Clinical Chemistry 65:3
462–472 (2019)

Detection and Characterization of Circulating Tumor Cells in Patients with Merkel Cell Carcinoma

Sabine Riethdorf,[1*] Lina Hildebrandt,[2] Lucie Heinzerling,[3] Ellen Heitzer,[4] Nicole Fischer,[5] Sonja Bergmann,[1] Oliver Mauermann,[1] Julie Waldispühl-Geigl,[4] Cornelia Coith,[1] Gerhard Schön,[6] Sven Peine,[7] Gerold Schuler,[3] Michael R. Speicher,[4] Ingrid Moll,[2] and Klaus Pantel[1]

BACKGROUND: Merkel cell carcinoma (MCC) is a rare, aggressive skin cancer with increasing incidence and high mortality rates. MCC has recently become the subject of immune checkpoint therapy, but reliable biomarkers for estimating prognosis, risk stratification, and prediction of response are missing.

METHODS: Circulating tumor cells (CTCs) were detected in peripheral blood from patients with MCC by use of the CellSearch® system. Moreover, CTCs of selected cases were characterized for Merkel cell polyomavirus (MCPyV), chromosomal aberrations, and programed death ligand 1 (PD-L1) production.

RESULTS: Fifty-one patients were tested at first blood draw (baseline), and 16 patients had 2 or 3 consecutive measurements to detect CTCs. At baseline, ≥1 CTC (range, 1–790), >1, or ≥5 CTCs/7.5 mL were detected in 21 (41%), 17 (33%), and 6 (12%) patients, respectively. After a median follow-up of 21.1 months for 50 patients, detection of CTCs correlated with overall survival (≥1, $P = 0.030$; >1, $P < 0.020$; and ≥5 CTCs/7.5 mL, $P < 0.0001$). In multivariate Cox regression analysis, the detection of ≥5 CTCs/7.5 mL adjusted to age and sex compared to that of <5 was associated with a reduced overall survival ($P = 0.001$, hazard ratio = 17.8; 95% CI, 4.0–93.0). MCPyV DNA and genomic aberrations frequently found in MCC tissues could also be detected in single CTCs. Analyzed CTCs were PD-L1 negative or only weakly positive.

CONCLUSIONS: The presence of CTCs is a prognostic factor of impaired clinical outcome, with the potential to monitor the progression of the disease in real time. Molecular characterization of CTCs might provide new insights into the biology of MCC.

Merkel cell carcinoma (MCC)[8] is a rare, highly aggressive cutaneous neuroendocrine neoplasm *(1)* for which there is uncertainty about the cell of origin *(2–6)*. Of particular note is the causal link between the oncogenic activity of Merkel cell polyomavirus (MCPyV) *(7)* and the occurrence of MCC. The estimated disease-associated mortality rate ranges between 33% and 46% *(4)*. Male sex, high age, immunosuppression, and the presence of concomitant malignancies are prognostic factors affecting the clinical outcome of MCC *(8, 9)*. Among various cell biologic markers, only a few putative biomarkers for MCC progression and prognosis have been identified *(4)*. Therapy depends on the stage of disease, with surgery and radiotherapy in early stages and immune checkpoint-inhibitor therapy in metastatic disease *(4)*. Thus, treatment of advanced stage MCC with pembrolizumab or avelumab, antibodies recognizing programed cell death 1 (PD-1) or programed cell death ligand 1 (PD-L1), resulted in clinical activity both in patients with MCPyV-positive and MCPyV-negative MCCs *(10, 11)*. Several candidate biomarkers aimed to predict response to these therapies including production of PD-1 or PD-L1 in tumor cells, tumor-infiltrating lymphocytes, and other microenvironmental cells have already been tested *(12)*. However, comprehensive validation in large clinical studies is still lacking. Sentinel node biopsy and radiologic imaging are used to determine the extent of metastatic disease and to monitor therapy response. Owing to high costs, limited availability of tumor tissue, and

[1] Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2] Department of Dermatology and Venereology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [3] Department of Dermatology, Universitätsklinikum Erlangen, Erlangen, Germany; [4] Institute of Human Genetics, Diagnostic and Research Center for Molecular BioMedicine, University of Graz, Graz, Austria; [5] Institute of Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [6] Institute of Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [7] Institute of Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

* Address correspondence to this author at: Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Fax +49-40-741056546; e-mail s.riethdorf@uke.de.

Received September 7, 2018; accepted November 28, 2018.
Previously published online at DOI: 10.1373/clinchem.2018.297028

[8] Nonstandard abbreviations: MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; PD-1, programed cell death 1; PD-L1, programed cell death ligand 1; CTCs, circulating tumor cells; UCB, urothelial carcinoma of the bladder; WGA, whole-genome amplification; CGH, comparative genomic hybridization; OS, overall survival.

insufficient diagnostic specificity and sensitivity, there is a need for new ultrasensitive biomarkers. Current treatment decisions in patients with MCC are usually based on staging and characteristics of the primary tumors. However, tumor cells can acquire new features during disease progression, and tissue material derived from recurrent or metastatic tumors is rarely accessible. Thus, strong emphasis is now put on establishing noninvasive biomarkers from liquid biopsies for real-time evaluation of recurrent disease *(13)*. Circulating tumor cells (CTCs) have recently proven to be prognostic markers in patients with different metastatic tumors and predictors of relapse in early stage disease *(14)*. Of note, CTCs have also been detected with high frequencies in patients with neuroendocrine tumors *(15, 16)*. First case studies reporting the presence of circulating MCC cells in blood and bone marrow of patients with metastatic disease were published for patients with autoimmune diseases or concomitant leukemia and myeloma *(17–19)*. So far, only 2 reports on CTC detection in small cohorts of MCC patients have been published *(20, 21)*.

Here, we present the results of the largest study to date on CTC detection in 51 patients including long-term follow-up data for 50 patients. Moreover, we focused on molecular characterization of individual CTCs regarding PD-L1 production, genomic aberrations, and detection of MCPyV DNA.

Materials and Methods

PATIENTS AND STUDY MATERIAL

All patients provided informed consent for participating in this study, which was approved by the ethical commissions of the Hamburger Ärztekammer (PV3779, PV5392) and the Friedrich Alexander University of Erlangen (Re.-No. 281_12B). Clinicopathological characteristics at the time of first blood examination are displayed in Table 1.

Blood samples from 51 patients treated at the University Medical Centers in Hamburg or Erlangen between 2012 and 2017 were collected in CellSave tubes (Menarini, Silicon Biosystems) and processed within 96 h after blood draw by the CellSearch system (Menarini). The patient cohort comprised patients with all stages of the disease and with or without evidence of tumor and/or recurrence and/or metastases. CTC enumeration was performed by the CellSearch system, which has been cleared by the Food and Drug Administration for CTC detection in blood from patients with metastatic breast, colorectal, and prostate cancer *(22)*.

