VEGF-A inhibition as a potential therapeutic approach in Merkel cell carcinoma

Thibault Kervarrec, Pauline Gaboriaud, A. Tallet, Valérie Leblond, Françoise Arnold, Patricia Berthon, S. Schweinitzer, Thibaut Larcher, Serge Guyétant, D. Schrama, et al.

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T Kervarrec MD MSc[1,2,3], P Gaboriaud[2], A Tallet PharmD, MSc[4], V Leblond PhD[2], F Arnorld PhD[2], P Berthon PhD[2], S Schweinitzer[3], T Larcher DVM[5], S Guyétant MD PhD[1,2], D Schrama PhD[3], R Houben PhD[3], M Samimi MD PhD[2,6*], A Touzé PhD[2*]

(1) Department of Pathology, Université de Tours, CHU de Tours, avenue de la République, 37170, Chambray-les-tours, France
(2) "Biologie des infections à polyomavirus" team, UMR INRA ISP 1282, Université de Tours, 31 avenue Monge, 37200, Tours, France
(3) Department of Dermatology, Venereology and Allergology, University Hospital Würzburg, Josef-Schneider-Straße 2, 97080 Würzburg, Germany.
(4) Molecular biology plateform, Université de Tours, CHU de Tours, avenue de la République, 37170, Chambray-les-tours, France
(5) APEX, INRA, ONIRIS, ENVN, Université de Nantes, Ecole vétérinaire de Nantes, 101 Route de Gachet, 44307 Nantes, France
(6) Dermatology Department, Université de Tours, CHU de Tours, avenue de la République, 37170, Chambray-les-tours, France

(*) M Samimi and A Touzé contributed equally to the present study

Corresponding author:
Pr Mahtab Samimi
Dermatology Department, Hospital of Tours, avenue de la République, 37170, Chambray-les-tours, France
Email: mahtab.samimi@univ-tours.fr
Fax: +33 247478247

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Abbreviations used: LTAg: Large T antigen; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus; VEGF-A: Vascular endothelial growth factor A

To the editor:

Merkel cell carcinoma (MCC) is an aggressive carcinoma of the skin with frequent metastasis and fatal outcome (Lemos et al. 2010). Until recently, despite rapid chemoresistance, platinum salt-based chemotherapy remained the first-line therapy for stage IV disease (Nghiem et al. 2017). Tumor progression is related to escape from the immune system and restoration of the T-cell response by inhibitors targeting the programmed cell death 1/programmed death-ligand 1 checkpoint is an emerging approach (Colunga et al. 2017; Kaufman et al. 2016). Thus, avelumab has recently been approved as second-line therapy in refractory advanced MCC (Colunga et al. 2017; Kaufman et al. 2016).

However, MCC tumor progression is also related to interactions with non-immune microenvironment components, notably by promoting angiogenesis. In this respect, high vascular density has been associated with decreased recurrence-free survival (Bob et al. 2017) and overall survival (Ng et al. 2008) in MCC. Indeed, vascular endothelial growth factor A (VEGF-A), a proangiogenic factor involved in the development of a wide range of neoplasms (Veeravagu et al. 2007), was previously detected in more than 90% of MCC tumors (Brunner et al. 2008). In addition, high intra-tumoral level of VEGF-A predicts metastasis (Fernández-Figueras et al. 2007). From these observations, we hypothesized that VEGF-A could represent a therapeutic target in MCC.

In a first validation step, VEGF-A expression was assessed by immunochemistry on a tissue microarray assay of 97 MCC cases from a French cohort previously described (Kervarrec et al. 2017), which were scored semi-quantitatively (null, low or high expression). VEGF-A staining was observed in 92 cases (95%), showing high VEGF-A expression in 38 cases (39%), low VEGF-A expression in 54 cases (56%) and no expression in 5 cases (5%). Cases with absent/low or high VEGF-A expression did not differ in age, sex, American Joint Committee on Cancer stage, tumor sample (primary vs metastases) or immunosuppression

