

## Antibodies to Merkel Cell Polyomavirus T Antigen Oncoproteins Reflect Tumor Burden in Merkel Cell Carcinoma Patients

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### Abstract

Merkel cell polyomavirus (MCPyV) is a common infectious agent that is likely involved in the etiology of most Merkel cell carcinomas (MCC). Serum antibodies recognizing the MCPyV capsid protein VP1 are detectable at high titer in nearly all MCC patients and remain stable over time. Although antibodies to the viral capsid indicate prior MCPyV infection, they provide limited clinical insight into MCC because they are also detected in more than half of the general population. We investigated whether antibodies recognizing MCPyV large and small tumor-associated antigens (T-Ag) would be more specifically associated with MCC. Among 530 population control subjects, these antibodies were present in only 0.9% and were of low titer. In contrast, among 205 MCC cases, 40.5% had serum IgG antibodies that recognize a portion of T-Ag shared between small and large T-Ags. Among cases, titers of T-Ag antibodies fell rapidly (~8-fold per year) in patients whose cancer did not recur, whereas they rose rapidly in those with progressive disease. Importantly, in several patients who developed metastases, the rise in T-Ag titer preceded clinical detection of disease spread. These results suggest that antibodies recognizing T-Ag are relatively specifically associated with MCC, do not effectively protect against disease progression, and may serve as a clinically useful indicator of disease status. *Cancer Res*; 70(21); 8388–97. ©2010 AACR.

### Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer with 35% to 50% disease-associated mortality (1). A recently discovered human polyomavirus [Merkel cell polyomavirus (MCPyV)] has been strongly linked to MCC and seems to participate in the causation of most MCC tumors. Indeed, MCPyV DNA is detectable in ~80% of MCC tumor lesions and has been shown to be integrated into MCC tumor DNA (2–5).

Similar to other polyomaviruses, MCPyV encodes two major families of genes (6). The early genes [tumor-associated

antigens (T-Ag)] promote cell cycle entry and replication of viral DNA and are expressed from a single transcript. The largest of the T antigen species, aptly named large T antigen (LT-Ag), is an oncoprotein that is persistently expressed in a truncated form in the majority of MCC tumors and likely promotes cell division in MCC cell lines by inhibition of the tumor suppressor pRb (7–10). Small T antigen (ST-Ag) shares the first 78 amino acids with LT-Ag, is thought to inhibit the tumor suppressor PP2A through its unique COOH-terminal domain, and is also expressed in MCCs (10). In contrast to the T antigens, the late genes [viral proteins (VP)] encode proteins that form the viral capsid, of which VP1 is the major constituent. Although the capsid is required for initial infection, VP1 expression cannot be detected in MCC tumors (11) and is thus thought to not play a role in tumor persistence.

Work from several groups, including our own, has shown that nearly all MCC patients have antibodies to the MCPyV capsid (11–13). This suggests that a history of infection with MCPyV is associated with MCC tumor development. However, infection with MCPyV is common, and 42% to 77% of subjects from the general population have antibodies to capsid proteins (12–15). Thus, there is limited potential for such antibodies to be used clinically as a marker for MCC.

We hypothesized that serum antibodies to MCPyV T antigens would be more specifically associated with MCC than those of the viral capsid. In contrast to capsid proteins that are highly visible to the humoral immune system, T antigen oncoproteins are not present in viral particles, are only expressed after viral entry into host cells, are located in the

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nucleus (16), and are thus less likely to trigger an antibody response except in the setting of dying or diseased tissue (such as a tumor that persistently expresses the T antigens). In this study, we used multiplex serology and a case-control design to characterize differences in frequency, titer, and specificity of anti-MCPyV antibodies between MCC cases and population-based controls.

## Materials and Methods

### Human subjects

All studies were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center and of the University Hospital of Wuerzburg and were performed in accordance with Helsinki Principles.

Serum samples from MCC cases used in the matched case-control analyses were from the Merkel Cell Carcinoma Repository of Patient Data and Specimens, Fred Hutchinson Cancer Research Center, and were collected between January 1, 2008 and May 1, 2010. Case patients ( $n = 139$ ) in this matched portion of the study were from the United States and ranged in age from 31 to 91 years at diagnosis and included 79 men and 60 women. Control subjects ( $n = 530$ ) were recruited from Western Washington by random digit dialing as previously described (17, 18) and frequency matched to case patients with MCC by age (within 5 years) and gender with a final ratio of 4 controls to 1 case.

A second set of sera from MCC cases (not matched to controls) was obtained from 66 MCC patients and was available for some but not all studies. These sera included 20 cases from the United States (sera collected 2009–2010) and 46 MCC patients from Germany (sera collected 2001–2010). The age (range, 37–96 years) and gender distributions (25 females and 41 males) of this population were similar to that of the MCC cases used in the matched analyses.

### Plasmids, cloning, site-directed mutagenesis, and preparation of fusion proteins

Fusion proteins, in which glutathione *S*-transferase was added to the NH<sub>2</sub> terminus and an 11-amino acid epitope tag was added to the COOH terminus (19), were used for all antibody binding assays. See Supplementary Data for details of generation of the fusion proteins.

### Multiplex antibody binding assay

These methods are a modification of the protocol described by Waterboer and colleagues (20, 21) and have been described in detail previously (13). In brief, polystyrene beads were covalently coupled to casein that had previously been linked with glutathione. Each fluorescently labeled bead set was loaded with a different fusion protein and, after washing to remove unbound fusion protein, pooled for an initial experiment using serum diluted at 1:100 to determine which samples were seroreactive for VP1, LT-Ag, and ST-Ag from each of BK polyomavirus and MCPyV. See Supplementary Data for details.

