Merkel Cell Polyomavirus Status Is Not Associated with Clinical Course of Merkel Cell Carcinoma

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The majority of Merkel cell carcinomas (MCCs) are associated with the recently identified Merkel cell polyomavirus (MCV). However, as it is still unclear to which extent the presence of MCV impacts tumor characteristics or clinical outcome, we correlated the MCV status of tumor lesions obtained from 174 MCC patients including 38 MCC patients from Australia and 138 MCC patients from Germany with clinical characteristics, histomorphology, immunohistochemistry, and course of the disease. MCV DNA was present in 86% of MCCs and, in contrast to previous reports, no significant difference in MCV prevalence was present between Australian and German MCC cases. When patients were stratified according to their MCV status, only tumor localization ($P=0.001$), gender ($P=0.024$), and co-morbidity, i.e., frequency of patients with previous skin tumors ($P=0.024$), were significantly different factors. In contrast, year of birth and diagnosis, age at diagnosis, or histological type and features representing the oncogenic phenotype such as mitotic rate or expression of p16, p53, RB1, and Ki67 were not significantly different between MCV-positive and MCV-negative MCCs. MCV status also did not influence recurrence-free, overall, and MCC-specific survival significantly. In summary, although MCV-positive and MCV-negative MCCs may have different etiologies, these tumors have comparable clinical behaviors and prognosis.

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INTRODUCTION

Merkel cell carcinoma (MCC) is a rare, aggressive neuroendocrine carcinoma of the skin. Currently, MCC accounts for only a small fraction of cutaneous malignancies, with age-adjusted incidence rates of 0.18 to 0.41 per 100,000 people (Albores-Saavedra *et al.*, 2009). Noteworthy, however, the incidence of MCC appears to have tripled from 1986 to 2001 (Hodgson, 2005). The typical clinical course of MCC progression includes a high rate of locoregional recurrences, nodal invasion, and distant metastases (Krasagakis and Tosca, 2003). In fact, MCC is the most aggressive skin malignancy with more than one-third of MCC patients dying from this cancer; i.e., it is at least twice as lethal as melanoma.

Recently, a new type of human polyomavirus was identified in MCC and named Merkel cell polyomavirus (MCV) (Feng *et al.*, 2008). Notably, the integration of MCV before clonal expansion and at distinct sites in different MCC tumors sustain the assumption that viral proteins are causal for tumorigenesis (Feng *et al.*, 2008; Sastre-Garau *et al.*, 2009). Moreover, we could recently demonstrate that MCV-positive MCC cells critically depend on the expression of the T antigens reflecting oncogene addiction to T-antigen expression (Houben *et al.*, 2010b).

Meanwhile, many reports have confirmed the presence of MCV DNA in tumor samples of most MCC patients (Kassem *et al.*, 2008; Becker *et al.*, 2009; Sastre-Garau *et al.*, 2009). Nevertheless, a few exceptions have also been reported: (1) in Australian patients, only approximately one-fourth of patients harbored detectable MCV DNA in one report (Garneski *et al.*, 2009); and (2) in two American patients, MCV DNA was detectable in the primary but not in a corresponding metastatic tumor (Andea *et al.*, 2009). Moreover, whether the presence of MCV DNA in MCC impacts tumor characteristics or clinical behavior remains controversial (Becker *et al.*, 2009; Sihto *et al.*, 2009; Handschel *et al.*, 2010; Bhatia *et al.*, 2010a, 2010b; Houben *et al.*, 2010a).

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Abbreviations: HR, hazard ratio; LT, large T-antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus

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The previous observations in combination with the assumption of a causal role of MCV for the etiology of MCC prompted us to compare the frequency of MCV in Australian and European MCC patients and to test the concordance of MCV status in multiple samples of individual patients. Furthermore, we compared clinical and histological parameters of virus-positive and -negative cohorts of this large MCC cohort comprising both German and Australian patients.

RESULTS

Patient characteristics and MCV status

A total of 185 samples of 136 European and 49 samples from 38 Australian MCC patients were analyzed. Detailed patient characteristics are given in Table 1. The PCR-based MCV status analysis revealed that 85.3% (116 of 136) of the European MCCs and 86.8% (33 of 38) of the Australian MCCs were positive, resulting in a combined total of 85.6% for all analyzed tumors (Table 1). Estimated copies of viral genome per cell was <0.1 in only 9 patients, ranged between 0.1 and 10 in 110, and was >10 for 30 of our 149 positive patients (data not shown). Notably, MCV-PCR-positive tumors were also positive for MCV large T-antigen (LT) protein expression (Figure 1).

