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Vascular invasion is an early event in pathogenesis of Merkel cell carcinoma

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This study investigated vascular and especially lymphovascular invasion in primary Merkel cell carcinoma and its value as a prognostic factor. Paraffin-embedded blocks prepared from tumor samples obtained from126 patients diagnosed with Merkel cell carcinoma in 1979–2004 were immunohistochemically stained using antibodies CD31 and D2-40 to detect intravascular tumor emboli. This finding was compared with the clinical data and the disease outcome. Intravascular tumor cells were observed in 117 (93%) of the samples. The majority, 83 (66%), showed only lymphovascular invasion. Only blood vascular invasion was seen in four (3%) samples. In all, 30 (24%) samples demonstrated both lymphovascular invasion and blood vascular invasion. In only nine (7%) samples, there was no invasion within the vascular structures. The tumors lacking invasion were significantly smaller ($P<0.01$ and $\alpha=0.050$) than those with vascular invasion, although lymphovascular invasion was observed even in the smallest tumor (0.3 cm) of this study. Already in the early stages of the disease, Merkel cell carcinoma seems to have the capacity to penetrate vessel walls. Our finding of the high frequency of lymphovascular invasion might therefore explain the extremely aggressive clinical behavior of Merkel cell carcinoma. This may support the role of sentinel node biopsy even in the case of very small primary Merkel cell carcinoma tumors.
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Merkel cell carcinoma is a rare and potentially aggressive neuroendocrine carcinoma of the skin. It has a poorer prognosis than epidermal skin tumors, entailing a high risk of local recurrences and early regional lymph node involvement and distant metastases.[1–3] The etiology of Merkel cell carcinoma may be multifactorial. The exposure to ultraviolet radiation,[4–6] as well as immunosuppression[7–10] is associated with Merkel cell carcinoma. Recently, a novel polyomavirus, named Merkel cell polyomavirus, was identified in Merkel cell carcinoma tumor tissue, suggesting that a viral infection might also be an etiological factor.[11]

Tumor size has been shown to be a strong prognostic factor.[12–16] The most commonly adopted four-level staging system divides patients into ones having either a local disease with a primary tumor <2 cm or ≥ 2 cm (stages I and II), a lymph node positive disease (stage III), or a distant metastatic disease (stage IV).[16–19] Patients with tumors <2 cm have better prognosis.[13,20]

Thus far, the most consistent predictor of survival in Merkel cell carcinoma is the presence or absence of lymph node metastases.[21] Pre-operative lymphoscintigraphy has been shown to reliably detect sentinel nodes also in Merkel cell carcinoma[22,23]; sentinel lymph node biopsy is thus recommended by many authors.[17,24,25]

Vascular invasion is frequently seen in Merkel cell carcinoma tumor samples. In routine hematoxylin-eosin staining, vascular invasion has been observed in 38–60% of the samples.[14,15,26] The presence of lymphovascular invasion has been reported to indicate a poorer prognosis,[16,27] although in these studies, the detection of lymphovascular structures was based on hematoxylin-eosin staining only[16] or on stainings with panvascular CD34 antibody.[27]

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CD31, also known as platelet endothelial cell adhesion molecule 1, is a type I integral membrane glycoprotein and a member of the immunoglobulin superfamily of cell surface receptors. It is found essentially on the surface of endothelial cells. It is a panvascular endothelial marker expressed in both lymphatic and blood endothelial cells.[28]

D2-40 is a monoclonal antibody directed against human podoplanin, a transmembrane mucoprotein expressed in lymphatic endothelial cells. It is a sensitive and specific marker of lymphatic endothelium, and does not react with blood vessel endothelium.[29,30]

This study focused on the vascular and especially lymphovascular invasion in primary Merkel cell carcinoma samples. Immunohistochemistry using antibodies CD31 and D2-40 was applied to analyze the frequency of this phenomenon and its value as a prognostic factor.

Materials and methods

Data on altogether 207 patients diagnosed with Merkel cell carcinoma during 1979–2004 were obtained from the Finnish Cancer Registry and the Helsinki University Hospital files. Detailed data on the course of the disease were obtained from the files of the district hospitals and primary health care centers. Only patients with a known primary tumor, sufficient clinical data, and a tissue sample of adequate quality were included in the study. The patients' clinical characteristics are shown in Table 1. The mean follow-up time was 52.3 (range 0–296) months.