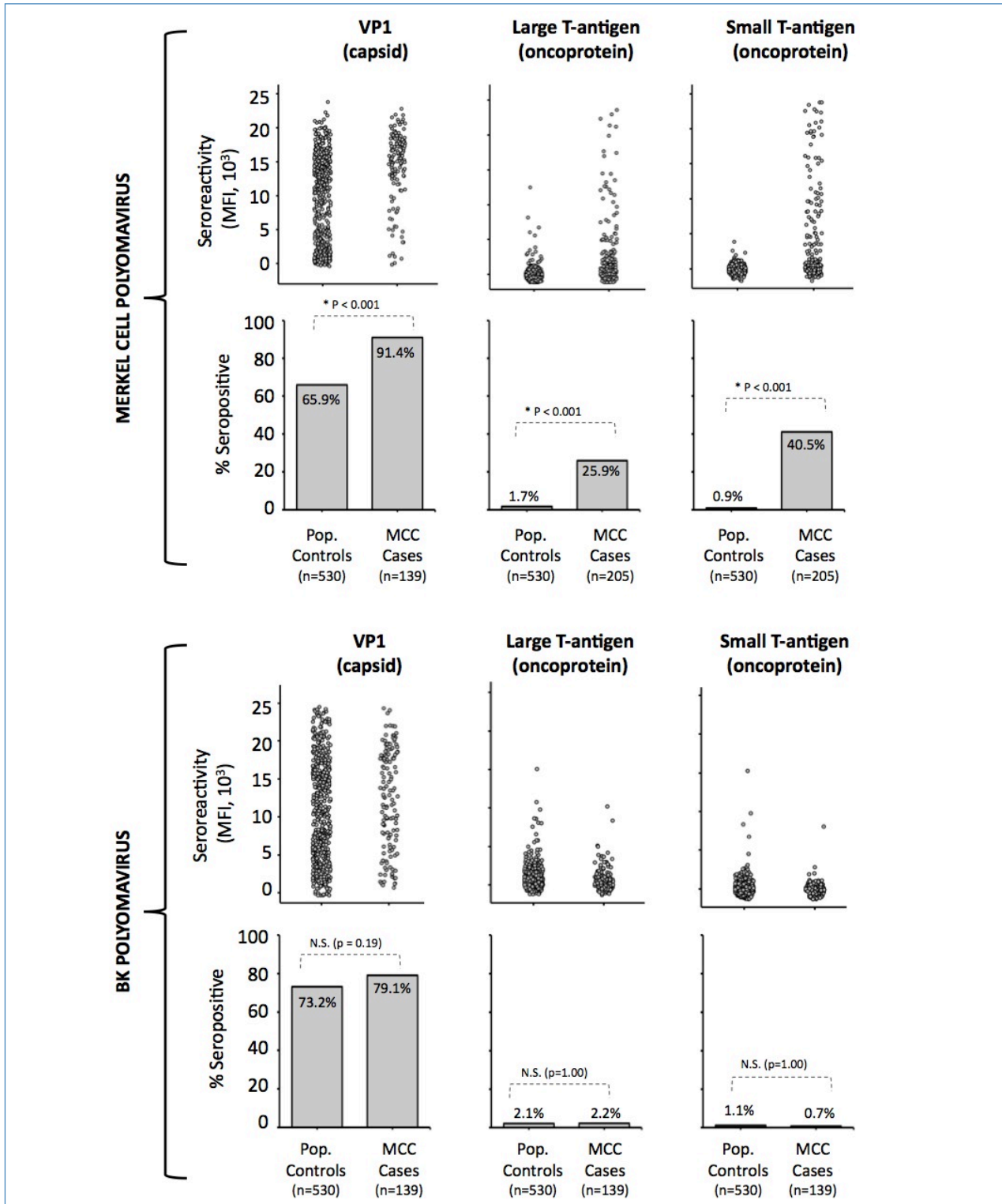
For titration experiments, human sera were diluted 1:50 in blocking buffer in 96-well polypropylene plates (Fisher

Scientific) and shaken for 20 minutes at room temperature. A 1:10 dilution of each sample was made in blocking buffer and the plate was shaken as before. A second 1:10 dilution was made and the plate was shaken. The dilute sera (50  $\mu$ L) were transferred to the wells of a 96-well filter plate that had a mixture of antigen-coated beads (50  $\mu$ L). Each plate had six wells used for the titration of a control (pooled positive sera). Serial dilutions of the control pool were made starting at 1:2 (concentration range, 1:100–1:3,200) on the first plate and ending at 1:6 (concentration range, 1:100–1:777,600) on the final plate. The subtracted median fluorescent intensity (MFI) values for each antigen were used to generate titration curves on GraphPad Prism (GraphPad Software, Inc.) using the Sigmoidal dose-response (with variable slope) program. A cutoff of MFI = 1,000 was used as the threshold between positive and negative because 1,000 approximated the border between signal and noise. These curves (separate for ST-Ag and LT-Ag; see Fig. 4A) were used to obtain titers (apparent titers) for MFIs of all experimental samples. The actual titers were then corrected for serum dilutions. Thus, the titer for each sample was estimated to be the dilution at which the sample would have an MFI of 1,000. Samples that were too low to be computed were given an arbitrary titer of 1. Several sera had titers too high to be calculated even at the 1:10,000 dilution and were assigned titers of 2,500,000 for ST-Ag and 1,200,000 for LT-Ag.

**MCPyV PCR detection in tumor tissue.** DNA from MCC was extracted and polyomavirus DNA was detected in tumor tissue by quantitative PCR (qPCR) using methods as previously described for U.S. (22) and European (4) cases.

**MCPyV immunohistochemistry.** Staining for MCPyV LT-Ag was performed with the CM2B4 antibody (Santa Cruz Biotechnology; ref. 7) at 1:100 dilution in EDTA buffer (pH 9) with 15% swine serum in serum-free protein block and incubated overnight at 4°C. Antigen retrieval was performed at pH 9 for 30 minutes in a preheated 95°C water bath, and then slides were slowly cooled for 30 minutes on the bench. All tumors were represented in triplicate on a single tissue microarray (TMA). Scoring was performed using the Allred method (23) by an observer blinded to serostatus, and the median score was used. Some off-target nuclear staining was observed in tonsil tissue; however, no peritumoral or intratumoral vessels, stroma, or lymphocytes were immunoreactive with the CM2B4 antibody. If more than one tissue from a particular tumor was represented on the TMA, a single specimen was chosen using the following ranking: primary > nodal metastasis > recurrence > other metastasis. Peritumoral and intratumoral vessels, stroma, and lymphocytes all were CM2B4 negative. Scores ranged from 0 to 8, and cut points used were as follows: negative, Allred 0 to 1.5; weak positive, Allred 2 to 4.5; strong positive, Allred 5 to 8.

**Statistical analyses.** Gender-specific cut points (3 SDs above the mean of population control sera) were used to determine seropositivity. Cut points used for men were as follows: MCST-Ag, 1,058; MCLT-Ag, 3,280; BKST-Ag, 3,683; BKLT-Ag, 6134. Cut points used for women were as follows: MCST-Ag, 818; MCLT-Ag, 2,110; BKST-Ag, 3,021; BKLT-Ag, 4,216. For VP1, the previously published cut point of 5,000



**Figure 1.** Prevalence of antibodies to Merkel cell and BK polyomavirus VP1 (capsid), LT-Ag, and ST-Ag proteins among MCC patients and age- and sex-matched controls. Population controls were random digit-dialed persons who were age and sex matched to 139 MCC cases in the United States. MCPyV LT-Ag and ST-Ag data ( $n = 205$ ) include these 139 initial cases on which the matching was performed (T-Ag data for the set of 139 available in Table 1) and an additional 66 MCC cases of similar age and sex distributions. First row, reactivity to MCPyV VP1 (left), LT-Ag (middle), and ST-Ag (right). Each spot represents one serum sample. Second row, proportion of control and case subjects seroreactive for each MCPyV antigen. Third and fourth rows, analogous studies carried out using BK polyomavirus antigens. N.S., nonsignificant.

was used (13). Odds ratios (OR) were sex and age adjusted. Proportion seropositive was compared between groups (e.g., cases and controls, virus-positive versus virus-negative cases) with Fisher's exact test. Linear regression was used to compare association between reactivity with ST-Ag to reactivity with LT-Ag and to compare association between BKV and MCPyV. Statistical analyses were performed with STATA software (StataCorp).

## Results

### MCC patients are more likely than controls to have antibody responses to MCPyV T-Ags

Sera from 139 MCC cases ("initial MCC cases") and 530 population controls (age and sex matched 4:1 to these cases) were screened for antibody reactivities to the MCPyV and BK polyomavirus (BKV) VP1, LT-Ag, and ST-Ag proteins in a single batch (Supplementary Fig. S1). Similar to prior reports, MCC case subjects were significantly more likely than control subjects to have antibody reactivity to the MCPyV capsid [OR, 5.5; 95% confidence interval (CI), 2.9–11.2] but not the BKV capsid (OR, 1.4; 95% CI, 0.9–2.3; Fig. 1; refs. 12, 13). However, the association with MCPyV T-Ag was even stronger. A total of 205 MCC cases were studied for T-Ag reactivity: 139 "initial cases" (Table 1) and an additional 66 cases from Europe and the United States with a similar age and gender distribution. MCC case subjects were more likely to have IgG antibody reactivity to the MCPyV LT-Ag (OR, 16.9; 95% CI, 7.8–36.7) and ST-Ag oncoproteins (OR, 63.2; 95% CI, 24.4–164.0; Table 1). Furthermore, and in contrast to VP1, antibodies to the T antigen were rarely detected among controls but commonly detected among cases. Indeed, 26% of MCC patients were seroreactive to LT-Ag compared with only 2% of population subjects ( $P < 0.001$ ; Fig. 1). Examining seropositivity to ST-Ag, the association grew even stronger, with 40.5% of MCC cases seroreactive compared with only 0.9% of control subjects ( $P < 0.001$ ; Fig. 1).

