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博士論文

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Doctoral Dissertation

胎盤生長因子於口腔鱗狀細胞癌之表現

Expression of Placenta Growth Factor (PlGF) in Oral Squamous Cell Carcinomas

鄭世榮

Shih-Jung Cheng

指導教授:江俊斌教授、郭彥彬教授

Advisor: Professor Chun-Ping Chiang

Professor Mark Yen-Ping Kuo

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國立台灣大學醫學院臨床牙醫學研究所
博士學位論文
口試委員會審定書

論文題目

胎盤生長因子於口腔鱗狀細胞癌之表現

Expression of Placenta Growth Factor (PlGF)

in Oral Squamous Cell Carcinomas

本論文係鄭世榮君（學號 D94422001）在國立臺灣大學醫學院臨床牙醫學研究所完成之博士學位論文，於民國一 0 一年六月七日承下列考試委員審查通過及口試及格，特此證明。

口試委員：

（簽名）

（指導教授）

系主任、所長 （簽名）

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背景: 胎盤生長因子(Placenta growth factor，PlGF)是屬於血管內皮生長因子家族(VEGF family)。PlGF 可藉由 VEGFR-1 引發腫瘤血管新生，PlGF 過度表現與全身很多癌症有關，亦包括口腔癌。之前，我們利用免疫組織化學染色法研究顯示，口腔鱗狀細胞癌 (OSCC) 組織中，有過度 PlGF 蛋白表現，則 OSCC 患者之預後較差。但並無文獻提到有關 OSCC 患者，其癌組織中 PlGF mRNA 量及血清中 PlGF 蛋白量，與 OSCC 患者預後之相關性。因此，本研究進一步檢測 OSCC 患者癌組織中 PlGF mRNA 量及血清中 PlGF 蛋白量，是否和 OSCC 患者之腫瘤發展、復發及存活有關。

材料及方法

本研究第一部分利用即時定量聚合酶鏈式反應(quantitative real-time reverse transcription-polymerase chain reaction; qRT-PCR)方法，測量 63 例 OSCC 癌組織及癌旁正常口腔黏膜 (non-SCC) 中 PlGF mRNA 量。Threshold cycle (C_T) 定義為 DNA 可被監測出之最少 PCR cycle 數目。C_T 數值大表示 PlGF mRNA copy number 少。OSCC 及 non-SCC 組織中，PlGF mRNA 的差異表現為 $-\Delta C_T = -$ (OSCC C_T – non-OSCC C_T) 。$-\Delta C_T$ 數值愈大，表示 OSCC 組織中 PlGF mRNA copy number 愈多。ANOVA、卡方檢定(Chi-square test)、Kaplan-Meier 存活率方法及 Cox proportional hazard regression model，來分析癌組織中 PlGF mRNA 量與 OSCC 患者臨床病理參數及存活率之相關性。本研究第二部分利用酵素連結免疫吸附法(enzyme-linked immunosorbent assay, ELISA)，探討 72 位 OSCC 患者術前及術後三個月及 30 位具正常口腔黏膜者，其血清中 PlGF 蛋白量。再利用 ANOVA、卡方檢定、Kaplan-Meier 存活率方法及 Cox proportional hazard regression model，來分析血清中 PlGF 蛋白量與 OSCC 患者臨床病理參數及存活率之相關性，並試圖尋找預測存活時間的獨立預後因子。

結果

本研究第一部分發現，PlGF mRNA $-\Delta C_T$ 的平均值較高和患者有較大腫瘤 ($P = 0.03$)、有

局部淋巴結轉移(P = 0.003)、有較高臨床分期 (P = 0.013) 及有局部復發 (P = 0.039) ，有統計學上有意義之相關。以 Cox regression model 進行多變數分析發現，淋巴結轉移情形 (P = 0.019)、PlGF mRNA -ΔC_T value > 2 (P = 0.016)為影響 OSCC 患者存活時間的獨立預測因子。Kaplan-Meier 存活分析發現， PlGF mRNA -ΔC_T value > 2 比 PlGF mRNA -ΔC_T value ≤ 2 之 OSCC 患者，有較差的無復發存活率 (log-rank test, P = 0.017)。

本研究第二部分發現，術前 OSCC 患者血清中 PlGF 蛋白量的平均值，比具正常口腔黏膜者的平均值高，且具統計學上有意義之差別 (19.1 ± 10.7 pg/ml vs. 10.1 ± 4.5 pg/ml，P < 0.001)。術前 OSCC 患者血清中 PlGF 蛋白量平均值較高和患者有較大腫瘤 (P = 0.015)、有局部淋巴結轉移 (P =0.001)、有較高臨床分期 (P = 0.002)及有局部復發(P = 0.037)，有統計學上有意義之相關。Cox regression model 進行多變數分析發現，血清中 PlGF 蛋白濃度為影響存活時間的獨立預測因子(P = 0.014)。Kaplan-Meier 存活分析發現，血清中 PlGF 蛋白 > 19.1 pg/ml 比血清中 PlGF 蛋白 ≦ 19.1 pg/ml 者，會有較差的無復發存活率 (log-rank test，P = 0.009)。當以血清中 PlGF 蛋白為 19.1 pg/ml (正常平均值加 2 個標準差) 為切點時, 預測腫瘤復發的敏感度 (sensitivity)、特異度 (specificity)及陽性預測值 (positive predictive value) 分別為 80%、56% 和 78%。

結論

本研究發現，OSCC 患者組織中 PlGF mRNA 量及血清中 PlGF 蛋白量，可以預測 OSCC 之腫瘤大小、有無局部淋巴結轉移、臨床分期、有無局部復發，及其無復發存活率，因此 OSCC 患者組織中 PlGF mRNA 量及血清中 PlGF 蛋白量，可以當作 OSCC 患者預後的指標。

關鍵詞: 胎盤生長因子、口腔癌、血管新生

Abstract

Background/Purpose

Placenta growth factor (PlGF) belongs to vascular endothelial growth factor (VEGF) family. PlGF could induce tumor angiogenesis through binding to VEGFR-1, which was highly expressed in a variety of human cancers, including oral cancer. Our previous study demonstrated a significant association of higher expression of PlGF protein with poor prognosis of patients with oral squamous cell carcinoma (OSCC). This study further examined whether the expression of PlGF mRNA in OSCC tissues and the serum PlGF protein level in OSCC patients could be used to predict the progression and prognosis of OSCCs in Taiwan.

Materials and methods

In the first part of this study, we used quantitative real-time reverse transcription-polymerase chain reaction (quantitative RT-PCR) to detect the PlGF mRNA levels in 63 paired OSCC and adjacent normal-looking oral mucosa (non-OSCC) tissues. Threshold cycle (C_T) was defined as the PCR cycle number needed to generate a pre-determined amount of DNA (threshold). For a chosen threshold, a smaller starting copy number of mRNA results in a higher C_T value. In this study, the relative expression level of tissue PlGF mRNA in each OSCC patient was expressed as $-\Delta C_T = -$ (OSCC C_T – non-OSCC C_T). Thus, the higher the $-\Delta C_T$, the greater the copy number of PlGF mRNA in tissues. In the second part of this study, serum samples were obtained from 72 OSCC

patients before and 3 months after surgical cancer excision and from 30 normal control subjects. Serum PlGF protein levels were determined by enzyme-linked immunosorbent assay (ELISA).

Results

In the first part of this study, we found that the higher mean PlGF mRNA $-\Delta C_T$ value was significantly associated with OSCCs with larger tumor size (P = 0.03), positive lymph node metastasis (P = 0.003), more advanced clinical stages (P = 0.013) or the presence of loco-regional recurrence (P = 0.039). Positive lymph node metastasis (P = 0.019) and PlGF mRNA $-\Delta C_T$ value > **2** (P = 0.016) were identified as two independent unfavorable prognosis factors by multivariate analyses with Cox regression model. Moreover, Kaplan-Meier curve showed that OSCC patients with a PlGF mRNA $-\Delta C_T$ value > 2 had a significantly poorer recurrence-free survival than those with a PlGF mRNA $-\Delta C_T$ value $\leqq$ 2 (log-rank test, P = 0.017). In the second part of this study, we found that **t**he mean serum PlGF protein levels were significantly higher in pre-surgery OSCC patients than in normal controls (19.1 ± 10.7 pg/ml vs. 10.1 ± 4.5 pg/ml, P < 0.001). Serum PlGF protein levels dropped to near the normal control levels after surgical cancer removal. Higher pre-surgery serum PlGF protein levels were significantly associated with OSCCs with larger tumor size (P = 0.015), positive lymph node metastasis (P = 0.001), more advanced clinical stages (P = 0.002), and loco-regional recurrence (P = 0.037). The serum PlGF protein level was identified as an independent unfavorable prognosis factor by multivariate Cox regression analyses (P = 0.014).

Kaplan-Meier curve showed that OSCC patients with a higher serum PlGF protein level had a significantly poorer cumulative recurrence-free survival than those with a lower serum PlGF protein level (log-rank test, $P = 0.009$). When we used the serum PlGF protein level of 19.1 pg/ml (mean normal control value plus 2 standard deviations) as a cutoff point, the sensitivity, specificity, and positive predictive value for tumor recurrence was 80%, 56% and 78%, respectively.

Conclusion

This study found that PlGF mRNA level in OSCC tissues and serum PlGF protein level in OSCC patients could be used to predict the tumor size, regional lymph node metastasis, clinical stage, and recurrence of OSCCs and the prognosis of OSCC patients. Therefore, we conclude that PlGF mRNA level in OSCC tissues and serum PlGF protein level in OSCC patients may be valuable biomarkers for prediction of progression, recurrence and prognosis of OSCC in Taiwan.

Key words: Placenta growth factor、oral cancer、angiogenesis

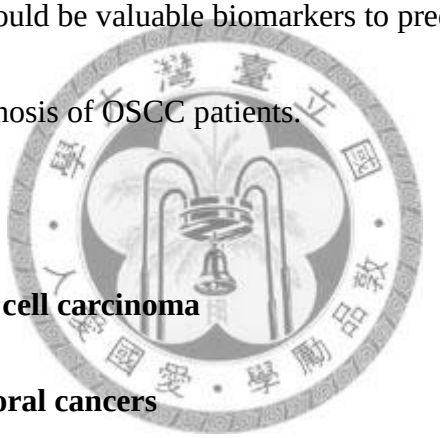
I. Introduction and literature review

In Taiwan, oral squamous cell carcinomas (OSCC) rank as the sixth most prevalent cancer in both sexes and account for the fourth most common cancers in males in 2010 (Cancer registry annual report in Taiwan area, 2010). Despite significant efforts committed in recent years in diagnosis and treatment of OSCC, the overall survival rate has remained approximately 50% (Forastiere et al., 2001). Tumor recurrence is one of the major causes resulting in a poor survival of OSCC patients. This suggests an urgent need for a novel biomarker to predict the progression, recurrence and prognosis of OSCCs.

Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family, which is related to angiogenesis and carcinogenesis (Fischer et al., 2008). PlGF was found to promote proliferation, differentiation, and survival of endothelial cells by binding to VEGF receptor 1 (VEGFR1, also known as FLT1) (Fischer et al., 2008). Clinically, overexpressed PlGF mRNA or protein level correlates with pathological angiogenesis (Fischer et al., 2008), tumor cell growth (Ikai et al., 2005; Lacal et al., 2000; Marcellini et al., 2006), positive lymph node metastasis (Chen et al., 2004; Marcellini et al., 2006; Parr et al., 2005), advanced clinical stage (Chen et al., 2004; Wei et al., 2005; Zhang et al., 2005), recurrence (Parr et al., 2005; Ho et al., 2007), and poor prognosis (Chen et al., 2004; Parr et al., 2005; Wei et al., 2005) of a variety of cancers. Recently, we reported that the higher mean PlGF labeling index assessed by immunohistochemical staining are

significantly associated with the more advanced progression and poorer prognosis of OSCC (Cheng et al. 2010). Several previous studies have also reported increased serum PlGF protein levels in patients with renal cell (Matsumoto et al., 2003), pancreatic (Chang et al., 2008; Sabbaghian et al., 2010) or colorectal carcinomas (Rahbari et al., 2002; Wei et al., 2009).

In this study, we further evaluated whether the PlGF mRNA level in OSCC surgical specimen measured by the quantitative real-time reverse transcription-polymerase chain reaction (quantitative RT-PCR) and the serum PlGF protein levels in OSCC patients measured by the enzyme-linked immunosorbent assay (ELISA) could be valuable biomarkers to predict the therapeutic effect, progression, recurrence and prognosis of OSCC patients.



1.1 Overview of oral squamous cell carcinoma

1.1.1 Epidemiology of global oral cancers

Oral cancer is the sixth most common cancer worldwide, with a high prevalence in South Asia. An estimated 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) were reported in 2008 worldwide. Generally, the highest oral cavity cancer rates are found in Melanesia, South-Central Asia, and Central and Eastern Europe and the lowest in Africa, Central America, and Eastern Asia for both males and females. Smoking, alcohol consumption, smokeless tobacco use, and HPV infections are the major risk factors for oral cavity cancer, with smoking and alcohol drinking having synergistic effects (Blot et al., 1988; Hashibe et al., 2009). Worldwide,

smoking accounts for 42% of deaths from cancers of the oral cavity (including the pharynx) and heavy alcohol consumption for 16% of the deaths; the corresponding percentages in high-income countries are about 70% and 30%, respectively (Danaei et al., 2005). The rise in the incidence rate of oral cancer in Taiwan may have been in part due to the increased consumption of areca quid and alcohol (Ho et al., 2002). Oral cavity cancer mortality rates among males decreased significantly in most countries, including those of Europe and Asia, over the past decades (Garavello et al., 2010; Mayne et al., 2006). However, rates continued to increase in several Eastern European countries, including Hungary and Slovakia (Garavello et al., 2010). The mortality rate increase in females in most European countries largely reflects the ongoing tobacco epidemic (Garavello et al., 2010). This contrasts with the decreasing trends at all ages in both males and females in the United States and United Kingdom, where the tobacco epidemic began and declined earlier (Garavello et al., 2010). However, incidence rates for oral cancer sites related to HPV infections, such as the oropharynx, tonsil, and base of the tongue, are increasing in young adults in the United States and in some countries in Europe, which is hypothesized to be in part due to changes in oral sexual behavior (D'Souza et al., 2009; Marur et al., 2010).

1.1.2 Epidemiology of oral cancers in Taiwan

With rapid aging of populations, cancer has become the first leading cause of death in Taiwan since 1982 (Chen et al., 2002). In 1982, the incidence rate of head and neck cancer was 5.12 per 100,000 people in males and 1.54 per 100,000 people in females. In 1991, the incidence rate of head

and neck cancer had not much changed, with the incidence rate being 6.02 and 1.51 per 100,000 people in males and females, respectively. However, in 2003, the incidence rate of head and neck cancer significantly increased to 35.08 and 3.56 per 100,000 people in males and females, an alarming 5.82-fold increase in men and 2.35-fold increase in woman in a decade was found (Chen et al., 2008). In 2008, the incidence rate of head and neck cancer significantly increased to 37.57 per 100,000 people in males. In 2010, oral cavity cancer had become the 5th most common cancer causing mortality in Taiwanese men. Similarly, the mortality rate also increased significantly, from 4.25 per 100,000 in 1995 to 9.6 per 100,000 in 2006, a 2.26-fold increase in the past decade. There has been a trend toward lower age at diagnosis of head and neck cancer over time. From 1989 to 1993, the peak of incidence rate was for people aged 50–59 years, but this shifted to ages 40–49 years between 1993 and 2000. A similar trend was also found in the mortality rate. During the period between 1991 and 1994, mortality rate peaked at age 50–59 years, but shifted to age 40–49 years between 1999 and 2002. These data are consistent with other regional reports from northern and southern Taiwan. In 2006, the median age at death from head and neck cancer was 54 years compared with 69 years in other forms of cancer. Gender differences in head and neck cancer have been described, with a marked male predominance. A study analyzing 703 OSCC patients between 1985 and 1996 in southern Taiwan found a 51:1 male-to-female ratio (Chen et al., 1999). The overall 5-year survival rate in patients with head and neck cancer is one of the lowest among common malignant neoplasms and has not significantly changed during the last two decades.

