Association of high cellular expression and plasma concentration of angiopoietin-like 4 with tongue cancer lung metastasis and poor prognosis

TAKUYA TANAKA[1,2], TAKAHISA IMAMURA[3,4], ATSUSHI IRIE[5], MASAKAZU YONEDA[6], RYUJI IMAMURA[7], KEN KIKUCHI[8], SAKI KITAGAWA[3], TATSUKO KUBO[3], HIDENAO OGI[9] and HIDEKI NAKAYAMA[1]

[1]Department of Oral and Maxillofacial Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Kumamoto 860-8556; [2]Department of Dentistry and Oral Surgery, Amakusa Central General Hospital, Amakusa, Kumamoto 863-0033; [3]Department of Molecular Pathology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Kumamoto 860-8556; [4]Department of Life Science, Shokei University, Kumamoto, Kumamoto 862-8678; [5]Department of Immunogenetics, Faculty of Life Sciences, Kumamoto University, Kumamoto, Kumamoto 860-8556; [6]Department of Oral Surgery, Oral and Maxillofacial Center, Kagoshima University Hospital, Kagoshima, Kagoshima 890-8520; [7]Department of Urology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Kumamoto 860-8556; [8]Sakurajyuji Hospital, Kumamoto, Kumamoto 861-4173; [9]Department of Oral Surgery, Minamata Medical Center, Minamata, Kumamoto 867-0041, Japan

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Abstract. Angiopoietin-like 4 (ANGPTL4) promotes cancer cell migration through vessels and has been implicated in cancer metastasis. Our previous study identified a robust increase in *ANGPTL4* mRNA expression in lung-metastasized tongue cancer (TC) cells. Therefore, the present study investigated the association of ANGPTL4 with lung metastasis and outcomes of patient with TC. ANGPTL4 expression in TC cells was investigated by immunohistochemical staining. Patients were classified into 'low (0-30%)' and 'high (>30%)' ANGPTL4-expression groups based on the proportion of ANGPTL4-positive TC cells. The high ANGPTL4-expression group included 15 of 48 patients with TC. Notably, a significantly greater proportion of patients with lung metastasis exhibited a high rate of ANGPTL4-expressing cancer cells compared with patients without lung metastasis (P=0.029). The overall 5-year survival rate was lower in the high (27%) ANGPTL4-expression group compared with the low (68%) ANGPTL4-expression group. Univariate and multivariate analyses revealed that patients with high ANGPTL4 expression in TC cells exhibited significantly lower overall survival (OS) rates [hazard ratio (HR), 2.99; 95% confidence interval (95% CI), 1.34-6.69; P=0.008 and HR, 2.72; 95% CI, 1.14-6.51; P=0.024, respectively]. High plasma ANGPTL4 concentrations as measured by ELISA were associated with lung metastasis (P<0.001). The optimal cut-point for prediction of TC lung metastasis was 9.1 ng/ml (P<0.001; 95% CI, 7.2-10.9). The OS of patients with plasma ANPTL4 above the cut-point was significantly lower than that of patients with plasma ANGPTL4 ≤9.1 ng/ml (P<0.001). These results suggest that a high level of ANGPTL4 in cancer cells and plasma may predict lung metastasis and/or a poor prognosis of patients with TC.

Correspondence to: Dr Takahisa Imamura, Department of Life Science, Shokei University, 2-7-78 Kuhonji, Chuo-ku, Kumamoto, Kumamoto 862-8678, Japan
E-mail: taka@shokei-gakuen.ac.jp

Abbreviations: ANGPTL4, angiopoietin-like 4; SCC, squamous cell carcinoma; TC, tongue cancer

