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ORIGINAL ARTICLE

Upregulation of vascular endothelial growth factor mRNA level is significantly related to progression and prognosis of oral squamous cell carcinomas

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Background/purpose: Vascular endothelial growth factor (VEGF) is a potent angiogenic factor. This study evaluated whether the VEGF mRNA level in oral squamous cell carcinoma (OSCC) tissue could be a biomarker to predict the progression and prognosis of OSCCs in Taiwan.
Methods: This study used quantitative real-time reverse transcription-polymerase chain reaction (quantitative RT-PCR) to detect the VEGF mRNA levels in 60 OSCC specimens. Threshold cycle (C_T) was defined as the PCR cycle number needed to generate a predetermined amount of DNA (threshold). The relative amount of tissue VEGF mRNA, standardized against the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, was expressed as ΔC_T = (VEGF C_T − GAPDH C_T). For a chosen threshold, a smaller starting copy number of mRNA results in a higher C_T value. Thus, the lower the ΔC_T, the greater the copy number of VEGF mRNA in tissues.
Results: The lower mean VEGF mRNA ΔC_T value was significantly associated with OSCCs with larger tumor size ($p = 0.040$), positive lymph node metastasis ($p = 0.023$), and more advanced clinical stages ($p = 0.008$). VEGF mRNA ΔC_T value < 4.2 ($p = 0.026$) was identified as an independent unfavorable prognosis factor using multivariate regression analyses. Moreover, Kaplan–Meier curve showed that OSCC patients with a VEGF mRNA ΔC_T value < 4.2 had a significantly poorer overall survival than those with a VEGF mRNA ΔC_T value ≥ 4.2 (log-rank test, $p = 0.0427$).

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Conclusion: The OSCC tissue VEGF mRNA level can be used to predict the progression and prognosis of OSCCs in Taiwan.

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent malignancy of the oral cavity and late-stage OSCCs with neck metastasis often associated with poor prognosis of patients.[1] In Taiwan, the mortality rate of head and neck cancers is in fifth place in the overall population and the fourth place in male populations in 2011.[2]

OSCC is an aggressive epithelial neoplasm. Despite the advances in early detection and treatment of OSCC in recent years,[3] the overall survival rate of OSCC is still not promising, probably due to the lack of a good marker for early diagnosis and prediction of the progression and prognosis of OSCC. Vascular endothelial growth factor (VEGF) can promote angiogenesis which is essential for cancer growth and metastasis.[4–6] Therefore, VEGF protein or mRNA may be a good marker for prediction of cancer progression and prognosis.

VEGF can increase vascular permeability, promote endothelial cell proliferation and migration, and inhibit endothelial cell apoptosis.[5,6] In humans, the gene encoding VEGF is located on the short arm of chromosome 6 (6p21.3).[7] VEGF is a mitogen for vascular endothelial cells and helps the migration and organization of vascular endothelial cells for neovascularization and tumor micrometastasis.[8,9] Overexpression of VEGF mRNA or protein has been associated with aggressive progression and poor prognosis in several human cancers, including colorectal,[10–12] gastric,[13–15] pancreatic,[16,17] and breast carcinomas[18–20] as well as melanoma.[21]

VEGF is also found to be an important angiogenic cytokine for neovascularization in head and neck cancers.[22–29] Overexpression of VEGF mRNA or protein has been reported to be significantly related to poor prognosis and shorter survival[22–27] as well as positive lymph node metastasis[23,28,29] in head and neck cancer patients. In this study, we evaluated whether the VEGF mRNA level of OSCC surgical specimens could be a crucial biomarker to predict the progression, recurrence, and prognosis of OSCCs.

Methods and materials

Patients and oral cancer specimens

Sixty OSCC patients (55 men and 5 women; mean age 56 years; range, 36–81 years) were included in this study. This study has been reviewed and approved by the Institutional Review Board of the National Taiwan University Hospital, Taipei, Taiwan. For each patient and each normal control patient, an informed consent was obtained before collection of surgical samples. Surgical samples of OSCC were collected from 60 OSCC patients. Moreover, 38 biopsy specimens of normal oral mucosa (NOM) were obtained from 38 controls (30 men and 8 women; mean age 33 years; range, 17–55 years) with no oral habits or any oral mucosal diseases during extraction of an impacted permanent lower third molar and these were used as the normal controls. All sample tissues were freshly embedded in optimum cutting temperature compound (Fisher Scientific, Hanover Park, IL, USA), snap frozen, and kept at −80°C until use. VEGF mRNA levels in both OSCC and NOM tissues were measured by quantitative real-time reverse transcription-polymerase chain reaction (quantitative RT-PCR). OSCC was diagnosed with histological examination of hematoxylin and eosin-stained tissue sections.

