# **Clinical Factors Associated With Merkel Cell Polyomavirus Infection in Merkel Cell Carcinoma**

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- **Background** Merkel cell carcinoma is a rare malignancy of the skin. Integration of Merkel cell polyomavirus (MCPyV) DNA to the tumor genome is frequent in these cancers. The clinical consequences of MCPyV infection are unknown.
  - **Methods** We analyzed formalin-fixed paraffin-embedded Merkel cell carcinoma tissue samples from 114 of 207 patients diagnosed with Merkel cell carcinoma in Finland from 1979 to 2004 for the presence of MCPyV DNA with the use of polymerase chain reaction (PCR), quantitative PCR, and DNA sequencing and examined associations between tumor MCPyV DNA status and histopathologic factors and survival. The median follow-up time after Merkel cell carcinoma diagnosis for subjects who were alive was 9.9 years (range = 4.9–21.9 years). All *P* values are two-sided.
  - Results MCPyV DNA was present in 91 carcinomas (79.8%). Compared with MCPyV DNA-negative cancers, MCPyV DNA-positive cancers were more often located in a limb (40.7% vs 8.7%, P = .015) and less frequent in patients who had regional nodal metastases at diagnosis (6.6% vs 21.7%, P = .043). Patients with MCPyV DNA-positive tumors had better overall survival than those with MCPyV DNA-negative tumors (5-year survival: 45.0% vs 13.0%, respectively; P < .001, two-sided log-rank test).</p>
- **Conclusions** MCPyV infection is associated with clinical outcomes in patients with Merkel cell carcinoma. These findings lend support to the hypothesis that viral infection is frequently associated with the pathogenesis of Merkel cell carcinoma.
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Merkel cell carcinoma is a relatively rare neuroectodermal carcinoma that arises from the mechanoreceptor Merkel cells in the skin, with an annual incidence rate of approximately four cases per million (1). According to Surveillance, Epidemiology, and End Results data, the age-adjusted incidence of Merkel cell carcinoma in the United States tripled between 1986 and 2001 (1). Merkel cell carcinoma affects mainly the white population with a median age at presentation of approximately 70 years, and it manifests more often in men than in women (1–4). It tends to recur locally and to give rise to regional nodal and distant metastases. Merkel cell carcinoma is usually treated surgically, either with or without postoperative radiation therapy. The reported 5-year relative and disease-specific survival rates are approximately 70% (2,3).

Results of several studies suggest that multiple factors contribute to the etiology of Merkel cell carcinoma. For example, exposure to ultraviolet radiation is associated with Merkel cell carcinoma (5–7). Like Kaposi sarcoma, Merkel cell carcinoma occurs more often than expected in immunocompromised individuals, such as those diagnosed with chronic lymphocytic leukemia, lymphoma, or a human immunodeficiency virus infection, and among organ transplant recipients (8–11).

The recent identification of a small novel polyomavirus with a genome consisting of double-stranded DNA, provisionally named Merkel cell polyomavirus (MCPyV), in Merkel cell carcinoma tumor tissue suggested a role for this virus in the pathogenesis of

the disease (12). MCPyV DNA was found to be clonally integrated in the genomes of Merkel cell carcinoma tumors, suggesting that viral infection may be an early event in Merkel cell carcinoma pathogenesis (12). In five recent studies (12–16), MCPyV DNA was found in 24%–89% of the Merkel cell carcinomas examined. One study suggested that the prevalence of MCPyV DNA in Merkel cell carcinomas may vary in different populations (16). However, little is known about whether the frequency with which MCPyV DNA is detectable in Merkel cell carcinomas varies with the age or sex of the patient, the size or histological subtype of carcinoma, or the time period during which the cancer was detected. To our knowledge, it is also not known whether integration of MCPyV DNA into the tumor genome is associated with

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the prognosis of subjects who are diagnosed with Merkel cell carcinoma.

In this study, we investigated the clinical features of MCPyV DNA-positive Merkel cell carcinomas that were diagnosed in a population-based series of individuals. To our knowledge, this is the first study to examine the clinical course of this disease according to tumor MCPyV DNA status.

## **Subjects and Methods**

#### Subjects

Individuals who were diagnosed with Merkel cell carcinoma in Finland from January 1, 1979, to October 24, 2004, were identified from the files of the Finnish Cancer Registry. The Finnish Cancer Registry was founded in 1952 and has coverage of more than 90% of all malignancies diagnosed in Finland (17). In addition, we searched the files of the largest hospital of Finland, the Helsinki University Central Hospital, to identify patients who had been diagnosed with Merkel cell carcinoma during this time but were not included in the registry. These efforts identified 207 individuals who had been diagnosed with Merkel cell carcinoma.