Key words: ANGPTL4, lung metastasis, plasma, survival, prognosis, tongue cancer

Introduction

The American Cancer Society estimated that 17,960 and 2,870 patients in the United States would be newly diagnosed with, and die as a result of tongue cancer (TC), respectively, in 2021 (1). While advances in surgical and radiation therapies have increased the average 5-year survival rate for patients with oropharyngeal cancer to 66%, this is still markedly lower than the >90% 5-year survival rate of patients with other cancer types, such as prostate and breast cancer (1). Most often, patient death is caused by regional and/or distant metastasis; thus, metastasis is indicative of a poor prognosis (2-5). Squamous cell carcinoma (SCC) accounts for approximately 90% of oral and oropharyngeal malignancies in the United States (6), and commonly develops in the tongue (7). Notably, the average rate of nodal metastasis has been reported to be approximately 30% among patients with TC at initial evaluation, which is markedly higher than that of patients with other oral cavity

cancers (8,9). Moreover, several studies have identified a high rate of occult nodal metastasis (20-40%) in patients with TC who showed no evidence of regional spread during clinical or radiographic evaluation (8,10-15). Combined with the fact that the rate of TC has increased among young women over the last 20-30 years (16-18), these data highlight the urgent need for a novel approach to predict metastasis and start treatments at the early stage in patients with TC.

Angiopoietin-like 4 (ANGPTL4) belongs to a family of proteins that are structurally similar to the angiopoietins but do not bind to the angiopoietin receptors, tyrosine kinase with immunoglobulin-like and EGF-like domain 1 (TIE 1) and endothelial-specific receptor tyrosine kinase (TEK or TIE 2) (19). ANGPTL4 is a critical mediator of transmigration (20), and promotes trans-endothelial migration by up-regulating the expression of vascular endothelial adhesion molecule-1 (VCAM-1) in endothelial cells (21). Increased VCAM-1 expression, in turn, promotes the attachment of circulating cancer cells to the vessel walls, and facilitates extravasation and tumor establishment in other tissues. Clinically, ANGPTL4 expression is correlated with venous and lymphatic invasion in human SCC (22), and increased ANGPTL4 gene expression has been reported to promote lung metastasis in breast cancer (23). Recently, we identified a robust increase in *ANGPTL4* mRNA expression in lung-metastasized TC cells (24) that we induced to become highly metastatic to lymph nodes by repeating the passage in which the cells were injected into a nude mouse tongue and harvested from metastasized cervical lymph nodes (25). Together, these data suggest that ANGPTL4 is associated with TC lung metastasis.

To determine whether ANGPTL4 levels are predictive of TC lung metastasis, we investigated the clinical association of ANGPTL4 with TC lung metastasis and prognosis of the patients.

Materials and methods

Tissue samples, immunohistochemistry, and retrospective patient analysis. TC tissue samples were obtained via surgical resection from 48 Japanese patients (male 27, female 21, ranging 23~91 years old) with TC who complained of mostly uncurable tongue aphtha or ulcer and were admitted to the Kumamoto University Hospital between 2003 and 2015. Tissue samples were used with the approval of the internal ethics committee and all patients provided written informed consent. ICD-10 codes of TC comprise 2 C020, 28 C021, 7 C022 and 1 C028. In total, 23 of the patients with TC subsequently had lung metastasis, of which 13 also had lymph node metastasis, as diagnosed via computed tomography (CT) scans and pathological tissue examination of resected lymph nodes, respectively.

Deparaffinized 3-μm-thick tissue sections were pretreated (20 min) with 0.3% H_2O_2 in methanol before treatment (20 min) with Serum-Free Protein Block (Dako Cytomation). Sections were then incubated (4˚C overnight) with a rabbit polyclonal antibody to ANGPTL4 (20 μg/m1; ab196746, Abcam) and stained at room temperature for 20 min using EnVision+ solution (Dako Cytomation) and 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.006% H_2O_2, according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. After counting TC cells in five random high-power fields (BX40, Olympus) in each tissue section, the percentage of TC cells expressing AGPTL4 was determined. Patients were resultantly classified into 'low' (0-30% positivity) and 'high' (>30% positivity) ANGPTL4 expression groups according to previous methods (26-28). Patients' clinical parameters were compared between the two groups.