(data not shown). However, we found an association between high VEGF-A level and presence of the Merkel cell polyomavirus (MCPyV), considered the main trigger in MCPyV-positive MCC oncogenesis. Indeed, high VEGF-A expression was observed in 27/57 cases (47%) showing immunohistochemical expression of large T antigen (LTAg) evaluated as described (Kervarrec et al. 2017) and only 8/34 cases (24%) showing no LTAg expression (**Figure 1a-b;** Fisher's exact test: p=0.027). Accordingly, quantitative PCR (primer specific for LTAg sequence) revealed higher viral loads in cases with high VEGF-A expression compare to the others (median: 16 copies/cell [Q1–Q3: 9.25–28.75] vs 9 copies/cells [Q1-Q3: 0-16]; Mann–Whitney U test: p<0.001; **Figure 1c)**. These results reveal increased VEGF-A level in MCPyV-positive tumors, but further investigations are required to clarify whether VEGF-A production is primarily driven by MCPyV oncoproteins. Then, we investigated VEGF-A production by MCC tumor cells. The VEGF-A expression was assessed in 5 MCPyV-positive MCC cell lines by RT-PCR and western blot analysis, and VEGF-A concentration was quantified in supernatants by ELISA (all described in **Supplemental Methods**). In accordance with our previous results, VEGF-A expression was detected at the RNA and protein levels in all investigated MCC cell lines and in supernatants (**Figure 1d-f**), thereby confirming VEGF-A production by MCC tumor cells. Notably, VEGF-A expression was generally higher than in HaCat cells **(Supplemental Figure S1)** an established VEGF-A expressing cell culture system (Cai et al. 2018).

In a second step, we investigated VEGF-A as a potential therapeutic target. Because of its high specificity for tumor-human derived VEGF-A and no recognition of the mouse counterpart (Liang et al. 2006), its acceptable toxicity, and its potential use in combination with immunotherapy (Manegold et al. 2017), we selected the humanized monoclonal antibody bevacizumab for VEGF-A inhibition in MCC. *In vitro* experiments confirmed that bevacizumab did not have a direct effect on MCC cell lines viability (data not shown). We

then tested the anti-tumor growth effect of bevacizumab on the previously established xenotransplantation mouse model using MCPyV(+) MCC cells line WaGa (Houben et al. 2012). Briefly, tumors were induced by subcutaneous injection of 10^7 tumor cells in 16 female NOD SCID mice (local ethics committee: Apafis 3973, 2016-020410139630-V2). The general state of the animal and the tumor volume were monitored every 2 days during the entire procedure. When tumor volume reached 25 mm^3, mice were randomly assigned to the experimental (n=8) or control group (n=8) and received an intraperitoneal injection of bevacizumab three times per week (2 mg/kg, injected volume 0.2 ml) (experimental group) or an equivalent volume of phosphate buffered saline (control group).

Tumor growth rates were significantly lower in the experimental than control mice (growth curve slope: median: 0.8 mm^3/day [Q1–Q3 -0.7–4.1] vs 130 mm^3/day [Q1–Q3 107–144]; Mann–Whitney U test: $p=1.5.10^{-4}$) (**Figure 2a-b-Supplemental Figure S2)**. Accordingly, final median tumor weight was significantly lower in the experimental than control mice (median: 0.4 g [Q1–Q3 0.2–0.5 vs 2.4 g [Q1–Q3: 2.1-2.6]; Mann–Whitney U test: $p=3.10^{-4}$). Intra-tumor vascular density, assessed by CD31 immunohistochemical staining, was significantly lower in experimental than control mice (mean value of vascular density: 0.49% [Q1–Q3 0.43-0.51] vs 0.98% [Q1–Q3 0.78-1.42]; Mann–Whitney U test: $p=3.10^{-4}$) showing a direct inhibition of blood vessel growth by bevacizumab (**figure 2c-d**). We observed no liver or lung metastasis in either group and no difference in necrosis (p=0.5).

One major limitation of MCC preclinical studies is the lack of an available tumor model with immunocompetent mice. Indeed, VEGF-A also acts on immune cells by inhibiting both lymphocytic and dendritic-cell maturation (Ohm et al. 2003). Thus, bevacizumab could reduce these immunosuppressive effects in combination with immunotherapy (Manegold et al. 2017). In addition, inclusion of an VEGF-A non-expressing cell line to exclude a stromal VEGF-A targeting by bevacizumab and a MCPyV-negative MCC cell line in a xenograft

model would be suitable but was not performed in the present study because of lack of appropriate or representative cell lines (Guastafierro et al. 2013).

