PLASMA LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN PATIENTS WITH HEAD AND NECK CANCERS

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Thesis

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PLASMA LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN PATIENTS WITH HEAD AND NECK CANCERS.

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M.Sc. (MEDICAL PHYSICS)

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a cytokine that plays an important role in tumor angiogenesis. VEGF is over expressed in many human cancers, including head and neck cancers. Plasma VEGF (p-VEGF) has been reported to be significantly raised in patients with prostate cancer, leukemia and gastric cancer and to be related to disease aggressiveness. But p-VEGF levels in patients with head and neck cancers have not been reported.

In this study, we measured VEGF concentrations in plasma by a highly sensitive enzyme-linked immunosorbent assay (ELISA) and analyzed p-VEGF in 66 patients with head and neck cancers and the results were compared with 31 normal subjects.

Fifty pg/ml of VEGF concentrations was used as a cut off level, 48.9% of cancers including hypopharyngeal, laryngeal and nasopharyngeal cancers released p-VEGF of >50 pg/ml in contrast to 22.0% of cancers in other locations ($p<0.05$). Seventy percent of poorly differentiated tumors showed p-VEGF of >50 pg/ml while 15.0% of the well and moderately differentiated tumors released high level of p-VEGF ($p<0.01$). There was no significant correlation between p-VEGF levels and lymph node involvement, tumor stage and overall stage grouping. In conclusion, plasma VEGF might serve as an noninvasive tool for characterization of head and neck cancers.

KEY WORDS:PLASMA VASCULAR ENDOTHELIAL GROWTH FACTOR / ENZYME-LINKED IMMUNOSORBENT ASSAY / HEAD AND NECK CANCER

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ระดับวาสคิวลาร์เอ็นโดทีเลียลโกรทแฟคเตอร์จากพลาสมาของผู้ป่วยมะเร็งบริเวณศีรษะและลำคอ
(PLASMA LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN PATIENTS WITH HEAD AND NECK CANCERS)

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บทคัดย่อ

วาสคิวลาร์เอ็นโดทีเลียลโกรทแฟคเตอร์ (VEGF) เป็นไซโตไคน์ ที่มีบทบาทสำคัญในขบวนการสร้างหลอดเลือดหล่อเลี้ยงก้อนมะเร็ง มีการตรวจพบการแสดงออกอย่างมากของ VEGF ในมะเร็งหลายชนิด รวมถึงมะเร็งบริเวณศีรษะและลำคอ ในพลาสมาของผู้ป่วยมะเร็งต่อมลูกหมาก มะเร็งเม็ดเลือดขาว และมะเร็งกระเพาะอาหาร พบว่ามีระดับความเข้มข้นของ VEGF ค่อนข้างสูง และมีความสัมพันธ์กับการลุกลามของโรค แต่ยังไม่มีรายงานถึงพลาสมา VEGF ในผู้ป่วยมะเร็งบริเวณศีรษะและลำคอ

การศึกษานี้ วัดความเข้มข้นของ VEGF ในพลาสมาด้วยวิธี Sandwich enzyme immunoassay ในพลาสมาของผู้ป่วยมะเร็งบริเวณศีรษะและลำคอ จำนวน 66 คนและทำการเปรียบเทียบกับค่า VEGF ในคนปกติจำนวน 31 คน

เมื่อใช้ความเข้มข้น 50 พิโคกรัมต่อมิลลิลิตรเป็นเกณฑ์ตัดสินระดับสูงและต่ำของการหลั่ง VEGF พบว่าร้อยละ 48.9 ของมะเร็งบริเวณคอหอยส่วนกล่องเสียง กล่องเสียง และโพรงหลังจมูก หลั่ง VEGF มากกว่า 50 พิโคกรัมต่อมิลลิลิตร ต่างจากมะเร็งในบริเวณช่องปากและคอหอยส่วนปาก ซึ่งมีเพียงร้อยละ 22.0 ที่หลั่ง VEGF ปริมาณความเข้มข้นสูง ความแตกต่างนี้มีนัยสำคัญทางสถิติ ร้อยละ 70.0 ของมะเร็งที่มีพยาธิแบบ poorly differentiation หลั่ง VEGF มากกว่า 50 พิโคกรัมต่อมิลลิลิตร ขณะที่ร้อยละ 15.0 ของมะเร็งที่มีพยาธิแบบ well และ moderately differentiation เท่านั้นที่หลั่ง VEGF ปริมาณความเข้มข้นสูง ในการศึกษานี้ไม่พบความสัมพันธ์ของการหลั่ง VEGF กับขนาดของมะเร็ง สภาวะการลุกลามไปยังต่อมน้ำเหลือง และระยะของโรค ผลการศึกษาในครั้งนี้สรุปว่า พลาสมา VEGF น่าจะเป็นประโยชน์ในการศึกษาพฤติกรรมของมะเร็งใน บริเวณศีรษะและลำคอ

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LIST OF ABBREVIATIONS

Abbreviation	Term
bFGF	Basic fibroblast growth factor
ANG	Angiogenin
VEGF	Vascular endothelial growth factor
p-VEGF	Plasma vascular endothelial growth factor
KDR	Kinase domain region
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene diamine tetraacetic acid
mRNA	Messenger ribonucleic acid
kDa	Kilodalton
ECM	Extracellular matrix
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor 1
HUVEC	Human umbilical-vein endothelial cell
VPF	Vascular permeability factor
VCAM-1	Vascular cell adhesion molecule 1
ICAM-1	Intercellular adhesion molecule 1
NK	Natural killer cell
VLA-4	Very late antigen 4
HIF-1α	Hypoxia-inducible factor-1α
TPS-1	Trehalose-phosphat synthase 1
VHL	Von Hippel-Lindau gene
ENT	Ear, nose and throat
rpm	Round per minute
C°	Degree celsius
ml	Milliliter
pg/ml	Picogram per milliliter

LIST OF ABBREVIATIONS (Continued)

Abbreviation	Term
μl	Microliter
nm	Nanometer
OD	Optical density
SD	Standard diviation
CI	Confidence interval
χ^2	Chi-square test

CHAPTER I
INTRODUCTION

Angiogenesis implies neovascularization or formation of new blood vessels from pre-existing microvessels (1,2). It involves several pathological conditions, including wound healing, chronic inflammatory diseases and tumor growth (3). It is generally assumed that microvessel formation around a tumor is stimulated by various angiogenic factors secreted by the tumor cells such as basic fibroblast growth factor (bFGF), angiogenin (ANG), endostatin and vascular endothelial growth factor (VEGF). Among these angiogenic factors, VEGF is one of the most potent and specific endothelial cell growth factor. VEGF may sometime be referred as vascular permeability factor because of its activity in increasing vascular permeability (4).

