

## Association of Merkel Cell Polyomavirus–Specific Antibodies With Merkel Cell Carcinoma

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- Background** Merkel cell polyomavirus (MCPyV) has been detected in approximately 75% of patients with the rare skin cancer Merkel cell carcinoma. We investigated the prevalence of antibodies against MCPyV in the general population and the association between these antibodies and Merkel cell carcinoma.
- Methods** Multiplex antibody-binding assays were used to assess levels of antibodies against polyomaviruses in plasma. MCPyV VP1 antibody levels were determined in plasma from 41 patients with Merkel cell carcinoma and 76 matched control subjects. MCPyV DNA was detected in tumor tissue specimens by quantitative polymerase chain reaction. Seroprevalence of polyomavirus-specific antibodies was determined in 451 control subjects. MCPyV strain-specific antibody recognition was investigated by replacing coding sequences from MCPyV strain 350 with those from MCPyV strain w162.
- Results** We found that 36 (88%) of 41 patients with Merkel cell carcinoma carried antibodies against VP1 from MCPyV w162 compared with 40 (53%) of the 76 control subjects (odds ratio adjusted for age and sex = 6.6, 95% confidence interval [CI] = 2.3 to 18.8). MCPyV DNA was detectable in 24 (77%) of the 31 Merkel cell carcinoma tumors available, with 22 (92%) of these 24 patients also carrying antibodies against MCPyV. Among 451 control subjects from the general population, prevalence of antibodies against human polyomaviruses was 92% (95% CI = 89% to 94%) for BK virus, 45% (95% CI = 40% to 50%) for JC virus, 98% (95% CI = 96% to 99%) for WU polyomavirus, 90% (95% CI = 87% to 93%) for KI polyomavirus, and 59% (95% CI = 55% to 64%) for MCPyV. Few case patients had reactivity against MCPyV strain 350; however, indistinguishable reactivities were found with VP1 from strain 350 carrying a double mutation (residues 288 and 316) and VP1 from strain w162.
- Conclusion** Infection with MCPyV is common in the general population. MCPyV, but not other human polyomaviruses, appears to be associated with Merkel cell carcinoma.

J Natl Cancer Inst 2009;101:1–13

Merkel cell carcinoma is a rare but aggressive skin cancer of neuroendocrine origin (1). The known risk factors for Merkel cell carcinoma include sun exposure, age more than 50 years, and immune suppression (2). The recent discovery of a novel polyomavirus, Merkel cell polyomavirus (MCPyV), in approximately 75% of Merkel cell tumor tissues (3) has led to the hypothesis that it is an etiologic agent for Merkel cell carcinoma. This hypothesis is biologically plausible because other members of the polyomavirus family have been shown to cause tumors in animals (4), and the T (tumor) antigen gene products, which inhibit the function of the retinoblastoma and p53 tumor suppressor proteins, are expressed in Merkel cell cancers (5). Importantly, although the large T antigen is often mutated in the cancers, the retinoblastoma protein-binding domain and the conserved region 1 domain, which are essential regions for cell transformation, are preserved in Merkel cell carcinoma (5). It is quite premature, however, to conclude that MCPyV causes Merkel cell carcinoma when so little is known about the natural history or the prevalence of the virus in the general population.

There are five known members of the polyomavirus family for which humans are the primary host. BK virus (BKV) and JC virus (JCV) were discovered in the 1970s (6,7), and WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) were identified in

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**See** “Funding” and “Notes” following “References.”

**DOI:** 10.1093/jnci/djp332

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## CONTEXT AND CAVEATS

### Prior knowledge

Approximately 75% of patients with the rare skin cancer Merkel cell carcinoma appear to carry Merkel cell polyomavirus (MCPyV).

### Study design

A retrospective case-control study was used to study levels of antibodies against polyomaviruses in plasma from 41 patients with Merkel cell carcinoma and 76 matched control subjects. Seroprevalence of polyomavirus-specific antibodies was determined in another 451 control subjects, who represented the general population. MCPyV DNA was detected in tumor tissue specimens.

### Contribution

The authors found that 36 (88%) patients with Merkel cell carcinoma carried antibodies against MCPyV compared with 40 (53%) control subjects. MCPyV DNA was detectable in 24 (77%) of the 31 Merkel cell carcinoma tumors available, with 22 (92%) of these patients also carrying antibodies against MCPyV. Among 451 control subjects from the general population, prevalence of antibodies against the five human polyomaviruses was 92% for BK virus, 45% for JC virus, 98% for WU polyomavirus, 90% for KI polyomavirus, and 59% for MCPyV.

### Implications

Although infection with MCPyV is common in the general population, MCPyV, but not the other four human polyomaviruses, appears to be associated with Merkel cell carcinoma.

### Limitations

The case-control study was small. Study subjects were primarily white, so that results may not be generalizable. There is no "gold standard" for determining MCPyV positive or negative status.

*From the Editors*

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respiratory infections in 2007 (8,9). Infections with BKV and JCV are common, usually acquired in childhood, and persist throughout adulthood. Earlier studies of the humoral response to BKV and JCV used hemagglutination inhibition assays, whereas more recent studies have used enzyme-linked immunosorbent assays to detect antibodies against the major capsid proteins (VP1s) of each type (10–12). Antibodies against BKV have been detected in 80%–90% of adults, and antibodies against JCV have been detected in 40%–60% of adults (13). There has been only one previous report on the prevalence of antibodies against WUPyV and against KIPyV (14). Humoral responses to human polyomaviruses are generally type specific; however, some antibody cross-reactivity has been reported between simian virus 40 VP1-specific antibodies and BKV VP1 and between BKV VP1-specific antibodies and simian virus 40 (15).

To determine whether an association exists between MCPyV-specific antibodies and Merkel cell carcinoma, it was important to evaluate the potential for antibody cross-reactivity against VP1 proteins from other human polyomaviruses. Waterboer et al. (16) developed a multiplex serological assay that uses glutathione S-transferase (GST)-VP1 fusion proteins tethered to spectrophotometrically distinguishable colored microbeads. We adapted this method to study the antibody response against the five polyomaviruses that infect humans and used it in two analyses. The first analysis

was a case-control study designed to test the hypothesis that case patients with Merkel cell carcinoma are more likely than control subjects to carry antibodies against MCPyV VP1. The second analysis was a population-based study designed to determine the prevalence of polyomavirus-specific antibodies in the general population.

## Subjects and Methods

### Study Subjects and Plasma, Serum, and Tumor Specimens

Merkel cell carcinoma plasma and serum samples were from the Merkel Cell Carcinoma Repository of Patient Data and Specimens, Fred Hutchinson Cancer Research Center, University of Washington. Consecutive patients ( $n = 41$ ) who agreed to participate and gave informed consent were enrolled between January 1, 2008, and August 31, 2008, and are the case patients for this analysis. Case patients ranged in age from 42 to 86 years at diagnosis and included 27 men and 14 women. Control subjects for the Merkel cell carcinoma case-control study, referred to as control group 1, were randomly selected from control subjects with available specimens (serum and plasma) in a previously reported case-control study (17) at the Fred Hutchinson Cancer Research Center. Control group 1 was frequency matched to case patients by age and sex and included 51 men and 25 women, for a total of 76 subjects. All serum and plasma specimens were stored at  $-70^{\circ}\text{C}$  until testing. Tissue samples of Merkel cell carcinomas that were tested for Merkel cell polyomavirus DNA were obtained as excess surgical tissue or extracted from archival formalin-fixed paraffin-embedded blocks, when the materials were available and the patient had given consent. All specimens contained at least 50% tumor cells as estimated by histology.

A second set of 451 women were studied as population-based control subjects and referred to as control group 2. These women ranged in age from 24 to 77 years and had previously participated in a study of anogenital cancer (17) to explore the age-specific prevalence and potential role of sexual transmission of polyomaviruses compared with human papillomavirus type 16 (HPV-16). HPV-16 serology from these women has been reported previously (17,18). Control group 2 was selected from the general population as previously described (17,18). Briefly, control subjects were women residing in King, Pierce, and Snohomish counties, which constitute metropolitan Seattle, who were recruited by use of random-digit dialing and matched to the age of men and women with various cancers. Written informed consent was obtained and all procedures and protocols were approved by Institutional Review Boards of the Fred Hutchinson Cancer Research Center and the University of Washington.

### Plasmids and Cloning, Preparation of Fusion Proteins, Immunoblot Analysis, and Site-Directed Mutagenesis

Chemicals were purchased from Sigma (Sigma Chemical, St Louis, MO) unless otherwise noted. The DNA constructs pGEX4t3.tag, pGEX.BKV, and pGEX.JCV were provided by Dr Michael Pawlita (German Cancer Research Center, Heidelberg, Germany) (16). pGEX.BKV and pGEX.JCV were used to produce fusion proteins between GST and the VP1 proteins from BKV and from JCV, respectively. Any sequence inserted in-frame into the *Bam*HI-*Sal*I sites of the pGEX4t3.tag plasmid created fusion

**Table 1.** Antibody positivity to polyomaviruses in women in the general population (control group 2) by age group\*

Age, y	No. in age group	No. positive (%)				
		BKV	JCV	WUPyV	KIPyV	MCPyV w162
<30	48	47 (97.9)	22 (45.8)	46 (95.8)	45 (93.8)	27 (56.9)
30–39	122	114 (93.4)	49 (40.2)	120 (98.4)	110 (90.2)	66 (54.3)
40–49	119	113 (95.0)	42 (35.3)	116 (97.5)	108 (90.8)	78 (65.7)
50–59	83	70 (84.3)	50 (60.2)	82 (98.8)	76 (91.6)	51 (60.9)
60–69	46	42 (91.3)	22 (47.8)	45 (97.8)	41 (89.1)	30 (65.8)
≥70	33	27 (81.8)	18 (54.5)	31 (93.9)	26 (78.8)	19 (57.8)
<i>P</i> <sub>trend</sub> †		.004	.04	.9	.2	.6

\* BKV = BK virus; JCV = JC virus; KIPyV = KI polyomavirus; MCPyV w162 = Merkel cell polyomavirus strain w162; WUPyV =WU polyomavirus.

