Immunohistochemical Study of VEGF Expression in Oral Squamous Cell Carcinomas: Correlation with the mTOR–HIF-1α Pathway

TOMOFUMI NARUSE, GORO KAWASAKI, SOUICHI YANAMOTO, AKIO MIZUNO and MASAHIRO UMEDA

Department of Clinical Oral Oncology, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto, Nagasaki, Japan

Abstract. *The aim of this study was to clarify the relationship between vascular endothelial growth factor (VEGF) expression and clinicopathological factors in oral squamous cell carcinoma (OSCC). We also examined the correlation between the VEGF expression and the mammalian target of rapamycin (mTOR)–hypoxia inducible factor-1α (HIF-1α) pathway. Formalin-fixed paraffin-embedded tissues from 120 OSCC cases and 10 samples of normal mucosa were stained immunohistochemically for VEGF-A, VEGF-C, p-mTOR and HIF-1α proteins. VEGF-A and VEGF-C protein expression was detected in 76 out of 120 (63%) and 81 of 120 (67.5%) OSCCs, respectively, and their expression was significantly higher in primary OSCC than in normal oral mucosa. VEGF-A expression was significantly associated with the tumor stage and age. VEGF-C expression was significantly associated with the cancer cell invasion. The cases with combined p-mTOR+/HIF-1α$^+$/VEGF-A$^+$ expression had a significantly higher tumor stage and invasion grade, and combined p-mTOR+/HIF-1α$^+$/VEGF-C$^+$ expression was significantly associated with tumor stage, regional lymph node metastasis and invasion grade. In a survival analysis, no obvious correlation was observed with any of the immunohistochemical results. This study indicated that the mTOR–HIF-1α–VEGF pathway affects the progression of OSCC, and inhibition of this pathway may be useful for the treatment of OSCC.*

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck region, and its incidence has recently been increasing. The primary treatment modality for OSCC is surgery. Recent advances in treating OSCC have led to the improvement of local tumor control, but fewes than half of patients with advanced OSCC survive for 5 years. Therefore, new treatment strategies are required (1).

Although angiogenesis, the formation of new blood vessels, is a normal physiological event, it is closely related to both tumor growth and metastasis. These newly-formed vessels provide the principal route by which tumor cells exit the primary tumor site and enter the circulation. For many types of tumors, vascular density is a prognostic indicator of metastatic potential, with highly vascular primary tumors having a higher incidence of metastasis than poorly vascularized tumors. We now know there are various molecular determinants of these different mechanisms of vascular growth (2-5).

The family of vascular endothelial growth factors (VEGFs), including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor and VEGF-F, play a key role in angiogenesis and lymphangiogenesis (6). Among them, VEGF-A is known to be a key angiogenic factor, and is the most frequently used by a tumor to switch on its angiogenic phenotype (7). In fact, VEGF-A overexpression has been reported in most types of cancer, including oral cancer, and it is thought to be a prognostic factor for survival (8-13). VEGF-C stimulates the proliferation of both vascular and lymphatic endothelial cells *via* the VEGF receptor (VEGFR)-2 and VEGFR-3 in many physiological and pathological processes. VEGF-C has been detected in several different types of cancer, and its level in some studies seems to correlate with nodal metastasis and patient survival (10, 15, 16). Recently, various drugs targeting VEGF-mediated signaling have been introduced into the treatment of terminal cancer to control tumor angiogenesis at the clinical level (17-25).

Several reports have indicated that tumor cells under hypoxic conditions have high neovascularity and aggressive behavior. Although cell growth is generally decreased under

Correspondence to: Dr. Goro Kawasaki, Department of Clinical Oral Oncology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1, Sakamoto, Nagasaki, Japan. Tel: +81 958497696, Fax: +81 958497700, e-mail: gkawa@net.nagasaki-u.ac.jp

Key Words: VEGF, mTOR, HIF-1α, angiogenesis, oral cancer.

