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Correlation between vascular endothelial growth factor expression and cervical lymph node carcinoma

(Running head: Vascular Endothelial Growth,Cervical Lymph Node Carcinomaand Nasopharyngeal Carcinoma)

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ABSTRACT*---Introduction, Recent experimental evidence indicates that angiogenesis affects tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is considered to be an important regulator of tumor angiogenesis. The present study was designed to examine the role of VEGF on cervical lymph node metastasis in primary nasopharyngeal carcinomas (NPCs).*

*Methods: Formalin-fixed paraffin-embedded biopsy specimens were obtained from 36 primary NPC with histologically undifferentiated carcinomas and clinically divided into four cervical lymph node status, i.e. N0, N1, N2 and N3. The expression of VEGF was observed with immunohistochemistry involving rabbit polyclonal antibody Anti Human VEGF-A (Biocare Medical, LLC, USA). Assessment of the staining was performed by two independent observers who had no knowledge of the clinical background of the patients. Mann-Whitney U test was used to determine the correlation between the expression of VEGF and cervical lymph node status. Statistical significance was defined as p < 0.05.****Results:****VEGF expression in NPC based on cervical lymph node status, for weak VEGF expression was 83.33% for all samples, followed by moderate VEGF expression with16.67%. From the Mann-Whitney U-test result, it was found that pwas0.058. There was no significant correlation between VEGF expression and cervical lymph node status (p > 0.05).* ***Conclusion:*** *There was no correlation between the increase of VEGF expression and cervical lymph node enlargement in nasopharyngeal carcinoma.*

Keywords---*Cervical lymph node, growth factor expression, nasopharyngeal carcinoma, vascular endothelial*

I. INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is a malignancy derived from nasopharyngeal epithelcells. In Indonesia, NPC ranks fifth of all malignancy after cervical, breast, lymph node and skin cancer. In head and neck malignancies, NPC ranks first with frequency of about 60%[(1)]. In Indonesia the mean of the prevalence was 6.2/100,000 population per year or 13,000 new cases per year. A research in Dr. CiptoMangunkusumo Hospital Jakarta in 1996-2005 obtained 1,121 new NPC patients [(2)].

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Nasopharyngeal carcinoma is known as a very malignant tumor which easily infiltrates local tissue and metastasis often occurred in the cases. Seventy-five percent of patients come with complaints of lumps in the cervical lymph node. The biological nature of NPC depends on the status of cervical lymph node.Patients with advanced cervical lymph node have a poor prognosis tendency NPC.One of the ways to determine the progression ofNPCis through cervical lymph node status[(3)].

Current predictions of NPC prognosis are generally still based on clinical staging assessments, ie by assessing primary tumor (T), cervical (N)) metastases and distant metastasis (M).Many NPC patients with the same clinical stages show different prognoses. This suggests that TNM assessments have not been able to provide accurate predictions for NPC prognosis, and thus it is necessary to develop molecular biomarkers on NPC that can help clinicians improve prognostic predictions and therapeutic interventions in NPC patients[(4)].

The process of invasion and metastasis of tumor cells in NPC is still a question to date. Several influential molecular biomarkers in the metastasis process have been identified in tumor specimens of KNF patients. A recent study suggested angiogenesis to be a major factor for tumor growth, invasion and metastasis [(4)]. The expression or increased expression of several receptors has been evaluated in NPC, i.e., the epidermal growth factor receptor (EGFR), cKIT c-erbB-2 (HER-2) and vascular endothelial growth factor (VEGF).Those are pro-angiogenics factors that play a role in angiogenesis [(3)].

VEGF protein is a pro-angiogenic factor as well as a major mediator that plays a role in angiogenesis for tumor growth, invasion and metastasis. VEGF expression in tumor tissue is affected by several factors including genetic, tissue hypoxia, cytokines and growth factors, on cogenes and tumor suppression genes[(3)]. An angiogenic phase switch causes angiogenesis initiation by expressing VEGF. Activation of VEGF neurons by VEGF receptors triggers multiple signal transduction resulting in survival, proliferation and migration of endothelial progenitor cells to peripheral circulation with clinical manifestations of rapid primary tumor growth, cervical lymph node and distant metastases[(2)].

Several studies assessing the association of VEGF expression with growth and metastasis in KNF through immunohistochemical examination of NPC tissue still yield different results. A study reported VEGF expression between tissue samples taken from normal nasopharynx, benign nasopharyngeal tumor and NPC had expression values of 10%, 40% and 80%[(15)]. 80.1% (59 of 73) immunohistochemical studies of KNF tissue gave positive VEGF expression and had a significant correlation with KNF growth and metastasis. VEGF played an important role in cervical lymph node metastasis through the induction of angiogenesis in KNF[(15)].