Paraffin-embedded blocks prepared from the tumor samples of 126 patients were available for re-evaluation. The diagnoses were confirmed in a blinded manner by two researchers (TB and HK). The diagnoses were based on typical histological morphology in the hematoxylin-eosin slides, and confirmed by immunohistochemical analysis using cytokeratin 20 and thyroid transcription factor 1 antibodies; the latter was negative in all samples. In five cases, cytokeratin 20 was negative, and the diagnosis was further confirmed with synaptophysin and chromogranin A, which gave positive results. Tumor size (the greatest surface dimension) was measured from the hematoxylin-eosin-stained slides.

Immunohistochemistry for cytokeratin 20, thyroid transcriptase factor 1, synaptophysin, and chromogranin A were performed according to the routines of the immunohistochemical laboratory of the Department of Pathology, Helsinki University Hospital. Immunohistochemical staining for detecting vascular structures was performed using antibodies CD31 and D2-40.

Formalin-fixed and paraffin-embedded tumor samples were cut into 4 μm thick sections. Before immunohistochemistry, the sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Slides for CD31 staining were treated in a microwave oven in Tris-EDTA buffer (pH 9.0) for 2×7 min and 2×5 min, and then cooled at room temperature for 20 min. The slides were then treated with 0.3% Dako REAL Peroxidase-Blocking Solution for 5 min to block endogenous peroxidases. The immunostaining was performed by adding monoclonal mouse anti-human CD31, endothelial cell, clone JC70A (DakoCytomation, Glostrup, Denmark) antibody (1:25 diluted in Dako REAL Antibody Diluent, Biohit, Helsinki, Finland) for 1 h, followed by incubating for 30 min with Dako REAL Envision/HRP detection system, and finally visualized by Dako REAL DAB + Chromogen for 10 min. Between each step, the slides were washed with PBS-0.04% Tween20. The slides were counterstained with Mayer's hematoxylin and mounted in mounting medium (Aquamount, BDH, Poole, UK).

Slides for D2-40 staining were treated in a microwave oven in Tris-EDTA buffer (pH 9.0) for 24 min and cooled at room temperature for 20 min. Immunostaining was performed adding monoclonal mouse anti-human D2-40 (code M3619, Dako, Glostrup, Denmark) antibody diluted 1:50 (Labvision) for 30 min, followed by 30 min incubation with the Envision (K5007, Dako) detection system, and finally visualized by DAB Chromogen for 10 min.

Table 1 Clinical characteristics of Merkel cell carcinoma patients at diagnosis

	No. of patients (n = *126*)	%
Sex		
Female	90	71
Male	36	29
Age (years)		
Mean	77.6	
Median	79	
Range	50–100	
Tumor location		
Head or neck	73	58
Upper extremities	21	17
Trunk	14	11
Lower extremities	18	14
Tumor size (cm)		
Mean	1.86	
Median	1.5	
Range	0.3–8.5	
Stage at diagnosis		
I Local only <2 cm	75	59
II Local only ≥ 2 cm	36	29
III Nodal[a]	11	9
IV Distant metastatic	4	3

[a]Nodal disease assessed by palpation or histological evaluation (when performed).

Interpretation of Immunohistochemical Stainings

One slide of each sample was observed under a light microscope for intravascular tumor invasion. The

observers were blinded regarding clinical data and disease outcome. Vascular invasion was defined as a cluster of tumor cells within a vascular lumen, identified by CD31 or D2-40 staining. Lymphovascular invasion was identified as a tumor embolus within a D2-40 positive lining (Figure 1a and b). Blood vascular invasion was identified as a tumor embolus within a CD31 positive structure that was negative in D2-40 staining (Figure 2a and b). Nikon DS-5M-L2 camera was used for microscope image acquisition.

The correlation between tumor size and vascular invasion status was calculated using analysis of variance after logarithmic transformation, because the study population was not in a normal distribution. A Bonferroni's comparison test was also used to compare the groups. The prognostic significance of the vascular invasion to the later metastases was assessed using Fisher's exact test. The cause of death was analyzed with a log rank test and overall survival analyses were performed using the Kaplan–Meier method (NCSS 2000, Kaysville, UT, USA).

The study was approved by the Ethics Committee of the Helsinki University Central Hospital. The Ministry of Health and Social Affairs granted permission to collect the patient data, and the National Authority for Medicolegal Affairs granted permission to collect the tissue samples for study purposes.