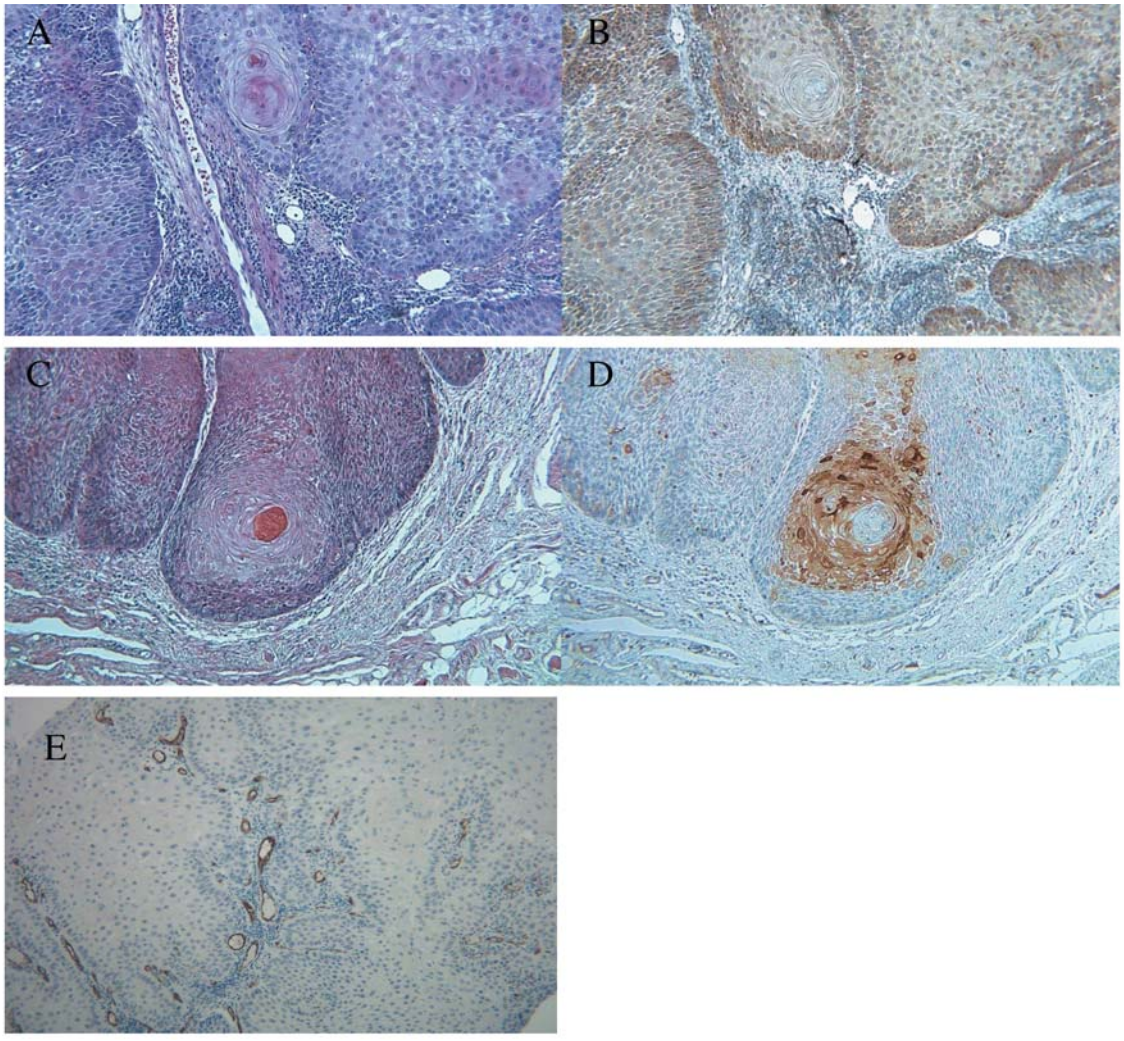

Figure 1. *Representative H&E and immunohistochemical staining for VEGF-A, VEGF-C and CD34 in well-differentiated OSCC. A: H&E (×100) and B: immunohistochemical staining of the same tumor demonstrating strong VEGF-A cytoplasmic expression (staining index of 3). C: H&E (×100) and D: immunohistochemical staining for VEGF-C demonstrating strong cytoplasmic expression in the cancer nest (staining index of 3) (×100). E: Immunohistochemical expression of CD34 (×100).*

hypoxic conditions, some tumor cells overcome inhibition of proliferation and adapt to growth under hypoxia. This is largely due to an increased level of hypoxia-inducible factor-1α (HIF-1α), which is stabilized and translocated to the nucleus, where it induces expression of the VEGFs and other various tumor growth factors (8, 10, 13).

Some researchers have indicated that the mammalian target of rapamycin (mTOR) is a positive regulator of HIF-1α expression and activity, and that the inhibition of HIF-mediated gene expression is considered to be related to the antitumor activity of mTOR inhibitors (9).

In the present study, we examined the expression levels of VEGF-A and -C, HIF-1α and phosphorylated-mTOR (p-mTOR) in oral SCCs. The aim of this study was to clarify the relationship between VEGF expression and clinicopathological factors. In addition, we also examined the correlation of these proteins with the mTOR-HIF-1α pathway.

Materials and Methods

Patients and specimens. A total of 120 patients with OSCC and 10 healthy individuals were enrolled in this study from 1998 to 2007 in the Department of Oral and Maxillofacial Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. The tumor stage was classified according to the TNM classification of the International Union Against Cancer (26). All samples from OSCC patients were biopsy specimens. The specimens of normal oral mucosa from 10 healthy individuals were used as controls. The specimens were fixed in 10% buffered