Cancer clinical stage is the major determinant of survival rate. The 5-year survival rates of oral cavity cancer patients in stages I, II, III and IV are 72–90%, 39–85%, 27–70% and 12–50%, respectively (Liao et al., 2008; Liao et al., 2007; Ko et al., 1995). Survival rates for head and neck cancer are significantly influenced by tumor size, lymph node involvement, distant metastasis, tumor differentiation and areca quid chewing (Liao et al., 2004; 2008). Areca quid chewing independently contributes to the risk of head and neck cancer, and the estimated prevalence of areca quid chewing in Taiwanese patients with head and neck cancer is approximately 85% (Liao et al., 2004; 2007; 2008). Approximately 50% of patients who were areca quid chewers are also alcohol drinkers and tobacco smokers (Liao et al., 2004; 2007; 2008). A cumulative effect from areca quid chewing, alcohol drinking and tobacco smoking has been observed, with a 123-fold increased risk of oral cancer when the three risk factors are present (Ko YC et al., 1995). With regard to the anatomical location of oral cavity cancers, approximately 30–40% of all cases occur in the tongue or in the buccal mucosa. Altogether, lesions at these sites account for approximately 70% of all oral cavity malignancies (Liao et al., 2008; Chen et al.,1999; Ko et al.,1995).

1.1.3 Risk factors related to oral cancers

Oral cancer was known to be associated with cigarette smoking, excessive alcohol consumption, areca quid chewing, viral or fungal infection (candida, human papilloma virus (HPV), Herpex simplex virus, etc.), nutrition deficiency (Plummer-Vinson Syndrome, Vitamin A deficiency, Vitamin C deficiency, etc.), family hereditary, immunodeficiency, gene mutation, and so on. Recent 2003 IARC monograph declared areca quid chewing, by itself, to be a Group 1 carcinogen. The

Taiwanese chewers commonly use fresh, unripe betel fruit with slaked lime as an essential ingredient. The composition of areca quid differs geographically; the areca quid used in Taiwan contains areca nut, lime and Piper betel inflorescence (Yang et al., 2001; 2005). Piper betel inflorescence contains high concentrations of hydroxychavicol and safrole, whereas arecoline, a major areca nut alkaloid, is considered to be the most important carcinogen in the areca nut. Arecoline has been shown to induce structural chromosomal aberration, sister chromatid exchange and micronuclei formation in different cell types (Jeng et al., 2001; Shirname et al., 1983). Studies in human oral cancer cells have shown that exposure to arecoline or areca nut extract (ANE) results in growth arrest in the late S and G2/M phases (Lee et al., 2006). Piper betel inflorescence, which contains safrole, is a unique ingredient of areca quid in Taiwan. Safrole–DNA adducts have been suggested to play an important role in oral carcinogenesis (Chen et al., 1999). A further report has provided evidence that alkaline saliva generated by chewing areca quid may play a role in cigarette-related nicotine-induced DNA damage, and reactive oxygen species may be involved in generating this DNA damage (Wu et al., 2005). These findings provide a molecular explanation for the synergistic effect of areca quid chewing and tobacco smoking in the development of head and neck cancer in Taiwan.

1.1.4 Precancerous lesions and conditions

Many OSCCs develop from premalignant conditions of the oral cavity (Silverman et al., 1984; 1968). The World Health Organization classifies oral precancerous/potentially malignant disorders into 2 general groups. A precancerous lesion is “a morphologically altered tissue in which oral cancer is more likely to occur than its apparently normal counterpart.” These precancerous lesions include leukoplakia, erythroplakia, and the palatal lesions of reverse smokers. A precancerous

condition is “a generalized state associated with significantly increased risk of cancer.” The precancerous conditions include submucous fibrosis, lichen planus, epidermolysis bullosa, and discoid lupus erythematosus. Intervention should be based on histopathological features of a biopsy of the lesions. In many cases, a biopsy is mandatory so that such lesions can be further evaluated. Currently, histological criteria (presence and degree of dysplasia) represent the gold standard in precancerous lesion risk evaluation (Blot et al., 1988). The latest WHO classification (Barnes et al., 2005) recommends a more objective gradings. The criteria for grading of oral epithelial dysplasia are summarized as follows:

Mild dysplasia (grade I) demonstrates proliferation or hyperplasia of cells of the basal and parabasal layers which does not extend beyond the lower third of the epithelium. Cytological atypia is generally slight with only mild pleomorphism of cells or nuclei. Mitoses are not prominent, and when present are usually basally located and normal. Architectural changes are minimal.

Moderate dysplasia (grade II) demonstrates a proliferation of atypical cells extending into the middle one-third of the epithelium. The cytological changes are more severe than in mild dysplasia and changes such as hyperchromatism, and prominent cell and nuclear pleomorphism may be seen. Increased and abnormal mitoses may be present, but these are usually located in the basal layers. Architectural changes may be seen in the lower half of the epithelium where there may be loss of basal polarity and hyperplasia leading to bulbous rete pegs. However stratification and maturation are relatively normal, often with hyperkeratosis.

Severe dysplasia (grade III) shows abnormal cell proliferation from the basal layer into the upper third of the epithelium. Cytological and architectural changes can be very prominent. All the changes seen in mild and moderate dysplasia are seen but in addition there is marked pleomorphism often with abnormally large nuclei with prominent or even multiple nucleoli. Prominent and suprabasal mitoses are usually evident and abnormal tripolar or star-shaped forms may be seen. Apoptotic bodies may also be prominent. Architectural changes are severe, often with complete loss of stratification and with deep abnormal keratinization and even formation of keratin pearls. Abnormal forms of rete pegs are usual and bulbous rete pegs are regarded as particularly significant in the diagnosis of severe dysplasia. Abnormal shaped rete pegs may also be seen, with lateral extensions or small branches. These are quite abnormal and may be the earliest signs of invasion. Occasional lesions may show prominent acantholysis with severe disruption of the architecture. Although the epithelium may be thickened, severe dysplasia is sometimes accompanied by marked epithelial atrophy.

Carcinoma *in situ* is the most severe form of epithelial dysplasia and is characterized by full thickness cytological and architectural changes. In the oral cavity, such changes are rare, and often, even in the presence of the most severe atypia, there is still an intact keratinized surface layer. Carcinoma *in situ* is thought by some to be a premalignancy but others regard it as evidence of actual malignant change but without invasion. Severe epithelial dysplasia has an overall malignant transformation rate of about 16% but studies show a wide range of 7% – 50% (Silverman et al.,

1984; Schepman et al., 1998; Gupta et al., 1990; Bouquot et al., 1988; JBanoczy et al., 1976; Amagasa et al., 1985; Vedtofte et al., 1987; Mincer et al., 1972; Pindborg et al., 1977; Lumerman et al., 1995; Jaber et al., 2003). Moderate dysplasia has a malignant transformation potential of 3% – 15%, whereas mild epithelial dysplasia shows a very low risk (less than 5%). It is always assumed, however, that there is a temporal progression of disease, analogous to multistage carcinogenesis, and that mild dysplasia will progress to severe dysplasia and then to carcinoma.

1.1.5 Clinicopathological classification and treatment modalities of oral squamous cell carcinoma

The WHO grading system (Pindborg et al., 1997) recommends 3 categories: well-differentiated, moderately-differentiated and poorly-differentiated. This usually depends on the subjective assessment of the degree of keratinization, cellular and nuclear pleomorphism, and mitotic activity (Woolgar, 2006). The influence of histologic grading as a prognostic factor in OSCC was assessed in 215 patients and was found to be a significant predictor of locoregional failure and tumour recurrence (Kademani et al., 2005).

Surgery is the most well-established mode of initial definitive treatment for a majority of oral cancers, with a longstanding history of being the accepted method of treatment for oral cancers over a century. Introduction of ionizing radiation, following the discovery of radium, became an important means of non-surgical treatment of oral carcinoma. However, in the majority of patients

with advanced cancer, radiotherapy is employed in conjunction with surgery, most often offered as post-operative treatment. Chemotherapy in the management of oral carcinoma was considered palliative in the 1950's, 60's and 70's. However with the introduction of Cis-platinum, clinical trials of induction chemotherapy demonstrated that response to chemotherapy is observed in a significant number of patients (Shah et al., 2009). For patients with advanced-staged disease, the current preference for the sequence of combined modality treatment program is surgical resection with immediate appropriate reconstruction followed by post-operative radiation therapy or post-operative concurrent chemoradiotherapy. The observations from two prospective randomized trials of adjuvant chemoradiotherapy have shown that patients who have extracapsular extension of disease in metastatic cervical lymph nodes and those who have positive margins have a significant improvement in local regional control and disease free survival by addition of chemotherapy to postoperative radiation therapy compared to post-operative radiation therapy alone (Bernier et al., 2005). Targeted therapies with epidermal growth factor receptor (EGFR) inhibitors are an active area of investigation at this time. Immunotherapy and gene therapy are also areas of research where further work needs to be done (Shah et al., 2009).

1.2 Angiogenesis

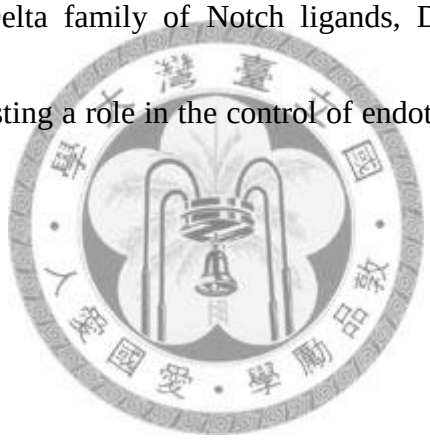
The dependence of tumor growth on the development of a neovasculature is now a well-established aspect of cancer biology (Folkman, 1971). Angiogenesis is important for supply of

oxygen, nutrients, growth factors, hormones, and proteolytic enzymes, influences on hemostatic factors that control the coagulation and fibrinolytic system, and dissemination of tumor cells to distal sites (Folkman, 1990; Fidler, 1994). The angiogenic process is a highly complex, dynamic process regulated by a number of pro- and anti-angiogenic molecules. The so called ‘‘switch’’ to an angiogenic phenotype is considered a hallmark of the malignant process whereby proangiogenic mechanisms overwhelm or circumvent negative regulators of angiogenesis (Hanahan, 2000). In general, increased tumor vascularization (eg, increased microvessel density) and tumor expression of proangiogenic factors has been associated with advanced tumor stage and poor prognosis in a variety of human cancers (Brown, 1997; Dvorak, 1995; Reinmuth , 2003).

1.2.1 Angiogenic growth factors

The known endothelial cell specific growth factors and their receptors can be classified into vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families. VEGF is a major inducer of angiogenesis and it is structurally related to PlGF (placenta growth factor), VEGF-B, VEGF-C, VEGF-D and Orf virus derived VEGF (also called VEGF-E) (Ferrara and Davis- Smyth, 1997; Erikson and Alitalo, 1999; Persico et al., 1999; Achen et al., 1998; Ogawa et al., 1998; Meyer et al., 1999). Loss of even a single VEGF allele results in embryonic lethality, suggesting that VEGF levels are critical during embryonic angiogenesis (Carmeliet et al., 1996). There are to date three known receptor tyrosine kinase that bind VEGFs (VEGFR-1, VEGFR-2 and VEGFR-3)

(Neufeld et al., 1999; Veikkola et al., 2000; Aprelikova et al., 1992). In adults, VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, while VEGFR-3 is mostly restricted to the lymphatic endothelium (Neufeld et al., 1999; Kaipainen et al., 1995). In addition to endothelial cell specific growth factors, there is also a wide range of other less specific angiogenic growth factors, including basic fibroblast growth factor (bFGF) and transforming growth factor- β (TGF-β) (Friesel and Maciag, 1995). Recently, Eph receptor tyrosine kinases and their cell-surface-bound ephrin ligands were found. Among nontyrosine kinase receptors, a recently characterized member of the Delta family of Notch ligands, Dll4 is expressed specifically in vascular endothelial cells, suggesting a role in the control of endothelial cell biology (Shutter et al., 2000).



1.2.2 Angiogenic inhibitors

A large and structurally diverse group of endogenous angiogenesis inhibitors has also been discovered, including thrombospondin-1 (Tolsma et al., 1993), interferon-α/β (Dvorak and Gresser, 1989), angiostatin (O'Reilly et al., 1994), endostatin (O'Reilly et al., 1997) and anti-thrombin III (O'Reilly et al., 1999). Some of these are fragments of proteins, that are either components of the extracellular matrix (ECM) or of enzymes of the blood clotting pathways.

1.2.3 Tumor angiogenesis

When a primary tumor first arises, proliferation of cancer cells may be balanced by apoptosis

and the tumor may remain undetectable for years (Hanahan and Folkman, 1996). Such a phenomenon, called tumor dormancy, may depend on a rate-limiting role of neovascularization (Holmgren, 1996). The onset of neovascularization in primary tumors can be relatively sudden, described as the angiogenic switch (Hanahan and Folkman, 1996; Folkman et al., 1989). The angiogenic switch in tumors is currently thought to be caused by a shift in the net balance of positively and negatively acting angiogenic mediators, angiogenic growth factors and angiogenesis inhibitors, respectively. The mechanisms underlying this shift of balance remain incompletely understood. Thus, some oncogenes and tumor-suppressor genes contribute to the angiogenic switch by causing up- or down-regulation of endogenous angiogenesis inhibitors or pro-angiogenic growth factors (Kerbel et al., 1998; Hanahan and Folkman, 1996). On the other hand, the control of bioavailability of angiogenic activators and inhibitors also has a role in the regulation of the angiogenic switch. A variety of proteases can release TGF-β or bFGF stored in the extracellular matrix (Taipale et al., 1998; Whitelock et al., 1996), whereas proteolytic cleavage of plasmin results in the formation of the inhibitor, angiostatin (Gately et al., 1997). Some studies have suggested that instead of growing as avascular masses, some tumors, especially metastases, may initially grow by coopting existing host vessels (Holash et al., 1999). This coopted host vasculature would not immediately undergo angiogenesis to support the tumor, but instead regresses, leading to secondary hypoxia, massive tumor cell loss, and further to hypoxia-induced angiogenesis (Holash et al., 1999). Furthermore, there is also evidence of mobilization of bone marrow-derived endothelial progenitor

cells (EPC) in various angiogenesis models, resulting inaugmented neovascularization (Asahara et al., 1999; Peichev et al., 2000; Takahashi et al., 1999).

