Induction of lymphangiogenesis through vascular endothelial growth factor-C/vascular endothelial growth factor receptor 3 axis and its correlation with lymph node metastasis in nasopharyngeal carcinoma

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Title: Induction of Lymphangiogenesis through Vascular Endothelial Growth Factor-C/Vascular Endothelial Growth Factor Receptor 3 Axis and its Correlation with Lymph Node Metastasis in Nasopharyngeal Carcinoma

Authors and affilations:

Naohiro Wakisaka[1], Kyoko Hirota[1], Satoru Kondo[1], Seiko Sawada-Kitamura[2], Kazuhira Endo[1], Shigeyuki Murono[1], Tomokazu Yoshizaki[1*]

[1]Division of Otolaryngology, Head and Neck Surgery, and 2Division of Human Pathology,

School of Medicine, Kanazawa University,

Takara-machi 13-1, Kanazawa, Ishikawa,

920-8640, Japan

Naohiro Wakisaka, MD, PhD

E-mail: wakisaka@med.kanazawa-u.ac.jp

Kyoko Hirota, MD

E-mail: VEZ07302@nifty.com

Satoru Kondo, MD, PhD

E-mail: ksatoru@med.kanazawa-u.ac.jp

Seiko Sawada-Kitamura, MD, PhD

E-mail: sawadase@med.kanazawa-u.ac.jp

Kazuhira Endo, MD, PhD

E-mail: endok@med.kanazawa-u.ac.jp

Shigeyuki Murono, MD, PhD

E-mail: murono@med.kanazawa-u.ac.jp

Corresponding author*:

Tomokazu Yoshizaki, MD, PhD

Mailing address: Division of Otolaryngology, Head and Neck Surgery, Graduate School

of Medicine, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-8640,

Japan

Phone: +81-76-265-2413

Fax: +81-76-234-4265

E-mail address: tomoy@med.kanazawa-u.ac.jp

Abstract

Objectives: The contribution of the lymphatic system to tumor metastasis is being increasingly appreciated through studies of human cancers. As the biological behavior of nasopharyngeal carcinoma (NPC) depends on its nodal status, patients with advanced nodal status show a higher tendency toward a poor outcome. Here, we examined the role of lymphangiogenesis on lymphatic spread of NPC. We also evaluated the involvement of vascular endothelial growth factor (VEGF)-C/VEGF receptor 3 (VEGFR3) signaling pathway on lymphangiogenesis in NPC. Furthermore, we tested whether Epstein-Barr virus (EBV)-latent membrane protein (LMP) 1 induces VEGF-C.

Materials and Methods: Forty-one patients with NPC were evaluated for expressions of VEGF-C and its receptor, VEGFR3, and LMP1 proteins and lymphatic vessel counts (LVC) highlighted by anti-podoplanin employing immunohistochemistry. The VEGF-C induction by LMP1 was then tested with Western blotting and enzyme-linked immunosorbent assay in vitro.

Results: The LVC and VEGF-C expression were significantly higher in cases with advanced regional lymph node metastasis (N2,3) than those with no or limited lymph node involvement (N0,1) (p = 0.0380 and p = 0.0109, respectively). In VEGF-C/VEGFR3-positive cases, the LVC were significantly increased compared with

VEGF-C/VEGFR3-negative cases (p = 0.0007). However, LMP1 expression did not show significant associations with LVC and VEGF-C-expression scores (p = 0.1210 and p = 0.1324, respectively). Induction of VEGF-C protein by LMP1 was not detected *in vitro*.

Conclusions: These results suggest the involvement of the VEGF-C/VEGFR3 axis in the induction of lymphangiogenesis which results in lymphatic spread of NPC. However, EBV-LMP1 was not associated with the mechanism.

Key Words: Lymphangiogenesis, lymph node metastasis, vascular endothelial growth factor-C, vascular endothelial growth factor receptor 3, nasopharyngeal carcinoma, Epstein-Barr virus, latent membrane protein 1.

