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Full Length Research Paper

Epstein Barr virus latent membrane protein-1 (LMP-1) expression and angiogenesis in Indonesian nasopharyngeal carcinoma (NPC)

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The pivotal role of Epstein Barr virus latent membrane protein 1 (LMP-1) in angiogenesis has been concluded from several *in vitro* experiments. However, there were very limited and inconsistent data on the correlation between LMP-1 and angiogenesis from clinical samples. Therefore, we aimed to investigate the correlation between LMP-1 and angiogenesis from Indonesian nasopharyngeal carcinoma (NPC). Forty cases of paraffin embedded tissues were stained by immunohistochemistry for the expressions of LMP-1, proangiogenic factors including vascular endothelial growth factor (VEGF) and interleukin (IL)-8, and von Willebrand factor (vWF) indicating microvessels density (MVD) as the final result of angiogenesis. The level of plasma $VEGF_{165}$ was determined by ELISA. The results showed that LMP-1 expression was neither correlated significantly with ^{10}log MVD, ^{10}log plasma and tissue VEGF, nor ^{10}log IL-8 (r=0.273, p=0.088; r=-0.109, p=0.504; r=0.140, p=0.390 and r=0.274, p=0.087, respectively). However, there were trends that a higher expression of LMP-1 was followed by a higher expression of MVD, tissue VEGF and IL-8. In conclusion, our results may be supportive to the experimental findings on the role of LMP-1 in angiogenesis. Not less important, our study that also showed significant correlation between tissue VEGF and MVD and between IL-8 and MVD suggests a possible role for antiangiogenic therapy in nasopharyngeal carcinoma.

Key words: LMP-1, angiogenesis, NPC.

INTRODUCTION

The role of angiogenesis or new blood vessels formation in the progression, invasion and metastasis of cancer has been widely investigated in nasopharyngeal carcinoma (NPC). Microvessels density (MVD) indicates the final result of angiogenesis that are promoted by proangiogenic factors. Vascular endothelial growth factor (VEGF) and Interleukin-8 (IL-8) are proangiogenic factors reported to be involved in angiogenesis of NPC (Qian et al., 2000; Krishna et al., 2005; Li et al., 2008; Kurnianda et al., 2009).

A strong association between Epstein-Barr virus (EBV) and the incidence of NPC has been accepted, as indicated by the presence of EBV DNA within the malignant cells in the majority of cases, particularly WHO type III (undifferentiated carcinoma). Latent Membrane Protein-1 (LMP-1) is the major oncoprotein of EBV involved in angiogenesis concluded from several *in vitro* experiments where LMP-1 gene had been introduced to EBV-negative cell lines and showed to promote angiogenesis via induction of several pathways such as COX-2, NFκβ and SIAH1, leading to downstream

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transcription of proangiogenic factors such as VEGF, IL-8 and HIF-1α (Yoshizaki, 2002, Murono et al., 2001; Yoshizaki et al., 2001; Kondo et al., 2006, Wakisaka et al., 2004,). In relation with suggested role of LMP-1 in angiogenesis, several preclinical studies investigated the possible development of LMP-1 vaccine to inhibit angiogenesis (Duraiswamy et al., 2003). Therefore, data from clinical samples are necessary to confirm the biological plausibility of LMP-1 role in angiogenesis concluded from the above mentioned experimental studies. So far, there were few and inconsistent clinical data on the correlation between LMP-1 and angiogenesis. One study from Yoshizaki et al reported a significant correlation between LMP-1 and MVD in 38 samples of NPC from Taiwan by immunohistochemistry, suggesting that LMP-1 might have induced some proangiogenic factors. They found significant correlation between LMP-1 and IL-8, but not with VEGF (Yoshizaki et al., 2001). Benders et al found no consistent relationship between LMP-1 and either HIF-1α expression or MVD in 18 NPC materials (11 Indonesians, 7 Americans) by sensitive fluorescent and signal amplification technologies (Benders et al., 2009). Furthermore, a consistent correlation between LMP-1 and angiogenesis in clinical samples might also be challenged by the concept of LMP-1 polymorphisms (Burrows et al., 2004).

In this study we intended to investigate the correlation between LMP-1 and proangiogenic factors including plasma and tissue VEGF, IL-8 and MVD in 40 cases of Indonesian NPC. The examination of both tissue and plasma VEGF was due to their different biological and functional properties. The data obtained from our study will be beneficial to add the lack of data from clinical samples on the correlation between LMP-1 and angiogenesis.