VEGF is a heparin-binding peptide which binds to two tyrosine-kinase receptors, flt-1and KDR / flk-1, almost exclusively expressing on endothelial cells (5). VEGF plays a central role in the regulation of tumor angiogenesis and is capable of promoting tumor growth and metastasis (6). It has been implicated in endothelial cell proliferation and migration, increased vascular permeability, and stromal degradation through the activation of proteolytic enzymes (7,8). VEGF expression has been demonstrated in a large variety of human malignancies, and its over expression in tumor tissue is related to increase in intratumoral microvessel density and poor prognosis (9-13).

Two isoforms of VEGF, i.e. $VEGF_{121}$ and $VEGF_{165}$ are soluble and are released by tumor cells into various body fluids (14). Enzyme-linked immunosorbent assay (ELISA) allows quantitative determination of VEGF in the circulation of patients. This could theoretically reflect the overall angiogenic activity of the tumor (8).

Attempts have been made to measure serum VEGF levels as a potential clinical indicator of prognostic value (15-17). Serum VEGF levels have been reported to be higher in cancer patients than in normal controls and to be positively correlated with the presence of metastatic disease (18,19). Serum VEGF levels have been

demonstrated to be correlated with disease stage in patients with colorectal cancer (20) and to be associated with a poor clinical outcome in patients with small cell lung cancer (17), esophageal cancer (21), colorectal cancer (20), hepatocellular cancer (22) including head and neck cancer (23-25). Moreover, elevated VEGF levels also correlated with survival rate in advanced laryngeal carcinoma (26).

The origin of the VEGF in the serum samples of cancer patients remains to be determined. It has been hypothesized that VEGF in the serum of cancer patients is originated from the cancer cells themselves as well as tumor infiltrating inflammatory cells. However, peripheral blood cells including platelets, neutrophils, and lymphocytes also express VEGF (27-29). Therefore, VEGF detected in the serum of cancer patients may be released from blood cells as well as from tumor itself. Banks, et al claimed that serum VEGF measurements were totally unsuitable and recommended the use of citrate or ethylenediaminetetraacetic acid (EDTA) plasma for VEGF quantitation in blood (30).

Presently, there are only a few published reports of plasma VEGF (p-VEGF) levels in tumor systems namely prostate cancer (31), leukemia (32) and gastric cancer (33). From these reports, elevated p-VEGF is related to disease aggressiveness.

Head and neck cancer is an important public health problem because of its relatively high incidence among Thai people. Cancer registry in Ramathibodi Hospital (34), reported that some cancer of head and neck cancer for instance nasopharyngeal carcinoma is ranked among the ten leading sites of cancers treated at Ramathibodi Hospital. Circulating VEGF in head and neck cancer was measured using serum which had been questioned for its reliability as platelet could also release VEGF during coagulation (24). There has been no study done using plasma sample for head and neck cancers. In this study, plasma samples from patients with head and neck cancers as well as healthy controls were measured for VEGF. This study was conducted to answer the following questions :

1. Did plasma from head and neck cancer patients show higher VEGF concentration in comparing to that of normal subjects ?
2. Did cancers from different anatomical locations of the head and neck secrete different VEGF levels ?

3. Did the p-VEGF levels differ among tumors with different degree of differentiation ?
4. Did p-VEGF levels correlate with nodal status, tumor stage or overall stage grouping ?

CHAPTER II
OBJECTIVES

Main objectives

1. To determine the p-VEGF concentration in patients with head and neck cancers and normal subjects.
2. To evaluate the associations between p-VEGF concentration with tumor location, degree of differentiation, lymph node involvement, primary tumor stage, and overall stage grouping.

CHAPTER III
LITERATURE REVIEW

1. VEGF in tumor angiogenesis

In situ hybridisation studies, performed by several investigators, have demonstrated that the VEGF mRNA is markedly upregulated in the majority of human tumors examined. These include lung (13), thyroid (35) , breast (36), gastro-intestinal tract (37,38), kidney and bladder (39), ovary (40)and uterine cervix (41) carcinomas, angiosarcoma (42) and several intracranial tumors (43-46). The VEGF mRNA is expressed in tumor cells but not in endothelial cells. In contrast, the mRNAs for Flt-1 and KDR (kinase domain region) are upregulated in tumor endothelial cells (37,38,44). This distribution is consistent with the hypothesis that VEGF is primarily a paracrine regulator (4). An exception may be angiosarcoma, where the VEGF and Flt-1mRNA are found to be co-expressed in angiosarcoma cells, raising the possibility that, in this malignancy, VEGF may be an autocrine factor (42). Freeman and associates have suggested that tumor infiltrating lymphocytes may also contribute bioactive VEGF (27). Immunohistochemical studies have localised the VEGF protein not only to the tumor cells but also to the vasculature (37,44). This discrepancy indicates that tumor–secreted VEGF accumulates in the target cell. Ultrastructural studies have localised VEGF bound to tumor endothelial cells to the abluminal plasma membrane and to the recently described vesiculovascular organelles, cystoplasmic structures which are thought to be involved in macromolecular transport across the tumor endothelium (47).

A correlation has been noted between VEGF expression and microvessel density in primary breast cancer sections (48). A postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than those with VEGF-poor tumors (48). A similar correlation has been described in gastric carcinoma patients (49). VEGF-positivity in tumor sections correlated with vessel involvement, lymph node metastasis and liver metastasis. Furthermore, patients

with VEGF-positive tumors had a worse prognosis than those with VEGF-negative tumors (49).

2.The characteristic of VEGF

VEGF is one of the most potent angiogenic factors. It has a specific mitogenic activity on endothelial cells and it is apparently devoid of mitogenic activity for other cell type (4). The active form of VEGF is a homodimeric cytokine of molecular weight 34-46 kDa (8).

The human VEGF gene is located on chromosome 6p21.3. Alternative exon splicing of the VEGF gene results in the production of four protein isoforms containing VEGFs 121, 165, 189 and 206 amino acid residues. All of them bind to heparin. $VEGF_{121}$ is acidic and therefore lacks heparin-binding activity. As a result , $VEGF_{121}$ is free to diffuse into the extracellular space. Although, $VEGF_{165}$ is also secreted from the cell, a significant proportion remains bound to the cell surface and extracellular matrix, and so does not move freely. While the larger isoforms ($VEGF_{189}$ and $VEGF_{206}$) contain increasingly basic and heparin-binding residues and are bound to the cell surface of sequestered in the extracellular matrix (ECM). Most tumor cell types produce several VEGF isoforms simultaneously but $VEGF_{121}$ and $VEGF_{165}$ are usually the predominant variants (5).