In all 33 MCV-positive and 4 MCV-negative MCC patients with multiple samples available for analysis, the MCV status was concordant (data not shown). Moreover, in one patient, sequence analysis of the LT gene in tumor cells derived from four different lesions demonstrated the same stop codon mutation in all four lesions (data not shown).

Comparison of MCV-negative and MCV-positive tumors

Next, we compared the histological and clinical characteristics of tumors with different viral status on the total patient population. This comparison clearly demonstrated that both cohorts were similar in many aspects: There were no significant differences in microscopic characteristics such as histological type, microanatomic compartment involved, mitotic rate, lymphocyte infiltrate, and cytokeratin 20 staining, or clinical features, i.e., stage at diagnosis, age at diagnosis, year of diagnosis, year of birth, and presence or absence of immune suppression (Table 1). However, body site distribution of primary MCC was significantly associated with the MCV status; the MCV-positive tumors were more frequently located on the extremities and less often located on the trunk than their virus-negative counterparts (Table 1; $P=0.001$). Furthermore, the percentage of female patients was significantly higher among the MCV-positive patients ($P=0.024$), and virus-negative patients had more often a previous history of other skin cancers ($P=0.024$).

To evaluate if oncogenic viral proteins encoded by MCV impact cell cycle regulation, apoptosis, or mitogenic signaling, we scrutinized MCV-positive and -negative tumors for expression and phosphorylation of a number of proteins representing these pathways. In detail, protein expression analyses of p16, p53, RB1, pERK, and Ki67 for a subgroup of tumors (45–53 randomly selected patients per staining) revealed that p16 and RB1 were highly frequent in tumor cells, pERK was rare, whereas the frequencies for p53 and Ki67 were quite variable between patients. Nevertheless, there was no obvious differences between the MCV-negative and MCV-positive tumors (Figure 2; Houben *et al.*, 2010a).

No impact of MCV status on clinical behavior

For 127 of the patients, detailed information was available to evaluate the impact of MCV status on recurrence-free, overall, as well as MCC-specific survival by univariate and multivariate analyses. For recurrence-free survival, there was no significant difference between the Kaplan–Meier curves of MCV-positive or -negative MCC patients, respectively (Figure 3a; log-rank (Mantel–Cox) test; $P=0.158$). Accordingly, the univariate hazard ratios (HRs) for virus-positive patients did not differ significantly (HR = 1.753; 95% confidence interval (CI) 0.794–3.870; $P=0.165$), or when adjusted to gender, age, and tumor stage at diagnosis (HR = 1.701; 95% CI 0.748–3.868; $P=0.205$) or additionally to localization of primary tumor, lymphocyte infiltrate, and microanatomic compartment involved (HR = 2.778; 95% CI 0.930–8.298; $P=0.067$)–all factors that are known to have an impact on clinical course (Andea *et al.*, 2008; Albores-Saavedra *et al.*, 2009; Table 2).

The overall median survival was 29.4 months for MCV-negative and 57 months for MCV-positive MCC patients (Figure 3b). But similar to recurrence-free survival, both the Kaplan–Meier curves ($P=0.468$) and the HRs were not significantly different (HR = 0.750; 95% CI 0.344–1.636 for virus-positive patients; $P=0.470$). In addition, both multivariate analyses, i.e., adjusted to gender, age, and tumor stage (HR = 0.981; 95% CI 0.423–2.273; $P=0.964$) or additionally for lymphocyte infiltrate, microanatomic compartment involved, and localization of the primary tumor (HR = 1.861; 95% CI 0.519–6.679; $P=0.341$) could not demonstrate a significant difference in overall survival between MCV-positive and -negative MCC patients. Next, we focused on MCC-specific survival, i.e., only patients who died with advanced disease were regarded as an event. Still, the Kaplan–Meier curves and the HRs of MCV-positive and -negative patients did not display a significant difference (Figure 3c and Table 3). Similarly, adjusting for the various factors discussed above in multivariate analyses did not result in significantly different HRs for MCV status (Table 2).

