Original Article

Viral Oncoprotein Antibodies as a Marker for Recurrence of Merkel Cell Carcinoma: A Prospective Validation Study

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BACKGROUND: Merkel cell carcinoma (MCC) is an aggressive skin cancer with a recurrence rate of >40%. Of the 2000 MCC cases per year in the United States, most are caused by the Merkel cell polyomavirus (MCPyV). Antibodies to MCPyV oncoprotein (T-antigens) have been correlated with MCC tumor burden. The present study assesses the clinical utility of MCPyV-oncoprotein antibody titers for MCC prognostication and surveillance. **METHODS:** MCPyV-oncoprotein antibody detection was optimized in a clinical laboratory. A cohort of 219 patients with newly diagnosed MCC were followed prospectively (median follow-up, 1.9 years). Among the seropositive patients, antibody titer and disease status were serially tracked. **RESULTS:** Antibodies to MCPyV oncoproteins were rare among healthy individuals (1%) but were present in most patients with MCC (114 of 219 patients [52%]; $P < .01$). Seropositivity at diagnosis independently predicted decreased recurrence risk (hazard ratio, 0.58; $P = .04$) in multivariate analyses adjusted for age, sex, stage, and immunosuppression. After initial treatment, seropositive patients whose disease did not recur had rapidly falling titers that became negative by a median of 8.4 months. Among seropositive patients who underwent serial evaluation (71 patients; 282 time points), an increasing oncoprotein titer had a positive predictive value of 66% for clinically evident recurrence, whereas a decreasing titer had a negative predictive value of 97%. **CONCLUSIONS:** Determination of oncoprotein antibody titer assists in the clinical management of patients with newly diagnosed MCC by stratifying them into a higher risk seronegative cohort, in which radiologic imaging may play a more prominent role, and into a lower risk seropositive cohort, in which disease status can be tracked in part by oncoprotein antibody titer. ***Cancer* 2016;000:000–000.** © *2016 American Cancer Society.*

KEYWORDS: Merkel cell carcinoma (MCC), Merkel cell polyomavirus (MCPyV), oncoprotein, serology, skin cancer, T antigen.

INTRODUCTION

Merkel cell carcinoma (MCC) is a neuroendocrine skin cancer with an incidence of 0.6 per 100,000,[1] corresponding to approximately 2000 new cases annually in the United States based on 2015 census data.[2] Age, sun exposure, and male sex are risk factors for MCC,[3] and immunosuppression portends a poorer outcome.[4,5] MCC has a recurrence rate of >40%.[6] This high recurrence rate indicates a need for data-driven surveillance approaches.

In 2008, a causative polyomavirus (Merkel cell polyomavirus [MCPyV]) was identified in 80% of MCCs[7] (Fig. 1A).[8] MCPyV is common worldwide, with 60% of adults demonstrating serologic evidence of prior infection.[9-13] Infection often occurs in childhood and is typically self-limited.[13-15] However, among patients who develop MCC, MCPyV integrates into the human genome; undergoes tumor-specific, truncating mutations; and thus can no longer replicate (Fig. 1B).[7,16] Instead, viral oncoproteins (T antigens) are persistently expressed in MCC tumors and help to promote cell cycle progression and tumorigenesis through multiple mechanisms,[17] including inhibition of the tumor-suppressor retinoblastoma protein (pRb),[18] stabilization of the oncoprotein c-Myc,[19] and evasion of innate immunity.[20,21] These oncoproteins are detectable by immunohistochemistry in 70% to 100% of MCCs.[18,19]

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Overall, 90% of individuals with MCC produce antibodies to the MCPyV capsid proteins.[10] High titers of anticapsid antibodies at presentation have been reported to be a favorable prognostic factor.[22,23] However, these antibodies (which mark previous exposure) are also detectable in >60% of healthy adults.[10,12] Furthermore, titers of antibodies to the MCPyV capsid protein do not vary with MCC tumor burden[9,23] and thus could not serve as biomarkers for recurrence. Given the limitations of anticapsid antibodies, instead, we focused on antibodies against MCPyV oncoprotein. These antibodies are rarely detectable in healthy individuals but are prevalent among patients with MCC.[9,23] In a discovery case series of 20 patients, we observed that titers increased with rising MCC burden and fell after tumor excision.[9] Similarly, others have demonstrated that patients with blood draws at the time of recurrence are more likely to have detectable antibodies than those with draws at the time of remission, although longitudinal, patient-specific data were not presented.[23]

In the current study, using a large, prospective validation cohort of 219 newly diagnosed patients who were followed over a 5-year period, we tested the clinical utility of MCPyV-oncoprotein antibodies in MCC management. To maximize clinical applicability, the assay was first established in a hospital-based laboratory. We tested 2 clinical roles for oncoprotein antibody quantification: for initial MCC prognostication and as a marker for disease recurrence after definitive therapy (Fig. 1C). Our results suggest that MCPyV-oncoprotein antibody titer is a biomarker that can assist in optimizing MCC management.