VEGF initiates an intracellular signalling cascade through two tyrosine kinase receptors, flt-1 and KDR / flk-1, which are located almost exclusively on the surface of vascular endothelial cells. Flt-1 and KDR have seven immunoglobulin-like domains in the extracellular region, a single transmembrane segment, and an intracellular tyrosine kinase domain. VEGF binds to flt-1 and KDR with a dissociation constant of about 20 pmol/L and 100 pmol/L, respectively. KDR is thought to be the principal receptor for VEGF signalling, whereas flt-1 functions as a decoy receptor to regulate the availability of VEGF (5). The VEGF and VEGFR family system brings about regulation of vascular and lymphatic endothelium, and also has a key role in tumor formation through promotion of angiogenesis and lymphangiogenesis (5,50).

3. Biological activities of VEGF

1. VEGF is a potent mitogen for micro and macrovascular endothelial cells derived from arteries, veins and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types (51-53).These in vitro findings have been corroborated by studies on tissue sections, which have demonstrated that high-affinity VEGF binding sites are localised on the vascular endothelium of large or small vessels, but not to other types (54-55).
2. VEGF promotes angiogenesis (the growth of endothelial cells) in vitro models synergistically with bFGF (56). Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel (57).
3. VEGF elicits a pronounced angiogenic response in a variety of in vivo models, including the chick chorioallantoic membrane, the primate iris etc (52,58,59-61).
4. VEGF induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor 1(PAI-1) in cultured bovine microvascular endothelial cells (62).
5. VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical-vein endothelial cells (HUVEC) (63).
6. VEGF promotes expression of the urokinase receptor in vascular endothelial cells (64).
7. VEGF stimulates hexose transport in cultured vascular endothelial cells (65).
8. VEGF is known also as vascular permeability factor (VPF), bases on its ability to induce vascular leakage in the guinea-pig skin (66,67). Dvork and colleagues (67) proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumors and wounds. According to this hypothesis, a major function of VPF/VEGF in the angiogenic process is the induction of plasma protein leakage. This effect would result in the formation of an extravascular fibrin gel, a substrate of endothelial and tumor cell growth.
9. VEGF may induce fenestrations in endothelial cells. Topical administration of VEGF resulted in the development of fenestrations in the endothelium of

small venules and capillaries, even in regions where endothelial cells are not normally fenestrated (68).

10. VEGF promotes expression of VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) in endothelial cells. This induction results in the adhesion of activated NK (natural killer) cells to endothelial cells, mediated by specific interaction of endothelial VCAM-1 and ICAM-1 with CD18 and VLA-4 (Very late antigen 4) on the surface of NK cells (69).
11. VEGF has certain regulatory effects on blood cells. Clauss, et al (70) reported that VEGF may promote monocyte chemotaxis, while Broxmeyer, et al (71) have shown that VEGF induces colony formation by mature subsets of granulocyte-macrophage progenitor cells. These findings may be explained by the common origin of endothelial and hematopoietic cells and the presence of VEGF receptors in progenitor cells as early as hemangioblasts in blood islands in the yolk sac.
12. VEGF may have an inhibitory effect on the maturation of host professional anti gen presenting cells, such as dendritic cells. VEGF was found to inhibit immature dendritic cells, without having a significant effect on the function of mature cells. These findings led to the suggestion that VEGF may also facilitate tumor growth by allowing the tumor to avoid the induction of an immune response (72).
13. VEGF induces dose-dependent vasodilatation in vitro (73), and produces transient tachycardia, hypotension and a decrease in cardiac output when injected systemically. Such effects appear to be mediated primarily by endothelial cell derived nitric-oxide (73,74).
14. In a study with a controllable expression vector for VEGF in a human breast carcinoma xenograft, suppression of VEGF from the time of inoculation with cancer cell almost completely inhibited tumor growth. However, when VEGF suppression was initiated after the tumor had reached a prespecified size, no growth inhibition was seen. This finding suggests that VEGF has a critical role in the initial phase of the tumor growth and neovascularisation (75) .

15. VEGF recruits bone marrow-derived progenitor endothelial cell and induces their differentiation (76,77). Interestingly, a clinical haematology study showed that normal donor-type endothelial cells were present in the vascular endothelium of a patient who had an allogenic stem-cell transplantation (78). Thus, bone marrow clearly provides progenitor endothelial cells to the systemic vascular system .
16. In patients with atherosclerotic diseases, VEGF can induce neovascularisation into ischaemic lesions through the recruitment of progenitor endothelial cell from the circulation. Therefore, VEGF is also likely to have a crucial role in regulation of tumor angiogenesis (8).

4. Regulation of VEGF expression

VEGF is produced by malignant and normal cells in response to hypoxia and inflammation, and by malignant cells that have undergone genetic changes. In both normal and pathological angiogenesis, hypoxia is the main force initiating the angiogenic process. Hypoxia induces the expression of VEGF and its receptor via HIF-1α (hypoxia-inducible factor-1α) (79) and is also an attractant for macrophages. In a tumor, the angiogenic phenotype can be triggerd by hypoxia resulting from the increasing distance of the growing tumor cells to the capillaries or from the inefficiency of the newly formed vessels. Also, several oncogenes such as v-ras, k-ras, v-raf, src, fos and v-yes (80,81) induce the up-regulation of angiogenic factors like VEGF and increase the production of cytokines and proteolytic enzymes (82). Moreover, oncogene products may act directly as angiogenic factors. In contrast, the tumor suppressor p53 has been found to cause degradation of HIF-1α (83), inhibition of VEGF production, and stimulation of the inhibitor TPS-1 (84). Finally, the VHL (Von Hippel-Lindau) gene product inhibits tumor growth and suppresses the expression of hypoxia–inducible genes (85,86). Consequently, inactivation of the VHL gene, as seen in VHL disease, an inherited cancer syndrome characterized by extensively vascularized tumors, results in stabilization and activation of HIF-1α (87).

5. VEGF in human cancer and clinical significance

Tumor expression of VEGF can be studied at protein level by techniques such as immunohistochemical staining and Western blot analysis, and at mRNA level by reverse transcriptase polymerase chain reaction or in situ hybridization. Quantitation of the expression of VEGF in the tumor provides an alternative to microvessel count in assessing tumor angiogenic activity. One of the main difficulties with the latter technique is to identify the most vascularized areas (hot spots) within the tumor. As a result, considerable interobserver variation is possible and may limit the reproducibility of microvessel density studies (8).