We excluded 93 (44.9%) of the 207 subjects from this study for the following reasons: 37 subjects had no formalin-fixed paraffinembedded archival tumor tissue available for review; 13 subjects were found not to have Merkel cell carcinoma upon histopathologic review of the diagnosis by a pathologist with a special interest in Merkel cell carcinoma pathology (T. Böhling); for eight subjects, the site of the primary tumor was not known; for 16 subjects, clinical information was not available; for 17 subjects, DNA of adequate quality could not be obtained from the archival tumor tissue; and for two subjects, the quantitative polymerase chain reaction (PCR) assay we used to detect MCPyV DNA was not informative. The remaining 114 subjects with confirmed Merkel cell carcinoma and with adequate tumor tissue DNA and clinical information available were included in this study (Figure 1).

For histopathologic confirmation of the diagnosis, we required that the tumor morphology was consistent with Merkel cell carcinoma on hematoxylin-eosin-stained tissue sections and that tumor sections were positive by immunostaining for cytokeratin 20 (Merkel cell carcinomas usually have paranuclear intermediate filaments that are immunopositive for cytokeratin 20) or, when cytokeratin 20 was not expressed (three cancers), positive by immunostaining for both synaptophysin and chromogranin A [markers of neuroendocrine differentiation (18)]. Negative immunostaining for thyroid transcription factor 1 was also required to exclude metastatic small cell lung carcinoma (another type of neuroendocrine tumor) from the differential diagnosis. Tumor histology was classified according to World Health Organization criteria (18) and as described elsewhere (19-21). Tumors with mixed morphology that contained regions of the trabecular subtype were included in the trabecular subtype in statistical analyses; tumors with both small cell and intermediate cell morphology were classified as tumors with intermediate cell morphology.

Clinical data were extracted from hospital case records and records of the primary care centers. Data on the date and cause of death were also obtained from the Finnish Cancer Registry and the Register Office of Helsinki. The longest diameter of the tumor was

# CONTEXT AND CAVEATS

## **Prior knowledge**

Merkel cell carcinoma is a rare malignancy of the skin in which Merkel cell polyomavirus (MCPyV) DNA is frequently found to be integrated into the tumor genome.

## Study design

Histopathologic and molecular biological analyses of tumor tissue and DNA from Finnish patients who were diagnosed with Merkel cell carcinoma.

## Contribution

MCPyV DNA–positive Merkel cell cancers tended to be located in a limb, have less frequent nodal metastases at the time of the diagnosis, and were associated with better Merkel cell carcinoma–specific and overall survival compared with MCPyV DNA–negative cancers.

#### Implications

MCPyV may have a role in the molecular pathogenesis of many Merkel cell carcinomas.

#### Limitations

Approximately 45% of the identified Merkel cell carcinoma patients were excluded from the analysis for various reasons, which might have resulted in selection bias.

From the Editors

measured from the hematoxylin–eosin-stained slides whenever feasible; otherwise, we used the diameter that was reported in the case records. All patients were treated with surgical excision of the primary tumor. Eighteen patients received postoperative radiation therapy; none received systemic adjuvant chemotherapy. In addition, as control samples, we obtained 22 formalin-fixed paraffinembedded tissue samples from the archives of the Department of Pathology (Helsinki University Central Hospital) comprising glioblastoma (n = 8), skin melanoma (n = 7), or histologically normal tissue (n = 7; one each of skin, stomach, colon, skeletal muscle, and lymph node, and two bone marrow samples); samples within each category were selected at random.



Figure 1. Subjects included in this study. PCR = polymerase chain reaction.

This study was approved by the ethics committee of the Department of Surgery of the Helsinki University Central Hospital. The Ministry of Social Affairs and Health in Finland granted permission for us to collect tumor tissue for this study.

## **MCPyV DNA Detection**

Five tissue sections (10 µm thick) cut from each tumor were deparaffinized in xylene, rehydrated in an ethanol series, and subjected to DNA extraction with the use of a QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The resulting genomic DNA (50 ng) was amplified for intron 2 of the protein tyrosine phosphatase gamma receptor gene (PTPRG) to ascertain that DNA quality was adequate for MCPyV DNA detection by using a FastStart Taq DNA Polymerase dNTPack kit (catalog number 04738381001; Roche Diagnostics GmbH, Mannheim, Germany) in a 20-µL PCR mixture that contained 1× PCR buffer, 2 mmol/L of MgCl<sub>2</sub>, 200 µmol/L of deoxynucleotide 5'-triphosphate solution, 1 U of FastStart DNA polymerase, and 0.3 µmol/L of forward and reverse primers (5'-TATGGGAGTGTGGGATGGTT-3' and 5'-TAAGCTGGGAGGATCGCTTA-3', respectively). Intron 2 of PTPRG (located on chromosome 3p14.2) is 20000 bp downstream from the 3' end of PTPRG intron 1, which has been reported to contain one MCPyV DNA genomic integration site in Merkel cell carcinoma (12). The PCR cycling conditions consisted of an initial denaturation step at 95°C for 4 minutes, followed by 50 cycles with denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 45 seconds. The final elongation step was performed at 72°C for 7 minutes.