† The test for trend was a nonparametric two-sided test that is an extension of the Wilcoxon rank sum test.

proteins with GST on the amino terminus and an 11-amino acid “Tag” epitope (so called because it targets a region of the simian virus 40 large T antigen) on the carboxyl terminus (19), so that fusion proteins could be recognized by anti-Tag antibodies (20). The MCPyV strain 350 expression plasmid (pGEX.MCPyV350) was provided by Dr Robert Garcea (University of Colorado, Boulder, CO) (14). Dr Tobias Allander (Karolinska Institute, Stockholm, Sweden) and Dr David Wang (Washington University, St Louis, MO) provided plasmids containing KIPyV (pGEM-KIPyVVP1-2) and WUPyV (pENTR-WUPyV) sequences, respectively (8,9). The VP1 open reading frames of KIPyV, WUPyV, and MCPyV (from tumor w162) were cloned into the modified pGEX4t3 plasmid after removal of the JCV VP1 sequence from pGEX.JCV by digestion with *Bam*HI and *Sal*I and gel isolation of the vector. Primers (listed in Supplementary Table 1, available online) were used to amplify the VP1 sequences by polymerase chain reaction (PCR) performed with pfu Ultra (Stratagene, La Jolla, CA) for KIPyV and WUPyV, with reaction conditions of 2 minutes at 94°C followed by 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 90 seconds; or AmpliTaq Gold (Applied Biosystems, Foster City, CA) for MCPyV, with reaction conditions of 5 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 90 seconds. All products were subcloned into the CR Blunt II Topo vector (Invitrogen Corp, Carlsbad, CA) following the manufacturer’s protocol. Clones containing a VP1 insert of the expected size were digested with *Bam*HI and *Sal*I and subcloned into the gel-purified pGEX4t3.tag plasmid. All pGEX clones were verified by BigDye sequencing (Applied Biosystems), and sequences were aligned by use of Vector NTI (Invitrogen). Sequences were compared by use of the ClustalW program (<http://align.genome.jp/>). The MCPyV w162 VP1 sequence (GenBank accession No. FJ392560) was predicted to express a protein that was identical to the MCPyV 339 VP1 sequence (GenBank accession No. EU375804), except at residue 422, which was mutated because the primers used for cloning were based on the MCPyV 350 VP1 sequence.

We sequenced seven additional Merkel cell carcinomas from the Merkel Cell Carcinoma Repository of Patient Data and Specimens, Fred Hutchinson Cancer Research Center, University of Washington. Partial VP1 sequences were obtained by amplification by PCR, with reaction conditions of 5 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 90 seconds and the sequencing primers listed in

Supplementary Table 1 (available online). PCR products were electrophoresed on a 1% agarose gel to confirm size and submitted to Functional Biosciences, Inc (Madison, WI) for ExoSAP cleanup (ie, enzyme-mediated removal of remaining primers, other single-stranded DNAs, and leftover deoxynucleoside triphosphates) and bidirectional sequencing to determine amino acid sequences and to analyze polymorphisms at positions 185, 288, 316, and 366.

Polyomavirus VP1 fusion proteins were expressed in Rosetta *Escherichia coli* (EMD Biosciences Inc, La Jolla, CA) by induction of a 1-L culture (optical density at 600 nm = 0.5) with 0.25 mM isopropyl β-D-1-thiogalactopyranoside, and bacteria were allowed to grow overnight at room temperature. Cells were lysed by two passes through a Microfluidizer (model M-110S; Microfluidics Corp, Newton, MA). Lysates were prepared as described by Sehr et al. (21), diluted with an equal volume of glycerol (Fisher Scientific, Pittsburgh, PA), and stored at –20°C.

For immunoblot analysis, the bacterial lysates were diluted in sample buffer containing 100 mM dithiothreitol and 1% sodium dodecyl sulfate and heated to 100°C for 5 minutes, and 4 μg of lysate protein per lane was electrophoresed on 10% EZ-Run (Fisher Scientific, Fair Lawn, NJ) polyacrylamide gels in a HoeferVE mini-electrophoresis apparatus (Hoefer Inc, Holliston, MA). Proteins were transferred from the polyacrylamide gel to a nitrocellulose (NitroBind; Micron Separations Inc, Westboro, MA) membrane in a HoeferVE mini-electrotransfer apparatus (2.5 hours at 300 mA in a buffer of 25 mM Tris base, 192 mM glycine, and 10% methanol), and the blot was blocked overnight with 1% nonfat dry milk in phosphate-buffered saline (PBS). VP1 expression in each bacterial lysate was confirmed by immunoblotting with a monoclonal antibody against the epitope Tag by use of antibody-containing supernatant from the KT3 hybridoma cell line provided by Michael Pawlita and by use of multiplex serological assays as described below. Immunoblot analysis was conducted by use of a Snap i.d. apparatus (Millipore Corp, Billerica, MA). KT3 supernatant (containing anti-Tag monoclonal antibodies) (20 μL) was diluted with 3 mL of PBS plus nonfat dry milk (0.5%) and the mixture was incubated with the blot for 10 minutes. The blot was washed three times with PBS–0.05% Tween 20 (Fisher Scientific), and 3 mL of PBS–0.5% nonfat dry milk containing 1 μL of horseradish peroxidase linked to anti-murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated with the blot for 10 minutes. The membrane was washed as described above and developed by use of Lumi-Light

reagents (Roche–Applied Biosystems, Indianapolis, IN) as described in the product protocol.

Site-directed mutagenesis was used to replace one, two, or three amino acids in the codon-modified VP1 sequence from MCPyV strain 350 in the pGEX4t3 vector with the corresponding amino acids in VP1 from MCPyV strain w162. The codon-modified sequence was used because the expression level of capsid proteins may depend on the match between the codon used and the tRNA that is available (22). Mutagenesis was performed by amplifying plasmid DNA (pGEX\_MCPyVw162) in two tubes each containing 5 ng of DNA and one of two complementary primers (Supplementary Table 1, available online) encoding the sequence changes. After three cycles of PCR (pfu Ultra, 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 15 minutes), the tubes were combined, an additional 1  $\mu$ L of polymerase was added, and 20 additional PCR cycles were performed under the same conditions. After amplification, *DpnI* restriction endonuclease was added (1  $\mu$ L) and the mixture was incubated for 2 hours at 37°C to digest the input plasmid DNA. After digestion, the amplified DNA was used to transform One Shot Top 10 cells (chemically competent *E. coli*, Invitrogen). The bacteria were grown overnight at 37°C on LB agar plates containing carbenicillin at 50  $\mu$ g/mL. The entire VP1 open reading frame from carbenicillin-resistant colonies was sequenced to identify clones with the desired sequence changes. To generate VP1 mutants that had more than one amino acid alteration, the changes were made sequentially by use of DNA that had one (or two) mutation(s) as the starting material to generate a sequence with two (or three) mutations. The mutations made were the replacement of histidine with asparagine at VP1 position 288 (H288D), the replacement of isoleucine with arginine at position 316 (I316R), and/or the replacement of asparagine with aspartic acid at position 366 (N366D). Although the MCPyV w162 VP1 amino acid sequence also differs from that of MCPyV 350 VP1 at position 185, that residue was not modified because it was not well conserved among the other polyomaviruses.

#### **Multiplex Antibody-Binding and Quantitative Multiplex Antibody-Binding Assays**

**Preparation of Antigen-Coated Beads.** Polyomavirus VP1–GST fusion proteins, GST, and the fusion protein containing L1 protein from HPV-16 and GST were bound to specific bead sets according to the methods of Waterboer et al. (16,23). Briefly, polystyrene microspheres or beads containing a unique combination of fluorescent dyes (MiraiBio, South San Francisco, CA) were covalently coupled with glutathione-linked casein. The beads are light sensitive and so were protected from the light during all incubations. The first experiment used VP1 fusion protein preparations (all in crude bacterial lysates) from the human polyomaviruses (BKV, JCV, WUPyV, and KIPyV as well as both MCPyV strains, w162 and 350) and a preparation of GST protein; a total of seven bead sets were included in this analysis. The second experiment used GST, the VP1 fusion proteins for the five human polyomaviruses (including MCPyV strain w162 but not MCPyV strain 350), and GST–L1 from HPV-16 (which was included to investigate potential associations between seropositivity to polyomavirus with age or number of sex partners) because the seroepidemiology of HPV-16 has been studied (18); a total of seven bead sets for

these seven proteins were included in this analysis. In the third experiment, which tested for reactivity against mutated MCPyV VP1 fusion proteins, the seven mutated VP1 proteins, wild-type VP1 fusion proteins from MCPyV strains w162 and 350, GST, and BKV VP1 fusion proteins were used; a total of 11 bead sets were included in this analysis. For all three experiments, 1 mg of a bacterial lysate containing a distinct fusion protein or GST was diluted to a final volume of 1 mL with PBS containing casein at 1 mg/mL (PBS–casein) and incubated with a spectrophotometrically distinct bead set that had previously been coupled with casein–glutathione for 1 hour at room temperature with moderate shaking (Thermo Scientific Barnstead Titer Plate Shaker; Barnstead International, Dubuque, IA). The number of beads (per set) used for each experiment varied and was calculated as 2750 beads times the number of wells (equal to the total number of plasma samples, serum samples, and monoclonal antibodies) to be tested. Unbound antigens were removed by three washes with 0.5 mL of PBS–casein and resuspended in the same buffer by three cycles of sonication followed by vigorous vortex mixing. Finally, all antigen-coated bead sets used in an experiment were combined and diluted in PBS–casein to a final concentration of 55 000 beads per milliliter for each bead set (ie, if seven bead sets were being used in an experiment, then  $7 \times 55\,000$  beads per mL = 385 000 beads per mL would be used).