DETERMINATION OF PD-L1 PRODUCTION IN CTCs

To analyze PD-L1 production in CTCs, the CellSearch CXC kit (Menarini) was used, which enabled immunofluorescent characterization of CTCs by a phycoerythrin (PE)-labeled antibody. Here we used the PE-labeled anti-PD-L1 antibody (E1L3N® XP® Rabbit mAb, PE conjugate, CellSignaling Technology) diluted 1:50 (working concentration, 3 μL antibody and 147 μL antibody diluent per sample) in antibody diluent (Dako Cytomation) in Menarini-supplied reagent cups. The intensity of PD-L1 immunofluorescence in the fourth channel of the CellSearch system was categorized into weakly positive, strongly positive, and negative.

TUMOR CELL LINES

MCC (MCPyV positive: MKL-1, MS-1, WaGa; MCPyV negative: UISO) *(23, 24)* and urothelial carcinoma of the bladder (UCB) cell line cells (5637) *(25)* were grown in RPMI 1640 medium (Gibco), and UCB cell lines RT4 and 647V *(25)* in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal calf serum, 100 U/mL penicillin, 0.1 g/L streptomycin, and 2 mmol/L glutamine. Cell lines were authenticated and tested to be free of *Mycoplasma* regularly.

WESTERN BLOT ANALYSIS

Cells were washed in phosphate-buffered saline, and cell extracts were prepared by lysis in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Separated proteins were subjected to Western blot analysis with the rabbit monoclonal antibody PD-L1 (E1L3N) XP (Cell Signaling Technology, CST) at a dilution of 1:1000.

ISOLATION OF SINGLE CTCS BY MICROMANIPULATION

After CellSearch processing, CTCs were isolated by micromanipulation with use of a micromanipulator (Eppendorf AG) supplemented with custom-made capillaries (40 μmol/L in diameter, capillary type III) *(26)*.

WHOLE-GENOME AMPLIFICATION OF DNA FROM SINGLE CTCs

For whole-genome amplification (WGA), the GenomiPhi DNA Amplification kit (GE Healthcare) *(26)* was applied. The quality of purified WGA products was tested by multiplex PCR *(26)*.

ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

Array comparative genomic hybridization (CGH) was performed at the Institute of Human Genetics, Diagnostic and Research Center for Molecular BioMedicine, University of Graz, Austria, with the Human Genome CGH Microarrays 60k (Agilent Technologies) following the instructions of the manufacturer. Briefly, 1 μg of GenomiPhi amplified test DNA digested with *Alu*I and *Rsa*I, 300 ng of undigested DNA isolated from paraffin-embedded primary tumor and metastasis tissue, and a commercially available male reference DNA (Promega) were labeled with the Sure Tag DNA Labeling Kit (Agilent Technologies, cyanine 5-dUTP for the test samples and cyanine 3-dUTP for the reference DNA). The

Table 1. CTC detection at time of first blood draw and clinicopathological characteristics.

Clinicopathological parameter	CTC-positive ≥1, %	CTC-negative 0, %	*P* value	CTC-positive >1, %	CTC-negative ≤1, %	*P* value	CTC-positive ≥5, %	CTC-negative <5, %	*P* value
Total number (n = 51)[a]	41	59		33	67		12	88	
Age, years									
≤75	12	24	NS	10	26	NS	4	32	NS
>75	28	36		22	42		6	58	
Mean: 74.8									
Median: 79									
Sex									
Men	18	25	NS	12	31	NS	2	41	NS
Women	24	33		22	35		10	47	
AJCC stage[b]									
I/II	16	50		8	58		0	66	
III	12	10	0.001	12	10	<0.0001	4	18	0.001
IV	12	0		12	0		6	6	
N[c]									
N0	16	50	0.002	8	58	<0.0001	0	66	0.003
N1	24	10		24	10		10	24	
M									
M0	28	60	0.002	20	68	0.001	4	84	0.009
M1	12	0		12	0		6	6	
Other malignancy									
Yes	12	24	NS	10	27	NS	2	35	NS
No	27	37		20	43		6	57	
MCPyV									
Positive	60	20	NS	60	20	NS	0	80	NS
Negative	10	10		10	10		0	20	

[a] Total number of patients with data for CTC and sex: 51.
[b] AJCC, American Joint Committee on Cancer; MCPyV, Merkel cell polyomavirus.
[c] Any N stage.

labeled DNA was purified and concentrated to a final volume of 9.5 μL. Then the DNA was mixed with Cot-1 DNA, Agilent blocking agent, and Agilent hybridization buffer and denatured at 96 °C for 3 min, followed by an annealing step at 37 °C for 30 min. Finally, DNA was hybridized to the Sure Print G3 Human Catalog 8 × 60K (Agilent p/n G4450A) CGH microarrays. After hybridization, the slides were washed and scanned with a microarray scanner. Images were preprocessed with Feature Extraction and DNA Workbench 5.0.14. Evaluation of our array CGH was done on the basis of a previously published algorithm *(27)*.

DETECTION OF MCPYV DNA

Analysis of tumor tissues for the presence of MCPyV DNA sequences was performed as described elsewhere *(7, 28)*. For the detection of MCPyV DNA sequences in CTCs, 150 ng of WGA GenomiPhi amplified DNA and the following 2 nested primer sets were used: I, LT1F-LT1R (outside PCR) LT1F: TACAAGCACTCCACCAAAGC; LT1R: TCCAATTACAGCTGGCCTCT /M1M2F-M1M2R (inside PCR), M1M2F: GGCATGCCTGTGAATTAGGA, M1M2R: TTGCAGTAATTTGTAAGGGGACT; II, LT5F-LT3R (outside PCR), LT5F: GCTCCTAATTGTTATGGCAACA, LT3R: ATATAGGGGCCTCGTCAACC /LT3F-LT5R (inside PCR), LT3F: TTGTCTCGCCAGCATTGTAG, LT5R: TGGGAAAGTACACAAAATCTGTCA *(7)*.

Analysis and evaluation of DNA sequences were performed on the Applied Biosystems 3130 Genetic Analyzer.

STATISTICAL ANALYSES

Correlations between CTC detection and clinicopathological parameters were analyzed by χ^2 or Fisher exact tests. Two-tailed P values <0.05 were considered statistically significant. Absolute CTC numbers were compared to American Joint Committee on Cancer tumor stages, lymph node, and distant metastases by the Mann–Whitney U and Kruskal–Wallis tests.

Overall survival (OS) was defined as the time between blood collection and death by any cause. Nonparametric Kaplan–Meier estimates of the survival function and log-rank test were used to compare CTC-positive and CTC-negative samples. A priori, no statistical power and/or size of patient population was calculated.