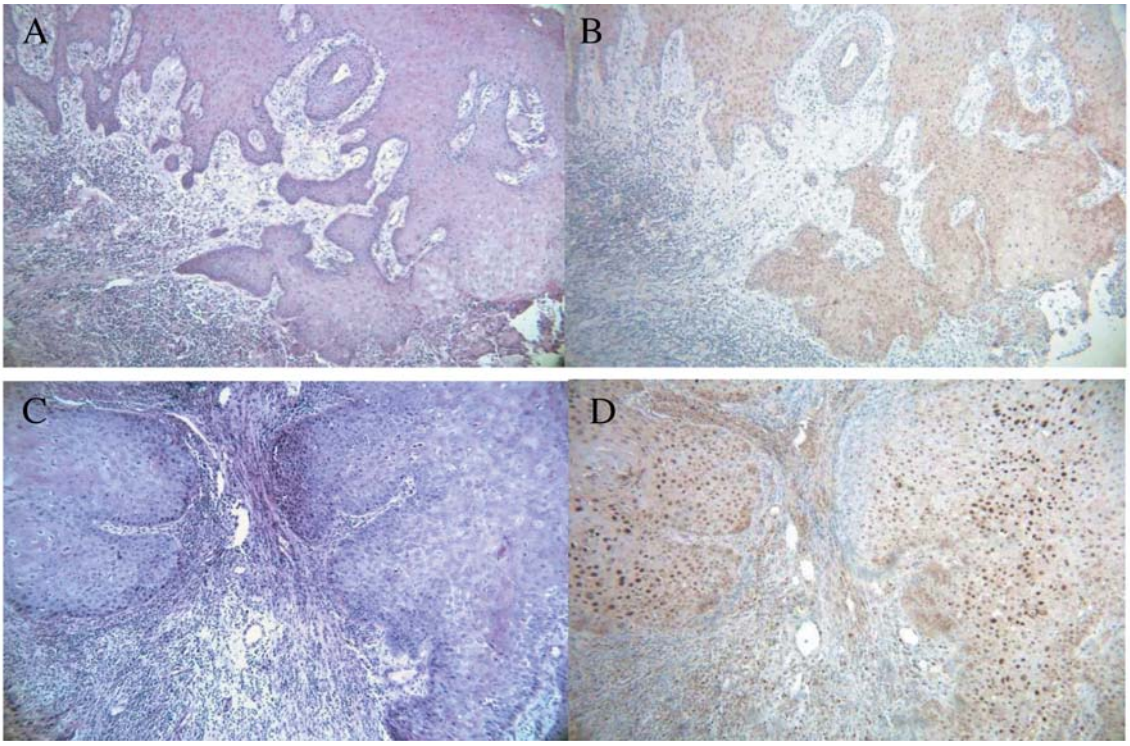

Figure 2. *Representative H&E and immunohistochemical staining for p-mTOR and HIF-1α in well-differentiated OSCC. A: H&E (×100) and B: immunohistochemical staining of the same tumor demonstrating strong cytoplasmic p-mTOR expression (staining index of 3). C: H&E (×100) and D: immunohistochemical staining of the same tumor demonstrating strong nuclear and cytopasmic expression (staining index of 3) (×100).*

formalin for 24-48 h and embedded in paraffin wax. Tumor histological differentiation was defined according to the World Health Organization classification (27) and the invasive grade was assessed by the Yamamoto-Kohama (YK) mode of invasion (28).

Immunohistochemistry. Deparaffinized sections in xylene were soaked in 10 mmol/l citrate buffer (pH 6.0) and placed in an autoclave at 121˚C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (ENVISION+; DAKO, Glostrup, Denmark). The primary antibodies used were against p-mTOR, HIF-1α, VEGF-A, VEGF-C, proliferating cell nuclear antigen (PCNA) and CD34 (DAKO). The sections were then washed in Dulbecco's phosphate-buffered saline (PBS), followed by incubation with the primary antibodies at 4˚C overnight.

The reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Meyer's hematoxylin and mounted. Results were evaluated by calculating the total immunostaining score as the product of the proportional score and the intensity score. As described previously, the proportional scores described the estimated fraction of positively-stained tumor cells (0, none; 1, <10%; 2, 10-50%; 3, 50-80%; 4, >80%). The intensity score represents the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranges from 0-12. Positive sections were defined as those with a total score >4 (29). Immunohistochemical overexpression was defined as a total score greater than 4, since the patient samples showed a bimodal distribution of immunohistochemical expression with the discriminating nadir at a total score value of 3 to 4.

Statistical analysis. The associations between the sample expression of target molecules and clinicopathological features were assessed by Fischer's exact test. Significance was assessed by the Mann-Whitney *U*-test. Survival analysis was calculated by the Kaplan-Meier method and compared using the log-rank test. *P*-values <0.05 were considered to be significant.

Results

Expression of p-mTOR, HIF-1α, VEGF-A and -C. VEGF-A and ¯C: Immunohistochemical staining of VEGF-A and -C was found in the cytoplasm of both normal tissue and OSCC samples. The proteins were found to be strongly expressed in the invasion front of the tumor. VEGF-A and -C protein expression was detected in 76 out of 120 and 81 out of 120 SCCs, respectively. Representative immunohistochemical staining is shown in Figure 1.

CD34: Immunohistochemical expression of CD34 represents a good method to quantify angiogenesis in carcinoma. The expression was significantly associated with VEGF expression (Figure 1).

Table I. *Correlation between VEGF-A and VEGF-C expression and clinicopathologic features.*

	VEGF-A		*P*-value	VEGF -C		*P*-value
	– (n=44)	+ (n=76)		– (n=39)	+ (n=81)	
Normal epithelium	10	0	<0.0001	10	0	<0.0001
Squamous cell carcinoma	44	76		39	81	
Gender						
Male	30	42	0.115	25	47	0.332
Female	14	34		14	34	
Age (years)						
68≤	11	39	<0.01	14	36	0.245
68>	33	37		25	45	
T Classification						
T1+T2	32	42	<0.05	28	46	0.08
T3+T4	12	34		11	35	
N Classification						
N0	33	59	0.454	28	64	0.379
N1+N2	11	17		10	18	
Differentiation						
Well	39	63	0.284	31	71	0.182
Moderate, poor	5	13		8	10	