To conclude, our results suggest VEGF-A as a potential therapeutic target in MCC. VEGF-A is frequently highly expressed in tumor cells, especially in MCPyV(+) cases. Because bevacizumab was found efficient for tumor growth inhibition in a preclinical model, it may be a promising therapeutic option in metastatic MCC, as an alternative or combined with current treatments.

Conflict of Interest: The authors declare no conflict of interest.

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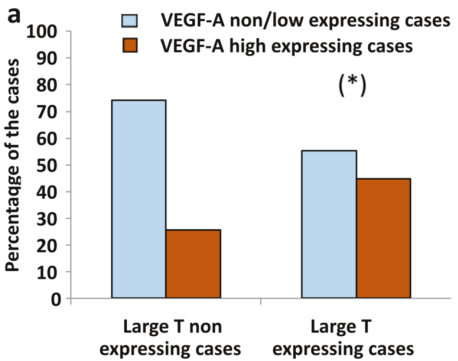
Legends

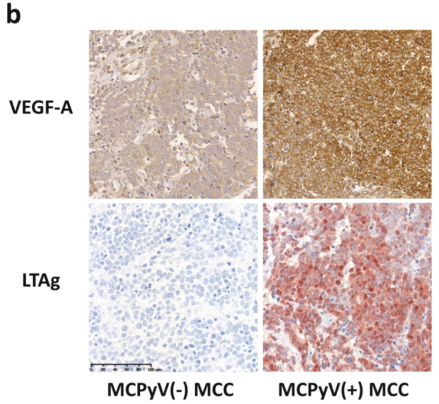
Figure 1: Vascular endothelial growth factor A (VEGF-A) expression in Merkel cell carcinoma (MCC) tumor samples and MCC cell lines (MKL-1, MKL-2, MS-1, WaGa and PeTa): a) Expression of VEGF-A in LTAg-expressing and non-expressing MCC tissue: VEGF-A expression was assessed semi-quantitatively and dichotomized as VEGF-A low (absent or low) and VEGF-A high expression. In parallel, an Allred score for MCPyV-LTAg expression was determined. Only cases with score > 2 were considered LTAg-expressing. Data are expressed as percentage of VEGF-A expressing and non/low-expressing cases, * p=0.027 comparing LTAg-expressing and non expressing cases by Fisher exact test; **b) Representative immunochemical detection of MCPyV large T antigen (LTAg) (CM2B4) and VEGF-A protein in MCPyV(-) and MCPyV(+) tumor tissue** (bar=100 μm)**; c) MCPyV viral load in VEGF-A -non/low and high expressing MCC groups**. Viral load was determined by quantitative PCR with WaGa cells as a control (Rodig et al. 2012). Horizontal line is median; box edges are Q1-Q3 and whiskers are range, ** $p<0.001$ Mann–Whitney U test; **d) VEGF-A mRNA level in MCC cell lines:** RT-PCR analysis of VEGF-A and RPLPO levels (the latter used as a control); **e) VEGF-A protein level in MCC cell lines:** western blot analysis of VEGF-A protein level (expected size: 22 kDa); **f) VEGF-A protein level in supernatant of MCC cell lines:** ELISA of VEGF-A secretion in conditioned culture media from 3.10^5 cells/ml cultured for 6 days. Non-conditioned culture media was used for normalization. Data are mean±SD of three independent experiments. All procedures are described in supplemental methods.