MATERIALS AND METHODS

Patients With MCC

Patients who had pathologist-verified MCC were prospectively enrolled in an institutional review board-approved natural-history cohort study and provided written informed consent. In total, 465 patients provided consent and had blood drawn, of whom 219 were newly diagnosed (≤90 days) and were included in further analyses. Blood was collected in red-top tubes and shipped overnight at ambient temperature to our Specimen-Processing Facility between June 2008 and October 2013. Sera were stored at −80°C. Grossly hemolyzed samples were excluded. Clinical follow-up was obtained through February 18, 2014.

Population Controls

Sera from Seattle-based blood-donors (Supporting Table 1; see online supporting information) were tested.

MCPyV Oncoprotein Antibody Detection

Serology assays were performed at the University of Washington Clinical Immunology Laboratory (available at: www.merkelcell.org/sero; viewed October 18, 2016). Glutathione-S-transferase (GST)-tagged MCPyV small T-antigen protein was produced recombinantly in Rosetta *Escherichia coli*, purified, and linked to a Luminex bead (Luminex Corporation, Austin, Tex).[9,10] GST was used as negative control and run concurrently. Sera were applied at 1:100, 1:1000, and 1:10,000 dilutions. Blocking was performed with superblock (Millipore CBS-K at 0.025%; EMB Millipore, Billerica, Mass), and antibodies were detected with a biotinylated antihuman immunoglobulin G (IgG) secondary antibody (1:1000 dilution; Kierkegaard & Perry Laboratory, Gaithersburg, Md) and streptavidin-phycoerythrin detection. Every plate included 24 dilutions of a standard pool (derived from 14 strongly positive patients). Titers for individual sera were calculated using weighted nonlinear regression. The threshold for a negative titer was set as <75 standard titer units (STU), because 99% of normal control participants without MCC had oncoprotein antibody titers below 75 STU. The positive threshold was set at ≥150 STU, because assay results above this level were highly reproducible. On the basis of these performance characteristics, titers <75 STU were defined as seronegative, those ≥150 STU were defined as seropositive, and those between 75 and 150 STU were defined as "borderline." Patients who had initial titers ≥75 STU were considered to be antibody producers.

Classification of Surveillance Draw Values

Serial blood draws were considered "rising" if the titer value was ≥150 STU and increased ≥ 20% from the prior draw. Draws were considered "falling" if the titer decreased by at least 20% from the prior draw or was <75 STU. All other draws were considered "stable." The 20% threshold for a change in titer was predetermined based on run-to-run variability across samples of various titers (Supporting Fig. 1A; see online supporting information). Draws occurred at 3-month to 6-month intervals, based on National Comprehensive Cancer Network guidelines[24] suggesting disease assessment at this interval. Supporting Table 2 details the timing of draws and the numbers of patients who were at risk at various time points (see online supporting information).

For patients who developed recurrent disease, all draws up to and including the time of first recurrence were included in the serial draw analysis. Only the first recurrence of MCC was considered for each patient, and draws

