Expression of Vascular Endothelial Growth Factor Receptor-2 in Merkel Cell Carcinoma

HELI KUKKO[1], VIRVE KOLJONEN[1], PATRIK LASSUS[1], ERKKI TUKIAINEN[1], CAJ HAGLUND[2] and TOM BÖHLING[3]

[1]Department of Plastic Surgery and [2]Gastroenterological and General Surgery, Helsinki University Hospital, Helsinki; [3]Department of Pathology, Helsinki University and HUSLAB, Helsinki, Finland

Abstract. *Background: The aim of this study was to examine the expression of vascular endothelial growth factor receptor-2 (VEGFR-2) in Merkel cell carcinoma (MCC) and to correlate the expression with tumour growth and the development of metastasis. Patients and Methods: The study included 21 patients treated for MCC at Helsinki University Hospital, Helsinki, Finland between 1987 and 2003. The VEGFR-2 expression was studied by immunohistochemistry. The correlations between the quantitative expression of VEGFR-2 and tumour size and metastatic dissemination were analyzed statistically. Results: VEGFR-2 was expressed in 91% of the large (≥2 cm) and 70% of the small (<2 cm) tumours. There was a stronger positive correlation between expression of VEGFR-2 and tumour size than between VEGFR-2 and metastatic potential. Conclusion: A correlation between the expression of pro-angiogenic marker and tumour size was established. Our results indicate that inhibiting angiogenesis could be a treatment option for MCC. The role of neovascularization in the metastatic process in MCC remains to be determined.*

Merkel cell carcinoma (MCC) is a rare primary neuroendocrine carcinoma of the skin affecting mainly the elderly of caucasian origin. Local recurrences are common as is early and frequent metastasis to the local lymph basin before progression to systemic disease (1).

Physiological and pathological angiogenesis is a complex process involving a balance between several pro- and anti-angiogenic molecules. Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen and a major regulator of normal and pathological angiogenesis (2). VEGF has two receptors that bind to it with high affinity, namely VEGFR-1 (Flt-1, fms-like tyrosine kinase) and VEGFR-2 (Flk-1/KDR, foetal liver kinase-1/ kinase domain region), both of which are expressed on endothelial cells. Of these two receptors, VEGFR-2 is the major mediator for several VEGF actions *in vivo*, including mitogenesis and chemotaxis in endothelial cells, the induction of angiogenesis and permeability, and the activation of antiapoptotic effects in endothelial cells (3).

The aims of the present study were to examine the expression of VEGFR-2 in primary MCC samples and further to investigate whether this expression correlates with tumour growth and the development of metastasis.

Patients and Methods

Immunohistochemistry was performed on 21 primary MCC tumour samples obtained from patients surgically treated at the Department of Plastic Surgery, Helsinki University Hospital between 1987 and 2003. None of the patients received chemotherapy or radiation therapy pre-operatively. The samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and kept in dry storage at room temperature. The diagnoses were confirmed with immunohistochemical analysis using cytokeratin 20 and thyroid transcription factor 1 antibodies; the latter was negative in all samples. Tumour size (the greatest surface dimension) was measured from hematoxylin-eosin-stained slides and documented as <2 cm or ≥2 cm, this cut-off point was chosen on the basis of our previous study results (4). For VEGFR-2 analysis, 5 μm/sections were cut on charged slides and dried overnight at 37˚C. The sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. The slides were first immersed in 1% hydrogen peroxide in methanol and then in blocking solution (0.01 M Tris, 0.1 M $MgCl_2$, 0.5% Tween, 1% BSA, and 5% normal goat serum) to block endogenous peroxidase activity and unspecific binding sites. The sections were then rinsed and incubated with the primary antibody (Human VEGF R2 / KDR Affinity Purified Polyclonal Ab, 1:60, AF357, R&D Systems, USA) overnight at +4˚C. Subsequent incubation in biotinylated anti-rabbit serum was performed with

Correspondence to: Heli Kukko, MD, Department of Plastic Surgery, Töölö Hospital, P.O. Box 266, FIN 0029 HUS, Finland. Tel: +358 50 427 1980, Fax: +358 9 471 87 217, e-mail: heli.kukko@hus.fi