Results

Intravascular tumor cells were observed in 117 (93%) of the samples. The majority, 83 (66%), showed only lymphovascular invasion. Only blood vascular invasion was seen in four (3%) samples. In

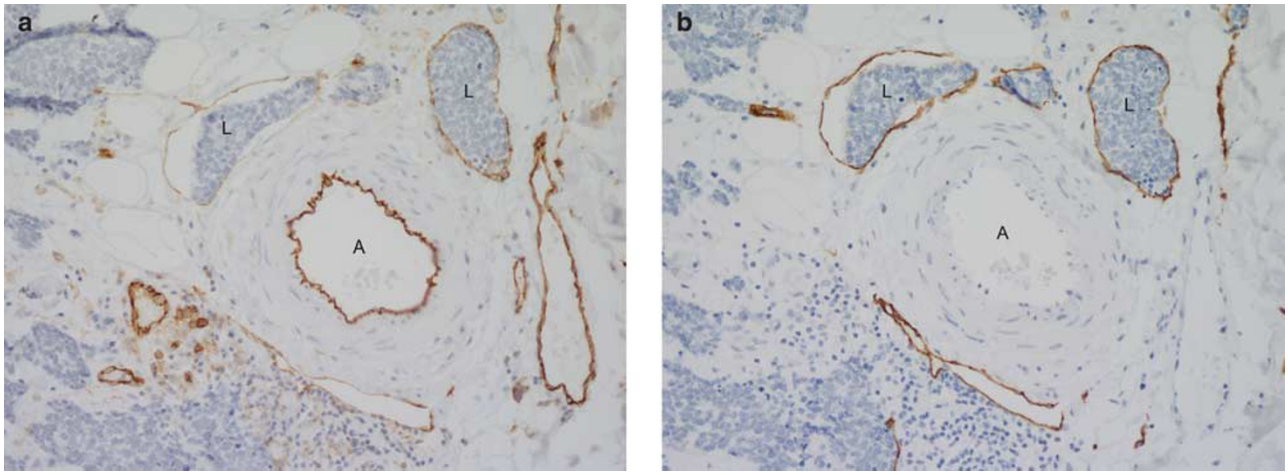

Figure 1 (**a**, **b**) Lymphovascular invasion. Staining with CD31 (**a**) and D2-40 (**b**). A cluster of tumor cells within a lymph vessel (L). Endothelial cells of an artery (A) stain with CD31, but remain negative with D2-40 staining. Original magnification ×200.

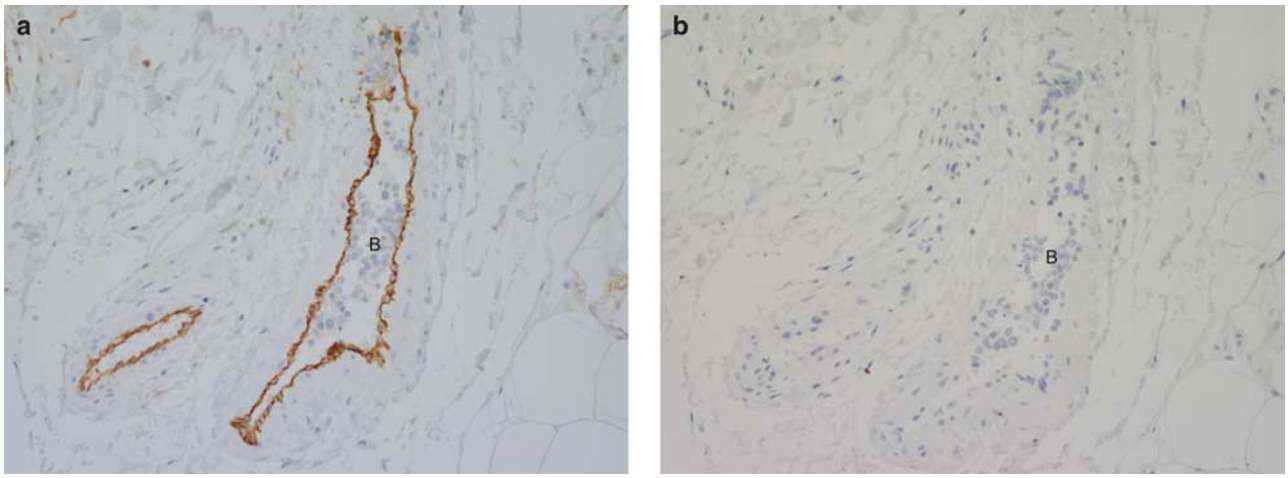

Figure 2 (**a**, **b**) Blood vascular invasion. Staining with CD31 (**a**) and D2-40 (**b**). A cluster of tumor cells within a blood vessel (B) identified by CD31 staining. The endothelial cells remain negative in D2-40 staining. Original magnification ×200.