Introduction

Seventy-five percent of patients with nasopharyngeal carcinoma (NPC) have regional lymph node involvement at diagnosis [1]. Even early primary tumors are often associated with advanced regional lymph node metastasis. As the biological behavior of NPC depends on its nodal status, patients with advanced nodal status have a higher tendency toward a poor outcome [2]. Another distinctive character of NPC is the consistent association with Epstein-Barr virus (EBV). EBV is considered to be strongly associated with the carcinogenesis of NPC [3].

Angiogenesis, the growth of new capillaries from pre-existing blood vessels, is essential for cancers to grow beyond minimal size [4]. Tumor angiogenesis is induced by increased secretion of angiogenic factors and by down-regulation of angiogenic inhibitors [5]. Previously, we showed a significant relationship between angiogenesis and advancement of nodal status in NPC tissues [6, 7]. Furthermore, we reported that latent membrane protein 1 (LMP1), an EBV-associated oncoprotein, induces angiogenic factors including vascular endothelial growth factor (VEGF)-A, interleukin (IL)-8, and fibroblast growth factor (FGF)-2 [6-8]. Thus, LMP1 plays a central role in angiogenesis, which results in progression of lymph node metastasis in NPC.

The contribution of the lymphatic system to tumor metastasis is being

increasingly appreciated through studies of human cancers, such as carcinoma of the breast, colon, and prostate as well as melanoma [9-12]. Tumor cells enter the lymphatic vasculature by invading pre-existing peritumoral lymphatic vessels or by eliciting lymphangiogenesis via growth factor production [11, 13, 14]. The density of lympatic vessels correlates with the progression of lymph node metastasis and poor outcome in some human cancers [10, 15]. One of the lymphangiogenic growth factors, named VEGF-C, that signals through its receptor, VEGF receptor 3 (VEGFR3), has been discovered. VEGF-C-expressions in tumor tissues have been shown to promote lymphangiogenesis through signaling of VEGFR3 expressed on lymphatic endothelial cells, which results in the lymphatic spread of the tumor cells [16-21].

Here, we counted the tumor lymphatic vessels, using podoplanin as a lymphatic marker, and examined its relationship with lymph node metastasis in NPC. We tested the presence of the VEGF-C/VEGFR3 pathway and its role in the increasing number of lymphatic vessels and lymph node metastasis using NPC tissues. The impact of EBV-LMP1 on lymphangiogenesis was also evaluated in NPC, and the induction of VEGF-C by EBV-LMP1 was examined *in vitro*.

Materials and Methods

Tissue samples.

Forty-one specimens were obtained from patients with NPC who underwent biopsy at Kanazawa University Hospital or Toyama Prefectural Central Hospital between 2000 and 2009. All specimens were fixed in 10% neutral formalin and embedded in paraffin. After a review of all H&E-stained slides of the specimens, they were classified into three histopathological groups. These were composed of 3 squamous cell carcinomas (WHO type I), 14 nonkeratinizing carcinomas (WHO type II), and 24 undifferentiated carcinomas (WHO type III). The clinical staging of NPC was evaluated based on the tumor-node metastasis classification of the Union Internationale Contre le Cancer. Pretreatment staging of N factor was performed with high-resolution computed tomography and/or 18F-fluorodeoxy-glucose positron emission tomography before starting the primary treatment.

Immunohistochemical analysis.

Consecutive 4-µm sections were cut from each block. Immunohistochemical staining was performed as described previously [7, 22]. The following antibodies were used as primary antibodies: monoclonal antibodies for podoplanin (dilution 1:100) and

LMP1 derived from mouse (dilution 1:50) (DAKO, USA), polyclonal antibody for VEGF-C derived from rabbit (dilution 1:100) (Invitrogen, USA), and polyclonal antibody for VEGFR3 derived from goat (dilution 1:50) (R&D systems, USA). The sections were color-developed with substrate/chromogen diaminobenzidine (DAKO, Copenhagen, Denmark). The sections were counterstained with hematoxylin. NPC specimens that had been used in previous studies [7, 22], normal lymph node, human placental tissue, and human melanoma specimens were used as positive controls for LMP1, podoplanin, VEGF-C, and VEGFR3 proteins, respectively. The specificity of staining was confirmed using non-immune serum instead of the primary antibody as a negative control.

