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LABORATORY INVESTIGATION

Endoglin (CD 105) is expressed on endothelial cells in the primary central nervous system lymphomas and correlates with survival

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Abstract Endoglin (CD105) is predominantly expressed on the cellular lineages within the vascular system and it is overexpressed on proliferating endothelial cells that participate in neoangiogenesis, with a weak or negative expression in the vascular endothelium of normal tissues. To investigate the correlation between the CD105 expression and possible prognostic markers or progression in the primary central nervous system lymphomas (PCNSLs), the present study assessed 26 cases of PCNSL by immunostaining for CD105 and CD34. Intratumoral microvessel density (IMVD) was determined in the hotspots and interfaces at a magnification of x200. According to the mean value, the patients were classified into lower-IMVD and higher-IMVD groups. When CD34 was used as a marker of angiogenesis, the survival rates of these two groups demonstrated no significant difference. In contrast, when CD105 was used as a marker of angiogenesis, the survival rate of the lower-IMVD group was significantly higher than that for the higher-IMVD group ($P < 0.01$). In the group of CD34-immunostained vessels, no difference was observed in IMVD between the hotspots and interfaces ($P = 0.31$). In the group with CD105-immunostained vessels, a greater IMVD was observed in the hotspots than in the interfaces ($P < 0.01$). These results suggested that the growth of PCNSLs was dependent on angiogenesis, that IMVD as determined by anti-CD105 monoclonal antibody was a reliable prognostic marker in PCNSLs, and that PCNSLs may therefore not require sufficient neoangiogenesis at the start of PCNSLs, however, it may instead require a higher rate of neoangiogenesis as they infiltrate and destroy the brain parenchyma.

Keywords CD34 · CD105 (endoglin) · Central nervous system lymphomas · Neoangiogenesis · Prognosis

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Introduction

The incidence of primary central nervous system lymphomas (PCNSLs) is quite low, currently accounting for 1–2% of all lymphomas, but this incidence has dramatically risen over the past two decades [1, 2]. An increasing incidence of PCNSLs has been seen not only in patients with acquired immunodeficiency syndrome (AIDS), but also in immunocompetent hosts. The majority of PCNSLs have been histopathologically confirmed to be diffuse large B-cell lymphoma (DLBCL) [3]. However, recent studies have demonstrated that PCNSLs have distinct characteristics which distinguish them from non-PCNSLs [3, 4].

Angiogenesis is the process by which tumors induce a blood supply, and it plays a crucial role in both

growth and progression. Therefore, angiogenesis has been proposed to be a prognostic marker in a variety of human neoplasms [5–23]. Although the importance of angiogenesis in solid tumors has been well established, its role in hematopoietic tumors has not [24]. Furthermore, only a few studies have investigated the clinical implications of tumor angiogenesis in central nervous system (CNS) lymphomas [15, 25].

Endoglin (CD105) is a receptor for transforming growth factor (TGF)-beta 1 and TGF-beta 3, and it modulates TGF-beta signaling by interacting with TGF-beta receptor I (TGF-beta RI) and/or TGF-beta receptor II (TGF-beta RII) [26]. Whereas antibodies against panendothelial cells, such as anti-CD31 and anti-CD34 antibodies, have usually been used in the evaluation of angiogenesis, these panendothelial antibodies react with not only newly forming vessels but also with normal vessels trapped within tumor tissues. On the other hand, endoglin (CD105) is predominantly expressed on cellular lineages within the vascular system and it is overexpressed on proliferating endothelial cells that participate in tumor angiogenesis, with either a weak or negative expression in the vascular endothelium of normal tissues [22, 26]. Investigators have recently shown that CD105 to be a more specific and sensitive microvessel marker than other commonly used panendothelial antibodies in malignant neoplasms of the brain, breast, colon, esophagus, urothelial bladder, and lung [6, 8, 13, 14, 16, 18, 20].

From this perspective, we studied a series of 26 PCNSLs in the present study to investigate the correlation between CD105 expression and the progression of PCNSLs.

Materials and methods

Cases

Surgical tissue samples (n = 26) from 26 patients with PCNSLs were used for the present study. All tumor specimens were retrieved from the archives of the Department of Surgical Pathology at Saga Medical School, Kurume University and affiliated hospitals between 1989 and 2005. The clinical information regarding these 26 patients was also retrieved from these archives.

