Prospective study of Merkel cell polyomavirus and risk of Merkel cell carcinoma

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Merkel cell carcinoma (MCC) is a rare type of skin cancer that has a characteristically increased incidence among immunosuppressed subjects. The DNA of Merkel cell polyomavirus (MCV) is regularly found in most MCC tumors. We investigated whether Merkel cell polyomavirus (MCV) infection increases the risk for future MCC. Two large biobank cohorts (Southern Sweden Microbiology Biobank and the Janus Biobank), containing samples from 856,000 healthy donors, were linked to the Cancer Registries in Sweden and Norway to identify cases of MCC occurring up to 30 years after donation of a serum sample. For each of the 22 cases (nine males and 13 females), four matched controls were included. The serum samples were analyzed with an MCV neutralization assay and for IgG antibodies to MCV pseudovirions, using JC polyomavirus and cutaneous human papillomaviruses as control antigens. An increased risk for future MCC was associated both with high levels of MCV antibodies [OR 4.4, 95% CI 1.3–17.4] and with MCV neutralizing activity (OR 5.3, 95% CI 1.3–32.3). In males, MCV seropositivity was not associated to MCC risk, whereas the risk was strongly increased in females, both for high levels of MCV antibodies (OR 7.0, 95% CI 1.6–42.8) and for MCV neutralizing activity (OR 14.3, 95% CI 1.7–677). In conclusion, we found prospective evidence that MCV infection is associated with an increased risk for future MCC, in particular among females.

Merkel cell carcinoma (MCC) is an aggressive form of skin cancer, with a disease-specific mortality rate up to 50%.[1] Clonally integrated Merkel cell polyomavirus (MCV) has been found in cellular DNA from MCC[2] tumors. MCV DNA is common on human healthy skin[3,4] and MCV-specific antibody responses are common in the general population.[5–8] MCC patients have higher levels of antibodies to MCV capsids than control subjects.[5,9] Antibodies to MCV T antigen oncoproteins appear to follow the tumor burden in MCC patients,[10] and it is therefore not known whether the association between MCV and MCC is induced by the tumor or by the infection. Prospective studies are considered an essential component of causality inference as they can provide information on the direction of causality of an association. Recently, an international evaluation classified MCV infection as "probably carcinogenic to humans."[11] Absence of prospective epidemiological studies was one of the reasons cited for considering the epidemiologic data as insufficient for more definite conclusions.

Presence of antibodies to MCV correlates strongly with the presence of the MCV DNA in the skin, both when using a neutralization assay[9] or an MCV pseudovirion binding assay.[12] The antibody assays correlate well with each other[12] and higher levels of antibodies correlate with higher viral load of MCV DNA in the skin.[12,13] Because well-validated serological markers of MCV infection exists, the use of case–control studies nested within biobank cohorts prospectively followed for incident MCC can provide information on whether MCV infection does increase the risk for future MCC.

Our objective was to investigate whether MCV infection would be associated with MCC also in a prospective study.

Material and Methods

Cohorts and study design

About 524,000 individuals donated about 1.5 million serum samples to the Southern Sweden Microbiology Biobank (SSMB), from 1969 and onward.[14] The major reasons for donating samples are viral diagnostics, screening of blood donors and screening of pregnant women.[14] Controls and cases were matched by reason for donating sample. Although

What's new?

Although the DNA of Merkel cell polyomavirus (MCV) is regularly found in most Merkel cell carcinoma tumors, it is also common in healthy skin. The presence of MCV-specific antibodies is higher among Merkel cell carcinoma patients, but the prospective value of this finding has not been formally established. Here, the authors demonstrate that the risk of future Merkel cell carcinoma is increased among subjects with high levels of MCV antibodies, especially in females, thus supporting the model that MCV is etiologically linked to Merkel cell carcinoma.

screening of pregnant women is a major reason for donating samples to SSMB, there were actually no cases (or matched controls) among the pregnant women—presumably because MCC primarily affects older subjects. About 332,000 Norwegian volunteers donated about 493,000 serum samples to the Janus Biobank, from 1972 and onward. Population-based invitations to health surveys enrolled most donors (90%), the remainder being blood donors.[14] Cases and controls were matched by enrolment method. The biobanks were followed up using linkages to the national cancer registries in Norway and Sweden using International Classification of Diseases (ICD) ICD7 code 191, ICD10 code C44 and SNOMEDO10 code 82473 to identify MCC cases. As the Norwegian cancer registry did not contain MCC morphology code 82473 before January 1, 1993, pathology reports of cases diagnosed with ICD7 code 191 before that date were re-read, which identified one MCC case. To be included as a case, at least one serum sample should have been taken at least 1 month before diagnosis. Because risk factors for MCV infection are unknown, specific analysis of confounding was not possible. However, the specificity of the association was investigated by comparison with multiple other viruses that also infect the skin.