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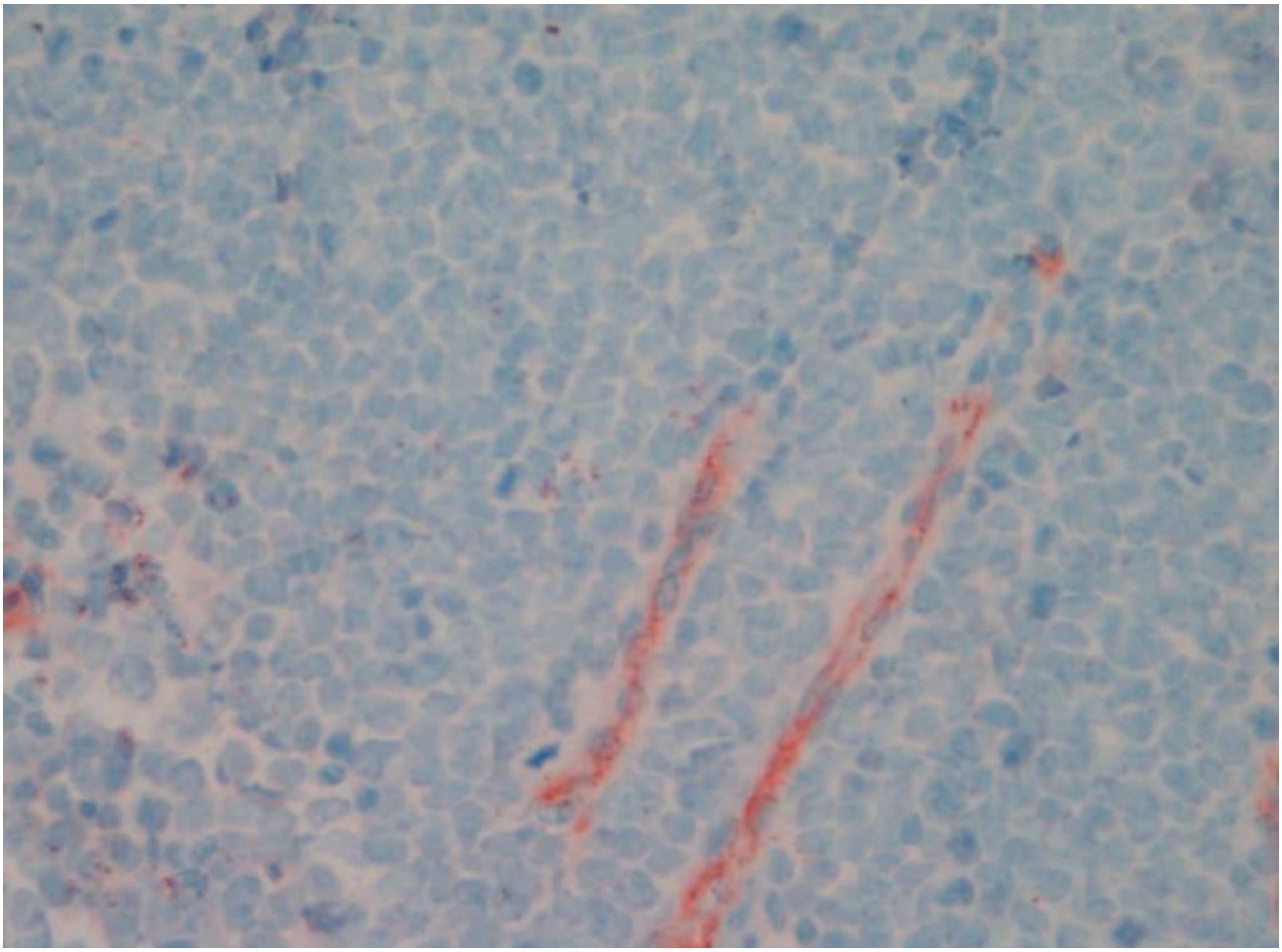

Figure 1. *Positive VEGFR-2 immunostaining in intratumoral vessels. Original magnification x400.*

reagents of the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was developed with 3-amino-9-ethyl carbazole. Finally, the sections were stained with hematoxylin. Negative controls were performed by omitting the primary antibody, and a known positive section for the antibody was included as a positive control.

One section of each tumor was analyzed and the staining pattern was recorded. The sample was considered positive irrespective of the location of the positive reaction within the tumour, that is whether in the tumour cells, stromal cells, or intratumoural vascular structures.

For quantification, the stained area was observed under a microscope and the staining intensity of the immunoreaction of VEGFR-2 was recorded as negative (-), weak (+), or strong (++). Statistical analysis was conducted with NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) software. Fisher's Exact Test was used to calculate the statistical significance between staining intensity and tumor size or clinical outcome. The level of significance was chosen as $p<0.05$, unless otherwise noted. The correlation between the expression of VEGFR-2 and tumour size and metastatic dissemination were calculated with Spearman's Rank Correlation (r).

Results

Positive staining for VEGFR-2 was seen in the endothelial cells of the intratumoral vessel walls in 17 (80%) of the 21 samples (Figure 1). The staining pattern in these cells was cytoplasmic. The tumour cells themselves showed no staining. Staining intensity was weak in 11 (65%) and strong in six (35%) of the 17 positive samples.

VEGFR-2 was expressed in 10/11 of the ≥2 cm tumors and in 7/10 of the <2 cm tumors. VEGFR-2 was expressed in 7/8 of the tumors that developed metastases afterwards and in 10/13 of the non-metastasized tumours.

The statistical analysis revealed that the expression of VEGFR-2 correlated with the tumour size (correlation coefficient r=0.268) indicating a weak positive correlation. This correlation was slightly clearer than the correlation with the development of metastases (r=0.178). There was no statistical significance of staining intensity and tumour size or the development of metastasis.

Discussion

The malignancy, morbidity, and mortality of a tumor are defined largely by its capacity to invade and metastasize. An essential component of the metastatic process is neovascularization, through which tumor cells extravasate to new locations and initiate growth and subsequently metastasis (5). Induction of angiogenesis is required for tumors to grow beyond 1 to 2 mm in diameter, the limit of simple diffusion of nutritives and oxygen (6). VEGF stimulates angiogenesis and the survival of endothelial cells in tumors, thereby enabling tumor expansion and metastasis (7). Decreased expression of VEGFR-2 has also been shown to decrease tumour growth *in vivo* in breast cancer cells (8). Consistent with this, our study showed that the protein expression of VEGFR-2 in Merkel cell carcinoma correlated positively with the tumour size. Another recent study, however, found no expression of angiogenic factors in MCC (9).

We have also studied the effect of simultaneous expression of VEGFR-2 and endostatin in our previous study (10). Tumours positive for VEGFR-2 and concurrently negative for endostatin seemed to be larger than tumours that expressed VEGFR-2 and endostatin simultaneously. Inhibiting angiogenesis with anti-VEGF antibodies has given some promising results with other carcinomas (11). Clinical trials on cancer patients show initial evidence that VEGF inhibition may result in an increase in the time to progression and even in the survival of patients with metastatic carcinomas (12-16). Although our study demonstrated a slightly stronger tendency towards greater VEGFR-2 expression with tumor size than with metastatic potential, these results suggest that anti-angiogenic therapy might be a possible treatment option for MCC patients too.

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