progression and nodal metastasis in OSCC[10]. Some of the main angiogenic and lymphangiogenic factors have been identified as vascular endothelial growth factor (VEGF)-A and VEGF receptor(VEGFR) 2, as well as the VEGF-C/-D and VFGFR3 systems, respectively[15,16].

Mesenchyme forkhead 1 (also known as Forkhead box protein C2, FOXC2) is a gene encoding a

transcription factor that controls the generation of mesodermal tissue such as vascular and lymphatic tissues[17,18]. FOXC2 was previously associated with EMT[19-23] and tumor angiogenesis[24,25] in various cancers. Although a relationship has already been reported between the expression of FOXC2 and poor prognosis in various cancers[21,26-29], those between the expression of FOXC2 and clinicopathological features in OSCC have not yet been investigated.

The purpose of this study was to determine the clinicopathological significance of FOXC2 in OTSCC and clarify the function of FOXC2 in OTSCC cell lines *in vitro*. We performed an immunohistochemical analysis to determine the relationships between the expression of FOXC2 and clinicopathological features in clinical OTSCC samples. We also examined the effects of FOXC2 expression on the proliferation and invasion potential of OTSCC cell lines.

Materials and methods

Patients

The study protocol was approved by the ethics committee of Nagasaki University Graduate School of Biomedical Sciences. Paraffin-embedded sections were obtained from biopsy specimens of 61 patients with tongue squamous cell carcinoma who underwent radical surgery in our department. Tumor stages were classified according to the TNM classification of the International Union against Cancer, histological differentiation was defined according to the WHO classification, and invasion patterns were determined according to Yamamoto's classification[30]. As controls, samples of a normal oral epithelium were obtained after informed consent from ten patients undergoing routine surgical removal of their third molars.

Cell lines

Five human tongue squamous cell carcinoma cell lines (OSC20, HSC3, HSC4, SAS, and OSC20), and human keratinocyte cell line, HEKa as the control, were obtained from the Human Science Research Resource Bank (Osaka, Japan). These cells were cultured in Dulbbeco's modified Eagle's medium (DMEM)(Wako Pure Chemical industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum(FBS)(Sigma Chemical Co., St. Louis, MO, USA) under the conditions of 5% CO_2 in air.

Immunohistochemical staining and evaluations

Serial 4-μm-thick specimens were taken from tissue blocks. Sections were deparaffinized in xylene, soaked in target retrieval solution buffer (Dako, Glostrup, Denmark), and placed in an autoclave at 121°C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubating sections with 0.3% H_2O_2 in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (Envision+, Dako, Carpinteria, CA). The primary antibody used was directed against FOXC2 (2H3, Abnova, Taipei City, Taiwan), VFGF-A, and VEGF-C(Santa Cruz Biotechnology, Inc., Texas, USA). Sections were incubated with the primary antibody overnight at 4°C. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Meyer's hematoxylin and mounted. Negative controls were prepared by replacing the primary antibody with phosphate-buffered saline. The expression of FOXC2 was defined as the presence of specific staining mainly in the cytoplasm of tumor cells. The immunoreactivity was scored by HSCORE[31]. The HSCORE was scored in a semiquantitative fashion incorporating both the intensity and the distribution of specific staining. The evaluations were recorded as percentages of positively stained target cells in each of five intensity categories which were denoted as 0(no staining), 1+(weak), 2+(distinct), 3+(strong), 4+(minimal light transmission through the staining). For each tissue, a value designated the HSCORE was determined by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of staining. The overexpression of immunoreactivity was defined as HSCORE ≥75[31].

RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was synthesized from 1 μg of total RNA using Oligo d (T) primer (Invitrogen) and ReverTra Ace (Toyobo,

Osaka, Japan). In the PCR analysis, cDNA was amplified by *Taq* DNA polymerase (Takara, Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR program involved a 3-min initial denaturation step at 94°C, followed by 25 cycles (for FOXC2), or 19 cycles (for GAPDH) at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, on a PCR Thermal Cycler MP (Takara). Primer sequences were as follows: 5'-TCACACATAGAGGCCAGCAG-3' for FOXC2 (F); 5'-CCCTCATCGCAGTGAAAAAT-3' for FOXC2 (R); 5'-ATGTCGTGGAGTCTACTGGC-3' for GAPDH (F); and 5'-TGACCTTGCCCACAGCCTTG-3' for GAPDH (R). The amplified products were separated by electrophoresis on ethidium bromide-stained 2% agarose gels. Band intensity was quantified by Image J software.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay

Cells were seeded in a 96-well plate at a concentration of 5 x 10^3 per well and incubated for 24h. In the

MTT assay, cells were incubated with 0.5 mg/ml MTT (Sigma Chemical Co.). Four hours later, the medium was replaced with 100 μl dimethylsulfoxide (DMSO) (Sigma Chemical Co.) and vortexed for 10 min. Absorbance (A) was then recorded at 570 nm using Easy Reader 340 AT (SLT Labinstruments, Salzburg, Austria). Cell viability (%) was calculated as the percentage of progression potential in tongue squamous cell carcinoma cell lines.

Invasion assay

A BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) was used for the invasion assay. This contained an internal chamber with an 8-μm porous membrane bottom that was coated with Matrigel. Six-well cell culture inserts and a 6-well multiwell companion plate were used for the experiment. The membranes were rehydrated with warm serum-free medium for 2 h. The internal chamber was filled with 1.25×10^5 cells in medium containing 10% FBS as a chemoattractant. Cells were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. After the incubation, noninvading cells were removed from the top of the wells with a cotton swab, and cells that transferred to the inverse surface of the membrane were subjected to Diff-Quick staining(Sysmex International Reagents Co., Ltd. Kobe, Japan). As the control, The HEKa cell line was examined(date not shown). Cells were counted under a microscope at 100× magnification. As a control, cells that passed through a control chamber without Matrigel were counted. All experiments were performed in triplicates, and cell numbers were counted in at least 4 fields/well. The ratio of the cell count

that passed through the Matrigel chamber to the control cell count was defined as the invasion index, and was expressed as a percentage.