For each case, four controls (alive and free of skin cancer when the case was diagnosed) were selected matched for age (±2 years), sex, county (in the Janus Biobank. The SSMB targets only a single county in Sweden), subcohort, number of serial samples and length of follow-up. With four controls per case, the study was estimated to be able detect associations that were 4-fold or greater, at conventional levels of significance (0.05) and power (0.8). The first (oldest) sample for the control was matched with the first (oldest) sample of the case for date of sampling (±2 months). If controls meeting the matching criteria could not be found, matching criteria were widened in successive steps of ±1 month of sampling date and ±1 year of age until a control was found. No samples taken after diagnosis were included.

Altogether 98 persons (22 cases and 76 controls) with 134 serum samples (49 samples from cases and 85 from controls) were included to the study. The 22 MCC cases included nine males and 13 females, and the 76 controls included 50 females and 26 males. From Sweden, 16 MCC cases and 62 controls were identified. Seven cases had only one sample, six cases had two samples, one had three, one had 10 and one had 11 prediagnostic serum samples. In total, 105 samples were retrieved. In Norway, six MCC cases and 14 matched

controls were identified. Including serial samples, 29 samples were retrieved. For 14 cases, formalin- fixed paraffin embedded tumor blocks could be retrieved.

MCV antibody assays

MCV pseudovirions with reporter vector were generated as described.[9,12] JCV pseudovirions were a gift from Kestutis Sasnaukas.[15] Expression vectors to produce MCV and Human Papillomavirus (HPV) type 5 were provided by C. B. Buck and J. T. Schiller, National Cancer Institute, Bethesda, MD. Nucleotide sequences of plasmids can be found at http://home.ccr.cancer.gov/lco/default.asp. Expression vectors for pseudovirions of HPV types 3, 15, 32, 38 and 76 were cloned using the same strategy with codon-modified nucleotides.[16,17]

The pseudovirion binding assay was performed as described,[12,17] except that heparin-coupled beads were stored at -80°C before use. Antibody levels were calculated relative to a standard serum using mean fluorescence intensities obtained at dilutions 1:3,160, 1:10,000 and 1:31,600 with the parallel line (PLL) method.[18]

MCV neutralization was performed as described,[9] except that we used 36 pg of pseudovirion per well and serum dilutions of 1:100, 1:316, 1:1,000, 1:3,160, 1:10,000 and 1:31,600 and analysis by chemoluminescence as described.[12] Only >50% neutralizing activity at 1:10,000 dilution was considered positive.

In the binding assay, a mean fluorescence intensity of >250 was, for all studied viruses, considered as presence of antibodies.[17] For MCV serology, we also investigated whether high antibody levels (PLL >1) were associated with MCC risk.[12] Antibody levels in the MCV binding assay were highly correlated to the neutralizing antibody titers ($R^2 = 0.83$) (data not shown).

Real-time PCR

Tumor tissue sections were deparaffinized with xylene, digested with Proteinase K[19] and tested for MCV with real-time PCR.[12] Positive samples were tested in triplicate and had to be positive in 2/3 runs to be considered positive. Cellular DNA was quantified by β-globin real-time PCR.[20]

Statistical analysis

Prism 5 Software (Graphpad) investigated agreement between serological methods. Two-tailed Spearman rank correlation coefficients, R square and associated *p* values were calculated.