1.2.4 Angiogenesis and tumor metastasis

Several studies since Weidner's first report in 1991 reveal that the higher the microvessel count is in areas of highest vessel density, the lower is the rate overall survival of the tumor patients (Weidner et al., 1991). For a tumor cell to metastasize, it must pass through several barriers and finally be able to survive and grow in the target tissue. First tumor cells must enter the vasculature in the primary tumor. VEGF and bFGF, secreted by the tumor cells, induce the expression of plasminogen activator and collagenases, contributing to the degradation of basement membranes (Kalebic et al., 1983; Nagy et al., 1989). In addition, proliferating tumor capillaries with fragmented and leaky basement membranes are easily penetrated by tumor cells. In fact, a considerable percentage of tumor blood flow is indirect contact with tumor cells (Hashizume et al., 2000). After overcoming the first vascular barrier, tumor cells have to survive the circulation, attach to the micro-vasculature of the target organ, exit from this vasculature (usually through postcapillary venular endothelium), and survive in the target tissue (Nicolson, 1988). Furthermore, in order to form macrometastases, micrometastatic cells must also be able to induce angiogenesis in their new target tissue. While angiogenesis is required for tumor growth, active lymphangiogenesis has not been detected within tumors. functional lymphatic vessels are often absent from the interior of solid

tumors, possibly due to collapse of the vessels by the interstitial pressure induced by the growing cancer cells and the leaky tumor blood vessels (Leu et al., 2000). Functional, enlarged lymphatics are, however, detected in the tumor periphery. VEGF-C is a growth factor for both lymphatic and blood vessels, and the expression of its specific receptor VEGFR-3 becomes restricted mainly to the lymphatic endothelium during development (Kaipainen et al., 1995; Kukk et al., 1996). Several recent reports have suggested a correlation between VEGF-C expression and lymphatic metastasis in tumors (Ohta et al., 1999; Tsurusaki et al., 1999; Yonemura et al., 1999). Even though the tumor itself lacks functional lymphatic vessels, the VEGF-C overexpression induced increase of peritumoral lymphatics makes the lymphatic vessels more accessible to the metastases.

1.3 VEGF and cancers

1.3.1 Vascular endothelial growth factor family, VEGF family

VEGF-A (commonly referred to as VEGF) was first identified by Dvorak et al. (2002) as a vascular permeabilitity-inducing factor secreted by tumor cells, and thus referred to as vascular permeability factor (VPF). Ferrara et al. (1996) later isolated and cloned VEGF-A as an endothelial specific mitogen. VEGF is a major inducer of angiogenesis and it is structurally related to PlGF (placenta growth factor), VEGF-B, VEGF-C, VEGF-D and Orf virus derived VEGF (also called VEGF-E) (Ferrara and Davis-Smyth, 1997; Erikson and Alitalo, 1999; Persico et al., 1999; Achen et al., 1998; Ogawa et al., 1998; Meyer et al., 1999). The biological functions of the VEGF family

are mediated by activation of three structurally homologous tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3 (Neufeld et al., 1999; Veikkola et al., 2000; Aprelikova et al., 1992)

1.3.1.1 VEGF-A

VEGF-A is a 45-kDa homodimeric glycoprotein with a diverse range of angiogenic activities. The VEGF-A gene undergoes alternative splicing to yield mature isoforms of 121, 165, 189, and 206 amino acids (Houck, 1991; Tischer et al., 1991). In addition, some less commonly expressed variants have also been identified (VEGF145 and VEGF183) (Neufeld et al., 1999).

VEGF121 is freely secreted, whereas the largest isoforms (VEGF189 and VEGF206) are sequestered in the extracellular matrix (ECM) and require cleavage by proteases for their activation (Dvorak, 2002). VEGF165 exists in both a soluble and an ECM-bound form (Keyt et al., 1996). The ECM-bound isoforms of VEGF-A, VEGF-C, and VEGF-D can be released in a diffusible form by plasmin cleavage at the C-terminus, which generates a bioactive fragment (Park et al., 1993; McColl et al., 2003). Alternatively, VEGF can be released from the ECM by MMP-9 to initiate the angiogenic switch (Bergers et al., 2000). VEGF165 is the predominant isoform and is commonly overexpressed in a variety of human solid tumors. In mice, homozygous or heterozygous deletion of the VEGF gene is embryonically lethal, resulting in defects in vasculogenesis and cardiovascular abnormalities, demonstrating that VEGF is essential for development (Carmeliet et al., 1996; Ferrara, 1996). VEGF-A is also important to a number of postnatal angiogenic processes, including

wound healing, ovulation, menstruation, maintenance of blood pressure, and pregnancy (Brown, 1992). VEGF-A has also been linked to several pathologic conditions associated with increased angiogenesis, including arthritis, psoriasis, macular degeneration, and diabetic retinopathy (Ferrara et al., 2003). In healthy conditions, endothelial cells survive for prolonged periods owing to the low level of VEGFA expression by the vasculature, which acts as a survival signal. Hence, when quiescent endothelial cells are deprived of this trophic VEGFA signal, they become dysfunctional (pro-thrombotic), lose their vasorelaxing activity (nitric oxide release) or even disappear altogether (which leads to bleeding) (Baffert et al., 2006; Gerber et al., 1999; Lee et al., 2007). Furthermore, large amounts of VEGFA are produced in healthy conditions, which suggest that VEGFA is needed for endothelial-cell homeostasis (Rudge et al., 2007). VEGFA binds to FLT1 with an affinity (dissociation constant; Kd ~2–10 pM) that is much higher than for FLK1 (Sawano et al., 2001). Yet, VEGFA induces weaker tyrosine-kinase activity in FLT1, possibly because of an inhibitory sequence in the juxtamembrane domain that represses FLT1 activity (Seetharam et al., 1995; Waltenberger et al., 1994; Gille et al., 2000). This weak tyrosine-kinase activity of FLT1 and its high affinity for VEGFA have led to the development of a model in which FLT1 acts as a decoy receptor and modulates angiogenesis through its ability to sequester VEGFA, and thereby reduces signalling through FLK1 (Park et al., 1994). Additional functional diversity may occur through the formation of dimers between VEGF family members. For example, VEGF-A may form heterodimers with either PlGF (Cao et al., 1996) or VEGF-B (Olofsson et al., 1996). In humans,

because of the alternative splice variants, this has the potential to create enormous diversity and presents a challenge to assessing the functional consequences of heterodimerization (Birkenhager et al., 1996). For example, the six human VEGF-A and four human PlGF isoforms could theoretically form 24 VEGF-PlGF heterodimers, and VEGF-A could theoretically form 12 different combinations with the two human VEGF-B variants.

1.3.1.2. VEGF-B and PlGF

VEGF-B and PlGF null mice display no defects in embryonic vasculogenesis or developmental abnormalities, suggesting that the role of PlGF and VEGF-B may be redundant (Carmeliet, 2001). However, loss of PlGF impairs angiogenesis, plasma extravasation, and collateral growth during ischemia, inflammation, wound healing, and tumor growth, suggesting a role for PlGF in pathologic states in the adult. VEGF-B exists as two isoforms (VEGF167 and VEGF 186) that bind to FLT1 and to neuropilin 1 (Neufeld, 2001). Neuropilin 1 is widely expressed in various tissues (Nash, 2006) including endothelial and mural cells, brown fat, skin, lung, placenta, brain and retina, but it is particularly abundant in the heart and skeletal muscle (Li, 2001). Despite some evidence for VEGFB activity *in vitro*, genetic studies have revealed that VEGFB-deficient mice are healthy and fertile, and do not display vascular defects, which indicates that VEGFB is redundant in angiogenesis in the developing embryo and healthy adult *in vivo* (Li et al., 2008; Aase et al., 2001; Bellomo et al., 2000). The role of VEGFB in tumor growth remains largely elusive. VEGFB has

been detected in a wide range of tumors, including meningioma, and colorectal, lung and breast cancer (Andre et al., 2000; Donnini et al., 1999; Niki et al., 2000). Moreover, VEGFB expression correlates with microvascular density in oral squamous cell carcinoma (Shintani et al., 2004).

1.3.1.3 VEGF-C and VEGF-D

The VEGF homologs VEGF-C and VEGF-D play key roles during embryonic and postnatal lymphangiogenesis. Homozygous deletion of the VEGF-C gene in mice is embryonically lethal and heterozygous deletion results in postnatal defects associated with defective lymphatic development (Karkkainen et al., 2004). Both factors induce lymphangiogenesis in transgenic mouse models (Jeltsch et al., 1997; Veikkola et al., 2001). VEGF-C and VEGF-D may also play a role in new blood vessel growth as well, especially during pathological states such as tumor growth.

1.3.1.4. VEGF-E

VEGF-E is not a mammalian VEGF homolog, but rather a viral protein encoded by the parapoxvirus Orf virus, which preferentially utilizes kinase-insert domain-receptor (KDR)/fetal liver kinase-1 (Flk-1) receptor and carries a potent mitotic activity without heparin-binding domain (Ogawa et al., 1998). VEGF-E shares 22% sequence identity to VEGF-A.

1.3.2 VEGF receptors

VEGF ligands mediate their angiogenic effects via several different receptors. Two receptors were originally identified on endothelial cells and characterized as the specific tyrosine kinase receptors VEGFR-1 (also referred to as fms-like tyrosine kinase 1 [Flt-1]) (Shibuya et al., 1990). VEGFR-2 (also referred to KDR, (Terman, 1992) and the murine homologue, Flk-1) (Matthews et al. 1991). These two receptors share 44% homology and possess a characteristic structure consisting of seven extracellular immunoglobulin-like domains, a single transmembrane domain, and a consensus tyrosine kinase domain interrupted by a kinase insert domain (Shibuya et al., 1990; Terman, 1991). More recently, an additional tyrosine kinase receptor, VEGFR-3 (also referred to as fms-like tyrosine kinase 4 [Flt-4]), was identified and has been found to be primarily associated with lymphangiogenesis (Kaipainen et al., 1995; Paavonen et al., 2000). The various members of the VEGF family have differing binding specificities for each of these receptors. All of the VEGF-A isoforms bind to both VEGFR-1 and VEGFR-2, whereas PlGF-1, PlGF-2 and VEGF-B are specific for VEGFR-1 binding and activation (Park et al., 1994; Olofsson et al., 1998; Silvestre et al., 2003). Naturally occurring heterodimers of VEGF-A and PlGF have also been identified that can bind to and activate VEGFR-2 (Cao et al., 1996; DiSalvo et al., 1995). VEGF-E specifically interacts with VEGFR-2 whereas VEGF-C and VEGF-D interact with both VEGFR-3 and VEGFR-2 (Shibuya et al., 2003; Achen et al., 1998; Joukov et al., 1996).

1.3.2.1 VEGF-R1

VEGFR-1 is a receptor for VEGF-A and has the unique ability to bind VEGF-B and PlGF. VEGFR-1 is critical for physiologic and developmental angiogenesis. VEGFR-1 null mice die in utero between days 8.5 to 9.5 due to excessive hemangioblast proliferation and poor organization of vascular structures (Fong et al., 1995). VEGFR-1 was initially thought to be a negative regulator of VEGF activity either by acting as a decoy receptor for VEGF (Hiratsuka et al., 1998) or by downregulating VEGFR-2–mediated signaling (Dunk et al., 2001). VEGF-mediated stimulation of VEGFR-1 autophosphorylation and signaling in endothelial cells is weak when compared to signaling through VEGFR-2 (Waltenberger et al., 1994). A repressor motif has been identified in the juxtamembrane region of VEGFR-1 that impairs phosphatidylinositol 3-kinase (PI3K) signaling and endothelial cell migration in response to VEGF stimulation (Gille et al., 2000; Zeng et al., 2001). However, other studies have indicated that VEGFR-1 has a positive, functional role in certain cell types, participating in monocyte migration (Barleon et al., 1996; Clauss et al., 1996), recruitment of endothelial cell progenitors (Lyden et al., 2001) increasing the adhesive properties of natural killer cells (Chen et al., 2002) and inducing growth factors from liver sinusoidal endothelial cells (LeCouter et al., 2003). A recent study (Autiero et al, 2003) showed that activation of VEGFR-1 by PlGF resulted in transphosphorylation of VEGFR-2 in endothelial cells co-expressing these receptors. Furthermore, VEGF/PlGF heterodimers are capable of activating intramolecular VEGFR cross talk through formation of VEGFR-1/VEGFR-2 heterodimers. Other recent studies have shown that during pathologic conditions such as tumorigenesis, VEGFR-1 is a potent, positive

regulator of angiogenesis (Hiratsuka et al., 2001). A naturally occurring, alternatively spliced soluble form of VEGFR-1 (sVEGFR-1) also exists (Kendall et al., 1993). sVEGFR-1 may function to reduce or modulate endogenous VEGF or PlGF activity.

1.3.2.2. VEGF-R2

VEGFR-2 mediates the majority of the downstream effects of VEGF-A in angiogenesis, including microvascular permeability, endothelial cell proliferation, invasion, migration, and survival (Zeng et al., 2001; Millauer et al., 1993). The importance of VEGFR-2 in vasculogenesis is demonstrated by the fact that hetero and homozygous knockout mice die in utero of defects in blood island formation and vascular development (Shalaby et al., 1995). VEGFR-2–mediated proliferation of endothelial cells involves activation of a phospholipase C gamma-protein kinase C-Raf-MAP kinase signaling pathway, whereas survival and migration is believed to involve PI3K and focal adhesion kinase, respectively (Veikkola et al., 2000; Abedi et al., 1997).

1.3.2.3 VEGFR3

VEGFR-3 is a receptor tyrosine kinase originally cloned from a human leukemia cell line and human placenta (Pajusola et al., 1993; Galland et al., 1993). VEGFR-3 preferentially binds VEGF-C and VEGFD. VEGFR-3 is expressed throughout the embryonic vasculature, but during development and in the adult, its expression is limited to lymphatic endothelial cells (Kaipainen et al., 1995). Homozygous deletion of the VEGFR-3 gene in mice leads to embryonic death at day 10

to 12.5, with an underdeveloped yolk sac, poor perineural vasculature, and pericardial fluid accumulation (Dumont et al., 1998). VEGFR-3 activation and upregulation of its ligands have been observed in certain neoplastic conditions, including breast cancer and melanoma with elevated levels of VEGF-C or VEGF-D associated with lymph node metastasis in patients (Achen et al., 2001; Valtola et al., 1999; Pepper et al., 2003).

1.3.3 Functions of VEGF

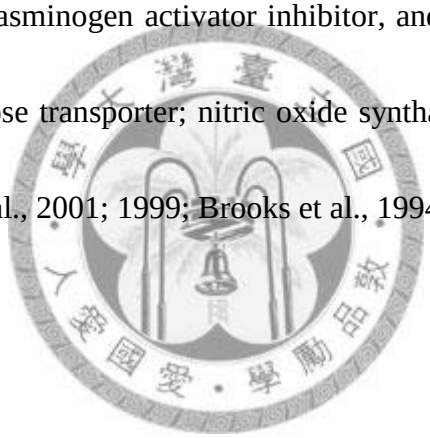
1.3.3.1 Vessels Permeability

VEGF is also termed vascular permeability factor (VPF) (Dvorak et al., 1995). In fact, VEGF is one of the most potent inducers of vascular permeability known - 50,000-fold more potent than histamine (Dvorak, 2002). This ability to enhance microvascular permeability is one of the most important properties of VEGF, especially with regards to the hyperpermeability of tumor vessels that is thought to be largely attributable to tumor cell expression of VEGF. It has been suggested that the increase in permeability results in the leakage of several plasma proteins, including fibrinogen and other clotting proteins. This can lead to the deposition of fibrin in the extravascular space, which subsequently retards the clearance of edema fluid and transforms the normally antiangiogenic stroma of normal tissues into a proangiogenic environment (Dvorak et al., 1995; 2002). VEGF increases permeability in a variety of vascular beds, including the skin, peritoneal wall, mesentery, and diaphragm, and can lead to pathologic conditions such as malignant ascites

and malignant pleural effusions (Yoshiji et al., 2001; Yuan et al., 1996).