Plasma ANGPTL4 assay. Blood samples were collected from 40 patients with TC who were admitted to the Kumamoto University Hospital between 2003 and 2017. Of these, 20 patients subsequently had lung metastasis later, and 13 also had lymph node metastasis. One patient developed lymph node, but not lung, metastasis. Heparinized plasma samples were obtained by centrifugation, and plasma ANGPTL4 levels were measured using a human ARP4 ELISA kit (ab99974, Abcam) according to the manufacturer's instructions. Briefly, ANGPTL4 standards and plasma samples were pipetted into each well of a 96-well plate precoated with a human ANGPTL4-specific antibody. After ANGPTL4 capture, the plate was washed, and biotinylated anti-human ANGPTL4 antibody was added to each well. The plate was again washed to remove unbound biotinylated antibody before HRP-conjugated streptavidin was added to each well. After further washing, a 3,3,5,5-tetramethylbenzidine (TMB) substrate solution was added to each well to initiate a color reaction that was proportional to the original amount of bound ANGPTL4. Stop Solution was used to change the resultant color from blue to yellow, and the intensity of the converted color was measured at 450 nm with a microplate reader (Model 550; Bio-Rad Laboratories).

Statistical analysis. Fisher's exact test was used to analyze potential associations between ANGPTL4 expression levels and all patients' clinicopathological parameters except age, which was instead analyzed via unpaired Student's t-test. Overall patient survival rates were evaluated using the Kaplan-Meier method and verified using the log-rank test. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for overall 5-year survival rate of patients in univariate and multivariate analyses. Plasma ANGPTL4 concentration values were analyzed using the unpaired Student's t-test. Values were expressed as the average ± standard deviation (SD) (n=20). An optimal cut-point of the plasma ANGPTL4 concentration for screening lung metastasis of TC was identified by bootstrapped ROC analysis under Liu's method using 1,000 bootstrap samples. The 95% confidence interval of the optimal cut-point was determined by normal distribution, under the ROC curve by binomial distribution, and sensitivity and specificity by binomial distribution. All statistical analyses were performed using the Stata Statistical Software: Release 17 for Windows (StataCorp LLC). A P-value <0.05 was considered to indicate statistically significance.

Results

ANGPTL4 expression in TC cells. To determine whether ANGPTL4 is involved in TC progression, tongue tissues were examined for ANGPTL4 expression in TC cells. Only a subset of TC cells expressed ANGPTL4. Of 48 patients analyzed,

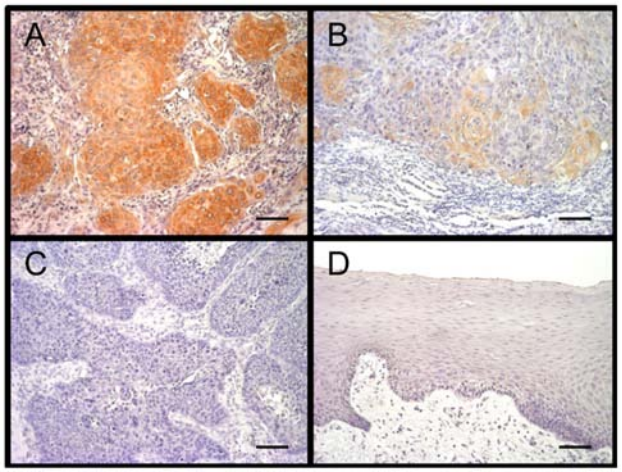


Figure 1. Immunohistochemical staining of ANGPTL4. Immunohistochemical staining for cancer tissues of patients with TC was performed using an anti-ANGPTL4 antibody. Representative images showing TC cells that were found to exhibit (A) a high (positive cell rate >30%) or a low [positive cell rate (B) 1-30% or (C) ≈0%) rate of ANGPTL4 expression. (D) Noncancerous tongue epithelial cells. Scale bars, 100 μm. ANGPTL4, angiopietin-like 4; TC, tongue cancer.