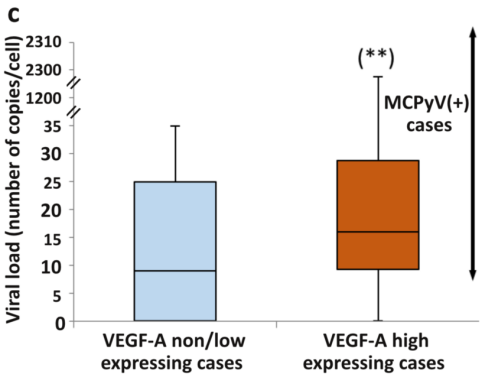
Figure 2: Efficacy of bevacizumab treatment on growth of WaGa xenotransplantation tumors in mice: a) tumor growth in bevacizumab and control groups (mean +/- SD tumor volume in mm^3)**.** Tumors were induced by subcutaneous injection of 10^7 WaGa MCC cells with 100 µg Matrigel in a final volume of 0.2 ml DMEM medium into mice. Mice received intraperitoneal injections of bevacizumab three times per week (2 mg/kg, injected volume 0.2 ml) (bevacizumab group, n=8) or an equivalent volume of phosphate buffered saline (controls, n=8); **b) End-point tumor volume (mm^3) in bevacizumab and control groups**, * $p<0.001$ Mann–Whitney U test; **c) Representative CD31 immunohistochemical staining of tumors in bevacizumab and control groups** (bar=250 µm) revealing lower vascular density ($p<0.001$ Mann–Whitney U test) and smaller vessel size ($p<0.01$ Mann–Whitney U test) in bevacizumab- versus control-treated tumors; **d) Vascular density at the end point in bevacizumab and control groups,** ** $p<0.001$ Mann–Whitney U test).

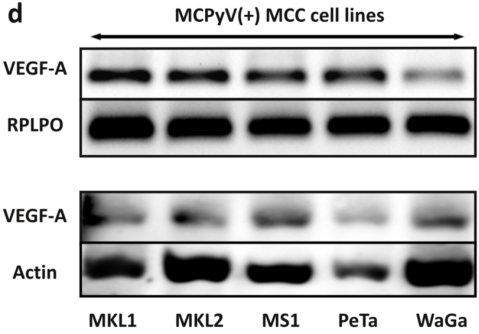
Supplemental figure S1: VEGF-A protein level in supernatants of HaCat and MCC cell lines: VEGF-A ELISA was performed with conditioned media from $3x10^5$ cells/ml cultured for 6 days.

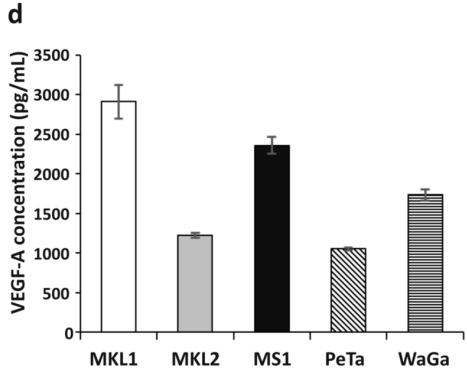
Supplemental figure S2: a) Growth of individual tumors in experimental and control mice (values are presented as tumor volume in mm^3). The blue-colored mice in the control group were sacrificed after 23 days due to reaching an endpoint (tumor ulceration); **b) Representative microscopy of tumors at the end of the experiment**. Tumors were cut in the larger axis, formalin-fixed and paraffin-embedded and stained with hematein, phloxin and safran. Microscopy photographs were taken at the same magnification, showing larger tumor size in controls than experimental mice.