Statistical analysis

Statistical analysis was performed using StatMate® (ATMS Co., Tokyo, Japan). The relationship between the expression of FOXC2 and clinicopathological features was assessed by Fischer's exact test. Continuous data are given as means ± standard deviation. Data sets were examined by a one-way analysis of variance (ANOVA) followed by Scheffe's post-hoc test. Survival analysis was carried out with Kaplan-Meier curves and the related log-rank tests. Prognosis factors were assessed by the Cox proportion hazard model. The relationship between FOXC2 mRNA expression and the invasion/growth index was determined using Person's correlation coefficient. P values less than 0.05 were considered significant.

Results

Relationships between FOXC2 expression and clinicopathological features

Immunohistochemistry with an anti-FOXC2 polyclonal antibody was performed on samples obtained from 61 patients with oral tongue squamous cell carcinoma. Representative immunohistochemical stainings are shown in Figure 1A. The overexpression of FOXC2 was undetectable in the normal epithelium. FOXC2 staining was mainly detected in the cytoplasm of tongue squamous cell carcinoma cells, and strong FOXC2 staining was observed at the invasive front and diffuse invasive area (Fig. 1B). The overexpression of FOXC2 was detected more frequently in OSCC (22 of 61, 36.1%) than in the normal oral epithelium (0 of 10, 0%; $p<0.05$). Furthermore, the overexpression of FOXC2 was more frequently observed in cancers with higher grades according to the pattern of invasion(grade 4 vs. 1-3; $p<0.05$, Table 1). Angiogenesis and lymphangiogenesis have been shown to play crucial roles in tumor progression and nodal metastasis in OSCC[10]. The family of vascular endothelial growth factors, including VEGF-A, VEGF-B, VEGF-C,

VEGF-D, VEGF-E, placental growth factor, and VEGF-F, was previously reported to be crucially involved in angiogenesis and lymphangiogenesis [33]. Of these, VEGF-A and -C expression levels were previously correlated with lymph node metastasis in esophageal squamous cell carcinoma[34]. We also examined the relationship between the expression of FOXC2 and that of VEGF-A and –C. Immunohistochemical staining of VEGF-A and –C was detected in the cytoplasm of both normal tissue and tumor cells (Fig. 1C and D). These proteins were found to be strongly expressed in the invasion front of the tumor. Correlations were also observed between the expression of FOXC2 and that of VEGF-A and –C (VEGF-A; $p<0.001$, VEGF-C; $p<0.001$).

These results strongly suggest that the overexpression of FOXC2 may be a potent predictor of survival through the invasion and angiogenesis potential.

.

Relationship between FOXC2 expression and survival analysis

The 5-year disease-specific survival rates of OTSCC patients according to FOXC2 expression were plotted (Fig. 2). The high-FOXC2 expression group had a significantly poorer prognosis with 45.9% than that of the low-expression group with 97.2% ($p<0.001$). Moreover, for the purpose of examining the relationships between the expression of FOXC2 and clinicopathological features, we performed a univariate analysis using the log-rank test and multivariate analysis by the Cox proportion hazard model. Univariate analysis revealed that the prognosis of OTSCC patients could be predicted by N classification(N 0 vs.1/2; $p<0.05$), stage classification (stage I/II vs. III/IV; $p<0.05$), pattern of invasion (grade 1-3 vs. 4; $p<0.01$), local recurrence (local recurrence (-)vs.(+); $p<0.05$), VEGF-A (VEGF-A (-)vs.(+); $p<0.05$), and FOXC2 overexpression (FOXC2 overexpression (-)vs.(+); $p<0.01$). Multivariate analysis also suggested that the overexpression of FOXC2 may also be an independent prognostic factor, similar to N classification(N0 vs 1/2;$p<0.05$), stage classification(stage I/II vs III/IV; $p<0.05$), pattern of

invasion(grade 1-3vs 4; $p<0.05$), local recurrence(local recurrence (+) vs (-);$p<0.01$), and the overexpression of FOXC2 (FOXC2 overexpression (-)vs.(+); $p<0.05$). These results also strongly suggest that the overexpression of FOXC2 may be a potent predictor of survival, similar to the clinicopathological features described above.

Effect of FOXC2 expression on the proliferation and invasion potential of OTSCC cells

Cell proliferation and invasion are basic characteristics of tumor progression and metastasis. To determine the effects of FOXC2 expression on proliferation and invasion potential in OTSCC cell lines, we performed the MTT and Matrigel invasion assays. At the mRNA level, the expression of FOXC2 was shown a tendency to be associated with invasion potential in OTSCC cell lines ($p=0.066$) (Fig. 3A and B). Moreover, a correlation was observed between the expression of FOXC2 and proliferation potential ($p<0.05$) (Fig. 3C). These results suggest that FOXC2 may play key roles in tumor proliferation and

invasion in OTSCC *in vitro*.

Discussion

Recent several studies reported the clinicopathological and functional significance of FOXC2 in various cancers. In esophageal squamous cell carcinoma, the strong expression of FOXC2 was previously associated with an advanced tumor stage, lymph node metastasis, and lymphatic invasion[26]. Zhu et al. reported that the strong expression of FOXC2 correlated with differentiation, invasion depth, lymph node metastasis, and tumor stage in gastric cancer[28]. Furthermore, FOXC2 expression was previously correlated with lymph node metastasis in extrahepatic cholangiocarcinoma and colorectal cancer[21,35]. In glioblastoma cells, FOXC2 expression was also shown to enhance proliferation and invasion[36]. In the present study, we performed immunohistochemical analyses to determine the relationship between FOXC2 expression and clinicopathological features in OTSCC patients. The results obtained showed that the overexpression of FOXC2 was also more frequent in cancers with higher grades according to the T classification (T 3/4 vs. 1/2; $p<0.05$), N classification (N0 vs. 1/2; $p<0.01$), staging (stage I/II vs. III/IV;

$p<0.05$), and invasive pattern (grade 4 vs. 1-3; $p<0.05$). The results of the immunohistochemical examination revealed that FOXC2 staining was mainly detected in the cytoplasm and strong FOXC2 staining was observed at the invasive front and diffuse invasive area. FOXC2 staining was also mainly detected in cytoplasmic cancer tissues in esophageal and gastric cancers and extrahepatic cholangiocarcinoma[21,26,28]. A previous study detected the nuclear, perinuclear, and cytosolic localization of FOXC2 in breast and colonic adenocaricinoma[25]. A previous study also demonstrated that FOXC2 staining patterns ranged from absent to faint cytoplasmic staining in 52% of cancer cases, moderate cytoplasmic staining in 37%, and strong cytoplasmic and/or nuclear staining (high) in 10%[19]. Therefore, further studies are needed to confirm the significance of the cytoplasmic staining of FOXC2 in OTSCC.