The main role of VEGF in tumor angiogenesis has been the focus on basic research and drug development in the field of oncology[(15)]. From several studies it has been concluded that an anti-VEGF combination with chemotherapy or radiotherapy produces a better anti-tumor effect than chemotherapy/radiotherapy alone[(13)]. One of the first-line therapy trials has been performed with bevacizumab of a humanized monoclonal antibody directed against VEGF in combination with chemotherapy providing survival benefits in metastatic colorectal cancer[(15)].

This study aims to analyze the relationship of increased VEGF expression with cervical lymph node enlargement (N) as a clinical manifestation of tumor angiogenesis that has not been done in RSUD Dr. Soetomo Surabaya.

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II. METHOD

This was an observational analytic study with cross-sectional design.The study was conducted from January to April 2013 at the One Stop Oncology Poly (POSA) Otolaryngology-HN, NPC in the laboratory of Anatomy Pathology Faculty of Medicine, UniversitasAirlangga-Dr. Soetomo Surabaya.This study involved samples of NPC patients without cervical lymph node N0 and had cervical lymph nodes N1, N2, N3 as well as nasopharyngeal histopathologic preparations that had been established as NPC of WHO type III (undifferentiated carcinoma).

The inclusion criteria in this study were NPC patients with cervical lymph nodes N0, N1, N2 and N3 as well as histopathologic preparations in the form of paraffin blocks of nasopharyngeal biopsy materials with sufficient tumor tissue for VEGF expression examination with immunohistochemical techniques.The exclusion criteria were patients who have received the definitive therapy before, i.e. radiotherapy, chemotherapy or a combination of both. The sampling technique used in this study was consecutive sampling with probability sampling technique, i.e. sampling without randomization according to the sample size.

This research procedure began with the first examination of NPC WHO type III patient in Otolaryngology-Head and Neck POSA of Dr. Soetomo General Hospital Surabaya according to the inclusion and exclusion criteria, followed by checking the patient's cervical lymph node status and collecting large sample consisted of nine patients for each group of N0, N1, N2 and N3, then collecting histopathological examination numbers of patients, looking for paraffin blocks of NPC network, and the selection of networks that were considered sufficient by anatomical pathologist consultant in Anatomical Pathology Laboratory Faculty of Medicine-Dr. SoetomoGeneral Hospital Surabaya.Immunohistochemical discovery was then conducted afterwards with VEGF antibody of rabbit polyclonal antibody anti HumanVEGF-A clone EP1176Y (Biocare Medical, LLC, USA), and VEGF expression was evaluated by consultant anatomical pathology doctors in AP laboratory in Faculty of Medicine, Gajah Mada University Yogyakarta, and then VEGF expression assessment was performed by consultant anatomical pathology doctors in Anatomical Pathology Laboratory Faculty of Medicine UniversitasAirlangga/ Dr. Soetomo General Hosptital Surabaya. After that, the results of assessment (score) VEGF expression were recorded on the data collection sheet.

All the collected data were arranged in the data collection sheet in tabular form and processed statistically using SPSS (SPSS. Inc. Chicago IL). The data analysis in this study used Mann-Whitney U-test to determine the relationship between the increase of VEGF expression with enlargement of cervical lymph node N0, N1, N2 and N3 on KNF. VEGF expression was rated as a nominal variable based on the scale described above. The level of significance (α) = 0.05.

III. RESULTS

Basic data

The result of data analysis was obtained if most of NPC patient was in the age group of 40-49 years old with 19 cases (52.77%), followed by the age group of 30-39 years oldwith 8 cases (22.22%) and 50-59 years old with to 6 cases (16.67%). The youngest age was 30 years old and the oldest was 72 years old (Table 1). Based on the analysis of the sexes, there were 22 respondents (61.11%) and 8 cases (38.89%).The ratio between men and women was 1.5:1 (Table 2).The results of analysis on the distribution of ethnic groups found that most NPC patients were Javanese with 31 patients (83.33%), Madurese with 5 patients (13.89%) and Dayak tribe with 1 patient (2.78%) (Table 3). In

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the distribution of the occupation, the results showed that most of the samples were farmers with 11 patients (30,55%) followed by unemployed with 10 patients (27,77%) (Table 4).