Evaluation of immunohistochemical staining for VEGF-C, VEGFR3, and LMP1 proteins.

The stained sections were independently examined by two authors (K. H. and N. W.). In each case, two arbitrary separate microscopic fields (200x) containing >200 tumor cells were evaluated. After counting both immunoreactive cells and the total number of tumor cells, the average percentages of immunoreactive cells were calculated without knowledge of the clinical data.

The results for VEGF-C protein were classified into scores from 0 to 10, depending

on the percentage of immunoreactive tumor cells: score 0, 0%; score 1, >0% and ≦10%; score 2, >10% and ≦20%; score 3, >20% and ≦30%; score 4, >30% and ≦40%; score 5, >40% and ≦50%; score 6, >50% and ≦60%; score 7, >60% and ≦70%; score 8, >70% and ≦80%; score 9, >80% and ≦90%; score 10, >90% and ≦100%. The data for LMP1 protein were classified into negative or positive, depending on the percentage of immunoreactive tumor cells: negative, <10%; positive, ≧10%[22].

The finding for VEGFR3-staining was considered positive whenever the vessel structures in the tumor stroma were highlighted. When the authors could not find any vessel structure highlighted in the tumor stroma, the specimen was considered negative.

VEGF-C, a member of the VEGF family of secreted glycoprotein, is a ligand of VEGFR3. We postulated that VEGF-C secreted by tumor cells stimulates lymphangiogenesis through binding with VEGFR3 expressed on lymphatic vessels in NPC. Thus, we need to evaluate the association of the set of VEGF-C and VEGFR3 expression with the lymphatic vessel counts or the progression of lymph node metastasis. To analyze the associations of VEGF-C/VEGFR3 axis with lymphatic vessel counts or regional lymph node status, VEGF-C/VEGFR3 expressions were classified into two categories, depending on the percentage of immunoreactive tumor cells:

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VEGF-C/VEGFR3-negative, VEGF-C-expression score <5 and VEGFR3-negative; and

VEGF-C/VEGFR3-positive, either VEGF-C-expression $\geqq 5$ or VEGFR3-positive.

Lymphatic vessel counting.

Lymphatic vessels were highlighted by staining lymphatic endothelial cells with

anti-podoplanin antibody. The area of highest lymphvascular density was found by light

microscopy in a 10x field followed by counting in a 200x field. Any lymphatic endothelial

cell or endothelial cluster staining positive for podoplanin that was clearly separate

from adjacent lymphatic vessels was considered to be a single, countable vessel. Results

were expressed as the highest number of lymphatic vessels identified within any single

200x field.

Plasmids, cell line, and transient transfection.

A pcDNA3-based LMP1-expression plasmid (pcLMP1) has been described

previously [23]. Ad-AH cells are an EBV-negative human nasopharyngeal epithelial cell

line [24]. Cells were maintained in DMEM with 10% fetal bovine serum, penicillin, and

streptomycin. For Western blot analysis, cells were transfected with 1.0 μg of the

appropriate plasmids using the Effectene transfection kit (QIAGEN) in accordance with

manufacturer's instruction. Forty-eight hours after transfection, Ad-AH cells were held

for 24 hours in DMEM with neither fetal bovine serum nor antibiotics.

Western blot analysis for VEGF-C and LMP1 proteins.

Whole-cell lysates were extracted in 100 μl radioimmunoprecipitation assay buffer

[50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40,

0.5% Na deoxycholate, 0.1% SDS, 25 mmol/L DTT, 0.2 mmol/L Na orthovanadate, 100

mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride], and the protein concentration

was determined by a Bio-Rad protein assay. Each cell lysate (100 μg) was solubilized for

10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham) by ATTO

semidry transfer apparatus. The membrane was incubated with an antibody to VEGF-C

(dilution 1:100) (Invitrogen, USA), or LMP1 (dilution 1:100) (DAKO, USA) protein at

4℃ overnight. α-tubulin was used as the loading control.

Enzyme-linked immunosorbent assay (ELISA).