Histologic and immunohistochemical studies

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and then were processed conventionally for histology and immunohistochemistry. The sections (5 μm) were stained using hematoxylin and eosin (HE) for the histological evaluations. The remaining unstained serial sections were used for immunohistochemistry. All specimens were histologically diagnosed according to the World Health Organization criteria for tumors of the nervous system [2]. Immunohistochemical studies were performed using peroxidase avidin–biotin methods (LASB kit, DakoCytomation, Carpinteria, CA, USA) on paraffin sections following heat-induced antigen retrieval. The primary antibodies were directed toward CD20 (dilution 1:50; DakoCytomation, Glostrup, Denmark), CD79a (dilution 1:50; DakoCytomation, Glostrup, Denmark), CD3 (dilution 1:50; DakoCytomation, Glostrup, Denmark), CD45RO (dilution 1:100; DakoCytomation, Glostrup, Denmark), endoglin (CD105, dilution 1:50; Novocastra, Newcastle, United Kingdom) and CD34 (dilution 1:30; Novocastra).

For double immunohistochemistry, after incubation with the first primary antibody CD105 and then washing with phosphate-buffered saline, the alkaline phosphatase-labeled secondary rabbit polyclonal antibody against murine IgG was reacted for 30 min at 37°C and visualized with the BCIP/NBT substrate system (DakoCytomation), which produces a bluish-purple color. After microwave heating in ethylene diamine tetra acetic acid (EDTA, pH 8) for 5 min, the specimens were then incubated with the second primary antibody (CD34) for 3 h. The same procedure used in the first step was repeated, and the expression of the second antibody was visualized with a New Fuchsin kit (Nichirei, Tokyo, Japan), which produces a red color.

Quantitation of CD-34 and CD-105 immunostained vessels

After scanning the immunostained sections at low magnification, three areas of tumors with the greatest number of distinctly highlighted areas (hotspots) or the interface areas between the tumor and normal brain tissue (interfaces) were counted on a ×200 field. Any single cell or spot that was stained by the immunohistochemical marker was thus counted as a vessel.

The average counts from the three areas were recorded as the CD105-intratumoral microvessel density (IMVD) or the CD34-IMVD for each tumor in order to measure the degree of angiogenesis. Immunohistochemical evaluations were performed by two observers in independent readings; including the cases that varied significantly between the readers were then re-evaluated to arrive at a consensus.

Statistical analysis

Comparisons of the expressions of CD 34 or CD105 were assessed by either Student's *t*-test or the Welch test (ATMS, Tokyo, Japan). All tests of significance were two-sided. The survival rate was calculated by the Kaplan–Meier method (HyperKaplan 3.6 software, presented by Dr. Michio Asano). To determine precisely how the survival rates are affected by other covariables and to control their confounding effect, a Cox's proportional hazard analysis was carried out (ATMS, Tokyo, Japan). The hazard ratios were estimated with their 95% confidence intervals.

Results

Clinical features

The clinical features of the 26 patients are summarized in Table 1. The follow-up data are available in 22 of the 26 cases. The legal limitations regarding personal information prevented us from analyzing the follow-up data of some cases. Therefore, the survival curves were established based on the data from only 22 patients. Most patients received adjuvant radiation and chemotherapy, while 2 patients (case No.19, 20) were not treated with either adjuvant radiation or chemotherapy due to their poor overall conditions. Eighteen patients (case No. 1–No. 18) received CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy, whereas 6 patients (case No. 21–No. 26) were treated by high doses of methotrexate.

Histologic and immunohistochemical features of PCNSLs

The histologic characteristics of the 26 PCNSLs are summarized in Table 1. Immunohistochemically, all cases expressed CD20 or CD79a, but were negative for CD3 and CD45RO (data not shown). In Case 10, the surgical tissue contained only the transitional zone between the tumor and normal brain, but it did not include any angiogenic hotspots. The endothelial cells