Studies on the expression of VEGF in gastric cancer (88), hepatocellular cancer (89), bladder cancer (90) and head and neck cancer (91) have found a strong association between high tumor VEGF expression and advanced tumor stage or poor survival. Such an association has also been extensively confirmed in other malignancies, such as osteosarcoma (92), melanoma (93), and even leukemia (32). In many cancers, VEGF expression in the tumor has been shown to be a significant prognostic factor of recurrence or survival independent of other conventional clinicopathologic prognosticators (32,94-98). In some studies, it was identified to be the strongest predictor of survival by multivariate analysis (94,98).

6. Circulating VEGF in cancer patients

VEGF is a soluble peptide that has been found to be secreted by tumors into various body fluids (50). It is detected in very high levels in malignant pleural and ascitic effusions, and its enhancing effect on vascular permeability is thought to be an important mechanism mediating the formation of malignant effusions (99). The finding of significantly higher serum VEGF levels in cancer patients than in normal subjects was firstly reported in 1994 (100). This was verified in a subsequent study in patients with various types of cancers (18). In the latter study, a significant correlation of serum VEGF level with tumor stage, microvessel density, and tumor VEGF expression was noted. A further study also demonstrated a higher serum VEGF level in disseminated disease than in localized disease among patients with a variety of cancers, irrespective of the histologic type (19). The findings of these pilot studies

sparked off great interest in research to determine the clinical value of circulating VEGF as a biomarker and prognostic indicator in various types of human cancer .

The results of studies in correlating circulating VEGF level with tumor progression or patient survival in different types of cancer (15,17,24,25,31,101-118) have been shown. Some of these studies have been performed using archived serum samples in storage and retrospective data (17,24,25,102,103,114-118), but other have been conducted with prospectively collected blood samples and clinical data (15,101,106,108,111). In many instances, however, it was not clearly stated whether the studies were retrospective or prospective in nature. With very few exceptions, these studies demonstrated a positive correlation of elevated serum or plasma VEGF level with advanced tumor stage or poor survival. This is in line with studies of the prognostic significance of tumor VEGF expression and suggests that circulating VEGF level is a good reflection of tumor angiogenic activity in various cancers.

It has been an important and attractive investigation that VEGF in the serum of cancer patient not only originated from the tumor cell themelves but also from platelets, granulocytes, monocytes, mast cell and lymphocytes (28-30,119-121). A direct relationship between both platelet count and serum VEGF concentration has been reported in several studies (28,119-121) Verheul, et al (119) showed that VEGF is transported by platelet and serum VEGF concentrations reflect platelet count rather than tumor burden. Maloney, et al (120) showed a clear association between platelet aggregation in vitro and VEGF release. This has been confirmed by Gunsilius, et al (29) who demonstrated that VEGF released from platelets during blood clotting is the main source of VEGF in serum samples. Banks, et al (30) claimed that serum VEGF measurments were totally unsuitable and recommended the use of citrate or EDTA plasma for VEGF quantitation in blood. Moreover, Dittadi, et al (122) investigated the procedure for sample collection with the objective to standardize a routine setting.

For plasma VEGF, a few studies have been reported. Correlation between p-VEGF and tumor vessel count (123) has demonstrated in liver metastasis of colorectal cancer. Significant elevation of p-VEGF was observed in hematological cancers (32). Patients with localized prostate cancers had lower p-VEGF than those with metastatic diseases (31,124).

CHAPTER IV
MATERIALS AND METHODS

4.1 Subjects

Blood samples were collected from patients who visited the ENT clinics, at Department of Otolaryngology, Ramathibodi Hospital. Of the 97 total blood samples, 66 were obtained from patients with histologically proven malignancies at the head and neck region, none of the patients received chemotherapy, radiotherapy or surgery, and the rest 31 samples were collected from normal healthy subjects without any evidences of diseases (e.g. liver dysfunction, diabetes, atherosclerosis etc.), without history of a known neoplasm , without recent trauma or surgery.

All studies were approved by the ethics committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University. All subjects had given written informed consents.

4.2 Preparation of plasma

The blood sample was obtained by cubital vein puncture in the same fashion for all subjects. The blood was collected by a sterile tube containing 12.15 mg of EDTA as an anticoagulant within 1 hour, the collected blood was centrifuged at 3000 rpm, 4 C° for 15 minutes. The plasma was immediately pipetted into a polypropylene tube and stored at ≤ - 20 C°. One freeze-thaw cycle was used for all samples.

4.3 Reagents

Enzyme-linked immunosorbent assay (ELISA) was the process that used to determine VEGF concentration in plasma samples. This study used a commercial

available ELISA kit (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis MN).

4.3.1 VEGF microplate

Ninety six wells polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against VEGF.

4.3.2 VEGF conjugate

Twenty one milliliter of polyclonal antibody against VEGF conjugated to horseradish peroxidase, with preservatives.

4.3.3 VEGF standard

Three vials (2000 pg/vial) of recombinant human $VEGF_{165}$ in a buffered protein base with preservatives, lyophilized.

4.3.4 Assay Diluent RD1W

Eleven milliliter of a buffer protein base with preservatives.

4.3.5 Calibrator Diluent RD6U

Twenty one milliliter of animal serum with preservatives.

4.3.6 Wash Buffer concentration

Twenty one milliliter of a 25-fold concentrated solution of buffered surfactant with preservative.

4.3.7 Color reagent A

Twelve point five milliliter stabilized hydrogen peroxide.

4.3.8 Color reagent B

Twelve point five milliliter stabilized chromogen (tetramethylbenzidine).

4.3.9 Stop solution

Six milliliter of 2N sulfuric acid.

4.3.10 Plate cover

Four adhesive strips.

4.4 Reagent preparation

4.4.1 Stock solution

Reconstitute the VEGF standard with 1 ml of Calibrator Diluent RD6U. This reconstitution produces a stock solution of 2000 pg /ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

4.4.2 VEGF standard dilutions

Use polypropylene tubes. Pipette 500 µl of Calibrator Diluent RD6U into each tube. Use the stock solution to produce a dilution series. Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (2000 pg/ml). Calibrator Diluent RD6U serves a the zero standard (0 pg /ml). Figure 1 demonstrates preparation of a dilution series of VEGF standard.