**Pretreatment of Serum or Plasma.** To reduce nonspecific binding, human serum and plasma samples were incubated with blocking buffer before being incubated with antigen-coated beads. Each human serum or plasma sample (2  $\mu$ L) was diluted 1:50 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 in PBS–casein) in 96-well polypropylene plates (Fisher). Plates were incubated for 1 hour at room temperature with shaking. When monoclonal antibodies were used in this experiment, they were not pretreated with blocking buffer but were diluted in PBS–casein, 1:100 dilution for anti-Tag monoclonal antibodies and 1:500 dilution for anti-GST monoclonal antibodies.

**Multiplex Antibody Binding Assay.** We transferred 50  $\mu$ L of pretreated antibody-containing serum or plasma or 50  $\mu$ L of dilute monoclonal antibodies from the polypropylene plate to the wells of a 96-well filter plate, which has a mesh backing to allow for repeated filtrations and incubations (Multiscreen<sub>HTS</sub>-BV; Millipore Corp, Bedford, MA). The antigen-coated bead mixture (containing all bead sets) (50  $\mu$ L) was added to each well containing pretreated serum or plasma or diluted monoclonal antibodies. With the exception of the cross-sectional study in which serum from control group 2 was studied, plates were incubated for 1 hour at room temperature with shaking. For the cross-sectional study, plates were incubated overnight at 4°C followed by a 1-hour incubation at room temperature with shaking; the longer incubation time in this study was used to improve detection of HPV-16 L1 by human antibodies that were included in this assay. Wells were washed three times with 100  $\mu$ L of PBS–casein. Either biotin-coupled anti-human IgG (KPL, Gaithersburg, MD) or biotin-coupled anti-mouse IgG (PerkinElmer Life and Analytical Sciences, Inc, Waltham, MA) was diluted 1:1000

in PBS–casein and 50 µL was added per well. Plates were incubated for 30 minutes with shaking and the wells were washed as described above. Streptavidin coupled to phycoerythrin (Invitrogen; diluted 1:1000 in PBS–casein) was added (50 µL per well) to detect biotin-coupled bound antibodies. Plates were incubated for 30 minutes and the wells were washed three times in PBS–casein. After 100 µL of PBS–casein was added to each well, the amount of phycoerythrin bound to beads was determined on a BioPlex 200 Instrument (BioRad Laboratories, Hercules, CA) as a reflection of the amount of bound antibody and expressed as the median fluorescent intensity. For each sample, the antigen-specific binding was obtained by subtracting the median fluorescent intensity for beads coated with GST alone from that for beads coated with each of the other fusion proteins.

**Cut-point Determination.** Cut points for antibody positivity were selected by visual inspection of the distribution of MCPyV values among control subjects for that study. For the case–control study, the cut point for antibody positivity was a median fluorescent intensity of more than 5000. To evaluate the robustness of the association between MCPyV antibody positivity and Merkel cell carcinoma, we used a sliding cut point. Statistically significant associations between MCPyV antibody positivity and Merkel cell carcinoma were observed for median fluorescent intensity values between –109 and 29062 (median fluorescent intensity values were corrected for background reactivity as measured by GST reactivity, which was subtracted from the crude median fluorescent intensity value for each antigen, resulting occasionally in corrected median fluorescent intensity values of less than 0). For the cross-sectional study, the cut point for antibody positivity was a median fluorescent intensity of more than 15 000. The cut point for HPV-16 antibodies was 820.25 and was based on the upper quartile of median fluorescent intensity values from control subjects.

**Normalization of Binding to Beads Coated With Mutated VP1 Proteins.** To compare reactivity against mutated VP1 proteins with reactivity against wild-type VP1 proteins, the median fluorescent intensity (MFI) values from each plasma (ie, one well) were normalized as:

$$[\text{MFI}(\text{VP1}_i) - \text{MFI}(\text{GST})]/[\text{MFI}(\text{VP1}_{\text{wt}}) - \text{MFI}(\text{GST})],$$

where VP1<sub>i</sub> is reactivity against MCPyV 350 VP1 or a mutated VP1, VP1<sub>wt</sub> is reactivity against wild-type MCPyV w162 VP1, and GST is reactivity against GST-coated beads. Normalized values from all plasma samples that were positive (ie, median fluorescent intensity of >5000 against MCPyV w162 VP1) were averaged. This experiment was conducted twice with similar results.

**Quantitative Multiplex Antibody Binding Assay.** Using a 1:100 dilution of antibodies resulted in median fluorescent intensity values that were higher than the linear range of the assay for some samples. To ensure that some data points from each positive sample fell within the linear range of the assay, serial dilutions of each antibody-containing sample (hybridoma supernatant, serum, or plasma) were made in blocking buffer. All other steps of the assay were the same as above, including a 1-hour incubation of bead mixture with serum,

plasma, or monoclonal antibody. In one experiment, a single serum with strong reactivity against BKV, JCV, WUPyV, KIPyV, and MCPyV w162 from a control subject without Merkel cell carcinoma was diluted 1:50 in blocking buffer, followed by a series of five 1:5 serial dilutions in wells of a 96-well polypropylene plate (final dilutions when mixed 1:2 [vol/vol] with bead mixture = 1:100 to 1:312 500). In another experiment, plasma samples from the case–control study (or anti-Tag monoclonal antibodies) were diluted 1:166.5 in blocking buffer, followed by seven 1:3 serial dilutions in blocking buffer (final dilutions with antigen-coated bead mixture = 1:300 to 1:729 000). The plates were incubated for 1 hour at room temperature with shaking. These pretreated samples were then transferred to 96-well filter plates, the appropriate antigen-coated beads were added, and all subsequent steps were conducted as described above for the multiplex antibody-binding assay with a 1-hour incubation of antibodies with antigen-coated beads.

The antibody dilution producing one-half of the maximal binding (EC<sub>50</sub> value) was computed by use of median fluorescent intensity values from the experiment with serial dilutions of plasma samples and the curve fitting function in the Prism program (GraphPad Software Inc, La Jolla, CA), which also was used to produce all graphs. For each sample, the median fluorescent intensity values and inverse natural logarithm of the plasma dilution were fit to the sigmoidal dose–response (with variable slope) equation:

$$\text{MFI}_i = y_0 + \frac{a}{1 + 10^{-\left(\frac{x-x_0}{b}\right)}},$$

where y<sub>0</sub> is the lowest median fluorescent intensity value, a is the highest median fluorescent intensity value minus the lowest median fluorescent intensity value, x is the natural logarithm of the antibody dilution, x<sub>0</sub> is the natural logarithm of the plasma dilution at the EC<sub>50</sub> value, and b is the slope of curve. For the 11 plasma samples tested, the R<sup>2</sup> was greater than .991 (95% confidence interval [CI] = 0.987 to 0.995), indicating that this equation was an excellent fit for these data.

To evaluate the reproducibility of this assay when serum vs plasma samples were used, the percentage of positive samples was compared between serum and plasma samples from each of the 75 control subjects and 16 case patients who had both serum and plasma available. A monoclonal antibody against GST (B-14; Santa Cruz Biotechnology, Santa Cruz, CA) was used to verify expression of the MCPyV strain 350 VP1–GST fusion protein (which lacked the epitope tag). The results were identical for 73 (97%) of 75 replicates among control subjects and all 16 replicates from case patients. Because there were fewer serum samples available for case patients and no statistical difference in the proportion positive by each test for control samples (57% and 55%), plasma samples were used for subsequent multiplex antibody assays.

### Amplification of MCPyV DNA From Merkel Cell Carcinoma Tumor Tissue

DNA was extracted from paraffin-embedded Merkel cell carcinoma tumor tissue of patients in the case–control study by use of a QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA) or from fresh Merkel cell tumor tissue with ALLprep DNA/RNA kits by the manufacturers' instructions. Tumor samples from 10 patients

were not available for DNA extraction. Quantitative PCR amplification of MCPyV DNA from tumor samples from 31 case patients with Merkel cell carcinoma was performed as previously described (24). Briefly, two primer sets were used that targeted MCPyV, but not the other human polyomaviruses, and another set of primers were used that targeted the *TPO* gene for comparison (for primer sequences, see Supplementary Table 1, available online). Reactions were conducted in triplicate along with a negative control sample.