All OSCC patients underwent total surgical excision of their tumors plus either selective or radical neck dissection based on clinically nodal metastasis at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital during the period from January 2002 to December 2009. Follow-up duration was defined as the period between the operation date and day of the last visit, according to the patient's chart. If involved surgical margin, perineural invasion, or lymphovascular permeation of OSCC, or extracapsular spread of metastatic cervical lymph node were detected histologically, concurrent postoperative chemoradiation therapies were also included in the treatment protocol. In this study, 17 patients underwent postoperative chemoradiation therapies due to the presence of aforementioned risk factors of recurrences. However, none of our patients had received any form of tumor-specific therapy or induction chemotherapy before total surgical excision of the lesion. Moreover, there were 33 (55.0%) patients with local recurrences or regional neck metastases and four (6.7%) patients with distant lung metastases. Salvage treatments for patients with local regional recurrences were excision of recurrent local cancers and neck dissection and those for patients with distant lung metastases were palliative chemotherapy. Of the 60 OSCC cases, 22 were buccal mucosa cancers, 17 tongue cancers, and 21 gingiva cancers, palate cancers, or floor of the mouth cancers. Histological features of OSCC were further classified into three different types (well-, moderately-, and poorly-differentiated OSCC). Of the 60 OSCC cases, there were 51 well-differentiated and nine moderately-differentiated OSCCs. Clinical staging and TNM status of OSCCs at initial presentation of the tumor were determined using clinical palpation, head-and-neck magnetic resonance imaging, chest X-ray, abdominal sonography, and whole body bone scan according to the guidelines from the American Joint Committee on Cancer (6th edition).[30]

Patients' oral habits

Details of patients' oral habits, including daily/weekly consumption of areca quid, cigarette, and alcohol, as well as the duration of these habits were recorded. OSCC patients were defined as areca quid chewers when they chewed two or more areca quids daily for at least 1 year, as cigarette smokers when they smoked every day for at least 1 year and consumed >50 packs of cigarettes per year, and as alcohol drinkers when they drank >4 days and consumed more than 20 g of pure alcohol per week for at least 1 year. According to these definitions, 49 (82%) patients were areca quid chewers, 48 (80%) patients were smokers, and 36 (60%) patients were drinkers. Furthermore, all of our OSCC patients stopped chewing areca quids after surgery, some of them stopped smoking completely after surgery, and some of them continued to smoke with a reduced number of cigarettes (<5 cigarettes per day) after surgery according to the inquisition from the patients and their family members.

Quantitative real-time RT-PCR

Total cellular RNA was isolated using an RNA extraction kit (Qiagen Inc., Alameda, CA, USA) from tissue homogenized with Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA) as recommended by the manufacturers. The mRNA expression levels after each treatment were determined by quantitative RT-PCR using the TaqMan Gene Expression Assays (Applied Biosystem, Foster City, CA, USA) for VEGF-A (ID: Hs00900055_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: Hs99999905_m1) on an ABI Prism real-time PCR system (Applied Biosystem) as previously described.[10] Threshold cycle (C_T) is the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed level above baseline. For a chosen threshold, a smaller starting copy number results in a higher C_T value. In this study, we chose GAPDH mRNA as an internal control. The relative amount of tissue VEGF mRNA, standardized against the amount of GAPDH mRNA, was expressed as ΔC_T = (VEGF C_T − GAPDH C_T).

Statistical analysis

The mean VEGF mRNA levels between OSCC and NOM samples and the correlation between the VEGF mRNA levels in OSCC samples and clinicopathological parameters of OSCC patients were analyzed using Student *t* test. Cumulative survival was analyzed with the Kaplan–Meier product-limit method. The duration of overall survival was measured from the beginning of surgery to the time of death (complete) or the last follow-up (censored). Comparison of cumulative survival between groups was performed using the log-rank test with the Statistica program 7.0 (StatSoft Inc., Tulsa, OK, USA). Univariate and multivariate survival analyses were performed with the Cox proportional hazard regression model to assess additional prognostic values of the different variables using SPSS version 22.0. (IBM SPSS, Armonk, NY: IBM Corp.). A *p* value <0.05 was considered statistically significant.