To determine assay reproducibility, a randomly selected subset of 116 sera (including cases and controls and positive and negative sera) was retested on a separate date in a blinded fashion. Seroreactivities were well correlated ( $R^2 = 0.73$  for ST-Ag; Supplementary Fig. S2). On a second additional date, 149 samples with an LT-Ag and/or ST-Ag MFI > 500 were retested. Correlation between this retest and the initial screen was also excellent (ST-Ag:  $R^2 = 0.83$ ; data not shown).

No relationship was observed between seroreactivity to BK T-Ag (Fig. 1) and MCC. Furthermore, seroreactivity to BK T-Ag was not associated with seroreactivity to MCPyV T-Ag, indicating that there was little cross-reactivity between assays (data not shown).

As shown in Fig. 1, reactive MCC cases seemed to have much stronger seroreactivity to the MCPyV T-Ags than the few reactive controls at the screening dilution. Multiple dilutions were further tested to confirm this higher antibody titer for case or control subjects with an MFI on screen of 500 or greater. The geometric mean titer (2,900) for antibodies recognizing LT-Ag among reactive "initial" MCC cases ( $n = 56$ ; 95% CI, 1,500–5,800) was more than 10-fold greater than the geometric mean titer of 200 among the reactive matched population controls ( $n = 36$ ; 95% CI, 100–300). Differences in ST-Ag reactivity were even more pronounced. Indeed, geometric mean titer for ST-Ag was several orders of magnitude greater in MCC cases (2,100; 95% CI, 800–5,500;  $n = 57$ ) than matched controls (5; 95% CI, 1–30;  $n = 10$ ).

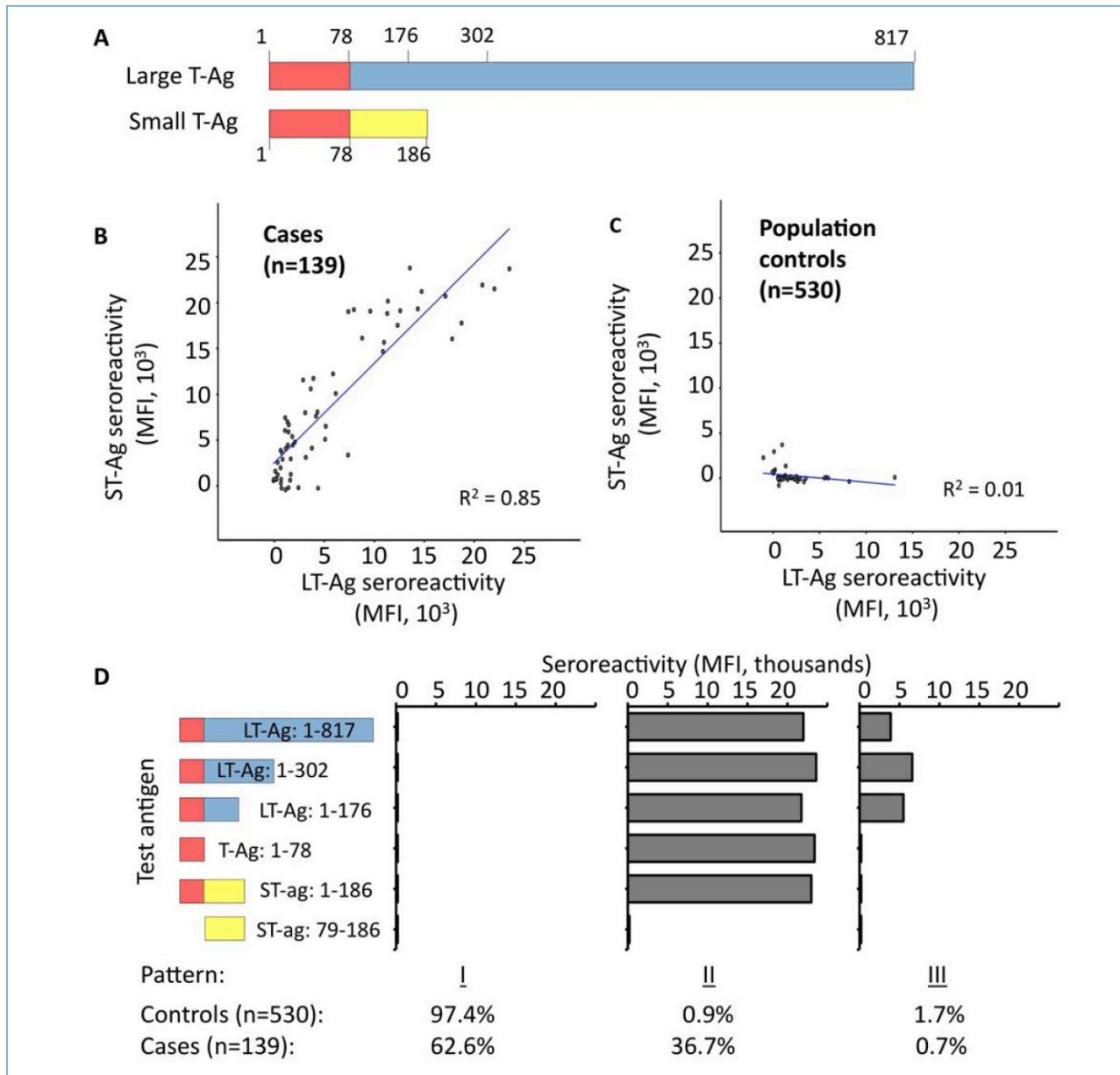
### Localization of reactive T antigen epitopes

The MCPyV LT-Ag and ST-Ag proteins result from splice variants of a single transcript. The two oncoproteins share a common NH<sub>2</sub>-terminal domain (78 amino acids), followed by regions unique to each protein (Fig. 2A; ref. 24). We observed similar seroreactivity to full-length LT-Ag and ST-Ag in the initial MCC cases ( $n = 139$ ;  $R^2 = 0.85$ ; Fig. 2B), but not matched population controls ( $n = 530$ ;  $R^2 = 0.01$ ; Fig. 2C). (LT-Ag and ST-Ag reactivities were also strongly correlated

**Table 1.** Risk of Merkel cell carcinoma associated with serostatus to MCPyV ST-Ag and LT-Ag

Antigen	Serostatus	Controls ( $n = 530$ )	Cases ( $n = 139$ )	OR (95% CI)
		$n$ (%)	$n$ (%)	
MCPyV ST-Ag	–	525 (99.1)	88 (63.3)	1.0 (reference)
	+	5 (0.9)	51 (36.7)	63.2 (24.4–164.0)
MCPyV LT-Ag	–	521 (98.3)	108 (77.7)	1.0 (reference)
	+	9 (1.7)	31 (22.3)	16.9 (7.8–36.7)
BKV ST-Ag	–	524 (98.9)	138 (99.3)	1.0 (reference)
	+	6 (1.1)	1 (0.7)	0.6 (0.1–5.3)
BKV LT-Ag	–	519 (97.9)	136 (97.8)	1.0 (reference)
	+	11 (2.1)	3 (2.2)	1.0 (0.3–3.8)

NOTE: One hundred and thirty-nine initial U.S. MCC cases are included along with 530 U.S. population controls matched to these cases in a 4:1 manner by 5-y age and gender. ORs have been adjusted for age at blood draw and gender. Cut points used to determine positivity (based on 3 SDs above the mean of controls): men: MCST-Ag, 1,058; MCLT-Ag, 3,280; BKST-Ag, 3,683; BKLT-Ag, 6,134; women: MCST-Ag, 818; MCLT-Ag, 2,110; BKST-Ag, 3,021; BKLT-Ag, 4,216.