Table 1 Summary of clinicopathological data and immunohistochemistry

Case No.	Patient's age/gender	Location of tumor	Histopathology	CD 34-IMVD		CD 105-IMVD		Follow up
				H	I	H	I	
1.	59/M	Lt. temporal lobe	DL, CBV	20.1	18.0	11.6	10.6	ANED 1 month
2.	56/F	Rt. frontal lobe	DL, CBV	60.3*	/	5.0	/	DOD 20 months
3.	56/M	Lt. temporal lobe	DL, CBV	51.3*	/	30.3**	/	ANED 10 months
4.	57/M	Lt. temporal lobe	DL, IMBV	27.3	/	5.0	/	Unknown
5.	83/F	Lt. thalamus	DL, IMBV	17.6	16.7	13.3	11.7	ANED 12 months
6.	83/F	Lt. thalamus	DL, CBV	10.0	/	5.1	/	ANED 17 months
7.	62/F	Lt. frontal lobe	DL, CBV	26.0	26.0	5.3	1.0	Unknown
8.	68/F	Bil. splenium	DL, CBV	20.0	/	5.1	/	Unknown
9.	61/M	Rt. parietal lobe	DL, CBV	24.0	/	5.0	/	ANED 94 months
10.	70/M	Rt. temporal lobe	DL, CBV	/	45.0	/	10.0	DOD 20 months
11.	48/M	Rt. frontal lobe	DL, CBV	39.6*	41.0	11.0	7.6	ANED 60 months
12.	54/F	Rt. occipital lobe	DL, CBV	24.0	/	20.0**	/	ANED 2 months
13.	70/F	Cerebellum	DL, CBV	66.0*	/	22.3**	/	ANED 1 month
14.	71/F	Rt. frontal lobe	DL, CBV	43.6*	22.0	31.0**	11.0	ANED 4months
15.	79/M	Rt. frontal lobe	DL, CBV	33.0	57.6	20.0**	12.0	DOD 5 months
16.	67/M	Rt. basal ganglia	DL, CBV	70.0*	16.0	10.0	5.7	ANED 18 months
17.	70/F	Lt. thalamus	DL, CBV	54.0*	57.7	36.6**	24.3	DOD 6 months
18.	59/M	Lt. basal ganglia	DL, CBV	26.0	25.3	16.3	7.3	DOD 6 months
19.	79/F	Rt. frontal lobe	DL, CBV	46.6*	/	19.0**	/	DOD 1 month
20.	70/M	Rt. basal ganglia	DL, IMBV	24.3	32.0	18.0**	11.3	DOD 1 month
21.	56/F	Cerebellum	DL, CBV	23.0	44.6	12.0	8.7	ANED 4 months
22.	66/M	Lt. temporal lobe	DL, CBV	22.0	/	13.0	/	ANED 5 months
23.	54/M	Lt. temporal lobe	DL, CBV	45.0*	18.2	35.2**	8.2	DOD 4 months
24.	60/F	Rt. thalamus	DL, CBV	40.0*	22.2	35.0**	5.0	DOD 10 months
25.	62/F	Lt. frontal lobe	DL, IMBV	22.3	/	15.2	/	DOD 11 months
26.	56/M	Rt. frontal lobe	DL, CBV	50.0*	27.0	16.0	5.0	DOD 14 months

ANED, alive and no evidence of disease; CBV, centroblastic variant; DL, diffuse large; DOD, died of disease; F, female; IMBV, immunoblastic variant; IMVD, intratumoral microvessel density; M, male

* Higher than the mean (35.4)