In the present study, we also found a correlation between FOXC2 expression and poor prognosis in the 5-year disease-specific survival rates. This result is consistent with previous findings in which FOXC2

expression was correlated with a poor prognosis in various cancers, including esophageal, gastric, and non-small cell lung cancer as well as extrahepatic cholangiocarcinoma[21,26-28]. In the present study, multivariate analysis with Cox proportion hazard model revealed that FOXC2 expression may be a significant independent prognostic factor similar to N classification, stage classification, pattern of invasion, local recurrence, and expression of VEGF-A. These results suggest that FOXC2 expression levels could be used as a prognostic factor in OTSCC patients.

In the OTSCC cell lines examined in the present study, FOXC2 expression was correlated with the proliferation potential and revealed a tendency to be associated with the invasion potential. Regarding FOXC2 expression and invasion potential, FOXC2 expression was has been associated with EMT[19-23]. The expression of matrix metalloproteinases 2 (MMP-2) and matrix metalloproteinases 9 (MMP-9) was found to be markedly higher in a high-FOXC2 expression group with esophageal cancer involving local invasion[26]. The knockdown of FOXC2 was previously shown to inhibit cell motility and invasion in

extrahepatic cholangiocarcinoma and also decrease the expression of EMT markers (N-cadherin, MMP-2, and Angiopoietin 2)[21]. A previous study demonstrated that the overexpression of FOXC2 activated lymphatic drainage and enhanced lymphatic invasion by metastasizing cells[26]. Additionally, FOXC2 expression was reported to be induced in cells undergoing EMT and was triggered by a number of signals, including TGF-β1 and several EMT-inducing transcription factors, such as Snail, Twist, and Goosecoid[21]. FOXC2 also promoted mesenchymal differentiation during EMT and was correlated with the highly aggressive basal-like subtype of breast cancer[21]. Although the expression of FOXC2 was mainly observed in cells at the invasive front and diffuse invasive area and was correlated with invasive features using immunohistochemical and *in vitro* analyses in the present study, the precise mechanisms responsible remain unclear in OTSCC. Therefore, further studies are needed to elucidate the relationship between FOXC2 and invasion potential in OTSCC.

Angiogenesis and lymphangiogenesis are known to be crucial for tumor progression and nodal metastasis in OSCC[10]. The family of vascular endothelial growth factors, including VEGF-A, VEGF-B,

VEGF-C, VEGF-D, VEGF-E, placental growth factor, and VEGF-F, play crucial roles in angiogenesis and lymphangiogenesis[34]. Of these, VEGF-A and -C expression levels have been correlated with lymph node metastasis in esophageal squamous cell carcinoma[34]. Naruse et al. reported that VEGF-A may be related to tumor growth and VEGF-C to invasion[32]. FOXC2 was previously shown to play an important role in the migration and tubular transformation of vascular endothelial cells and also in tumor angiogenesis, which are known to be elicited by the activation of VEGF-A signaling[24,25]. In OSCC, Prospero homeobox 1 (Prox 1) and FOXC2 were shown to act as oncogenes by inducing lymphangiogenesis and angiogenesis, respectively[29]. FOXC2 was also found to be involved in the regulation of Prox 1 expression[29]. In the present study, we revealed that the expression of FOXC2 correlated with that of VEGF-A and -C. These results suggest that FOXC2 may play pivotal roles in tumor proliferation and invasion through VEGF signaling. However, further studies are needed to clarify the relationships between FOXC2-VEGF signaling, tumor proliferation, and invasion potential.

In conclusion, the present study demonstrated that FOXC2 was associated with tumor proliferation and invasion via the FOXC2-VEGF signaling pathway and may be an independent prognostic factor in OTSCC patients. Further studies on the expression and function of FOXC2 may offer additional indicators

for the diagnosis and treatment of OTSCC patients.

Acknowledgments

This study was supported in part by Grant 24792236 from the Ministry of Education, Culture, Sports, Science and Technology, Japan for S. Yamada.

Funding: None

Competing interests: None declared

Ethical approval: Not required

References

1. Johnson NW, Jayasekara D, Amarasinghe AA (2011) Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology. Periodontology 2000 57(1):19-37

2. Hicks WL Jr, North JH Jr, Loree TR, Maamoun S, Mullins A, Orner JB, Bakamjian VY, Shedd DP (1998) Surgery as a single modality therapy for squamous cell carcinoma of the oral tongue. Am J Otolaryngol 19(1):24-28

3. Sessions DG, Spector GJ, Lenox J, Parriott S, Haughey B, Chao C, Marks J, Perez C (2003) Analysis of treatment results for base of tongue cancer. Laryngoscope 113(7):1252-1261

4. González-García R, Naval-Gías L, Rodríguez-Campo FJ, Sastre-Pérez J, Muñoz-Guerra MF, Gil-Díez Usandizaga JL (2008) Contralateral lymph neck node metastasis of squamous cell carcinoma of the oral cavity: a retrospective analytic study in 315 patients. J Oral Maxillofac Surg 66(7):1390-1398

5. Ziober BL, Silverman SS Jr, Kramer RH (2001) Adhesive mechanisms regulating invasion and

metastasis in oral cancer. Crit Rev Oral Biol Med 12(6):499-510

6. Bánkfalvi A, Krassort M, Buchwalow IB, Végh A, Felszeghy E, Piffkó J (2002) Gains and losses of adhesion molecules (CD44, E-cadherin, and beta-catenin) during oral carcinogenesis and tumour progression. J Pathol 198(3):343-351

7. Arora S, Kaur J, Sharma C, Mathur M, Bahadur S, Shukla NK, Deo SV, Ralhan R (2005) Stromelysin 3, Ets-1, and vascular endothelial growth factor expression in oral precancerous and cancerous lesions: correlation with microvessel density, progression, and prognosis. Clin Cancer Res 11(6):2272-2284

8. Yanamoto S, Kawasaki G, Yoshitomi I, Iwamoto T, Hirata K, Mizuno A (2007) Clinicopathologic significance of EpCAM expression in squamous cell carcinoma of the tongue and its possibility as a potential target for tongue cancer gene therapy. Oral Oncol 43(9):869-877

9. Brinkman BM, Wong DT(2006) Disease mechanism and biomarkers of oral squamous cell carcinoma. Curr Opin Oncol 18(2):228-233