**Figure 2.** Localization of T antigen regions containing immunoreactive antibody epitopes. A, LT-Ag and ST-Ag proteins share a common domain, amino acids 1 to 78, followed by portions unique to each protein. Amino acids indicated in the figure reflect end points of various truncations tested. B and C, correlation between LT-Ag and ST-Ag seroreactivity among the 139 initial MCC cases and 530 matched population controls. Note that ~500 controls overlap in the region of no reactivity to either antigen. D, using the indicated T-Ag truncations, three major patterns of reactivity were observed. Pattern I is consistent with no reactivity. Pattern II is consistent with recognition of an epitope within the first 78 amino acids shared between LT-Ag and ST-Ag. Pattern III is consistent with recognition of an LT-Ag epitope located between amino acids 78 and 176, in a region of LT-Ag that is not shared with ST-Ag.

in the additional 66 case sera, but these sera were not available at the time of detailed epitope localization analyses.) Thus, we hypothesized that MCC cases recognized a T-Ag epitope contained within the shared domain.

Several truncations of large and ST-Ag were used to map reactive antibody epitopes in MCPyV T-Ags (Fig. 2D). The most common pattern for both cases and controls ("I") showed no reactivity to any T-Ag epitope. Among seroreac-

tive subjects, two general patterns, "II" and "III," were observed. Pattern II was consistent with recognition of the common/shared domain and observed among 36.7% of patients and 0.9% of controls. In general, reactivity to full-length LT-Ag was reduced in this assay, likely due to lower amounts of LT-Ag on the beads compared with the shorter T-Ag products (data not shown). Sera with pattern III responses recognized epitopes unique to LT-Ag (between

LT-Ag amino acids 78 and 176 or between 176 and 302) and were seen among 1.7% of controls and in a single MCC case (0.7%). Interestingly, the MCC patient with this LT-only recognition pattern had no detectable MCPyV DNA in their tumor lesion, suggesting that this pattern may be nonspecific and may represent cross-reactivity to another protein. In accordance with these results, the remaining experiments were performed using full-length ST-Ag as the target T-Ag.

### MCC cases with MCPyV-positive tumor lesions more likely to be seropositive

Cytokeratin-20 (CK20) is a canonical marker for MCC and has been reported to be associated with virus status, with CK20<sup>-</sup> tumors unlikely to have detectable virus (7). CK20 data were available for 123 MCC cases from the initial case group. None of the 6 (0%) CK20<sup>-</sup> cases had antibodies to ST-Ag, whereas 45 of 117 (38%) of the CK20<sup>+</sup> cases were seropositive. However, this trend did not reach statistical significance ( $P = 0.08$ ; Fig. 3A).

Next, we investigated whether patients with detectable MCPyV DNA in their tumors were more likely to have antibodies recognizing MCPyV antigens. For 37 MCC cases in the initial case group, MCPyV DNA copy number was characterized by qPCR (22) as absent, low/borderline (more than five-cycle difference between MCPyV and control gene; corresponding to 1 copy per 16 cells or fewer), or moderately/strongly positive based on MCPyV DNA copy number ( $n = 37$ ). Consistent with the findings in the general population, a significant fraction of virus-negative cases ( $n = 8$ ) had antibodies recognizing the VP1 capsid (Fig. 3B). However,

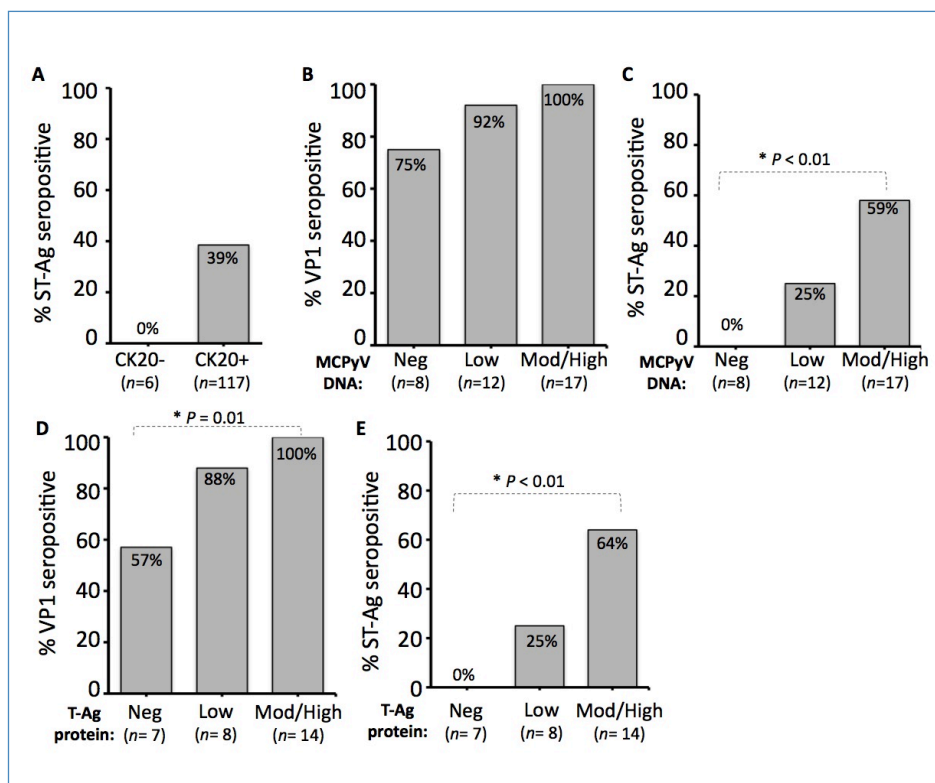
serology for the MCPyV T-Ag much more closely reflected the virus status of the tumor. Indeed, none of the eight cases with DNA-negative (0%), 3 of 12 (25%) of cases with DNA-low, and 10 of 17 (59%) cases with DNA strong positive tumors were ST-Ag seropositive ( $P = 0.01$ , comparing strong positives with absent; Fisher's exact test; Fig. 3C). Similar results were observed among 33 European MCC cases [qPCR done by different method (4)]. Among five cases with no detectable MCPyV DNA, there were no ST-Ag-reactive sera (0%). In contrast, 19 of 28 cases (68%) with MCPyV DNA-positive tumors were seroreactive to ST-Ag ( $P < 0.01$ , Fisher's exact test; data not shown).

Similar analyses were performed based on MCPyV T-Ag protein expression ( $n = 29$  cases with available formalin-fixed, paraffin-embedded tumor for study; see Materials and Methods for details). Using immunohistochemistry of tumor lesions (antibody directed at LT-Ag), 0 of 7 (0%) MCPyV LT-Ag protein nonexpressers, 2 of 8 (25%) weak expressers, and 9 of 14 (64%) strong expressers were ST-Ag seropositive ( $P = 0.01$ , comparing strong positives with absent, Fisher's exact test; Fig. 3E).