For these analyses, the IBM SPSS Statistics 21 program was used. In addition to univariate analyses, in a multivariate Cox model, different covariates [age 10 (age divided by 10) and sex] were used to adjust for the influence of ≥5 CTCs on OS. This analysis was performed by the program R version 3.4.3. For reporting of results, the REMARK criteria *(29)* were followed.

Results

PATIENT CHARACTERISTICS

Association of CTC detection and clinical data available for 50 patients are provided in Table 1. Representative CTC images are shown in Fig. 1. Thirty-five patients had 1 blood draw, and for 16 and 4 patients CTC results from second and third visits, respectively, were available (Table 2). Primary tumor sites were head and neck (n = 12), arm (n = 18), leg (n = 13), trunk (n = 4), and in 3 cases the primary tumor site was unknown.

Eighteen patients additionally suffered from other malignancies. One patient developed a second MCC and 17 others suffered from different malignancies: breast cancer (n = 1), chronic lymphatic leukemia (n = 3), bladder carcinoma (n = 2), colon carcinoma (n = 1), polycythemia vera (n = 1), lymphoma (n = 1), prostate cancer (n = 1), basal cell carcinoma (n = 2), squamous cell carcinoma (n = 1), melanoma (n = 2), uterine carcinoma (n = 1), and carcinoid (n = 1). The other malignancies were diagnosed before or after diagnosis of MCC. To the best of our knowledge the other tumors were not active during this study.

Before first blood draw (at baseline) all patients underwent surgery and/or resurgery with large margins (often 3 cm), mostly radiotherapy to the tumor bed and local lymph node area (n = 18), and some patients (n = 10) underwent sentinel node biopsy. Only a small number of patients (n = 6) received chemotherapies, mostly etoposide. This first blood draw was either at first diagnosis or during the follow-up of the patients.

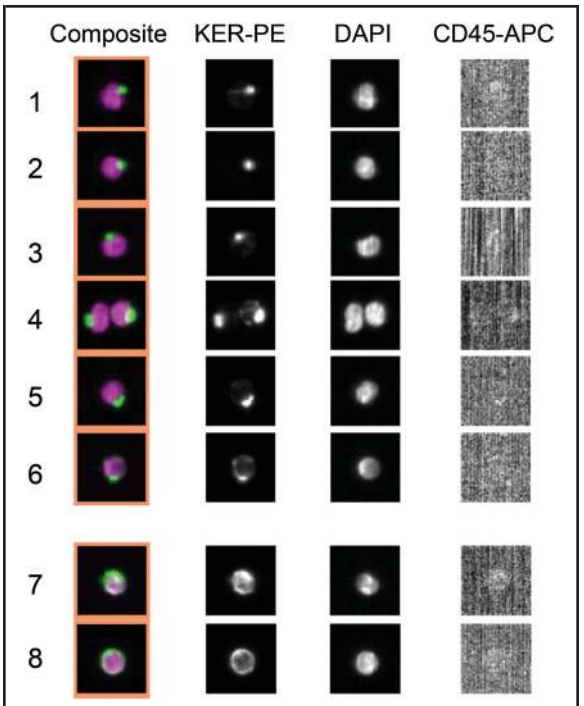

Fig. 1. CTCs detected with the CellSearch system.

CTCs were identified as round to oval, keratin-positive (phycoerythrin, KER-PE), CD45-negative (allophycocyanin CD45-APC), DAPI-positive (4`,6-diamidino-2-phenylindole, dihydrochloride) cells. Cells 1–6 have a paranuclear dot-like keratin pattern, whereas cells 7 and 8 present with a typical cytoskeleton keratin pattern.

DETECTION OF CTCs

The presence of 5 and 3 CTCs/7.5 mL was recently defined as the cutoff for discriminating unfavorable from favorable outcome in patients with metastatic breast or prostate as well as colorectal cancer, respectively *(22)*, but thus far no cutoff has been clinically validated for MCC. Applying ≥1 CTC/7.5 mL as cutoff, at time of first blood draw, 21/51 (41%) patients were detected CTC positive (range, 1–790, mean 66.9, median 2). More than 1 or ≥5 CTCs/7.5 mL at first blood collection were found in 17 (33%) or 6 (12%) patients, respectively (Table 1 and see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue3). Considering blood analyses at any time point, ≥1, >1, and ≥5 CTCs/7.5 mL were identified in 28 (55%), 22 (43%), and 10 (20%) patients, respectively (see Table 2 in the online Data Supplement), with 1960 being the highest CTC count measured (Table 2).

"Dot-like" paranuclear keratin immunofluorescence patterns indicative most probably of neuroendocrine

Table 2. Absolute numbers of CTCs detected at different time points during follow-up (CTC1, CTC2, CTC3).

Pat. ID	Stage, N, M at 1st blood draw[a]	CTC1	Time from 1st blood draw, days	CTC2	Time from 1st blood draw, days	CTC3
6	I/II, 0, 0	0	190	1		
11	I/II, 0, 0	0	30	2		
13	I/II, 0, 0	2	278	1		
18	I/II, 0, 0	0	97	1	373	1
24	I/II, 0, 0	0	7	0		
26	I/II, 0, 0	0	944	1		
27	I/II, 0, 0	0	133	0	233	0
28	I/II, 0, 0	0	320	0		
32	I/II, 0, 0	0	96	43		
33	I/II, 0, 0	0	523	2	258	95
35	IV, 1, 1	2	220	0		
36	III, 1, 0	2	162	0		
41	III, 1, 0	4	8	1	241	1320
42	III, 1, 0	0	280	64		
44	III, 1, 0	0	38	0		
47	IV, 1, 1	790	14	1960		

[a] N, absence (0) or presence (1) of regional lymph node metastasis. M, absence (0) or presence (1) of distant metastasis.

MCC origin were detected in CTCs from 24/28 CTC-positive patients, with percentages ranging between 33% and 100% (see Table 3 in the online Data Supplement). In most cases both CTCs with dot-like (Fig. 1, CTCs 1–6) and those with typical cytoskeleton patterns (Fig. 1, CTCs 7 and 8) could be identified.

The presence of CTCs at first blood draw and any cutoff values was significantly associated with tumor stage, lymph node involvement, and distant metastases (Table 1). All patients with distant metastasis (M1) exhibited at least 2 CTCs/7.5 mL. Also, the absolute CTC counts measured in 7.5 mL at time of first blood collection (Fig. 2 and see Table 1 in the online Data Supplement) were significantly associated with the tumor stage, lymph node, and distant metastases at time of first blood collection ($P < 0.0001$). On the other side, no correlation was evident between CTC positivity and age, sex, and the presence of other malignancies (Table 1 and see Table 2 in the online Data Supplement). The presence of MCPyV DNA was tested in 11 tumor tissues with 9 tumors being positive. CTCs were found in patients with virus-positive and virus-negative MCCs (Table 1).