### Statistical Methods

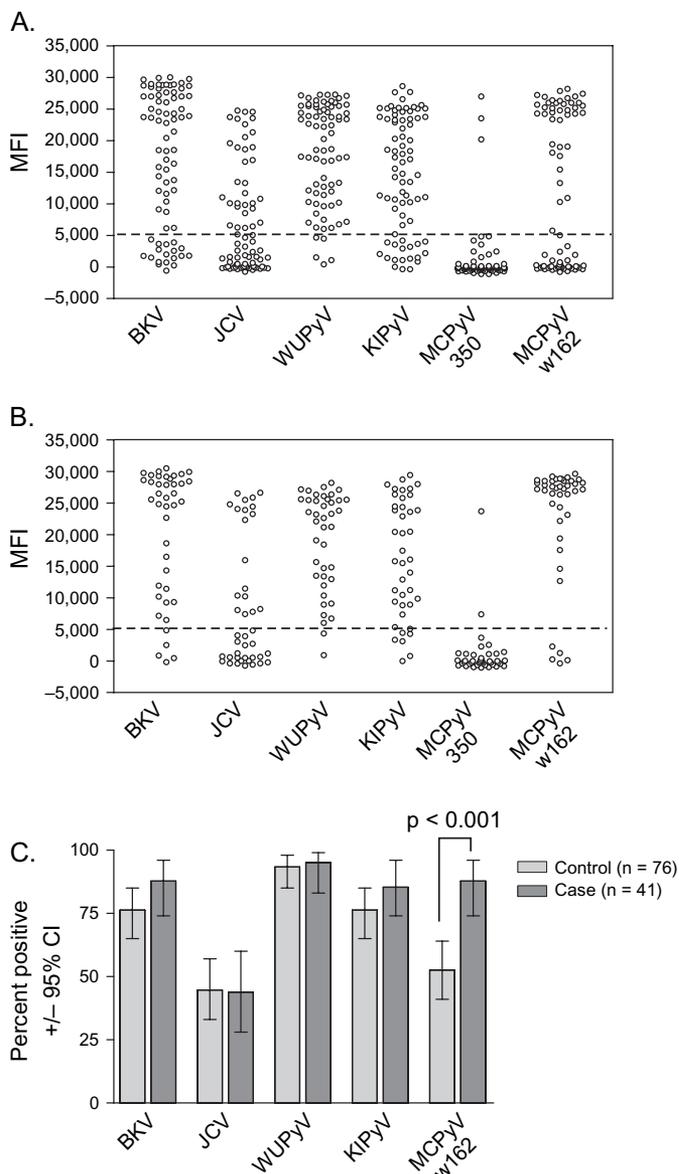
The prevalence of polyomavirus antibodies by age and number of sexual partners were compared by use of trend tests to investigate changes in the prevalence of these factors in case patients and control subjects. Relative risk of Merkel cell cancer was approximated by use of odds ratios (ORs) and 95% confidence intervals derived from logistic regression models. All odds ratios were adjusted for the matching factors, age, and sex.

Two-sided *t* tests were used to compare average median fluorescent intensity values across groups for all analyses. Statistical significance of trends across ordered groups in control group 2 was tested by use of a nonparametric test for trend that is an extension of the Wilcoxon rank sum test with a correction for ties (25). Virus-specific seropositivity rates were compared across control groups (limiting control group 1 to women to match the sex of control group 2) by use of  $\chi^2$  tests. A McNemar test was used to examine the difference between plasma and serum samples and a Fisher exact test was used to compare antibody results to DNA results from case patients. A Mann-Whitney test was used to compare EC<sub>50</sub> values between case patients and control subjects. All statistical tests were two-sided, with a *P* value less than .05 being considered statistically significant.

## Results

### Prevalence of Antibodies Against Polyomaviruses in Case Patients With Merkel Cell Carcinoma and Control Subjects

We assessed levels of antibodies against the five known human polyomaviruses (BKV, JCV, WUPyV, KIPyV, and MCPyV) by use of a multiplex antibody-binding assay and recombinant proteins containing VP1 from these five human polyomaviruses fused to GST. To determine whether human antibody responses were restricted to certain MCPyV strains, we constructed VP1 fusion proteins from MCPyV strains w162 and 350, whose sequences differ at four amino acid positions. For the first experiment, we used plasma samples from 41 case patients with Merkel cell carcinoma and from 76 age- and sex-matched population-based control subjects. Across samples from control subjects, levels of antibodies against MCPyV strain w162 appeared dichotomous (Figure 1). By use of a cut point of 5000 median fluorescent intensity units to define antibody positivity (Figure 1, A), 40 (53%) of the 76 control subjects carried antibodies against MCPyV w162 compared with 36 (88%) of the 41 case patients with Merkel cell carcinoma (OR adjusted for age and sex = 6.6, 95% CI = 2.3 to 18.8) (Figure 1, B and C). Among the 76 control subjects in control group 1, 58 (76%) carried antibodies against BKV, 34 (45%) against JCV, 71 (93%) against WUPyV, and 58 (76%) against KIPyV. No associations were observed between antibody positivity against BKV, JCV, WUPyV, or KIPyV and Merkel cell



**Figure 1.** Reactivity against the five human polyomaviruses in serum from 41 case patients with Merkel cell carcinoma and from 76 age- and sex-matched population-based control subjects in control group 1. **A)** Reactivity of plasma samples from all 76 control subjects in control group 1 against major capsid proteins (VP1 proteins) from five polyomaviruses: BK virus (BKV), JC virus (JCV), WU polyomavirus (WUPyV), KI polyomavirus (KIPyV), and Merkel cell carcinoma polyomavirus (MCPyV strains 350 and w162). Antibody reactivity specific for polyomavirus VP1 proteins was examined by use of a multiplex antibody-binding assay. Each circle represents the median fluorescent intensity (MFI) value from a plasma sample against the fusion protein indicated. **B)** Reactivity of plasma samples from 41 case patients with Merkel cell carcinoma against VP1 proteins from the five polyomaviruses. These plasma samples were tested as described for the control subjects. **C)** Percentage of seropositive case patients and control subjects. Percentages of subjects in panels **A** and **B** who were seropositive for antibodies against each polyomavirus were determined by use of a cut point for seropositivity of more than 5000 MFI units.

carcinoma case status (Figure 1, C), and no associations were observed between MCPyV w162 VP1 reactivity and the reactivity against VP1 proteins from any of these four polyomaviruses. Linear regression analyses also detected no correlation between reactivity against MCPyV w162 VP1 and the reactivity against

VP1 proteins from BKV, JCV, WUPyV, or KIPyV (range of  $r^2$  values = .001–.065) (Figure 2).

### Association of MCPyV Viral DNA in Merkel Cell Carcinomas With MCPyV Antibody Positivity

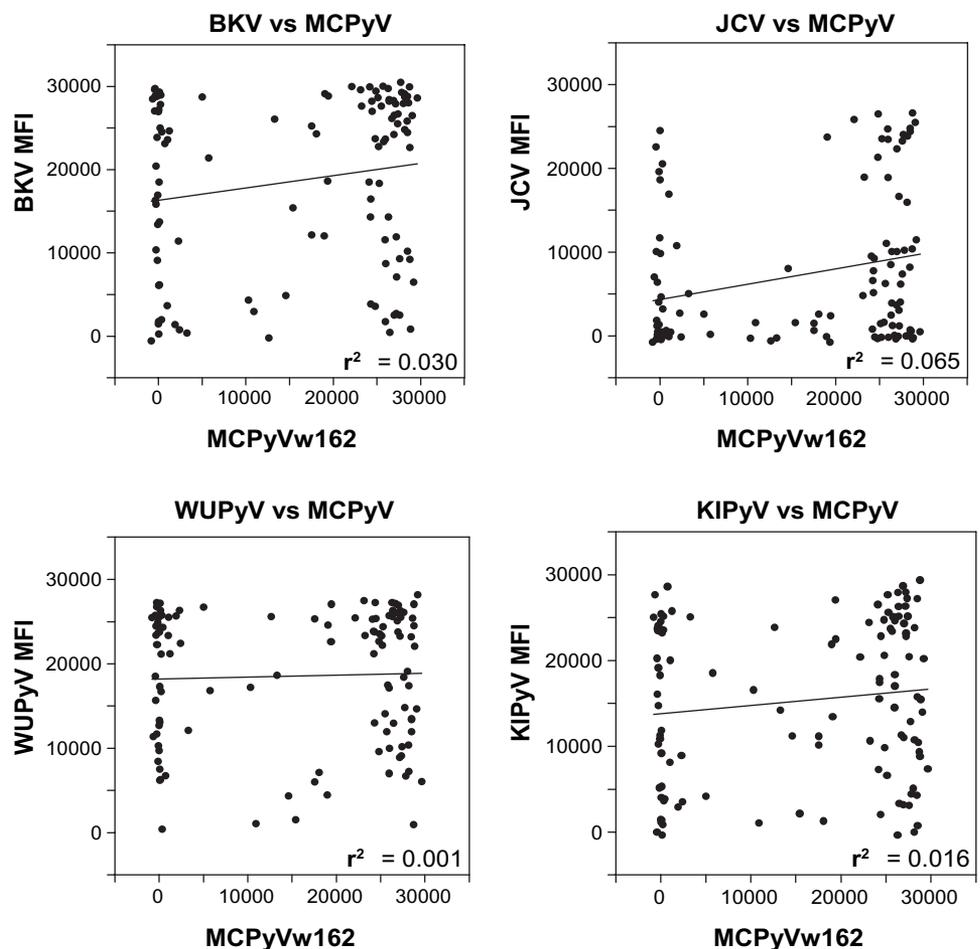
We investigated whether MCPyV viral DNA could be detected in Merkel cell tumors by quantitative PCR and investigated the relationship between positivity for MCPyV w162 VP1 antibody and positivity for viral DNA. Tumor tissue was available from 31 of the 41 case patients with Merkel cell carcinoma. We found that 24 (77%) of these 31 tumors contained MCPyV DNA. Among the 24 case patients who were positive for MCPyV DNA, 22 (92%) were positive for MCPyV w162 antibodies. Among the seven case patients who were negative for MCPyV DNA, all were positive for MCPyV w162 antibody. Thus, the proportion of case patients with MCPyV antibodies was high regardless of the MCPyV DNA status of the tumor.