MATERIALS AND METHODS

Forty specimens consisted of paraffin embedded tissue biopsies and frozen plasma derived from peripheral venous blood samples obtained from NPC patients were collected randomly from our archives of NPC project in Faculty of Medicine Gadjah Mada University, Sardjito General Hospital Yogyakarta Indonesia from 2003 to 2007. This study was performed under the umbrella project of NPC research in our institution, and was approved by the institutional ethics committee. All patients were required to provide written informed consent before starting treatment. They were composed of nil squamous cell carcinomas (WHO type I), 6 nonkeratinizing carcinomas (WHO type II), and 34 undifferentiated carcinomas (WHO type III). Twenty-nine patients were male, and 11 patients were female (mean age, 43.8±12.48 years; range, 19 to 72 years). Twenty three patients were stage III, 3 patients were stage IVa and 14 patients were stage IV B according to the International Union against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) 1997 criteria.

Immunohistochemical analysis

Immunohistochemical studies were performed using the avidin-biotin-complex method. Biopsies contained a substantial area of necrosis were excluded from the study. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 10 minutes 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 primary antibodies. Primary antibodies against LMP-1 (CS1-4; DAKO, Copenhagen, Denmark), IL-8 (BMS136, Bender Medsystem, California, US), VEGF (RB-9031-P, Thermo Scientific, California, US), and von Willebrand Factor (vWF) (clone 36B11, Novocastra Laboratories, Newcastle, UK) were diluted 1:50. After that, 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. Scoring system for LMP-1 was done according to Khabir et al. (Khabir et al., 2005). LMP-1 scoring was done in 5 fields and the result was presented as a mean. The same scoring system was used for IL-8. VEGF expression was evaluated by counting the mean number of cells stained in 5 views of 400x magnifications including 100 cells preview, and micro-vessels density was counted as mean number of cells stained with vWF within 5 views of 100x magnifications (Kurnianda et al., 2009).

Determination of plasma VEGF-A level

Determination of plasma VEGF A level was done based on method used in a previously published study by our group (Kurnianda et al., 2009). It was done by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine DVE00, R&D Systems, Minneapolis, USA) recognizing VEGF-A 165 bp splicing variant, according to the manufacturer's instructions. 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 9.0 pg/mL-1. Each assay well measured 100 µl plasma. All analyses and calibrations were carried out in duplicate. Optical density was determined using a microtitreplate reader at 450 nm/540 nm and concentrations are given in pg/ml.

Statistical analysis

Data was analyzed using SPSS software version 16.0. Statistical analysis was performed by Spearman's correlation coefficient by rank test and Pearson's correlation coefficient analysis, with $p < 0.05$ considered to be statistically significant.

RESULTS

LMP-1,IL-8,VEGF and MVD immunohistochemical features and expression scores

The expression patterns of LMP-1, IL-8 dan VEGF were found both on the surface and cytoplasms. Microvessels remarked by vWF staining were scattered on the tumor tissues and showed similar distribution with LMP-1, VEGF and IL-8 under 100x magnification. Expression scores of 16 samples were calculated by 2 pathologists blind to patient diagnosis separately and produced a Kappa score of 0.86. Based on this value, the following examinations were done by a pathologist. The data of expression scores are shown in Table 1.

Table 1. Expression scores of LMP-1, VEGF, IL-8, MVD, and level of plasma VEGF.

	Mean	Standard deviation	Minimum	Maximum
LMP-1	5.9	3.4	0	11.6
VEGF (pg/ml)	958.3	600.7	180.0	3056.4
VEGF	24.4	17.3	2	65.8
IL-8	4.9	3.1	0.2	9.6
MVD	23.8	21.5	2.8	84.2

Note: * Pearson's correlation coefficient analysis, Spearman's correlation coefficient by rank test.

Table 2. Correlations between LMP-1 and angiogenesis in NPC.

	r	p
LMP-1 and ^{10}log MVD*	0.273	0.088
LMP-1 and ^{10}log plasma VEGF *	-0.109	0.504
LMP-1 and ^{10}log tissue VEGF **	0.140	0.390
LMP-1 and ^{10}log IL-8**	0.274	0.087
^{10}log plasma VEGF and ^{10}log MVD*	0.031	0.850
^{10}log tissue VEGF and ^{10}log MVD**	0.612	0.000
^{10}log IL-8 and ^{10}log MVD**	0.317	0.046
^{10}log tissue VEGF and ^{10}log plasma VEGF	0.255	0.113

Note: * Pearson's correlation coefficient analysis, **Spearman's correlation coefficient by rank test.