all, 30 (24%) samples displayed both lymphovascular invasion LVI and BVI. In only nine (7%) samples, no invasion was observed within the vascular structures.

Lymphatic vessels (detected with D2-40 staining only) were found in all the samples, and they were located invariably outside the main boundaries of the tumor. CD31 displayed vascular structures (ie blood vessels) also inside the tumors.

The mean size of all the tumors was 1.86 cm. The tumors that lacked invasion were significantly smaller ($P<0.01$ and $\alpha=0.050$) than those showing vascular invasion (Table 2). Lymphovascular invasion was, however, observed even in the smallest tumor (0.3 cm) in this study.

The distribution of the clinical stages at the time of diagnosis in relation to invasion is presented in Table 2. All of the patients in the group of no observed intravascular invasion had a local disease, whereas all samples from patients with nodal or metastatic disease had either lymphatic or blood vascular invasion.

At the time of diagnosis, 111 (88%) of all patients had a local disease. The vascular invasion status and development of later metastases of these stages I and II cases are presented in Table 3. There was a trend in correlation between vascular invasion and later development of metastatic disease. None of the patients without detected vascular invasion developed lymph node or distant metastases during the follow-up (mean 5.2 years in this subgroup).

The overall survival figures for 2 and 5 years are given in Table 2. Even though none of the patients without detected invasion died of Merkel cell carcinoma during the follow-up, the overall survival time was not statistically significant when compared with the patients with detected invasion.

Discussion

This is the first study demonstrating the high capacity of Merkel cell carcinoma to invade vascular, predominantly lymphovascular structures. Using specific endothelial markers, we found that intravascular tumor invasion is very common in Merkel cell carcinoma; lymphovascular invasion and/or blood vascular invasion was seen in 93% of the samples. It seems that lymphovascular invasion

Table 2 Detected lymphovascular and blood vascular invasion in Merkel cell carcinoma samples in relation to clinical characteristics and outcome

	LVI+, BVI+	*LVI+, BVI−*	*LVI−, BVI+*	*LVI−, BVI−*
$n=126$	30 (24%)	83 (66%)	4 (3%)	9 (7%)
Tumor size (cm)				
Mean	2.2	1.8	2.6	0.9
Range	0.5–7.5	0.3–8.5	1.5–3.6	0.5–2.0
Stage at diagnosis				
I Local <2 cm	16 (53%)	50 (60%)	1 (25%)	8 (89%)
II Local ≥ 2 cm	10 (33%)	23 (28%)	2 (50%)	1 (11%)
III Nodal	4 (13%)	7 (8%)	0	0
IV Distant metastases	0	3 (4%)	1 (25%)	0
Overall survival				
2 years	40%	64%	50%	78%
5 years	20%	40%	50%	44%
Died of disease	8 (27%)	23 (28%)	2 (50%)	0
Died of other cause	18 (60%)	44 (53%)	0	5 (56%)
Alive[a]	4 (13%)	16 (19%)	2 (50%)	4 (44%)

LVI+, lymphovascular invasion detected; LVI−, lymphovascular invasion not detected; BVI+, blood vascular invasion detected; BVI−, blood vascular invasion not detected.
[a]At end of study period 31 July 2008.

Table 3 Development of regional or distant metastases in case of local disease at the time of diagnosis ($N=111$)

	LVI+, BVI+ n = *26*	*LVI+, BVI−* n = *73*	*LVI−, BVI+* n = *3*	*LVI−, BVI−* n = *9*
Regional lymph node	3 (12%)	14 (19%)	0	0
Regional and distant	2 (8%)	2 (3%)	0	0
Distant only	1 (4%)	3 (4%)	1 (33%)	0
Total	6/26	19/73	1/3	0/9

LVI+, lymphovascular invasion detected; LVI−, lymphovascular invasion not detected; BVI+, blood vascular invasion detected; BVI−, blood vascular invasion not detected.

is an early event in Merkel cell carcinoma tumorigenesis, observed already in very small, <5 mm tumors.