Table II. *Correlation between p-mTOR and HIF-1α expressions and clinicopathologic features.*

	p-mTOR		*P*-value	HIF-1α		*P*-value
	– (n=71)	+ (n=49)		– (n=35)	+ (n=85)	
Normal epithelium	10	0	0.0131	10	0	<0.0001
Squamous cell carcinoma	71	49		35	85	
Gender						
Male	46	26	0.136	19	53	0.268
Female	25	23		16	32	
Age (years)						
68≤	28	22	0.341	18	32	0.117
68>	43	27		17	53	
T Classification						
T1+T2	47	27	0.15	30	44	<0.001
T3+T4	24	22		5	41	
N Classification						
N0	57	35	0.181	33	59	<0.01
N1+N2	14	14		2	26	
Differentiation						
Well	60	42	0.535	30	72	0.57
Moderate, poor.	11	7		5	13	

p-mTOR: In normal oral tissue, p-mTOR protein expression was absent or minimal in the cytoplasm of epithelial cells. In OSCC specimens, the expression was detected in 49 out of 120 samples. p-mTOR was expressed mainly in the cytoplasm of the tumor cells, ranging from low to strong intensity. Representative immunohistochemical staining is shown in Figure 2.

HIF-1α: In normal oral tissue, HIF-1α protein expression was absent or minimal in the cytoplasm of epithelial cells, and was not detected in the nucleus of epithelial cells. In OSCC samples, HIF-1α protein expression was detected in 85 out of 120 samples. HIF-1α was expressed in tumor cell nuclei and/or cytoplasm, ranging from low to strong intensity.

Correlation between VEGF-A and -C and clinicopathological factors. We examined the expression of VEGF-A and -C in oral carcinoma as a function of the clinicopathological factors and invasion grade. As shown in Table I , VEGF-A expression was significantly associated with tumor stage and age (Table I), but VEGF-C expression was not significantly associated with any of the clinicopathological factors including metastasis to regional lymph nodes (Table I).

The 120 cases were classified according to the YK mode of invasion: YK-1, n=9; YK-2, n=28; YK-3, n=48; YK-4C, n=23; YK-4D, n=12. Although there was no significant correlation between VEGF-A expression and the invasion grade, there was a significant correlation between VEGF-C expression and the invasion grade (Table IV).

Correlation between p-mTOR, HIF-1α and clinicopathological factors. The p-mTOR and HIF-1α expression levels in oral carcinoma were examined as a function of the clinicopathological factors. For the p-mTOR protein, there was no significant correlation between p-mTOR expression and any of the clinical factors. However, HIF-1α expression was significantly correlated with the tumor stage and regional lymph node metastasis (Table II).

With regard to oral cancer cell invasion, there was no significant correlation with either p-mTOR or HIF-1α expression.

Correlation between the activity of the mTOR–HIF-1α pathway, clinicopathological factors, and PCNA labeling index. Cases having an active mTOR–HIF-1α pathway were defined as those that were positive for both p-mTOR and HIF-1α expression. Combined p-mTOR$^+$/HIF-1α$^+$ cases were significantly associated with the tumor stage and the presence of regional lymph node metastasis (Table III).

Immunohistochemical PCNA expression levels were examined in the cancer cells to determine the interaction between tumor cell proliferation and the function of the mTOR–HIF-1α pathway. PCNA expression was detected immunohistochemically in the nucleus of the tumor cells. The average PCNA labeling index (LI) was 54.9% in p-mTOR$^+$/HIF-1α$^+$ cases and 27.1% in p-mTOR$^-$/HIF-1α$^-$ cases. Although this difference was significant, no correlation was observed for the other combinations of p-mTOR and HIF-1α (Figure 3).

Table III. *Association of p-mTOR /HIF-1α and p-mTOR /HIF-1α/VEGF immunohistochemical phenotype and YK mode of invasion.*

	p-mTOR/HIF-1 (n)				*P*-value	p-mTOR/HIF-1α/ VEGF-A (n)		*P*-value	p-mTOR/HIF-1α/ VEGF-C (n)		*P*-value
	–/– (32)	+/– (3)	–/+ (39)	+/+ (46)		–/–/– (19)	+/+/+ (42)		–/–/– (16)	+/+/+ (44)	
Gender											
Male	17	2	28	25	>0.05	10	23	0.547	8	24	0.49
Female	15	1	11	21		9	19		8	20	
Age (years)											
68≥	15	1	12	22	>0.05	7	19	0.371	6	20	0.401
68<	21	2	24	26		12	23		10	24	
T Classification											
T1+T2	27	3	22	23	<0.001	17	22	<0.01	15	22	<0.01
T3+T4	5	0	18	23		2	20		1	22	
N Classification											
N0	30	3	27	32	<0.01	18	30	<0.05	15	31	0.054
N1+N2	2	0	12	14		1	12		1	13	
Differentiation											
Well	28	2	32	40	0.51	17	36	0.51	13	39	0.36
Moderate, poor	4	1	7	6		2	6		3	5	

Correlation between the activity of the mTOR–HIF-1α–VEGF pathway and clinicopathological factors. Cases defined as having an active mTOR–HIF-1α–VEGF pathway were those which were positive for p-mTOR, HIF-1α and VEGF. As shown in Table IV, combined p-mTOR$^+$/HIF-1α$^+$/VEGF-A$^+$ expression was significantly associated with tumor stage and invasion grade, as well as the presence of regional lymph node metastasis.