Ad-AH cells transfected with pcDNA3 with or without pcLMP1 were cultured with

serum-free DMEM for 24 h. The VEGF-C content of the supernatants was then

measured using a specific ELISA assay kit (R&D systems, USA) according to the

manufacturer's protocol.

Statistical analysis.

The data were analyzed with Stat View software (Abacus Concepts, Berkley, CA). Correlations between the VEGF-C-expression scores and the lymphatic vessel counts were evaluated by Spearman's rank test. The progression of N factor and the expressions of VEGFR3 and LMP1 proteins in relation to the lymphatic vessel counts and also the LMP1-expression in relation to VEGF-C-expression were analyzed using the Mann-Whitney U test. Associations among progression of N factor and the expressions of LMP1, VEGF-C or VEGFR3 proteins were analyzed with Fisher's exact t-test. P values <0.05 were considered significant.

Results

Expressions of podoplanin, VEGF-C, VEGFR3, and LMP1 in NPC tissues.

Podoplanin-expression was essentially restricted to thin-walled vessel structures but was occationally seen in tissue macrophages and in some connective tissues. Most lymphatic vessels highlighted by podoplanin-staining were detected as scattered in tumor cell nests at the tumor stroma (Figure 1A). In NPC tissues, VEGF-C-expression was observed as cytoplasmic staining in tumor cells (Figure 1B). In the stroma of the NPC tissues, VEGFR3-positive lymphatic vessels with small diameters could be observed (Figure 1C). LMP1 was localized at the cytoplasm and the cell membrane of the tumor cells (Figure 1D).

Detailed results showing the lymphatic vessel counts and VEGF-C scores in relation to the clinicopathological data are summarized in Additional Table S1. Lymphatic vessel counts in relation to progression of N factor are shown in Table 1. The N classification was subdivided into two categories (N0,1 and N2,3). In 16 cases with advanced lymph node metastasis category (N2,3), the mean lymphatic vessel count was significantly higher than in twenty-five cases of the N0,1-category ($p = 0.0380$). A significant relationship was observed between VEGF-C-expression scores and lymphatic vessel counts ($p = 0.0114$) (Figure 2), which suggests that expression of

VEGF-C protein is closely associated with lymphangiogenesis in NPC. VEGFR3-positive cases had significantly higher lymphatic vessel counts than VEGFR3-negative cases (p = 0.0012) (Table 1). When VEGF-C/VEGFR3-positive cases were compared with VEGF-C/VEGFR3-negative cases, the lymphatic vessel counts were significantly higher in VEGF-C/VEGFR3-positive cases (p = 0.0007) (Table 1).

VEGF-C-positive cases showed also significant progression in N factor compared with negative cases (p = 0.0109) (Table 2). Positive VEGFR3 protein expression showed a trend toward more advanced N factor-progression, but the result did not achieve statistical significance (p = 0.0965) (Table 2). However, VEGF-C/VEGFR3-positive cases showed significant progression of N factor compared with VEGF-C/VEGFR3-negative category (p = 0.0285) as shown in Table 2. These results suggest that the VEGF-C and its ligand, VEGFR3, -associated pathway is closely related with the progression of lymph node metastasis in NPC.

Associations between LMP1 expression and progression of N factor is also shown in Table 2. In LMP1-positive cases, N factor was more significantly progressed than in LMP1-negative cases (p = 0.0057) (Table 2), which was consistent with our previous reports [7, 22]. Association between LMP1-expression, and lymphatic vessel counts and VEGF-C-expression are shown in Table 3. Although LMP1-positive cases tended to

show higher numbers of lymphatic vessels and higher VEGF-C-expression scores, the differences were not statistically significant (p = 0.1210 and p = 0.1324, respectively) (Table 3).

These results suggest a significant role of VEGF-C/VEGFR3 axis in the progression of N factor through induction of lymphangiogenesis in NPC. However, the role of LMP1 in induction of lymphangiogenesis was not identified as a result of immunohistochemical analysis using NPC tissues.

Expression of VEGF-C protein was not induced by LMP1 *in vitro*.