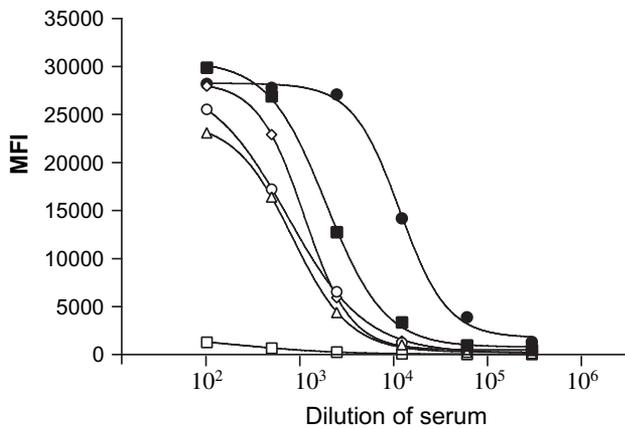
### Identification of Amino Acids Responsible for Low Antibody Binding to MCPyV Strain 350

Although frequent and strong antibody reactivity was observed against the VP1 from MCPyV strain w162 among the serum samples from the subjects in the case–control study, little activity was found against the VP1 from MCPyV strain 350 (Figure 1). In a serum sample with strong MCPyV w162 reactivity, the level of antibodies against VP1 from MCPyV w162 was 6–14 times higher than those of antibodies against VP1 protein from BKV, JCV,

WUPyV, or KIPyV; however, this serum had no reactivity against the VP1 from MCPyV strain 350 (Figure 3). Low reactivity against MCPyV was not explained by lower expression of the MCPyV 350 fusion protein (Supplementary Figure 1, B, available online). Sequences of the VP1 proteins from MCPyV strains w162 and 350 differ by four amino acids. To determine which, if any, of these residues may be responsible for the difference in antibody reactivity, we used site-directed mutagenesis to create a series of recombinant VP1 proteins in which one, two, or three amino acids in the VP1 sequence of strain 350 were replaced with corresponding amino acids in the VP1 sequence of strain w162 (Figure 4, A). We selected residues 288, 316, and 366 because they are evolutionarily conserved among human polyomaviruses under the hypothesis that mutations in conserved regions would disrupt the correct folding of the VP1 proteins. The mutations introduced were a histidine to aspartic acid change at position 288 (H288D), an isoleucine to arginine change at position 316 (I316R), and/or an asparagine to aspartic acid change at position 366 (N366D). Residue 185 also differed between sequences of MCPyV strains 350 and w162 but it was not conserved among the other polyomaviruses and so not investigated further. To determine which amino acids in the VP1 from MCPyV w162 were responsible for reactivity in human plasma, plasma samples from 26 subjects (12 case patients with Merkel cell carcinoma and 14 control subjects) with strong reactivity against MCPyV w162 VP1 (Figure 1) were tested for reactivity against each of the seven recombinant mutant VP1–GST fusion protein and wild-type VP1–GST fusion

**Figure 2.** Correlation between reactivity against the major capsid protein (VP1) of Merkel cell polyomavirus (MCPyV) strain w162 and against VP1 proteins of the four other polyomaviruses: BK virus (BKV), JC virus (JCV), WU polyomavirus (WUPyV), and KI polyomavirus (KIPyV). Reactivity was determined in a multiplex antibody-binding assay. Data are expressed as median fluorescent intensity (MFI) for reactivity against VP1 protein of the polyomaviruses as indicated from 76 control subjects from control group 1 and from 41 case patients with Merkel cell carcinoma. No distinctions were made between results from control subjects and from case patients, and each dot represents one plasma sample. Lines = data from linear regression analyses. Correlation coefficients ( $r^2$ ) are at the lower right corners.





**Figure 3.** Quantitative multiplex binding assay. An antigen-coated bead mixture was incubated with serum that was highly reactive against the major capsid protein (VP1) from MCPyV strain w162 and other polyomaviruses. Before mixing with the antigen-coated beads, the serum was serially diluted 1:5, starting at a dilution of 1:100. Data are the median fluorescent intensity (MFI) values, and the curves were generated by fitting the data to a sigmoidal curve by use of the computer program GraphPad PRISM. BK virus (solid squares), JC virus (open triangles), WU polyomavirus (open circles), KI polyomavirus (open diamonds), Merkel cell polyomavirus strain w162 (solid circles), and Merkel cell polyomavirus strain 350 (open squares). The data are from a representative experiment of two experiments. Results of both experiments were similar.

proteins for both MCPyV strains w162 and 350. Reactivity against MCPyV fusion proteins, expressed as median fluorescent intensity, was normalized to reactivity against MCPyV w162 and an average normalized value was calculated for MCPyV 350 VP1 and each mutated VP1 fusion protein. Although binding of human antibodies to the recombinant VP1 proteins with a single mutation H288D or I316R was no greater than the binding to MCPyV 350 VP1, binding to the VP1 recombinant protein with two mutations H288D and I316R was indistinguishable from binding to MCPyV w162 VP1 (Figure 4, B). The N366D mutation did not affect antibody binding, either as a single mutation or in combination with other mutations. Similar levels of fusion protein expression were confirmed by use of an anti-Tag antibody (Supplementary Figure 2, available online).

Partial sequencing of the MCPyV VP1 open reading frame from DNA isolated from seven additional Merkel cell tumors showed that amino acids at positions 288, 316, and 366 in all seven VP1 sequences were identical with those in the VP1 sequences from viruses MCPyV w162 and MCPyV 339 (Figure 4, C). Among the seven tumors, four had a glutamine at position 185 and three had an aspartate. It should be noted that the MCPyV VP1 from tumor 179 had a stop codon at position 123 (Genbank accession No. FJ649206). Thus, the nonconserved amino acids at positions 288, 316, and 366 found in MCPyV strain 350 do not appear to be typical of MCPyV VP1 proteins found in tumors.

### Quantitative Assessment of Anti-MCPyV Antibodies

To determine whether levels of antibodies against all five human polyomaviruses were different between antibody-positive case patients and antibody-positive control subjects and to explore potential cross-reactivity further, reactivity against VP1 proteins from all five polyomaviruses was determined in the plasma from the 24 subjects with the highest MCPyV w162 reactivity (12 case

patients and 12 control subjects) by use of a quantitative antibody-binding assay (Figure 5). Levels of antibody against all VP1 fusion proteins were compared by use of their median EC<sub>50</sub> values, which are expressed as a dilution of the plasma. The case patients tended to have higher levels of anti-MCPyV VP1 antibodies (median EC<sub>50</sub> = dilution of 1:24 100) than the 12 control subjects (median EC<sub>50</sub> = dilution of 1:7457); however, this association did not achieve statistical significance (Mann-Whitney  $P = .31$ ). When reactivity against MCPyV VP1 was compared with reactivity against the other polyomavirus VP1 proteins, we found that the level of antibodies against MCPyV was an order of magnitude higher than those of antibodies against the other four polyomavirus VP1 proteins (eg, average EC<sub>50</sub> values for BKV: case patients = 1:1470 and control subjects = 1:1170) (Figure 5). This observation argues against the possibility that MCPyV reactivity resulted from cross-reactivity against other known polyomaviruses.

### Prevalence of Antibodies Against Polyomaviruses in the General Population

To better understand the prevalence of antibodies to MCPyV and other polyomaviruses in individuals who do not have Merkel cell carcinoma, we tested serum from the 451 control subjects from the general population (control group 2) for reactivity against VP1 proteins from MCPyV w162 and the other four polyomaviruses and for reactivity against the L1 protein from HPV-16. [The prevalence of L1 antibodies in these 451 subjects has been reported previously (18).] Among these 451 control subjects, levels of antibodies against MCPyV VP1 fell into a distinctly bimodal distribution (Figure 6). A cut point of 15 000 median fluorescent intensity units was chosen as described above and used to identify 268 (59.4%) of the 451 control subjects as seropositive for MCPyV VP1; this percentage was similar to that among women in control group 1 (ie, 53%). Among these 451 control subjects, the prevalence of antibodies against VP1 proteins from the other four types of human polyomaviruses was 413 (92%, 95% CI = 89% to 94%) for BKV, 203 (45%, 95% CI = 40% to 50%) for JCV, 440 (98%, 95% CI = 96% to 99%) for WUPyV, and 406 (90%, 95% CI = 87% to 93%) for KIPyV (Figure 7, A). When results from control group 2 (all women) were compared with results from the women in control group 1, only BKV prevalence was statistically significantly different (ie, in the control group 2, 413 [92%, 95% CI = 89% to 94%] BKV-positive subjects of 451 control subjects, and in control group 1, 19 [79%, 95% CI = 58% to 93%] BKV-positive subjects of 24 control subjects;  $P = .02$ ).