1.3.3.2 Endothelial cell activation

VEGF exerts a number of different effects which include changes in endothelial cell morphology, cytoskeleton alterations, and stimulation of endothelial cell migration and growth. VEGF causes increased expression of a variety of different endothelial cell genes, including procoagulant tissue factor and fibrinolytic pathway proteins, such as urokinase, tissue-type plasminogen activator, type 1 plasminogen activator inhibitor, and urokinase inhibitor; and matrix metalloproteases; GLUT-1 glucose transporter; nitric oxide synthase; and integrins (Dvorak et al., 2002; Zachary, 2001; Eliceiri et al., 2001; 1999; Brooks et al., 1994).



1.3.3.3 Endothelial cell survival

VEGF was first shown to act as a survival factor for retinal endothelial cells (Alon et al., 1995). *In vitro*, VEGF has been shown to inhibit apoptosis by activating the PI3K-Akt pathway (Gerber et al., 1998; Gerber et al., 1998; Thakker et al., 1999) in addition to upregulating antiapoptotic proteins such as bcl-2 and A1. VEGF has also been shown to activate focal adhesion kinase (FAK) and associated proteins that have been shown to maintain survival signals in endothelial cells (Abedi et al., 1997; Zachary et al., 2001).

1.3.3.4 Endothelial cell proliferation

VEGF is a mitogen for endothelial cells. This endothelial cell proliferation appears to involve VEGFR-2–mediated activation of extracellular kinases Erk1/2 in addition to another member of the MAP kinase family, JNK/SAPK (Zachary et al., 2001; Meadows et al., 2001).

1.3.3.5 Endothelial cell invasion and migration

Degradation of the basement membrane is necessary for endothelial cell migration and invasion and is an important early step in the initiation of angiogenesis. VEGF induces a variety of enzymes and proteins important in the degradation process, including matrix-degrading metalloproteinases, metalloproteinase interstitial collagenase, and serine proteases such as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (TTPA) (Zachary et al., 2001; Choong et al., 2003). Activation of these various compounds leads to a prodegradative environment that facilitates migration and sprouting of endothelial cells (Ferrara et al., 1997). The intracellular mechanisms by which VEGF leads to increased endothelial cell migration are not entirely clear, but appear to involve FAK-associated signaling leading to focal adhesion turnover and actin filament organization as well as p38 MAPK-induced actin reorganization (Abedi et al., 1997; Zachary et al., 2001)

1.3.4 Expression of VEGF on tumor cells

Several studies have reported the presence of VEGFRs on liquid and solid tumor cells,

including those of non−small cell lung carcinoma, melanoma, prostate carcinoma, leukemia, mesothelioma, and breast carcinoma (Dias et al., 2001; Bellamy et al., 1999; Decaussin et al., 1999; Dias et al., 2000; Ferrer et al., 1999; Hayashibara et al., 2001; Lacal et al., 2000; Price et al., 2001). Various VEGF ligands support tumor growth, not only by inducing angiogenesis, but also by acting directly through VEGFRs expressed by tumor cells. Moreover, since the vast majority of solid tumors and a variety of hematologic malignancies have the capacity to express VEGF, expression of VEGFRs by tumor cells implicates a potential role for VEGF/VEGFR autocrine loops in these tumors.

1.3.5 Expression of VEGF on OSCCs

VEGF expression is up-regulated significantly during the transition from NOM, through dysplasia to OSCC. For the dysplastic lesions, no correlation is found between VEGF expression and grade of dysplasia (Johnstone et al., 2007). The head and neck squamous cell carcinoma (HNSCC) tumor cells express VEGFR-1, VEGFR-2, and VEGFR-3 in all specimens evaluated. Staining for all 3 receptors is also found on tumor associated macrophages and fibrobasts, except that VEGFR-2 is not present on fibroblasts. Staining intensity for VEGFR-1 and VEGFR-2 is significantly higher in tumor cells and macrophages than in vascular endothelial cells (VECs) stained for the same receptor. In HNSCC, as well as other tumor systems, VEGF has been shown to be expressed by tumor cells and to induce proliferation of adjacent VECs via a paracrine

mechanism (Benefield et al., 1996; Petruzzelli et al., 1997). Recent evidence has demonstrated that VEGF may also have a direct effect on tumor cell activity (Masood et al., 2001). These data suggest a possible autocrine mechanism for VEGF-regulated vasculogenesis and tumorigenesis.

1.4 PlGF and cancers

1.4.1. Placenta growth factor, PlGF

PlGF, shares 53% identity with the platelet-derived growth factor (PDGF)-like region of VEGF (Nissen et al., 1998). The human PlGF gene has been mapped to chromosome 14q24 (Mattei et al., 1996). PlGF is anticipated to have 149 amino acids and is encoded by 7 exons that span 800 kb (Roy et al., 2006). By alternative splicing, 4 forms of PlGF protein are generated: PlGF-1, PlGF-2, PlGF-3, and PlGF-4 (Maglione et al., 2000). Only PlGF-2 is capable of binding heparin (Hauser and Weich, 1993). Unlike VEGFA, which binds to both FLT1 and FLK1, PlGF binds to FLT1 but not FLK1, and it also binds to neuropilin 1 and 2 (Persico et al., 1999; Migdal et al., 1998)

1.4.2. Distribution of PlGF in tissues

PlGF is abundant in the trophoblasts of the placenta at the middle to late stages of pregnancy. The amount of PlGF persistently increases toward the terminal development of the placenta, and its

biosynthesis seems to be limited to trophoblasts and stromal cells (Maglione et al., 1993; Hauser et al., 1993). This temporal pattern of PlGF production may reflect a role in preventing excessive neovascularization and overgrowth of the placenta tissue by downregulating angiogenesis. In addition to the placenta, the gene is abundantly expressed in the thyroid under normal physiological conditions (Viglietto et al., 1995).

1.4.3. Impact of PlGF gene knockout mice

Knockout of only one allele of the Vegfa gene, encoding VEGF-A, is lethal in the mouse embryo (heterozygous lethality) with impaired angiogenesis and blood island formation leading to developmental abnormalities (Ferrara et al., 1996; Carmeliet et al., 1996). In contrast, several studies suggest that genetic deletion of either Vegfb or PlGF genes does not result in obvious impairment of the vascular system (Luttun et al., 2002; Aase et al., 2001; Bellomo et al., 2001). Although PlGF knockout mice under normal physiological conditions do not exhibit obvious phenotypic changes, these animals show vascular defects under several pathological settings (Luttun et al., 2002; Fischer et al., 2007; Carmeliet et al., 2001; Luttun et al., 2002). The response to VEGF-A is also impaired, including reduced angiogenesis and vascular permeability under ischemic insult in a hind limb model and recruitment of monocytes and macrophages in a skin wound assay. Transplantation of wild-type bone marrow restores angiogenesis and collateral growth in this knockout model under ischemic conditions, which suggests that PlGF may contribute to

vessel growth in adult animals through the mobilization of bone marrow–derived cells (Carmeliet et al., 2001).

1.4.4 Mechanisms of PlGF

Several studies have analysed the molecular mechanisms of PlGF. This growth factor stimulates angiogenesis, in part by enhancing VEGF signalling. It displaces VEGFA from FLT1, which liberates VEGFA and allows it to activate FLK1 and enhance VEGF-driven angiogenesis (Park et al., 1994). PlGF also upregulates the expression of VEGFA, fibroblast growth factor 2 (FGF2), platelet-derived growth factor-β (PDGFB), matrix metalloproteinases (MMPs), and other angiogenic factors (Marcellini et al., 2006; Roy et al., 2005). Moreover, the binding of PlGF to FLT1 leads to intermolecular crosstalk between FLT1 and FLK1, which amplifies FLK1 signalling and consequently enhances VEGF-driven responses (Autiero et al., 2003). These effects of PlGF suggest that endothelial cells enhance their own responsiveness to VEGFA by producing PlGF (Autiero et al., 2003). However, PlGF is also capable of inducing its own signals through FLT1 independently of VEGFA. Indeed, gene expression profiling of endothelial cells has revealed that PlGF signals directly through FLT1 and switches on a number of pro-angiogenic genes (Autiero et al., 2003; Schoenfeld et al., 2004).

1.4.5. PlGF in primary human tumors

The first investigation conducted on PlGF expression in tumors was undertaken by Takahashi et al. (1994), who demonstrated that PlGF is expressed in hypervascular renal cell carcinoma. PlGF levels correlate with serosal invasion, lymph-node metastasis, tumor stage, and survival in gastric cancer (Chen et al., 2004); with disease progression and survival in colorectal cancer (Wei et al., 2005); with tumor stage in non-small cell lung carcinoma (Zhang et al., 2005); with recurrence, metastasis and mortality in breast cancer (Parr et al., 2005); and with post-surgical early recurrence of hepatocellular carcinoma (Ho et al., 2007). Plasma PlGF protein levels are also upregulated and correlate with tumor grade and survival in patients with renal cell carcinoma (Matsumoto et al., 2003). PlGF is not only produced by malignant cells, but also by endothelial cells, smooth muscle cells, pericytes, cancer-associated fibroblasts, tumour-associated macrophages and various other inflammatory cells in the tumor stroma (Fischer et al., 2007; Luttun et al., 2002; Carmeliet et al., 2001; Yonekura et al., 1999). Tumor cells can also induce PlGF expression by fibroblasts via crosstalk between tumor cells and the stroma. However, not all studies have found increased PlGF levels in tumors. PlGF mRNA is downregulated in thyroid tumors (Viglietto et al., 1995), and is undetectable in ovarian tumors (Sowter et al., 1997). PlGF expression is apparently lower in prostatic tumors than in normal prostatic tissue (Matsumoto et al., 2003). A similar situation occurs in the thyroid, where strong PlGF expression has been noted in normal tissue and its production is decreased in thyroid tumors (Viglietto et al., 1995).

1.4.6 PlGF as a positive regulator of tumorigenesis and metastasis

In the transgenic PLGF-expressing mice, overexpression of PlGF in keratinocytes is also linked to an increase in tumor growth, invasiveness, and the numbers and sizes of metastases in animals inoculated intradermally with B16-BL6 melanoma cells (Marcellini et al., 2006). Experiments with cultured cancer cell lines suggest that PlGF has a role in controlling cell motility and invasiveness. In breast cancer cell lines, exogenous addition of PlGF-2, but not VEGF-A, to culture medium stimulates two key characteristics associated with metastatic potential: motility (detected by cell migration assay) and invasiveness (detected by Matrigel spheroid assay) (Taylor et al., 2007). The PlGF-2–associated stimulation of motility and invasion is suppressed in an *in vitro* system by the addition of a peptide that blocks the heparin-binding site of VEGFR1 or by an antibody to PlGF (Taylor et al., 2007).

1.4.7 PlGF as a negative regulator of tumor growth angiogenesis.

Xu and colleagues (2006) reported that overexpression of PlGF impairs tumor growth in xenograft models. In above study, a full-length PlGF-2 plasmid expression construct was stably transfected into three human cell lines (lung carcinoma, colon carcinoma, and glioblastoma) that produced high amounts of VEGF-A, and clones with the highest amounts of PlGF-2 were selected for implantation (Xu et al., 2006). Although there was no effect on the growth rate of the cell lines in culture, subcutaneous or orthotopic implantation of the PlGF-overexpressing cell lines into mice

revealed an inhibition of tumor growth and a reduction in tumor-associated angiogenesis. The antagonism of VEGF-induced angiogenesis by production of PlGF within the same population of cells has also been demonstrated in a murine model of fibrosarcoma (Eriksson et al., 2002). VEGF-A–PlGF-1 heterodimers fails to activate VEGFR2-mediated signaling and fails to induce angiogenesis *in vitro* and *in vivo*. Although overexpression of human PlGF-1 in murine fibrosarcoma cells does not alter the growth rate of the cells in culture, forced production of PlGF-1 markedly reduces the rate of growth of tumors arising from subcutaneously implanted inoculates.

1.4.8 Epression of PlGF in non-cancerous disease

Pre-eclampsia is characterized by high blood pressure and elevated protein levels in urine during pregnancy. In a normal pregnancy, angiogenesis, and vascular transformation lead to normal placental development, whereas pre-eclamptic pregnancies are subject to abnormal angiogenesis and vascular transformation. Since PlGF is a factor in angiogenesis, testing of the PlGF levels between 15 and 18 weeks allows a doctor to determine whether a patient is at high risk, since the PlGF level in a patient with pre-eclampsia will be lower than average PlGF levels during a normal pregnancy (Schmidt et al., 2009). The new tests identify PlGF in blood earlier and more effectively than do traditional testing measures.

The pathogenesis of chronic obstructive pulmonary disease (COPD) is hypothesized to result from an imbalance of proteases and antiproteases in the lung. The expression of PlGF increases as a

response of airway epithelial cells to proinflammatory cytokines. The sustained stimuli of cytokines and PlGF subsequently reduce VEGF expression and promote the apoptosis of airway epithelial cells through VEGFR. The apoptosis of epithelial cells is considered essential for the pathogenesis of pulmonary emphysema (Cheng et al., 2008).

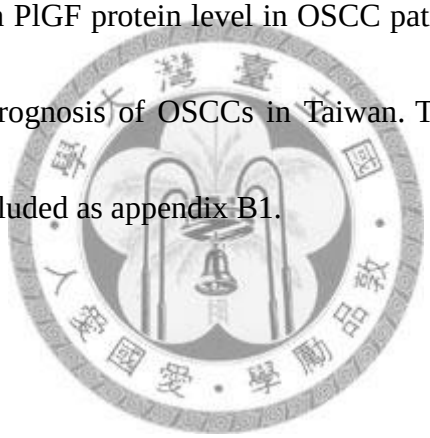
As a risk-predicting biomarker in patients with acute coronary syndrome (ACS), placental growth factor (PlGF) (Lenderink et al., 2006) was recently shown to be important in early and advanced atherosclerotic lesions and coronary plaque rupture. This biomarker could be up-regulated in the crucial mechanism of atherosclerosis in ACS patients (Heeschen et al., 2004) based on subsequent platelet aggregation and systematic thrombosis, which may lead to the acute myocardial infarction (AMI) or sudden cardiac death. Hence, as a member of the vascular endothelial growth factor family (VEGF) (Carmeliet et al., 2001) PlGF may be a primary inflammatory instigator of atherosclerotic plaque instability during the acute phase of ACS.

In our previous study, we had used an immunohistochemical technique to examine the expression of PlGF in 100 specimens of oral squamous cell carcinoma (OSCC) (Cheng et al., 2010). We found that the higher mean PLGF labeling index was significantly associated with OSCCs with positive lymph node metastasis or with more advanced clinical stages. Positive lymph node metastasis and PlGF labeling index > 40% are identified as independent unfavorable prognosis factors. Several previous studies have reported an increased PlGF mRNA expression in tumor tissues and increased serum PlGF protein levels in patients with several human cancers. Thus, in

this study we assessed whether the PlGF mRNA expression in OSCC tissues and serum PlGF protein level in OSCC patients could be valuable biomarkers to predict the therapeutic effect, progression, recurrence and prognosis of OSCC patients.

Specific goals:

1. To assess whether the expression of PlGF mRNA in OSCC tissues could be used to predict the progression, recurrence and prognosis of OSCCs in Taiwan.
2. To evaluate whether the serum PlGF protein level in OSCC patients could be used to predict the progression, recurrence and prognosis of OSCCs in Taiwan. This work was published by Oral Oncology and the article is included as appendix B1.