Prognostic significance of p-mTOR, HIF-1α, VEGF-A and-C expression. We calculated the 5-year survival by the Kaplan-Meier method and compared the survival using the log-rank test. There was no significant correlation between survival and combined p-mTOR$^+$/HIF-1α$^+$/VEGF-A$^+$ and –C$^+$ cases or with the other types of combinations (Figure 4).

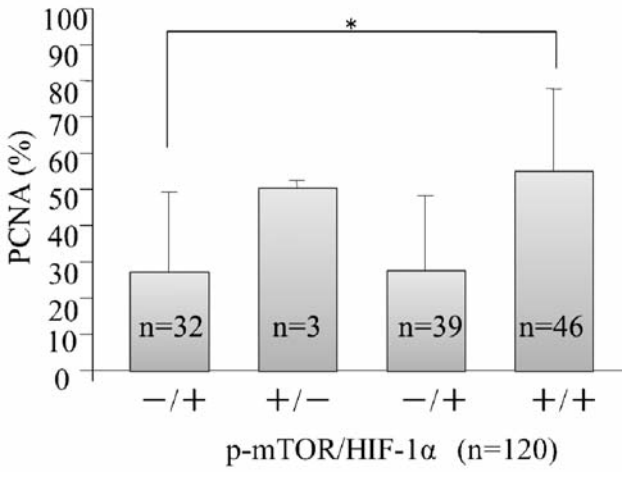

Figure 3. *Correlation between p-mTOR/HIF-1α expressions and PCNA –labeling index. Values are means±SD. *p<0.05.*

Discussion

VEGF is a key regulator of angiogenesis, vasculogenesis, and developmental hematopoiesis. VEGF overexpression has been known to be associated with tumor growth, metastasis and the survival rate (2-5). Recently, some reports have indicated that VEGF expression and activity is critically regulated mainly by mTOR and HIF-1α, and overexpression of p-mTOR and HIF-1α has been reported in many types of human cancer (8, 9). mTOR-dependent translation is known to control a number of specific cell-growth regulators including HIF-1α transcriptional factor, which functions in diverse processes including cell growth, glycolysis and angiogenesis, all contributing to enhanced tumorigenesis (9). Hence, we hypothesized that the mTOR–HIF-1α–VEGF pathway might have an important role in oral cancer progression. We therefore examined the relationship between the protein expression levels and various clinicopathological factors.

Overexpression of VEGF-A and -C has also been reported in various types of cancer. In this study, we observed VEGF-A and -C expression in 76 out of 120 SCCs (63%) and 81 out of 120 SCCs (67.5%) respectively, whereas their expression was not observed in any normal tissue samples. Therefore, we suggest that VEGF-A and -C expression is associated with