From 16 patients, blood samples were collected at 1 or 2 points during follow-up observation. Time intervals between blood draws are shown in Table 2. Although a strong increase in CTC counts from <5/7.5 mL to ≥5/7.5 mL was observed in patients 32, 33, 41, and 42; patient 47 already presented with a high CTC number at first blood draw (Table 2). Patient 41's disease was ini-

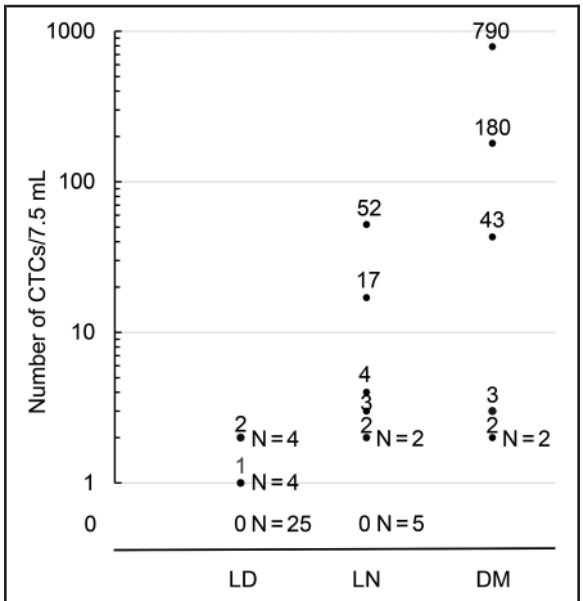

Fig. 2. Absolute numbers of CTCs in individual patients with local disease (LD), lymph node metastasis (LN), and distant metastasis (DM).

N, number of patients with the same CTC result.

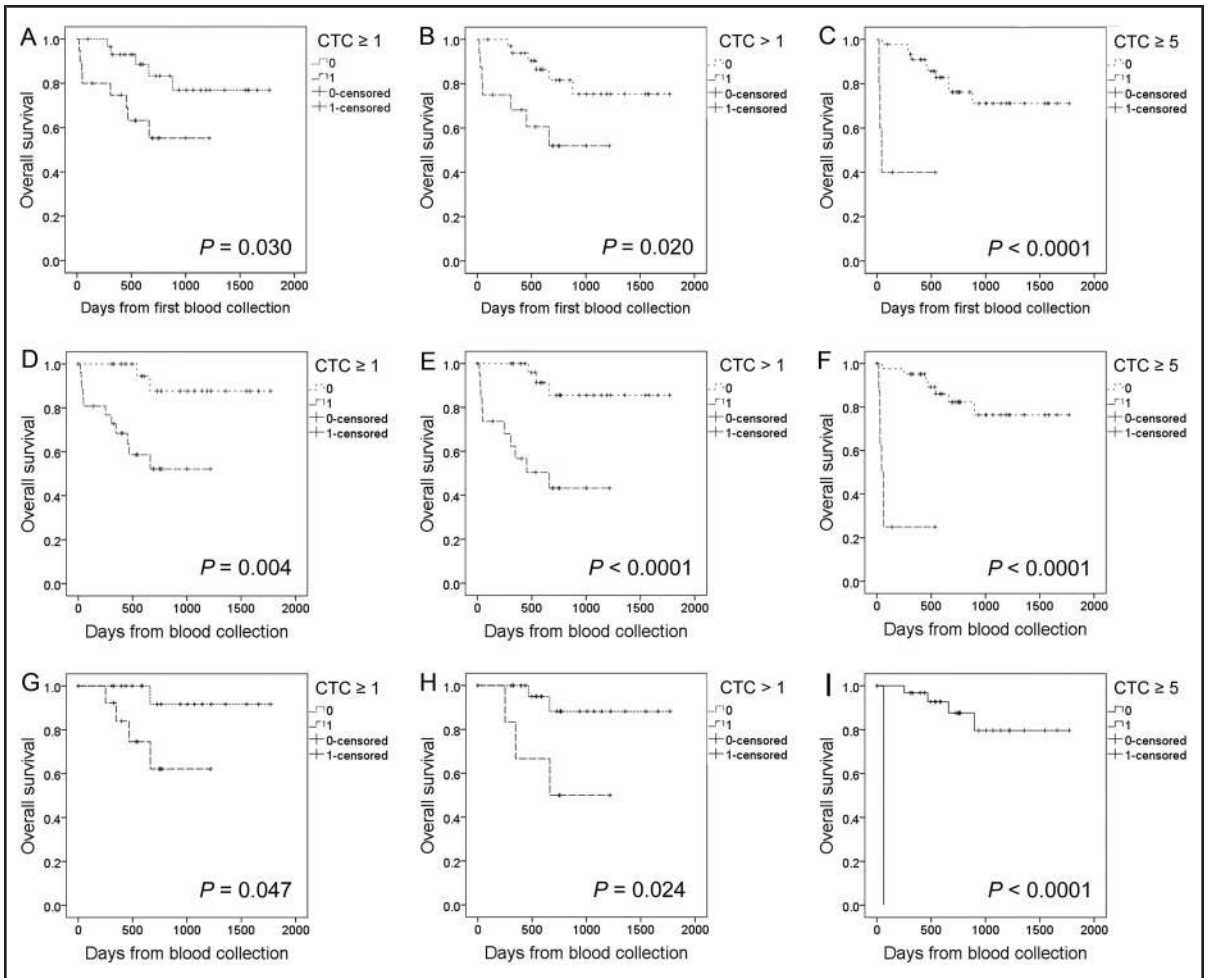

Fig. 3. Kaplan–Meier plots for OS.

Kaplan-Meier plots according to CTC detection at first blood collection for all patients (A–C), CTC cutoffs ≥1 (A), >1 (B), and ≥5 CTCs/7.5 mL blood (C). Kaplan–Meier plots for OS according to CTC detection at any time point for all patients (D–F), CTC cutoffs ≥1 (D), >1 (E), and ≥5 CTCs/7.5 mL blood (F). Kaplan–Meier plots for OS according to CTC detection at any time point for patients without lymph node and distant metastases at first blood collection (G–I), CTC cutoffs ≥1 (G), >1 (H), and ≥5 CTCs/7.5 mL blood (I).

tially diagnosed as a primary tumor in the left lower arm with a positive axillary sentinel lymph node and lymph node metastases when he was included in the study. The patient then showed an enormous increase of CTCs within 8 months from 4 to 1320/7.5 mL and died 61 days after the third blood draw. Patient 47, for whom an increase of CTC counts from 790 to 1960/7.5 mL within 14 days was observed, died 29 and 13 days after the first and second CTC detection, respectively. Moreover, we were able to sequentially investigate patient 33 over a period of 26 months. Seventeen months after a CTC-negative result, we detected 2 CTCs/7.5 mL, and 9 months later we detected 95 CTCs/7.5 mL. The patient died 2 months after the last CTC measurement. Interestingly, patient 36 (Table 2) was determined to be CTC-positive, with 2 CTCs/7.5 mL, and turned to be CTC-negative 7 months later. Twenty-four months later this patient still is under avelumab therapy and has stable disease.