10. Sasahira T, Kirita T, Kurihara M, Yamamoto K, Bhawal UK, Bosserhoff AK, Kuniyasu H (2010) MIA-dependent angiogenesis and lymphangiogenesis are closely associated with progression, nodal metastasis and poor prognosis in tongue squamous cell carcinoma. Eur J Cancer. 46(12):2285-2294

11. Sakamoto K, Imanishi Y, Tomita T, Shimoda M, Kameyama K, Shibata K, Sakai N, Ozawa H, Shigetomi S, Fujii R, Fujii M, Ogawa K (2012) Overexpression of SIP1 and downregulation of E-cadherin predict delayed neck metastasis in stage I/II oral tongue squamous cell carcinoma after partial glossectomy. Ann Surg Oncol 19(2):612-619

12. Berx G, Raspe E, Christofori G, Thiery JP, Sleeman JP(2007) Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. Clin Exp Metastasis 24(8):587–597

13. Kalluri R, Weinberg RA(2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119(6):1420–1428

14. Baranwal S, Alahari SK(2009) Molecular mechanisms controlling E-cadherin expression in breast

cancer. Biochem Biophys Res Commun 384(1):6–11

15. Kopfstein L, Veikkola T, Djonov VG, Baeriswyl V, Schomber T, Strittmatter K, Stacker SA, Achen MG, Alitalo K, Christofori G (2007) Distinct roles of vascular endothelial growth factor-D in lymphangiogenesis and metastasis. Am J Pathol 170(4):1348-1361

16. Lohela M, Bry M, Tammela T, Alitalo K(2009) VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol 21(2):154-165

17. Kume T(2008) Foxc2 transcription factor: a newly described regulator of angiogenesis. Trends Cardiovasc Med 18(6):224-228

18. Kriederman BM, Myloyde TL, Witte MH, Dagenais SL, Witte CL, Rennels M, Bernas MJ, Lynch MT, Erickson RP, Caulder MS, Miura N, Jackson D, Brooks BP, Glover TW (2003) FOXC2 haploinsufficient mice are a model for human autosomal dominant lymphedema-distichiasis syndrome. Hum Mol Genet 12(10):1179-1185

19. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, Weinberg RA (2007) Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci USA 104(24):10069-10074.

20. Mortazavi F, An J, Dubinett S, Rettig M (2010) p120-catenin is transcriptionally downregulated by FOXC2 in non-small cell lung cancer cells. Mol Cancer Res 8(5):762-774

21. Watanabe A, Suzuki H, Yokobori T, Altan B, Kubo N, Araki K, Wada S, Mochida Y, Sasaki S, Kashiwabara K, Hosouchi Y, Kuwano H (2013) Forkhead box protein C2 contributes to invasion and metastasis of extrahepatic cholangiocarcinoma, resulting in a poor prognosis. Cancer Sci 104(11):1427-1432

22. Hollier BG, Tinnirello AA, Werden SJ, Evans KW, Taube JH, Sarkar TR, Sphyris N, Shariati M, Kumar SV, Battula VL, Herschkowitz JI, Guerra R, Chang JT, Miura N, Rosen JM, Mani SA (2013) FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. Cancer

Res 73(6):1981-1992

23. Liu B, Han SM, Tang XY, Han L, Li CZ (2014) Overexpressed FOXC2 in ovarian cancer enhances the epithelial-to-mesenchymal transition and invasion of ovarian cancer cells. Oncol Rep 31(6):2545-2554

24. Kume T(2012) The Role of FoxC2 Transcription Factor in Tumor Angiogenesis. J Oncol doi: 10.1155/2012/204593.

25. Sano H, Leboeuf JP, Novitskiy SV, Seo S, Zaja-Milatovic S, Dikov MM, Kume T(2010) The Foxc2 transcription factor regulates tumor angiogenesis. Biochem Biophys Res Commun 392(2):201-206

26. Nishida N, Mimori K, Yokobori T, Sudo T, Tanaka F, Shibata K, Ishii H, Doki Y, Mori M(2011) FOXC2 is a novel prognostic factor in human esophageal squamous cell carcinoma. Ann Surg Oncol 18(2):535-542

27. Jiang W, Pang XG, Wang Q, Shen YX, Chen XK, Xi JJ(2012) Prognostic role of Twist, Slug, and Foxc2 expression in stage I non-small-cell lung cancer after curative resection. Clin Lung Cancer

13(4):280-287

28. Zhu JL, Song YX, Wang ZN, Gao P, Wang MX, Dong YL, Xing CZ, Xu HM(2013) The clinical significance of mesenchyme forkhead 1 (FoxC2) in gastric carcinoma. Histopathology 62(7):1038-1048

29. Sasahira T, Ueda N, Yamamoto K, Kurihara M, Matsushima S, Bhawal UK, Kirita T, Kuniyasu H(2014) Prox1 and FOXC2 act as regulators of lymphangiogenesis and angiogenesis in oral squamous cell carcinoma. PLoS One doi: 10.1371/journal.pone.0092534

30. Yamamoto E, Kohama G, Sunakawa H, Iwai M, Hiratsuka H(1983) Mode of invasion, bleomycin sensitivity, and clinical course in squamous cell carcinoma of the oral cavity. Cancer 51(12):2175-2180.

31. McCarthy KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA, Creasmna WT, Seigler HF, McCarthy, KS Sr(1986) Use of a Monoclonal Anti-Estrogen Receptor Antibody in the Immunohistochemical Evaluation of Human Tumors. Cancer Res. 46(8):4244-4268.

32. Naruse T, Kawasaki G, Yanamoto S, Mizuno A, Umeda M(2011) Immunohistochemical study of VEGF

expression in oral squamous cell carcinomas: correlation with the mTOR-HIF-1α pathway. Anticancer Res 31(12):4429-4437

33. Otrock ZK, Makarem JA, Shamseddine AI(2007) Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol Dis 38(3):258-268

34. Ding MX, Lin XQ, Fu XY, Zhang N, Li JC(2006) Expression of vascular endothelial growth factor-C and angiogenesis in esophageal squamous cell carcinoma. World J Gastroenterol 12(28):4582-4585

35. Watanabe T, Kobunai T, Yamamoto Y, Matsuda K, Ishihara S, Nozawa K, Iinuma H, Kanazawa T, Tanaka T, Konishi T, Ikeuchi H, Eshima K, Muto T, Nagawa H(2011) Gene expression of mesenchyme forkhead 1 (FOXC2) significantly correlates with the degree of lymph node metastasis in colorectal cancer. Int Surg 96(3):207-216

36. Li W, Fu X, Liu R, Wu C, Bai J, Xu Y, Zhao Y, Xu Y(2014) FOXC2 often overexpressed in glioblastoma enhances proliferation and invasion in glioblastoma cells. Oncol Res 21(2):111-120

Figure Legends

Figure 1

Representative immunohistochemical staining for FOXC2, VEGF-A and -C in well-differentiated OTSCC.