In most earlier studies on vascular invasion in Merkel cell carcinoma, vascular invasion has been observed only in hematoxylin-eosin-stained slides at rates of 38–60%.[14,15,31] Here, we found a much higher rate of vascular invasion. Our series confirms that immunohistochemistry, using specific marker, increases the sensitivity of detecting vascular invasion in Merkel cell carcinoma, as has been shown in other types of cancer as well.[32–34]

Furthermore, in the earlier studies on Merkel cell carcinoma and vascular invasion, no distinction was made between lymphovascular and blood vascular structures. We used D2-40, which is a sensitive and specific marker for lymphatic endothelia. Thus far, our study is the largest Merkel cell carcinoma study focusing specifically on cancer cell invasion in lymphovascular structures. In a small series of six Merkel cell carcinoma cases, blood vascular invasion was found to be more frequent (100%) than lymphovascular invasion (33%) when CD31 and lymphatic vessel endothelial hyaluronan receptor 1 were used[35]; this observation is contradictory to our finding. In our series of 126 Merkel cell carcinoma cases, blood vascular invasion was found in 27% of the cases, and lymphovascular invasion in 90% of the samples.

Although CD31 is an established panvascular endothelial marker, there is evidence that it is more specific for blood vessels than lymphatic vessels. Podgrabinska *et al* reported that CD31 is expressed in both lymphatic and blood endothelial cells, but that it is less pronounced in the former. CD31 may therefore fail to show lymphatic invasion in some tumors.[28]

In another earlier study reporting (lympho)vascular invasion in Merkel cell carcinoma,[27] immunohistochemistry was performed only with CD34, a panvascular marker such as CD31. Therefore, we suggest that some of the invaded vessels might have been blood vessels, not only lymphovascular structures.

(Lympho)vascular invasion has been reported to have prognostic value in Merkel cell carcinoma.[16,27] Our results indicate that the very high frequency of observed vascular invasion reduces its value as a prognostic tool. Nevertheless, also in our series, those patients whose tumor samples were negative for both lymphovascular invasion and blood vascular invasion seemed to have a better prognosis. Whether this implies true negativity remains unclear, as only one slide per tumor sample was examined. In a clinical setting, multiple samples and sections of each tumor have to be examined to ensure that no invasion exists, thus making it impractical as a prognostic tool.

It has been shown that lymphatic invasion is strongly associated with sentinel lymph node metastases in malignant melanoma.[36,37] In our study, 19% of the patients with a local disease at the time of diagnosis developed regional lymph node metastases, and in all of them, lymphovascular invasion was seen in the primary tumor sample. During our study period, sentinel node biopsy was not in routine use for Merkel cell carcinoma, and thus we could not investigate the correlation of lymphovascular invasion and sentinel node biopsy metastases. Future studies will clarify this correlation.

In the case of malignant melanoma, positive nodes are only rarely found in patients with thin melanomas.[38] Therefore, sentinel node biopsy is not recommended for melanomas that are 0.75 mm or less in depth, and with no adverse features. Lymphatic invasion seems to occur more frequently in the later stages of melanoma.[36] In this study, we found lymphovascular invasion to be very common, occurring already in very small (0.3 cm) Merkel cell carcinoma tumors. Perhaps such a cutoff point of tumor size for sentinel node biopsy as used in the case of malignant melanoma cannot therefore be used in Merkel cell carcinoma.

Hematogenic spread has been reported in Merkel cell carcinoma. Also in our series, there were five patients with distant metastases without regional lymph node involvement, although blood vascular invasion was not detected in three of them.

In conclusion, Merkel cell carcinoma seems to have the capacity to invade through the vessel walls in the early stages of the disease. The high frequency of lymphovascular invasion demonstrated in this study may explain the extremely aggressive clinical behavior of Merkel cell carcinoma. This finding supports the notion that sentinel node biopsy has a function even in very small primary Merkel cell carcinoma tumors. It consequently emphasizes the importance of treating Merkel cell carcinoma tumors by wide local excision and possible radiation therapy to the tumor bed to sterilize the residual disease in the invaded vessels.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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