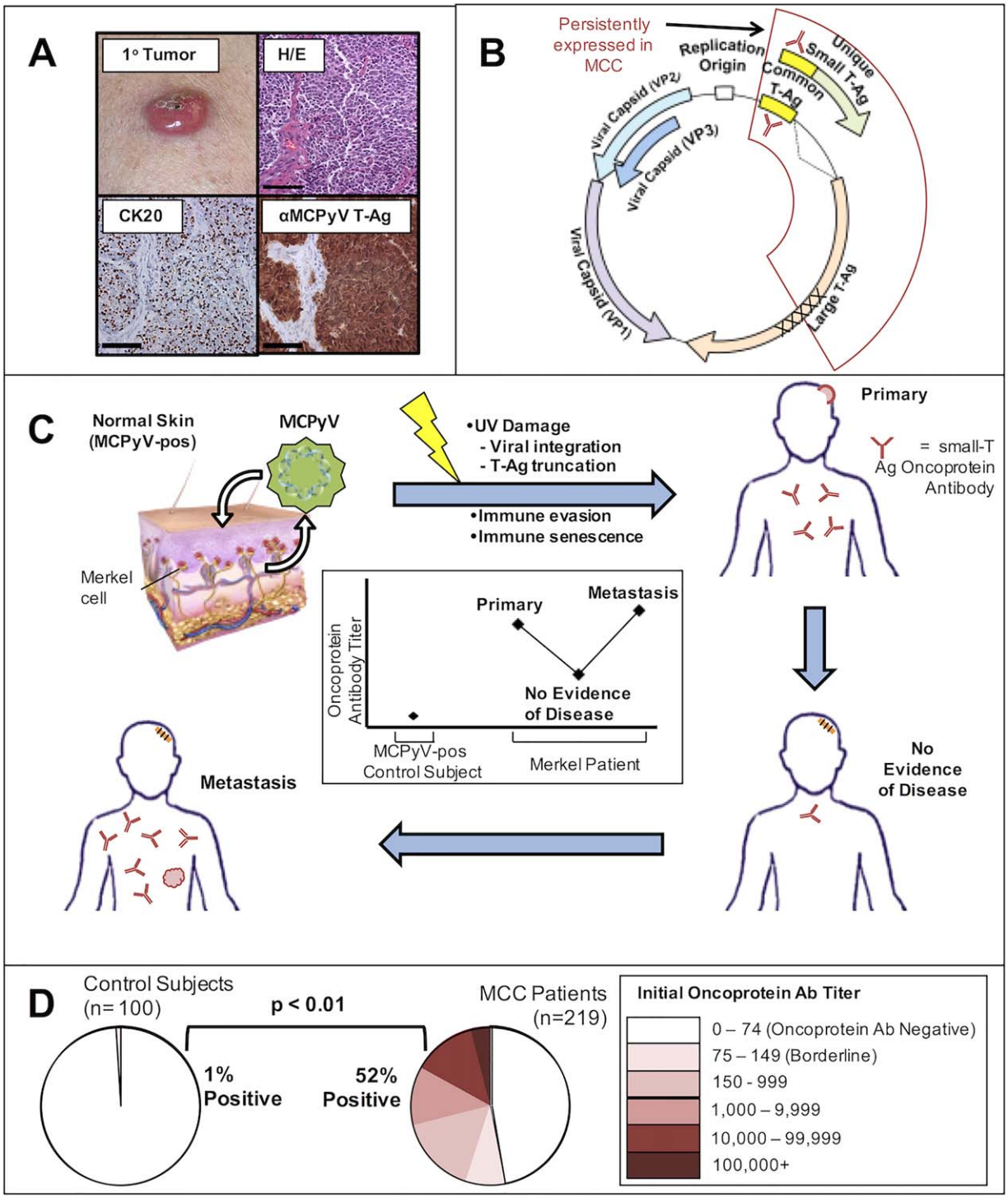

Figure 1. The rationale for a viral serologic assay for Merkel cell carcinoma (MCC) recurrence is illustrated. (A) The clinical and microscopic characteristics of a Merkel cell polyomavirus (MCPyV)-positive MCC arising on sun-exposed skin are illustrated. Tumor sections that contain stroma (pink on H&E staining) demonstrate MCC-specific expression of cytokeratin-20 (CK20) in a perinuclear, dot-like pattern and express viral large T-antigen (T-Ag) oncoprotein (CM2B4 antibody[8]. Scale bar = 50 μm. (B) This is a schematic of the MCPyV genome[7] and oncoproteins persistently expressed in human MCCs. The small and large T-Ag oncoproteins share an amino-terminal domain (common T-Ag) that is recognized by antibodies produced by the majority of patients with MCPyV-positive tumors.[9] The X symbols indicate the region in which truncating mutations clonally occur in individual tumors. (C) This is a schematic of MCC development and relative MCPyV-oncoprotein antibody titers. Pos indicates positive; UV, ultraviolet. (D) The distribution of antibody titers is illustrated among control participants and patients with MCC. One percent of healthy blood donors (n = 100) were seropositive versus 52% of patients with MCC (n = 219) at the time of diagnosis.