** Higher than the mean (16.7)

of the blood vessels in the non-neoplastic tissue specimens stained positive for CD34, but negative for CD105 (Fig. 1A, B). Regarding the CD34-immunostained vessels, no apparent difference was observed in the staining distribution between the interfaces and hotspots (Fig. 1C, Interfaces; Fig. 2A, Interfaces; Fig. 2C, Hotspots). However, in the case of the CD105-immunostained vessels, there were fewer vessels in the interfaces than in the hotspots (Fig. 1D, Interfaces; Fig. 2B, Interfaces; Fig. 2D, Hotspots). The vessels that were double immunostained with CD34 and CD105 demonstrated a different distribution (Fig. 3A, Interfaces; Fig. 3B, Hotspots; Fig. 3C, Interfaces; Fig. 3D, Hotspots). Namely, almost all CD105-immunostained vessels were surrounded by lymphoma cells in both the hotspots and interfaces. However, the CD 34-immunostained vessels were not surrounded by lymphoma cells in the interfaces, but they were surrounded by lymphoma cells in the hotspots. To gain a better understanding of the distributions of the CD34- and CD105-immunostained vessels, statistical analyses were performed among the relevant groups (Table 2). The means CD34 IMVD of the hotspots was 35.4 ± 16.4, while that of the interfaces was 30.3 ± 14.4. The means CD105 IMVD of the hotspots was 16.7 ± 10.2, while that of the interfaces was 9.3 ± 5.0. In the group containing the CD34-immunostained vessels, no difference was observed in the IMVD between the hotspots and the interfaces ($P = 0.31$, Student's t-test). In the group containing the CD105-immunostained vessels, there was greater IMVD in hotspots than in the interfaces ($P < 0.01$, Welch). The IMVD was higher in the hotspots of the CD34-immunostained vessels than in hotspots of CD105-immunostained vessels ($P < 0.001$, Student's t-test). From the means of IMVD of the hotspots, the patients were classified into lower-IMVD and higher-IMVD groups (Table 1). When CD34 was used as a marker of angiogenesis, the survival rate between these two groups demonstrated no significant difference (Cox–Mantel test). In contrast, when CD105 was used as a marker of angiogenesis, the survival rate of the lower-IMVD group of the patients was significantly higher than that of the higher-IMVD group of patients (Cox–Mantel test, $P < 0.01$) (Fig. 4). On a multivariate analysis, a high CD 105-IMVD demonstrated an independent prognostic impacts ($P < 0.01$) (Table 3).

Discussion

Tumor angiogenesis and its clinical significance have been investigated in a variety of solid tumors [6, 7–10, 13–16, 18, 20–22]. Recently, several studies have suggested that the growth of hematopoietic neoplasms is also dependent on angiogenesis [5, 11, 23]. However, there are conflicting descriptions as to whether the degree of angiogenesis as measured by the microvessel density has a prognostic value in lymphomas. For example, Zhao et al. reported that vascular endothelial growth factor-A was expressed both on lymphoma cells and endothelial cells in angioimmunoblastic T-cell lymphomas and it was thus related to lymphoma progression [23]. Salven et al. also demonstrated the

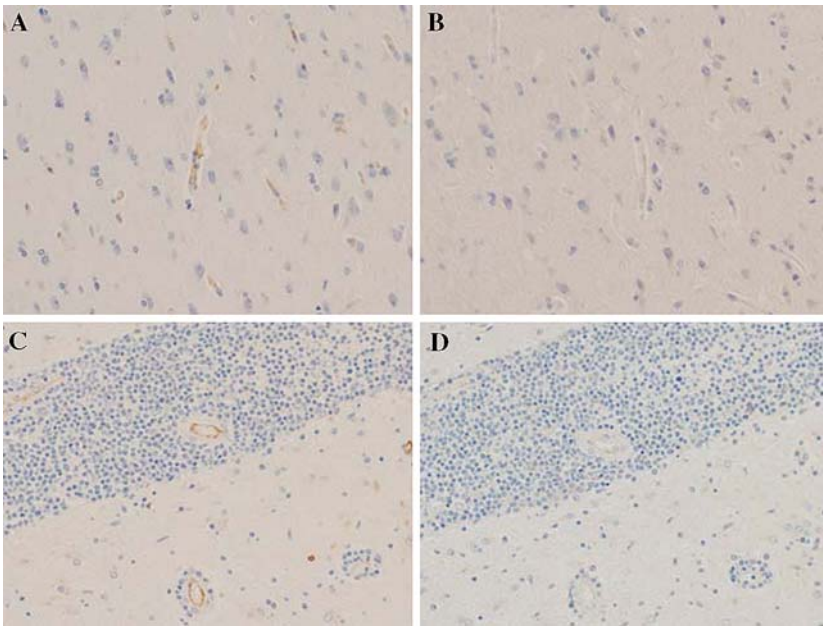

Fig. 1 Illustration of the immunohistochemical findings for CD34 and CD105 (Case 10). The endothelial cells of blood vessels in the non-neoplastic brain tissue showed positive CD34 staining (**A**), whereas the endothelial cells of blood vessels in the non-neoplastic brain tissue showed negative CD105 staining (**B**). The endothelial cells of blood vessels in the subarachnoid lymphoma cells showed positive CD34 staining (**C**), whereas the endothelial cells of blood vessels in the subarachnoid lymphoma cells showed negative CD105 staining (**D**)