33 (69%) and 15 (31%) were then classified into 'low' and 'high' ANGPTL4 expression groups, respectively (Fig. 1A-C), according to the percentage of ANGPTL4-expressing TC cells. ANGPTL4 was not expressed in noncancerous tongue epithelial cells (Fig. 1D).

Association of TC ANGPTL4 expression with lung metastasis and poor prognosis. To evaluate the impact of ANGPTL4 on TC lung metastasis, TC cells from patients with or without subsequent metastasis were examined for ANGPTL4 expression. No significant differences in patient age, sex, tumor histological grade, vascular invasion, or lymph node metastasis were observed between the high and low ANGPTL4 expression groups (Table I). In addition to patients at advanced pathological stage (P=0.031) and clinical stage (P=0.043), a significant greater proportion of patients with lung metastasis exhibited a high percentage of ANGPTL4 expressing cancer cells as compared to those without lung metastasis (P=0.029) (Table I). These findings suggested an association between high level of TC ANGPTL4 expression and lung metastasis.

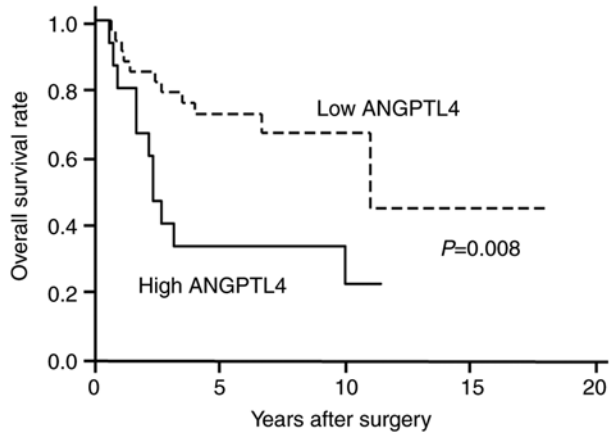
Furthermore, the overall survival (OS) rate of patients with TC high rate of ANGPTL4 expression was significantly lower than that of patients with low ANGPTL4 expression (Fig. 2). The overall 5-year survival rate was more than twofold higher in patients in the low (68%) as compared to the high (27%) ANGPTL4 expression group (Table II). The median survival period of the patients in the two groups was 132 and 28 months, respectively. Univariate and multivariate analyses revealed that the OS rate of patients with high ANGPTL4-expressing TC was significantly lower than that of the patients with low ANGPTL4-expressing TC [hazard ratio (HR), 2.99; 95% confidence interval (CI), 1.34-6.69; P=0.08 and HR, 2.72; 95% CI, 1.14-6.51; P=0.024, respectively]. However, no significant difference in OS rate was identified in pathological and clinical stages in multivariate analysis. These results indicated that high expression of ANGPTL4 in TC cells is an independent predictor for poor prognosis and may suggest that ANGPTL4 promotes lung metastasis and poor patient outcomes in TC.

Table I. Association between cancer-cell ANGPTL4 expression and patient clinicopathological parameters in tongue cancer.

	ANGPTL4 expression		
Parameter	Low	High	P-value
Patients, n	33	15	
Average age ± SD, years	61.0±15.4	61.8±14.3	0.860[a]
Sex, n			
Male	16	11	0.129[b]
Female	17	4	
Histological grade[c], n			
Well	30	13	0.642[b]
Moderate	2	2	
Poor	1	0	
Pathological stage[d], n			
T1	21	2	0.031[b]
T2	7	6	
T3	4	5	
T4	1	2	
Clinical stage[e], n			
I	21	2	0.043[b]
II	5	5	
III	6	4	
IV	1	4	
Vascular invasion, n			
(+)	4	1	0.497[b]
(-)	29	14	
Lymph node metastasis, n			
(+)	6	7	0.077[b]
(-)	27	8	
Lung metastasis,n			
(+)	12	11	0.029[b]
(-)	21	4	

P-values were calculated using [a]an unpaired Student's t-test or [b]Fisher's exact test. [c]Well vs. moderate/poor; [d]T1/T2 vs. T3/T4; [e]I/II vs. III/IV. ANGPTL4, angiopietin-like 4.