DETECTION OF CTCs AND OS

OS correlated with American Joint Committee on Cancer stage, lymph node, and distant metastases at first blood collection (see Fig. 1, A–C, in the online Data Supplement). After a median follow-up of 21.1 months for 50 patients (mean, 23.7; range, 0.6–59), the presence of CTCs/7.5 mL at first blood draw was associated with OS in univariate Kaplan–Meier analysis (≥1, $P = 0.030$; >1, $P < 0.020$; and ≥5 CTCs/7.5 mL, $P < 0.0001$, Figs. 3A-C). In multivariate Cox regression analysis, the

presence of ≥5 CTCs/7.5 mL at first blood draw adjusted to age 10 (age divided by 10), and sex was significantly associated with reduced OS ($P = 0.001$, hazard ratio = 17.8; 95% CI, 4.0–93.0). At least 9/13 patients died because of MCC, and the death of 1 patient was caused by a lymphoma.

Including CTC positivity at any time, the presence of CTCs at all 3 cutoff values (≥1, $P = 0.004$; >1, $P < 0.0001$; and ≥5 CTCs/7.5 mL, $P < 0.0001$) correlated significantly with OS (Fig. 3, D–F). This correlation was also found when only patients without lymph node and distant metastases were analyzed (≥1, $P = 0.047$; >1, $P < 0.024$; and ≥5 CTCs/7.5 mL, $P < 0.0001$, Fig. 3, G–I).

DETECTION OF PD-L1 PRODUCTION ON CTCs

Cell line cells with known PD-L1 production were added into blood samples from healthy donors, and PD-L1 production was determined in the CellSearch system. The MCPyV-positive and MCPyV-negative MCC cell lines and RT4 did not show PD-L1-specific bands in Western blot analysis, whereas the UCB cell lines 5637 and 647V cells were PD-L1 positive (Fig. 4A). They also displayed strong PD-L1-specific immunofluorescence and served as positive controls in the CellSearch system. As negative control, RT4 cells were used (Fig. 4B).

Detected CTC numbers can vary between CTC and CXC kit detection in the CellSearch system. With the CXC kit, we analyzed blood samples from 4 patients with overt metastases [number of CTCs/7.5 mL by the CXC kit: patient 33, 50 (time point 3); patient 34, 37 (time point 1); patient 47, 1960 (time point 2); patient 51, 138 (time point 1)] for PD-L1 production. Although most analyzed CTCs revealed to be PD-L1 negative (e.g., CTCs 1–3, Fig. 4C), only in case 47 we detected <1% CTCs with very weak PD-L1-specific immunostaining (e.g., CTCs 4–6, Fig. 4C). Thus, the overall frequency of PD-L1 production in CTCs obtained from MCC was very low.

DETECTION OF MCPyV DNA

Because it is still unknown whether MCPyV has an influence on tumor cell dissemination and clinical outcome of MCC, we first analyzed primary tumor tissues for MCPyV DNA. Using Sanger sequencing, we did not observe a correlation of MCPyV positivity and the detection of CTCs (Table 1). WGA products from CTCs of 3 patients with more than 100 CTCs/7.5 mL were analyzed for MCPyV large T encoding sequences. All 7 CTCs analyzed from 1 patient, and 9/12 CTCs from another patient contained MCPyV DNA sequences. From a third patient, 3 single CTCs and 1 pool of 5 CTCs were revealed to be MCPyV DNA positive.

SINGLE-CELL ARRAY CGH

Subsequent copy number aberration analysis was performed to analyze the genomic profiles of CTCs obtained from an index patient with overt metastases (patient 47). Array CGH was performed with WGA products from 2 single CTCs and from a CTC pool consisting of 5 CTCs (P5). Fig. 4D displays gains on chromosomes 1, 6, 7, 11, 16 p, and 19p in 2 CTCs (CTCs 1 and 2) and in the CTC pool. CGH profiles of DNA derived from primary tumor tissue and bone metastasis of this patient are also shown in Fig. 4D. CGH profiles of CTCs 1, 2, and P5 collectively are more similar to the profile of the metastasis than to that of the primary tumor. Gains of chromosome 6p and losses of 10q are not detectable in DNA isolated from the primary tumor but are visible in the profiles of the bone metastasis and of the CTCs.

Discussion

Liquid biopsies play a central role in cancer biomarker development, but they have been under-investigated in MCC so far. Here we detected 1 or more CTCs/7.5 mL in up to 55% of patients with MCC. Both the rate of CTC positivity and the absolute number of CTCs were significantly associated with the tumor stage, lymph node involvement, and distant metastases. Remarkably, CTCs were detected in 70% of patients with lymph node metastases and in all patients with distant metastases. Moreover, CTCs were significantly associated with OS, and prognostic relevance of higher CTC counts (cutoff, ≥5 CTCs/7.5 mL) held true in multivariate Cox regression analysis adjusted for age and sex ($P = 0.001$, hazard ratio = 17.8; 95% CI, 4.0–93.0). Prognostic relevance might be biased by the fact that most patients were older than 75 years, thereby having an increased risk of death from causes other than MCC. MCC-specific survival was not available for all patients, but most documented causes of deaths (n = 9 patients) were MCCs.

In our study the CellSearch system was used, which has demonstrated associations of CTCs with poor clinical outcome for patients with different metastatic and nonmetastatic carcinomas *(30)*. So far only 1 study applied the same platform for a small study cohort and found CTCs with prognostic relevance in 14/34 patients with MCC (41%); however, further molecular characterization of these cells was not performed *(20)*. Another study used the Maintrac method and detected EpCAM-positive cells in a total of 29/30 patients with MCC (97%). Addition of anti-CD56 or antikeratin 20 antibodies improved the diagnostic specificity of this assay. The authors described an association of CTC counts with disease burden, but no association of CTC detection and outcome of the disease was observed *(21)*.

One characteristic feature of MCC cells is their paranuclear dot-like production pattern of keratins.