We previously showed the induction of comprehensive set of cellular factors that mediates steps in the process of invasion and metastasis of tumor cells, as well as angiogenesis. Contrary to our expectations, in the immunohistochemical study using NPC tissues, we could not show an association between expressions of LMP1 and VEGF-C proteins. To confirm whether LMP1 is really irrelevant in the induction of VEGF-C expression, we investigated whether LMP1 protein could induce VEGF-C protein production *in vitro*.

Ad-AH cells were transiently transfected with pcDNA3 with or without pcLMP1. VEGF-C protein was detected by Western blot analysis of whole-cell extract. As shown

in Figure 3A, VEGF-C protein expression was not upregulated by increasing the amount of LMP1 protein expression. We also examined the VEGF-C-concentration in supernatant from Ad-AH cells transfected with pcDNA3 with or without pcLMP1 by ELISA. As shown in Figure 3B, the concentrations of VEGF-C in supernatant were similar between cells with or without pcLMP1-transfection. These results support the data from immunohistochemical analysis that LMP1 is irrelevant in VEGF-C-expression in NPC tissues.

Discussion

A close relationship between tumor angiogenesis and regional lymph node metastasis has been shown in malignant tumors [25-27]. Increased tumor angiogenesis may have an indirect influence on nodal metastasis by increasing the number of cells entering the blood. Tumor cells then pass through the junctions between blood and lymphatic circulations and can be arrested in the lymph node. Previously, we reported a relationship between angiogenesis and the progression of regional lymph node metastasis in NPC [6, 22]. We also showed that an EBV-encoded oncoprotein, LMP1, plays a key role in anigiogenesis through the induction of angiogenic factors including FGF-2, IL-8, and VEGF-A [6-8, 22, 28-30]. However, lymphangiogenesis studies have been limited by the lack of specific lymphatic endothelial markers. Recently, monoclonal antibody for podoplanin was shown in breast and tonsillar tissues to selectively detect lymphatic vessels [31, 32]. Thus, it has remained unresolved whether lymphangiogenesis can be related to lymph node metastasis in clinical NPC specimens.

A clinically detectable lymph node metastasis is the result of complex multistep processes, including the dissemination of tumor cells from the primary site to the lymphatic vessels, the transport of tumor cells through the lymphatic system to the lymph nodes, the settlement of tumor cells in lymph nodes, and the growth of the

metastatic lesion to a detectable mass [9]. Some retrospective analyses of human cancer samples have shown a positive correlation between tumoral lymphatics and lymph node metastasis [27, 33]. Tumor cells produce multiple lymphangiogenic factors that might stimulate tumor lymphangiogenesis. Various studies using animal tumor models have provided evidence that overexpression of VEGF-C in tumor cells can increase peri- and/or intratumoral lymphangiogenesis as evidenced by both increased lymphatic vessel density and/or diameter and enhanced proliferation rates in tumor-associated lymphatic vessels [11]. Thus, in the current study, as a preliminary report on the relationship between the lymphangiogenesis and the progression of N factor in NPC, we highlighted VEGF-C, which is expressed in the tumor cells, and its receptor, VEGFR3, of which the major site of expression is the lymphatic endothelium. We showed that counting the lymphatic vessels highlighted with anti-podoplanin antibody significantly correlated with the progression of N factor. Furthermore, we showed that the presence of VEGF-C/VEGFR3 axis in NPC tissues was significantly associated with both increasing lymphatic vessel counts and the progression of N factor. These results indicate that the VEGF-C/VEGFR3 axis (composed of tumor-secreted VEGF-C and VEGFR3-expression in lymphatic endothelial cells) is involved in the lymphatic spread of tumor cells through induction of lymphangiogenesis in NPC.