Increase of plasma ANGPTL4 concentrations in TC patients with lung metastasis and poor prognosis. To further explore the relationship between ANGPTL4 and TC lung metastasis,



Number at risk	0	5	10	15	20
Low ANGPTL4	33	19	8	1	0
High ANGPTL4	15	4	3	0	0

Figure 2. Association of overall patient survival rate with ANGPTL4 expression in TC. The overall survival rate was evaluated for groups of patients with TC classified by high (n=15; solid line) or low (n=33; dashed line) rates of ANGPTL4 expression in their collected TC tissue samples. Statistical analysis was performed using the Kaplan-Meier method and verified using the log-rank test. The table of numbers at risk at the bottom of the figure indicates the number of patients living just before the time point. ANGPTL4, angiopietin-like 4; TC, tongue cancer.

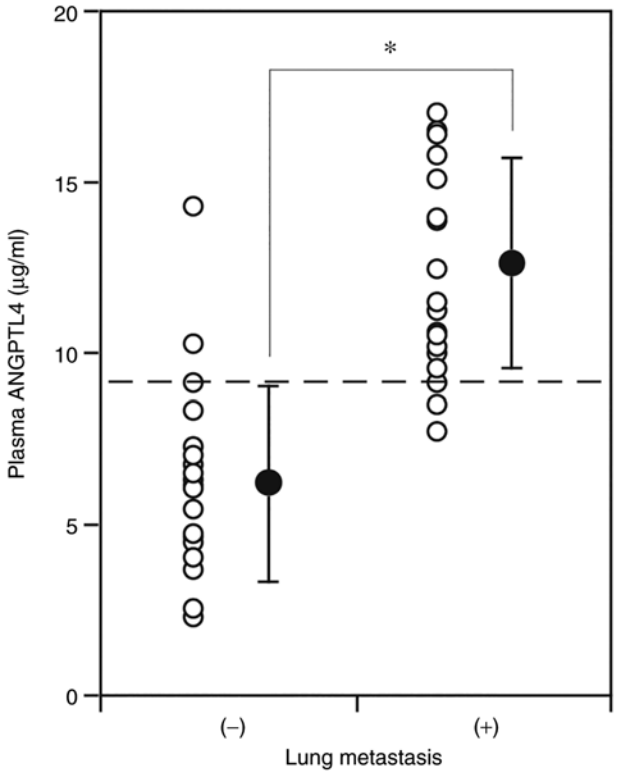


Figure 3. ANGPTL4 plasma concentrations in patients with TC with or without lung metastasis. ANGPTL4 plasma concentrations were compared between patients with TC with and without lung metastasis. Open circles indicate individual ANGPTL4 plasma concentrations of patients with TC (n=20 each). Closed circles and bars denote averages and standard deviation values, respectively. Statistical analysis was performed using an unpaired Student's t-test. *P<0.001. The dashed line indicates the optimal cut-point of the plasma ANGPTL4 concentration for prediction of TC lung metastasis. The optimal cut-point was determined by bootstrapped receiver operating characteristic analysis under Liu's method using 1,000 bootstrap samples. ANGPTL4, angiopietin-like 4; TC, tongue cancer.

ANGPTL4 concentrations in plasma obtained on the first day of admission was measured. The plasma ANGPTL4 concentrations of the patients who subsequently developed lung metastasis later (12.6±3.1 ng/ml) were significantly higher than those of the patients without lung metastasis (6.2±2.8 ng/ml) (P<0.001) (Fig. 3). This result supports the likely association of high ANGPTL4 concentrations with lung metastasis in TC. ANGPTL4 levels in plasma/serum of controls (individuals without cancer or other disease) varied in reports. ANGPTL4 concentrations in the present study were comparable to those reported by Smart-Halajko *et al* (29); the median concentration was 7.7 (interquartile range, 5.9 to 11.0) ng/ml.