Correlation ofIncreased Expression of Vascular Endothelial Growth Factor and Enlarged Cervical Lymph Node in Nasopharyngeal Carcinoma

The result of VEGF examination on NPC with cervical lymph node N0 found weak expression on 9 samples. In NPC with cervical lymph node N1 there were 8 samples with weak expression and 1 with medium expression. In NPC with cervical lymph node N2 there were 7 samples with weak expression and 2 samples with medium expression. As in NPC with cervical lymph node N3, 6 samples were obtained with weak expressions and 3 samples with medium expression (Table 5).

VEGF expression was weak found weak in 83.33% of all samples.VEGF expression was 16.67% of all samples, and there was an increase in the number of cells giving medium expression on N0, N1, N2 and N3.VEGF positive 2 and positive 3 expressions which were considered as strong and very strong expression were not found in this study.The result of Mann Whitney U-test obtained p value = 0.058. This implied that the correlation between increased VEGF expression in NPC and cervical lymph node enlargement N0, N1, N2 and N3 was found to be non-significant ($p > 0.05$).

Immunohistochemistry results in KNF tissue were identified by the presence of dark brown color in cell membranes and tumor cell cytoplasm. Observation and analysis of VEGF expression were performed with a binocular microscope with 400x magnification.

IV. DISCUSSION

In distribution of NPC patients based on age, the incidence begins to increase at age 45-54 years, then decreases.NOC was mostly found in the productive age of 30-59 years (about 80%), with peak age between 40-49 years and the highest incidence at age 40-60 years [1, 5]. The group of age was productive ages for workers and they were often being outside of the house which increased the exposure to carcinogenic substances or more pollution[6]. Cancer cells arise from normal cells undergoing malignant transformation, since a contact with carcinogens to the onset of cancer cells is required for a sufficiently long induction time, up to 15-30 years [7]. EBV infection as a risk factor of NPC had the latency period in infected nasopharyngeal cells, about 20-25 years without symptoms of primary EBV infection in childhood and asymptomatic. Malignant process in NPC occurs after latent infection was found at age 40-60 years [8].

The previous research obtained the ratio with men and women of 4:1 [9]. Meanwhile, a research from other center in Adam Malik Hospital obtained the ration of men and women of 2.5:1 (10). In distribution of NPC patients based on gender, most of the patients were male (70%) with the ratio between men and women of 3: 1[1, 5]. High incidence in men might be due to differences in living habits and occupations that caused men to be more in contact with carcinogens that cause NPC Life habits such as smoking increase the risk of NPC to 2-6 times.Exposure to formaldehyde in the workplace increases the risk of NPC to 2-4 times.Increased risk also occurs in workers who inhale firewood smoke, and the risk increases 2 times in workers exposed to industrial heat and combustion products [11].

The previous studies of NCP cases found the most tribe was the Javanese with 73.64% followed by the Madurese of 13.94% [12]. A research from other centers obtained the most results from Batak 42.9%, followed by Java

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28.6% and the rest were Malay, Minang, Aceh and Banjar [10]. NPC incidence remained high among the descendants of South China resident in another country. Thus, this became the predisposition to the disease, and was combined with the triggers from the environment[13].

Indonesia which is belonged to the Malayo Polynesian group of the Mongoloid race had a fairly high frequency[14]. In this study there were differences in the distribution of ethnic groups due to the largest population in Surabaya as the location of study was Javanese. A study on Batak tribe found a potential gene allele as the cause of susceptibility of NPC, i.e. HLA-DRB * 08 [15].

Based on the literature the exposure that may occur to workers associated with NPC events was exposure to dust or particles of moderate size (5-10 μm). This was because particles were readily absorbed by the nasopharyngeal mucosa. Several epidemiologic studies showed increased risk factors for NPC in workers exposed to wood dust in certain periods and doses.Other studies have also found an increased risk of NCP occurring in workers working in combustion-burning environments (ash, charcoal). The ratio of worker exposure could not be determined because it was dependent on frequencies and endemic areas [16].Smoking and occupational exposure to formaldehyde and wood dust also became the risk factors. Formaldehyde was known as carcinogenic cavity of rice in rats.Smoke particles derived from burning coal, wood and other materials were mostly stored in the nasopharynx [17]. There were several major chemicals known to cause KNF based on occupational exposure, including bleaching agents, acids and bases, sulfuric acid, inks, formaldehyde and pesticides, and the exposure risk of 10-20 years [18].