Shapiro Wilk test for data distribution normality resulted in normal distribution only for LMP-1, and abnormal for the rest of variables. Logaritmic data transformation resulted in normal distribution for ^{10}log VEGF plasma and ^{10}log MVD, but not for ^{10}log IL-8 and ^{10}log tissue VEGF. Pearson's correlation coefficient analysis was used when the two data were normally distributed, and Spearman's correlation coefficient by rank test was used when one or two data remained abnormally distributed.

Correlations between LMP-1 and angiogenesis in NPC

Correlations between LMP-1 and MVD as well as with proangiogenic factors: plasma and tissue VEGF, and IL-8 were shown in Table 2. There was neither significant correlation between LMP-1 and ^{10}log MVD, or LMP-1 and proangiogenic factors namely ^{10}log plasma VEGF, ^{10}log tissue VEGF and ^{10}log IL-8. However, there was a trend that an increase in LMP-1 expression score was accompanied by an increase in ^{10}log MVD score, as shown in Figure 1.

Correlation between proangiogenic factors and angiogenesis in NPC

There were significant correlations between proangiogenic factors namely ^{10}log tissue VEGF and ^{10}log IL-8 with ^{10}log MVD, but not for ^{10}log plasma VEGF and ^{10}log MVD, as shown in table 2. The significant correlations between ^{10}log tissue VEGF and ^{10}log IL-8 with ^{10}log MVD were shown in Figures 2 and 3.

DISCUSSION

Although there was not statistical significant correlation between LMP-1 and ^{10}log MVD in our NPC cases ($r = 0.273$, $p = 0.088$), we could still observe a trend that an increase in LMP-1 score expression was accompanied by an increase in MVD score. This positive trend may still be supportive for the experimental data on the role of LMP-1 in angiogenesis. Our result is different from a study by Yoshizaki et al. (2001) that reported a significant correlation between LMP-1 and MVD in NPC samples from 38 patients in Taiwan. This difference may be caused by several factors such as pathological technique including different clones of antibodies and scoring system employed. We could also presume a possibility that polymorphisms factor in LMP-1 gene might have an implication in angiogenesis pattern affected, at least in Indonesian population compared to Taiwan. A basic experiment introducing at least two polymorphic variants to EBV negative cell lines is required to verify this presumption. However, since we should not overlook a possible different mechanism of LMP-1 in angiogenesis in an in vitro and in vivo, a more conclusive clinical data on the effect of LMP-1 to angiogenesis in NPC also

