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Elevation of Vascular Endothelial Growth Factor in Indonesian Advanced Stage Nasopharyngeal Carcinoma

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Studies on the role of vascular endothelial growth factor (VEGF) as the most potent angiogenic factor in disease progression of nasopharyngeal carcinoma (NPC) remain limited, partly be due to the geographical distribution of the disease. However, it has never been reported from Indonesian population despite its common incidence. Therefore, we aimed to study the possible prognostic value of VEGF in Indonesian advanced stage NPC.

A clinical examination, CT scan, and the tumor tissue and plasma collection were performed before a combined therapy, and a local control rate was reassessed every 3 months to determine progression-free survival (PFS). Plasma VEGF-A was measured in 40 patients by ELISA, and VEGF and vWF expressions were examined in 30 patients by immunohistochemistry. Survival curves were plotted based on plasma VEGF-A level, VEGF, and microvessel density (MVD) count indicated by vWF expressions vs PFS.

The median follow up was 16 months. Patients with high VEGF-A level (≥834pg/ml) presented shorter survival rate, as compared to those of low level (<834pg/ml) (41.2% vs 82.6%; p=0.009, log rank 6.81). Patients with overexpressed VEGF (≥25%) showed shorter survival than those with low expression (<25%) (45.5% vs 79.6%; p=0.05, log rank 3.84). Patients with higher MVD count (≥25%) also had shorter survival than those with lower MVD count (53.4% vs 80%; p=0.101, log rank=2.70).

In conclusion, an elevated plasma VEGF level predicts shorter survival for Indonesian advanced stage NPC in this study. However, more samples may be required to draw a better conclusion on a possible role of VEGF as a prognostic factor in the disease progression of NPC. This data is also substantial for the development of anti-VEGF as a possible targeted therapy for NPC.

Nasopharyngeal carcinoma (NPC) is a malignancy originating from nasopharyngeal epithelial cells. This disease is pathologically, epidemiologically and clinically distinct from other head and neck cancers. Endemic areas include Southern China, Alaska and Southeast Asia. There are estimated to be 10,000 new cases per year in Indonesia. It was also the most common malignancy in males visiting Tulip Integrated Cancer Unit of Sardjito Hospital Yogyakarta, Indonesia between 2001─2005, among whom 80% were diagnosed at an

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advanced stage and who mostly had type III WHO pathological classification, which has a strong correlation with Epstein-Barr virus (EBV) latent infection.[1]

Angiogenesis is a fundamental process in tumor growth and metastasis, the degree of which depends on the net balance of the effects of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor (VEGF), widely known as vascular permeability factor (VPF) or vasculotropin, is a glycoprotein possessing potent angiogenic and mitogenic as well as increasing vascular permeability activity specific to endothelial cells.[2] 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. In addition, some less commonly expressed variants have also been identified (VEGF145 and VEGF183). 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. VEGF165 exists in both a soluble and an ECM-bound form. 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. Alternatively, VEGF can be released from the ECM by MMP-9 to initiate the angiogenic switch. VEGF165- which is a mature isoform of VEGF-A - is the predominant isoform and is commonly overexpressed in a variety of human solid tumors.[2]

Data on the possible role of VEGF in head and neck cancer, including NPC, are lacking when compared to other malignancies, and the prognostic value of circulating VEGF and VEGFR expressions is not yet clear. A study from China showed elevated circulating VEGF in male patients with metastatic NPC; however these levels did not correlate with the locoregional progression of NPC, and the usefulness of detecting serum-VEGF in the early diagnosis of NPC appeared to be limited.[3] A study from India reported a correlation between circulating VEGF and disease reccurence.[4]

Polymorphisms of LMP-1, LMP-2 and EBNA genes of EBV have been reported in different ethnicities, and strongly suggest different patterns of infection that induce NPC.[5] These variations may also affect the various patterns of VEGF expression among different ethnicities.

In the current study, we aimed to determine the level of plasma VEGF-A, expression of VEGF and microvessel density (MVD) by von Willebrand Factor (vWF) in tumor biopsy specimens of advanced stage NPC in the Indonesian population, and to study their correlations with the disease outcome. The results will contribute to further consideration and research into VEGF development as a targeted therapy for solid tumors, particularly NPC.