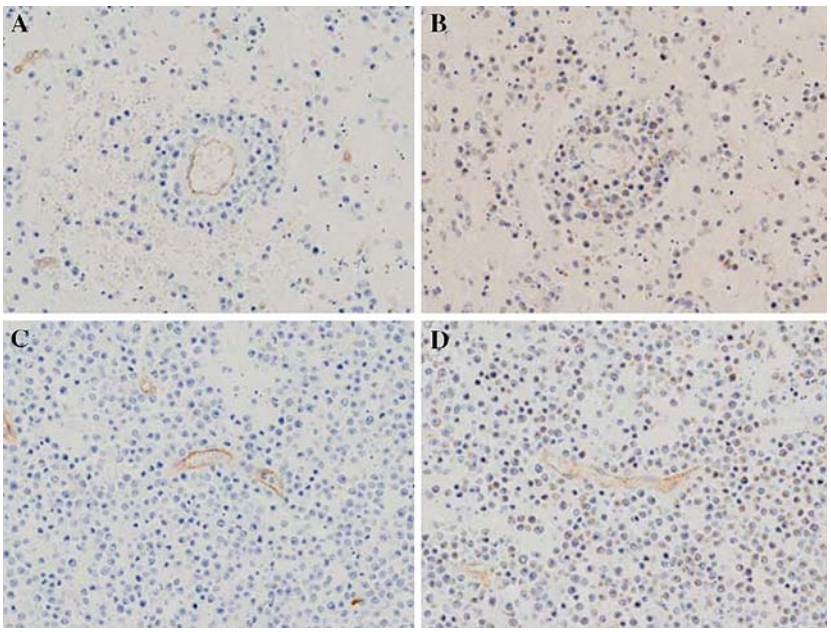

Fig. 2 Illustration of the immunohistochemical findings for CD34 and CD105 (Case 20). The endothelial cells of blood vessels in the interface showed positive CD34 staining (**A**), whereas the endothelial cells of the blood vessels in the interface showed negative CD105 staining (**B**). The endothelial cells of the blood vessels in the hotspot showed positive CD34 staining (**C**) and positive CD105 staining (**D**)

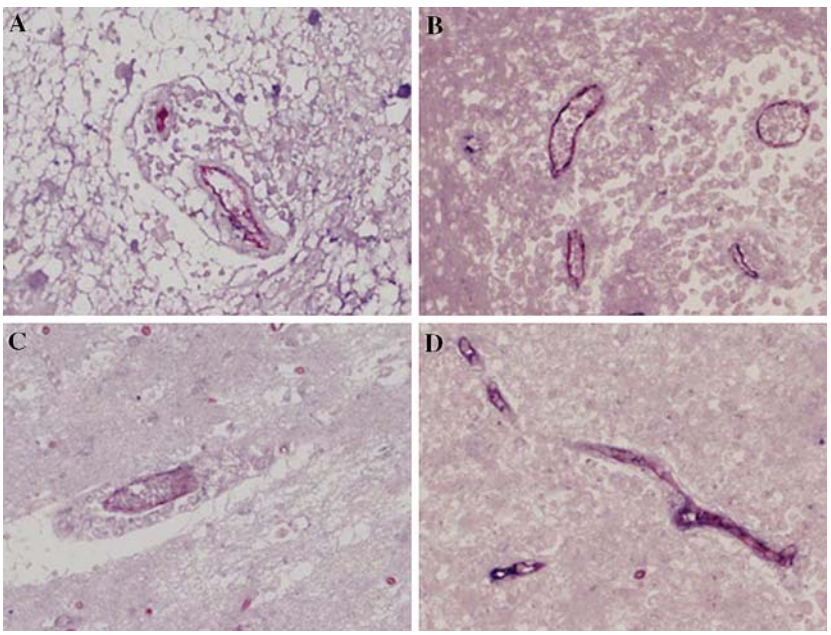

Fig. 3 Double immunostaining of CD105 (*blue-purple*) and CD34 (*red*) (Case 18, **A**, **B**; Case 15, **C**, **D**). Apparent double staining of CD105 and CD34 in the vessels (Hotspots) (**B**, **D**). The endothelial cells of the blood vessels in the interface showed both positive CD34 staining and negative CD105 staining (Interfaces) (**A**, **C**)