DISCUSSION

Until the recent description of the MCV, the pathogenesis of MCC was poorly understood. The demonstration that the MCV genome was integrated in the tumor cell DNA in six of eight tumors positively tested rendered MCC the first cancer with an integrated polyomavirus genome (Feng *et al.*, 2008). Noteworthy, this integration indicated a causative role of MCV in the induction of MCC, especially as the patterns of integration demonstrate that viral infection precedes clonal expansion of the tumor cells (Feng *et al.*, 2008). Indeed, we also observed concordant MCV results and sequences (performed only for one patient) for different samples of the same patient sustaining that the integration of the viral genome into the host cell is an early event occurring before

Table 1. Patient and tumor characteristics

Variable	MCC total	MCV positive	MCV negative	*P*-value*
Number of patients	174	149 (85.6%)	25 (14.4%)	
Analyzed samples	234	203	31	
Mean age (IQR) (years)	73.3 (66–81)	73 (66–80)	75.1 (68.5–82.5)	0.354[6]
Mean follow-up (IQR) (months)	24.9 (4.8–34)	24.9 (4.5–34.1)	25.2 (5.2–30.9)	0.894[7]
Mean year of birth (IQR)	1929 (1922–1937.5)	1929.4 (1922–1938)	1927.5 (1919–1934)	0.450[6]
Mean year of diagnosis (IQR)	2002.6 (1999.8–2006)	2002.6 (2000–2006)	2002.9 (1999–2007)	0.710[7]
Gender				
Male	88 (56.4%)	70 (52.6%)	18 (81%)	0.024[8]
Female	68 (43.6%)	63 (47.4%)	5 (19%)	
Stage at diagnosis[1,2]				
Stage I	80 (61.1%)	69 (62.7%)	11 (52.3%)	0.170[8]
Stage II	47 (35.9%)	39 (35.5%)	8 (38.1%)	
Stage III	4 (3.1%)	2 (1.8%)	2 (9.5%)	
Localization[1]				
Head/neck	52 (46.4%)	41 (44.6%)	11 (55%)	0.001[8]
Trunk	16 (14.3%)	9 (9.8%)	7 (35%)	
Extremities	44 (39.3%)	42 (45.7%)	2 (10%)	
Histological type				
Small cell	9 (8.4%)	6 (6.6%)	3 (18.8%)	0.175[8]
Intermediate	67 (62.6%)	59 (64.8%)	8 (50%)	
Trabecular	31 (29%)	26 (28.6%)	5 (31.3%)	
Microanatomic compartment				
Dermis	8 (7.7%)	8 (9.2%)		0.454[8]
Subcutis	95 (91.3%)	78 (89.7%)	17 (100%)	
Deeper	1 (1%)	1 (1.1%)		
Mitotic rate[1]				
Intermediate	48 (43.6%)	43 (46.2%)	5 (29.4%)	0.288[8]
High	62 (56.4%)	50 (53.8%)	12 (70.6%)	
Lymphocyte infiltrate[1,3]				
Yes	46 (41.4%)	39 (41.5%)	7 (41.2%)	0.999[8]
No	65 (58.6%)	55 (58.5%)	10 (58.8%)	
Immunosuppression[1,4]				
Yes	8 (10.5%)	6 (9.7%)	2 (14.3%)	0.635[8]
No	68 (89.5%)	56 (90.3%)	12 (85.7%)	
Previous history of skin cancer[1,5]				
Yes	22 (25.6%)	14 (20%)	8 (50%)	0.024[8]
No	64 (74.4%)	56 (80%)	8 (50%)	
Cytokeratin 20[7]				
Positive	165 (98.8%)	144 (99.3%)	21 (95.5%)	0.247[8]
Negative	2 (1.2%)	1 (0.7%)	1 (4.5%)	

Abbreviations: IQR, interquartile range; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus.
[1]The respective characteristics were not documented for all patients.
[2]Staging was performed according to Boyle *et al.* (1995): stage I: localized disease; stage II: local–regional metastases; stage III: distant metastases.
[3]The presence of a lymphocyte infiltrate was determined by hematoxylin and eosin (HE) staining. Only lymphocytes infiltrating the tumor were considered.
[4]Patients were regarded immunosuppressed when they had received immunosuppressing drugs due to organ transplantation or HIV infection.
[5]The most common skin cancers, i.e., basal cell carcinoma, squameous cell carcinoma, and melanoma, were considered.
**P*-values were calculated by:
[6]*t*-test (when values were normally distributed) or
[7]Mann–Whitney test to compare values of two independent groups or
[8]Fisher's exact test for contingency tables.

acquiring metastatic potential. The causative role of MCV in the pathogenesis of MCC was further substantiated by our recent finding that MCV-positive MCC cell lines require T-antigen expression to maintain proliferation and survival (Houben *et al.*, 2010b). Although the transforming capacity of MCV has still to be proven formally, these observations fueled the discussion if MCV-positive and MCV-negative MCCs can still be regarded as the same tumor entity.