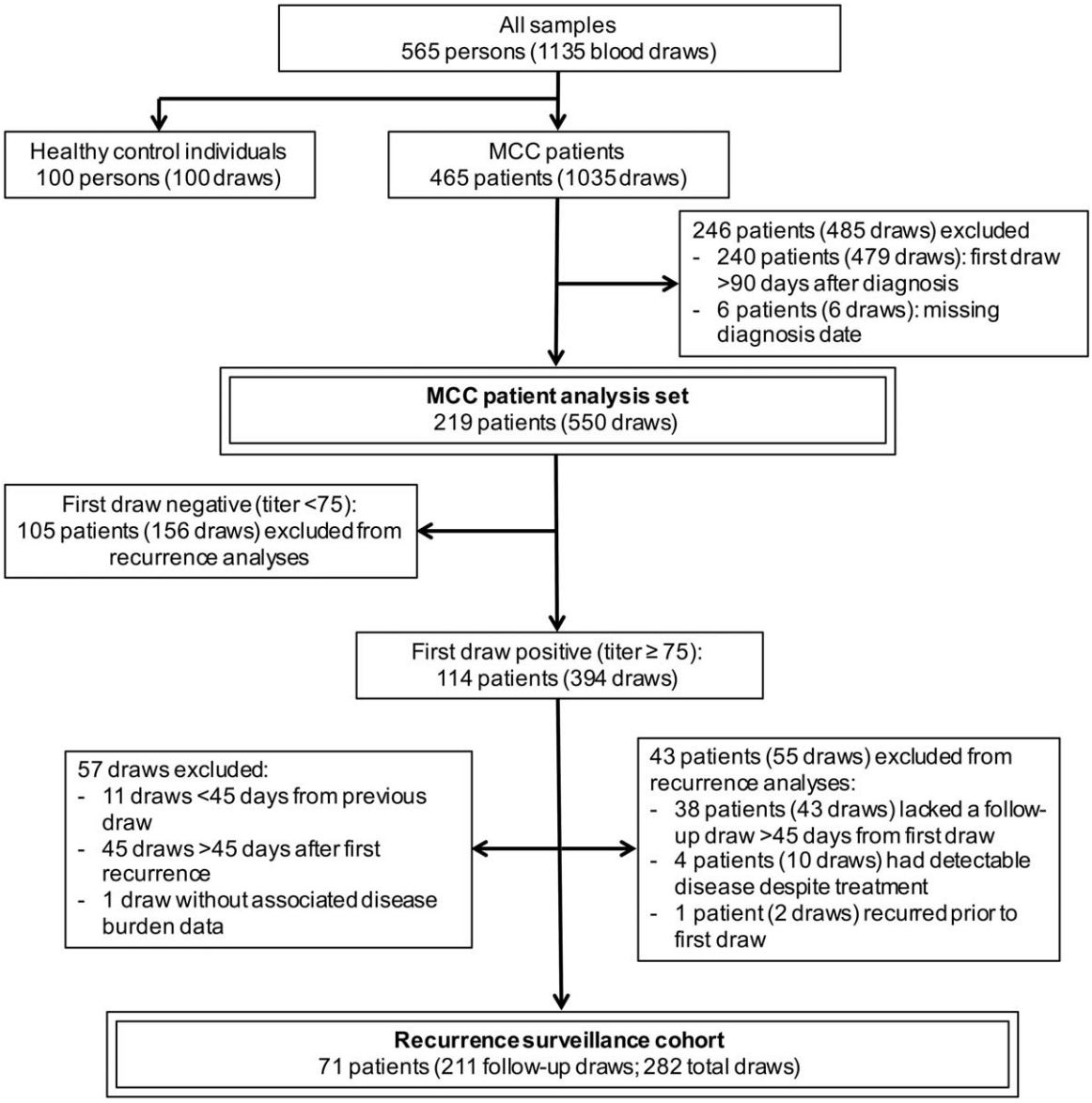

Figure 2. This is a patient-inclusion schematic for demographic and outcomes analyses. In total, 219 patients were evaluable for demographic and survival analyses, and 71 were both seropositive and had serial draws and thus could be included in recurrence analyses. An additional 43 patients were seropositive at diagnosis but were excluded from survival analyses because of the lack of a follow-up draw (38 patients), inability to receive definitive therapy (4 patients), or recurrence before the first draw (1 patient). MCC indicates Merkel cell carcinoma.

that occurred >45 days after the first recurrence were excluded. A blood draw value was paired with recurrence status if the recurrence was clinically or radiologically detected within a 45-day window before or after the draw.

Statistical Analysis

Comparisons between the proportion of patients with MCC and healthy individuals who were seropositive for MCPyV were performed using the Fisher exact test. Demographic factors between seropositive and seronegative patients with MCC were compared using logistic regression.

Recurrence-free survival was defined as the interval from the date of initial diagnostic biopsy to the date of first disease recurrence, last follow-up, or death. The risk of recurrence associated with clinical prognostic factors (age, sex, stage, and immune suppression) was estimated using a Cox proportional hazards model. A multivariate Cox model was used to compare recurrence-free survival between antibody-positive and antibody-negative patients.

TABLE 1. Patient Characteristics and Merkel Cell Polyomavirus Oncoprotein Antibody Status Among 219 Patients With Merkel Cell Carcinoma[a]

Characteristic	MCPyV Oncoprotein Antibody Status at MCC Diagnosis: No. of Patients (%)		*P*
	Seronegative, n = 105	Seropositive, n = 114	
Sex			.98
Women	44 (47.3)	49 (52.7)	
Men	61 (48.4)	65 (51.6)	
Age at diagnosis, y			.01[b]
≤65	35 (38.5)	56 (61.5)	
>65	70 (54.7)	58 (45.3)	
Immune suppressed			.004[b]
No	86 (43.9)	110 (56.1)	
Yes	19 (82.6)	4 (17.4)	
Primary site			.03[b]
Head and neck	25 (42.4)	34 (57.6)	
Buttock	1 (12.5)	7 (87.5)	
Upper limb	45 (62.5)	27 (37.5)	
Lower limb	14 (46.7)	16 (53.3)	
Trunk	10 (76.9)	3 (23.1)	
Occult primary	10 (27)	27 (73)	
Stage at MCC diagnosis			.001[b]
I	60 (65.2)	32 (34.8)	
II	7 (25.9)	20 (74.1)	
III	35 (38.5)	56 (61.5)	
IV	3 (37.5)	5 (62.5)	

Abbreviations: MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus.
[a] In total, 219 patients with newly diagnosed MCC were followed prospectively, of whom 114 (52%) were MCPyV-oncoprotein antibody positive at diagnosis. Patients with sun-protected or occult primary tumors, higher stage at diagnosis, or younger age at diagnosis were significantly more likely to be seropositive, whereas immunosuppressed patients were significantly less likely to be seropositive. One patient lacked staging information.
[b] This *P* value indicates a statistically significant difference.

To evaluate whether changes in oncoprotein titer could be used to detect first recurrences of MCC, sensitivity, specificity, and positive and negative predictive values were determined using the generalized estimating equations approach. A linear model with an autocorrelation structure was used to account for multiple observations within an individual. Comparisons between fractions of patients who developed recurrent disease within 45 days of falling, rising, and stable titers were performed with the Fisher exact test. Analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC) or STATA version 11.0 (Stata Corporation, College Station, Tex), and a statistical significance threshold of 5% was used.