simultaneous serum elevation of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) levels to be an independent predictor of a poor prognosis in non-Hodgkin's lymphoma [17]. On the other hand, Stewart et al. reported that non-Hodgkin's lymphoma may be less angiogenic than most solid tumors [24]. Regarding PCNSLs, Rubenstein et al. speculated that angiotropism in PCNSLs may constitute an important paracrine growth mechanism for lymphoma infiltration [15]. However, Roser et al. commented that the lack of a correlation between apoptosis and vascularity may indicate that PCNSLs growth occurs independently of vessel formation, even though the high microvessel density in PCNSLs could play a significant role in the growth kinetics and infiltrating potential of the tumor. They also commented that this could be due to the various techniques in the microvessel counting used by different laboratories rather than due to truly different results [25]. In fact, whereas several comparative studies on the usefulness of panendothelial cell antibodies in lung carcinoma have been conducted, different results have been reported [9, 10]. In this respect, Tanaka et al. considered that because the vascular endothelial

Table 2 Statistical difference in CD34 and CD105 immunostained vessels of primary central nervous system lymphomas (PCNSLs)

Immunostained vessels	Accounts of vessels		
	No of cases	Range	Mean ± SD
CD34			
H	25	10.0–70.0	35.4 ± 16.4[a,c]
I	15	16.0–57.7	30.3 ± 14.4[a]
CD105			
H	25	5.0–36.6	16.7 ± 10.2[b,c]
I	15	1.0–24.3	9.3 ± 5.0[b]

H, hotspots; I, interfaces

[a] Hotspots of CD34 stained vessels versus interfaces of CD34 stained vessels, not significant, $P = 0.31$ (Student's *t*-test)

[b] Hotspots of CD105 stained vessels versus interfaces of CD105 stained vessels, significant, $P < 0.01$ (Welch)

[c] Hotspots of CD34 stained vessels versus hotspots of CD105 stained vessels, $P < 0.001$ (Student's *t*-test)

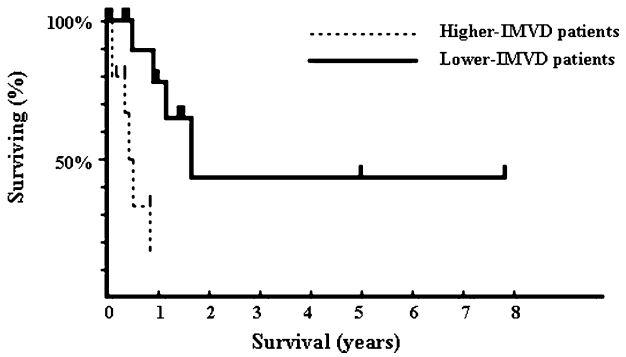

Fig. 4 Survival curves (Kaplan–Meier method) for patients with (**A**) higher intratumoral microvessel density (IMVD) ($n = 10$) and (**B**) lower IMVD ($n = 12$) determined by an anti-CD105 monoclonal antibody. Significant differences were observed in the 5-year survival rate between these two groups ($P < 0.01$, Cox–Mantel test)

cells of both tumors and normal tissues show a remarkable heterogeneity, panendothelial antibodies may not be an ideal reagent to visualize tumor-associated blood vessels [20].

In the present study, CD105 staining reduced the false-positive staining of blood vessels when in comparison to the commonly used panendothelial marker CD34. In addition, the survival rate of the lower-IMVD patients was significantly higher than that for the higher-IMVD patients when CD105 was used as a marker of angiogenesis. In contrast, when CD34 was used as a marker of angiogenesis, the survival rates did not significantly differ between these two groups. In addition, using a multivariate analysis, CD105-IMVD demonstrated independent prognostic impacts on in

Table 3 Multivarate analysis of prognostic factors for primary central nervous system lymphomas (PCNSLs) ($n = 22$)

Variables	Risk ratio	*P* value
CD105 IMVD		
>16.7	1	
<16.7	23.1	0.01
CD 34 IMVD		
>35.4	1	
<35.4	0.40	0.32
KS		
<60	1	
>60	0.97	0.98
Age		
<60 years	1	
>60 years	0.81	0.88
Gender		
Female	1	
Male	1.04	0.96
MTX therapy		
Yes	1	
No	0.38	0.19