II. Materials and methods

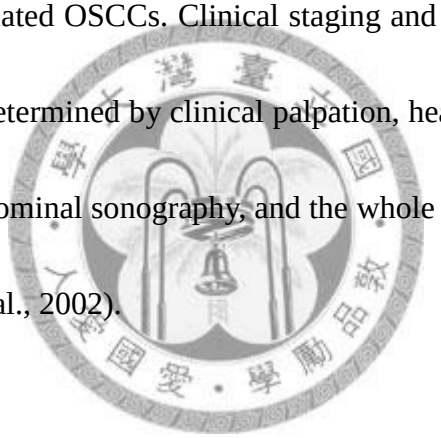
Part I: Tisssue placenta growth factor mRNA

2.1.1 Patients and oral cancer specimens

This study has been reviewed and approved by the Institutional Review Board and an informed consent was obtained from each patient before collection of surgical samples. Sixty-three OSCC patients (59 men and 4 women; mean age 56 years; range 33-81 years) were included in this study. Paired surgical samples were collected from OSCC and adjacent normal-looking oral mucosal (non-OSCC) tissues. All tissues were freshly embedded in OCT compound, snap frozen, and kept at -80˚C until use. PlGF mRNA levels in both OSCC and non-OSCC tissues were measured by quantitative RT-PCR. Diagnosis of OSCC was based on histological examination of hematoxylin and eosin-stained tissue sections.

All OSCC patients received total surgical excision of their tumors plus either selective (39 patients) or radical neck dissection (24 patients) at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan during the period from January 2008 to December 2011. 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, postoperative concurrent chemoradiation therapies were also included in the treatment protocol. In this study, 4 patients received adjuvant radiotherapy and another 33 patients received both radiotherapy and chemotherapy after surgery. None of the patients had received any form of tumor-specific therapy before total surgical excision of the lesion. Of the 63 cases of OSCC, 23 were buccal mucosa, 19 tongue, 10 gingiva, 6 palate, and 5 floor of the mouth cancers. Histological features of OSCC were further classified into three different types (well-, moderately-, and poorly-differentiated OSCC). Of the 63 OSCC cases, there were 59 (94%) well- and 4 (6%) moderately-differentiated OSCCs. Clinical staging and TNM status of OSCCs at initial presentation of the tumor were determined by clinical palpation, head-and-neck magnetic resonance imaging (MRI), chest X-ray, abdominal sonography, and the whole body bone scan according to the guidelines from AJCC (Sobin et al., 2002).



2.1.2 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 2 or more areca quids daily for at least one year, as cigarette smokers when they smoked every day for at least one year and consumed more than 50 packs of cigarettes per year, and as alcohol drinkers when they drank more than four days and consumed more than 20 g of pure alcohol per week for at least one year. According to these definitions, 56

(88%) patients were areca quid chewers, 56 (88%) were smokers, and 51 (81%) 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.

2.1.3 Quantitative real-time reverse transcription-polymerase chain reaction

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 PlGF (ID: Hs00182176_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: Hs99999905_m1) on an ABI Prism real-time PCR system (Applied Biosystem) as previously described (Wei et al., 2005). 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 PlGF mRNA, standardized against the amount of GAPDH mRNA, was expressed as $-\Delta C_T = -$ (PlGF C_T – GAPDH C_T). Then, the relative expression level of tissue PlGF mRNA in each

OSCC patients was expressed as $-\Delta C_T = -$ (OSCC C_T – non-OSCC C_T).

2.1.4 Statistical analysis

The mean PlGF mRNA levels between OSCC and non-OSCC tissues were compared by paired t-test. The correlation between the PlGF mRNA levels in OSCC samples and clinicopathological parameters of OSCC patients was analyzed by Student's t-test. Cumulative survival was analyzed with the Kaplan-Meier product-limit method. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (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 SAS 9.1 (SAS Institute Inc., Morrisville, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

Part II: Serum placenta growth factor level

2.2.1 Patients and serum samples

This study has been reviewed and approved by the Institutional Review Board and an informed consent was obtained from each patient before collection of serum samples. Serum samples were collected from 72 patients (64 men and 8 women; mean age 54 years; range 28-77 years) with

OSCC before and 3 months after surgical removal of OSCCs and from 30 healthy control subjects (26 men and 4 women; mean age 29 years, range 23-50 years) before extraction of impacted mandibular third molars. Diagnosis of OSCC was based on histological examination of hematoxylin and eosin-stained tissue sections. All OSCC patients received total surgical excision of their tumors plus either selective (39 patients) or radical neck dissection (33 patients) at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan during the period from January 2007 to December 2010. If involved surgical margin, perineural invasion or lymphovascular permeation of OSCC, or extracapsular spread of metastatic cervical lymph node were detected histologically, postoperative concurrent chemoradiation therapies were also included in the treatment protocol. In this study, 5 patients received adjuvant radiotherapy and another 34 patients received both radiotherapy and chemotherapy after surgery. None of the patients had received any form of tumor-specific therapy before total surgical excision of the lesion. Of the 72 cases of OSCC, 30 were buccal mucosa, 16 tongue, 16 gingiva, 5 palate, and 5 floor of the mouth cancers. Histological features of OSCC were further classified into three different types (well-, moderately-, and poorly-differentiated OSCC). Of the 72 OSCC cases, there were 64 (89%) well- and 8 (11%) moderately-differentiated OSCCs. Clinical staging and TNM status of OSCCs at initial presentation of the tumor were determined by clinical palpation, head-and-neck magnetic resonance imaging (MRI), chest X-ray, abdominal sonography, and the whole body bone scan according to the guidelines from AJCC (Sobn et al., 2002).

2.2.2 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 2 or more areca quids daily for at least one year, as cigarette smokers when they smoked every day for at least one year and consumed more than 50 packs of cigarettes per year, and as alcohol drinkers when they drank more than four days and consumed more than 20 g of pure alcohol per week for at least one year. According to these definitions, 60 (83%) patients were areca quid chewers, 59 (82%) were smokers, and 58 (81%) 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. In addition, all the subjects in the control group were selected according to strict criteria and none of them have areca quid chewing and cigarette smoking habits.

2.2.3 Enzyme-linked immunosorbent assay (ELISA)

Serum PlGF protein levels were measured by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) in triplicate according to the manufacturer's protocol. Serum concentrations of PlGF were expressed as pg/ml of protein.

2.2.4 Statistical analysis

The mean pre- and post-surgery serum PlGF protein levels of OSCC patients and mean serum PlGF protein levels of normal control subjects were compared first by analysis of variance (ANOVA) among groups and then by Student's t-test or paired t-test between any two groups, where appropriate. The correlation between the serum PlGF protein levels and clinicopathological parameters of OSCC patients was analyzed by Student's t-test. Cumulative survival was analyzed with the Kaplan-Meier product-limit method. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). Comparison of cumulative survival between groups was performed using the log-rank test with the Statisca program (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 SAS 9.1 (SAS Institute Inc., Morrisville, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

III. Results

Part I: Increased placenta growth factor mRNA level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma

3.1.1 PlGF mRNA levels in oral cancer tissues

The mean PlGF mRNA levels in the paired OSCC and non-OSCC tissues of 63 OSCC patients are presented in Table 1.1. The lower C_T value is interpreted as having higher initial copy numbers of PlGF mRNA in tissues. We found that the mean PlGF mRNA C_T value was significantly lower in OSCC (7.2 ± 0.3) than in control counterpart of non-OSCC tissues (9.1 ± 0.6, $P < 0.001$) (Table 1.1).

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

In this study, the higher the $-\Delta C_T$, the greater the copy number of PlGF mRNA in tissues. Correlation between the mean PlGF mRNA $-\Delta C_T$ value in 63 paired surgical samples and clinicopathological parameters of 63 OSCC patients is shown in Table 1.2. We found that the mean PlGF mRNA $-\Delta C_T$ value was significantly higher in OSCC patients with larger tumor size (P =

0.030), positive lymph node metastasis (P = 0.003), more advanced clinical stages (P = 0.013), and the presence of loco-regional recurrence (P = 0.039) (Table 1.2). No significant correlation was found between the mean PlGF mRNA $-\Delta C_T$ value and patients' age, gender, cancer location, and histology of OSCC. The PlGF mRNA $-\Delta C_T$ value was also not associated with the areca quid chewing, cigarette smoking, or alcohol drinking habit (data not shown).

3.1.3 Survival analyses

Univariate analysis performed by Cox proportional hazard regression model identified larger tumor size (P = 0.027), positive lymph node metastasis (P = 0.003), advanced clinical stage (P = 0.032), and PlGF mRNA $-\Delta C_T$ value > 2 (P = 0.008, the median PlGF mRNA $-\Delta C_T$ value was 1.9) as correlating with poorer survival of OSCC patients. Multivariate analyses with Cox regression model further identified positive lymph node metastasis (P = 0.019) and PlGF mRNA $-\Delta C_T$ value > 2 (P = 0.016) as two independent unfavorable prognosis factors (Table 1.3). The Kaplan-Meier curve showed that OSCC patients with a PlGF mRNA $-\Delta C_T$ value > 2 had a significantly poorer cumulative recurrence-free survival than those with a PlGF mRNA $-\Delta C_T$ value $\leqq 2$ (P = 0.017, log-rank test, Figure 1.1). In addition, the Kaplan-Meier curve showed that OSCC patients with larger tumor size, nodal metastasis, and advanced clinical stage also had a significantly poorer cumulative recurrence-free survival (Figures 1.2~1.4).

Part II: Increased serum placenta growth factor level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma

3.2.1 Serum PlGF levels in normal and cancer patients

The mean serum PlGF protein levels in 30 normal control subjects and in 72 OSCC patients before and 3 months after surgical excision of the OSCC are presented in Table 2.1. The mean serum PlGF protein level was significantly higher in pre-surgery OSCC patients (19.1 ± 10.7 pg/ml) than in normal control individuals (10.1 ± 4.5 pg/ml, $P < 0.001$). Three months after total surgical removal of the tumors, the mean serum PlGF protein level dropped to 11.0 ± 6.6 pg/ml which was significantly lower than the mean pre-surgery serum PlGF protein level ($P < 0.001$) but had no significant difference to the normal control serum PlGF protein level ($P = 0.519$) (Table 2.1).

3.2.2 Correlation between the serum PlGF protein levels in pre-surgery and clinicopathological parameters of OSCC patients

Correlation between the mean serum PlGF protein levels in pre-surgery OSCC patients and clinicopathological parameters of OSCC patients is shown in Table 2.2. We found that the mean serum PlGF protein level was significantly higher in pre-surgery OSCC patients with larger tumor size ($P = 0.015$), positive lymph node metastasis ($P = 0.001$), more advanced clinical stages ($P = 0.002$), and loco-regional recurrence ($P = 0.037$). No significant correlation was found between the

mean serum PlGF protein level and patients' age, gender, cancer location, and histology of OSCC. The serum PlGF protein level was also not associated with the areca quid chewing, cigarette smoking, or alcohol drinking habit (Table 2.2).

3.2.3 Survival analysis

Univariate analysis performed by Cox proportional hazard regression model identified larger tumor size (P = 0.029), positive lymph node metastasis (P = 0.004), advanced clinical stage (P = 0.031), and serum PlGF protein level > 19.1 pg/ml (P = 0.007) as correlating with poorer survival of OSCC patients. Multivariate analysis with Cox regression model further identified positive lymph node metastasis (P = 0.018) and high serum PlGF protein level (> 19.1 pg/ml, P = 0.014) as independent unfavorable prognosis factors (Table 2.3). The Kaplan-Meier curve showed that OSCC patients with a serum PlGF protein level > 19.1 pg/ml had a significantly poorer cumulative recurrence-free survival than those with a serum PlGF protein levels ≦ 19.1 pg/ml (P = 0.009, log-rank test, Figure 2.1). In addition, the Kaplan-Meier curve showed that OSCC patients with larger tumor size, positive nodal metastasis, more advanced clinical stage had a significantly poorer cumulative recurrence-free survival (Figures 2.2~2.4).

To further investigate whether the serum PlGF protein level could be a biomarker for prediction of the recurrence of OSCC, we chose the serum PlGF protein level greater than 19.1 pg/ml (which was equal to the mean normal control value plus 2 standard deviations) as a cutoff

point for identification of the presence of cancer recurrence. By this definition, the sensitivity, specificity, and positive predictive value for tumor recurrence was 80%, 56%, ad 78%, respectively. This work was published by Oral Oncology and the article is included as appendix B1. Other publication related to this dissertation is included as appendix B2.

IV. Discussion

Part I: Increased placenta growth factor mRNA level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma

In this study, we measured the PlGF mRNA level in 63 paired OSCC and non-OSCC tissue samples. The relatively lower mean PlGF mRNA C_T value in OSCC than in non-OSCC tissues indicated an increased PlGF mRNA expression in OSCC tissues than in non-OSCC tissues. The finding of PlGF mRNA expression in normal-looking oral mucosal (non-OSCC) tissue adjacent to OSCC also suggests that the PlGF mRNA expression is an early event in oral carcinogenesis. Takahashi et al. (1994) found a significantly higher PlGF mRNA expression in hypervascular renal cell carcinoma tissues than in adjacent normal kidney tissues. Chen et al. (2004) reported a significantly higher PlGF protein level in gastric cancer tissues than in the corresponding non-cancerous mucosal tissues. Parr et al. (2005) also demonstrated that the PlGF protein expression is dramatically increased in breast cancer tissues compared with normal breast tissues. Actually, overexpression of PlGF mRNA or protein has been demonstrated in a variety of human carcinomas including breast (Parr et al., 2005), gastric (Chen et al., 2004), lung (Zhang et al., 2005), colorectal (Wei et al., 2005), renal cell (Takahashi et al., 1994), uterine cervical (Kodama et al., 1997), and hepatocellular carcinomas (Ho et al., 2007).

A significant association of higher PlGF mRNA and protein levels with regional nodal metastasis of OSCC was shown in the present and our previous studies (Cheng et al., 2010). The similar findings were also demonstrated in human breast cancer (Parr et al., 2005). The reasons why PlGF increased cancer metastasis could be explained as follows. First, angiogenesis is a pivotal factor for tumor growth and metastasis (Carmeliet et al., 2000). PlGF can promote vessel growth and maturation directly by affecting endothelial and mural cells, as well as indirectly by recruiting pro-angiogenic cell types. PlGF can also increase the expression of VEGFA which is a potent angiogenic factor (Fischer et al., 2008). Furthermore, PlGF displaces VEGFA from FLT1, which liberates VEGFA and allows it to activate FLK1 (also known as VEGFR2) and augment VEGF-driven angiogenesis (Park et al., 1994). PlGF also activates and attracts macrophages that release angiogenic and lymphangiogenic molecules (Selvaraj et al., 2003). In addition, PlGF can promote tumor angiogenesis, lymphangiogenesis, and the formation of the premetastatic niche (Fischer et al., 2008). Second, PlGF promotes the growth, survival and migration of metastatic tumor cells. Third, PlGF enhances the expression of matrix metalloproteinase 9 (MMP9) which facilitates the cancer cell invasion and metastasis (Fischer et al., 2008). Fourth, PlGF directly regulates the motility of human non-small cell lung cancer cells (Chen et al., 2008) and also stimulates *in vitro* motility and invasion of the human breast tumor cell lines (Taylor et al., 2007). Moreover, an antagonistic PlGF/FLT1 peptide can inhibit the growth and metastasis of human breast cancer xenografts (Taylor et al., 2007). Fifth, PlGF inhibits the differentiation of dendritic

cells and in turn suppresses the antigen recognition and anti-tumor immune defense (Fischer et al., 2008). In summary, PlGF 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 showed a significant association of PlGF mRNA overexpression in OSCCs with larger tumor size and positive lymph node metastasis. Because higher T and N statuses always result in more advanced clinical stages of OSCC, it is not difficult to explain why OSCCs with the higher expression of PlGF mRNA are prone to have the more advanced clinical stages. Indeed, high expression of PlGF mRNA or protein is significantly associated with an advanced clinical stage of gastric (Chen et al., 2004), lung (Zhang et al., 2005), and colorectal cancers (Wei et al., 2005).