MATERIALS AND METHODS

Materials

Patients admitted to the Tulip Integrated Cancer Unit of Sardjito Hospital Yogyakarta, Indonesia between January 2001 to December 2005, with biopsy-proven NPC in stage III and IV according to the American Joint Committee on Cancer (AJCC) VI staging system, were eligible for this study.[6] Patients had no history of previous radiotherapy or chemotherapy. Other eligibility criteria were Karnofsky performance status $\geq$ 60%; Hb level $\geq$ 10g/dl, WBC count of greater than $4x10^9$/L and platelet count of greater than $100x10^9$ /L; serum creatinine level less than 1.5 mg/dL; liver function with AST/ALT < 5 x upper limit, and total bilirubin less than 2.5mg/dL.

This study was performed after approval from the institutional ethics committee. All patients were required to provide written informed consent before starting treatment.

All patients underwent fibreoptic nasopharyngoscopy and biopsy to obtain specimens for pathologic diagnosis. Pre-treatment staging evaluations included clinical examination of the head and neck; computed tomography (CT) scan or magnetic resonance imaging (MRI) from the skull base to the whole neck; chest radiography; whole-body bone scan; abdominal sonography; complete blood count with differential count, platelet count, and biochemical profile.

Locally advanced disease patients received combination therapy of neo-adjuvant chemotherapy and radiotherapy. The chemotherapy regimen consisted of Cisplatin 100mg/m2 day 1 and 5FU 1000mg/m2 day 1─5, with a 21-days cycle given 3 times. Four weeks after the last chemotherapy, external beam radiotherapy 70Gy was given to the local lesion followed by 20Gy of brachytherapy.[7] Metastatic disease patients were treated with platinum-based chemotherapy.

All patients were subjected to physical examination, complete blood count, and platelet count during each week of therapy. After completion of treatment, patients were followed weekly until acute side effects resolved. Patients were then evaluated every 3 months with clinical examination, CT scan, chest radiography, abdominal sonography, blood count, and biochemistry tests. Tumor response with regard to disease outcome was assessed based on WHO criteria.[8] Median of follow up duration for survival analysis was 16 months. Patients Progression free survival (PFS) duration was determined from informed consent until disease progression defined clinically, pathologically or by imaging, and death due to any cause.

Control group included 20 normal individuals who did not have any chronic illness, since it may cause the pathologic elevation in any angiogenic factors.[3]

Methods

Determination of plasma VEGF-A level

Peripheral venous blood samples were drawn from each subject and collected in sterile test tubes, centrifuged at 2,000 g for 10 min and stored at -20°C until assayed collectively for the VEGF-A level. Plasma VEGF-A concentration was determined as plasma immunoreactivity by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine DVE00, R&D Systems, Minneapolis, USA) recognizing VEGF-A 165bp splicing variant, according to the manufacturer's instructions as described previously.[9] The assay has been reported to recognize both natural and recombinant human VEGF and not to exhibit cross reactivity with a series of growth factors and cytokines. The manufacturer claims a sensitivity of 9.0pg/mL^{-1}. Each assay well measured 100µl plasma. All analyses and calibrations were carried out in duplicate. Optical density was determined using a microtitreplate reader at 450 nm/540nm and concentrations are given in pg/ml.

VEGF and vWF expression

Immunohistochemistry for VEGF and vWF from tumor biopsies was performed by a streptavidin biotin complex procedure. When the biopsies contained a substantial area of necrosis, it was excluded from the study. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were then incubated with 10% normal sheep plasma in phosphate-buffered saline (PBS) solution for 30 min, followed by overnight incubation at 4°C with rabbit anti-human VEGF polyclonal antibody (Thermo Scientific #RB-9031-P, California, US), specifically recognizing 165, 189 and 121 amino acid splice variants of VEGF of human. This antibody was diluted 1:50. We used an antibody against VEGF other than to VEGFR in order to compare the result of the measurement of VEGF level of expression in the plasma and on the tissue, since these variants are found both in soluble and an extra cellular matrix-bound form.[2] Next, each slide was treated with biotinylated anti-rabbit immunoglobulin for 10 min, and then incubated

with streptavidin-peroxidase complex for 45 min. Aminoethylnorbazole (AEC) was used as a chromogen, and nuclear counterstaining was performed with Mayer's hematoxylin solution. VEGF expression was evaluated by counting the mean number of cells stained in 5 views of 400x magnification including 100 cells perview. Data is presented in percentage, and mean of ≥ 25% was defined as overexpression. The expression of von Willebrand factor (vWF) indicating microvessel density was assessed using in a similar method by a mouse anti-human vWF monoclonal antibody (clone 36B11, Novocastra Laboratories, Newcastle, UK), which was also diluted 1:50. Microvessels density was counted as mean number of cells stained within 5 views of 100x magnification, and mean number of ≥ 25 was defined as higher count of MVD. Immunohistochemical staining was assessed by an investigator without knowledge of the clinicopathological findings of the patients studied. Immunoreactivity of VEGF expression was evaluated semi-quantitatively using the following grading range: samples with low staining <25% were graded '+', samples with medium staining between 25-50% were graded '++' and samples with strong staining >50% were graded as '+++', as previously described.[9]