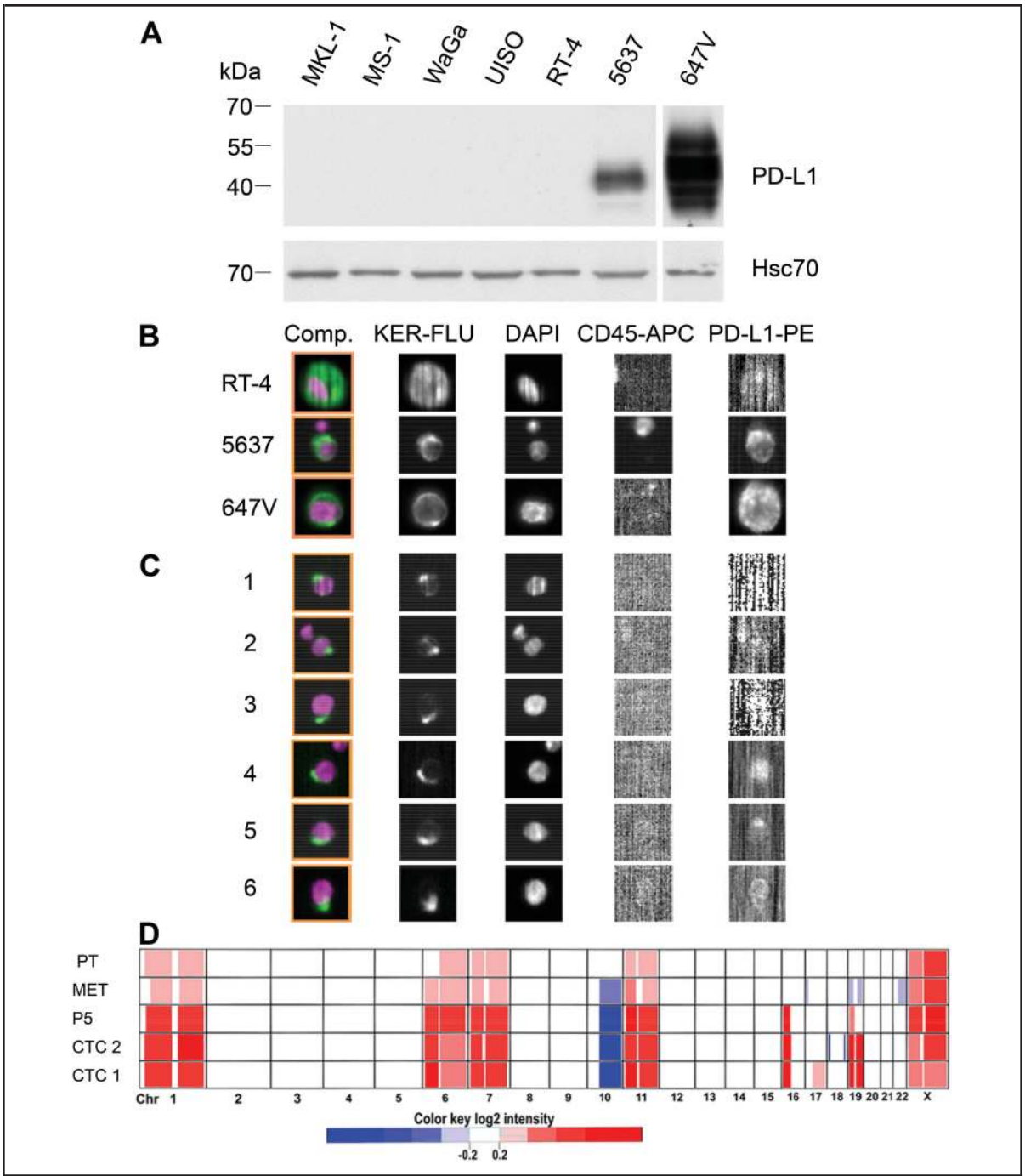

Fig. 4. PD-L1 production and Array CGH profiles.

(A), Western blot analysis of PD-L1 production in different cell lines (MCC cell lines: MKL-1, MS-1, WaGa, UISO; cell lines from urothelial carcinomas of the bladder: RT4, 5637, and 647V. HSC70, loading control. (B, C), Determination of PD-L1 production on cell line cells and CTCs in the fourth channel of the CellSearch system. (B), Strongly PD-L1-positive (5637 and 647V) and PD-L1-negative (RT4) cell line cells. (C), 1–3 are PD-L1-negative CTCs and 4–6 are weakly PD-L1-positive CTCs from the same MCC patient. (D), Array CGH profiles from CTCs and tumor tissues of a patient with metastatic MCC: single CTCs (1 and 2), a CTC pool consisting of 5 cells (P5), and tumor tissues derived from the primary tumor (PT) and a bone metastasis (MET). Red shows gains and blue losses of chromosomal regions. Comp, composite image; KER-FLU, antikeratin antibody labeled with fluorescein; DAPI, 4`,6-diamidino-2-phenylindole, dihydrochloride; APC, allophycocyanin; PE, phycoerythrin; Chr, chromosome.

Accordingly, in most CTC-positive cases we identified CTCs with this pattern as a hint for their MCC origin. It has been suggested that the keratin staining pattern of CTCs might be helpful to exclude other malignancies as a source of CTCs *(20)*. However, this dot-like keratin pattern has also been observed in CTCs originating from other neuroendocrine tumors in the gastrointestinal tract *(31)* and in CTCs derived from aggressive prostate cancer with neuroendocrine differentiation *(32)*.

Molecular CTC analysis at the single-cell level has the potential to provide insights into the biology of MCC. Therefore, we established single-CTC analyses *(33)* and applied them for CTCs isolated from patients with MCC. However, molecular characterization of single CTCs is challenging and there is almost no information on the molecular nature of circulating MCC cells. Here we provided proof of principle data that CTCs from patients with MCC are amenable to in-depth molecular characterization. We showed associations of genomic aberrations between the primary tumor and CTCs, although a stronger similarity was observed between CTCs and the bone metastasis. Thus, WGA of single-CTC DNA performed in our study for index cases opens the way for deeper molecular analyses of CTCs.

Because causal association with clonally integrated MCPyV has been described for the majority of MCCs, viral DNA sequences detected in CTCs can substantiate the suspicion of an MCC origin. In the WGA products of 19/22 analyzed CTCs from 3 patients, we detected MCPyV DNA sequences; in 3 CTCs, these sequences could not be detected. We cannot exclude that CTCs might also be derived from an additional tumor other than MCC. However, typical keratin immunostaining pattern (seen as a dot-like or paranuclear production pattern) in most cases suggests that the CTCs are derived from a neuroendocrine tumor like MCC.

In an additional approach, CTCs were analyzed for copy number aberrations. We identified genomic aberrations that are characteristic for MCC such as gains in chromosomes 1, 6, or 7 or losses in chromosome 10, underlining the MCC origin of these CTCs *(34)*. Interestingly, besides remarkable similarities, differences between the primary tumor and CTCs (and a bone metastasis) collected from the same patient were detected (e.g., in chromosomal regions 6p and 10q). Notably, losing chromosomal regions of 10q might be associated with a loss of the phosphatase and tensin homolog tumor suppressor gene (*PTEN*)[9] that is frequently observed in MCC *(35)*. Thus, the exploration of genomic profiles of CTCs could support retracing clonal origins of individual tumor cells and uncover their potential to initiate metastasis. Furthermore, single-CTC analyses might contribute to the elucidation of primary tumor sites in patients with MCC who have cancers of unknown primary primarily detected in the lymph nodes or other secondary organs *(36)*. Moreover, mutational load in MCC is frequently high *(36, 37)*, and unraveling a mutational phenotype by analyzing CTCs might add value for therapeutic decisions.