Up to date, only a few studies have addressed the differences between MCV-positive and MCV-negative MCCs with respect to tumor and patients characteristics or course of disease (Garneski *et al.*, 2009; Sihto *et al.*, 2009; Andres *et al.*, 2009b). As MCC is a rare cancer, patient numbers in the reported studies are limited. Thus, to establish the impact of MCV on MCC, several studies are needed to eventually allow a meta-analysis. Therefore, we analyzed the presence of MCV in 174 MCC patients from Europe and Australia and compared their clinical and immunohistological features according to their MCV status. We included Australian patients in this study as it was previously reported that the presence of MCV DNA is much lower in Australia when compared with North America or Europe (Garneski *et al.*, 2009).

In the presented cohort, 86% of the MCCs from either continent harbored MCV DNA. For the European patient cohort, the observed frequency is in accordance with the reported frequencies (Kassem *et al.*, 2008; Sastre-Garau *et al.*, 2009). In contrast, the frequency for the Australian patients is much higher than previously reported: in the study by Garneski *et al.* (2009), only 5 (24%) of 21 Australian MCC specimens were virus positive. Notably, these samples were obtained from the same group who generated a series of MCC cell lines of which all four tested cell lines were negative for MCV (Shuda *et al.*, 2008; and own observation). Analysis of the expression of MCV-derived LT on the protein level confirmed the high frequency of MCV-positive patients in our Australian patient cohort. These different frequencies in the two studies can neither be explained by the geographical regions from which the samples were obtained (Brisbane vs. Sydney) nor the kind of MCC tumors (in both series, ~50% of the samples were metastases). Thus, these differences may be either due to (1) chance, (2) technical factors such as different MCV assays or quality of DNA, or (3) differences in the year

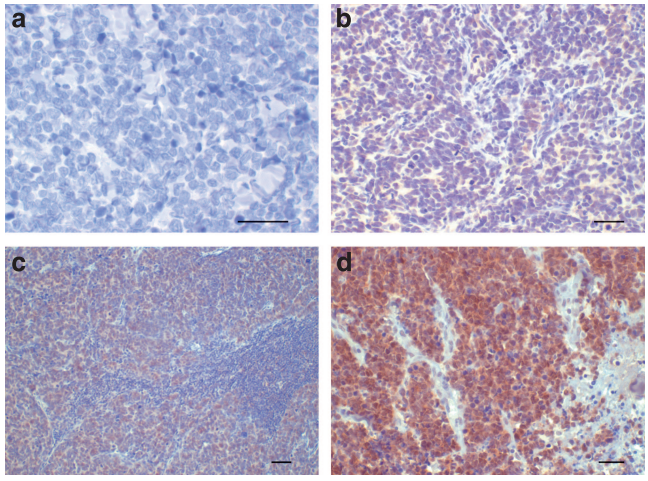

Figure 1. Immunohistological staining for large T-antigen in Merkel cell carcinoma (MCC) tumors. Sections of paraffin-embedded tumors were stained for large T-antigen expression. Examples of (**a**) a Merkel cell polyomavirus (MCV)-negative tumor and (**b–d**) different levels of large T-antigen protein expression in Australian patients are depicted. Bar = 100 μm.