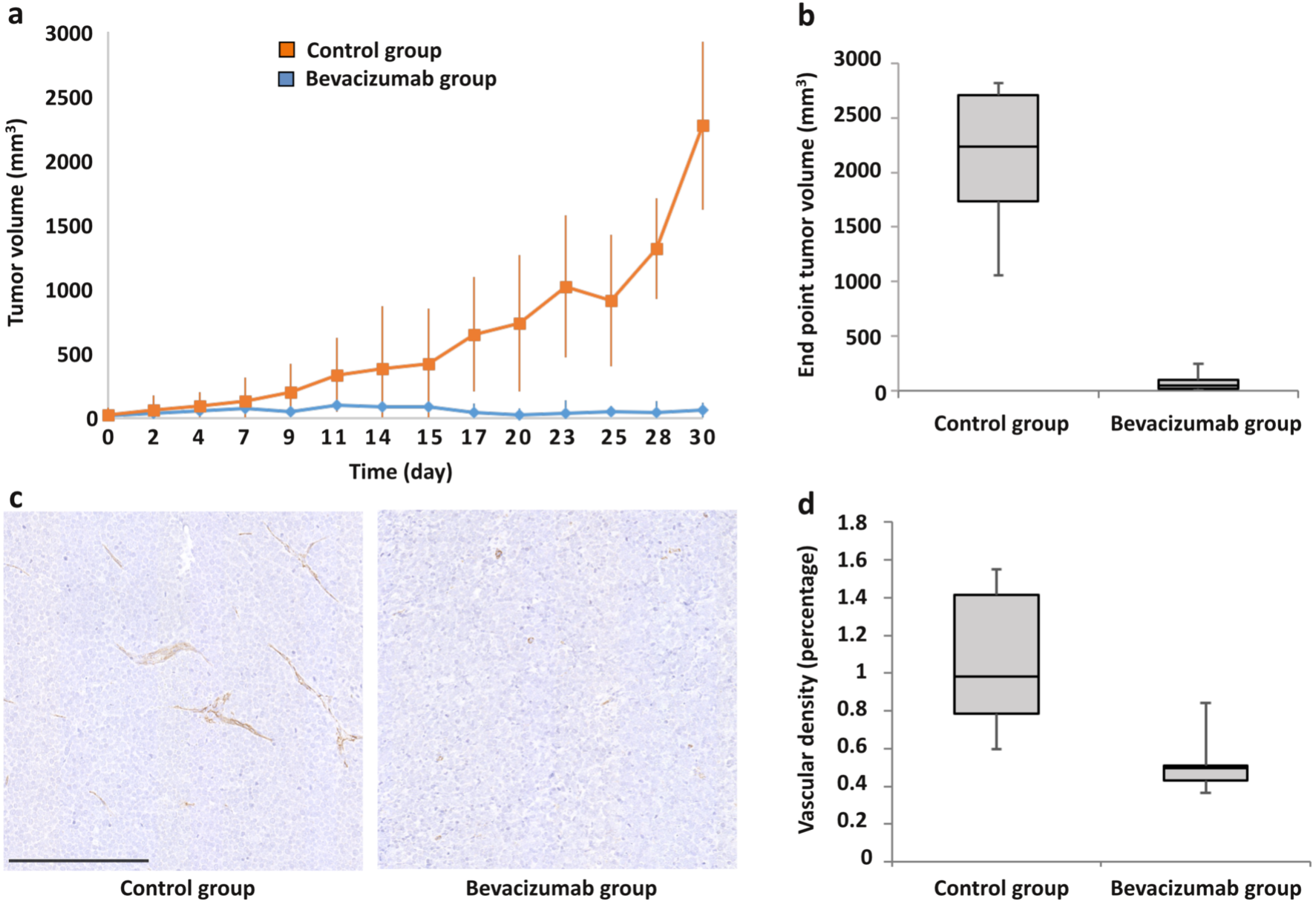

a
VEGF-A non/low expressing cases
VEGF-A high expressing cases
(*)
Percentage of the cases
100
90
80
70
60
50
40
30
20
10
0
Large T non expressing cases
Large T expressing cases


b
VEGF-A
LTAg
MCPyV(-) MCC
MCPyV(+) MCC


c
Viral load (number of copies/cell)
2310
2300
1200
35
30
25
20
15
10
5
0
(**)
MCPyV(+) cases
VEGF-A non/low expressing cases
VEGF-A high expressing cases


d
MCPyV(+) MCC cell lines
VEGF-A
RPLPO
VEGF-A
Actin
MKL1
MKL2
MS1
PeTa
WaGa


d
VEGF-A concentration (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
MKL1
MKL2
MS1
PeTa
WaGa


a
Control group
Bevacizumab group
Tumor volume (mm³)
3000
2500
2000
1500
1000
500
0
0 2 4 7 9 11 14 15 17 20 23 25 28 30
Time (day)
b
End point tumor volume (mm³)
3000
2500
2000
1500
1000
500
0
Control group
Bevacizumab group
c
Control group
Bevacizumab group
d
Vascular density (percentage)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control group
Bevacizumab group