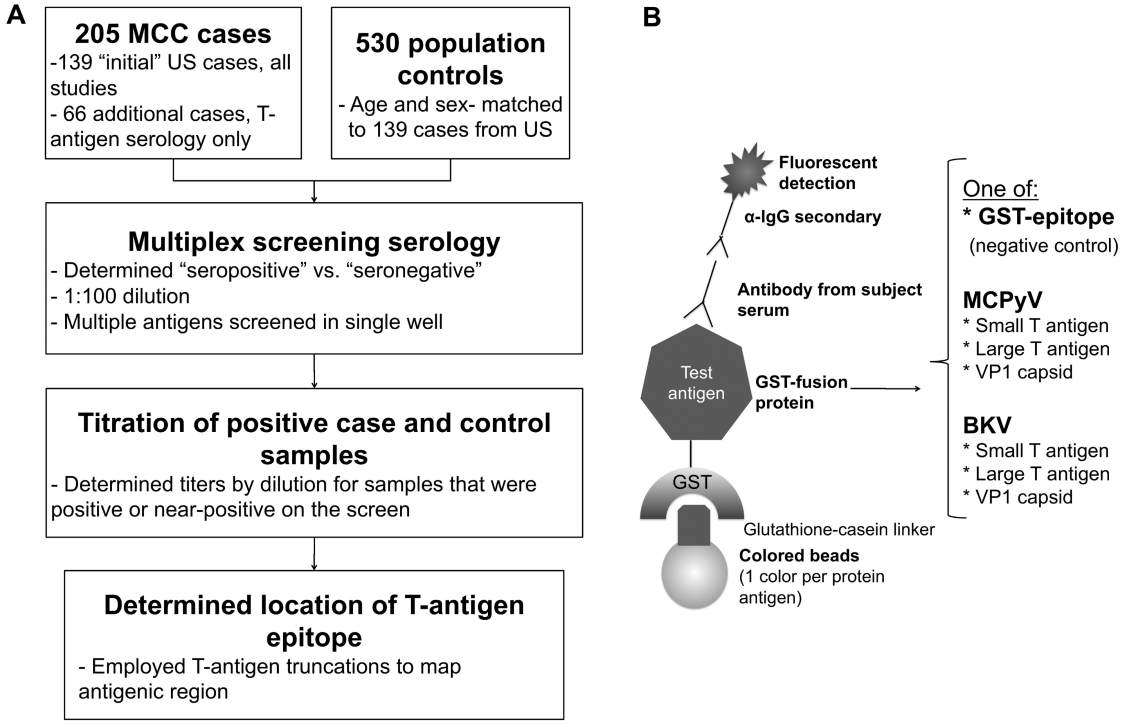
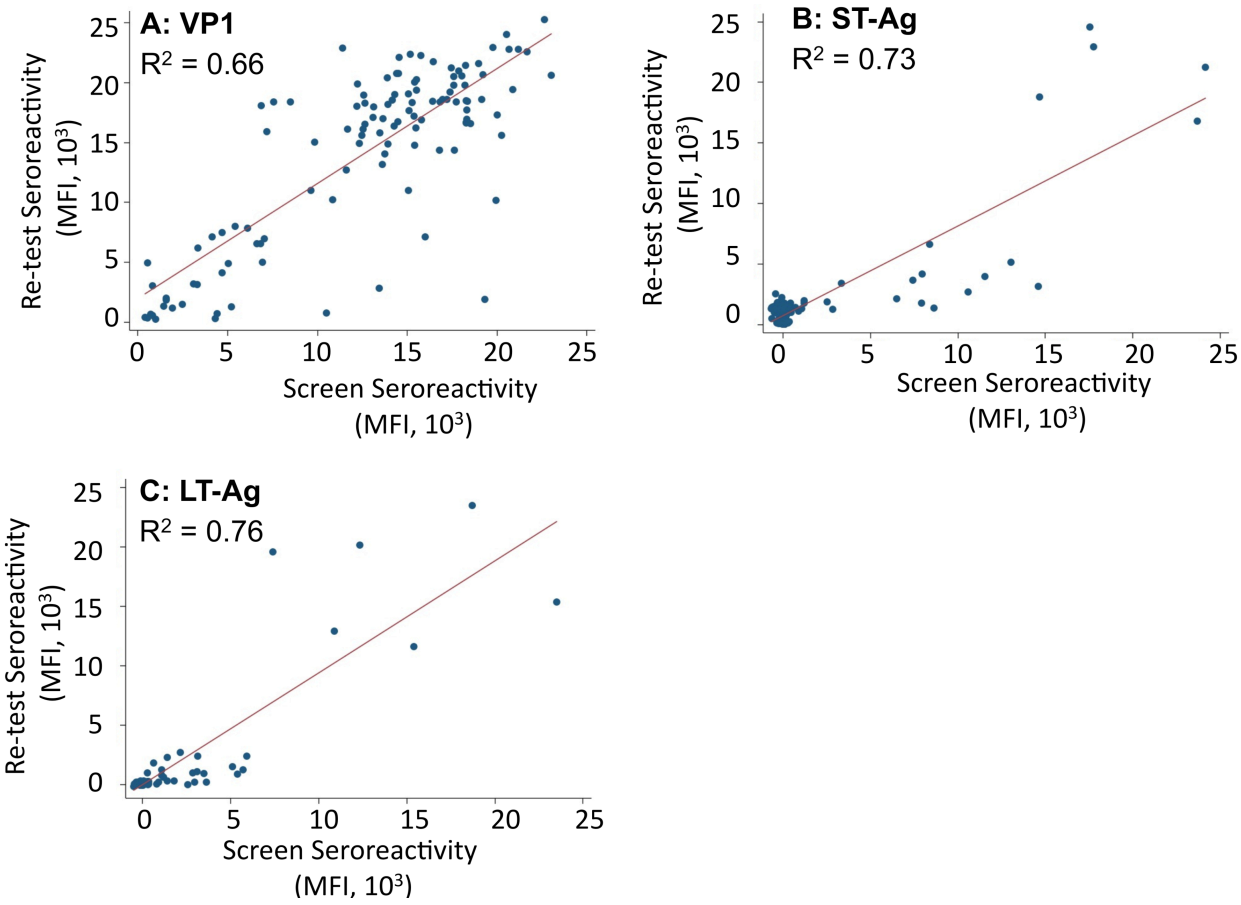
Paulson KG, Carter JJ et al.

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-ags, 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 SalI, 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: actagtaccatggatttagtcctaaatagg, ggtcccatatataggggcctcgtcaacctcatcaaacatcgagaagtcacttcgaagtgacttctctatgtttgatgaggttgacgaggcccctatatatcggacc and actagtttattgagaaaaagtaccagaatcttgg. 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- cells (New England Biolabs, Ipswich, MA). DNA encoding LT was gel isolated from dam- cells following digestion with BclI (compatible end with BamHI digested DNA), SalI 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-agusing 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-agnot 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 SalI 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/SalI 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 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 superblock [Chemicon International, Temedula, 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.

Supplemental references

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3. Sehr P, Muller M, Hopfl R, Widschwendter A, Pawlita M. HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. J Virol Methods 2002;106:61-70.

4. Pogulis RJ, Vallejo AN, Pease LR. In vitro recombination and mutagenesis by overlap extension PCR. Methods Mol Biol 1996;57:167-76.

5. Waterboer T, Sehr P, Michael KM, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. Clin Chem 2005;51:1845-53.

6. Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. J Immunol Methods 2006;309:200-4.