RESULTS

Prevalence of MCPyV Oncoprotein Seropositivity

In total, 465 patients with MCC were enrolled in our prospective natural-history cohort study and had at least 1 associated blood draw (n = 465 patients, 1035 blood-draws). Of these 465 patients, 219 had a blood draw ≤90 days after diagnosis and were included in further analyses (Fig. 2). Analyses were limited to newly diagnosed patients to reduce late-entry enrollment bias and because titers fall quickly after therapy.

Among 219 patients with newly diagnosed MCC, 114 (52%) were MCPyV-oncoprotein–seropositive at the time of diagnosis. This was markedly increased compared with the population prevalence of 1% MCPyV-oncoprotein seropositivity determined from screening 100 healthy blood-bank donors ($P < .01$) (Fig. 1D). In addition, titers were higher among patients with MCC: No control participant had an antibody titer ≥150 standard titer units (STU), compared with 45% of patients with MCC at diagnosis ($P < .01$).

Characteristics of Patients With MCC Who Produced Oncoprotein Antibodies

Demographics of the seropositive and seronegative patients with MCC are compared in Table 1. Immune-suppressed individuals were less likely to produce detectable antibodies ($P < .01$). Serostatus was associated with the location of the primary lesion ($P = .03$). There were high rates of oncoprotein antibody seropositivity among patients with sun-protected MCCs (buttocks, 88% seropositive) and those with occult primary lesions (73%).

TABLE 2. Merkel Cell Carcinoma Recurrence Free Survival and MCPyV Oncoprotein Serostatus at Diagnosis[a]

	Univariate			Multivariate		
Variable	HR	95% CI	Global *P*	HR	95% CI	Global *P*
Stage			< .01[b]			< .01[b]
I	1.00	Reference		1.00	Reference	
II	2.49[c]	1.18-5.25		3.13[c]	1.42-6.90	
III	2.48[c]	1.43-4.30		3.00[c]	1.68-5.33	
IV	6.89[c]	2.87-16.52		8.46[c]	3.34-21.43	
Sex			< .01[b]			.02[b]
Women	1.00	Reference		1.00	Reference	
Men	2.11[c]	1.29-3.47		1.79[c]	1.08-2.95	
Age, y			.18			.19
≤65	1.00	Reference		1.00	Reference	
>65	1.38	0.86-2.20		1.40	0.85-2.32	
Immune suppression			.18			.21
No	1.00	Reference		1.00	Reference	
Yes	1.54	0.81-2.93		1.56	0.78-3.12	
Oncoprotein antibody			.1			.04[b]
Seronegative	1.00	Reference		1.00	Reference	
Seropositive	0.68	0.44-1.08		0.58[c]	0.36-0.97	

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a] HRs are shown for the risk of recurrent Merkel cell carcinoma (MCC). On multivariate Cox regression analysis, seropositive Merkel cell polyomavirus oncoprotein antibody status at diagnosis was associated with significantly improved recurrence-free survival. Stage and sex were also significant (N = 219 patients with newly diagnosed MCC).
[b] This *P* value indicates a statistically significant difference.
[c] This is a statistically significant HR.

Stage at diagnosis was significantly associated with MCPyV-oncoprotein seropositivity. Specifically, patients diagnosed with stage II or III MCC were more likely to be seropositive than those diagnosed with stage I MCC.

MCPyV Antibody Seropositivity at Diagnosis and Recurrence-Free Survival

Of the 219 patients included in the analysis of oncoprotein antibodies, 67 had an observed recurrence, with 52 of 67 (78%) recurring within 12 months of diagnosis. There were 51 deaths (35 attributable to MCC and 16 from other causes). The median follow-up for patients who were still alive at last contact was 681 days (1.9 years). The analyses of recurrence-free survival, prognostic factors and MCPyV antibody serostatus are presented in Table 2. Higher stage and male sex were associated with an increased risk of recurrence ($P < .01$ and $P = .02$, respectively). Age and immune suppression were not significantly associated with the risk of recurrence ($P = .19$ and $P = .21$, respectively). It is noteworthy that MCPyV antibody-seropositive status was independently associated with a 42% decreased risk of recurrence (hazard ratio, 0.58; 95% confidence interval [CI], 0.36-0.97) in the multivariate model adjusting for known prognostic factors (Table 2).

MCPYV-ONCOPROTEIN SEROLOGY QUANTITATIVE ASSAY PERFORMANCE

We hypothesized that MCPyV-oncoprotein antibodies might be useful not only for initial prognostication but also as an ongoing biomarker in seropositive patients. For this to be possible, the assay needs to be both readily clinically available and reproducible. We established the assay in a Clinical Laboratory Improvement Amendments 1988 (CLIA)-certified clinical laboratory and measured its performance and quantitative reproducibility. After assay optimization at a hospital clinical immunology laboratory, the coefficient of variation for MCPyV-oncoprotein serology ranged from 17% to 27% (Supporting Fig. 1A; see online supporting information), and the assay was highly linear (Supporting Fig. 1C; see online supporting information). Potentially confounding factors were assessed. Storage at ambient temperature for up to 14 days had no effect on titer (Supporting Fig. 1B; see online supporting information), suggesting that a delay in shipping of sera does not meaningfully affect the results. We compared red-top versus gold-top "serum-separator" tubes and observed no effect of tube type (Supporting Fig. 1D; see online supporting information). Finally, to determine whether various serum conditions affected titer, we mixed sera of defined titer with various amounts of sera containing