Chemotherapy has been used to treat patients with MCC who have locally restricted, recurrent, or metastatic disease, but despite responses, the disease frequently recurs *(11)*. Moreover, a considerable number of mostly elderly patients present with severe adverse effects but without benefit for OS *(11)*. Thus, checkpoint-inhibitor therapy is currently the standard of care in metastatic disease. Therefore, it is important to note that causal association of MCC with immunosuppression, MCPyV integration, and UV-induced high mutational burden *(37)* provides a rationale for the application of these therapies. Three different anti-PD-1 and anti-PD-L1 antibodies tested so far in patients with advanced MCC showed durable responses *(10, 11)*. In an international multicenter, single-group, open-label, phase 2 trial, treating 88 patients with stage IV MCC who had progressive disease after chemotherapy with the anti-PD-L1 antibody avelumab *(38)* resulted in durable responses with low rates of adverse effects *(11)*. This result led to the first FDA approval for a therapy in patients with MCC *(39)*. In this study neither PD-L1 production of tumor tissues nor MCPyV status was used as inclusion criteria. Although most MCPyV-negative tumors were shown to be PD-L1 negative *(12, 40)*, responses were observed irrespective of MCPyV status and of PD-L1 production retrospectively investigated on most recent biopsies *(11)*.

Prior studies have demonstrated that PD-L1 production in MCC is correlated with improved outcome *(12, 41)*, and PD-L1 production of tumor and immune cells in primary tumor tissues has been used as candidate biomarker for prediction of response in several studies *(42)*. However, robust predictive values for MCC and cutoff values for the number of PD-L1-positive tumor or immune cells have not been demonstrated yet *(11)*. Therefore, we questioned whether PD-L1 production of CTCs could add value for real-time monitoring of the PD-L1 status on tumor cells in advanced tumor stages. The first results of studies investigating PD-L1 production on CTCs are promising *(43–45)*. Our study is the first to analyze PD-L1 production of CTCs derived from MCC. Counter to our expectation, the MCPyV-positive or MCPyV-negative MCC cell lines tested here and the vast majority of CTCs from patients with metastatic MCC turned out to be PD-L1 negative. However, further prospective studies should include determination of

[9] Human Gene: *PTEN*, phosphatase and tensin homolog.

PD-L1 production of CTCs before, during, and after immunotherapy in larger patient cohorts.

To be captured with the CellSearch system, CTCs must be epithelial cell adhesion molecule (EpCAM) positive. In contrast to other malignant skin tumors, MCCs frequently produce EpCAM *(46)*. Nevertheless, the failure to detect CTCs in a certain percentage of patients might be caused by a loss of EpCAM production, for example, through epithelial to mesenchymal transition.

In conclusion, our results demonstrate significant correlations between CTC counts and aggressiveness of MCC. Future clinical studies should investigate whether sequential liquid biopsies for CTCs might help to improve the management of MCC patients. Moreover, future MCC trials should include the measurement of circulating cell-free DNA as complementary liquid biopsy biomarker to identify patients at risk of progression or detect progression earlier than staging procedures *(13)*.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements:* (*a*) *significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data;* (*b*) *drafting or revising the article for intellectual content;* (*c*) *final approval of the published article; and* (*d*) *agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

S. Riethdorf, statistical analysis; L. Heinzerling, provision of study material or patients; G. Schön, statistical analysis; S. Peine, provision of study material or patients; G. Schuler, financial support, provision of study material or patients; I. Moll, administrative support; K. Pantel, financial support, administrative support.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: K. Pantel, the ERC-2010-AdG_20100317 grant DISSECT.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: The authors thank Petra Keikavoussi for coordinating sample logistics and Malgorzata Stoupiec for technical assistance. Moreover, the authors thank Dr. R. Stoehr (Institute of Pathology, University of Erlangen-Nürnberg, Erlangen, Germany), Dr. Katharina Röck (Pharmacology and Clinical Pharmacology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany), and Dr. Michael Rink (Department of Urology, University Medicla Center Hamburg-Eppendorf, Hamburg, Germany) for kindly providing the bladder cancer cell lines RT4, 5637, and 647V, respectively.

References

1. Tang CK, Toker C. Trabecular carcinoma of the skin: an ultrastructural study. Cancer 1978;42:2311–21.
2. Van Keymeulen A, Mascre G, Youseff KK, Harel I, Michaux C, De Geest N, et al. Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis. J Cell Biol 2009;187:91–100.
3. Tilling T, Moll I. Which are the cells of origin in Merkel cell carcinoma? J Skin Cancer 2012;2012:680410.
4. Schadendorf D, Lebbe C, Zur Hausen A, Avril MF, Hariharan S, Bharmal M, et al. Merkel cell carcinoma: epidemiology, prognosis, therapy and unmet medical needs. Eur J Cancer 2017;71:53–69.
5. Sauer CM, Haugg AM, Chteinberg E, Rennspiess D, Winnepenninckx V, Speel EJ, et al. Reviewing the current evidence supporting early B-cells as the cellular origin of Merkel cell carcinoma. Crit Rev Oncol Hematol 2017;116:99–105.
6. Becker JC, Stang A, DeCaprio JA, Cerroni L, Lebbe C, Veness M, et al. Merkel cell carcinoma. Nat Rev Dis Primers 2017;3:17077.
7. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 2008;319:1096–100.
8. Brewer JD, Shanafelt TD, Otley CC, Roenigk RK, Cerhan JR, Kay NE, et al. Chronic lymphocytic leukemia is associated with decreased survival of patients with malignant melanoma and Merkel cell carcinoma in a SEER population-based study. J Clin Oncol 2012;30:843–9.
9. Paulson KG, Iyer JG, Blom A, Warton EM, Sokil M, Yelistratova L, et al. Systemic immune suppression predicts diminished Merkel cell carcinoma-specific survival independent of stage. J Invest Dermatol 2013;133:642–6.
10. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, et al. PD-1 blockade with pembrolizumab in advanced Merkel-cell carcinoma. N Engl J Med 2016;374:2542–52.
11. Kaufman HL, Russell J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, et al. Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. Lancet Oncol 2016;17:1374–85.
12. Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Luber BS, Wang H, et al. PD-L1 expression in the Merkel cell carcinoma microenvironment: association with inflammation, Merkel cell polyomavirus and overall survival. Cancer Immunol Res 2013;1:54–63.
13. Bardelli A, Pantel K. Liquid biopsies, what we do not know (yet). Cancer Cell 2017;31:172–9.
14. Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479–91.
15. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 2012;30:525–32.
16. Khan MS, Tsigani T, Rashid M, Rabouhans JS, Yu D, Luong TV, et al. Circulating tumor cells and EpCAM expression in neuroendocrine tumors. Clin Cancer Res 2011;17:337–45.
17. Carey RW, Taft PD, Bennett JM, Kaufman S. Carcinocythemia (carcinoma cell leukemia): an acute leukemia-like picture due to metastatic carcinoma cells. Am J Med 1976;60:273–8.
18. Nemoto I, Sato-Matsumura KC, Fujita Y, Natsuga K, Ujiie H, Tomita Y, et al. Leukaemic dissemination of Merkel cell carcinoma in a patient with systemic lupus erythematosus. Clin Exp Dermatol 2008;33:270–2.
19. Hartley MA, Tao J, Baz R. Merkel cell carcinoma in the peripheral blood of a patient with concomitant chronic lymphocytic leukemia and multiple myeloma. J Clin Oncol 2010;28:e113–4.
20. Blom A, Bhatia S, Pietromonaco S, Koehler K, Iyer JG, Nagase K, et al. Clinical utility of a circulating tumor cell assay in Merkel cell carcinoma. J Am Acad Dermatol 2014;70:449–55.
21. Gaiser MR, Daily K, Hoffmann J, Brune M, Enk A, Brownell I. Evaluating blood levels of neuron specific enolase, chromogranin A, and circulating tumor cells as Merkel cell carcinoma biomarkers. Oncotarget 2015;6:26472–82.
22. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. J Oncol 2010;2010:617421.
23. Guastafierro A, Feng H, Thant M, Kirkwood JM, Chang Y, Moore PS, et al. Characterization of an early passage Merkel cell polyomavirus-positive Merkel cell carcinoma cell line, MS-1, and its growth in NOD scid gamma mice. J Virol Methods 2013;187:6–14.
24. Knips J, Czech-Sioli M, Spohn M, Heiland M, Moll I, Grundhoff A, et al. Spontaneous lung metastasis formation of human Merkel cell carcinoma cell lines transplanted into scid mice. Int J Cancer 2017;141:160–71.
25. Fogh J. Cultivation, characterization, and identification