### Anti-T antigen IgG antibody levels reflect MCC tumor burden

To better quantitate the degree of seropositivity among reactive cases and controls, we retested reactive specimens at multiple dilutions to determine antibody titer (the dilution at which a serum goes from reactive to nonreactive; Fig. 4A). Titers closely correlated with screening MFI values at the 1:100 dilution ( $R^2 = 0.79$ ; Fig. 4B).

**Figure 3.** Relationship between MCPyV detection in tumor lesion and patient serostatus. A, seroreactivity based on expression of CK20, a canonical marker of MCC that is positively associated with virus presence. B and C, strongly virus DNA-positive tumors have a higher rate of seropositivity to MCPyV VP1 (not significant) and T-Ag than cases in which MCPyV DNA cannot be detected in the tumor lesion. Cases were sorted into three bins based on amount of MCPyV DNA detected in the tumor relative to a control gene (Materials and Methods). D and E, MCPyV LT-Ag protein expression (CM2B4 antibody) in the patient's MCC tumor was significantly associated with seropositivity for both VP1 and T-Ag.



Interestingly, seroreactive patients presenting with advanced disease (regional or metastatic,  $n = 12$ ) had significantly higher anti-T antigen antibody titers at the time of presentation (within 90 days; geometric mean titer, 53,000; 95% CI, 5,100–553,000) than those presenting with local-only disease ( $n = 10$ ; geometric mean, 1,000; 95% CI, 300–3,800). When these cases are further broken down by extent of disease, a trend toward increased titer at presentation with increased burden of disease was apparent (Fig. 4C).

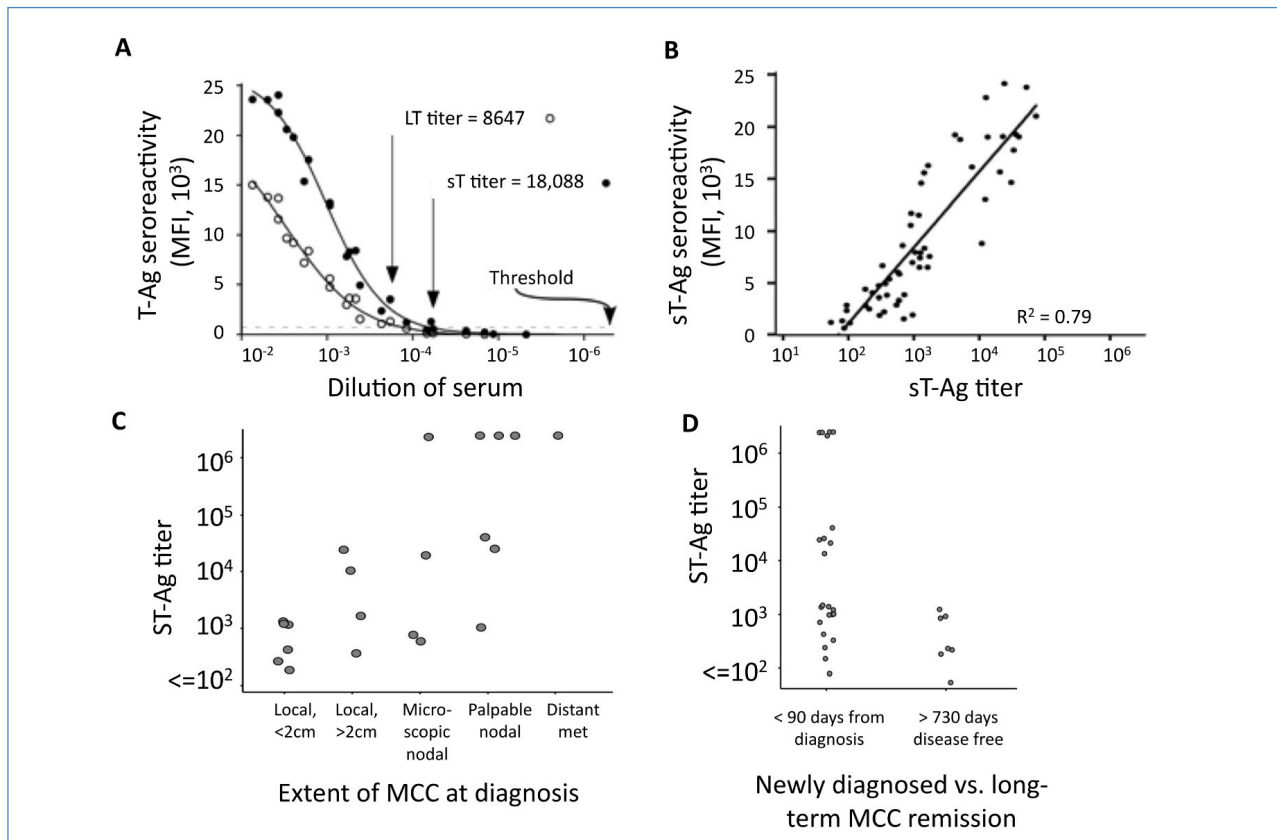
Furthermore, the titer of T-Ag antibodies among patients at or near the time of presentation (within 90 days;  $n = 23$ ; geometric mean, 8,150; 95% CI, 1,810–36,700) was an order of magnitude greater than that of patients who were >2 years since being rendered disease-free with no recurrences ( $n = 7$ ; geometric mean, 330; 95% CI, 120–920; Fig. 4D). In contrast, titers to VP1 were similar among recently diagnosed and long-term tumor-free cases, and a similar fraction was VP1 seroreactive (87% versus 100%, respectively). These observations led us to wonder whether IgG antibodies to the T-Ag oncoproteins were related to MCC tumor burden and whether they change with changing tumor burden.

There were 20 MCC cases that were informative on this issue because they met the following criteria: (a) blood draws

were available from two or more dates and (b) the T-Ag titer was  $\geq 100$  for at least one of the blood draws. All 43 blood draws available from these 20 cases are depicted in Fig. 5.