Results

VEGF mRNA levels in oral cancer tissues

The mean VEGF mRNA levels in 60 OSCC samples and 38 NOM samples are presented in Table 1. The lower ΔC_T value is interpreted as having higher initial copy numbers of VEGF mRNA in tissues. We found that the mean VEGF mRNA ΔC_T value was significantly lower in 60 OSCC samples (4.2 ± 2.4) than in 38 NOM samples (6.2 ± 2.1, $p < 0.0001$, Table 1). Furthermore, the mean VEGF mRNA expression level of the OSCC group was 4-fold higher than that of the NOM group ($p < 0.0001$, Figure 1).

Correlation between the VEGF mRNA levels in OSCC samples and clinicopathological parameters of OSCC patients

In this study, the lower the ΔC_T, the greater the copy number of VEGF mRNA in tissues. Correlation between the mean VEGF mRNA ΔC_T value in 60 OSCC samples and

Table 1 The mean vascular endothelial growth factor (VEGF) mRNA ΔC_T value in 60 oral squamous cell carcinoma (OSCC) samples and 38 normal oral mucosa (NOM) samples.

Groups	Mean VEGF mRNA ΔC_T value ± SD	*p*
NOM ($n = 38$)	6.2 ± 2.1	< 0.0001*
OSCC ($n = 60$)	4.2 ± 2.4	

* A significant difference in the mean VEGF mRNA ΔC_T value was found between NOM and OSCC samples using Student *t* test with $p < 0.0001$.

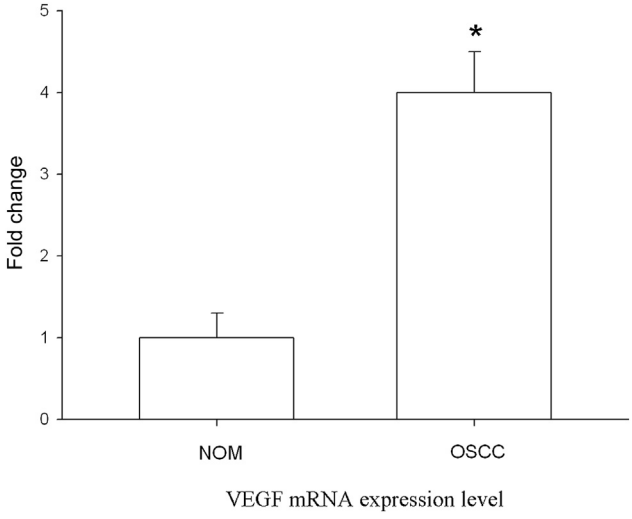

Figure 1 The vascular endothelial growth factor (VEGF) mRNA expression levels in the oral squamous cell carcinoma (OSCC) group and the normal oral mucosa (NOM) group. It was found that the mean VEGF mRNA expression level of the OSCC group was 4-fold higher than that of the NOM group ($p < 0.0001$).

Table 2 Correlation between the mean vascular endothelial growth factor (VEGF) mRNA ΔC_T values in OSCC samples and clinicopathological parameters of 60 OSCC patients.

Parameter	Mean VEGF mRNA ΔC_T value ± SD	*p*
Patients' age (y)		
≤50 ($n = 21$)	3.7 ± 2.0	0.238
>50 ($n = 39$)	4.5 ± 2.6	
Patients' sex		
Men ($n = 55$)	4.1 ± 2.4	0.578
Women ($n = 5$)	5.0 ± 3.1	
Cancer location		
Buccal mucosa ($n = 22$)	4.8 ± 1.9	0.220
Tongue ($n = 17$)	4.3 ± 3.0	
Other oral mucosal sites ($n = 21$)	3.5 ± 2.4	
T status		
T1 ($n = 20$)	5.1 ± 2.5	0.040
T2 ($n = 13$)	4.5 ± 2.1	
T3 ($n = 11$)	4.1 ± 2.2	
T4 ($n = 16$)	2.9 ± 2.0	
N status		
N0 ($n = 44$)	4.6 ± 2.4	0.023
N1 ($n = 5$)	3.5 ± 2.0	
N2 ($n = 11$)	2.5 ± 1.6	
N3 ($n = 0$)	0 ± 0	
Clinical staging		
Stage 1 + 2 ($n = 29$)	5.1 ± 2.5	0.008
Stage 3 + 4 ($n = 31$)	3.4 ± 2.3	
Loco-regional recurrence		
Without ($n = 27$)	3.7 ± 2.3	0.185
With ($n = 33$)	4.6 ± 2.5	
Histology of OSCC		
Well-differentiated ($n = 51$)	4.3 ± 2.4	0.236
Moderately-differentiated ($n = 9$)	3.3 ± 2.7	

clinicopathological parameters of 60 OSCC patients is shown in Table 2. We found that the mean VEGF mRNA ΔC_T value was significantly lower in OSCC patients with a larger tumor size ($p = 0.040$), positive lymph node metastasis ($p = 0.023$), and more advanced clinical stages ($p = 0.008$) (Table 2). No significant correlation was found between the mean VEGF mRNA ΔC_T value and patients' age, sex, cancer location, loco-regional recurrence, or histology of OSCC. The VEGF mRNA ΔC_T value was also not associated with the areca quid chewing, cigarette smoking, or alcohol drinking habit (data not shown).