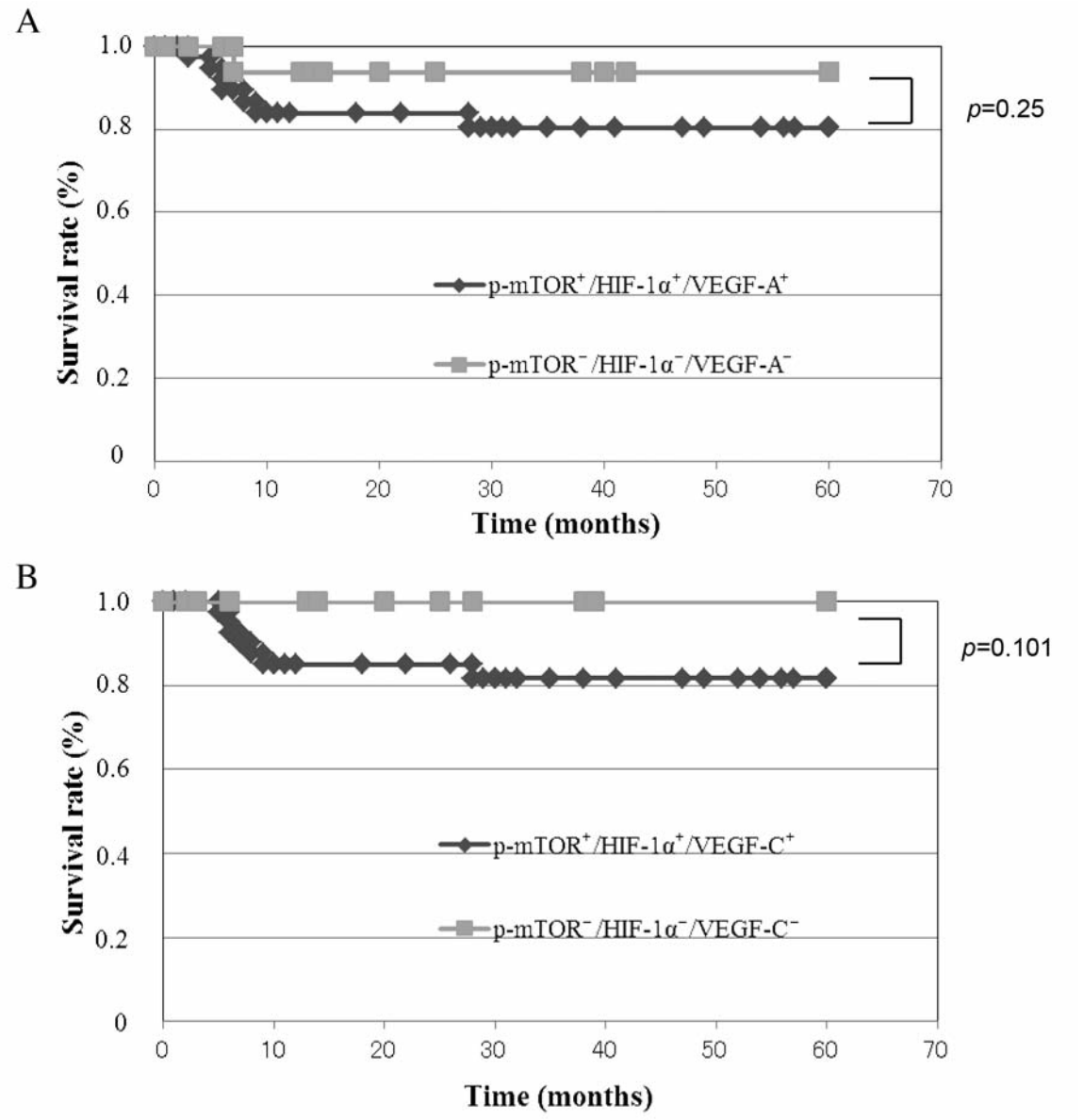

Figure 4. *Kaplan-Meier estimates of overall survival rate of oral cancer patients with positive and negative expression of p-mTOR/HIF-1α/VEGF. No significant difference was observed between the two groups for p-mTOR/HIF-1α/VEGF. Differences between the two groups were evaluated with the log-rank test.*

oral carcinoma. In clear cell renal cell carcinoma, non-small cell lung cancer, colorectal cancer, and head and neck cancer, the overexpression of VEGF-A and -C is correlated with a poorer prognosis (10-13). VEGF-A and -C expression levels are also significantly correlated with lymph node metastasis in esophageal squamous cell carcinoma (15). Tumors expressing high levels of VEGF also led to significantly poorer survival in patients with ovarian cancer (17). On the other hand, there was a report that showed no association of VEGF-A and -C expression with survival in patients with gastric cancer (14). In agreement with most of the previous reports, the present data showed that increased expression of VEGF-A was significantly correlated with patient age and tumor stage in OSCC. Although VEGF-C expression was not significantly correlated with any of the clinicopathological factors, it was significantly correlated with the YK mode of invasion. These results suggest that VEGF-A may correlate with tumor growth, while VEGF-C correlates with invasion. As reported in studies examining the clinicopathological impacts of VEGF-A and -C in carcinomas of other tissues,

Table IV. *Correlation between VEGF expression and p-mTOR/HIF-1α/VEGF expressions and YK mode of invasion of OSCC.*

	VEGF-A		*P*-value	VEGF-C		*P*-value
	– (n=44)	+ (n=76)		– (n=39)	+ (n=81)	
YK Grade 1, 2, 3	35	50		33	52	
YK Grade 4C, 4D	9	26	0.08	6	29	<0.05
	I		*P*-value	II		*P*-value
	– (n=19)	+ (n=42)		– (n=16)	+ (n=44)	
YK Grade 1, 2, 3	17	26		14	27	
YK Grade 4C, 4D	2	16	<0.05	2	17	<0.05

I: –, Triple negative for p-mTOR/HIF-1α/VEGF-A; +, triple positive. II: –, Triple negative for p-mTOR/HIF-1α/VEGF-C; +, triple positive. YK mode of invasion: grade 1: well defined borderline; grade 2: cords, less marked borderline; grade 3: groups of cells, no distinct borderline; grade 4C: diffuse invasion of cord-like type; grade 4D: diffuse invasion of diffuse type.

VEGF-A and -C may be ubiquitously expressed in various malignant tumors, where they are involved in tumor growth and metastasis. In some studies, overexpression of VEGF-A and -C were reported to be correlated with a poorer prognosis. In our study, since there was no correlation between survival and the overexpression of VEGF-A and -C, their expression levels were not considered to be prognostic factors in patients with OSCC.

The mTOR pathway plays a central role in regulating protein synthesis, ribosomal protein translation, and cap-dependent translation. Deregulations of mTOR signaling is frequently associated with tumorigenesis, angiogenesis, tumor growth and metastasis (8, 9). In this study, we observed p-mTOR expression in 49 out of 120 SCCs (41%), whereas it was not observed in any of the normal epithelial tissue samples. In gastric cancer, p-mTOR overexpression was significantly correlated with lymph node metastasis, stage differentiation and PCNA expression (30). Cytoplasmic p-mTOR expression correlates with a poorer survival in patients with cervical adenocarcinoma (31). High expression of p-mTOR correlates with a poor outcome in glioblastoma (32). However, there was no correlation between p-mTOR and clinicopathological factors in lung cancer (33). The clinicopathological significance of p-mTOR expression is therefore controversial. In our study, no obvious correlation of p-mTOR expression with survival, tumor stage or lymph node metastasis was observed.

