A short-term in vivo model for Merkel Cell Carcinoma

Abstract
In vivo tumor models are essential for studying the biology of cancer, identifying tumor targets and evaluating antitumor drugs. Considering the request for the minimisation of animal experiments and following the “3R”-rule (“replacement,” “refinement,” “reduction”), it has become crucial to develop alternative experimental models in cancer biology. Several studies have already described the avian chorioallantoic membrane (CAM) model as an alternative to rodents, suitable to investigate growth, progression and metastasis of various types of cancer. In the present work, we grafted three Merkel cell carcinoma (MCC) cell lines onto the avian CAM and monitored tumor growth and development of solid tumor nodules. Morphology of xenograft was characterised histologically and immunohistochemically. Our results demonstrate CAM assay as a useful tool to study MCC pathophysiology.

1 | BACKGROUND
Merkel Cell Carcinoma (MCC) is a rare, highly aggressive neuroendocrine tumor of the skin with poor prognosis that typically occurs in elderly and immunosuppressed patients.[1] The MCC is characterised by the presence of cytokeratin 20 (CK-20) and neuroendocrine granules. The outcome of immune surveillance suggested viral carcinogenesis, which was indeed demonstrated in the majority of cases.[2] UV radiation exposure is an additional epidemiologic risk factor for MCC.[3] Due to the development of immune checkpoint inhibitors, a new therapeutic window opened for MCC patients.[4-6] Recently, treatment with three humanized antibodies, namely avelumab, pembrolizumab and nivolumab targeting PD-L1/PD-1 pathway have shown durable responses in MCC patients.[7-9] We did not observe any significant difference within MCC cell lines with respect to tumor area or macroscopic blood vessels.

2 | QUESTION ASKED
We investigated whether the CAM system is suitable as a short-term in vivo model for MCC to study tumor growth, proliferation and angiogenesis.

3 | EXPERIMENTAL DESIGN
We grafted three MCPyV-positive MCC cell lines (MKL-1, PeTa, WaGa), that clearly differ in their phenotype and growth behaviour in cell culture in vitro, onto the CAM and monitored tumor growth and development of solid tumor nodules. Morphology of xenograft was characterised histologically and immunohistochemically. Our results demonstrate CAM assay as a useful tool to study MCC pathophysiology.

4 | RESULTS
All cell lines formed tumors within 3 days after transplantation; progression of tumor formation was monitored by photo-documentation throughout the incubation period (Figure S2A). MKL-1, PeTa and WaGa tumors revealed a reproducible growth pattern. MCC cells developed into solid nodules from day 3 after transplantation; vascularisation steadily progressed, and avian vessels developed radially towards the tumors (Figure 1A-C, Figure S2A). On day 5 posttransplantation, the tumor area (mm2) was determined by ImageJ (Figure S2B), and the number of vessels surrounding the xenografts was counted manually (Figure S2C) according to Ribatti et al, 2006.[14] We did not observe any significant difference within MCC cell lines with respect to tumor area or macroscopic blood vessels.

At day 5 post transplantation, xenografted MCC tumors were excised, fixed, paraffin embedded and stained for haematoxylin and eosin (H and E); for details, see supplementary material. The tumors showed strong interaction of MCC cells with the CAM mesenchyme and invasion of tumor cells from the primary onplant site into the surrounding CAM tissue, thereby disrupting the CAM upper epithelium (Figure 1D-F arrows). The histological appearance of the tumors...
was similar to those of MCC, composed of strands or nests of uniform, small round cells with marginal cytoplasm and round nucleus (Figure 1G-I, arrows).\textsuperscript{[15]}

Furthermore, using immunohistochemistry method (IHC), the sections were analysed for the expression of MCC marker CK-20, MCPyV-LT antigen and the proliferation marker Ki-67 (Figure 2). Expression of specific neuroendocrine tumor markers such as chromogranin-A (CGA) and synaptophysin (p-38) are shown in Figure S3.\textsuperscript{[16,17]}

CK-20 was present in all MCC xenografts in a typical dot-like perinuclear staining pattern (Figure 2A-C).\textsuperscript{[18]} The MCPyV-LT antigen was detected in all three MCC cell lines. The IHC showed nuclear staining of LT antigen in MKL-1 (Figure 2D) and cytoplasmic staining for PeTa and WaGa (Figure 2E-F) due to the differences in truncating mutation in LT antigen.\textsuperscript{[19]} This staining of MCPyV LT antigen could be used as a valuable marker for drug screening of virus-positive cells. The neuroendocrine marker CGA and p-38 were strongly expressed in MKL-1, PeTa and WaGa cells and allowed identification of single evaded tumor cells (Figure S3A-F).

Ki-67 was used to stain the proliferating cells distributed throughout the tumor mass. The positive Ki-67 staining was seen in all three MCC cell lines indicating the tumor growth and proliferation (Figure 2G-I). This will be useful to study the response of MCC cell lines to drugs under in vivo conditions.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

VKB, CK and EB performed the research, NGTW designed the research study, JCB provided cell lines, NGTW contributed essential reagents and tools, VKB analysed the data. VKB, CK, JCB, WS and NGTW wrote the paper. VKB, EB, WS and NGTW revised the manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

FIGURE S1 Schematic workflow of ex ovo CAM assay. Fertilized eggs were incubated for 3 days, the egg shell was then cracked into plastic dishes, following further incubation for 7 days. MCC cells were applied on vascular branches of the CAM and incubated for 3-7 days. The CAM with the attached grafts was excised, followed by FFPE tissue embedding and sectioning. The tumour morphology was analysed by histology and immunostaining.

FIGURE S2 Photo-documentation of growth behaviour of xenografted MCC cell lines. (A) MKL-1 (upper panel), PeTa (middle panel) and WaGa (lower panel) were monitored for 5 days upon engraftment. Bars equal 1 mm. (B) Tumour area per CAM was measured using Image J software. (C) Angiogenesis was measured by counting macroscopic blood vessels (MBV) manually. Results were plotted as mean ± SD using GraphPad prism software. (N = 6 tumours). One-way ANOVA was used for statistical analysis.

FIGURE S3 Immunohistochemical characterizations of xenografted MCC cell lines with neuroendocrine specific marker. (A-F) All MCC cell lines express the neuroendocrine tumour specific markers synaptophysin (p38) and chromogranin A (100× and 400× magnification, scale bar = 100 µm and 20 µm respectively).