Survival analysis

In this study, the median follow-up duration was 47 months for 60 OSCC patients. Univariate analysis performed using Cox proportional hazard regression model identified larger tumor size ($p = 0.026$), positive lymph node metastasis ($p = 0.009$), more advanced clinical stages ($p = 0.022$), and VEGF mRNA ΔC_T value < 4.2 ($p = 0.018$, the median VEGF mRNA ΔC_T value was 3.9) as correlating with poorer survival of OSCC patients. Multivariate analyses with Cox proportional hazard regression model further identified positive lymph node metastasis ($p = 0.029$) and VEGF mRNA ΔC_T value < 4.2 ($p = 0.026$) as two independent unfavorable prognosis factors (Table 3). In this study, the median overall survival was 37 months for the patients with VEGF mRNA ΔC_T value <4.2 and 41 months for the patients with VEGF mRNA ΔC_T value ≥4.2 [HR (95% CI), 3.275(1.078−11.167)]. The Kaplan−Meier curve showed that OSCC patients with a VEGF mRNA ΔC_T value <4.2 had a significantly poorer cumulative overall survival than those with a VEGF mRNA ΔC_T value ≥4.2 ($p = 0.0427$, log-rank test, Figure 2).

Discussion

This study measured the VEGF mRNA level in 60 OSCC and 38 NOM samples. We found a significantly lower mean VEGF mRNA ΔC_T value in OSCC samples than in NOM samples, indicating an increased VEGF mRNA expression in OSCC samples than in NOM samples. Similarly, Berse et al[31] discovered a greater VEGF mRNA expression in renal cell carcinomas and colonic adenocarcinomas than in normal kidney and normal bowel mucosa tissues, respectively. Brown et al[32] also reported a significantly higher VEGF mRNA expression in gastrointestinal adenocarcinoma tissues than in normal epithelium. In addition, Guidi et al[33] demonstrated an elevated VEGF mRNA and protein expression in endometrial carcinomas than in normal endometrial tissues. The significantly higher expression of VEGF mRNA in cancerous tissues than in corresponding

Table 3 Univariate and multivariate overall survival analyses of the VEGF mRNA expression and clinicopathological parameters in 60 patients with oral squamous cell carcinoma using Cox proportional hazard regression model.

Factor	Hazard ratio (95% CI)	*p*
Univariate		
T status (T1 + T2 vs. T3 + T4)	3.473 (1.085−11.119)	0.026
N status (N0 vs. N1 + N2 + N3)	4.317 (1.432−11.947)	0.009
Clinical stage (Stage 1 + 2 vs. stage 3 + 4)	3.374 (1.759−8.131)	0.022
VEGF mRNA ΔC_T value (≥4.2 vs. < 4.2)	3.275 (1.078−11.167)	0.018
Multivariate		
N status (N0 vs. N1 + N2 + N3)	5.890 (1.202−28.865)	0.029
VEGF mRNA ΔC_T value (≥4.2 vs. < 4.2)	2.285 (1.556−14.234)	0.026

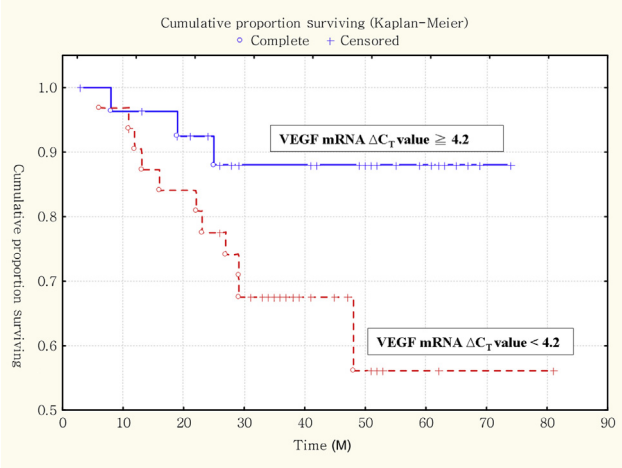

Figure 2 Kaplan-Meier survival curve showing a significant relation between cancer tissue vascular endothelial growth factor (VEGF) mRNA levels in 60 patients with oral squamous cell carcinoma (OSCC) and the overall survival of these 60 OSCC patients. The duration of overall survival was measured from the beginning of surgery to the time of death (complete) or the last follow-up (censored). The cumulative overall survival for OSCC patients with VEGF mRNA $\Delta C_T < 4.2$ was significantly poorer than that for OSCC patients with VEGF mRNA $\Delta C_T \geq 4.2$ ($p = 0.0427$, log-rank test).