IMVD, intratumoral microvessel density; KS, Karnofsky scale; MTX, methotrexate

this investigation. This was consistent with previous studies in which CD105 was expressed mainly in proliferating blood vessels and in which CD105 staining showed a positive correlation with the survival in malignant neoplasms [6, 8, 13, 16, 18, 20, 22, 26]. Although the prognostic significance of microvessel density in PCNSLs remains controversial, we considered that the growth of PCNSLs was dependent on angiogenesis and that IMVD determined by anti-CD105 monoclonal antibody was a reliable prognostic marker in patients with PCNSLs. Chang et al. reported that because traditional prognostic markers in non-PCNSLs, such as staging and International Prognostic Index scores, were not applicable to PCNSLs, the evaluation of p53, c-Myc, and Bcl-6 by immunohistochemistry might be warranted as part of a prognostic evaluation in patients with PCNSLs [1]. Taken together with our data, the combination of IMVD as determined by anti-CD105monoclonal antibody and an evaluation of p53, c-Myc, and Bcl-6 may therefore be a potentially more reliable prognostic marker in patients with PCNSLs.

In general, PCNSLs are typically patchy, poorly demarcated, and angiocentric at low magnification. Angiocentricity and angioinvasion, although most apparent in less cellular regions, is also present in densely cellular portions of the lesion [2, 27]. From these perivascular cuffs, tumor cells invade the neural parenchyma, either with compact cellular aggregates and a well-delineated invasion front or with single diffusely infiltrating tumor cells resembling encephalitis.

In the periphery and the adjacent brain of PCNSLs, perivascular lymphocytic cuffing, which is usually composed of small, non-neoplastic-looking lymphocytes admixed with a minority of plasma cells and which often form an inner collar that is quite distinct from the more diffuse parenchymatous infiltration by lymphoma cells and thus is often conspicuous [27]. Based on the monoclonality of normal-appearing plasma cells in these perivascular cuffs, Houthoff et al. considered that the cuffs to be part of the tumor [12].

In the present series, all cases showed the same pattern of the above described PCNSLs infiltration. We also performed statistical analyses among the relevant groups to gain a better understanding of the distribution of the CD34- and CD105-immunostained vessels in PCNSLs.

In the group of CD34-immunostained vessels, no difference was observed in IMVD between the hotspots and interfaces. In the group of CD105-immunostained vessels, a greater IMVD was observed in hotspots than in the interfaces ($P < 0.01$, Welch). Namely, the microvessels of the hotspots of PCNSLs are abundant with newly forming vessels, whereas the interface, including the subarachnoid or Virchow–Robin spaces of PCNSLs, may mainly contain normal vessels trapped within the tumor tissues rather than in the newly forming vessels. The wide range of both CD34 and CD105 staining and the large standard deviation from mean were also considered to be due to the characteristics of the infiltration style of PCNSLs as mentioned above.

Namely, small neurosurgical samples did not always contain large areas of hotspots. Some investigators have speculated that perivascular lymphocyte cuffing might be akin to a preneoplastic stage of the lymphoid elements that are presumed to have migrated from the vascular lumen into the perivascular spaces. The neoplastic transformation itself may, especially at the periphery of the tumor, remain at first relatively confined to the Virchow–Robin spaces, but thereafter may acquire a more florid expression as it infiltrates and destroys the neural parenchyma [12, 27]. In view of our study on the angiogenesis of PCNSLs, there may be some validity to this theory. In other words, PCNSLs may not need sufficient neoangiogenesis at the start of PCNSLs, but instead may require a higher rate of neoangiogenesis as they infiltrate and destroy the brain parenchyma at an advanced stage.

Endoglin (CD105) is emerging as a prime vascular target of antiangiogenetic cancer therapy [13, 26]. Recent studies have shown the systemic administration of naked antihuman endoglin monoclonal antibody to suppress established tumors, and its efficacy was markedly enhanced by combination with a chemotherapeutic drug using an antiangiogenic schedule of drug dosing [19, 26]. Therefore, the results of endoglin staining in PCNLs in the present study could thus eventually lead to the performance of therapeutic trials on antiangiogenic treatment for patients with PCNSLs.

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