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Table 1. Presence of antibodies to MCV and to control viruses in relation to the risk for MCC in the future

All individuals	Cases positive, N (%) (total 22)	Controls positive, N (%) (total 76)	OR	95% CI
MCV neutralization	10 (45)	12 (16)	5.3	1.3–32.3
High MCV antibody level[1]	11 (50)	14 (18)	4.4	1.3–17.4
Any level of MCV antibodies	19 (86)	57 (75)	2.6	0.66–15.0
HPV3	2 (9)	2 (3)	3.9	0.3–54.8
HPV5	3 (14)	11 (14)	1.2	0.2–6.0
HPV15	2 (9)	6 (8)	1.5	0.1–11.5
HPV32	0 (0)	2 (3)	1.8	0.0–23.8
HPV38	4 (18)	14 (18)	1.1	0.2–4.0
HPV76	5 (23)	19 (25)	0.8	0.2–3.3
JC polyomavirus	17 (77)	55 (72)	1.5	0.4–6.9
Males	**Cases positive, N (%) (total 9)**	**Controls positive, N (%) (total 29)**	**OR**	**95% CI**
MCV neutralization	2 (22)	4 (14)	1.3	0.08–19.9
High MCV antibody level[1]	2 (22)	4 (14)	1.3	0.08–19.9
Any level of MCV antibodies	7 (78)	24 (83)	1.0	0.1–12.6
Females	**Cases positive, N (%) (total 13)**	**Controls positive, N (%) (total 47)**	**OR**	**95% CI**
MCV neutralization	8 (62)	8 (17)	14.3	1.7–677
High MCV antibody level[1]	9 (69)	10 (21)	7.0	1.6–42.8
Any level of MCV antibodies	12 (92)	33 (70)	6.0	0.8–277

[1]Antibody level >1 unit.

Exact conditional logistic regression models were used to assess the relationship between virus antibodies and the risk of future MCC, using baseline serum sample data and Log-Xact version 6 (Cytel Software). p Values <0.05 were considered statistically significant. Sex-stratified analysis was performed following the same method.

Results

In total 22 MCC cases, nine males and 13 females and 76 controls were included in the study. As some cases and controls had several prediagnostic samples available, the study contained altogether 134 serum samples (49 samples from cases and 85 from controls). Age at sampling varied from 30 to 90 years, mean age 61. Mean age at MCC diagnosis was 70 years (range 47–91 years) and the baseline samples available for testing were obtained on average 12 years (range 1–26 years) before the cancer diagnosis. Total follow-up time for cases was 247 person-years.

The risk for future MCC was associated both with baseline presence of MCV neutralizing antibodies (OR 5.3, 95% CI 1.3–32.3) and with presence of high levels of antibodies against MCV (OR 4.4, 95% CI 1.3–17.4) (Table 1). Baseline presence of any level of MCV antibodies tended to associate with MCC risk, but not significantly (Table 1). To investigate whether acquisition of MCV antibodies after the baseline serum donation would affect MCC risk, we tested all

prediagnostic serum samples available and estimated the MCC risk in relation to seropositivity in any one of the prediagnostic samples. The risks were similar to those estimated using only the baseline samples. OR for MCC associated with high levels of MCV antibodies in any prediagnostic sample was 3.6 (95% CI 1.1–12.7) and OR for MCC associated with MCV neutralizing activity was 4.8 (95% CI 1.3–21.8). There were four cases that were seronegative at baseline and had multiple prediagnostic samples available. Only one case seroconverted (acquired MCV antibodies in a more recent prediagnostic sample). For none of the studied control virus antigens (JC polyomavirus and six HPV types that infect skin) was presence of antibodies associated with MCC (Table 1).

In sex-stratified analysis, we did not observe any significant association of MCV antibodies with MCC risk among males, whereas the future MCC risk among females was associated with MCV antibodies (Table 1), in particular the presence of baseline neutralizing antibodies (OR = 14.3, 95% CI 1.7–677) (Table 1). High MCV antibody levels in the first prediagnostic sample was significantly more common among female cases than males cases ($p = 0.025$).

Analysis stratified by long or short lag between sampling and MCC diagnosis found no noteworthy differences in risk, neither for neutralization (OR for <10 years 4.2 (95% CI 0.55–50.8), for >10 years 7.33 (95% CI 0.81–368) nor for

Table 2. Prediagnostic MCV antibodies and presence of MCV DNA in subsequent MCC tumors

Sex	MCV antibodies, any level	High MCV antibody level	MCV neutralization	MCV DNA	MCV DNA copies/cell	Pathology review
M	+	–	–	–	0	Epithelial
M	+	–	–	–	0	Lymphatic
M	+	+	+	+	0.6	Epithelial
M	–	–	–	–	0	Lymphatic
M	–	–	–	–	0	Epithelial
M	+	–	–	+	56.5	Lymphatic
F	+	+	+	+	0.3	Epithelial
F	+	+	+	+	5.0	Epithelial
F	+	–	–	+	2.6	Lymphatic
F	+	+	–	+	1.7	Epi+lymph
F	+	–	+	–	0	Epithelial
F	+	+	+	+	0.1	Epi+lymph

high antibody levels (OR for <10 years 4.0 (95% CI 0.68–28.1), for >10 years 5.0 (95% CI 0.79–55.5).