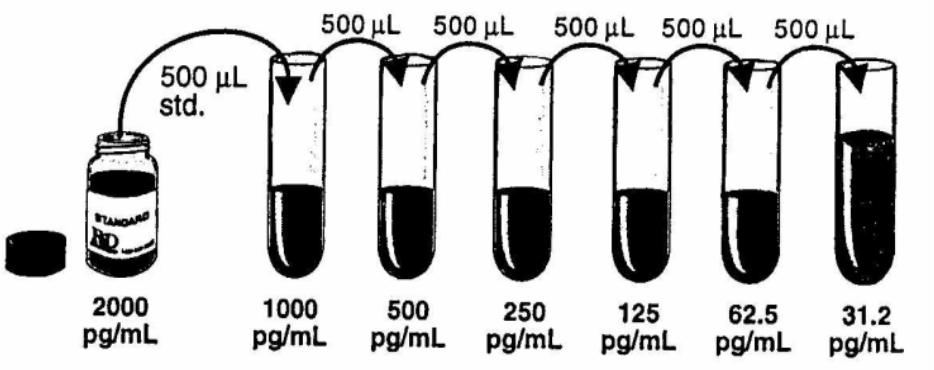

Figure 1. Preparation of a dilution series of VEGF standard (125).

4.5 Principle of ELISA (126)

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After washing away any unbounds substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells and incubats for 2 hours. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is stopped and the intensity of the color is measured.

4.6 Plasma VEGF immunoassay (125)

A quantitative, ELISA was performed in duplicate on each sample to measure the concentration of soluble p-VEGF.

1. Add 100 μl of assay diluent RD1W to each well of VEGF microplate.
2. Add 100 μl of standard or sample per well. Cover with the adhesive tape and incubated for 2 hours at room temperature. A plate layout was provided to record the standards and samples assayed.
3. Aspirate each well and washed, repeating the process twice for a total of three washes. Wash by filling each well with wash buffer (360 μl) using multi-channel pipette. Complete removal of liquid at each step was essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towel.
4. Add 200 μl of VEGF conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
5. Repeat the aspiration and washing as in step 3.
6. Add 200 μl of substrate solution to each well. Incubate for 25 minutes at room temperature.
7. Add 50 μl of stop solution to each well. When color change did not appear uniformly, gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. Every standard or sample was corrected for background with the subtraction of blank optical density (OD).

4.7 Calculation of plasma VEGF in samples

The data was linearized by plotting the ln of the VEGF standard concentrations versus the ln of OD, and the best fit line can be determined by regression analysis.

VEGF in plasma sample was determined using the standard curve. The concentration of VEGF in pg/ml was calculated from the formular below.

$$OD = AX^h$$

Take natural logarithm $\ln OD = \ln A + h \ln X$

$$\ln X = \frac{\ln OD - \ln A}{h}$$

$$X = e^{\frac{\ln OD - \ln A}{h}}$$

Where X was the VEGF concentration of sample, OD was the optical density of sample, A was the y-axis intercept, and h was the slope of standard curve.

4.8 Statistical analysis

Nonparametric test was employed to analyse the data. Subjects were categorized according to the levels of VEGF concentrations as 1-25, 26-50 and >50 pg/ml. Contingency table of frequency data was constructed. Test was performed to test the differences Chi-squared in plasma VEGF between normal subjects and cancer patients; tumor sites; tumor differentiation; nodal status and overall stage grouping.

CHAPTER V
RESULTS

The sample size for this study was 97 which consisted of 31 normal subjects and 66 patients with head and neck cancers. Details of the studied population is shown in Table 1.

The concentration of VEGF in plasma from normal subjects and cancer patients in this study varied greatly (Table 2). The range of its concentration in normal subjects varied between 7.1 pg/ml and 228.7 pg/ml. While cancer patients varied between 1.3 pg/ml and 1121.9 pg/ml. The VEGF concentration in each studied subjects did not distribute normally (Figures 2, 3, 4, 5, 6 and 7). The description of data by mean did not represent VEGF concentration due to large standard diviation (SD). Therefore the median value of p-VEGF concentration was a more reasonably measurement because of its smaller 95 % confidence interval (95%CI).

On the basis of observed distribution patterns, VEGF concentrations were categorized as follows: 1-25, 26-50 and >50 pg/ml. Percentages of subjects with specified ranges of VEGF concentrations are presented in Table 3. Contingency tables were constructed and chai-squared test was performed to reveal a non-significant difference between the profiles of VEGF concentrations in norml subjects and cancer patients (χ^2= 0.86; p >> 0.2). By inspection of median VEGF concentrations, cancers from hypopharynx, larynx and nasopharynx had higher medians than normal subjects and patients with cancers in oral cavity and oropharynx (Table 2). Chi-squared analysis confirmed the significant difference (Table 3). Greater than 50% of the normal subjects, patients with oral and oropharyngeal cancers released VEGF in the range of 1-25 pg/ml. On the contrary, patients with hypopharyngeal, laryngeal and nasopharyngeal cancers tended to release VEGF of greater than 50 pg/ml.

On the basis of tumor differentiation, enhanced VEGF concentration was significantly associated with poorly differentiated tumors (Table 2 and 4). Seventy

percent of poorly differentiated tumors released VEGF of greater than 50 pg/ml. While well and moderlately differentiated tumors had VEGF profiles which were similar to these of normal subjects.

Increased VEGF concentration in plasma was reported to be associated with disease aggressiveness (31,124). Profiles of p-VEGF concentrations for normal subjects, cancer patients with negative and positive lymph nodes are presented in Table 5. Although the median VEGF concentrations in patients with positive lymph nodes was higher than those of the patients with negative nodes and normal subjects (Tables 2 and 5), but the difference was small and did not reach the significant level.

Tables 6 and 7 show the profiles of VEGF concentrations in patients with early and advanced tumor stages as well as overall stage groupings. There was an increasing tend, although not significant, to observe enhanced VEGF concentrations in patients with advanced staging.

Table 1. Studied population.

Subject and tumor characteristic	Number of subject
Normal subjects	31
Age:mean = 30, range = 21-51	
Sex:men = 9, women = 22	
Patients with head and neck cancers	66
Age:mean = 62, range = 28-83	
Sex:men = 52, women = 14	
Clinical staging	
- Early stage (I,II)	19
- Advance stage (III,IV)	47
Primary tumor stage	
- T 1,2	26
- T 3,4	40
Lymph node involvement	
- Negative node	38
- Positive node	28
Degree of differentiation	
- Well	30
- Moderately	17
- Poorly	17
Tumor site	
- Oral cavity	21
- Oropharynx	9
- Hypopharynx	15
- Larynx	13
- Nasopharynx	4
- Miscellaneous (ear, nose, synchronous disease at supraglottic and soft palate, etc.)	4

Table 2. Plasma VEGF concentrations (pg/ml) in the studied subjects.