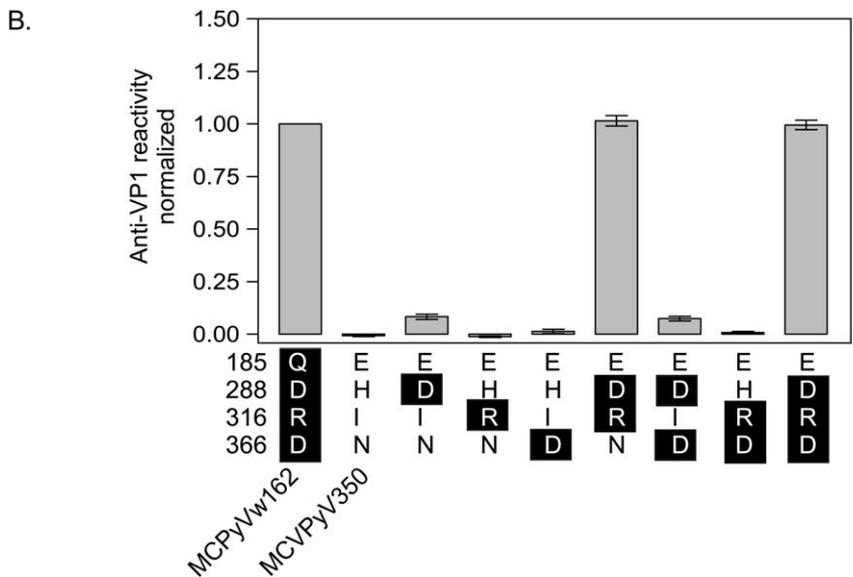
Among the 47 control subjects in the youngest age group (ie, those aged 18–29 years) of control group 2, 26 (57%) were seropositive for MCPyV w162 VP1 antibodies (Figure 7, B). In addition, the percentage of MCPyV VP1 antibody-positive individuals did not vary by age group ( $P_{\text{trend}} = .6$ , comparing age groups in Figure 7, B), indicating that most MCPyV w162 infections occurred before age 30 years. For HPV-16, the highest percentage of those seropositive for HPV-16 L1 antibodies was observed in the youngest age group of women (42.6%) and the percentage declined with increasing age ( $P_{\text{trend}} = .01$ , comparing age groups in Figure 7, B); this pattern is consistent with the sexual transmission of HPV-16. For BKV, more women in the youngest age group (98%) than those in other age groups were positive for antibody against BKV VP1, and there was a statistically significant decline in the percentage of BKV

**Figure 4.** Identification of amino acid residues in the major capsid protein (VP1) of Merkel cell polyomavirus (MCPyV) 350 that confer low seroreactivity. **A)** Alignment of VP1 sequences of three MCPyV strains and of the four other human polyomaviruses: BK virus (BKV), JC virus (JCV), KI polyomavirus (KIPyV), and WU polyomavirus (WUPyV). Sequences were obtained from GenBank and aligned with ClustalW (<http://align.genome.jp/>). Sequences containing residues corresponding to the four divergent residues in the MCPyV VP1 sequence are shown, with divergent residues boxed. Residue numbers refer to those in the MCPyV sequence. GenBank accession numbers for the VP1 sequences shown were FJ392560 for MCPyV w162, EU375803 for MCPyV 350, EU375804 for MCPyV 339, NC\_001538 for BKV, NC\_001699 for JCV, EF127906 for KIPyV, and EF444549 for WUPyV.

**A.**

Virus	Amino acid position																			
	185				288				316				366							
MCPyV350	Q	T	E	Y	P	C	A	H	I	V	R	K	I	W	V	P	G	N	P	D
MCPyV339	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
MCPyVw162	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
BKV	R	S	K	Y	P	A	A	D	I	C	R	K	R	S	V	P	G	D	P	D
JCV	R	T	K	Y	P	A	V	D	V	C	R	K	R	R	V	P	G	D	P	D
KIPyV	K	Y	K	G	P	C	A	D	M	L	R	Q	R	R	V	P	T	S	V	N
WUPyV	K	Y	K	G	P	C	A	D	L	L	R	Q	R	R	V	P	T	T	S	N

**B)** Identification of VP1 residues essential for antibody recognition of MCPyV strain w162. Point mutations in the MCPyV 350 VP1 sequence and reactivity in plasma against the mutant proteins are shown. Point mutations at positions 288, 316, and 366 were inserted in the MCPyV 350 VP1 sequence either alone or in combination by use of site-directed mutagenesis. All mutant proteins were expressed as GST-VP1 fusion proteins in bacteria and tested in a multiplex antibody-binding assay with 28 plasma samples that had been shown to have reactivity against MCPyV w162 VP1 (Figure 1). The letters listed vertically on the x-axis are the one-letter amino acid abbreviations for residues at the sequence position in the VP1 protein listed on the left. Letters that are white on a black background indicate that the sequence is the same as the MCPyV w162 VP1 sequence and letters that are black on a white background indicate that the sequence is the same as that of MCPyV 350 VP1. Data are the average median fluorescent intensity (MFI) of the 28 plasma samples with reactivity against MCPyV w162 VP1 (one test per sample) after subtracting background and normalizing to the MFI for MCPyV w162. **Error bars** = 95% confidence intervals. The data are from a representative experiment of an experiment conducted twice.



**C)** Comparison of VP1 sequences from nine MCPyV strains. Partial sequences from seven Merkel cell tumors were compared with the full-length sequences of VP1 proteins from MCPyV strains w162 and MCPyV350 (obtained from GenBank). Sequences around the nonconserved residues 185, 288, 316, and 366 are shown. Immunoreactive amino acids at positions 288 and 316 (**shaded**) are conserved in all sequences except for that of MCPyV strain 350. The amino acid sequences were identical to those of strains w162 and 339 (data not shown), except for a D221N substitution at position 235 and a stop codon at position 123 in sequence 179 (GenBank accession numbers = FJ649201–FJ649207).

**C.**

MCPyV strain	Amino acid position																			
	185				288				316				366							
350	Q	T	E	Y	P	C	A	H	I	V	R	K	I	W	V	P	G	N	P	D
162	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
244	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
235	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
250	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
205	Q	T	Q	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
160	Q	T	E	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
179	Q	T	E	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D
234	Q	T	E	Y	P	C	A	D	I	V	R	K	R	W	V	P	G	D	P	D

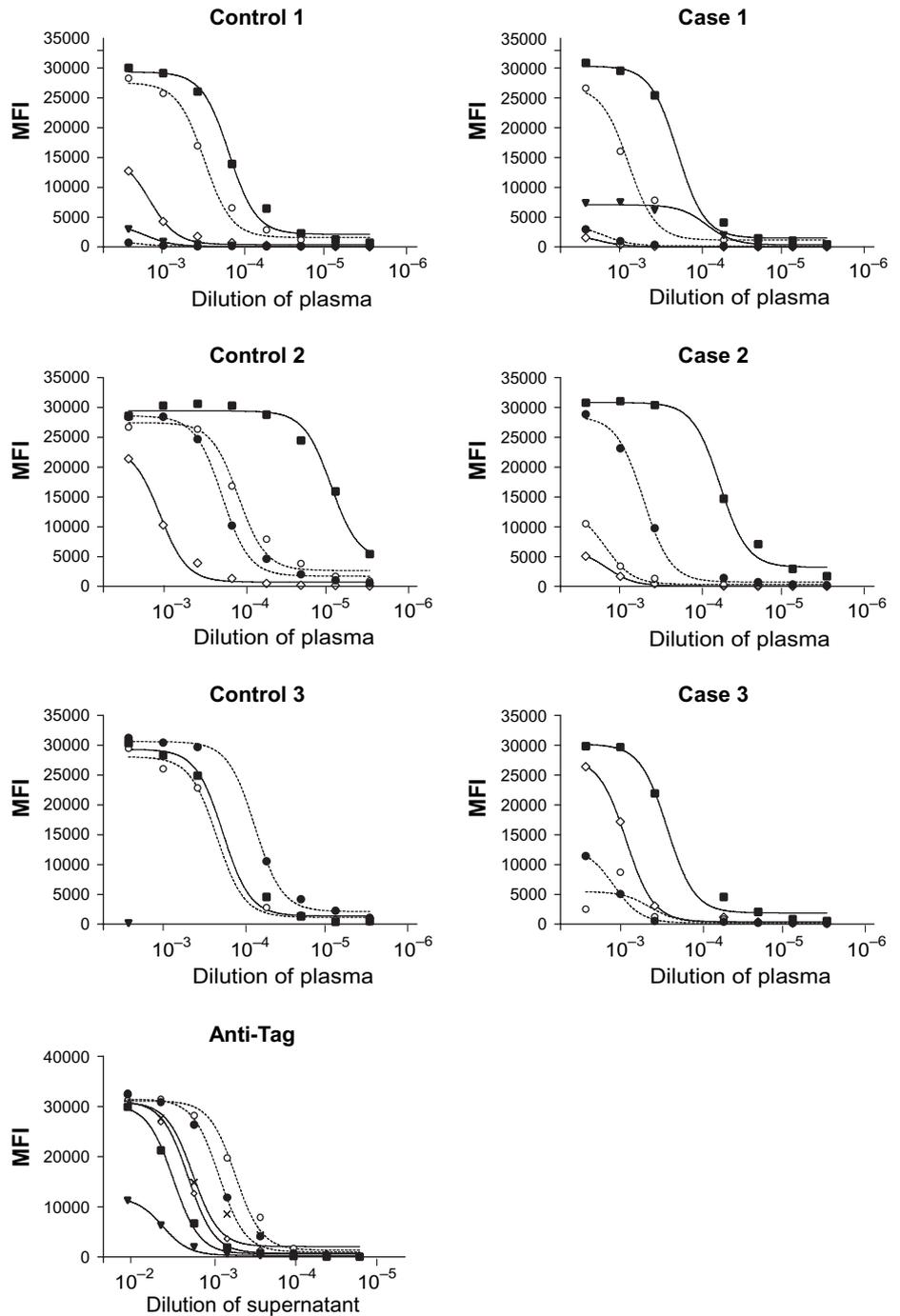
VP1 antibody-positive women with increasing age ( $P_{\text{trend}} = .004$ ; Table 1). For JCV, there was a small but statistically significant increase in the percentage of JCV antibody-positive women with increasing age ( $P_{\text{trend}} = .04$ ). No associations were found between carrying antibody against WUPyV or KIPyV and increasing age, indicating that most of those infections also occurred during childhood. In addition, antibody against VP1 proteins from WUPyV or KIPyV persisted until at least age 70 years.