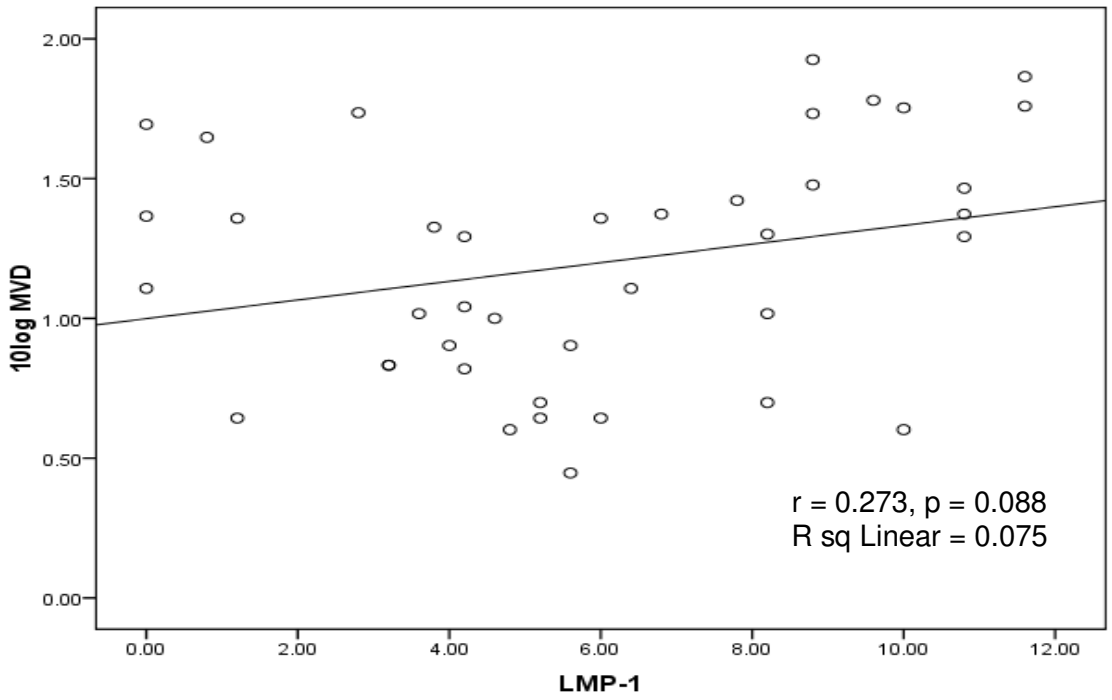

Figure 1. Correlation regression analysis between LMP-1 and ^{10}log MVD.

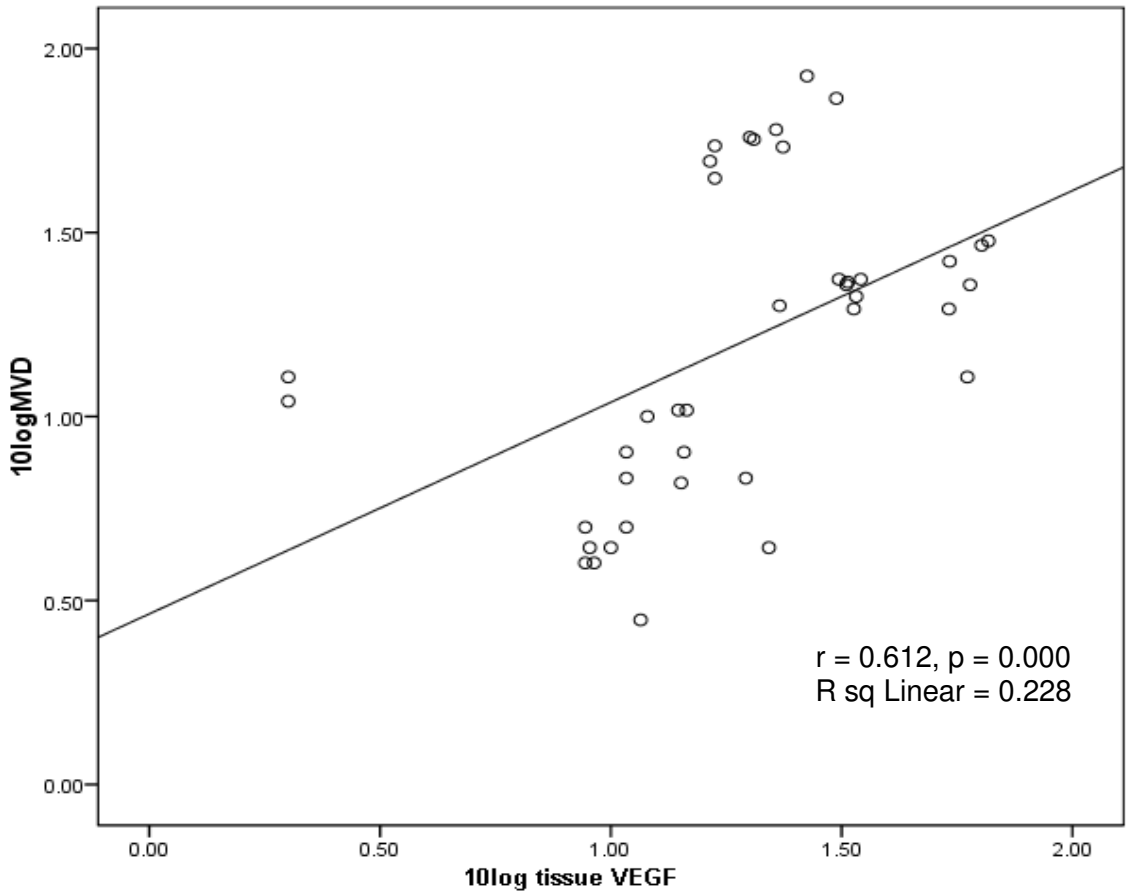

Figure 2. Correlation regression analysis between ^{10}log tissue VEGF and ^{10}log MVD.