Subject	Number of subject	Mean ±SD	Median (95%CI)	Range
Normal	31	44.0 ± 50.6	22.9 (18.4 -39.7)	7.1 - 228.7
Cancer				
- Oral cavity, oropharynx	30	37.1 ± 39.6	23.2 (20.1-29.9)	1.3 -184.4
- Hypopharynx, larynx, nasopharynx	32	120.2 ± 199.1	45.2 (28.5 -143.3)	10.2 -1121.9
- All cancers	66	76.2 ± 146.6	28.9 (23.6 -38.5)	1.3 - 1121.9
Staging				
- Stage I,II	19	49.2 ± 63.2	24.1 (18.7-43.0)	10.2 - 271.6
- Stage III,IV	47	87.2 ± 168.4	29.4 (24.1-46.1)	1.3 - 1121.9

Table 2. Plasma VEGF concentrations (pg/ml) in the studied subjects (continued).

Subject	Number of subject	Mean ± SD	Median (95%CI)	Range
Primary tumor stage				
- T 1,2	26	93.0 ± 221.0	23.0 (19.3 -38.5)	10.2 - 1121.9
- T 3,4	40	65.3 ± 64.7	34.0 (24.1-56.6)	1.3 - 226.1
Nodal status				
- Negative node	38	53.1 ± 60.7	25.6 (20.1-43.0)	1.3 - 271.6
- Positive node	28	107.7 ± 211.9	32.0 (24.1-79.6)	13.4 - 1121.9
Differentiation				
- Well	30	66.7 ± 200.8	24.1 (19.3 -29.9)	10.2 - 1121.9
- Moderately	17	49.0 ± 57.1	25.8 (18.7 -43.5)	1.3 - 184.4
- Poorly	17	125.0 ± 84.7	121.2 (44.3 -194.2)	21.3 - 271.6

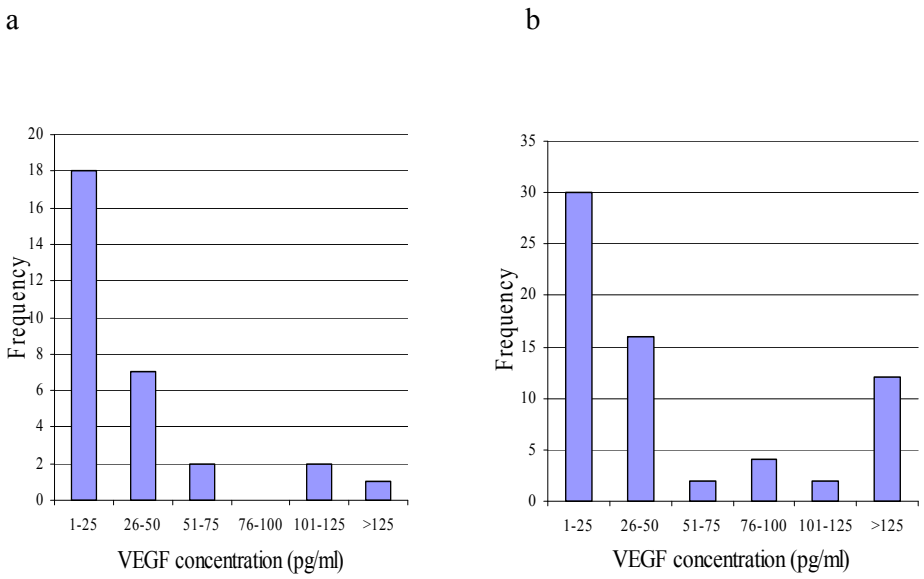

Figure 2. Distribution of plasma VEGF concentration in (a) normal subjects and (b) head and neck cancers.

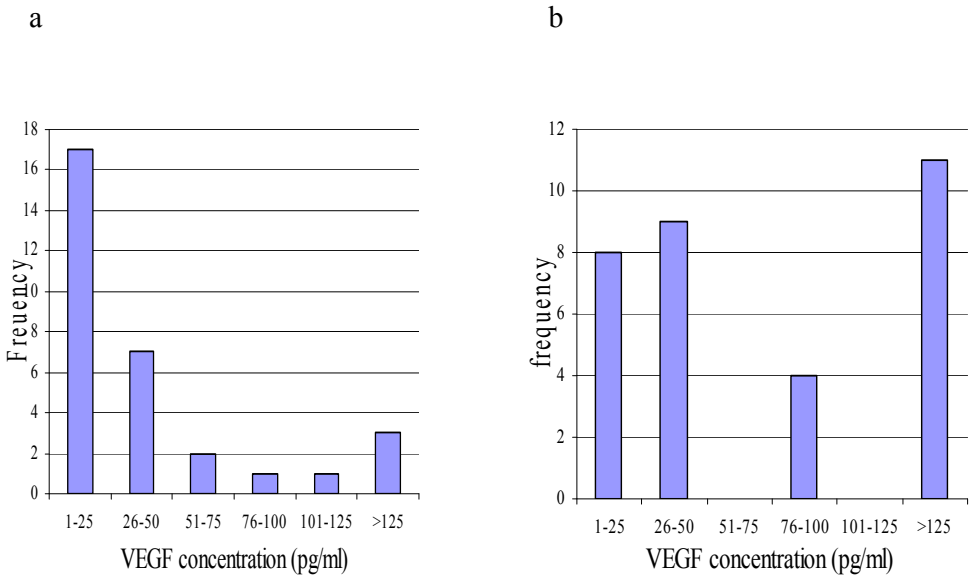

Figure 3. Distribution of plasma VEGF concentration in patients with cancers in (a) oral cavity and oropharynx (b) hypopharynx, larynx and nasopharynx.

a b

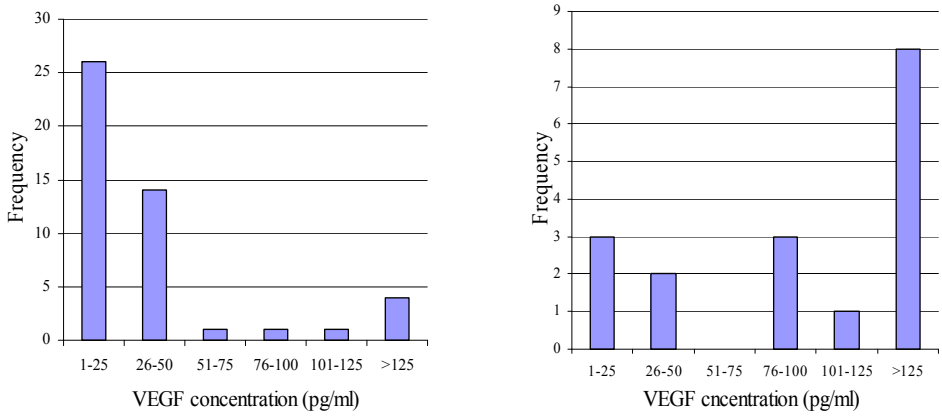

Figure 4. Distribution of plasma VEGF concentration in tumors with (a) well and moderately differentiation (b) poorly differentiation .