A significant correlation between the higher PlGF mRNA expression (higher $-\Delta C_T$ value) in OSCCs and the poorer recurrence-free survival in OSCC patients was demonstrated in this study. In addition, PlGF mRNA $-\Delta C_T$ value > 2 was identified as an independent unfavorable prognosis factor by multivariate analyses with Cox regression model. Previous study also showed a significant association of higher level of PlGF mRNA with a shorter survival in patients with colorectal carcinoma (Wei et al., 2005). In addition, an increased level of PlGF protein is significantly related to poor prognosis in patients with breast (Parr et al., 2005) or gastric carcinomas (Chen et al., 2004). Furthermore, high PlGF protein or mRNA level is significantly associated with recurrence of breast

cancer (Parr et al., 2005). PlGF protein or mRNA level can also predict the early recurrence after radical resection of hepatocellular carcinoma (Ho et al., 2007). The above findings indicate that PlGF protein or mRNA level may be an important prognostic indicator for patients with certain types of human carcinomas including OSCC.

Our results showed a significant increase in the PlGF mRNA expression in OSCC tissues compared to that in non-OSCC tissues. We also found that the PlGF mRNA level in OSCC samples was significantly correlated with tumor size, N status, clinical staging, and loco-regional recurrence of OSCCs. Moreover, OSCC patients with a higher PlGF mRNA expression had a poorer cumulative recurrence-free survival than those with lower PlGF mRNA levels. PlGF mRNA $-\Delta C_T$ value > 2 was also identified as an independent unfavorable prognosis factors for OSCCs by multivariate analyses. These results indicate that the PlGF mRNA level may be a biomarker for prediction of the progression of OSCCs and the prognosis of OSCC patients in Taiwan.

Part II: Increased serum placenta growth factor level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma

We recently found that the higher PlGF protein expression in OSCC tissues is significantly associated with the more advanced tumor progression and shorter patient survival. Because PlGF is

a soluble peptide, its serum level may indicate the angiogenic activity of the tumor and may serve as a predictive biomarker for cancer progression, recurrence and prognosis. In this study, we measured the pre-surgery and post-surgery serum PlGF protein levels in 72 OSCC patients and found that the higher serum PlGF protein level could return to near normal control value 3 months after total excision of the cancer. The prominent rise of serum PlGF protein value in pre-surgery OSCC patients and the significant fall of serum PlGF protein value after surgical removal of tumor tissue indicate that the OSCC tissue is the major source of PlGF proteins. Our recent study showed the expression of PlGF in OSCC tumor cells with the mean labeling index of 51 ± 19 (Cheng et al., 2010). Thus, at least part of the cancer cell-produced PlGF proteins may secrete into stromal tissue, diffuse into adjacent vascular or lymphatic vessels, and drain into systemic circulation, finally resulting in an increase in serum PlGF protein levels in pre-surgery OSCC patients. A previous study demonstrated a persistent increase in plasma PlGF protein levels on postoperative day 1, 3, and 7-20 after minimally invasive resection of colon cancer. The plasma PlGF protein level dropped to the preoperative baseline level on postoperative day 21-27 (Shantha ei al., 2011). Although the cause of the postoperative increase in plasma PlGF protein level was unclear, this study also suggested that the cancer tissue is the major source of PlGF proteins.

In addition, the higher pre-surgery serum PlGF protein levels were significantly associated with larger tumor size, positive lymph node metastasis, more advanced clinical stages, loco-regional recurrence, and poorer recurrence-free survival in this study. This suggests that the serum PlGF

protein level can also predict the progression, recurrence and prognosis of OSCC. Indeed, an elevated serum PlGF protein level has been reported in a variety of human carcinomas including renal cell (Matsumoto et al., 2003), pancreatic (Chang et al., 2008; Sabbaghian et al., 2010), and colorectal carcinomas (Rahbari et al., 2011; Wei et al., 2009). Furthermore, the plasma PlGF protein level is an independent prognostic factor for renal cell carcinoma (Matsumoto et al., 2003), and higher preoperative serum PlGF protein levels are associated with higher risk of recurrence and poorer prognosis of stage III colorectal cancer (Wei et al., 2009). However, Chang et al. (2008) showed no correlation between the serum PlGF protein concentration and overall survival in patients with pancreatic cancer. Rahbari et al. (2011) found that circulating PlGF protein levels were correlated inversely with recurrence-free survival in patients undergoing curative resection of colorectal liver metastases by multivariate analysis.

To the best of our knowledge, this is the first report demonstrating that serum PlGF protein levels are significantly correlated with the progression, recurrence and prognosis of OSCC. There are several reasons that can explain why the higher serum PlGF protein level is significantly associated with larger tumor size and positive lymph node metastasis. First, angiogenesis is a key factor for tumor growth and metastasis (Carmeliet et al., 2000). PlGF can stimulate vessel growth and maturation directly by increasing the proliferation and differentiation of endothelial and mural cells, as well as indirectly by recruiting pro-angiogenic cell types (Fischer et al., 2008). Second, PlGF can activate and attract macrophages that release angiogenic and lymphangiogenic factors

(Selvvaraj et al., 2003). Third, PlGF promotes the growth, survival and migration of metastatic tumor cells (Fischer et al., 2008). Fourth, PlGF increases the expression of matrix metalloproteinase 9 which facilitates the cancer cell invasion and metastasis. Fifth, PlGF inhibits the differentiation of dendritic cells and subsequently suppresses the antigen recognition and anti-tumor immune defense. Therefore, the serum PlGF protein may act as a hormone-like molecule that circulates to appropriate sites and in turn augments tumor angiogenesis and lymphangiogenesis, recruits angiogenic and lymphangiogenic factors-producing macrophages to the tumor microenvironment, increases tumor cell survival, migration and invasion, and inhibits anti-tumor immune activity, finally promoting the OSCC cell growth and metastasis (Fischer et al., 2008).

PlGF expression increases significantly in early gestation, peaks at around 26-30 weeks, and decreases as term approaches (Torry et al., 1998). The reduced PlGF mediates the genesis of preeclampsia and cigarette smoking can reduce the risk of preeclampsia (Mehendale et al., 2007). The serum PlGF protein levels have been reported to be significantly higher among smokers compared to non-smokers among diabetic women and women with a history of preeclampsia (Jeyabalan et al., 2010). Moreover, the serum PlGF protein levels are significantly higher in smokers with chronic obstructive pulmonary disease than in control smokers without chronic obstructive pulmonary disease and control non-smokers (Cheng et al., 2008). The results of aforementioned studies suggest that cigarette smoking can increase the secretion of PlGF proteins into the blood stream (Cheng et al., 2008; Jeyabalan et al., 2010; Mehendale et al., 2007). Although

the difference was not significant, our previous study showed a slightly elevated expression of PlGF protein in OSCC tissues of areca quid chewers and smokers (Cheng et al., 2008) and the present study also revealed a slightly increased serum PlGF protein level in areca quid chewers and smokers compared to non-chewers and non-smokers. Further studies are needed to clarify the exact relation between areca quid chewing and PlGF protein expression or secretion.

Our results showed a significantly higher serum PlGF protein levels in pre-surgery OSCC patients than in normal control subjects. Serum PlGF protein levels dropped to near the normal control levels after surgical removal of the cancer. We also found that higher pre-surgery serum PlGF protein levels were significantly associated with T status, N classification, clinical staging, and loco-regional recurrence. Moreover, OSCC patients with a higher serum PlGF protein level had a significantly poorer cumulative recurrence-free survival than those with a lower serum PlGF protein level. We conclude that the serum PlGF protein level may be a valuable biomarker for prediction of therapeutic effect, progression, recurrence, and prognosis of OSCC.

V. Conclusion

1. There is a significant increase in the PlGF mRNA expression in OSCC tissues compared to that in non-OSCC tissues.
2. Higher PlGF mRNA level in OSCC samples was significantly correlated with larger tumor size, positive lymph node metastasis, more advanced clinical stages, and loco-regional recurrence of OSCCs.
3. OSCC patients with a higher PlGF mRNA expression in cancer tissues had a poorer cumulative recurrence-free survival than those with lower PlGF mRNA levels in cancer tissues.
4. PlGF mRNA $-\Delta C_T$ value > 2 was identified as an independent unfavorable prognosis factors for OSCCs by multivariate analyses.
5. The PlGF mRNA level in cancer tissues may be a biomarker for prediction of the progression of OSCCs and the prognosis of OSCC patients in Taiwan.
6. There is a significantly higher serum PlGF protein levels in pre-surgery OSCC patients than in normal control subjects. Serum PlGF protein levels dropped to near the normal control levels after surgical removal of the cancer.

7. Higher pre-surgery serum PlGF protein levels were significantly associated with larger tumor size, positive lymph node metastasis, more advanced clinical stages, and loco-regional recurrence of OSCCs.

8. OSCC patients with a higher serum PlGF protein level had a significantly poorer cumulative recurrence-free survival than those with a lower serum PlGF protein level.

9. The serum PlGF protein level may be a valuable biomarker for prediction of therapeutic effect, progression, recurrence, and prognosis of OSCC.

VI. Tables

Table 1.1

The mean placenta growth factor (PlGF) mRNA C_T (threshold cycle) value in paired oral squamous cell carcinoma (OSCC) and non-OSCC tissue samples

Groups	Mean PlGF mRNA C_T value ±SD	Mean PlGF mRNA *-ΔC_T value ± SD	p-Value
Non-OSCC (n=63)	9.1 ± 0.6		
OSCC (n=63)	7.2 ± 0.3		
OSCC – non-OSCC (n=63)		1.9 ± 0.7	#$P < 0.001$

#A significant difference in the mean PlGF mRNA C_T value was found between paired OSCC and non-OSCC tissue samples with $P < 0.001$

*-ΔC_T = - (OSCC C_T – non-OSCC C_T)

Table 1.2

Correlation between the mean placenta growth factor mRNA $-\Delta C_T$ values in 63 paired surgical samples and clinicopathological parameters of 63 OSCC patients

	Mean PlGF mRNA $-\Delta C_T$ value ± SD	***P*-Value**
Patients' age (year)		
≦ 50 (n = 21)	1.4 ± 1.1	0.537
> 50 (n =42)	1.8 ± 0.7	
Patients' gender		
Men (n =59)	2.1 ± 1.1	0.136
Women (n = 4)	1.8 ± 0.8	
Cancer location		
Buccal mucosa (n = 23)	1.7 ± 1.1	0.678
Tongue (n = 19)	1.7 ± 0.8	
Other oral mucosal sites (n = 21)	1.8 ± 1.1	
T status		
T1 + T2 (n = 39)	1.0 ± 0.3	**0.030**
T3 + T4 (n = 24)	2.3 ± 0.2	
N status		
N0 (n = 41)	1.1 ± 0.1	**0.003**
N1 + N2 + N3 (n = 22)	2.5 ± 0.3	
Clinical staging		
Stage 1 + 2 (n = 29)	1.1 ± 0.2	**0.013**
Stage 3 + 4 (n = 34)	2.3 ± 0.3	
Loco-regional recurrence		
Without (n =52)	1.2 ± 0.2	**0.039**
With (n = 11)	2.1 ± 0.4	
Histology of OSCC		
Well-differentiated (n = 59)	2.1 ± 0.2	0.131
Moderately-differentiated (n = 4)	1.8 ± 0.5	

* $-\Delta C_T$ = (OSCC C_T – non-OSCC C_T)

Table 1.3

Univariate and multivariate recurrence-free survival analyses of the placenta growth factor mRNA expression and clinicopathological parameters of OSCC patients by Cox proportional hazard regression model

Factor	Hazard ratio (95% CI)	*p*-Value
Univariate		
T status (T1 + T2 vs. T3 + T4)	6.049 (2.963-10.266)	**0.027**
N status (N0 vs. N1 + N2 + N3)	11.552 (5.745-21.081)	**0.003**
Clinical stage (Stage 1 + 2 vs. stage 3 + 4)	5.349 (1.076-8.495)	**0.032**
PlGF mRNA $-\Delta C_T$ value (≦ 2.0 vs. > 2.0)	9.275 (3.578-18.167)	**0.008**
Multivariate		
N status (N0 vs. N1 + N2 + N3)	8.294 (3.059-16.685)	**0.019**
PlGF mRNA $-\Delta C_T$ value (≦ 2.0 vs. > 2.0)	7.285 (3.566-18.274)	**0.016**

Table 2.1

The mean serum placenta growth factor (PlGF) levels in normal control subjects and in oral squamous cell carcinoma (OSCC) patients before and 3 months after total surgical excision of OSCCs

Groups	Mean serum PlGF ± SD (pg/ml)	*P*-Value
Normal controls (n = 30)	10.1 ± 4.5	
Pre-surgery OSCC patients (n = 72)	19.1 ± 10.7	$^{*}P < 0.001$
Post-surgery OSCC patients (n = 72)	11.0 ± 6.6	$^{**}P < 0.001$

The mean serum PlGF level was significantly higher in pre-surgery OSCC patients than in normal controls ($^{*}P < 0.001$) or in post-surgery OSCC patients ($^{**}P < 0.001$). No significant difference in the mean serum PlGF level was found between normal controls and post-surgery OSCC patients ($P = 0.519$).