Statistical analysis

Data was analyzed using SPSS11.5 software. Kaplan-Meier analysis and logrank test were used to assess survival rate and to compare the difference of survival rate. Survival curves were then plotted based on plasma VEGF-A level, VEGF, and microvessel density (MVD) count indicated by vWF expressions vs PFS.

RESULTS

Patient characteristics

Characteristics of the patients are shown in Table I. The median age was 46 years; with mean of age was 45.5 years, with male predomination (82.5%). WHO classification of type 3 was mostly found (82.5%), while most of the patients were in the stage of locally advanced (87.5%). The median follow up for the patients was 16 months.

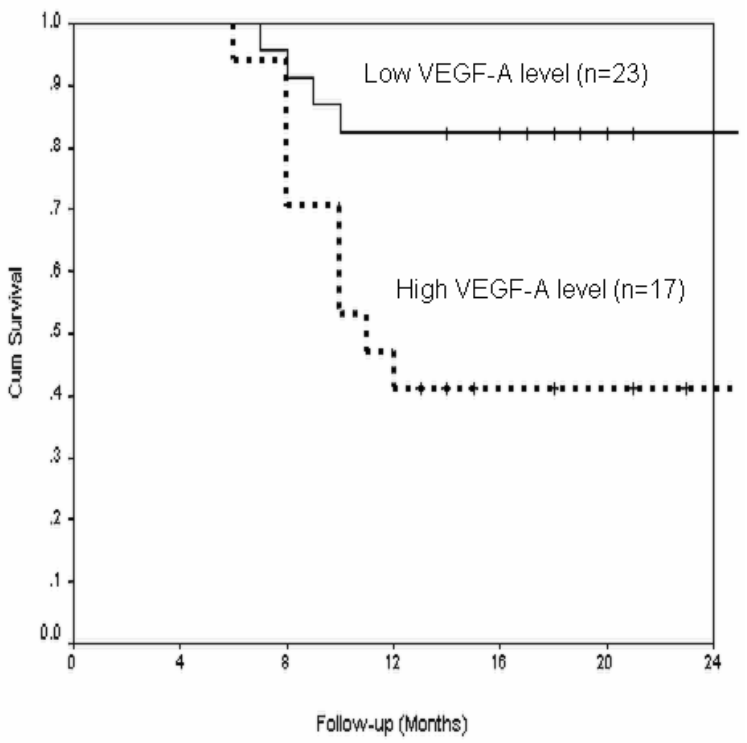
Table I. Patient Characteristics

Characteristic	Number (%)
Age, years	
Range	15─67
Median	46
Mean	45.5
Sex	
Male	33 (82.5)
Female	7 (17.5)
WHO classification	
Type 1	0 (0)
Type 2	7 (17.5)
Type 3	33 (82.5)
Stage AJCC VI	
Locally advanced disease	35 (87.5)
Metastatic disease	5 (12.5)

Plasma VEGF-A level

The level of plasma VEGF in 40 patients was 821.88±353pg/ml, while the control group, including 20 control subjects, was 541±341.24pg/ml. To study the effectiveness of the plasma VEGF level in predicting disease progression, a four-fold table was used to calculate the sensitivity, specificity, positive predictive value, and negative predictive value of plasma VEGF-A at the level of 834 pg/mL. At this level, the sensitivity was 71.3%, specificity was 73.1%, the positive predictive value was 58.8%, and the negative predictive value was 82.6%.

The Kaplan-Meier curve showed that in group of high VEGF-A level (≥834pg/ml) (n=17), progression occurs in 10 patients and not in the other 7 patients (41.2%). While in group of low VEGF-A level (<834pg/ml) (n=23), progression was found only in 4 patients and not in the other 19 patients (81.26%). The difference of survival rate between the 2 groups was significant (41.2% vs 81.26%; p=0.009, log rank=6.81) (Figure 1).