(A): Immunohistochemical staining for FOXC2 demonstrating the strong cytoplasmic expression of FOXC2 (staining intensity of 3)(x20) and diffuse invasion (B)(x20). (C): Immunohistochemical staining for VFGF-A demonstrating the strong cytoplasmic expression of VEGF-A (staining intensity of 3)(x20). (D): Immunohistochemical staining for VFGF-C demonstrating the strong cytoplasmic expression of VEGF-C (staining intensity of 3)(x20).

Figure 2

Kaplan-Meier curves for 5-year disease-specific survival analysis.

The 5-year disease-specific survival rates according to FOXC2 expression in OTSCC patients were plotted (Fig. 2). The high-FOXC2 expression group had a significantly poorer prognosis than that of the low-expression group ($p<0.001$).

Figure 3

(A) Representative RT-PCR analysis for FOXC2 in OTSCC cell lines (SAS, SCC25, OSC20, HSC-3, HSC-4). The FOXC2/GAPDH intensities are the mean ± SD of triplicate experiments. (B) Relationship between FOXC2 mRNA expression and the invasion index (%). FOXC2 expression was associated with the invasion index (Pearson's correlation, r=0854, P=0.066). ●, SAS cells; *, OSC20 cells; ■, HSC-3 cells; ▲, HSC-4 cells; ◆, SCC25 cells. (C) Relationship between FOXC2 mRNA expression and the growth index (%). A correlation was observed between FOXC2 expression and the growth index (Pearson's correlation, r=943, $p<0.05$). ●, SAS cells; *, OSC20 cells; ■, HSC-3 cells; ▲, HSC-4 cells; ◆, SCC25 cells.

Characteristics	Number of samples	FOXC2 overexpression(-)	FOXC2 overexpression(+)	P value
Normal epithelia	10	10	0	
squamous cell carcinoma	61	39	22	P＜0.05
Gender				
Male	41	26	15	0.776
Female	20	13	7	
Age				
<65	31	20	11	0.923
≧65	30	19	11	
T classification				
T1/T2	53	36	17	0.124
T3/T4	8	3	5	
N classification				
N0	47	32	15	0.315
N1/2	14	7	7	
stage classification				
I/II	45	31	14	0.229
III/IV	16	8	8	
Differentiation				
well	55	37	18	0.176
moderate/poor	6	2	4	
Pattern of invasion				
Grades1/2/3	51	36	15	P＜0.05
Grades 4	10	3	7	
Local recurrence				
(-)	54	35	19	0.695
(+)	7	4	3	
Secondary cervical lymph node metastasis				
(-)	46	31	15	0.365
(+)	15	8	7	
VEGF-A				
overexpression (-)	31	29	2	P＜0.001
overexpression (+)	30	10	20	
VEGF-C				
overexpression (-)	38	34	4	P＜0.001
overexpression (+)	23	5	18	

Table 1 Correlation between FOXC2 expression and clinicopathologic features.

Characteristics	Univariate analysis			Multivariate analysis		
	Risk ratio	95%CI	P value	Risk ratio	95%CI	P value
Gender (Male vs. Female)	0.4751	0.119-1.902	0.293			
Age (<65 vs. ≧65)	0.5772	0.138-2.421	0.452			
T classification (T1/T2 vs. T3/T4)	3.832	0.913-16.086	0.066			
N classification (N0 vs. N1/2)	4.727	1.164-19.195	P＜0.05	0.009	0.000-0.787	P＜0.05
Stage classification (stage I/II vs. III/IV)	5.859	1.389-24.711	P＜0.05	220.0	2.595-18652	P＜0.05
Differentiation (well vs. poor/moderate)	3.009	0.603-15.023	0.179			
Pattern of invasion (grade1-3 vs. grade 4)	11.625	2.692-50.196	P＜0.01	32.642	1.325-804.1	P＜0.05
Local recurrence (+ vs. -)	6.361	1.509-26.807	P＜0.05	1.313	0474-4.680	P＜0.01
Secondary cervical lymph node metastasis (+ vs. -)	3.478	0.866-13.966	0.079			
VEGF-A expression (+ vs. -)	8.243	1.013-67.073	P＜0.05	3.683	0.185-73.306	0.371
VEGF-C expression (+ vs. -)	3.155	0.750-13.272	0.117			
FOXC2 overexpression (+ vs. -)	17.171	2.089-141.12	P＜0.01	31.233	1.936-503.8	P＜0.05