An optimal cut-point of plasma ANGPTL4 concentration for prediction of TC lung metastasis was determined to be 9.1 ng/ml (P<0.001; 95% CI: 7.2-10.9) with a sensitivity of 90.0% and specificity of 90.0% (Fig. 3; Figs. S1 and S2; Tables SI-SV). The OS rate of patients with plasma ANGPTL4 concentrations above the cut-point was significantly lower than that of patients with plasma ANGPTL4 less than or equal to the cut-point (Fig. 4). Twenty-eight of the TC patients were examined both cellular expression and plasma concentration of ANGPTL4. These were no significant difference in plasma ANGPTL4 concentrations of patients whose TC cells had high (10.51±4.70 ng/ml; n=9) or low (9.36±4.75 ng/ml; n=19) ANGPTL4 expression (P=0.55).

Discussion

ANGPTL4 has been reported to be involved in various processes required for cancer progression and metastasis. For example, ANGPTL4 mediates the induction of neovascularization (30) and increases cancer cell proliferation and tumor growth (31,32) through enabling cancer cells to evade apoptosis and acquire anoikis resistance (33). Moreover, ANGPTL4 has been reported to enhance vascular invasion (22,34,35). In fact, cancer cell ANGPTL4 expression has been reported to correlate with lymph node metastasis in esophageal (22,36), gastric (34), and oral squamous cell cancers (37). Consistent with these findings, we herein demonstrated for the first time that both a high rate of ANGPTL4 expression in TC cells and high plasma ANGPTL4 concentration of TC patients are associated with lung metastasis (Table I and Fig. 3).

Only cancer cells in the collected TC tissues expressed ANGPTL4 (Fig. 1); accordingly, *ANGPTL4* mRNA expression in TC tissues is derived from TC cells. The low survival rate of TC patients with high cellular ANGPTL4 protein expression (Fig. 2) agrees with a previous report of poor prognosis in patients with high ANGPTL4 mRNA expression in TC tissues (38). Given that ANGPTL4 has been shown to possess multiple cancer promoting effects, the high ANGPTL4 expression rates reported herein in TC, and the high *ANGPTL4* mRNA expression levels previously identified in lung-metastasized breast cancer cells (23) and TC cells (24), strongly suggest that AMPTL4 promotes the metastasis of cancer cells, which is supported by finding that the OS rate of patients with high TC cell expression of

Table II. Univariate and multivariate analysis of overall survival in 48 patients with tongue cancer.

Parameter	5-year survival rate	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Pathological stage (T1-T2/T3-T4)	0.71/0.08	4.45	1.98-9.98	<0.001	0.27	0.07-1.12	0.071
Clinical stage (I-II/III-IV)	0.73/0.08	5.87	2.60-13.3	<0.001	1.05	0.23-4.86	0.954
Lymph node metastasis (-/+)	0.78/0.07	6.67	2.89-15.4	<0.001	18.9	2.17-163	0.008
ANGPTL4 expression (low/high)	0.68/0.27	2.99	1.34-6.69	0.008	2.72	1.14-6.51	0.024

Kaplan-Meier analysis method and Cox-proportional hazards regression were used to calculate the 5-year survival rate and the HR and 95% CI, respectively. T1-T2/T3-T4, T1+T2 vs. T3+T4; I-II/III-IV, I+II vs. III+IV; -/+, negative vs. positive; low/high, low expression vs. high expression; HR, hazard ratio; 95% CI, 95% confidence interval; ANGPTL4, angiopoietin-like 4.