For 14 cases, formalin- fixed paraffin embedded tumor blocks could be retrieved. Four 5 μm sections were used to extract DNA and two sections taken before and after the DNA sections were haematoxylin-eosin stained for histopathological re-review. Only 12 blocks contained MCC tumor tissue in both sections (six epithelial MCC, four lymphatic MCC and two mixed tumor type MCC). Among the 12 case tumors available, seven (58%) were positive for MCV DNA with a viral load varying from 0.1 to 56 copies per cell (Table 2). The MCV DNA-positive cases were all MCV seropositive (Table 2).

Discussion

We report prospective evidence that MCV infection is associated with increased MCC risk, particularly among females. The MCC risk was seen in particular for presence of neutralizing and high antibody levels to MCV. Presence of any level of antibodies to MCV was not significantly associated with MCC risk, although the point estimate of risk was elevated.

Because the antibody response to the MCV virion is well correlated to presence of MCV on the skin, with subjects with low amounts of virus or being presently negative (past infection) having lower antibody levels, our findings of an elevated risk associated with high prediagnostc antibody levels suggest that an MCV infection with high viral load is a risk factor for MCC, that may act already many years before the diagnosis.

The use of case-control studies nested within prospectively followed biobank cohorts has been instrumental in delineating whether associations seen in cross-sectional case–control studies may be of etiological significance or not, with positive associations reported for, *e.g.* HPV and cervical cancer.[21] The prospective design reduces several major sources of biases and when cohorts are followed using nationwide and comprehensive registries, biases due to selective loss to follow-up are minimized.[22] The major limitation is the difficulty in studying rare diseases, as this requires very large cohorts and very long follow-up times.[22]

As there are no known risk factors for MCV infection, specific analysis of possible confounding factors was not possible. Instead, we used comparison with a related polyomavirus (JC virus) and several different cutaneous papillomaviruses that, like MCV, also are common viruses that infect the human skin. As the association with MCC risk was specific to MCV, our findings suggest that the finding is not related to some more general susceptibility to polyomaviruses or to skin infections in general. UV light is a risk factor for MCC, but is not known to affect MCV infection. Also, as the study was matched by county in Norway and in Sweden derived from only one county, the uncontrolled variability in UV exposure is likely to be limited. The main study limitation was the low numbers of cases of this rare disease that occurred in the cohorts. Thus, although statistically significant excess risks were found, the confidence intervals were wide. Another limitation of the study was that not all tumor blocks from MCC cases were available for MCV DNA detection.

The major risk factor for MCC is immunosuppression. Increased cancer risk after immunosuppression is in particular seen for virus-associated cancers.[23] Data from the cancer registries in the Nordic countries have also pointed to female gender as an important risk factor.[24] In addition to the increased MCC incidence among females, MCC patients are at an increased risk for secondary cancers, an excess risk that is higher for female MCC patients.[24] The reason for these gender-specific differences in the epidemiology of MCC is unknown, but our findings suggest that a female susceptibility to high viral load MCV infection could be a possible explanation.

Although we obtained only few of the corresponding MCC tumors for MCV DNA testing, our data support that a

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majority of MCC tumors are MCV-positive. It has been shown that MCC tumors free of MCV have a different morphology compared to MCV-positive tumors, suggesting that MCC may exist in two different entities with different etiologies.[25,26] Several studies have demonstrated that MCV-negative MCC cases have worse prognosis[27–29] and that MCC patients with high MCV antibody levels have better survival,[30] also suggesting that MCV-associated and MCV-negative MCC are biologically different. Reports that prognosis of MCC is better among females would also be consistent with existence of an MCV-associated form of MCC that has better prognosis and tends to associate with female gender.[31] Previous case–control studies have consistently found that MCC patients have higher MCV-specific antibody levels.[9,30] The comparison with other studies thus suggests that the association between MCV and MCC that we find is generalizable. We also extend the previous studies to showing that the high MCV antibody level appears already long before the tumor. The fact that the association was found also in a prospective study contributes to the bulk of the evidence supporting that MCV infection may be carcinogenic to humans and increase the risk for a subset of MCC tumors.

Acknowledgements

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