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# **ANTIBODIES TO MERKEL CELL POLYOMAVIRUS CORRELATE TO PRESENCE OF VIRAL DNA IN THE SKIN**

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## **Abstract**

To validate whether Merkel cell polyomavirus (MCV) serology correlates with MCV infection, we compared realtime PCR for MCV DNA on fresh-frozen biopsies from various skin lesions and healthy skin from 434 patients to MCV serology using virus-like particles (VLP) and MCV neutralisation assays. 65% of subjects were MCV seropositive and 18% were MCV DNA positive. Presence of antibodies was correlated with presence of virus DNA [OR: 27.85 (95% CI=6.6-166.5)], with 97% of patients positive for MCV DNA being MCV seropositive. VLP antibody levels correlated to neutralisation titres ( $r=0.72$ ) and high antibody levels correlated to high MCV viral load ( $p<0.01$ ).

**Key words:** MCV, seroprevalence, high-throughput serology, multiplex technology, pseudovirions, Merkel cell carcinoma

## **Introduction**

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 MCC patients have higher levels of MCV-specific serum antibodies than control subjects [2-4].

The continued elucidation of the epidemiology of MCV 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 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 subjects suffering from non-melanoma skin cancer (NMSC) or non-malignant skin lesions were tested in both assays. Biopsies of skin lesions and healthy skin from the same patients were tested in MCV DNA-specific real-time PCR assays, allowing quantitative comparison of serology to MCV DNA loads.

## **Materials and methods**

### **Patients**

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

All subjects donated a serum sample and two skin biopsies, one 2 mm biopsy from the lesion and another from healthy skin of the same patient, approximately 10-15 cm from the lesion. Before taking the biopsy, 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 (SCC, n=5; BCC, n=1; AK n=3; SK, n=1; other benign, n= 1). The study adhered to the Declaration of Helsinki and was approved by the Ethical Review Committees of Karolinska Institute and of Lund University (Sweden) and Medical University Vienna (Austria). All patients provided written informed consent.

### **Real-time PCR**

DNA was extracted as described [8]. Primers and probes for qPCR were as described [9]. Standard curves used serial dilutions from 100,000 copies to one copy of purified MCV DNA (plasmid pCR.MCV, kind gift from Yuan Chang and Patrick Moore from University of Pittsburgh Cancer Institute). The method routinely detected 1 copy/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 mM Tris, pH8, 1 mM EDTA)], 1x Buffer II (Applied Biosystems, Foster City, CA), 3.5 mM MgCl<sub>2</sub> (Applied Biosystems), 0.2% BSA (Fraction V, Sigma-Aldrich), 0.2 mM dNTP (Fermentas), 0.2 μM of each primer (Cybergene, Huddinge, Sweden), 0.04 μM of the probe (Cybergene) and 0.625U of AmpliTaq Gold (Applied Biosystems). The real-time PCR was carried out in a GeneAmp® 5700 SDS (Applied Biosystems), using 2 min at 50°C, 10 min at 95°C and then 50 cycles of 15 sec at 95°C and 1 min at 60°C. All samples were verified and had to be positive in 2/3 runs to be considered positive. Negative samples were also repeat tested 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].

### **Generation of pseudovirions and VLPs**

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 website <<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. JCV VLPs produced in *Saccharomyces cerevisiae* were a kind gift from Kestutis Sasnauskas. These VLPs consist of the major capsid protein VP1 [11].

### **Luminex**

Luminex COOH beads (Bio-rad, CA) were activated as recommended by the manufacturer and conjugated with heparin at 0.75 mg/ml (Sigma-Aldrich, no. H4784) in sterile water at room temperature for 16h. The assay then followed the protocol by Faust et al [5].

Fluorescence signals were recorded using a Bio-Plex 200 Luminex (setting “low”). Beads without VLPs were used as control and background median fluorescent intensity (MFI) of each serum was subtracted from the VLP reactivity. A signal of >250 MFI for at least one serum dilution was used to separate antibody positive samples from negative ones. A human serum sample with a high antibody titre 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 (PLL) method [12].

### **Neutralization assay**

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[3]. We used 36 pg of VP1 per well, combined with serum diluted in final concentrations of 1:10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup> and 10<sup>6</sup>. Chemoluminescence was read for 0.2s per well using a Wallac Victor 1420 Multilabel counter. 50% neutralizing titers were calculated using Prism version 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 r-squared values of less than 0.9 were excluded from the analysis.

### **Statistics**

Epi-info version 3.5.1 estimated odds ratios (OR) and 95% confidence intervals (95% CI) using multivariate logistic regression.

## **Results**

### **Correlation of MCV neutralization and heparin-Luminex assays**

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 (27/434 samples) and 28 samples with antibody levels ranging from 0,9 to 8 (3-4 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 ( $R=0.72$ ,  $p<0.0001$ ).