Among women in control group 2, MCPyV w162 antibody positivity was not associated with the lifetime number of sexual partners ( $P = .2$ ) (Figure 7, C), supporting a nonsexual route of

transmission. As expected for the sexual transmission of HPV-16, we observed a strong association between the lifetime number of sex partners and antibody positivity for HPV-16 ( $P < .001$ ). In addition, antibody positivity against any of the other four human polyomaviruses was not associated with the number of sex partners (data not shown).

## Discussion

We found that antibodies specific for MCPyV were detected in statistically significantly more case patients with Merkel cell

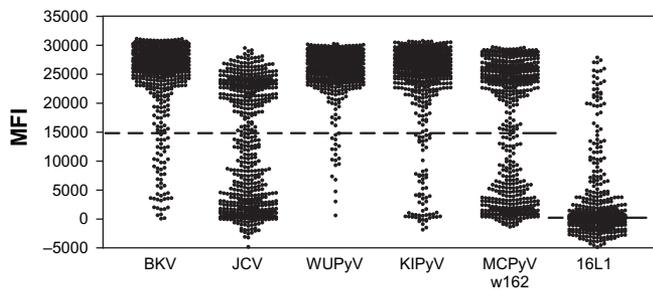


**Figure 5.** Quantitative measurement of antibodies against the major capsid protein (VP1) of human polyomaviruses. A quantitative multiplex antibody-binding assay was used to measure antibodies in plasma from case patients with Merkel cell carcinoma and control subjects (control group 1) who had tested strongly positive in the multiplex antibody-binding assay (Figure 1). Plasma (or hybridoma supernatant) was diluted to 1:333 followed by eight 1:3 serial dilutions (with the highest dilution =  $1:7.29 \times 10^5$ ) and tested for reactivity against VP1 proteins from the following polyomaviruses: BK virus, JC virus, WU polyomavirus, KI polyomavirus, and Merkel cell carcinoma polyomavirus virus (MCPyV). Data from one representative case patient with Merkel cell carcinoma or one representative control subject are shown in each panel. Data from other subjects were similar. Data points are the median fluorescent intensities (MFIs) for each antigen, which is a relative measure of the amount of human IgG bound to the fusion proteins. Curves were generated (and the antibody dilution producing one-half of the maximal binding [ $EC_{50}$  value] was calculated) by fitting the data to an equation for a sigmoidal curve. The plasma samples used in this experiment had been identified previously as having high reactivity against the VP1 of MCPyV w162. The data are from a representative experiment of two experiments. Results of both experiments were similar.

carcinoma than control subjects. This association was found to be specific for MCPyV and not due to the presence of cross-reactive antibodies against other polyomaviruses. These findings support a role for MCPyV in the etiology of this rare skin carcinoma. However, exposure to MCPyV, as measured by the presence of antibodies, like exposure to the other known human polyomaviruses, was found to be common in the general population. The high concentration of antibodies against MCPyV suggests that there may be a viremic or systemic stage of MCPyV infection as described for BKV or JCV (26). This result is in contrast with the low concentrations of antibodies against papillomaviruses whose infections are restricted to the epithelium.

Also similar to other polyomaviruses, infection with MCPyV occurred before young adulthood, and the prevalence was stable at older ages. We have not had an opportunity to examine serum from children to pinpoint the age of infection more precisely, but we have found that seropositivity was not more common in young adults than in older adults or in those with a higher numbers of sex partners than in those with a lower number.

Evidence for the specificity of these antibody-binding assays was based on the following observations: 1) No association was observed between seropositivity to MCPyV and to any other polyomavirus. 2) Patterns of responses to the polyomavirus VP1s varied greatly between subjects. 3) High levels of MCPyV antibodies did not



**Figure 6.** Distribution of seroreactivity against the major capsid proteins (VP1) of all five human polyomavirus (BK virus [BKV], JC virus [JCV], WU polyomavirus [WUPyV], KI polyomavirus [KIPyV], and Merkel cell carcinoma polyomavirus virus [MCPyV]) and human papillomavirus (HPV) 16 L1 protein among 451 women in population-based control group 2. The multiplex antibody-binding assay was used to assess seroreactivity in 451 samples previously tested for HPV-16 L1 reactivity (18). The background median fluorescent intensity (MFI) values were subtracted from MFI values for each VP1 and for HPV-16 L1 and data were plotted. **Dotted lines** indicate the cut points of 15000 for polyomaviruses and of 820.25 for the HPV-16 L1. The HPV-16 L1 cut point was the highest quartile of values.

correlate with high levels of antibodies against VP1s from other polyomaviruses. 4) Antibody positivity to MCPyV but not the other polyomaviruses was strongly associated with Merkel cell carcinoma.

The higher antibody prevalence among case patients with Merkel cell carcinoma is consistent with the hypothesis that MCPyV plays a role in the development of Merkel cell carcinoma. The lack of association between Merkel cell carcinoma and antibody positivity to the other polyomaviruses indicates that these patients are not globally susceptible to polyomavirus infection or reactivation. Although immunosuppression has been associated with Merkel cell carcinoma (2) and immunosuppression often leads to reactivation of polyomaviruses, higher levels of antibody positivity against other polyomaviruses were not observed.

Studies (3,24,27,28) that have tested for the MCPyV genome in Merkel cell carcinoma have found that between 15% and 30% of the tumors had no detectable viral DNA. Indeed, in this study, we detected MCPyV DNA in 24 (77%) of 31 case patients. Intriguingly, all seven case patients with Merkel cell carcinoma whose tumors were negative for MCPyV DNA had plasma that was positive for MCPyV VP1 antibodies. This result raises the possibility that MCPyV plays a role in cancer initiation but is not required for maintenance of the tumorigenic phenotype in all case patients and may be one explanation as to why some Merkel cell carcinomas are negative for MCPyV DNA. Alternatively, because MCPyV infections are common, there may be MCPyV-independent pathways that lead to Merkel cell carcinoma.

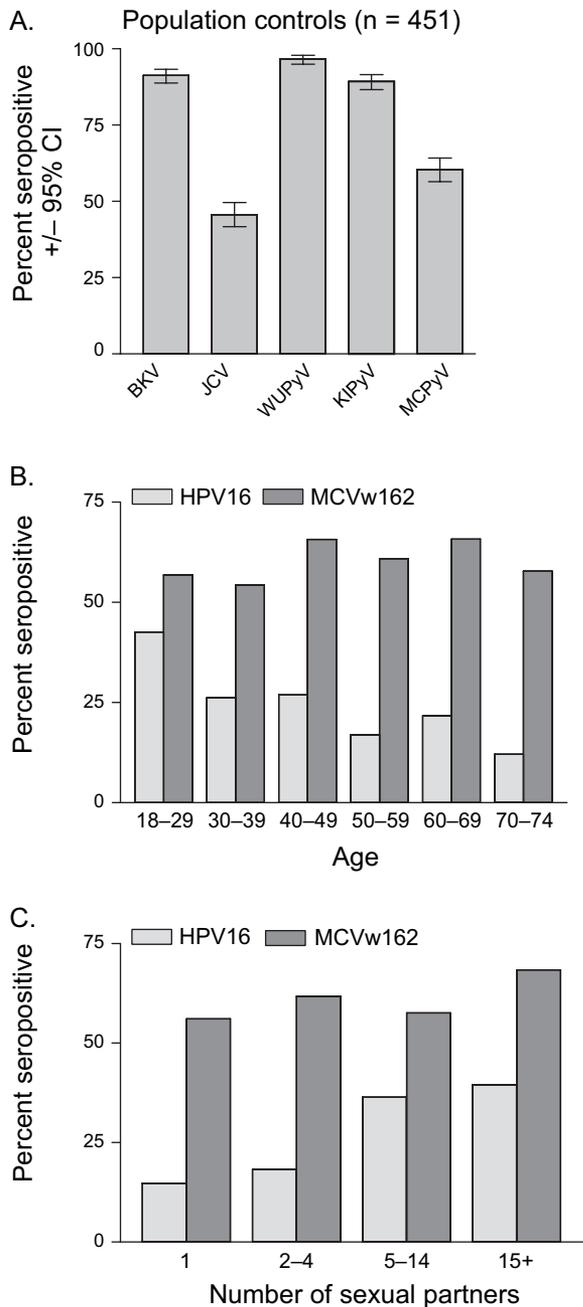
MCPyV has been shown to be integrated into the DNA of some Merkel cell carcinoma tumors (3), analogous to the manner in which high-risk HPV DNA is integrated into the DNA of cervical cancers (29). However, because some Merkel cell carcinomas do not appear to contain MCPyV DNA and other Merkel cell carcinoma tumors contain much less than one viral genome per cell, the role that this virus plays in cancer development might be quite different from that of HPV in the development of cervical cancer (30). Another apparent difference in the devel-

opment of these cancers is the longer time interval between initial exposure to MCPyV (before age 30 years) and development of Merkel cell carcinoma (median age = 70 years). For cervical cancer, the interval is approximately 30 years (eg, from HPV infection at age 20 years to diagnosis of cervical cancer at age 50 years) (31). The long time interval for the development of Merkel cell carcinoma suggests that rare genetic event(s) might be occurring in addition to the common event of MCPyV infection (32).