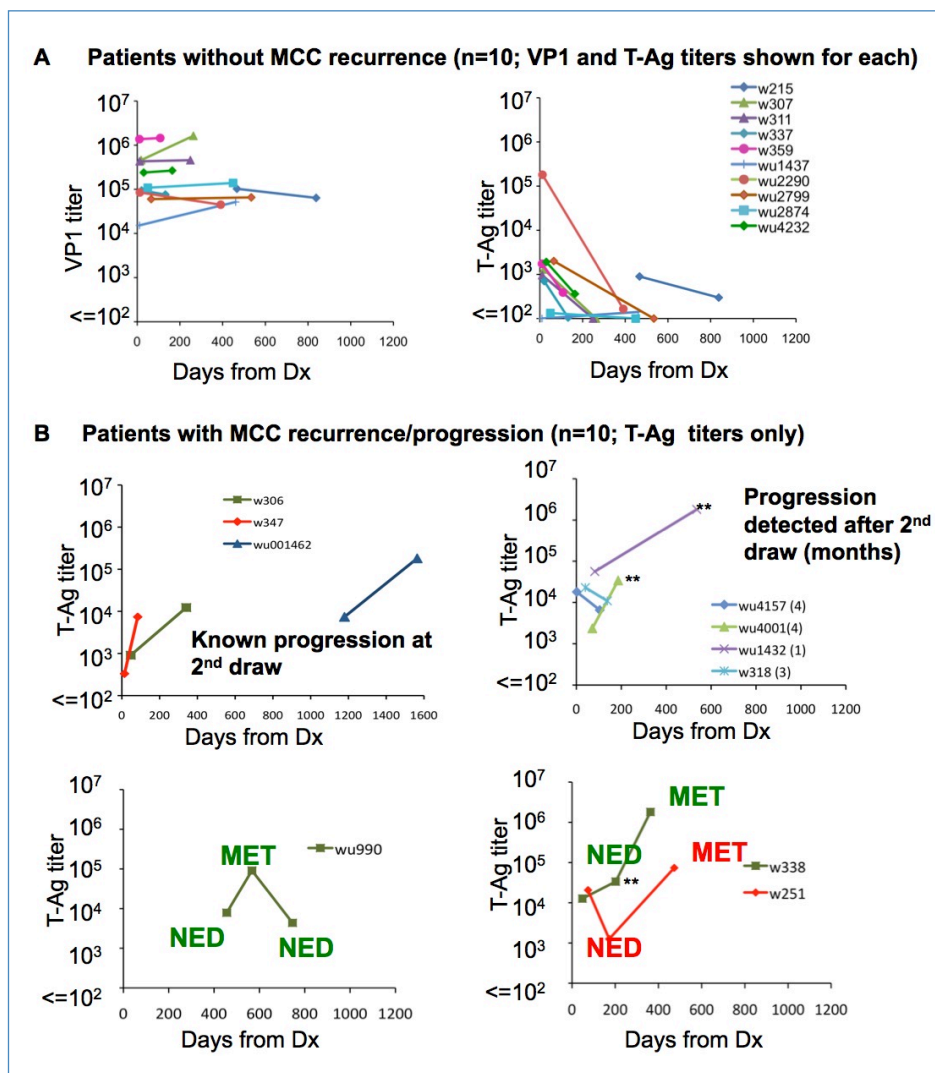
Among 10 patients whose disease did not progress in the follow-up period (all were rendered disease-free before the second draw), VP1 titer remained stable and did not significantly differ between draws (Fig. 5A, left). In contrast, we observed a rapid drop in T-Ag titer in 8 of these 10 patients, whereas the titer in the remaining two patients remained low (Fig. 5A, right). Indeed, among these 10 patients whose disease did not progress, the geometric mean T antigen titer was 1,290 at the initial draw (95% CI, 300–5,520) and diminished by 8-fold to 160 (95% CI, 110–240) by the time of the follow-up draw (98–469 days later, median of 308 days). The median rate of change in T-Ag titer was  $-10\%$  per month (range,  $-24\%$  to  $+3\%$ ) compared with  $1.5\%$  for VP1 (range,  $-5\%$  to  $+32\%$ ).

Importantly, anti-T-Ag titer not only reflected decreases in disease burden, but also increased in cases with recurrent disease (Fig. 5B). In all six draws from patients whose disease was known to have progressed, there were increased titers at the time of progression. Furthermore, in three of six cases with a blood draw that occurred several months before



**Figure 4.** Quantitative titration of antibodies recognizing MCPyV T-Ag. A, determination of titer by serial dilutions. A pool of sample sera selected from among T antigen seropositives is shown. The threshold of positive versus negative was set at an MFI of 1,000 (dotted line). B, ST-Ag titers, determined from one to three additional dilutions, compared with the initial screening ST-Ag MFI of the specimen. All 58 sera with a titer in quantitative range are shown. C, ST-Ag titer within 90 d of diagnosis compared with extent of MCC at presentation. D, ST-Ag titers comparing MCC patients within 90 d of diagnosis with those who were >2 y after diagnosis, with no recurrence.

**Figure 5.** Titers of IgG to MCPyV T-Ag, but not VP1, dynamically reflect MCC disease burden. All cases are shown that had available disease status information and an ST-Ag titer (performed if ST-Ag MFI > 500). There were 20 cases with a T-Ag titer of >100 for at least one draw and for whom multiple draws were available. Each line represents one case. Note the logarithmic scale. A, patients without MCC recurrence (these 10 cases represented in both the VP1 and T-Ag graphs). These patients did not progress in the time between draws or subsequently develop MCC recurrence after the second blood draw (available follow-up range, 0–6 mo). B, patients with progression. Importantly, for three cases (wu1432, wu4001, and w338), a rise in titer preceded the clinical detection of metastasis by 1 to 6 mo. Disease status at time of draw is indicated on the graph. NED, no evidence of disease; MET, metastasis/disease progression.



clinical recognition of MCC relapse (denoted in the figure by \*\*), a rise in T-Ag titer preceded the development of clinically appreciable MCC recurrence by 1 to 6 months.

## Discussion

MCPyV is a recently described polyomavirus (2) and MCPyV T antigen oncoproteins are persistently expressed in MCC tumors (7–9). These oncoproteins are capable of eliciting a humoral immune response and are more prevalent and of higher titer in MCC cases than controls. Furthermore, there is a strong association between T-Ag antibodies and virus detection in the MCC tumor. Finally, antibodies to MCPyV T-Ag oncoprotein (but not MCPyV capsid) vary greatly over time in MCC patients and reflect the burden of disease.

The strong association between antibodies to MCPyV T-Ags and MCC is similar to prior VP1 studies in this cancer in their support of a link between MCPyV and MCC and the

concept that MCC patients have long-term, high-level, and/or recent exposure to MCPyV antigens (11–13). Furthermore, and unlike anti-VP1 antibodies, T-Ag antibodies were only rarely detected among controls. This means that T-Ag antibodies are more specifically associated with MCC than antibodies to the capsid protein.

Interestingly, and in contrast to controls, MCC cases almost exclusively recognize a T-Ag epitope located in the common domain shared between MCPyV LT-Ag and ST-Ag, whereas the rare seropositive population controls usually recognized epitopes unique to LT-Ag. We hypothesize that large T reactive controls may represent cross-reactions to a different protein.

MCC patients with detectable MCPyV in their tumor by PCR or immunohistochemistry were more likely to have anti-VP1 and anti-T-Ag antibodies than those without detectable MCPyV. Interestingly, no case with a MCPyV-negative tumor seroreacted to MCPyV T-Ag. This suggests that there might be a population of MCC that develop without MCPyV involvement.

Importantly, we observed rapid changes in T-Ag titer that vacillated with disease burden. Indeed, titer often varied >10-fold in a 1-year period. Importantly, titers were highest when patients were sickest, suggesting that antibodies to T-Ag are not protective against disease progression. Similar to our observations, in some other cancers, antibodies recognizing human cancer antigens (such as p53) or papillomavirus proteins have been reported to be volatile and change with cancer burden (25, 26).

The rapid turnover of IgG antibodies recognizing T-Ag in MCC patient sera suggests instability within the B-cell population. Chemotherapy was not the cause of observed IgG decreases, as no patients in Fig. 5 had ever received chemotherapy. Instead, variation in titer is likely due to changes in antigen burden reducing or increasing stimulation of effector B lymphocytes. It is also possible that the memory B-cell response is not generated properly, perhaps due to ineffective priming within the tumor microenvironment.

It is possible that tracking the antibody titer to the viral T-Ag holds promise as an MCC disease marker. Computed tomography imaging is expensive and has rather poor sensitivity and specificity for MCC (27). In three cases, a rise in serologic ST-Ag titer preceded clinical detection of MCC metastasis by 1 to 6 months. For patients with a positive baseline MCPyV T-Ag titer, it may be appropriate to follow T-Ag titer at subsequent clinic visits to better inform clinicians of possible occult recurrence.

Our study has several limitations. The first is that serial blood draws were only available for 20 seropositive cases. The second is that it is currently impossible to know which if any "NED" patients may have slowly growing, clinically occult MCC. A third limitation is that given the very small number of control subjects with antibodies to ST-Ag (5 of

530), our estimate of the hazard ratio associated with seropositivity to ST-Ag is imprecise.