The HIF-1α transcriptional factor plays an essential role in oxygen homeostasis and high expression of HIF-1α protein has been found to be associated with both tumor aggressiveness and an unfavorable prognosis of various types of cancers (8). In the present study, we observed HIF-1α expression in 85 out of 120 SCCs (71%), whereas it was not observed in any of the normal epithelial samples. In renal cell carcinoma, ovarian cancer, head and neck cancer, and breast cancer, the overexpression of HIF-1α was shown to be a factor of worse prognostic and shorter patient survival (10, 21-23). A high HIF-1α expression is significantly associated with tumor stage and lymph node metastasis in colorectal cancer and esophageal squamous cell carcinoma (13, 24). In gastric cancer, overexpression of HIF-1α is significantly associated with tumor stage, TNM stage, and poor survival (25). There have not been any reports indicating that HIF-1α is not associated with any clinicopathological factors. In our study, we demonstrated that HIF-1α expression in OSCC reflected a large tumor size and the presence of regional lymph node metastasis.

Moreover, we examined the relationship between mTOR and HIF-α in our study and found that p-mTOR$^+$/HIF-1α$^+$ cases were significantly correlated with a higher tumor stage and more frequent regional lymph node metastasis. In addition, the PCNALI of cases with combined p-mTOR$^+$/HIF-1α$^+$ expression was significantly higher than that of p-mTOR$^-$/HIF-1α$^-$ tumors. These results strongly support the conclusion that the mTOR–HIF-1α pathway affects the progression of OSCC.

Finally, we examined the relationship between VEGF and the mTOR–HIF-1α pathway. In the present study, combined p-mTOR$^+$/HIF-1α$^+$/VEGF-A$^+$ expression was significantly associated with tumor stage, lymph node metastasis, and invasion grade. These results strongly support the conclusion that the mTOR–HIF-1α–VEGF pathway affects the progression of OSCC.

Recent studies have shown that temsirolimus and everolimus, which are novel mTOR inhibitors, have antioangiogenic potential, and these are in clinical development for the treatment of several tumors. However their effects against OSCC are still unclear (34-38).

Therefore, further investigations of this pathway should be performed both *in vivo* and *in vitro* to determine whether these agents might be useful in the clinic for treatment of patients with OSCC.

References

1 Mao L, Hong WK and Papadimitrakopoulou VA: Focus on head and neck cancer. Cancer Cell *5*: 311-316, 2004.

2 Peter C and Rakesh KJ: Angiogenesis in cancer and other diseases. Nature *407*: 249-257, 2000.

3 Zetter BR: Angiogenesis and tumor metastasis. Annu Rev Med *49*: 407-24, 1998.

4 Carmeliet P: Angiogenesis in health and disease. Nat Med *6*: 653-660, 2003.

5 Risau W: Mechanisums of angiogenesis. Nature *386*: 671-674, 1997.

6 Otrock ZK, Makarem JA and Shamseddine AI: Vascular endothelial growth factor family ligands and receptors: review. Blood Cells Mol Dis *38*: 258-268, 2007.

7 Ferrara N and Kerbel RS: Angiogenesis as a therapeutic target. Nature *438*: 967-974, 2005.

8 Pouysségur J, Dayan F and Mazure NM: Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature *441*: 437-443, 2006.

9 Shaw RJ and Cantley LC: Ras, PI(3)K and mTOR signaling controls tumour cell growth. Nature *441*: 424-430, 2006.

10 Dorević G, Matusan-Ilijas K, Babarović E, Hadzisejdić I, Grahovac M, Grahovac B and Jonjić N: Hypoxia-inducible factor-1alpha correlates with vascular endothelial growth factor A and C indicating worse prognosis in clear cell renal cell carcinoma. J Exp Clin Cancer Res *28*: 40, 2009.

11 Carrillo de Santa Pau E, Arias FC, Caso PE, Muñoz MGM, Sánchez HI, Muguruza TI, Moreno BR, Sacristán LS, Gómez PA and del Val Toledo LM: Prognostic significance of the expression of vascular endothelial growth factors A, B, C, and D and their receptors R1, R2, and R3 in patients with non-small cell lung cancer. Cancer *115*: 1701-1712, 2009.

12 Kyzas PA, Cunha IW and Ioannidis JP: Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis. Clin Cancer Res *11*: 1434-1440, 2005.

13 Cao D, Hou M, Guan YS, Jiang M, Yang Y and Gou HF: Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. BMC Cancer *9*: 432-440, 2009.

14 Lee SJ, Kim JG, Sohn SK, Chae YS, Moon JH, Kim SN, Bae HI, Chung HY and Yu W: No association of vascular endothelial growth factor-A (VEGF-A) and VEGF-C expression with survival in patients with gastric cancer. Cancer Res Treat *41*: 218-223. 2009.

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

16 Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL and Jackson DG: Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. Cancer Res *62*: 1315-1320, 2002.

17 Duncan TJ, Al-Attar A, Rolland P, Scott IV, Deen S, Liu DT, Spendlove I and Durrant LG: Vascular endothelial growth factor expression in ovarian cancer: a model for targeted use of novel therapies? Clin Cancer Res *14*: 3030-3035, 2008.

18 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med *350*: 2335-2342, 2004.

19 Abou-Alfa GK, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De Greve J, Douilard JY, Lathia C, Schwartz B, Taylor I, Moscovici M and Salth LB: Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. J Clin Oncol *24*: 4293-4300, 2006.