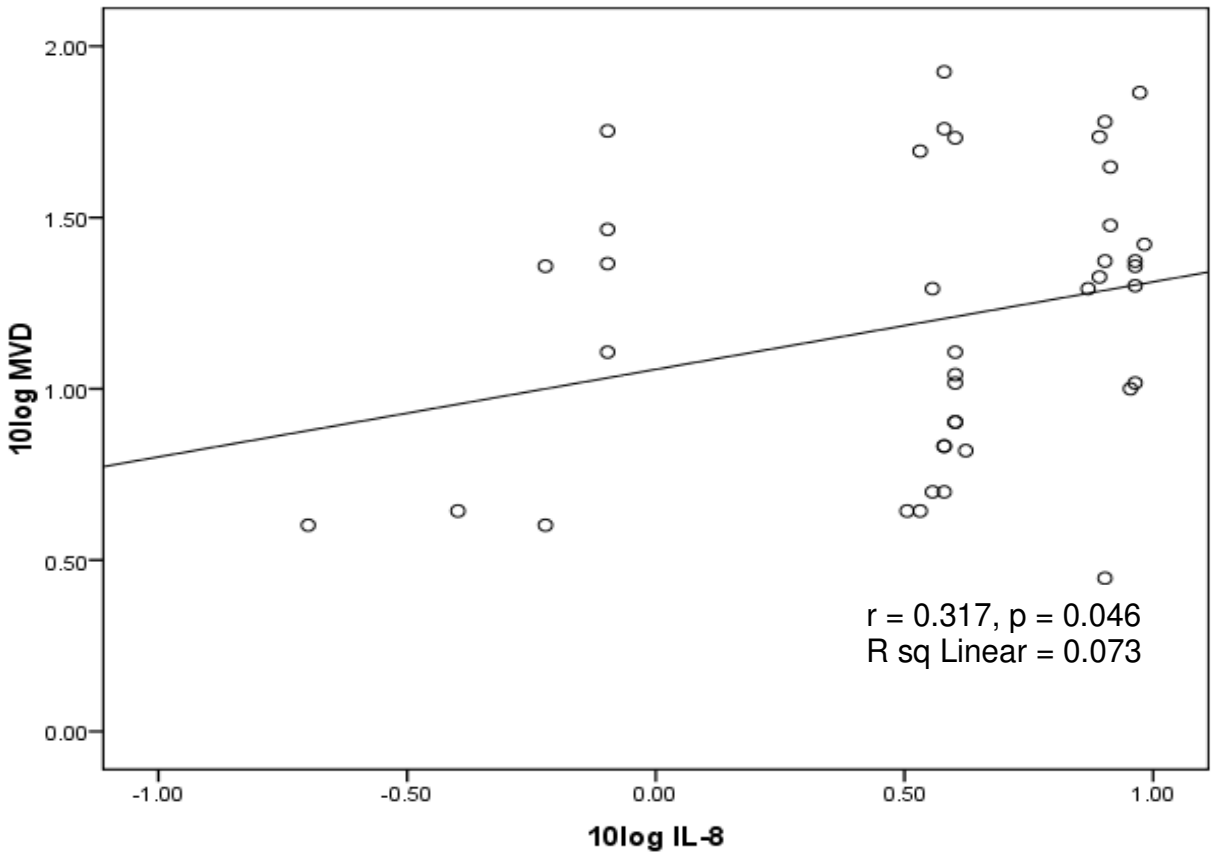

Figure 3. Correlation regression analysis between ^{10}log IL-8 and ^{10}log MVD.

requires further works involving more samples either from endemic or non endemic areas where the concept of LMP-1 polymorphisms had been reported (Burrows et al., 2004).

We did not find a significant correlation between LMP-1 and ^{10}log plasma and tissue VEGF in this study (r = -0.109; p = 0.504 and r = 0.140; p = 0.390, respectively). These results do not support an *in vivo* study which directly introduced LMP-1 into EBV-negative cell lines resulting in an induction of COX-2 that subsequently promoted VEGF production (Murono et al., 2001). Other experimental study using similar technique concluded that VEGF induction by LMP-1 was achieved via HIF-1α and NFκβ pathway (Wakisaka et al., 2004). Nevertheless, our result is in accordance with Yoshizaki et al (2001) who reported no significant correlation between LMP-1 and tissue VEGF in clinical setting. The data on the correlation between LMP-1 and plasma VEGF has never been reported to the best of our knowledge. Such data is necessary considering different biological properties of membrane bound and soluble VEGF. It is known that soluble VEGF originated from the VEGF bound to cell membrane or extracellular matrix (ECM) that is processed by protease enzyme, to initiate angiogenesis. Nonetheless, the role of bound VEGF in maintaining vascular endothelial survival should not be overlooked for an ultimate angiogenesis process (Hutchings et al., 2003; Hicklin and Ellis, 2005).