a b

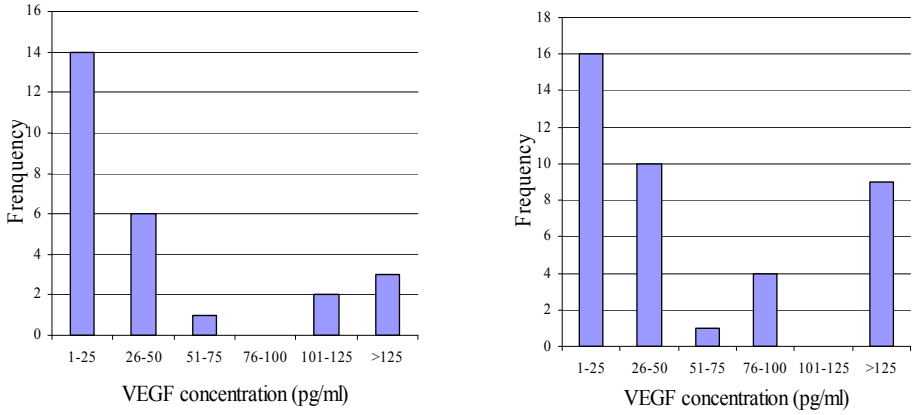

Figure 5. Distribution of plasma VEGF concentration in tumors with (a) tumor stage T 1,2 (b) tumor stage T 3,4.

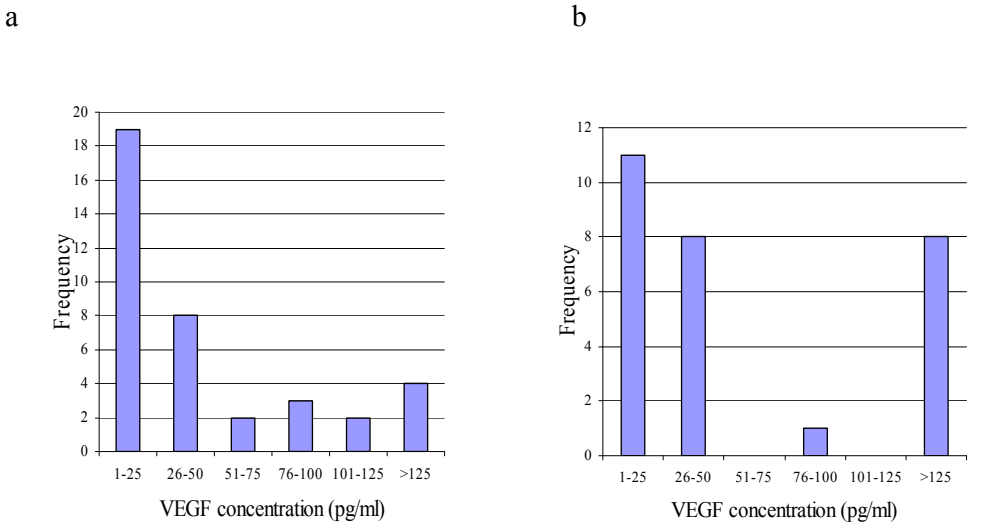

Figure 6. Distribution of plasma VEGF concentration in patients with (a) negative node (b) positive node.

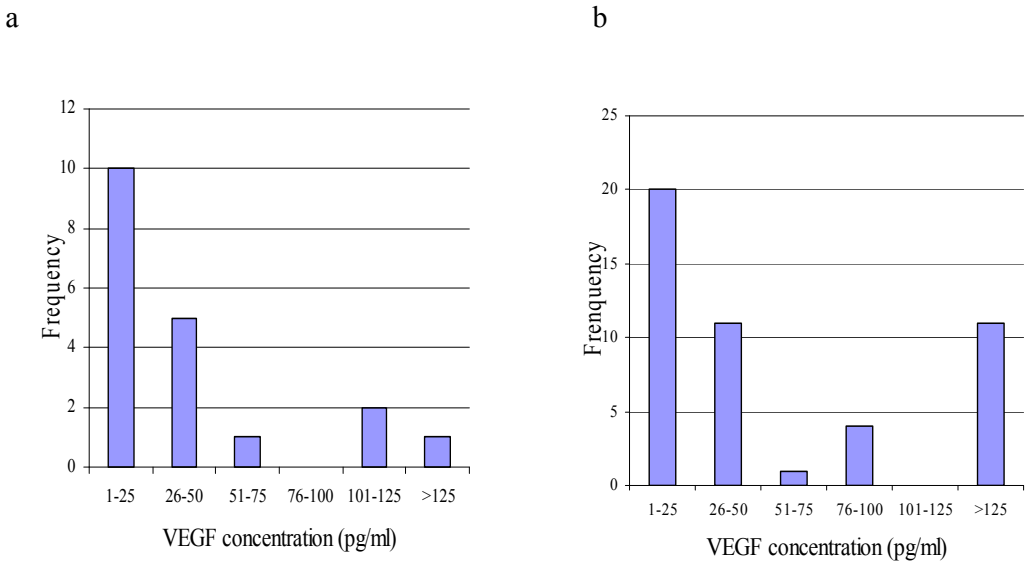

Figure 7. Distribution of plasma VEGF concentration in patients with (a) stage I,II (b) stage III,IV.

Table 3. Profiles of plasma VEGF concentrations in normal subjects and cancer patients. The data represent the percentage of subjects with specified VEGF concentration.

Subject	Number of subject	Plasma VEGF concentration (pg/ml)		
		1-25	26-50	>50
Normal	31	55	22.5	22.5
All cancers	66	46	24	30
Oral cancer	21	62	24	14
Oropharyngeal cancer	9	56	22	22
Hypopharyngeal cancer	15	7	40	53
Laryngeal cancer	13	46	15	39
Nasopharyngeal cancer	4	25	25	50
Miscellaneous	4	100	0	0

χ^2 (Normal vs all cancers) = 0.86 ; p-value >> 0.2

χ^2 (Normal vs oral cancer + oropharyngeal cancer) = 0.35 ; p-value >> 0.2

χ^2 (Normal vs hypopharyngeal cancer + laryngeal cancer + nasopharyngeal cancer) = 6.39; p-value < 0.05

Table 4. Profiles of plasma VEGF concentration in cancer patients as categorized by degree differentiation including normal subjects. The data represent the percentage of subjects with specified VEGF concentration.