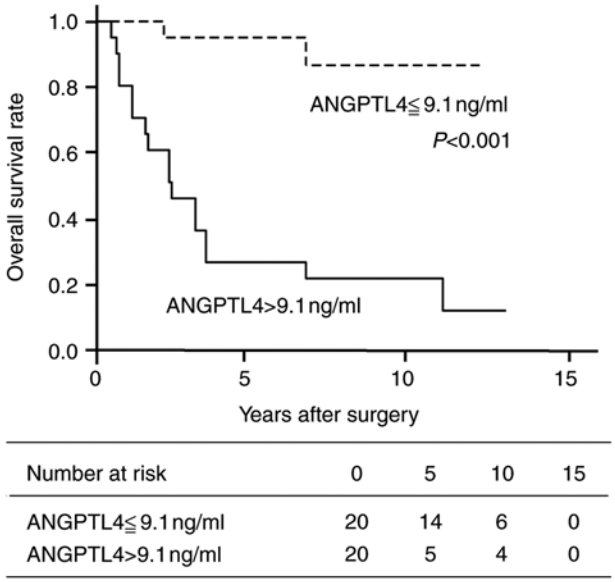


Figure 4. Correlation of overall patient survival rate with plasma ANGPTL4 concentration in TC. The overall survival rate was evaluated for groups of patients with TC classified by ANGPTL4 concentration >9.1 ng/ml (n=20; solid line) or ≤9.1 ng/ml (n=20; dashed line). Statistical analysis was performed using the Kaplan-Meier method and verified using the log-rank test. The table of numbers at risk at the bottom of the figure indicates the number of patients living just before the time point. ANGPTL4, angiopietin-like 4; TC, tongue cancer.

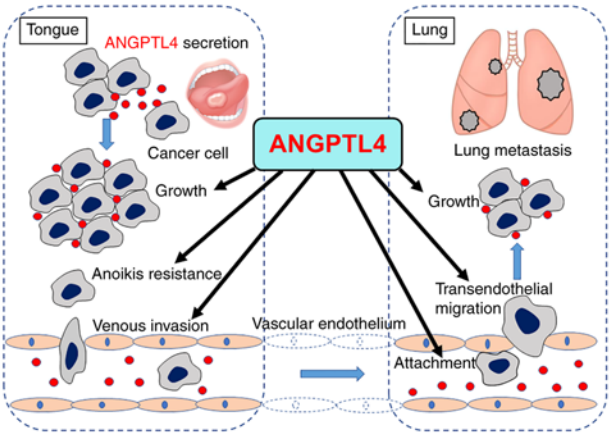


Figure 5. Promotive effects of ANGPTL4 on various stages of tongue cancer cell metastasis from the tongue to lungs. Black arrows indicate the stages of metastasis promoted by ANGPTL4. ANGPTL4, angiopoietin-like 4.

ANGPTL4 was significantly lower than that of patients with low TC cell expression in multivariate analysis (Table II). Thus, high ANGPTL4 expression is likely an indicative marker for lung metastasis and poor prognosis in TC. Furthermore, OS rate of patients with plasma ANGPTL4 concentrations above the cut-point 9.1 ng/ml was significantly lower than that of patients with plasma ANGPTL4 concentrations at or below 9.1 ng/ml (Fig. 4). Using the tentative cut-point identified in the present study, TC patients with a plasma ANGPTL4 concentration above 9.1 ng/ml may be treated at an earlier stage, thereby enhancing survival with a lessened risk of lung metastasis.

Previous studies have shown that serum ANGPTL4 concentrations are approximately threefold higher in patients with esophageal cancer than in those with benign esophageal diseases. Furthermore, serum ANGPTL4 levels in esophageal cancer patients are ameliorated after surgical resection of the cancer tissues (36). Similarly, serum ANGPTL4 concentrations in patients with renal cell cancer have been reported to be twofold higher than in healthy controls and are associated with advanced clinical disease stages and metastasis (39). It is likely that in both cases, increased serum concentrations of ANGPTL4 enhanced cancer cell proliferation and tumor growth (31). Thus, ANGPTL4 concentrations may be indicative of disease progression in other types of cancers besides TC lung metastasis. This may also be supported by the fact that plasma ANGPTL4 levels were higher in cachectic cancer patients than in weight-stable cancer patients (40).

