Antibodies to Merkel Cell Polyomavirus Correlate to Presence of Viral DNA in the Skin

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To validate whether Merkel cell polyomavirus (MCV) serology correlates with MCV infection, we compared real-time polymerase chain reaction results for MCV DNA on fresh-frozen biopsy specimens from various skin lesions and healthy skin from 434 patients to MCV serology results using viruslike particles (VLPs) and MCV neutralization assays. Sixty-five percent of participants were MCV seropositive and 18% were MCV DNA positive. The presence of antibodies was correlated with the presence of virus DNA (odds ratio, 27.85 [95% confidence interval, 6.6–166.5]), with 97% of patients who tested positive for MCV DNA being MCV seropositive. VLP antibody levels correlated to neutralization titers (r = .72), and high antibody levels correlated to high MCV load (P < .01).

Merkel cell polyomavirus (MCV) is a likely etiologic agent of Merkel cell carcinoma (MCC) [1]. MCV-specific antibody responses are very common in the general population, but patients with MCC have higher levels of MCV-specific serum antibodies than control participants [2–4].

The continued elucidation of the epidemiology of MCV infection will require high-throughput serological methods validated as a marker for MCV infection. We developed a high-throughput serology method using heparin-coated Luminex beads coated with MCV viruslike particles (VLPs), similar to the assay previously used for human papillomavirus serology [5]. We also used a neutralization assay employing MCV-based reporter vectors [3]. Serum specimens from participants with nonmelanoma skin cancer or nonmalignant skin lesions were tested in both assays. Biopsy specimens of skin lesions and healthy skin from the same patients were tested in MCV DNA-specific real-time polymerase chain reaction (PCR) assays, allowing quantitative comparison of serology to MCV DNA loads.

METHODS

Overall, 434 immunocompetent patients attending dermatology clinics in Sweden (n = 400) or Austria (n = 34) were included [6]. Seventy-two patients had squamous cell carcinoma (SCC; mean age, 80 years; range 50–94 years), 160 patients had basal cell carcinoma (BCC; mean age, 73 years; range, 34–93 years), and 81 patients had actinic keratoses (AK; mean age, 75 years; range, 53–95 years). As hospital-based controls (N = 121; mean age, 71 years; range, 29–97 years), we enrolled patients attending the same clinics who had nonmalignant skin lesions, the most common being seborrheic keratosis (SK; N = 62).

All participants donated a serum sample and two skin biopsy specimens, one 2-mm biopsy specimen from the lesion and another from healthy skin of the same patient, 10–15 cm from the lesion. Before the biopsy was performed, the skin was anesthetized and stripped with tape to avoid surface contaminations [7], as distinguishing true skin infection from skin surface contamination of virus merely deposited on the skin may otherwise be difficult. Eleven samples were excluded from DNA testing because of insufficient material left (patients with SCC, n = 5; patients with BCC, n = 1; patients with AK, n = 3; patients with SK, n = 1; patients with other benign lesions, n = 1). The study adhered to the Declaration of Helsinki and was approved by the ethical review committees of Karolinska Institute and Lund University (Sweden) and Medical University Vienna (Austria). All patients provided written informed consent.

DNA was extracted as described elsewhere [8]. Primers and probes for quantitative PCR (qPCR) were the same as those described elsewhere [9]. Standard curves used serial dilutions from 100,000 copies to 1 copy of purified MCV DNA (plasmid pCR.MCV; gift from Yuan Chang and Patrick Moore, University of Pittsburgh Cancer Institute). The method routinely...
detected 1 copy per sample. PCR mixtures were prepared in a clean room. The 25-μL PCR mix contained 2.5 μL of sample (diluted 1:2 in TE-buffer [10 mmol/L Tris; pH, 8; 1 mmol/L ethylenediaminetetraacetic acid]), 1× Buffer II (Applied Biosystems), .35 mmol/L magnesium chloride (Applied Biosystems), .2% bovine serum albumin (Fraction V; Sigma-Aldrich), .2 mmol/L dNTP (Fermentas), .2 μmol/L of each primer (Cybergene), .04 μmol/L of the probe (Cybergene), and .625 U of AmpliTaq Gold (Applied Biosystems). The real-time PCR was performed in a GeneAmp 5700 sequence detection system (Applied Biosystems), using 2 min at 50°C, 10 min at 95°C, and then 50 cycles of 15 s at 95°C and 1 min at 60°C. All samples were verified and had to be positive in 2 of 3 runs to be considered positive. Negative samples were also retested with more concentrated (undiluted) samples. The number of copies of cellular DNA in the sample was determined by qPCR for the β-globin gene [10].

MCV-based reporter vector (pseudovirion) stocks were generated by transfection of 293TT cells with MCV VP1/2 expression plasmids pwM and ph2m, respectively, together with or without (for VLP production) a reporter plasmid, phGluc, as described elsewhere [3]. Detailed vector production protocols are available at the Web site http://home.ccr.cancer.gov/LCO. The quality of purified capsids was verified by transmission electron microscopy of capsid stocks adsorbed to carbon-coated grids and stained with uranyl formate. JC virus (JCV) VLPs produced in Saccaromyces cerevisiae were a gift from Kestutis Sasnaukas. These VLPs consist of the major capsid protein VP1 [11].