Subject	Number of subject	Plasma VEGF concentration (pg/ml)		
		1-25	26-50	>50
Well	30	57	30	13
Moderately	17	53	29	18
Poorly	17	18	12	70
Normal	31	55	22.5	22.5

χ^2 (Well + moderately vs normal) = 0.97 ; p-value >> 0.2

χ^2 (Normal vs poorly) = 10.71 ; p-value < 0.01

χ^2 (Well+moderately vs poorly) = 18.55 ; p-value < 0.001

Table 5. Profiles of plasma VEGF concentration in cancer patients as categorized by lymph node involvement including normal subjects. The data represent the percentage of subjects with specified VEGF concentration.

Subject	Number of subject	Plasma VEGF concentration (pg/ml)		
		1-25	26-50	>50
Negative node	38	50	21	29
Positive node	28	39	29	32
Normal	31	55	22.5	22.5

χ^2 (N(-)vs N(+)) = 0.84 ; p-value >> 0.2

χ^2 (Normal vs N(-)) = 0.38 ; p-value >> 0.2

χ^2 (Normal vs N(+)) = 1.46 ; p-value > 0.2

Table 6. Profiles of plasma VEGF concentration in cancer patients as categorized by primary tumor stage including normal subjects. The data represent the percentage of subjects with specified VEGF concentration.

Subject	Number of subject	Plasma VEGF concentration (pg/ml)		
		1-25	26-50	>50
T 1,2	26	54	23	23
T 3,4	40	40	25	35
Normal	31	55	22.5	22.5

χ^2 (T 1,2 vs T 3,4) = 2.34 ; p-value > 0.2

χ^2 (Normal vs T1,2) = 0.00 ; p-value >> 0.2

χ^2 (Normal vs T3,4) = 1.78 ; p-value > 0.2

Table 7. Profiles of plasma VEGF concentration in cancer patients as categorized by staging including normal subjects. The data represent the percentage of subjects with specified VEGF concentration.

Subject	Number of subject	Plasma VEGF concentration (pg/ml)		
		1-25	26-50	>50
Stage I,II	19	53	26	21
Stage III,IV	47	43	23	34
Normal	31	55	22.5	22.5

χ^2 (Stage I,II vs Stage III,IV) = 1.10 ; p-value > 0.2

χ^2 (Normal vs Stage I,II) = 0.09 ; p-value >> 0.2

χ^2 (Normal vs Stage III,IV) = 1.42 ; p-value > 0.2

CHAPTER VI
DISCUSSION

VEGF is one of the most potent factors responsible for inducing tumor angiogenesis and its over expression in tumor tissue is related to increases in intratumoral microvessel density and poor prognosis (11-13). Two isoforms, $VEGF_{121}$ and $VEGF_{165}$, are soluble and can be assay in serum by ELISA. Serum VEGF (s-VEGF) has been shown to be significantly elevated in patients with many solid tumors for instance breast cancer (94), lung cancer (101), colorectal cancer (20) and head and neck cancer (23-26,127). This indicates some promise of s-VEGF as a potentially useful tumor marker. Teknos, et al (26) observed significantly elevated s-VEGF in patients with advanced laryngeal carcinoma with respect to healthy control. They noted the association of increased s-VEGF with more aggressive disease state and a poorer overall survival. Qian, et al (25) also reported the elevation of s-VEGF in metastatic nasopharyngeal carcinoma. Despite the significant increase in s-VEGF in head and neck cancer patients versus healthy controls as reported by many investigators, no significant correlation of s-VEGF level with either tumor stage or lymph node metastasis (23-25). The reliability of s-VEGF in predicting tumor growth and progression has been questioned because of the release of VEGF by platelet during coagulation. Because of this, Bank, et al (30) suggested the use of citrated or ethylenediaminetetraacetic acid (EDTA) plasma for the measurement of circulating VEGF.

There has been quite a few reports demonstrate the correlation of plasma VEGF (p-VEGF) with clinical courses. Aguayo, et al (32) patients with reported the prognostic significant of p-VEGF in acute myeloid leukemia but not in patients with myelodysplastic syndrome. Duque, et al (31) suggested the use of p-VEGF level to differentiate patients with metastatic prostate cancer from those with localized disease or healthy controls. Ohta, et al (33) observed the elevated p-VEGF in gastric cancer patients with venous invasion and lymph node metastasis. Presently, there has been

no report on p-VEGF in head and neck cancers. In this study, patients with cancers different sites of the head and neck were recruited for study. Elevation of p-VEGF appeared to be related with site of the disease. Plasma from patients with tumors in the hypopharynx, larynx and nasopharynx showed significant increased VEGF in comparing to those from healthy controls. As opposed to patients with tumors in oral cavity and oropharynx, the p-VEGF profile appeared the same as that of normal subjects. When 50 pg/ml was used as a cut off level, 47.0 % of hypopharyngeal, laryngeal and nasopharyngeal cancers in contrast to 17.0 % of oral and oropharyngeal cancers and also normal control released VEGF above the cut off point.

Enhanced p-VEGF was also associated with tumor differentiation. Seventy percent of poorly differentiated tumors showed p-VEGF >50 pg/ml while 15.0 % of the well and moderately differentiated tumors released high level of p-VEGF. Elevated p-VEGF in hypopharyngeal, laryngeal and nasopharyngeal cancers tended to be related to tumor differentiation. Since 43.8 % of these tumors were characterized as poorly differentiated tumors.

With respect to nodal status and tumor stage, slight but not significant increase in p-VEGF was oberved in patient with positive node or T3,T4 tumors. In our series, it was quite interesting to note that 34.0 % of stage III,IV cancers belonged to oral and oropharyngeal cancers and 57.5 % were those arising from the hypopharynx, larynx and nasopharynx. However, on the basis of tumor differentiation, there were only 34.0 % of these advanced cancers being characterized as poorly differentiated tumors. This could be the possible explanation why nonsignificant increased p-VEGF in patients with advanced diseases. Our study suggested enhanced p-VEGF rather related to tumor biology possibly tumor differentiation but not tumor growth and progression. Plasma VEGF might serve as an noninvasive tool for characterization of head and neck cancers.

CHAPTER VII
CONCLUSION

1. Elevated p-VEGF could be observe in cancers of the hypopharynx, nasopharynx and larynx.
2. No significant increase in p-VEGF could be observed in cancers of the oral cavity and oropharynx.
3. Elevated p-VEGF was significantly correlated with tumor differentiation.
4. Elevated p-VEGF was not related to tumor stage, lymph node involvement and overall stage grouping.

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