Previously, VEGF-A was considered only a tumor angiogenesis factor, signaling through VEGFR2 and VEGFR1, which are expressed on tumor-associated blood vessels [34]. FGF-2 is an angiogenic molecule that has potent stimulatory activity on vascular endothelial cells *in vitro* and on angiogenesis *in vivo* [9, 35]. The role of cyclooxygenase (COX)-2 in promoting tumor-associated angiogenesis is also well documented [36]. However, Hirakawa et al. identified VEGF-A as a novel tumor lymphangiogenesis factor [37]. Chang, et al. reported that FGF-2 stimulates the proliferation, migration and tube formation of isolated lymphatic endothelial cells, indicating a direct role in lymphatic vessel growth [38]. In addition, COX-2 demonstrates a positive regulatory role in lymphangiogenesis [39]. Interestingly, all these factors are induced by the EBV-associated oncoprotein, LMP1, in NPC [8, 29, 30]. Thus, we expected that LMP1 should have a key role in lymphoangiogenesis, as well as in angiogenesis, in NPC. However, we could not find a significant association between expression of LMP1 and lymphatic vessel counts in NPC tissues. In addition, although VEGF-C-expression was associated with both lymphatic vessel counts and the progression of N factor in NPC tissue, its expression was not associated with the expression of LMP1. We also failed to determine the upregulation of VEGF-C-production or -secretion by LMP1 *in vitro* at least at the protein level using Western blot analysis or ELISA. Thus, we conclude here

that EBV-LMP1 is not involved in the regulation of lymphangiogenesis in NPC. Although the detection of the key lymphangiogenesis regulator in NPC is beyond the scope of this paper, the roles of other lymphangiogenesis factors such as VEGF-D and platelet-derived growth factor-BB, and other EBV-associated proteins, such as LMP2A, need to be fully examined in the next step of our study. Tumor-associated macrophages producing lymphangiogenic factors such as VEGF-C and VEGF-D may also be of widespread significance.

In conclusions, we reported here that the VEGF-C/VEGFR3 axis promotes lymph node metastasis through the induction of lymphangiogenesis in NPC. The mechanism of lymph node metastasis through lymphangiogenesis was independent of EBV-associated oncoprotein, LMP1, and was distinct from lymph node metastasis dependent on tumor-angiogenesis in which LMP1 plays a key role through the induction of angiogenic factors. The pathway for lymphangiogenesis-induction should be fully elucidated, because the mechanism could be a new target for therapies to suppress lymph node metastasis in the treatment of NPC.

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Declaration of competing interests

The authors declare that they have no competing interests.

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Figure Captions

Figure 1. Immunohistochemical detection of podoplanin (Original magnification, x200) (A), VEGF-C (Original magnification, x400) (B), VEGFR3 (Original magnification, x200) (C), and LMP1 (Original magnification, x200) (D) in NPC tissues. (A) Podoplanin expression was essentially restricted to vessel structures. Lymphatic vessels highlighted with anti-podoplanin detected as a scattered pattern were seen at the tumor stroma. (B) VEGF-C protein was localized at the tumor cytoplasms. (C) VEGFR3 was detected at the lymphatic endothelium at the stroma of the tumor nest. (D) LMP1 was recognized at the cytoplasm and cell membrane of the tumor cells.

Figure 2. Correlation between VEGF-C score and lymphatic vessel counts in 41 cases of nasopharyngeal carcinoma. Shown is the plot of the lymphatic vessel counts according to the VEGF-C-expression score. Regression line: Lymphatic Vessel Counts (Y) = 7.209 + 1.062 x VEGF-C Score (X). Spearman's rank correlation coefficient = 0.414 (P = 0.0114).

Figure 3. (A) LMP1 does not induce VEGF-C protein detected by Western blotting in Ad-AH cells. Lysates of Ad-AH cells transfected with pcDNA3 with or without pcLMP1 are loaded on to corresponding lanes and examined by Western blotting with antibodies

to VEGF-C, LMP1, or α-tubulin proteins as described in Materials and Methods. Two different amounts of LMP1-expression plasmid were transiently transfected. α-tubulin was used as the loading control. **(B) LMP1 does not induce VEGF-C protein secretion detected by ELISA in Ad-AH cells.** pcDNA3 and pcLMP1 plasmids were transiently transfected in Ad-AH cells, and the VEGF-C concentration in supernatant of culture medium was measured by ELISA. The concentrations of VEGF-C in supernatant of cells transfected with pcDNA3 with or without pcLMP1 are shown. The results of triplicate experiments are shown.