Luminex COOH beads (Bio-rad) were activated as recommended by the manufacturer and conjugated with heparin at .75 mg/mL (Sigma-Aldrich; no. H4784) in sterile water at room temperature for 16 h. The assay then followed the protocol by Faust et al [5]. Fluorescence signals were recorded using a Bio-Plex 200 Luminex (at low setting). Beads without VLPs were used as controls, and the background median fluorescent intensity (MFI) of each serum sample was subtracted from the VLP reactivity. An MFI signal of >250 for at least 1 serum dilution was used to separate antibody positive samples from negative ones. A human serum sample with a high antibody titer was given an arbitrary antibody level of 10 units. The antibody levels of other samples were calculated relative to the standard, using the parallel line method [12].

An MCV reporter vector stock carrying an encapsidated plasmid encoding a Gaussia luciferase reporter gene New England Biolabs was used to perform neutralization assays, as described elsewhere [3]. We used 36 pg of VP1 per well, combined with serum diluted in final concentrations of 1:10³, 1:10⁴, 1:10⁵, and 1:10⁶. Chemoluminescence was read for .2 s per well using a Wallac Victor 1420 Multilabel counter. Fifty-percent neutralizing titers were calculated using Prism 5 software (Graphpad) to fit a sigmoidal dose-response curve with top and bottom values of 100% or 0% neutralization, respectively. Dose-response curves with r² values of <.9 were excluded from the analysis. Epi Info software (version 3.5.1; Centers for Disease Control and Prevention) was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) with multivariate logistic regression.

RESULTS

We compared the antibody levels detected in the MCV VLP-heparin-Luminex assay with the MCV neutralizing activity for 55 serum samples selected to have a wide range of antibody levels. All samples with antibody levels of >9 units (27 of 434 samples) and 28 samples with antibody levels of .9–8 units (3 or 4 samples for each unit level) were selected. The antibody levels detected in the VLP-heparin-Luminex assay and the neutralizing activity had a good correlation (R = .72; P < .001).

Overall, 18% of the 434 patient samples were MCV DNA positive in the qPCR analysis. The proportion of MCV DNA positive samples showed little variation between the patient groups (Table 1). The prevalence of MCV DNA in different skin lesions was remarkably uniform: from 14% positivity for the patients with BCC to 15% for those with AK and 20% for those with SK (Table 1). MCV DNA positivity in healthy skin varied from 5% for the patients with AK or SK to 13% for those with SCC. The viral copy numbers averaged .07 copies per cell among all study participants and .44 copies per cell among infected ones. One patient had a viral load of >1 copy per cell (28 copies per cell in a SK biopsy specimen), and the median viral load was highest for patients with SK (.0016 copies per cell) (Table 1). We also tested 4 formalin-fixed blocks of tumor tissue from a patient with MCC that were found to contain .3–.67 MCV copies per cell.

The MCV antibody levels among the 434 patients varied from 0 to 106 units (mean, 2.7 units; median, 2 units). The average antibody levels were similar between different patient groups (Table 1). The single patient with MCC had a high level of MCV antibodies (18 units).

There was a very high correlation between presence of MCV DNA load and MCV seropositivity (OR, 27.85 [95% CI, 6.6–166.5]) (Table 2). A similar correlation was observed between presence of MCV DNA load and MCV-neutralizing activity (Table 2). In contrast, there was no correlation between MCV DNA presence and presence of antibodies to the control human polyomavirus JCV (OR, 1.39 [95% CI, 0.8–2.43]) (Table 2). Overall seroprevalences were similar for the 2 different human polyomaviruses: 283 (65%) of 434 patients were MCV seropositive and 276 (64%) of 434 patients were JCV seropositive; 193 (44%) of 434 patients were positive for both viruses and 51 (12%) of 434 patients were double negative. The sensitivity of the MCV serology test to detect qPCR-proven MCV infection was very high at 97%, with an apparent
specificity of 40%. Presence of high MCV antibody levels (>1 unit) had a specificity of 81%, but the sensitivity was lower (70%). The association between MCV DNA and high MCV antibody levels was strong (OR, 10.5 [95% CI, 4.94–22.48]). Very high antibody levels (>5 units) were found in 40 (9%) of 434 patients. Presence of very high MCV antibody levels had a very high specificity for the presence of MCV DNA (ongoing MCV infection) of 96%, but the sensitivity was lower (32%) (Table 2).

All 79 MCV DNA positive participants and 80 of 355 randomly selected MCV DNA negative participants were also tested in MCV reporter vector-based neutralization. Fifty-three serum samples from the 79 DNA positive participants (67%) neutralized MCV at a 1:104 dilution and 41 (52%) neutralized MCV at a 1:105 dilution (Table 2). Only 12 (15%) of 80 serum samples from MCV DNA negative participants neutralized MCV at a 1:104 dilution and only 4 (5%) neutralized MCV at a 1:105 dilution (Table 2). By comparison, the average MCV antibody level as determined by the VLP-heparin-Luminex assay was 9 times higher among MCV DNA positive participants compared with the MCV DNA negative participants (8.3 units vs .9 units, respectively) (Table 2).