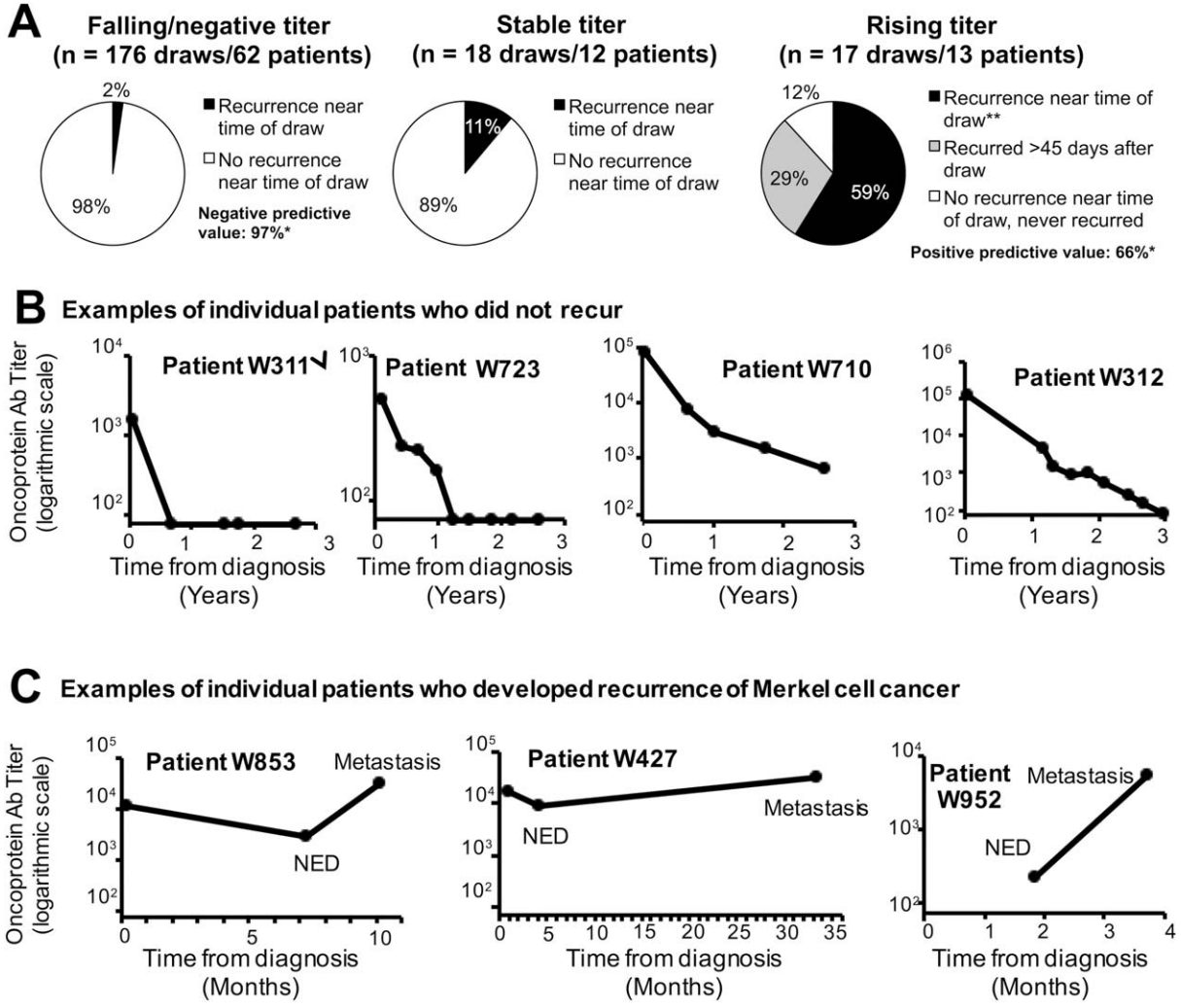

Figure 3. Illustrations depict prospective validation of serial Merkel cell polyomavirus (MCPyV) antibody levels as a marker of recurrence among patients who were positive for the antibody at diagnosis. In total, 71 patients were antibody-positive at diagnosis, received definitive treatment, and had serial follow-up draws (n = 282 draws, including 71 initial draws and 211 follow-up draws) during the study period. These patients were followed prospectively to determine whether the trend in MCPyV antibody levels between draws could be clinically useful as a tumor marker/biomarker for recurrence. (A) The association of titer trend with recurrence is illustrated. In total, 176 draws were falling by >20% compared with the previous draw. Ninety-eight percent of patients were without evidence of disease on clinical assessment (examination and/or scans). An asterisk indicates that, after statistically accounting for multiple draws in an individual patient, the negative predictive value (the likelihood that a falling titer represented no progression) was 97%. Conversely, 17 draws from 13 patients were associated with rising titers. In 59% of patients, recurrence could be detected at the time of the positive/rising titer, whereas an additional 29% of patients developed overt metastasis after the study period. The positive predictive value for detectable recurrence at the time of rising titer was 66%. $P < .05$ for the proportion of patients who had recurrent disease in a comparison between patients who had rising titers (59%) versus those who had falling/negative titers (2%). The number of patients in the 3 groups (with stable, failing, or rising titers) was > 71 because some patients were evaluable in more than 1 category (eg, a patient who initially had a falling titer later had a rising titer). (B) Examples of individual patients who did not develop recurrent disease are illustrated. Four of the 54 patients who did not develop recurrent disease during the study period are shown. Approximately one-half of patients who did not develop recurrent disease became seronegative during the study period at a median of 9 months (note the logarithmic y-axis [titer] and the x-axis [time in years]). Ab indicates antibody. (C) Examples of individual patients who had a recurrence are illustrated. Three of the 17 patients who developed a recurrence during the study period are shown. Note that the x-axis (time) is depicted here in months. NED indicates no evidence of disease.

high levels of rheumatoid factor and polyclonal immunoglobulins or sera that were lipemic, icteric, or hemolyzed. No reproducible interference was observed. This suggests that the viral protein antibody titer is both reproducible and quantitative, allowing for serial measurement over time.

Changes in Viral Oncoprotein Antibody Titer as a Biomarker of MCC Recurrence

On the basis of prior observations that patients with MCC who did not develop recurrent disease typically had a rapid and sustained fall in MCPyV-oncoprotein titer, we hypothesized, that among MCPyV antibody-positive patients, serial analyses of titers over time would be an informative marker for recurrence. To allow for a consistent analysis population, we focused on first recurrences among patients who were seropositive at diagnosis and had been rendered free of detectable disease. In total, 71 oncoprotein-seropositive patients with MCC met criteria (Fig. 2) for serial observation. Among these patients, a total of 282 blood draws were performed, including 71 initial *time-of-diagnosis* draws and 211 subsequent *surveillance* draws. Seventeen patients recurred during the study period, of which 16 patients had serology testing within 45 days of the recurrence. The median time to recurrence was 273 days (range, 81-1146 days).