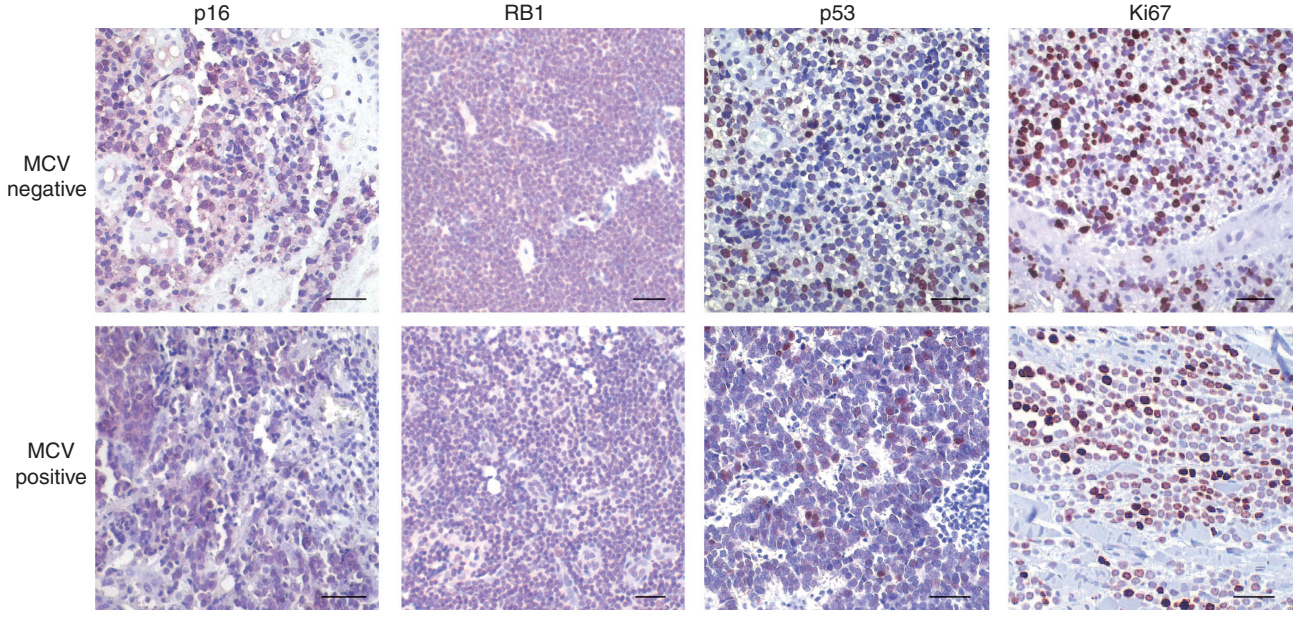

Figure 2. Immunohistochemical characteristics of virus-negative and -positive Merkel cell carcinoma (MCC) tumors. Exemplary results of p16, RB1, p53, and Ki67 staining in Merkel cell polyomavirus (MCV)-negative and MCV-positive MCC tumors. Generally, almost all Merkel carcinoma cells express p16 and RB1, whereas the expression of p53 and Ki67 varies between patients, however, irrespective of the MCV status. Bar = 100 μm.