Table 2.2

Correlation between the mean serum placenta growth factor (PlGF) levels in pre-surgery oral squamous cell carcinoma (OSCC) patients and clinicopathological parameters of 72 OSCC patients

Groups	**Mean serum PlGF level ± SD**	***P*-Value**
Patients' age (year)		0.059
≦ 50 (n = 32)	19.8 ± 12.0	
> 50 (n = 40)	14.9 ± 9.7	
Patients' gender		0.978
Men (n = 64)	19.1 ± 11.0	
Women (n = 8)	19.0 ± 8.9	
Cancer location		0.964
Buccal mucosa (n = 30)	19.1 ± 11.7	
Tongue (n = 16)	18.1 ± 8.8	
Other oral mucosal sites (n = 26)	19.7 ± 10.6	
T status		**0.015**
T1 + T2 (n = 47)	16.9 ± 8.7	
T3 + T4 (n = 25)	23.2 ± 12.9	
N status		**0.001**
N0 (n = 50)	16.4 ± 8.3	
N1 + N2 + N3 (n = 22)	25.0 ± 13.3	
Clinical staging		**0.002**
Stage 1 + 2 (n = 38)	15.4 ± 7.5	
Stage 3 + 4 (n = 34)	23.2 ± 12.3	
Loco-regional recurrence		**0.037**
Without (n = 62)	17.9 ± 12.0	
With (n = 10)	26.1 ± 4.6	
Histology of OSCC		0.558
Well-differentiated (n = 64)	19.7 ± 12.2	
Moderately-differentiated (n = 8)	18.4 ± 8.9	
Areca quid chewing		0.431
Chewers (n = 60)	19.5 ± 11.4	
Non-chewers (n = 12)	16.8 ± 6.5	
Cigarette smoking		0.588
Smokers (n = 59)	19.4 ± 11.3	
Non-smokers (n = 13)	17.6 ± 8.0	
Alcohol drinking		0.877
Drinkers (n = 58)	19.2 ± 11.4	
Non-drinkers (n = 14)	18.7 ± 7.8	

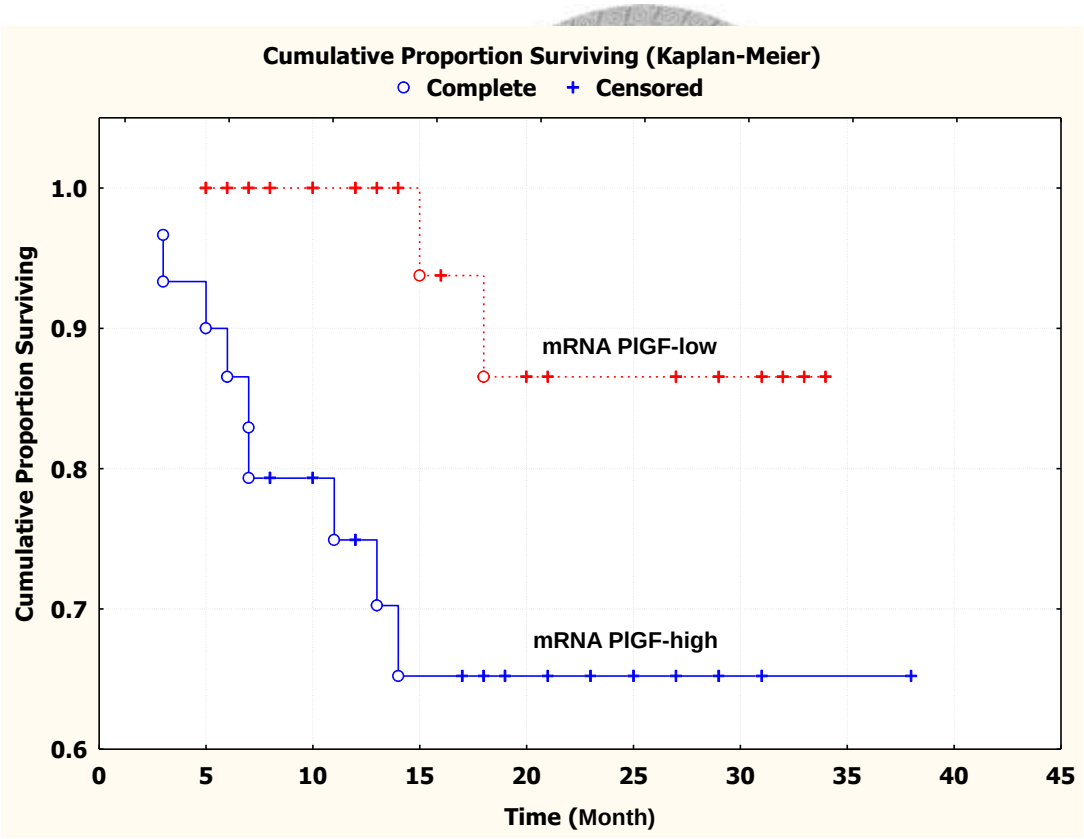
Table 2.3

Univariate and multivariate recurrence-free survival analyses of the serum placenta growth factor (PlGF) protein levels and clinicopathological parameters of OSCC patients by Cox proportional hazard regression model

Factor	Hazard ratio (95% CI)	*P*-Value
Univariate		
T status (T3 + T4 vs T1 + T2)	5.049 (2.872-9.266)	**0.029**
N status (N1 + N2 + N3 vs N0)	9.552 (4.745-19.081)	**0.004**
Clinical stage (Stage 3 + 4 vs Stage 1 + 2)	4.349 (1.056-8.485)	**0.031**
Serum PlGF level (> 19.1 pg/ml vs ≦19.1 pg/ml)	9.235 (3.568-17.167)	**0.007**
Multivariate		
N status (N1 + N2 + N3 vs N0)	8.274 (3.029-16.385)	**0.018**
Serum PlGF level (> 19.1 pg/ml vs ≦19.1 pg/ml)	6.284 (3.536-15.264)	**0.014**

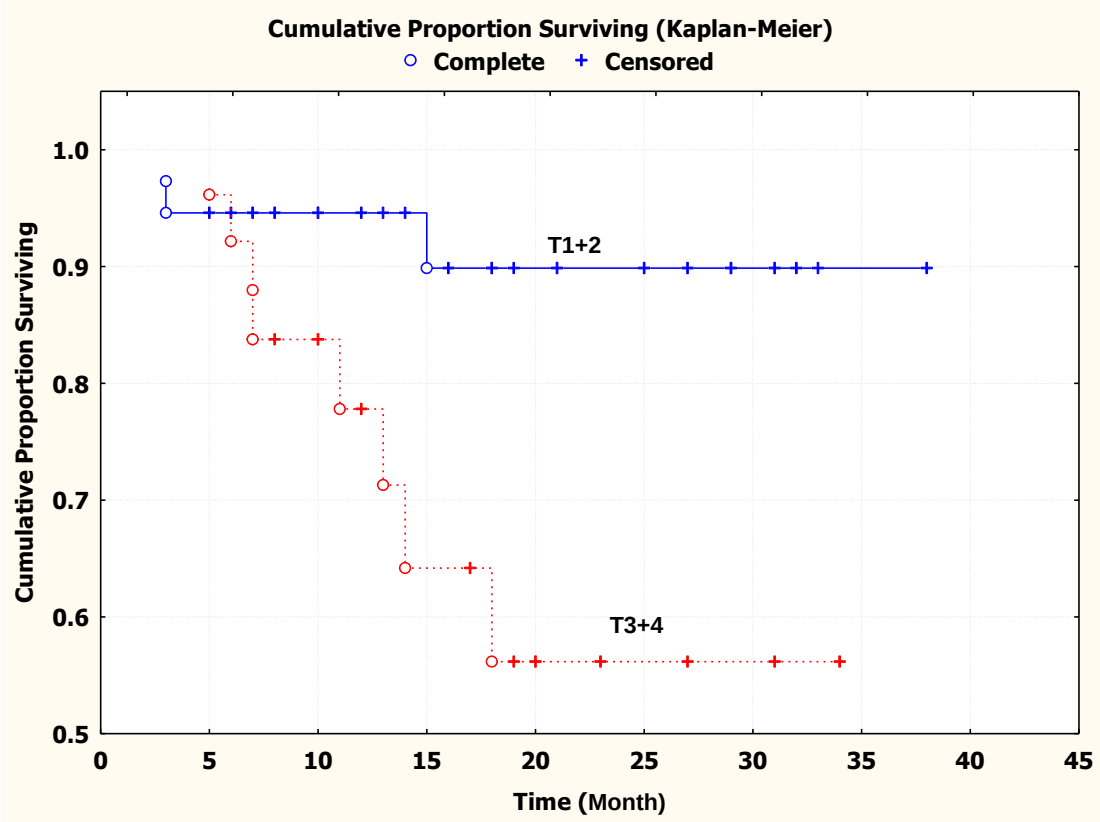
VII. Figures

Figure 1.1 Kaplan-Meier survival curve showing relation between tissue PlGF mRNA levels in 63 OSCC patients and recurrence-free survival in these 63 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with PlGF mRNA $-\Delta C_T$ value > 2 was significantly poorer than that for patients with PlGF mRNA $-\Delta C_T$ value $\leqq$ 2 (log-rank test, P = 0.017).



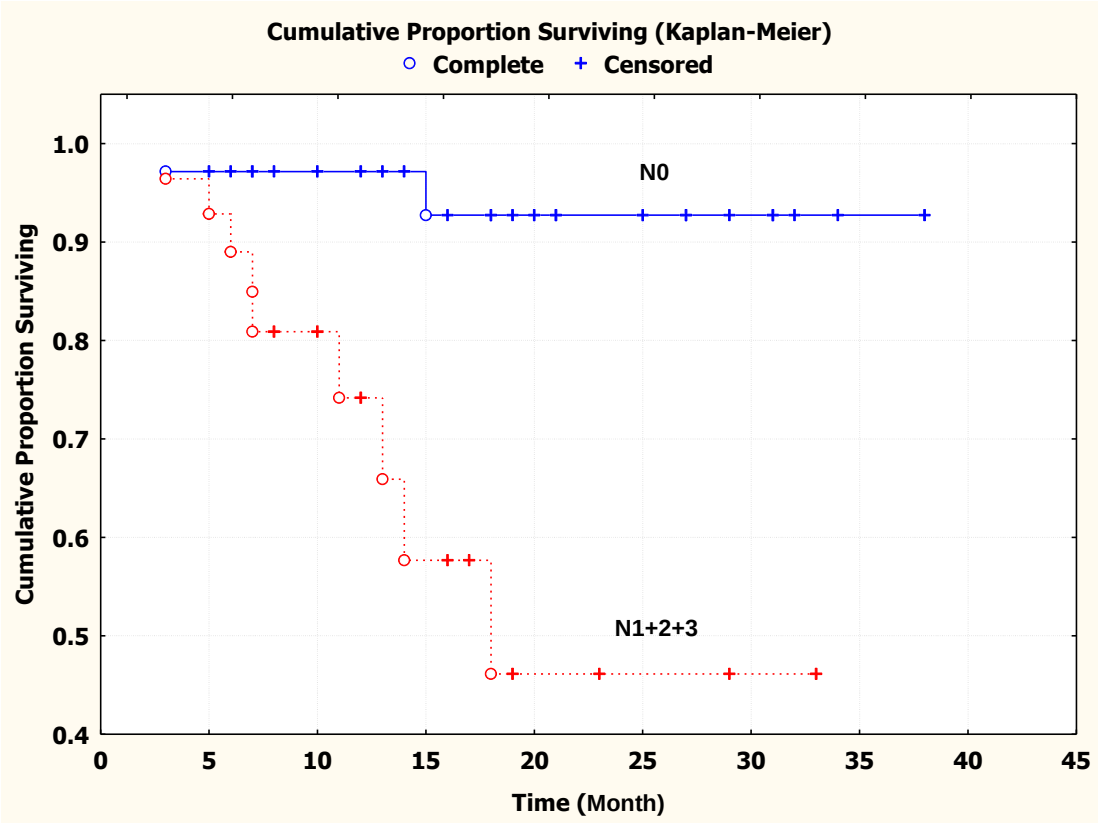
Log-Rank test, P = 0.017

Figure 1.2 Kaplan-Meier survival curve showing relation between tissue PlGF mRNA levels in 63 OSCC patients and recurrence-free survival in these 63 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with larger tumor size (T3+T4) was significantly poorer than that for patients with smaller tumor size (T1+T2) (log-rank test, $P = 0.028$).



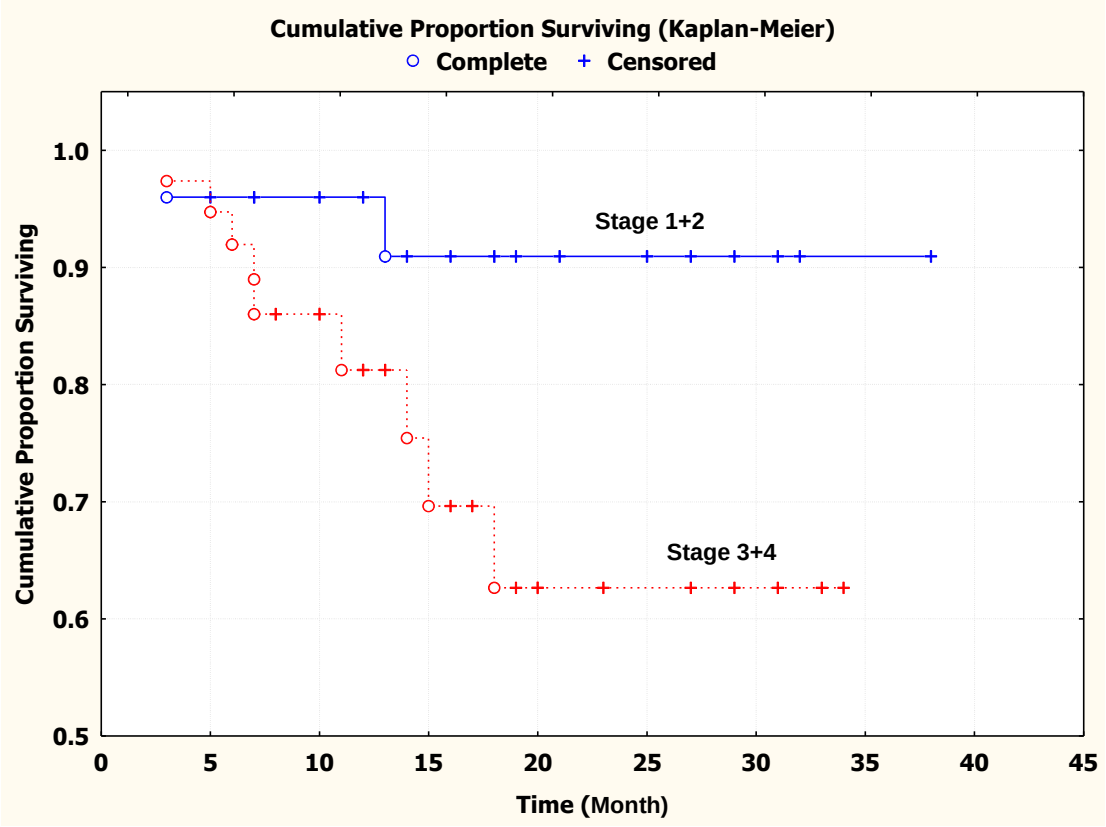
Log-Rank test, $P = 0.028$

Figure 1.3 Kaplan-Meier survival curve showing relation between tissue PlGF mRNA levels in 63 OSCC patients and recurrence-free survival in these 63 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with regional lymph node metastasis (N1+N2+N3) was significantly poorer than that for patients without regional lymph node metastasis (N0) (log-rank test, $P = 0.003$).



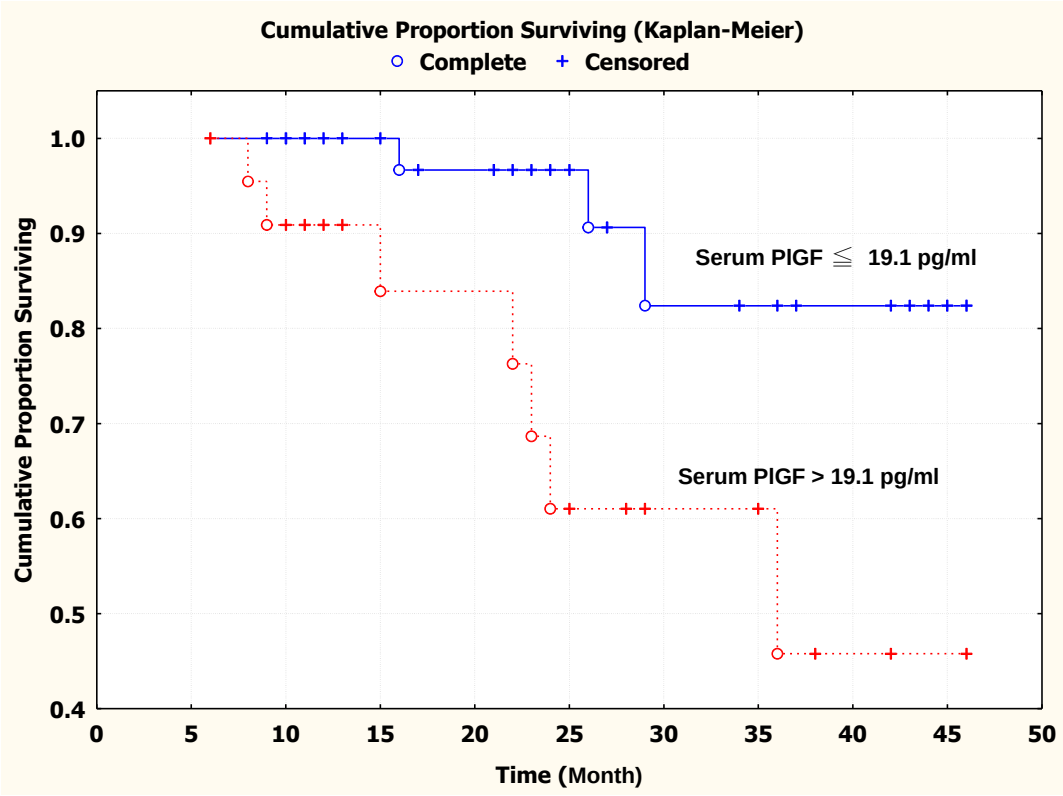
Log-rank test, $P = 0.003$

Figure 1.4 Kaplan-Meier survival curve showing relation between tissue PlGF mRNA levels in 63 OSCC patients and recurrence-free survival in these 63 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with higher clinical stages (stage 3+4) was significantly poorer than that for patients with lower clinical stages (stage 1+2) (log-rank test, $P = 0.033$).