Table 2 Univariate and Multivariate analysis of different prognostic parameters

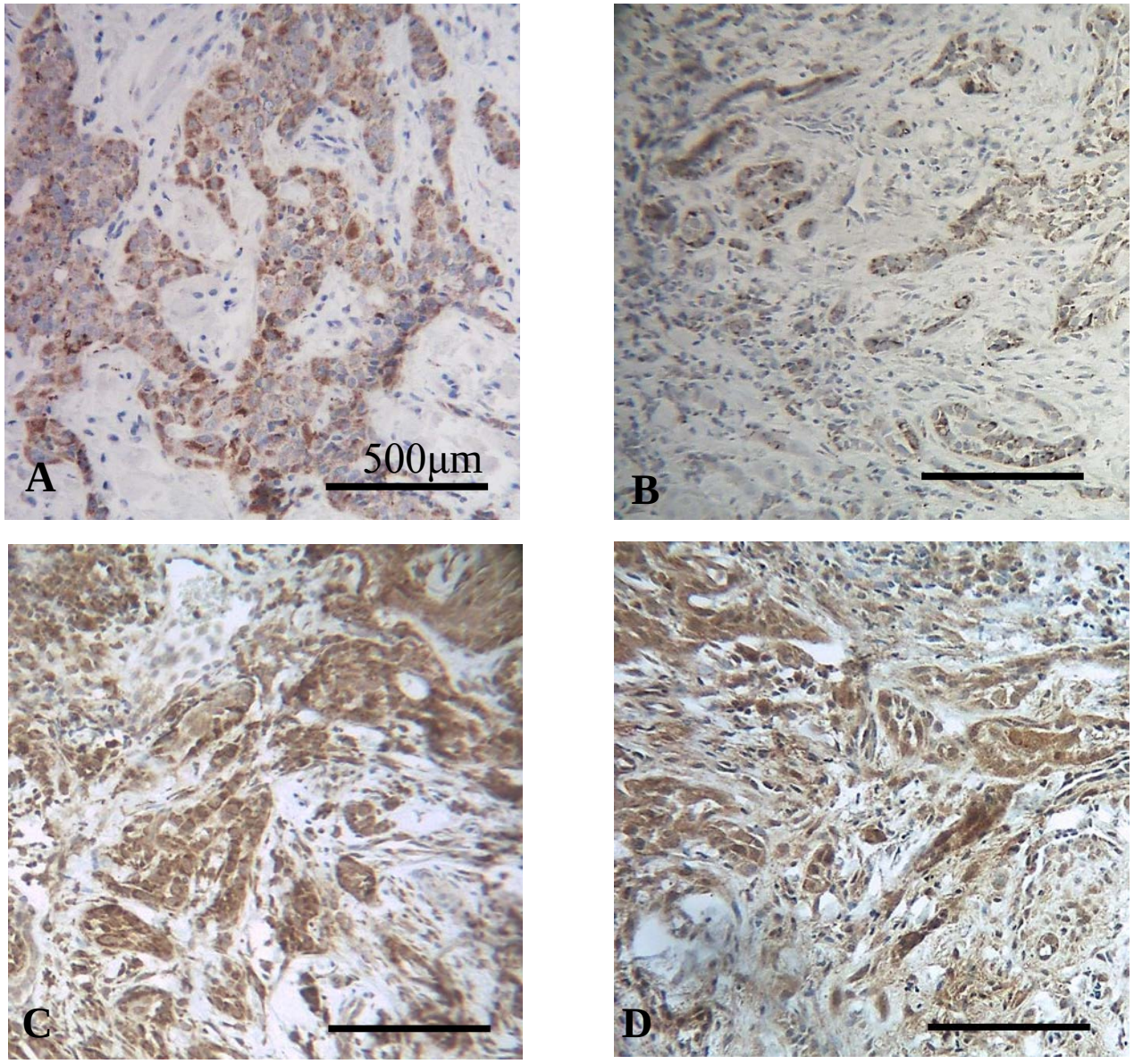

Fig. 1

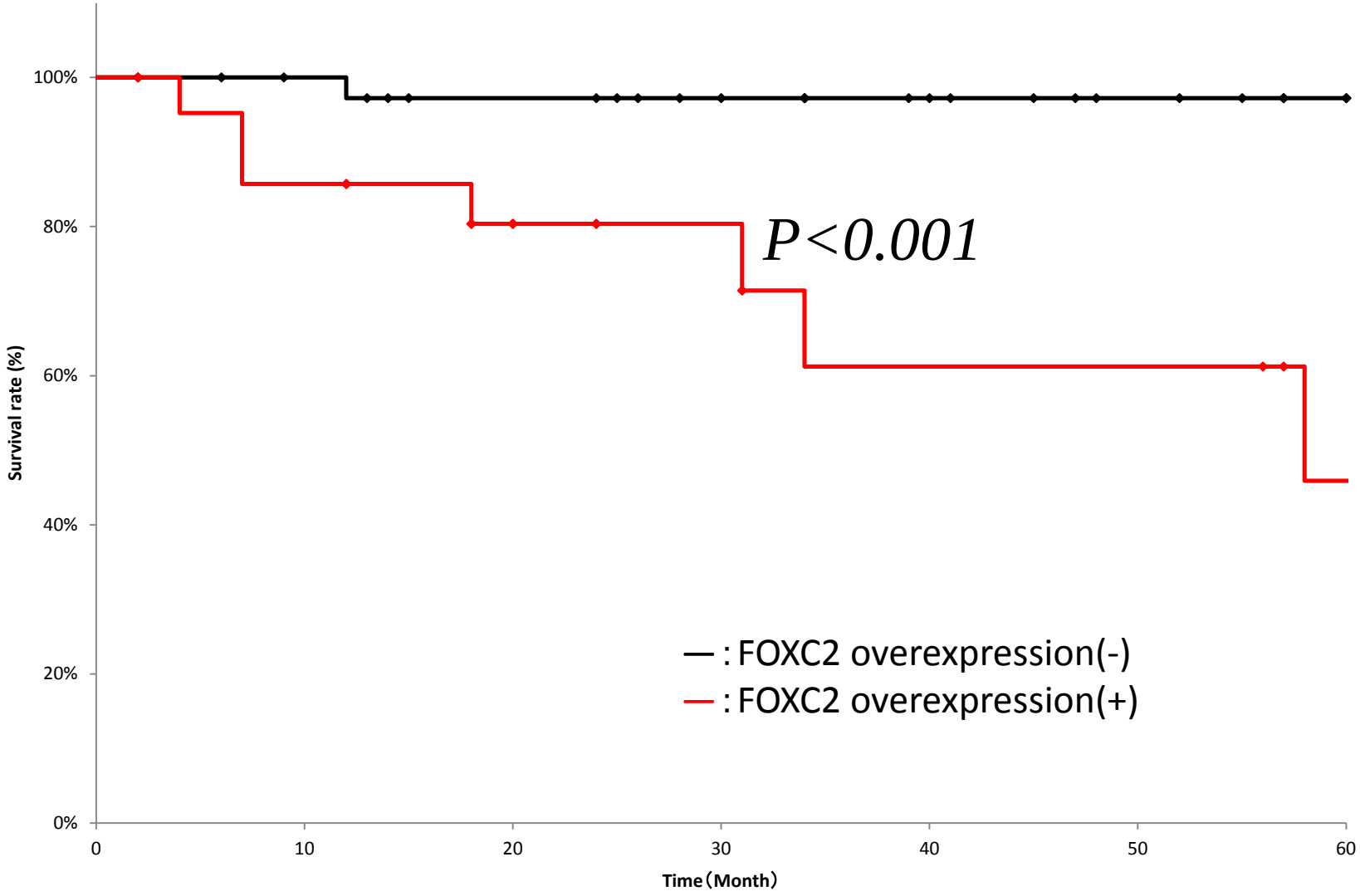
100%
80%
60%
40%
20%
0%
Survival rate (%)
P<0.001
— : FOXC2 overexpression(-)
— : FOXC2 overexpression(+)
0
10
20
30
40
50
60
Time（Month）