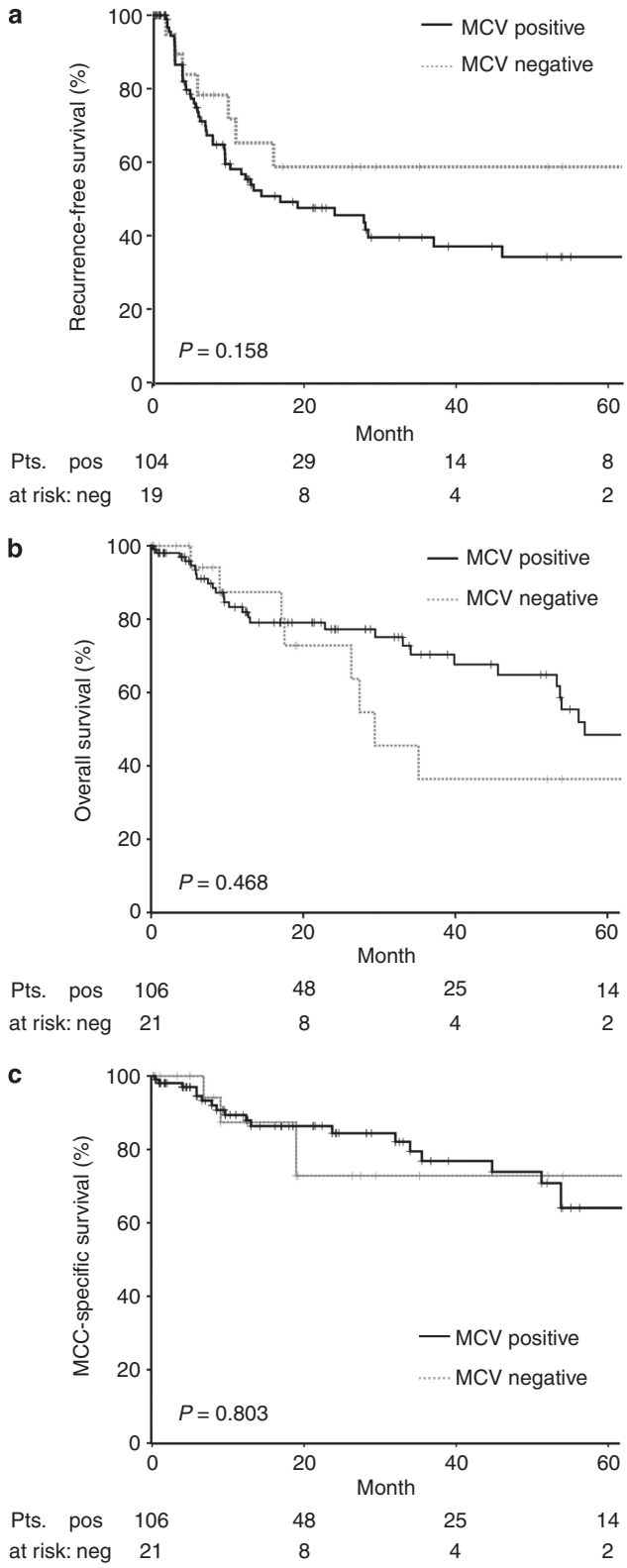

Figure 3. Kaplan–Meier curves for recurrence-free, overall, and Merkel cell carcinoma (MCC)-specific survival. MCC patients were stratified upon the molecular detection of Merkel cell polyomavirus (MCV) in their tumors. The (**a**) recurrence-free, (**b**) overall, and (**c**) MCC-specific survival rates are depicted for 60 months. Beneath each curve the patients at risk are given.

of diagnosis, as the samples in the study of Garneski *et al.* (2009) were obtained in the 1990s, whereas our samples were more recently obtained (median year of diagnosis for the Australian patient cohort is 1999).

The comparative study of 149 MCV-positive and 25 MCV-negative MCC patients revealed three significant differences, of which body site distribution of the primary MCCs is the most significant factor. This observation is in accordance with the report of Sihto *et al.* (2009) that MCV-positive tumors were less often localized at the trunk. These authors speculated that MCV might be transmitted by physical contact. An alternative explanation that would explain the high frequency of MCV-positive tumors located on the head and the extremities, i.e., sun-exposed areas, is that UV irradiation is involved in MCV-driven oncogenesis: integrated MCV genomes in MCC harbor mutations prematurely truncating the LT protein, thereby eliminating the helicase domain that should stabilize the integration of the viral genome. A large study of whether these truncating mutations bear a UV signature could help to clarify this notion.

Another significant difference between virus-positive and virus-negative cases in our cohort is the higher frequency of females among the virus-positive patients; however, when adjusted for multiple testing, this factor is not significant anymore ($P = 0.308$). Nevertheless, it should be noted that when all studies with reported gender distribution for the MCV status—including this study—are combined, 249 (76.9%) of 324 male and 268 (85.3%) of 314 female MCC patients were virus positive (Feng *et al.*, 2008; Foulongne *et al.*, 2008; Kassem *et al.*, 2008; Busam *et al.*, 2009; Nakajima *et al.*, 2009; Sastre-Garau *et al.*, 2009; Sihto *et al.*, 2009; Varga *et al.*, 2009; Andres *et al.*, 2009a; Fischer *et al.*, 2010; Handschel *et al.*, 2010; Bhatia *et al.*, 2010b). Although a sex imbalance could be biologically explained, more studies are needed to confirm or decline an association of MCV status with sex.

In a recently published study with 114 patients from Finland, patients with MCV-positive MCCs had a better overall survival than those with MCV-negative tumors (Sihto *et al.*, 2009). Similarly, in a small study of 23 patients, it was observed that patients with more MCV copies per cell were characterized by a better survival (Bhatia *et al.*, 2010a). In contrast, in a recent study of 44 patients (Handschel *et al.*, 2010), as well as in the large patient cohort reported here, this association of MCV status with survival could not be confirmed. Indeed, we could not detect significant differences in regression-free survival, overall survival, or MCC-specific survival when analyzed either in a univariate or multivariate fashion. The discrepancy regarding prognostic impact of the MCV status between the study of Sihto *et al.* (2009) can at least in part be explained by the circumstance that most of the MCC patients from their cohort were female (70%), a surprising gender distribution as all previous reports observed a male predominance in MCC patients. Accordingly, in our study cohort, only 43.6% of the patients were women. Thus, the patient cohort analyzed by Sihto *et al.* (2009) seems somehow exceptional that might be, for example, because of geographic or lifestyle aspects; a