Despite these limitations, this study represents a significant advance in our understanding of the humoral immune response to MCPyV and MCC. In summary, there are striking differences between MCC cases and matched population controls in anti-MCPyV T-Ag antibody prevalence, titer, and specificity. Further study of anti-MCPyV T-Ag antibodies in an enlarged population of MCC patients is indicated to determine their potential for clinical utility in assessing disease status of this cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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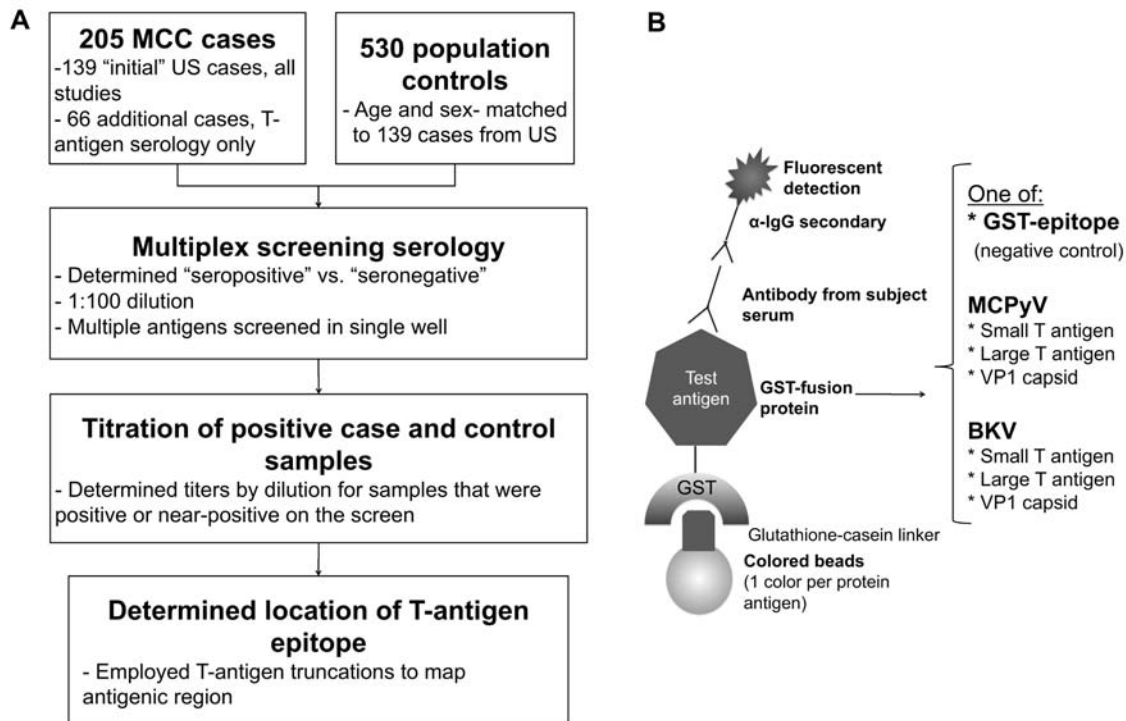
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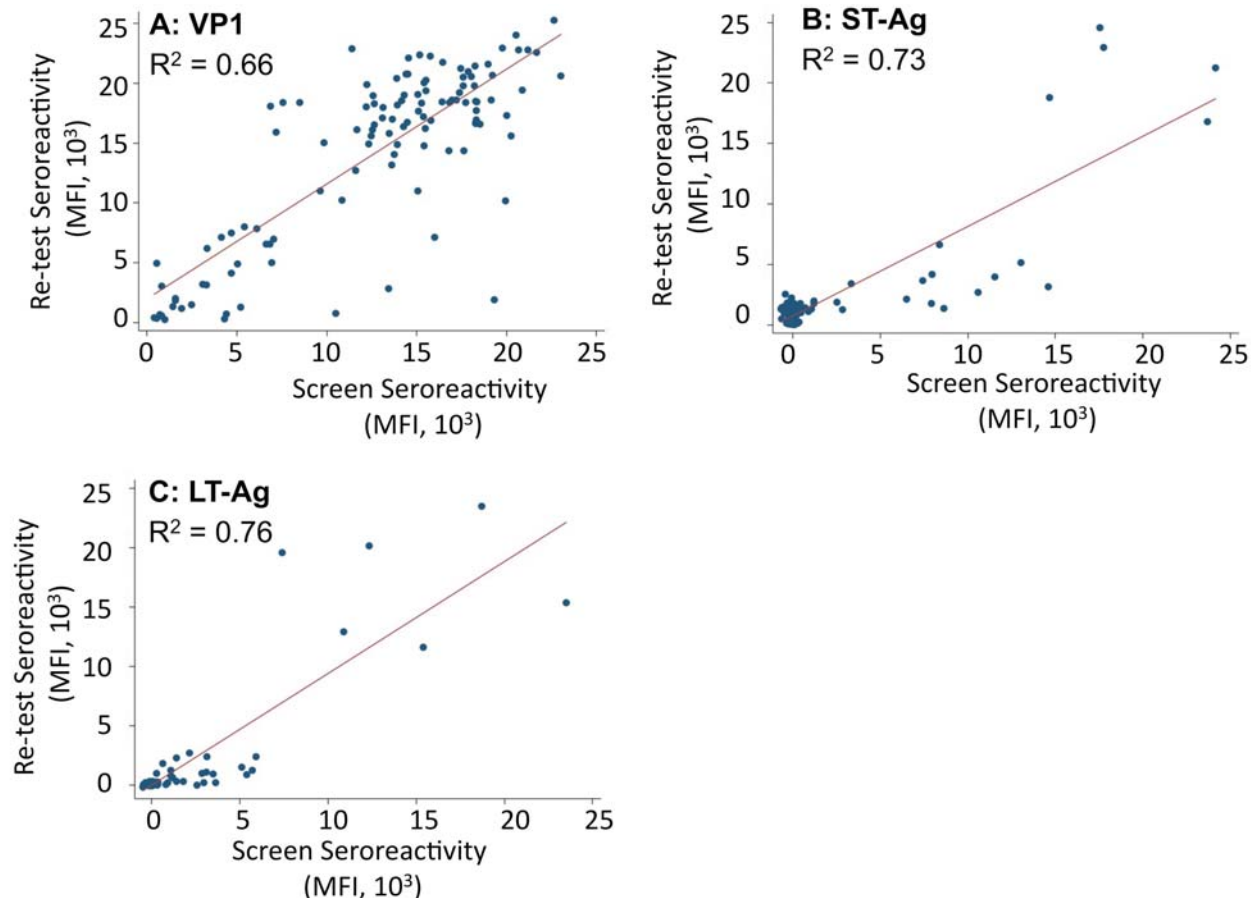
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Supplemental data associated with: **Antibodies to Merkel cell polyomavirus oncoproteins reflect Merkel cell carcinoma tumor burden**

**Supplemental figure 1. Experimental flow. A)** A case-control design was employed. 205 Merkel cell carcinoma cases were included: 139 "initial" cases from the United States were age- and sex-matched with population controls (1 case: 4 controls) and used for all studies. 66 additional cases became available at a later time point, were from the US and Germany, and were used for MCPyV small and large T antigen serology alone. For cases with multiple blood draws, only the earliest chronological draw was considered for analyses in Figures 2-4. **B)** Overview of multiplex serology method. IgG antibodies to the capsid and early proteins of MCPyV (associated with MCC) and BK polyomavirus (not associated with MCC) were studied & seroprevalences compared. Although large T-Ag is truncated in many MCC tumors, full-length large T-Ag was employed for screening purposes.