### **Presence of MCV DNA in relation to titer of MCV antibodies**

Overall, 18% of the 434 patients were MCV DNA positive in the qPCR. The proportion of MCV DNA positive subjects 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 BCC patients to 15% for AK and 20% for SK patients (Table 1). MCV DNA positivity in healthy skin varied from 5% for AK and SK patients to 13% for SCC patients. The viral copy numbers averaged 0.07 copies/cell among all study subjects and 0.44 copies/cell among infected ones. One patient had a viral load of more than 1 copy per cell (28 copies/cell in a SK biopsy) and the median viral load was highest for SK (0.0016 copies/cell) (Table 1). We also tested 4 formalin-fixed blocks of tumor tissue from an MCC patient that were found to contain 0.3 to 0.67 MCV copies per cell.

The MCV antibody levels among the 434 patients varied from 0 to 106 units (average 2.7 units; median 0.2 units). The average antibody levels were similar between the different patient groups (Table 1). The single MCC patient 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: 283/434 (65%) subjects were MCV seropositive and 276/434 (64%) were JCV seropositive. 193/434(44%) patients were positive for both viruses and 51(12%) were double negative. The sensitivity of the MCV serology to detect q-PCR-proven MCV infection was very high: 97%, with an apparent specificity of 40%. Presence of high MCV antibody levels (>1 units) had a specificity of 81%, but 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 9% of subjects (40/434). Presence of very high MCV antibodies had a very high specificity for the presence of MCV DNA (ongoing MCV infection): 96%, but the sensitivity was lower (32%) (Table 2).

### **MCV neutralizing activity in MCV DNA positive and negative populations**

All 79 MCV DNA positive subjects and 80/355 randomly selected MCV DNA negative subjects were also tested in MCV reporter vector-based neutralization. Fifty-three serum samples from the 79 DNA positive subjects (67%) neutralized MCV at a 1:10<sup>4</sup> fold dilution and 41 (52%) at a 1:10<sup>5</sup> dilution (Table 2). Only 12/80 (15%) serum samples from MCV DNA negative subjects neutralized MCV at a 1:10<sup>4</sup> fold dilution and only 4 (5%) at a 1:10<sup>5</sup> dilution (Table 2).

By comparison, the average MCV antibody level as determined by the VLP-heparin-Luminex assay was about 9 times higher among MCV DNA positive subjects compared to the MCV DNA-negative group (8.3 versus 0.9 units, respectively) (Table 2).

### **Correlation between MCV viral load and MCV antibody levels**

When considering the MCV DNA positive subjects 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 number of subjects having very strong neutralizing activity or very high antibody level (>5 Units) was significantly higher in highest viral load quartile compared to the lowest one [OR=5.2 (1.1-26.56) and OR= 8.67 (1.66-50.6 95%CI) respectively] (Table 2). There was a strong trend for increasing neutralizing activity and increased antibody level by viral load (both  $p < 0.01$ ), (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 biopsies.

Most apparently MCV DNA-negative subjects were seropositive for MCV. Our study had an ambitious sampling scheme where all patients donated 2 biopsies from different sites, but it is impossible to sample the entire skin of the subject and we can't thus not exclude that some MCV DNA negative subjects might be infected at a skin site not sampled. Compared to other studies, we have 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 collection may have removed some presence of MCV DNA that may have reflected surface contamination rather than infection.

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

Both Pastrana *et al.* and Carter *et al.* have demonstrated higher titers of MCV antibodies in MCC patients compared to control groups [2, 3]. In the current 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 MCC patients may be the result of increased replication of MCV in MCC patients. 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 human disease.

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## **Footnotes**

Conflict of interest: None declared.

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## Tables

Table 1. Presence of MCV DNA among patients with different skin diseases.

SCC= squamous cell carcinoma of the skin, BCC= basal cell carcinoma, AK= actinic keratosis

<b>Skin Disease (Number of patients)</b>	<b>MCV DNA in skin lesion N pos (%)</b>	<b>MCV DNA viral load in infected skin lesion (median copies/cell)</b>	<b>MCV DNA in healthy skin N pos (%)</b>	<b>MCV DNA viral load in infected healthy skin (median copies/cell)</b>	<b>MCV DNA in any biopsy N pos (%)</b>	<b>MCV DNA in both biopsies N pos (%)</b>	<b>MCV antibodies in Luminex assay units (range)</b>
<b>SCC N=67</b>	11 (16 %)	0.0005 ( $2 \times 10^{-5}$ to 0.086)	9 (13%)	0.0039 (0.001 to 0.04)	14 (21%)	6 (9%)	2.5 (0 to 32)
<b>BCC N=159</b>	22 (14%)	0.0005 ( $2 \times 10^{-7}$ to 0.003)	17 (11%)	0.0019 ( $3 \times 10^{-5}$ to 0.08)	30 (19%)	9 (6%)	2.5 (0 to 43)
<b>AK N=78</b>	12 (15%)	0.0006 ( $5 \times 10^{-6}$ to 0.21)	4 (5%)	0.0016 ( $4 \times 10^{-4}$ to 0.02)	12 (15%)	4 (5%)	2.5 (0 to 107)