Table 2. Univariate and multivariate HRs for MCV status

	Univariate HR (95% CI)	*P*-value	Multivariate 1 HR (95% CI)[1]	*P*-value	Multivariate 2 HR (95% CI)[2]	*P*-value
Recurrence-free survival						
MCV negative	1		1		1	
MCV positive	1.753 (0.794–3.870)	0.165	1.701 (0.748–3.868)	0.205	2.778 (0.930–8.298)	0.067
Overall survival						
MCV negative	1		1		1	
MCV positive	0.750 (0.344–1.636)	0.470	0.981 (0.423–2.273)	0.964	1.861 (0.519–6.679)	0.341
MCC-specific survival						
MCV negative	1		1		1	
MCV positive	1.054 (0.362–3.066)	0.924	1.601 (0.477–5.371)	0.446	3.664 (0.665–20.183)	0.136

Abbreviations: CI, confidence interval; HR, hazard ratio; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus.
[1]HR of MCV status was adjusted to gender, age, and stage; *n*=122.
[2]HR of MCV status was adjusted to gender, age, stage, lymphocyte infiltrate, involved microanatomic compartment, and localization of the primary tumor; *n*=76.

Table 3. Univariate and multivariate HRs for MCC-specific survival

	Univariate HR (95% CI)	*P*-value	Multivariate HR (95% CI)[1]	*P*-value
Gender				
Female	1		1	
Male	1.615 (0.673–3.874)	0.283	1.963 (0.769–5.001)	0.158
Age[2]	0.978 (0.938–1.019)	0.281	1.014 (0.972–1.057)	0.530
Stage				
I	1		1	
II	3.838 (1.597–9.22)	0.003	4.286 (1.659–11.076)	0.003
III	17.943 (4.29–75.042)	<0.001	26.587 (5.38–131.373)	<0.001
MCV status				
Negative	1		1	
Positive	1.054 (0.362–3.066)	0.924	1.601 (0.477–5.371)	0.446

Abbreviations: CI, confidence interval; HR, hazard ratio; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus.
[1]HR was adjusted to all the other factors.
[2]Age was analyzed as a continuous variable.

notion strengthened by a study of Danish MCC patients in which the majority, i.e. 60%, were female (Kaae *et al.*, 2010).

There are a couple of limitations to our study. As for most retrospective studies, complete clinical information was not available for all patients. Notably, the scrutinized patient cohort displays similar characteristics as the recently published large study on MCC patients by Albores-Saavedra *et al.* (2009). Indeed, our patient cohort is only slightly younger (73.3 compared with a calculated age of 74.6) and has a little higher frequency of females (43.6% compared with 38.5%). Importantly, in both studies the 5-year survival rate is ~60%. The second problem, which is also inherent for studies on rare diseases analyzing an uneven distributed factor, is the limited power of the performed statistics. In fact, the power of our MCC-specific survival analysis is, for example, only 65%. Notably, to increase the power to over 80%, we would have to include at least another 80 events, which is for such a rare cancer an almost impossible challenge. Nevertheless, the power to observe the same effect of MCV status on survival as

described by Sihto *et al*. (2009) is over 80% for our patient cohort (data not shown).

In most of the factors analyzed, we could not observe significant differences between virus-negative and -positive MCCs. Accordingly, it seems prudent to state that the MCC viral status does not have a major impact on the course of MCC. This notion is further corroborated by the fact that all established prognostic factors apart from gender and body side distribution were equally distributed between MCV-positive and MCV-negative patients. Moreover, when scrutinizing the expression of p16, RB1, p53, or the proliferation marker Ki67, no relevant differences were observed. In summary, although MCV-positive and -negative MCCs are likely to have different etiologies, they seem to share a very comparable clinical behavior, prognosis, and aberrations in signal transduction. Thus, future studies are warranted to establish the etiology of both MCV-positive and -negative MCCs. In this respect, the very recent discovery of new polyomavirus members also present in human skin should be noted (Schowalter *et al.*, 2010; van der Meijden *et al.*, 2010).

MATERIALS AND METHODS

Patients

Archived paraffin-embedded tumor samples from a total of 174 MCC patients were collected from Germany (136 patients) or Australia (38 patients). All tumors had been excised for therapeutic reasons. MCCs were defined by histology and immunohistochemistry using antibodies to CK20, CD56, and TTF1. The presence of MCC in the samples had been demonstrated by routine histology and confirmed by an independent pathologist. The study was approved by the respective institutional review boards, and was in accordance with the Declaration of Helsinki Principles.