of human tumor cells with emphasis on kidney, testis, and bladder tumors. Natl Cancer Inst Monogr 1978;5–9.
26. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, et al. Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. Clin Chem 2013;59:252–60.
27. Geigl JB, Obenauf AC, Waldispuehl-Geigl J, Hoffmann EM, Auer M, Hormann M, et al. Identification of small gains and losses in single cells after whole genome amplification on tiling oligo arrays. Nucleic Acids Res 2009;37:e105.
28. Fischer N, Brandner J, Fuchs F, Moll I, Grundhoff A. Detection of Merkel cell polyomavirus (MCPyV) in Merkel cell carcinoma cell lines: cell morphology and growth phenotype do not reflect presence of the virus. Int J Cancer 2010;126:2133–42.
29. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
30. Riethdorf S, O'Flaherty L, Hille C, Pantel K. Clinical applications of the CellSearch platform in cancer patients. Adv Drug Deliv Rev 2018;125:102–21.
31. Khan MS, Kirkwood A, Tsigani T, Garcia-Hernandez J, Hartley JA, Caplin ME, et al. Circulating tumor cells as prognostic markers in neuroendocrine tumors. J Clin Oncol 2013;31:365–72.
32. Beltran H, Jendrisak A, Landers M, Mosquera JM, Kossai M, Louw J, et al. The initial detection and partial characterization of circulating tumor cells in neuroendocrine prostate cancer. Clin Cancer Res 2016;22:1510–9.
33. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res 2013;73:2965–75.
34. Carless MA, Griffiths LR. Cytogenetics of melanoma and nonmelanoma skin cancer. Adv Exp Med Biol 2008;624:227–40.
35. Cohen PR, Tomson BN, Elkin SK, Marchlik E, Carter JL, Kurzrock R. Genomic portfolio of Merkel cell carcinoma as determined by comprehensive genomic profiling: implications for targeted therapeutics. Oncotarget 2016;7:23454–67.
36. Vandeven N, Lewis CW, Makarov V, Riaz N, Paulson KG, Hippe D, et al. Merkel cell carcinoma patients presenting without a primary lesion have elevated markers of immunity, higher tumor mutation burden, and improved survival. Clin Cancer Res 2018;24:963–71.
37. Carter MD, Gaston D, Huang WY, Greer WL, Pasternak S, Ly TY, et al. Genetic profiles of different subsets of Merkel cell carcinoma show links between combined and pure MCPyV-negative tumors. Hum Pathol 2018;71:117–25.
38. Kaufman HL, Hunger M, Hennessy M, Schlichting M, Bharmal M. Nonprogression with avelumab treatment associated with gains in quality of life in metastatic Merkel cell carcinoma. Future Oncol 2017;14:255–66.
39. Colunga A, Pulliam T, Nghiem P. Merkel cell carcinoma in the age of immunotherapy: facts and hopes. Clin Cancer Res 2018;24:2035–43.
40. Mitteldorf C, Berisha A, Tronnier M, Pfaltz MC, Kempf W. PD-1 and PD-L1 in neoplastic cells and the tumor microenvironment of Merkel cell carcinoma. J Cutan Pathol 2017;44:740–6.
41. Kervarrec T, Gaboriaud P, Berthon P, Zaragoza J, Schrama D, Houben R, et al. Merkel cell carcinomas infiltrated with CD33(+) myeloid cells and CD8(+) T cells are associated with improved outcome. J Am Acad Dermatol 2018;78:964–72.
42. Patel SP, Kurzrock R. PD-L1 expression as a predictive biomarker in cancer immunotherapy. Mol Cancer Ther 2015;14:847–56.
43. Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, et al. Frequent expression of PD-L1 on circulating breast cancer cells. Mol Oncol 2015;9:1773–82.
44. Anantharaman A, Friedlander T, Lu D, Krupa R, Premasekharan G, Hough J, et al. Programmed death-ligand 1 (PD-L1) characterization of circulating tumor cells (CTCs) in muscle invasive and metastatic bladder cancer patients. BMC Cancer 2016;16:744.
45. Nicolazzo C, Raimondi C, Mancini M, Caponnetto S, Gradilone A, Gandini O, et al. Monitoring PD-L1 positive circulating tumor cells in non-small cell lung cancer patients treated with the PD-1 inhibitor Nivolumab. Sci Rep 2016;6:31726.
46. Garcia-Caballero T, Pintos E, Gallego R, Parrado C, Blanco M, Bjornhagen V, et al. MOC-31/Ep-CAM immunoreactivity in Merkel cells and Merkel cell carcinomas. Histopathology 2003;43:480–4.