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Expression of VEGF and vWF from tumor tissue

The levels of VEGF and vWF expression were determined in 30 patients. In group of patients with over expressed VEGF(≥25%) (n=11), progression occurred in 6 patients and not in the other 5 patients (45.5%). While in group of low VEGF expression(<25%) (n=19), progression was found only in 4 patients and not in the other 15 patients (79.6%). Although there was a trend of shorter survival rate in group of VEGF over-expression than in low expression one, the difference between the 2 groups was not significant (45.5% vs 79.6%; p=0.05, log rank 3.84) (Figure 2).

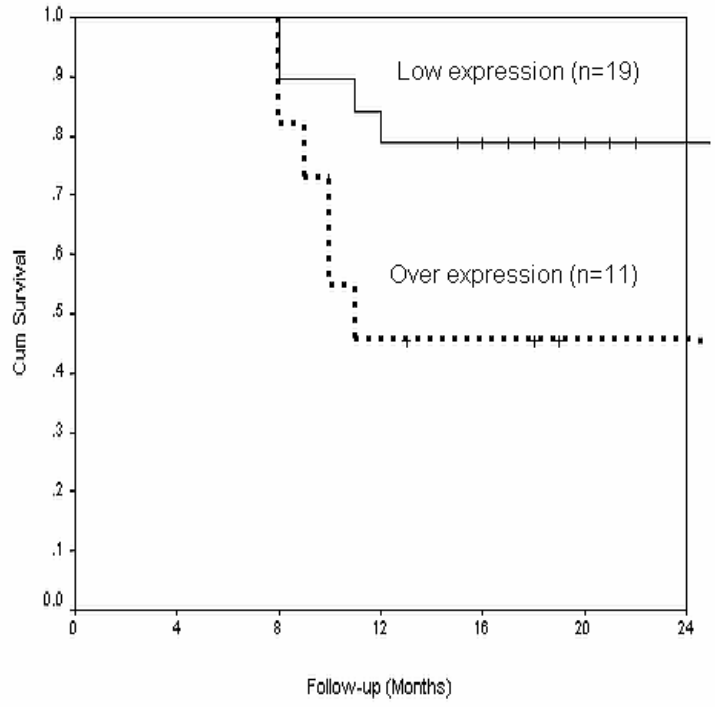

Figure 2. Kaplan-Meier curve showing that in group of patients with over expressed VEGF (≥25%) (n=11), progression occurred in 6 patients and not in the other 5 patients (45.5%). While in group of low VEGF expression(<25%) (n=19), progression was found only in 4 patients and not in the other 15 patients (79.6%). Although there was a trend of shorter survival rate in group of VEGF over-expression than in low expression one, the difference between the 2 groups was not significant (45.5% vs 79.6%; p=0.05, log rank 3.84).

In fifteen patients having vWF overexpression or higher MVD count (≥25), progression occurred in 7 patients and not in the other 8 patients (53.4%). While in group of low vWF expression or lower MVD count (n=15), progression was noted only in 3 and not in the other 12 patients (80%). Although there was also a trend of shorter survival rate in group of more MVD count than in lower one, the difference between the 2 groups was not significant (53.4% vs 80%; p=0.101, log rank=2.70) (Figure 3).