We have previously shown that a higher proportion of patients with Merkel cell carcinoma from North America than from Australia were positive for MCPyV DNA (24). This difference may be due to differences in sun exposure, the presence of an undetectable MCPyV strain, or heterogeneous distributions of MCPyV worldwide. Future studies should examine the prevalence of MCPyV antibodies in different geographic populations. Furthermore, if the findings in our study are similar to those of other case-control studies, a prospective study with samples collected before diagnosis of Merkel cell carcinoma would be an important step to establishing causality. In this study, samples from case patients were collected after diagnosis of Merkel cell carcinoma, so the disease process may have had an impact on the antibody level. However, this study was matched on sex and age, which reduces possible confounding by these factors.

In comparison to the results from MCPyV strain w162, only two of 41 case patients had detectable levels of antibodies against MCPyV strain 350. By mutating each of three discordant residues in the VP1 sequence for MCPyV strain 350 (residues 288, 316, and 366) to those of strain w162, alone or in combination, we found that both residues 288 and 316 were required to confer MCPyV w162-like binding by human serum to the strain 350 VP1 sequence. It is likely that these two mutations altered the folding of VP1 from MCPyV 350 to ablate the formation of conformation-dependent epitopes recognized by human serum, but it was not possible to determine whether MCPyV-specific antibodies recognize only native VP1 protein because denatured GST-VP1 fusion proteins did not bind to the polystyrene beads (results not shown). Sequences of the open reading frames of MCPyV VP1 from seven additional tumors were all identical with VP1 proteins from MCPyV strains 339 and w162 at positions 288, 316, and 366 (position 185 was either glutamine or glutamic acid). This finding is reminiscent of the first cloned HPV-16 L1 sequence from a cervical cancer, which had a non-conservative mutation that inhibited capsid assembly and binding by antibodies against L1 that were specific to conformation-dependent epitopes (33).

This is the first study, to our knowledge, to test for antibodies against all five polyomaviruses simultaneously. The prevalence of antibodies against BKV and JCV, which we reported, is consistent with previous observations (10,13,34). A previous study (34) reported that the prevalence of BKV decreased with increasing age and that JCV antibody prevalence peaked at approximately age 60 years. Approximately 90% of people in our control groups were positive for antibody against WUPyV and/or KIPyV, the two polyomaviruses that were recently discovered in respiratory infections (8,9). The prevalence of antibodies against these



**Figure 7.** Association of seropositivity against the major capsid protein (VP1) from Merkel cell polyomavirus (MCPyV) w162 or L1 protein from human papillomavirus 16 (HPV-16) with age or number of sexual partners. **A)** Percentage of women in the general population who are seropositive for polyomaviruses. Reactivity against the major capsid proteins (VP1) of the five human polyomaviruses (BK virus [BKV], JC virus [JCV], WU polyomavirus [WUPyV], KI polyomavirus [KIPyV], and Merkel cell carcinoma polyomavirus virus [MCPyV]) and HPV-16 L1 protein was assessed in serum from 451 control subjects (in the population-based control group 2) by use of the multiplex antibody-binding assay. Data are the proportion of seropositive samples for each polyomavirus type tested. **Error bars** = 95% confidence intervals (CIs). **B)** Association of MCPyV and HPV-16 seropositivity with age. Age-stratified seropositivity for HPV-16 (lightly shaded bars) and MCPyV w162 (solid bars). The following numbers of serum were evaluated in each group: 47 in the age group of 18–29 years (MCPyV seropositivity = 55.3%, 95% CI = 40.1% to 69.8%; HPV-16 seropositivity = 42.6%, 95% CI = 28.3% to 57.8%), 122 in the age group of 30–39 years (MCPyV = 55.7%, 95% CI = 46.5% to 64.7%; HPV-16 = 26.2%, 95% CI = 18.7% to 35.0%), 119 in the age group of 40–49 years (MCPyV = 64.7%, 95% CI = 55.4% to 73.2%; HPV-16 = 26.9%, 95% CI = 19.2% to 35.8%), 83 in the age group of 50–59 years (MCPyV = 59.0%,

polyomaviruses was not statistically significantly different in the age groups tested. Future prospective studies should examine serum from younger populations to determine the time most people become infected with MCPyV and the other newly identified polyomaviruses.

This study had several limitations, including retrospective enrollment of case patients and control subjects. A long-term prospective study that could track antibody response to the virus and cancer development as they develop over time would theoretically be ideal. In such a study, the numbers of case patients with Merkel cell carcinoma among those exposed and unexposed to the virus could directly be compared. However, given the high prevalence of MCPyV antibodies starting at young ages and the rarity of the cancer outcome among older adults, this ideal study would be inefficient. The efficient case–control study design that we used and the strong risk of Merkel cell carcinoma that we found to be associated with MCPyV support the expectation that these findings are likely to be accurate. Another potential limitation is that the study subjects were primarily white, which might constrain how widely these results can be generalized. However, in US Surveillance, Epidemiology, and End Results data, more than 95% of Merkel cell carcinomas were reported in white patients (35). Another limitation of this study was lack of gold standards for MCPyV antibody positivity and negativity. Any nondifferential misclassification would tend to reduce the association; therefore, the robust association observed here between MCPyV w162 antibody reactivity and Merkel cell carcinoma supports the likelihood of the principal findings of this study.

We conclude that MCPyV is a relatively common infection but the higher prevalence among case patients compared with control subjects is consistent with the hypothesis that this virus has a role in the development of Merkel cell carcinoma. It should be remembered that Merkel cell cancer is rare, with an age-adjusted incidence rate of 0.44 per 100 000 individuals (36). Thus, although antibody reactivity against MCPyV was associated with an increased risk of developing Merkel cell cancer, the absolute risk of cancer development in MCPyV antibody-positive individuals is exceedingly low.

95% CI = 47.7% to 69.7%; HPV-16 = 16.7%, 95% CI = 9.5% to 26.7%), 46 in the age group of 60–69 years (MCPyV = 65.2%, 95% CI = 49.8% to 78.6%; HPV-16 = 21.7%, 95% CI = 10.9% to 36.4%), and 33 in the age group of 70–74 years (MCPyV = 51.5%, 95% CI = 33.5% to 69.2%; HPV-16 = 12.1%, 95% CI = 3.4% to 28.2%). For the association between seropositivity for HPV-16 L1 and age,  $P_{\text{trend}} = .002$ . For the association between seropositivity for MCPyV and age,  $P_{\text{trend}} = .6$ . **C)** Association of MCPyV and HPV-16 seropositivity with number of sex partners. **Lightly shaded** = seropositivity against HPV-16 L1, **solid bars** = MCPyV w162 VP1. The following numbers of serum samples were evaluated in each group: 123 in the one-partner group (MCPyV seropositivity = 55.7%, 95% CI = 46.5% to 64.7%; HPV-16 seropositivity = 14.8%, 95% CI = 9.0% to 22.3%), 131 in the two- or three-partner group (MCPyV = 61.8%, 95% CI = 52.9% to 70.2%; HPV-16 = 18.3%, 95% CI = 12.1% to 26.0%), 151 in the five- to 14-partner group (MCPyV = 57.6%, 95% CI = 49.3.5% to 65.6%; HPV-16 = 28.8% to 44.6%), and 38 in the group with 15 or more partners (MCPyV = 68.4%, 95% CI = 51.3% to 82.5%; HPV-16 = 39.5%, 95% CI = 24.0% to 56.6%). For the association between seropositivity against HPV-16 L1 and number of sex partners,  $P_{\text{trend}} < .001$ . For the association between seropositivity against MCPyV VP1 and number of sex partners,  $P_{\text{trend}} = .5$ .

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## Funding

National Institutes of Health (AI038382, CA042792 to D.A.G.) (T32 CA80416-10 to K.P.), American Cancer Society (RSG-08-115-01-CCE to P.N.), ACS Jerry Wachter Merkel cell carcinoma Fund (to P.N.), and University of Washington Merkel cell carcinoma Patient Gift Fund (to P.N.).

## Notes

P. Nghiem is the co-senior author.

Analyses were conducted by M. M. Madeleine, L. G. Johnson, and J. J. Carter.

The authors were fully responsible for the design of the study, the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

We thank Drs Michael Pawlita, Robert Garcea, Tobias Allander, and David Wang for providing us with the plasmids used for expressing polyomavirus proteins. The assistance of Joleen Weese and Elizabeth Jensen in the sequencing facility and Renee Thibodeau and Elizabeth Donato for preparation of paraffin-embedded tissue is appreciated. These studies would not have been possible without the participation of the study subjects.

Notes in added proof: Since the submission of this article, Kean et al. (14) reported on a seroepidemiological study of human polyomaviruses that used virus-like particles. Their results were largely consistent with our findings; however, no serum was tested from patients with Merkel cell carcinoma. Tolstov et al. (37) have reported an association between MCPyV-neutralizing antibodies and Merkel cell carcinoma by use of a pseudovirus neutralization assay.

Manuscript received January 30, 2009; revised July 27, 2009; accepted August 25, 2009.