normal tissues suggests that VEGF mRNA level can be used as a biomarker for oral, renal cell, gastrointestinal, and endometrial cancers. Indeed, upregulation of VEGF mRNA or protein has been detected in a variety of human cancers.[10–29,34]

VEGF can promote angiogenesis which is a pivotal factor for tumor growth and metastasis.[8,9] This study demonstrated a significant association of upregulation of VEGF mRNA in OSCC specimens with larger tumor size and positive lymph node metastasis. Our previous study also found a positive correlation between higher VEGF protein expression in OSCC tissues and positive regional lymph node metastasis.[26] A positive correlation between overexpression of VEGF protein or mRNA in cancer tissues and lymph node metastasis has also been reported in head and neck SCCs[23,29] as well as oral and pharyngeal SCCs.[28]

VEGF can promote tumor growth because it can increase neovascularization. We further explained why VEGF can augment lymph node metastasis as follows. Firstly, VEGF is a mitogen for vascular endothelial cells and helps the migration and organization of vascular endothelial cells for neovascularization and tumor micrometastasis.[8,9] VEGF-A and -B are mainly angiogenic factors while VEGF-C is a lymphangiogenic factor.[35,36] VEGF can inhibit endothelial cell apoptosis, increase endothelial cell proliferation and migration, enhance vascular permeability, and thus promote metastasis.[5,6] Secondly, VEGF augments the expression of matrix metalloproteinase 9 (MMP9) which facilitates the cancer cell invasion and metastasis.[8,37] Additionally, VEGF-induced Akt phosphorylation leads to the increased motility of human OSCC cell line.[37,38] Thirdly, VEGF also mediates tumor evasion of normal immune surveillance by inhibiting the development of dendritic cells, and in turn suppresses the antigen recognition and antitumor immune defense.[37,38] Therefore, VEGF may promote lymph node metastases through multiple mechanisms such as an increase in tumor angiogenesis and lymphangiogenesis, an increase in tumor cell survival, motility, migration and invasion, an elevated expression of MMP9, and an inhibition of the immune surveillance by dendritic cells.

This study displayed a significant association of greater VEGF mRNA expression in OSCCs with a larger tumor size and positive lymph node metastasis. Because higher T and N statuses always indicate an advanced clinical stage, OSCC patients with a higher expression of VEGF mRNA are prone to have more advanced clinical stages. Actually, higher VEGF protein expression has been found to be related to the more advanced clinical stages of colon and gastric carcinomas.[11,15]

The present study demonstrated a significant correlation between higher VEGF mRNA expression in OSCCs and poorer overall survival in OSCC patients. In addition, VEGF mRNA ΔC_T value <4.2 was identified as an independent unfavorable prognosis factor using multivariate regression analyses. A significant association of elevated VEGF m-RNA or protein expression with disease-free or overall survival has also been discovered in head and neck SCC patients including OSCC patients.[22–27] Previous studies also showed a significant association of higher levels of VEGF mRNA or protein with a shorter survival in patients with colorectal,[10] gastric,[14] pancreatic,[17] and breast[18–20] carcinomas. The above findings indicate that VEGF protein or mRNA level may be an important prognostic factor for patients with certain types of human carcinomas including OSCC.

Immunohistochemistry and quantitative RT-PCT are two excellent techniques to confirm a histopathological diagnosis of a tumor or to find a biomarker for prediction of cancer progression and prognosis.[39–49] This study showed an intimate and significant relationship between the elevated VEGF mRNA level and larger tumor size, higher N status, and more advanced clinical stage of OSCCs. Moreover, OSCC patients with a higher VEGF mRNA expression had a poorer cumulative overall survival than those with lower VEGF mRNA levels. VEGF mRNA ΔC_T value <4.2 was also identified as an independent unfavorable prognosis factor for OSCCs using multivariate regression analyses. However, the sample size of this study is relatively small, thus a large-scale study with an adequate number of OSCCs is needed to confirm the results of the present study. We conclude that the VEGF mRNA level may be a biomarker for prediction of the progression and prognosis of OSCCs in Taiwan.

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