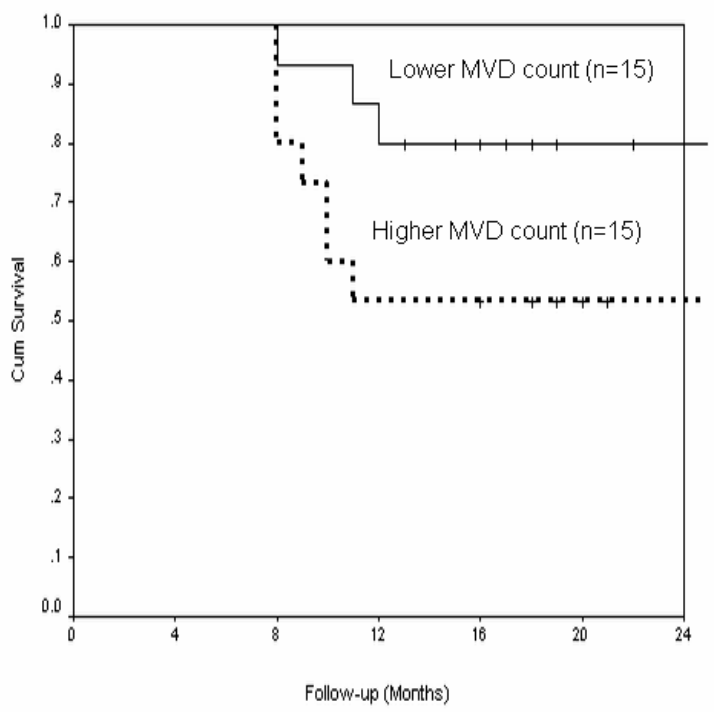

Figure 3. Kaplan-Meier curve showing that in fifteen patients having vWF overexpression or higher MVD count (≥25), progression occurred in 7 patients and not in the other 8 patients (53.4%). While in group of low vWF expression or lower MVD count (n=15), progression was noted only in 3 and not in the other 12 patients (80%). Although there was also a trend of shorter survival rate in group of more MVD count than in lower one, the difference between the 2 groups was not significant (53.4% vs 80%; p=0.101, log rank=2.70).

DISCUSSION

VEGF is a soluble peptide secreted by tumors into various body fluids. The finding of significantly higher serum VEGF levels in cancer patients than in normal subjects was firstly reported by Kondo et al in 1994, and was verified in subsequent studies of patients with various types of cancer. In the latter study, a significant correlation of the 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.[8,10] Elevation of serum-VEGF levels has been reported to be correlated with poor disease-free survival and poor progression-free survival in patients with urothelial carcinoma, with unfavorable survival in patients with small-cell lung carcinoma, with a poor 5-year survival rate in patients with non-Hodgkin lymphoma, and with an advanced tumor stage in patients with colorectal carcinoma; however, data on the role of VEGF in the disease progression of NPC are still lacking compared to other malignancies.[11]

In this study, we found a significant difference in survival rate between patients with an elevated level of VEGF plasma and a low level of VEGF plasma. Such a study which correlates the level of plasma VEGF and disease outcome in NPC is the novel finding of this study, which has not been reported previously from the Indonesian population to the best of

our knowledge. This result is relevant to other studies on the correlation of the VEGF level with disease progression and patient survival in other solid tumors. Qian et al reported an increase in the level of serum VEGF in non-metastatic NPC compared to the normal control, although the difference was not significant. They also reported a significant increase in the level of serum VEGF in metastatic NPC compared with normal controls and non-metastatic NPC.[3] Furthermore, the significant difference in disease progression between patients with an elevated and low level of plasma VEGF patients in this study possibly supported the theory of tumor hinderance by cytotoxic agents and the reduction of tumor oxygenation required for effective radiotherapy, which is caused by interstitial hypertension and poor blood flow, causing hypoxic and acidic microenvironments within tumors with excessive proliferation of endothelial cells and abnormal vessel maturation.[12,13]

VEGF expression on tumor tissue is affected by several factors such as tissue hypoxia, growth factors and cytokines, oncogenes and tumor suppressor genes. These factors may contribute to variations in the results of studies on the expression of angiogenic factors on tumor tissues. VEGF expression is found to be highest in hypoxic regions of the tumor near necrotic areas; hence, the site of tumor tissue may have a profound influence on the results when evaluating VEGF expression in a certain tumor. The level of circulating VEGF in serum is related with the higher expression of VEGF receptors on tumor tissue as well as microvessel density, which shows the important role of VEGF in the angiogenesis process of tumor growth.[13] In this study, we found a shorter survival in patients with overexpressed VEGF or vWF on tumor tissues, than in patients with a lower expression of the two markers. Although the differences were not significant, the trends were still obvious, as seen from the survival curves. These results supported the importance of VEGF and MVD examinations in angiogenesis, which affects tumor growth. Our biased results might be caused by the small sample size for tumor tissues.

In summary, elevation in the level of plasma VEGF which correlated with shorter survival in Indonesian advanced stage NPC subjects in this study lends weight to the positive role of VEGF in the disease progression of NPC. Although conducted in small size of samples, we believed that these results are substantial for the future development of VEGF as a prognostic factor and targeted therapy for NPC through inhibition of the VEGF-mediated signaling pathway. Investigation with larger samples is required to draw a more precise conclusion on the prognostic value of VEGF in NPC. Further studies to understand the role of Epstein Barr virus in the disease progression of NPC through VEGF-mediated angiogenesis are also needed.

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