**Supplemental figure 2. Test for assay reproducibility.** 116 sera (originating from 57 Merkel cell carcinoma cases and 59 population controls) were selected at random to be re-tested on a separate date for reactivity to Merkel cell polyomavirus antigens in order to confirm assay reproducibility. The experimenter was blinded to the serum identification numbers, case status, and previous results. A: Merkel cell polyomavirus VP1. B: Merkel cell polyomavirus ST-Ag. C: Merkel cell polyomavirus LT-Ag.  $R^2$  values for correlation as determined by linear regression are as indicated.

**Supplemental methods:****Plasmids, Cloning, Site-Directed Mutagenesis, and Preparation of Fusion Proteins**

Fusion proteins, in which glutathione S-transferase (GST) was added to the N-terminus and an 11 amino acid epitope tag added to the C-terminus (1), were used for all antibody binding assays. The DNA plasmid used to produce MCPyV (clone w162) and BKV VP1 fusion proteins have been described previously (2). To express the MCPyV T-ag, the entire MCPyV genome was amplified from MCC tumor tissue (tumor 156) using polymerase chain reaction (PCR) with pfu Ultra (Stratagene, La Jolla Ca) in three pieces and reassembled in a cloning vector (pBS+) at the BamHI site. Sequencing (BigDye, Applied Biosystems, Foster City, CA) revealed no truncating mutations in the T-antigen sequence (Accession Number: HM355825). The small T (sT) antigen sequence was amplified using primers: forward sT-ag (GGA TCC gat tta gtc cta aat agg aaa gaa; lower case letters are coding sequence) and reverse sT-ag (GTC GAC gaa aag gtg cag atg). Products were TOPO cloned into a CR Blunt II vector (Invitrogen Corporation, Carlsbad, CA). Following digestion with BamHI and Sall, inserts of the expected size were isolated and ligated (T4 DNA ligase, Invitrogen) into the modified pGex4t vector (3 Widschwendter A, capsid protein L1, 2002). Clones containing the MCPyV sT-ag coding sequence (pGEX.MCVsT) were identified by restriction digestion and verified by sequencing. Because the pBS+ plasmid interrupted the Large T (LT) sequence at the BamHI site, vector sequence was excised by BamHI digestion, the MCPyV genome was gel isolated (Qiagen, Valencia, CA), circularized with T4 ligase and used as a template to amplify the early region using the primers (ACTAGTACC atg gat tta gtc cta aat agg) and (ACTAGT tta ttg aga aaa agt acc aga atc ttg g). The PCR product was cloned into CR Blunt II and sequenced. To remove intronic sequence, site directed mutagenesis was performed (4) using the primers: actagtaccatggatttagtctctaaatagg, ggtcccatataggggctcgtaacctcatcaaacatcgagaagtgcacttogaagtgacttctctatggttgatgaggttgacgaggcccctatat atcggacc and actagttattgagaaaagtaccagaatcttg. The LT sequence was PCR amplified from this plasmid (primers: TGA TCA gat tta gtc cta aat agg aaa gaa aga and GTC GAC ttg aga aaa agt acc aga

atc) and cloned into the CR Blunt II vector. Plasmid DNA from clones containing inserts of the expected size, were used to transform dam<sup>-</sup> cells (New England Biolabs, Ipswich, MA ). DNA encoding LT was gel isolated from dam<sup>-</sup> cells following digestion with BclI (compatible end with BamHI digested DNA), Sall and NcoI (only cut in vector), gel isolated and ligated into pGex4t (pGex.MCVLT). Plasmids containing BKV sT-ag and LT-ag sequences were obtained from Mike Imperiali (University of Michigan Medical School, Ann Arbor, MI) and cloned into GST-expression plasmids employing the procedures described above for creating pGEX.MCVsT-ag using the primers: forward GGATCC gat aaa gtt ctt aac agg gaa gaa tcc a, reverse sT-GTCGACagg ctt tag atc tct gaa ggg agt ttc and reverse LT-GTCGACttt tgg ggg tgg tgt ttt agg.

To identify the regions of MCPyV T-antigen recognized by human antibodies, five additional plasmids were created: The first encoded the sequence of MCPyV shared between sT-ag and LT-ag using the MCPyV sT-ag forward primer listed above and the reverse primer: GTCGAC gac ctc atc aaa cat aga gaa gtc act t. The second plasmid encoded the region of MCPyV sT-ag not included in LT. This was performed using the reverse primer previously used for amplifying the entire MCPyV sT-ag with the forward primer: GTCGAC gac ctc atc aaa cat aga gaa gtc act t and cloned into the pGex plasmid as described above. The other three constructs were deletions of the pGex MCPyVLT plasmid. To create a truncating mutation at the BamHI site, pGex.MCVLT plasmid DNA was digested with BamHI and Sall and religated using DNA linkers (GATCCAAGCTTG and TCGACAAGCTTG). To create truncation mutants corresponding to mutations identified in tumors, the forward primer (GGA TCC agt agc aga gag) and two reverse primers 350 (GTC GAC tgt aaa ctg aga tga cga gg) 339 (GTC GAC gtc tag ctc ata ttc aca agc) were used to amplify LT sequences from the pGEX.MCVLT plasmid. These sequences were subcloned through the CR Blunt II vector and ligated into BamHI/Sall digested pGex.MCVLT (gel isolated). Clones were identified and sequenced as before.

Polyomavirus fusion proteins were expressed in Rosetta Escherichia coli (EMD Biosciences Inc. La Jolla, CA) and soluble protein lysates prepared as described previously (2). Immunoblots were made

using 1 µg of crude protein extract per lane, run on 10% Nupage gels (Invitrogen) in 1X NuPage buffer (MOPS). Proteins were transferred to nitrocellulose and immunoblotted using an antibody to the C-terminal epitope (KT3) as previously described (2).

### **Multiplex Antibody Binding Assay**

These methods are a modification of the protocol described by Waterboer et al (5) and have been described in detail previously (2). In brief, polystyrene beads were covalently coupled to casein that had previously been linked with glutathione. Each fluorescently labeled bead set was loaded with a different fusion protein and, after washing to remove unbound fusion protein, pooled for an initial experiment using serum diluted at 1:100 to determine which samples were sero-reactive for VP1, Large T and small T from each of BK polyomavirus and MCPyV. Human sera were diluted (final conc. 1:100 unless otherwise noted) in blocking buffer (0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone, 0.025% CBS-K superbloc [Chemicon International, Temecula, CA] and GST-containing bacterial lysate at 2 mg/mL of PBS–casein) to inhibit non-specific reactivity (6) and incubated with a mixture of antigen coated beads overnight at 4°C for T-Ag experiments and 1hr at room temperature for VP1 experiments (preliminary experiments found that binding of human IgG to T-antigens was slow in comparison with binding to MCPyV VP1 and required overnight binding to achieve maximal sensitivity). For T-Ag assays, plates were additionally incubated for 1 hr at room temperature with shaking (Thermo Scientific Barnstead Titer Plate Shaker, Barnstead International, Dubuque, IA). Human antibodies bound to beads were detected using biotinylated goat anti-IgG (gamma) (KPL, Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD) and Streptavidin phycoerythrin. Plates were read on a BioPlex 200 after calibrating using the low RP1 target. The mean fluorescent intensity (MFI) for beads coated with GST alone was subtracted from the MFI of all other bead sets. Screen plates included 39 pairs of identical serum from population controls; concordance was perfect among pairs and a single serum was chosen at random for study inclusion.

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