Hypoxia-inducible factor-1α (HIF-1α) induces ANGPTL4 expression in hepatocellular carcinoma (21) and HIF-1α expression is positively correlated with advanced clinical

stages and metastasis in TC (41). Thus, it is presumed that HIF-1α-driven upregulation of TC cell ANGPTL4 secretion in combination with an increase of ANGPTL4-secreting TC cells synergistically elevated plasma ANGPTL4 concentrations in TC patients with lung metastasis (Fig. 3). ANGPTL4 promotes cancer cell growth (31,32) but there is a delay from an elevation of TC cell ANGPTL4 secretion to an increase in TC cells, and even a low level of ANGPTL4 secretion can lead to a significant increase in TC cells after a relatively long time. This may explain why there is no correlation of plasma ANGPTL4 concentrations with TC cell ANGPTL4 expression levels. Thus, assessing ANGPTL4 levels in both TC cells and in plasma may increases confidence in predicting lung metastasis and poor outcomes of patients with TC.

The present study demonstrated that lung metastasis and low OS rate in TC are associated with high rates of ANGPTL4 expression in TC cells (Table I and Fig. 2). This finding suggests that ANGPTL4 secretion promotes lung metastasis and mortality in TC patients, leading to increased plasma ANGPTL4 concentrations (Fig. 3), which induce an increase in ANGPTL4-driven cancer promoting effects (20-22,31-35). A schematic illustrating the possible promoting effects of ANGPTL4 on TC lung metastasis are presented in Fig. 5. ANGPTL4 secreted from TC cells promotes TC growth and anoikis resistance in the tongue facilitating TC cell migration to blood vessels and venous invasion into the circulation, followed by attachment to endothelial cells in the lung vessels, transendothelial migration and cancer nest growth in the lung. Future mechanistic studies to delineate the role of ANGPTL4 in TC *in vitro* using TC cells and *in vivo* using a mouse model would elucidate the association between ANGPTL4 and lung metastasis. Interestingly, a higher histological grade is a well-established predictor of low overall survival rates in TC (42); however, while the OS rate of the high ANGPTL4 expression group was much lower than that of the low ANGPTL4 expression group (Fig. 2), the histological grade exhibited by patients in the two groups were not different (Table I). Thus, high rates of ANGPTL4-expressing cancer cells and high plasma ANGPTL4 concentrations may be reliable predictive factors for lung metastasis and poor patient prognosis in TC. ANGPTL4-driven cancer promoting activities suggest a therapeutic effect of lowering AGPTL4; therefore, AGPTL4 is a potential therapeutic target for TC.

There are some limitations on the present study. Data from patients who had heterogeneous therapies were analyzed, which may affect prognostic evaluation for AGPTL4. Because the survey consisted of a homogenous ethnic group and in a relatively small patient number, the generalizability of the present results is potentially limited.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TT and TI made substantial contributions to the conception, design and intellectual content of the present study. RI and TK performed immunohistochemistry. TI, MY and HO interpreted immunohistochemical staining results for patient classification. TT, MY, HO and HN collected tongue cancer tissue samples and patients' plasmas, and analyzed patients' clinicopathological data. TT, AI and SK contributed to the ELISA of ANGPTL4 in plasma. TT and KK performed statistical analysis of data. TT prepared the manuscript and TI and HN revised it critically for important intellectual content. MY, HO and HN confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent for tissue usage was obtained from the patients, and the use of these tissues was approved by The Internal Review Board of Kumamoto University Hospital (Rinri no. 1427; Kumamoto, Japan).

Patient consent for publication

Written informed consent for publication was obtained from the patients.

Competing interests

The authors declare that they have no competing interests.

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