Fig. 2

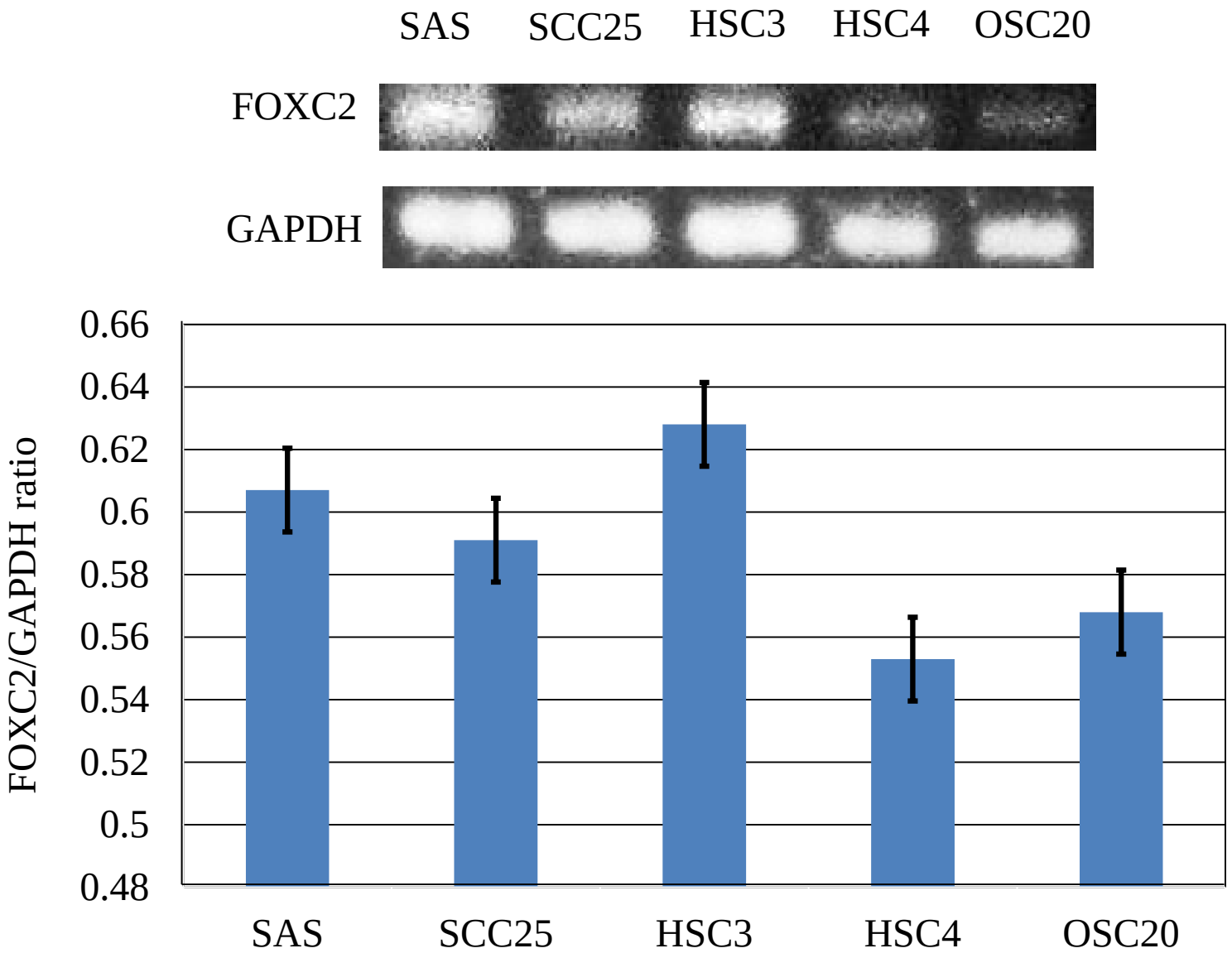
SAS
SCC25
HSC3
HSC4
OSC20
FOXC2
GAPDH
FOXC2/GAPDH ratio
0.66
0.64
0.62
0.6
0.58
0.56
0.54
0.52
0.5
0.48
SAS
SCC25
HSC3
HSC4
OSC20