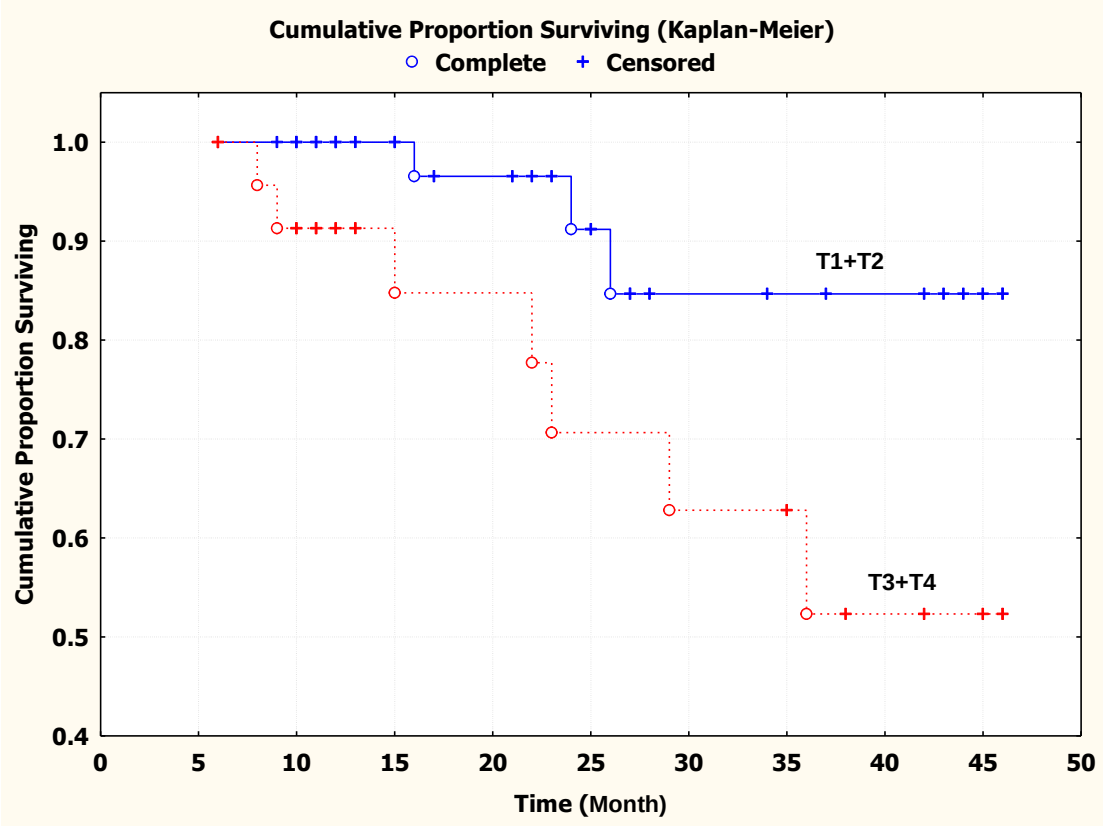
Log-rank test, $P = 0.033$

Figure 2.1 Kaplan-Meier survival curve showing relation between serum PlGF protein levels in 72 OSCC patients and recurrence-free survival in these 72 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with low serum PlGF protein level (≦ 19.1 pg/ml) was significantly higher than that for patients with high serum PlGF protein level (> 19.1 pg/ml) (log-rank test, $P = 0.009$).



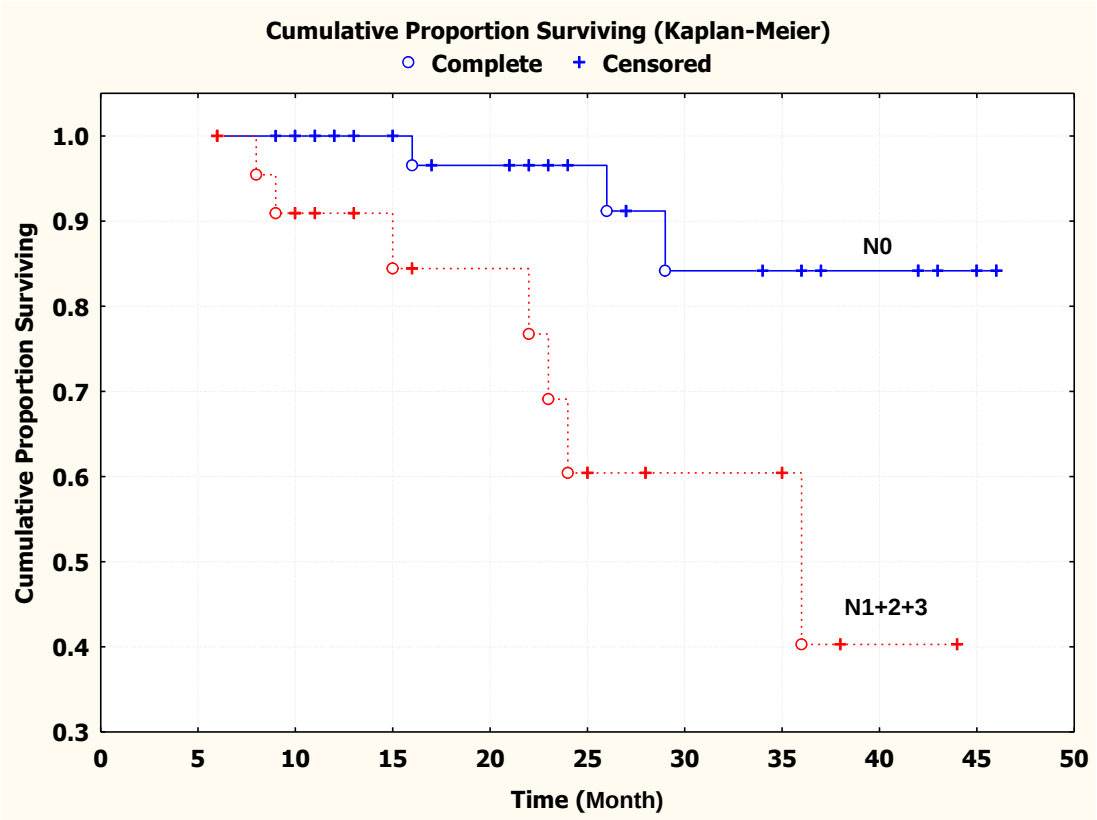
Log-rank test, $P = 0.009$

Figure 2.2 Kaplan-Meier survival curve showing relation between serum PlGF protein levels in 72 OSCC patients and recurrence-free survival in these 72 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with smaller tumor size (T1+T2) was significantly higher than that for patients with larger tumor size (T3+T4) (log-rank test, $P = 0.030$).



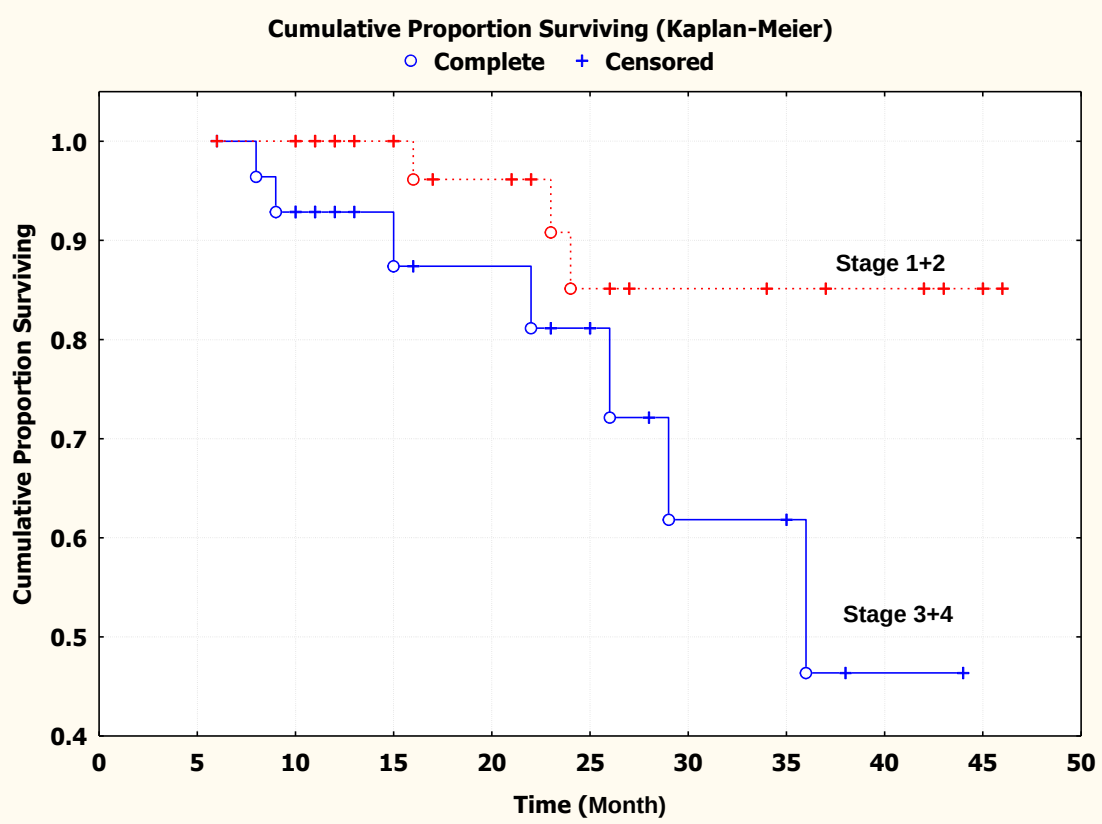
Log-rank test, $P = 0.030$

Figure 2.3 Kaplan-Meier survival curve showing relation between serum PlGF protein levels in 72 OSCC patients and recurrence-free survival in these 72 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients without regional lymph node metastasis (N0) was significantly higher than that for patients with regional lymph node metastasis (N1+N2+N3) (log-rank test, $P = 0.004$).



Log-rank test, $P = 0.004$

Figure 2.4 Kaplan-Meier survival curve showing relation between serum PlGF protein levels in 72 OSCC patients and recurrence-free survival in these 72 OSCC patients. The duration of recurrence-free survival was measured from the beginning of surgery to the time of recurrence (complete) or the last follow-up (censored). The cumulative recurrence-free survival for patients with lower clinical stages (stage 1+2) was significantly higher than that for patients with higher clinical stages (stage 3+4) (log-rank test, $P = 0.032$).



Log-rank test, $P = 0.032$

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IX. Appendix

A: Curriculum Vitae

NAME: Shih-Jung Cheng **BIRTHDAY**: April 3, 1967 **GENDER**: Male

ADDRESS: Department of Oral Maxillofacial Surgery, National Taiwan University Hospital, No. 1, Chang-Te Street, Taipei, Taiwan, 10048

TELEPHONE: (02) 2312-3456 ext. 67509

FAX: (02) 2383-1346

E-MAIL: sjcheng56@ntu.edu.tw

POSITION TITLE:

Asistant Professor, School of Dentistry, National Taiwan University

Attending Doctor, Department of Oral Maxillofacial Surgery, National Taiwan University Hospital

EDUCATION:

Chung-Shan Medical University, Taichung, Taiwan D.D.S. 1993 Dentistry

National Taiwan University, Taipei, Taiwan M.S. 1997 Oral & Maxillofacial Surgery

PROFESSIONAL EXPERIENCE

Employment

2010-present Director of Oral & Maxillofacial Surgery Ward, National Taiwan University Hospital, Taipei, Taiwan

2010-present Asistant Professor, School of Dentistry, National Taiwan University, Taipei, Taiwan

2006-present Attending Doctor, Department of Dentistry, National Taiwan University Hospital, Taipei, Taiwan

2006-2010 Lecturer, School of Dentistry, College of Medicine, National Taiwan University, Taipei, Taiwan

2002-2006 Attending Doctor, Department of Dentistry, Koo Foundation Sun Yat-Sen Cancer

Center, Taipei, Taiwan

1997-2002 Resident, Department of Dentistry, National Taiwan University Hospital, Taipei, Taiwan

Academic position

2011-present Specialist of Taiwan Association of Head and Neck Surgery

2011-present Editor of "Oral Care for Dental Hospitalized Patients", Taiwan Association of Family Dentistry

2010-present Specialist of Taiwan Association of Facial Plastic and Reconstruction

2010-2011 Editor of Editorial Board of "Clinical Treatment Guideline for Oral Cancer", Taiwan Clinical Oncology Group, National Health Research Institute

2008-2009 Associate Editor of Editorial Board of Taiwan Association of Oral & Maxillofacial Surgery

2008-present Specialist of Taiwan Association of Oral Implant

B1: Publication in Dissertation

1. **Cheng SJ**, Lee JJ, Cheng SL, Chen HM, Chang HH, Wang YP, Kok SH, Kuo MYP, Chiang CP. Increased serum placenta growth factor level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma. Oral Oncol 2012;48:424-428.
2. **Cheng SJ**, Cheng SL, Lee JJ, Chen HM, Chang HH, Kok SH, Ming Ling Chiang, Kuo MYP. Increased placenta growth factor mRNA level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma. J Formos Med Assoc 2012 accepted for publication.

B2: Other Publication related to Dissertation

3. **Cheng SJ,** Lee JJ, Kok SH, Chou CH, Chang HH, Chiang ML, Chen HM, Kuo MY, Chiang CP. Expression of placenta growth factor – an independent factor for prediction of progression and prognosis of oral cancer. Head Neck 2010;32(10):1363-1369.

B3:Publication (2007-2012)

1. **Cheng SJ**, Lee JJ, Cheng SL, Chen HM, Chang HH, Wang YP, Kok SH, Kuo MYP, Chiang CP. Increased serum placenta growth factor level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma. Oral Oncol 2012;48:424-428.
2. **Cheng SJ**, Cheng SL, Lee JJ, Chen HM, Chang HH, Kok SH, Ming Ling Chiang, Kuo MYP. Increased placenta growth factor mRNA level is significantly associated with progression, recurrence and poor prognosis of oral squamous cell carcinoma. J Formos Med Assoc 2012 accepted for publication.
3. **Cheng SJ**, Kok SH, Lee JJ, Kuo MYP, Cheng SL, Huang YL, Chen HM, Chang HH, Chiang

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