20 Goodman VL, Rock EP, Dagher R, Ramchandani RP, Abraham S, Gobburu JV, Booth BP, Verbois SL, Morse DE, Liang CY, Chidambaram N, Jiang JX, Tang S, Mahjoob K, Justice R and Pazdur R: Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. Clin Cancer Res *13*: 1367-1373, 2007.

21 Daponte A, Ioannou M, Mylonis I, Simos G, Minas M, Messinis IE and Koukoulis G: Prognostic significance of hypoxia-inducible factor 1alpha (HIF-1alpha) expression in serous ovarian cancer: an immunohistochemical study. BMC Cancer *8*: 335-344, 2008.

22 Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S and Harris AL: Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res *62*: 2493-2497, 2002.

23 Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, Jalesz R, Kubista E, Birner P and Oberhuber G: Austrian Breast and Colorectal Cancer Study Group: Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clin Cancer Res *8*: 1831-1837, 2002.

24 Kurokawa T, Miyamoto M, Kato K, Cho Y, Kawarada Y, Hida Y, Shinohara T, Itoh T, Okushiba S, Kondo S and Katoh H: Overexpression of hypoxia-inducible factor 1alpha (HIF-1alpha) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Br J Cancer *89*: 1042-1047, 2003.

25 Oh SY, Kwon HC, Kim SH, Jang JS, Kim MC, Kim KH, Han JY, Kim CO, Kim SJ, Jeong JS and Kim HJ: Clinicopathologic significance of HIF-1alpha, p53, and VEGF expression and preoperative serum VEGF level in gastric cancer. BMC Cancer *8*: 123-129, 2008.

26 Sobin LH and Wittekind CH: International Union Against Cancer: TNM Classification of Malignant Tumours, 5th edn., NewYork, Wiley, pp. 1-227, 1997.

27 Wahi PN: (WHO) Histological Typing of Oral and Oropharyngeal Tumours, International Histological Classification of Tumours, 4th edn. Geneva, WHO, pp. 1-28, 1971.

28 Yamamoto E, Kohama G, Sunakawa H, Iwai M and Hiratsuka H: Mode of invasion, bleomycin sensitivity, and clinical course in squamous cell carcinoma of the oral cavity. Cancer *51*: 2175-80, 1983.

29 Yanamoto S, Kawasaki G, Yoshitomi I, Iwamoto T, Hirata K and Mizuno A: 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*: 869-877, 2007.
30 Yu G, Wang J, Chen Y, Wang X, Pan J, Li G, Jia Z, Li Q, Yao JC and Xie K: Overexpression of phosphorylated mammalian target of rapamycin predicts lymph node metastasis and prognosis of Chinese patients with gastric cancer. Clin Cancer Res *15*: 1821-1829, 2009.
31 Dobashi Y, Suzuki S, Matsubara H, Kimura M, Endo S and Ooi A: Critical and diverse involvement of AKT/mammalian target of rapamycin signaling in human lung carcinomas. Cancer *115*: 107-118, 2009.
32 Faried LS, Faried A, Kanuma T, Aoki H, Sano T, Nakazato T, Tamura T, Kuwano H and Minegishi T: Expression of an activated mammalian target of rapamycin in adenocarcinoma of the cervix: A potential biomarker and molecular target therapy. Mol Carcinog *47*: 446-457, 2008.
33 Pelloski CE, Lin E, Zhang L, Yung WK, Colman H, Liu JL, Woo SY, Heimberger AB, Suki D, Prados M, Chang S, Barker FG 3rd, Fuller GN and Aldape KD: Prognostic associations of activated mitogen-activated protein kinase and Akt pathways in glioblastoma. Clin Cancer Res *12*: 3935-3941, 2006.
34 Miyazawa M, Yasuda M, Fujita M, Kajiwara H, Hirabayashi K, Takekoshi S, Hirasawa T, Murakami M, Ogane N, Kiguchi K, Ishiwata I, Mikami M and Osamura RY: Therapeutic strategy targeting the mTOR–HIF-1α–VEGF pathway in ovarian clear cell adenocarcinoma. Pathol Int *59*: 19-27, 2009.
35 Stefansson IM, Salvesen HB and Akslen LA: Vascular proliferation is important for clinical progress of endometrial cancer. Cancer Res *66*: 3303-3309, 2006.
36 Uchida S, Shimada Y, Watanabe G, Tanaka H, Shibagaki I, Miyahara T, Ishigami S and Imamura M: In oesophageal squamous cell carcinoma vascular endothelial growth factor is associated with *p53* mutation, advanced stage and poor prognosis. Br J Cancer *77*: 1704-1709, 1998.
37 Miyazawa M, Yasuda M, Fujita M, Hirabayashi K, Hirasawa T, Kajiwara H, Muramatsu T, Miyazaki S, Harasawa M, Matsui N, Ogane N, Murakami M, Mikami M, Yanase T and Osamura RY: Granulosa cell tumor with activated mTOR–HIF-1alpha–VEGF pathway. J Obstet Gynaecol Res *36*: 448-453, 2010.
38 Del Bufalo D, Ciuffreda L, Trisciuoglio D, Desideri M, Cognetti F, Zupi G and Milella M: Antiangiogenic potential of the mammalian target of rapamycin inhibitor temsirolimus. Cancer Res *66*: 5549-5554, 2006.

Received May 12, 2011
Revised October 31, 2011
Accepted November 2, 2011