DNA isolation and MCV detection

Genomic DNA was isolated from serial sections using a DNA Isolation Kit (Qiagen, Hilden, Germany). The samples were analyzed for the presence of MCV using a TaqMan assay specific for the MCV T antigen and VP1 gene. Taqman primer and probe for T antigen were described previously (Becker *et al.*, 2009); for VP1, they were designed with Primer Express (Applied Biosystems, Darmstadt, Germany): fw: 5′-GCCTTTTGAGGTCCTTTCAGTG-3′, rv: 5′-ACTGT TTACCCAAAGCCCTCTG-3′ and the probe: FAM-5′-CGCCTTGCCC TTATCCTGCTGATTACTTTG-3′-BHQ1. LINE1, a highly repetitive DNA element, served as DNA control. The relative presence of MCV genome in the samples was determined by the ΔΔCt method where DNA of a MCV-positive cell line with at least two copies of MCV genome served as calibrator allowing only an estimation of copy numbers (Houben *et al.*, 2010a). MCV positivity was concluded when the relative expression of LT was larger than our cut point determined by bystander positivity in other cancers (Becker *et al.*, 2009). Real-time PCR for VP1 did not have to be positive, as this region might be lost by integration into the genome (Schrama, own unpublished observation).

Immunohistochemistry

For immunohistochemical detection of LT in paraffin-embedded tissues, slides were deparaffinized, and endogeneous peroxidase was blocked with hydrogen peroxide. Subsequently, epitope retrieval was performed using EDTA antigen retrieval buffer (Sigma-Aldrich, Schnelldorf, Germany). After blocking with protein block (Dako, Hamburg, Germany), samples were incubated with CM2B4 (1:2,000; Shuda *et al.*, 2009) for 30 minutes at room temperature, washed twice with Tris-buffered saline buffer, incubated with Mouse Envision Polymer (Dako) for 30 minutes, and incubated with Nova Red (Vector, Burlington, Canada). Finally, slides were rinsed with deionized water, counterstained with hematoxylin (DAKO S3309), and mounted in Shandon Ez Mount (Thermo Scientific, Braunschweig, Germany).

Antigen retrieval for RB1, p16, p53, pERK, and Ki67 staining was performed by incubation with citrate buffer (DAKO), pH 6.0 (RB1, p16, pERK, and Ki-67) or pH 9.0 (p53), for 20 minutes at 90 °C, followed by rinsing with distilled water and twice with phosphate-buffered saline (DAKO, S3024). After incubation with the Blocking Solution (DAKO, S2023) for 10 minutes at room temperature and two washing steps with phosphate-buffered saline, anti-RB1 (RB-1441-PO, DAKO), anti-p16 (Clone 16P04, Neomarkers, Fremont, CA), anti-p53 (DO-7, Dako), anti-pERK (clone E10; Cell Signaling, Beverly, CA), or anti-Ki67 antibodies (MIB-1, DAKO) were added to the sections and incubated overnight at 4 °C (pERK), at room temperature for 60 minutes (RB1) or 25 minutes (all others). After two washes with phosphate-buffered saline, biotinylated multispecies-specific secondary antibodies (Dako, K5003) were applied for 30 minutes. Bound antibodies were visualized using streptavidin–horseradish peroxidase (DAKO K5003) and Vector Vip (Vector) as peroxidase substrate according to the manufacturer's guidelines. Finally, the nuclei were stained with hematoxylin.

Statistical analysis

Statistical analysis was performed with PASW 18 (IBM, Munich, Germany), and the bases for the analyses were the patients. Distributions between MCV-positive and MCV-negative patients were compared with the *t*-test, when the respective factor passed the Shapiro–Wilk test for normality or alternatively with the Mann–Whitney *U*-test. *P*-values for contingency tables were calculated using Fisher's exact test. *P*-values were not corrected for multiple testing and were considered significant when <0.05. Kaplan–Maier curves were compared by the Mantel–Cox log-rank test. Univariate and multivariate HRs were calculated by Cox proportional hazards survival regression analysis (Lawless, 1982; Kleinbaum, 1996). All applied models were tested for and passed the proportional-hazard assumption based on Schoenfeld residuals. The power calculations were performed in R (www.r-project.org) using the cpower function based on George–Desu method from the Hmisc package. Mortality rates and percent reduction in mortality were calculated from the studies and the power evaluated for the present study size at 60 months.

Sequencing of large T-antigen stop codon region

For sequence information, the T-antigen region was amplified using LongAmp Taq (New England Biolabs, Frankfurt, Germany) with the primers 5′-TCTGCGATGAATCACTTT-3′, and 5′-GTTGTATCAGGC AAGCAC-3′ and according to the manufacture's protocol. The amplicons were purified with the PCR purification kit (Qiagen) and sequenced by the commercial vendor Seqlab (Goettingen, Germany).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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