The correlation between increased VEGF expression with cervical lymph node enlargement N0, N1, N2 and N3 on KNF was found to be non-significant ($p > 0.05$).Thus, the hypothesis of this study is not proven.The results of this study indicated that the statistical value is not significant, with value of $p = 0.058$.The p value itself had a very small difference of 0.008 of the p values ($p < 0.05$).This could be influenced by several factors, one of which was the selection of a cross sectional study design.In an analytic research, the level of validity of the research became the main consideration of research design selection.The same results were reported if in a meta-analysis of 12 studies and 1002 patients with head and neck squamous carcinoma cells (HNSCC) in which 7% of cases were NPC. The results of the meta-analysis showed no association between VEGF expression with cervical lymph node metastasis in HNSCC [19]. Another study in assessing the expression of VEGF on 28 NPC networks obtained positive VEGF expression at an early stage of 7.1% and at an advanced stage of 67.9%.The results of statistical tests on the study did not show any significant correlation between VEGF expression and NPC stage [10].

Differences in the results of the study might be influenced by many other things such as tumor angiogenesis assessment depending on the size of the tumor, the size of the tissue sample, the location of the measurement (on the hot spots of the tumor or average calculation), immunohistochemical techniques, the scoring system, and the method of assessment [17].Other studies have reported an association between VEGF expression and cervical lymph nodes in NPC patients. The conclusion of the study is that VEGF had an important role in cervical lymph node metastasis through induction of angiogenesis in NPC. In the study, different calculation systems were used, i.e. scale 1 and 2[3].

The VEGF positive ratio was rated on a scale of 1 with a mean value of 76%, and a scale of 2 with a value of more than 76%. Weak, moderate, strong and very strong score system was used in this study. VEGF immunoreactivity observation by Wakisaka et al. performed on the cytoplasm of tumor cells. Strong painting intensity could be found in peripheral invasive tumors.The observations were performed with a light microscope with

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200x magnification.Calculation was then performed in three different areas to obtain the mean to be then multiplied by 100, and the value obtained was the positive ratio value.Different observational techniques were carried out in this study, where calculations were performed at 400x enlargement cell calcuatio was performed with VEGF immunoreactivity in three different areas to calculate the mean of the cell amount.

The angiogenesis process in NPC was regulated by EBV.Epstein-Barr Virus encoded the latent membrane protein 1 (LMP1) to initiate angiogenesis in NPC by induction of interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2).Epstein-Barr Virus also coded EBNA 1 which also induces angiogenesis in NPC.Some other proteins that influence the angiogenesis process in NPC included VEGF, hepatocyte growth factor and macrophage migration inhibitory factor (Du, 2012). KNF WHO types II and III were strongly associated with EBV [(8)]. This study used a sample of KNF WHO type III that was strongly related with EBV.

Studies showing the relationship between VEGF and IL-8 expression with LMP1 in angiogenesis process indicated 39 NPC networks. The study showed that LMP1 expression had a significant relationship with IL-8 expression, but the expression of LMP1 with VEGF and bFGF showed statistically insignificant relationship [(20)].

V. CONCLUSION

There was no correlation between inceasedVEGF expression and cervical lymph node enlargement in NPC.

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Tables

Table 1: Patients' age distribution

Age (year)	N	Percentage (%)
30 – 39	8	22.22
40 – 49	19	52.77
50 – 59	6	16.67
60 – 69	1	2.78
≥ 70	2	5.56

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Total	36	100.00

Table 2: Patients' gender distribution

Gender	N	Percentage (%)
Male	22	61.11
Female	14	38.89
Total	36	100.00

Table 3: Patients' ethnicity distribution

Ethnicity	N	Percentage (%)
Java	30	83.33
Madura	5	13.89
Dayak	1	2.78
Total	36	100.00

Table 4: Patients' occupation distribution

Occupation	N	Percentage (%)
Farmer	11	30.55
Factory worker	5	13.89
Entrepreneur	9	25.00
Stonemason	1	2.78
Unemployed	10	27.77
Total	36	100.00

Table 5: The examination results of VEGF value and cervical lymph node enlargement (N0, N1, N2, N3) in patients with NPC

Skor	N0	N1	N2	N3	%
-	9	8	7	6	83.33
+	0	1	2	3	16.67
++	0	0	0	0	0
+++	0	0	0	0	0
Total	9	9	9	9	100.00

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Note: Score - : Weak expression (tumor cells of <25 cells/LPB)

\+ : Medium expression (tumor cells of 25-50 cells/LPB)

++ : Strong expression (tumor cells of 50-75 cells/LPB)

+++ : very strong expression (tumor cells of >75 cells/LPB)

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