Again, we observed that most patients with MCC who were rendered free of disease had a rapid and sustained fall in MCPyV-oncoprotein titer. Among patients who were initially seropositive, the median interval between the time of MCC diagnosis and when antibodies were no longer detectable was 8.4 months. Only 4 of 30 (13%) evaluable recurrence-free patients still had detectable MCPyV-oncoprotein antibodies at 2 years.

In our prospective cohort, we tested whether a falling titer was clinically reassuring. There were 176 falling-titer samples from 62 patients; among these 176 samples, there were only 4 false-negative blood draws that occurred within 45 days of recurrence (2.2%) (Fig. 3A,3B). We statistically determined specificity and negative predictive value using a generalized estimating equations approach (which can account for multiple observations within the same individual; see Statistical Analysis, above). With this approach, the overall specificity of a falling titer was 89% (95% CI, 82%-96%), and the negative predictive value was 97% (95% CI, 94%-100%) (Supporting Table 3; see online supporting information).

It is noteworthy that, among the patients who had *false-negative* results with falling titers but clinical progression, 3 of 4 had developed small metastases immediately after the removal of large-volume primary and/or bulky lymph node disease. In fact, those 3 patients had overall decreases in tumor burden that were accurately reflected by the serologic test. Thus, in high-risk patients, an initial post-treatment scan should be considered.

There was a small population of patients who had a readily detectable, stable (<20% change) MCPyV-oncoprotein titers (12 patients; 18 of 211 follow-up draws). Only 2 of these patients (11%) had recurrence/progression detected within 45 days of the draw. This was not statistically different from the patients who had falling titers ($P = .10$) (Fig. 3A).

Compared with a falling or stable titer, which was clinically reassuring, a majority of patients with rising titers had recurrence/progression identified within 45 days of the rising titer. There were 17 blood draws from 13 patients that were classified as rising (the titer value was $\geq$150 standard titer units (STU) and increased $\geq$ 20% from the prior draw). In 59% of cases (10 draws/10 patients), recurrence/progression was detected within 45 days of the blood draw ($P < .01$ compared with patients who had falling titers) (Fig. 3A,C). Of the 7 remaining *false-positive* draws (from 3 patients), 5 (29%) came from a single patient (W-763) who had an ^{18}F-2-fluoro-2-deoxy-D-glucose–avid lymph node that was concerning for recurrence (but inaccessible to biopsy during study period) and then developed additional sites of metastatic disease that were biopsy-proven to represent MCC recurrence after the study closed. This patient is depicted by the gray shading in Figure 3A. If this patient is counted as false-positive, then the sensitivity is 63% (95% CI, 39%-87%), and the positive predictive value is 66% (95% CI, 33%-98%). Instead, if this patient's result is considered to be true-positive, then the positive predictive value improves to 83%.