<b>Seborrhoeic keratosis N=61</b>	12 (20%)	0.0016 ( $10 \times 10^{-5}$ to 28)	3 (5%)	0.004 ( $4 \times 10^{-4}$ to 0.01)	12 (20%)	3 (5%)	2.9 (0 to 104)
<b>Other benign diagnoses N=58</b>	10 (17%)	0.0001 ( $2 \times 10^{-5}$ to 0.004)	5 (9%)	0.001 ( $7 \times 10^{-5}$ to 0.006)	11 (19%)	4 (7%)	2.6 (0 to 12)

Table 2. Correlation of MCV specific antibodies with presence of MCV DNA.

Luminex assay	MCV DNA positive	MCV DNA negative	Total	OR (95% CI)	p-value	Sensitivity	Specificity	1st quartile # 2..53x10 <sup>-7</sup> - 1.8x10 <sup>-4</sup> copies/cell	2nd quartile # 2..2x10 <sup>-4</sup> - 11.8x10 <sup>-4</sup> copies/cell	3rd quartile # 12.3x10 <sup>-4</sup> - 6x10 <sup>-3</sup> copies/cell	4th quartile # 7x10 <sup>-3</sup> - 28 copies/cell	P for trend
Seropositive	77/79 (97%)	206/355 (58%)	283/434 (65%)	<b>27.85</b> (6.6-166.5)	>1x10 <sup>-4</sup>	0.97	0.4	20/20 (100%) NA	18/20 (90%) NA	20/20 (100%) NA	19/19 (100%) NA	NA
High antibody level*	55/79 (70%)	65/355 (18%)	120/434 (28%)	<b>10.22</b> (5.71-18.42)	>1x10 <sup>-4</sup>	0.7	0.81	14/20 (70%) Reference	13/20 (65%) OR=0.8 (0.17- 3.63 95%CI)	14/20 (70%) OR=1 (0.21- 4.73 95%CI)	16/19 (84%) OR=2.29 (0.39- 14.49 95%CI)	0.3
Very high antibody level**	25/79 (32%)	15/355 (4%)	40/434 (9%)	<b>10.5</b> (4.94-22.48)	>1x10 <sup>-4</sup>	0.32	0.96	4/20 (20%) Reference	4/20 (20%) OR=1 (0.17-5.98 95%CI)	4/20 (20%) OR=1 (0.17-5.98 95%CI)	13/19 (68%) <b>OR=8.67</b> (1.66-50.6 95%CI)	<b>25x10<sup>-4</sup></b>
Mean antibody level in units (range)	8.3 (0 to 107)	0.9 (0 to 10)	2.6 (0 to 107)	NA	NA	NA	NA	2.9 (0 to 12)	3.2 (0 to 15)	6.5 (0 to 43)	21.2 (0 to 107)	NA
JCV Luminex	55/79 (67%)	221/355 (62%)	276/434 (64%)	1.39 (0.8-2.43)	0.18	NA	NA	NA	NA	NA	NA	NA
<b>Neutralization assay</b>												
Strongly neutralizing***	53/79 (67%)	12/80 (15%)	65/159 (41%)	<b>11.55</b> (5.02-27.1)	>1x10 <sup>-4</sup>	0.67	0.85	13/20 (65%) Reference	12/20 (60%) OR=0.8 (0.18-3.51 95%CI)	13/20 (65%) OR=1 (0.22-4.45 95%CI)	16/19 (84%) OR=2.87 (0.51-17.82 95%CI)	19x10 <sup>-2</sup>

Very strongly neutralizing****	41/79 (52%)	4/80 (5%)	45/159 (28%)	<b>20.5</b> (6.38-73.1)	$>1 \times 10^{-4}$	0.52	0.95	7/20 (35%) Reference	8/20 (40%) OR=1.24 (0.29-5.42 95%CI)	12/20 (63%) OR=2.79 (0.65-12.44 95%CI)	14/19 (74%) <b>OR=5.2</b> (1.1-26.56 95%CI)	<b><math>76 \times 10^{-4}</math></b>
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. \* $>1$  Unit \*\* $>5$ Units, Neutralizing at  $\geq 1:10^4$  dilution.\*\*\* Neutralizing at  $\geq 1:10^5$  dilution\*\*\*\*. NA=Not applicable. # MCV viral load