When the MCV DNA positive subjects were considered by quartiles of viral load, the average MCV antibody levels were found to be increasing monotonously from 2.9 units in the lowest copy number quartile up to 21.2 units in the highest copy number quartile (Table 2). The neutralizing activity was increasing together with increasing viral load. The proportion of strongly seropositive subjects was significantly higher in the highest viral load quartile compared with the lowest one (OR 5.2 [95% CI, 1.1-26.56] for neutralization >1:105 and OR 8.67 [95% CI, 1.66-50.6] for Luminex antibody level >5 units [Table 2]). There was a strong trend of increasing neutralizing activity and increased antibody level by viral load ($P < .01$ for both), (Table 2).

## DISCUSSION

We have established a high-throughput serology assay for MCV antibodies and validated it as a sensitive marker for ongoing MCV infection. The MCV VLP-heparin-Luminex method was found to produce results comparable to a previously established MCV reporter vector-based neutralization assay. MCV antibody levels in both the MCV VLP-binding assay and the neutralization assay were found to correlate well with the viral load of MCV DNA in skin biopsy specimens.

Most apparently MCV DNA negative participants were seropositive for MCV. Our study had an ambitious sampling scheme in which all patients donated 2 biopsy specimens from different sites, but it is impossible to sample the entire skin of the participant, and thus we cannot exclude the possibility that some MCV DNA negative participants might be infected at
Table 2. Correlation of Merkel Cell Polyomavirus (MCV)–specific Antibodies With Presence of MCV DNA

<table>
<thead>
<tr>
<th>Assay, result</th>
<th>No. (%) of positive specimens</th>
<th>No. (%) of negative specimens</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<tr>
<td><strong>Luminex assay</strong></td>
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<tr>
<td>Seropositive</td>
<td>77/79 (97)</td>
<td>206/355 (58)</td>
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<tr>
<td>High antibody level&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55/79 (70)</td>
<td>65/355 (18)</td>
<td>10.22 (5.71–18.42)</td>
<td>&gt;1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.97</td>
<td>.4</td>
</tr>
<tr>
<td>Very high antibody level&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25/79 (32)</td>
<td>15/355 (4)</td>
<td>10.5 (4.94–22.48)</td>
<td>&gt;1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.32</td>
<td>.96</td>
</tr>
<tr>
<td>Mean antibody level, units (range)</td>
<td>8.3 (0–107)</td>
<td>.9 (0–10)</td>
<td>2.6 (0–107)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>JCV Luminex</td>
<td>55/79 (67)</td>
<td>221/355 (62)</td>
<td>1.39 (1.8–2.43)</td>
<td>.18</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td><strong>Neutralization assay</strong></td>
<td></td>
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<tr>
<td>Strongly neutralizing&lt;sup&gt;c&lt;/sup&gt;</td>
<td>53/79 (67)</td>
<td>12/80 (15)</td>
<td>11.55 (5.02–27.1)</td>
<td>&gt;1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.67</td>
<td>.85</td>
</tr>
<tr>
<td>Very strongly neutralizing&lt;sup&gt;d&lt;/sup&gt;</td>
<td>41/79 (52)</td>
<td>48/80 (5)</td>
<td>20.6 (6.39–73.1)</td>
<td>&gt;1 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.52</td>
<td>.95</td>
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<td><strong>NOTE.</strong> CI, confidence interval; NA, not applicable; OR, odds ratio.</td>
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<sup>a</sup> Antibody level of >1 unit.
<sup>b</sup> Antibody level of >5 units.
<sup>c</sup> Neutralizing at >1:10<sup>4</sup> dilution.
<sup>d</sup> Neutralizing at >1:10<sup>5</sup> dilution.
a skin site that was not sampled. Compared with other studies, our study had somewhat lower MCV DNA prevalences [13, 14] and a strong correlation of MCV DNA to MCV serology. Possibly, the tape stripping of the skin surface that we applied prior to biopsy specimen collection may have removed some presence of MCV DNA that may have reflected surface contamination rather than infection.

Our finding that 19% of patients with SCC have MCV DNA in tumor biopsy specimens is similar to previous studies that have reported MCV DNA in 15% of biopsy specimens from patients with SCC [15]. Our finding of an overall MCV seroprevalence of 65% is also in accordance with previous studies [2, 4].

Both Pastrana et al [3] and Carter et al [2] have demonstrated higher titers of MCV antibodies in patients with MCC compared with those in control groups. In the present study, we show that high levels of MCV antibodies correlate with higher burdens of MCV DNA. Thus, the high levels of MCV-specific antibodies found among patients with MCC may be the result of increased replication of MCV in patients with MCC. This model is consistent with the proposed causal role of MCV in MCC, suggesting that increased MCV replication imparts a greater (albeit still quite small) risk of MCC.

In conclusion, the availability of high-throughput MCV serological methods amenable to multiplexing is likely to be useful in the continuing elucidation of the epidemiology of MCV infection and its role in disease among humans.

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**References**