We found no significant correlation between LMP-1 and ^{10}log IL-8 (r = 0.274, p = 0.087). However, we could still observe a trend that an increase in LMP-1 expression score was accompanied by an increase in ^{10}log IL-8 expression score. This result is not in accordance with the result from Yoshizaki et al. (2001) that showed a significant correlation between LMP-1 expression and IL-8. Moreover, this group also proved that the induction of IL-8 by LMP-1 occured via activations of NFκβ and AP-1 pathways (Yoshizaki et al., 2001). However, a recent study by Benders et al. (2009) on the expression of LMP-1 in relationship with HIF1α which is a major transcription factor in angiogenesis, and with MVD, in 18 NPC samples with a more advanced technique called chromogenic and immunofluorescent histochemical stains methods found an inconsistency either between LMP-1 and HIF-1α or MVD. The difference between their result with in vitro models where LMP-1 directly induced HIF-1α (Wakisaka et al., 2004, Kondo et al., 2006) might show that *in vivo* settings can be expected to behave in a much more complicated fashion (Benders et al., 2009). This assumption may also be considered to explain the difference between our result and *in vivo* data from

Yoshizaki et al. (2001). Still, we could not exclude a possible factor of LMP-1 polymorphisms to explain such difference.

The sub analysis of relationships between ^{10}log tissue VEGF and MVD, and ^{10}log IL-8 and MVD revealed significant correlations (p=0.000, p=0.046, respectively), but not for ^{10}log plasma VEGF and ^{10}log MVD (p=0.850) (Table 2). Regression analysis showed a significant correlation between ^{10}log tissue VEGF and ^{10}log MVD (r=0.612, p=0.000) (Figure 2). This confirms the literatures explaining about the important role of VEGF in various malignancies (Felmeden et al., 2003; Hicklin and Ellis, 2005). In particular, this result adds important data on the relationship between tissue VEGF and MVD from Indonesian population. Our data is in accordance with previous study from Taiwan that reported a significant correlation between tissue VEGF expression and MVD (Yoshizaki et al., 2001). The relationship between plasma VEGF and MVD in NPC had never been reported before. This data supports previous clinical studies showing the important role of VEGF as prognostic factors in NPC and lends positive weight to application of anti VEGF therapy in NPC.

The correlation between tissue VEGF and plasma VEGF revealed no statistical significance (p=0.133) (Table 2). This is in accordance with theory that acknowledges the difference between measurements of bound and soluble VEGF is greatly affected by local condition of the tumor, especially tissue hypoxia factor. This local factor induces an effect to increase VEGF as a compensatory mechanism for more angiogenesis (Hicklin and Ellis, 2005; Wakisaka et al., 2004; Kondo et al., 2006). Such important role of tumor local factor may also explain a very strong correlation between tissue VEGF and MVD observed in this study, but not between plasma VEGF and MVD.

A significant correlation was also found between ^{10}log IL-8 and ^{10}log MVD (r = 0.317, p = 0.046) (Figure 3). This result shows that IL-8 contributed in angiogenesis in NPC and is in accordance with several studies either from basic or clinic on the role of IL-8 as a potent proangiogenic factor (Poon et al., 2001). This data is also in accordance with the report from NPC patients from Taiwan (Yoshizaki et al., 2001).

In conclusion, although correlations between LMP-1 and MVD, LMP-1 and VEGF (tissue and plasma), LMP-1 and IL-8 were not significant, trends that a higher expression of LMP-1 were followed by higher MVD, and higher expressions of tissue VEGF and IL-8 may still be supportive to the experimental data on the role of LMP-1 in angiogenesis. Further research including basic experiment regarding concept of LMP-1 polymorphisms and studies involving more samples are required to draw a better conclusion. Different factors in clinical setting than in experimental one should not also be neglected. Not less important, sub analysis data that showed significant correlation between tissue VEGF and MVD, and between IL-8 and MVD lend positive weights to application of anti angiogenic therapy in NPC.

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