Among the 10 patients who had rising titers and contemporaneous recurrences, 3 had locally recurrent disease, and 7 had distant metastatic disease. It is noteworthy that all 7 patients who had distant metastatic disease had new metastatic disease identified on scans but were clinically asymptomatic and had no palpable disease on physical examination.

DISCUSSION

MCC is an aggressive cutaneous malignancy with a recurrence rate of >40%. Here, we report in a large, prospective validation cohort a clinically available, virus-directed assay that can identify 2 populations of patients at diagnosis: an MCPyV-oncoprotein–seronegative group at higher risk of recurrence, who may benefit from closer imaging surveillance, and an MCPyV-oncoprotein–seropositive group, for whom serial MCPyV antibody titer assessment may assist in ongoing surveillance.

Recurrence-free survival was decreased in oncoprotein antibody-negative patients in a stage-independent fashion. Immune suppression[4,5,25] has consistently been reported as an adverse prognostic factor for MCC, and

the absence of identifiable MCPyV in tumor tissue is sometimes reported to be an adverse prognostic factor (our multivariate models did account for known immunosuppression).[26,27] The worsened outcome observed among MCPyV-oncoprotein antibody-negative patients is similar to that reported for poor outcomes among patients with low or absent titers of antibodies that recognize MCPyV capsid.[22,23] Capsid antibodies were not tested directly in the current study. However, antioncoprotein antibodies have several advantages: They are more specific to MCC (because these antibodies are detected only rarely in healthy individuals); and, most notably, antioncoprotein antibodies vary with disease burden (compared with capsid antibodies, which typically remain constant). Increased clinical and radiographic surveillance for MCPyV-oncoprotein antibody-negative patients may be warranted given their higher risk MCC and the inability to use serology to monitor them for recurrence.

Among patients who made MCPyV-oncoprotein antibodies, we observed that a rising antibody titer frequently indicated MCC recurrence (positive predictive value, 66%), whereas a falling titer was highly reassuring (negative predictive value, 97%). This negative predictive value provides significant clinical utility and may help to best direct the use of other testing, such as scans. It is worth noting that there were only 4 false-negative findings, 3 of which occurred in the immediate postexcision setting, during an interval in which there had been an overall decrease in disease burden. This suggests that, with the first falling titer (approximately at the 3-month time point), a scan should be considered. After this, scans for *antibody makers* could mostly be reserved for a change in clinical symptoms or a rising MCPyV-oncoprotein antibody titer. Although the current study focused on the detection of first recurrences, available longitudinal data suggest that this test may also be useful for detecting later MCC recurrences if the titer decreases markedly after treatment of the first recurrence.

This study had several limitations. Patients received several treatment modalities, including surgery, radiotherapy, and a combination thereof, so that therapy was not uniform but did reflect *real-world* variation. Blood samples were collected at diverse centers across the United States and were shipped with a delay in processing of up to 3 days. It is important to note that, according to our data, this delay should not affect assay results based on tests of serum antibody stability at room temperature (Supporting Fig. 1B; see online supporting information). Finally, as a necessity of study design and to maximize enrollment and minimize bias from delayed entry, we report only on first recurrences.

There is controversy regarding whether the early detection of asymptomatic distant metastatic cancer improves outcomes. Such early detection is only beneficial if available therapies are more effective for low-burden disease. Indeed, early detection of metastatic MCC may not have been particularly beneficial when therapy was limited to cytotoxic chemotherapy (which is typically only palliative in nature). However, the viral etiology of most MCC tumors and the strong association of MCC with immune suppression suggest great potential for effective immunotherapy (with the associated possibility for long-term disease control). Indeed, several promising immunotherapy trials targeting MCC are being conducted, and a recently reported trial indicated a $>50\%$ response rate to immune-checkpoint blockade in patients with advanced MCC, with a median durability of response greater than that achieved using cytotoxic chemotherapy.[28-30] In patients with melanoma, higher response rates to immune-checkpoint inhibitors have been observed, and longer progression-free survival was reported in patients who had lower disease burden at the time of treatment.[31] Therefore, the early detection of distant metastasis in patients with MCC offers an opportunity to change clinical management in ways that are likely to lead to improved patient outcomes, particularly in the emerging era of immune therapy for MCC.

Since the end of follow-up of this cohort in February 2014, we have observed that this assay is useful in several aspects of clinical care in the Seattle MCC program. Our patients are now routinely tested for MCPyV-oncoprotein antibodies at diagnosis; and, if antibodies are detected, then testing is typically carried out every 3 months while the patient is at significant risk of recurrence (approximately 3-4 years). A rising titer prompts clinical and radiographic evaluation. Oncoprotein-negative patients are often followed with imaging studies during their first 2 or 3 years. Providers from other centers can readily access this assay as a clinical *send-out* test.

In summary, greater than 50% of patients with MCC make MCPyV-oncoprotein antibodies, and those who do not are at higher risk for recurrence and may benefit from closer follow-up with imaging. Viral oncoprotein antibodies have clinical utility for the early detection of occult recurrent or distant metastatic disease. Prompt recognition and treatment of metastatic disease may be associated with better outcomes by allowing patients to start immunotherapy at a time of lower disease burden.

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CONFLICT OF INTEREST DISCLOSURES

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