Fig. 3A

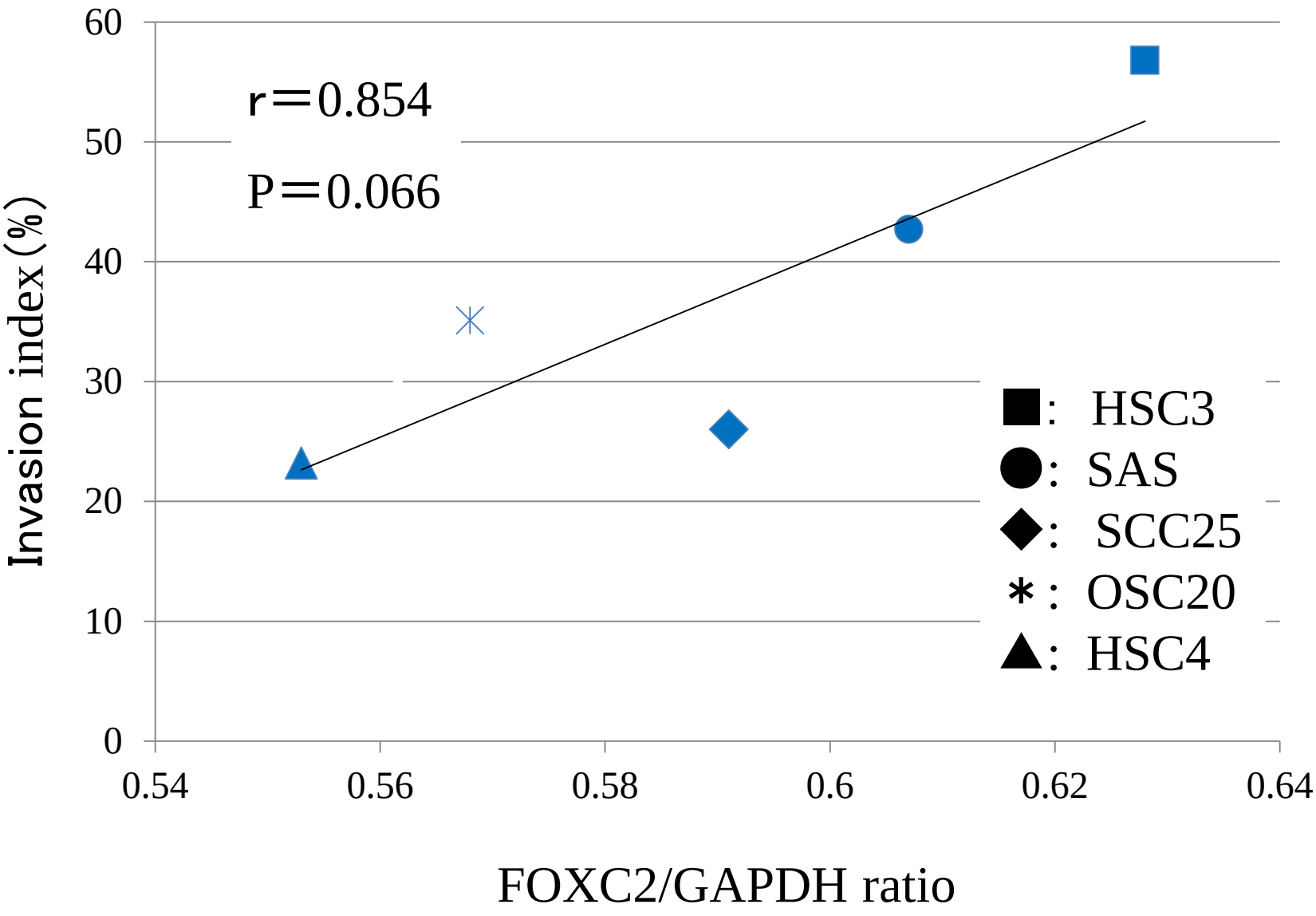
r=0.854
P=0.066
Invasion index (%)
60
50
40
30
20
10
0
0.54
0.56
0.58
0.6
0.62
0.64
FOXC2/GAPDH ratio
■: HSC3
●: SAS
◆: SCC25
∗: OSC20
▲: HSC4

Fig. 3B

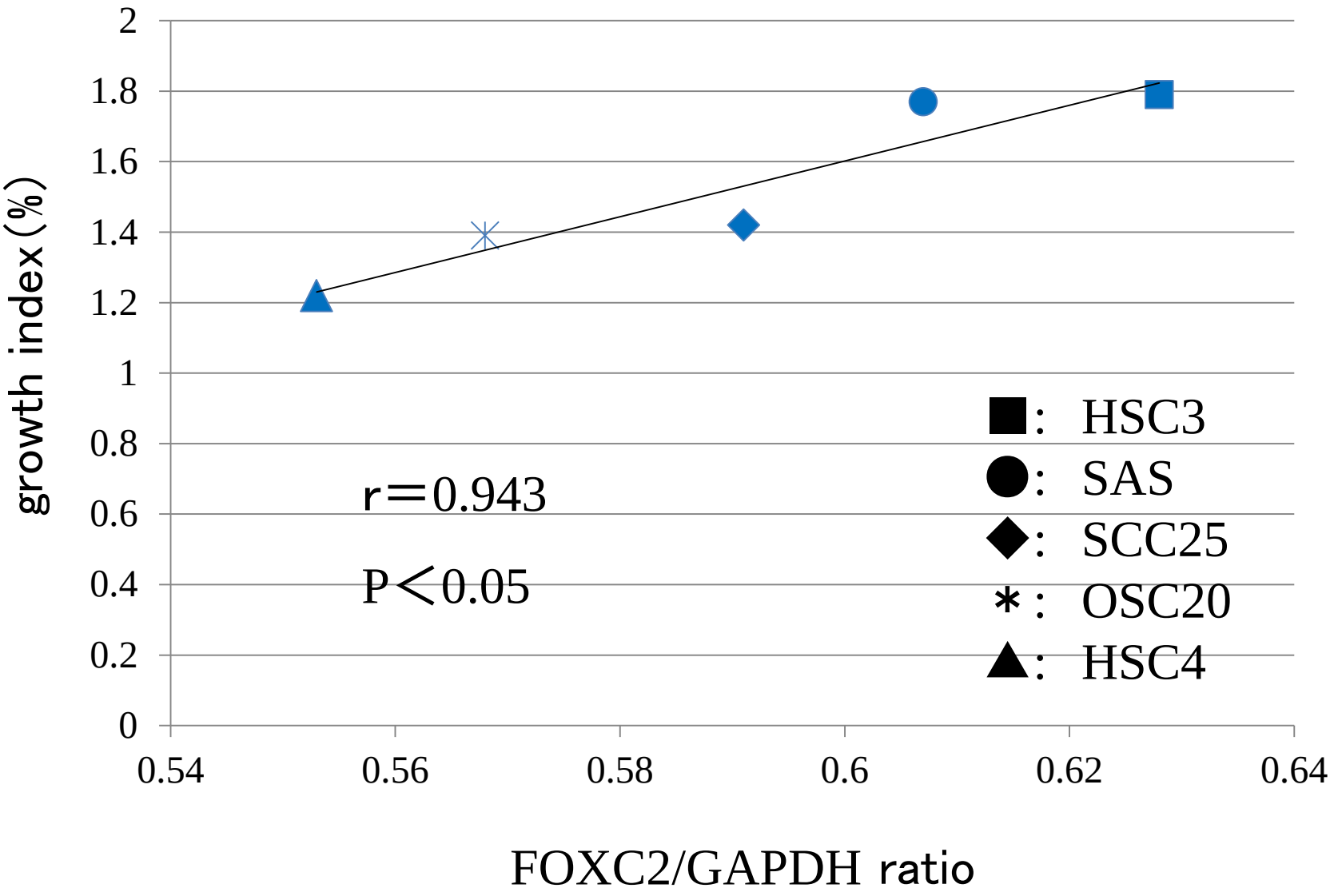
growth index (%)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.54
0.56
0.58
0.6
0.62
0.64
FOXC2/GAPDH ratio
r＝0.943
P＜0.05
■: HSC3
●: SAS
◆: SCC25
＊: OSC20
▲: HSC4

Fig. 3C