MCPyV DNA was next detected by subjecting the tumor genomic DNA to standard PCR and quantitative PCR. In the standard PCR analyses, we used two sets of primers that were designed to amplify the predicted coding regions for the viral large T antigen (LT1 primer pair [forward and reverse]: 5'-TAC-AAGCACACTCCACCAAAGC-3' and 5'-TCCAATTACAG-CTGGCCTCT-3', respectively, and LT3 primer pair [forward and reverse]: 5'-TTGTCTCGCCAGCATTGTAG-3' and 5'-ATATAGGGGGCCTCGTCAACC-3', respectively) and one primer set for amplifying the viral capsid protein 1 (VP1; VP1 primer pair [forward and reverse]: 5'-TTTGCCAGCTTACA-GTGTGG-3' and 5'-TGGATCTAGGCCCTGATTTTT-3', respectively) (12). Each PCR assay included one pair of primers and used the cycling conditions described above. The PCR products were resolved by agarose gel electrophoresis and detected with ethidium bromide staining.

Quantitation of MCPyV DNA was carried out by performing real-time PCR with hydrolysis probes (ie, hybridization probes labeled with a reporter dye and a quenching dye) in a LightCycler 480 instrument (Roche Diagnostics GmbH). Genomic DNA (50 ng) was amplified in a 20-µL PCR mixture by using LightCycler 480 Probes Master reagents (Roche Diagnostics GmbH) and fluoresceinlabeled locked nucleic acid (LNA) hydrolysis probe 6 (5'-TTC-CTCTG-3') or LNA hydrolysis probe 65 (5'-CTGGAGGA-3') from a Universal ProbeLibrary Set (Roche Diagnostics GmbH). The PCR mixture contained 1× PCR buffer, 100 nmol/L of probe (probe 6 for viral DNA detection or probe 65 for *PTPRG* DNA detection), and 200 nmol/L of each primer specific for the viral LT3 coding region (forward: 5'-GCATCTGCACCTTTTC-TAGACTC-3'; reverse: 5'-TTTTGCCTTATAGACTTTT-CCATATCT-3') or for *PTPRG* exon 19 sequence as a reference DNA (forward: 5'-CTACATTGCCACCCAAGGA-3'; reverse: 5'-TCCAGTGTTTTGTTCCCAAAT-3'). The primers and the probes were designed by using the ProbeFinder program at Assay Design Center of Universal ProbeLibrary (www.universalprobelibrary.com; Roche Diagnostics GmbH).

The cycling conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles with denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 45 seconds. The collected data were analyzed by using E-Method software (Roche Diagnostics GmbH), which takes into consideration the difference in amplification efficiency between the target gene and the reference gene when quantifying their relative DNA sequence copy number (22). The relative DNA sequence copy number for each tissue sample was expressed as a ratio of MCPyV DNA to PTPRG DNA. An MCPyV DNA to PTPRG DNA ratio of 0.01 (an arbitrary cutoff value) denotes the presence of one MCPyV DNA sequence per 100 PTPRG DNA sequences, a ratio of 0.1 denotes the presence of one MCPyV DNA sequence per 10 PTPRG DNA sequences, and a ratio of 1.0 denotes the presence of one MCPyV DNA sequence per one PTPRG DNA sequence. PCR amplification of LT3 was analyzed in 88 randomly selected Merkel cell carcinomas in a preliminary series of real-time PCR assays; one Merkel cell carcinoma DNA sample that contained MCPyV based on DNA sequencing and had a crossing point (Cp) value near the median Cp of the series was selected as the calibrator sample for further analyses and was subsequently used to construct the standard curve for the target and the reference gene (22). Whenever MCPyV DNA was detected (ie, when the MCPvV DNA to PTPRG DNA ratio was >0), the sample was considered positive.

Each PCR product generated using primers designed to detect MCPyV DNA was treated with an ExoSAP-IT enzyme mix (product number 78201; USB Corporation, Cleveland, OH) according to the manufacturer's protocol and then sequenced by using BigDye3 termination chemistry and an ABI 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA). The sequences were compared with the reference sequences of MCPyV isolates MCC350 (gb1EU375803.1) and MCC339 (gb1EU375804.1) obtained from the National Center for Biotechnology Information Entrez Nucleotide database by using LaserGene 7.2 software (DNASTAR, Inc., Madison, WI).

## **Statistical Analysis**

Associations between tumor MCPyV DNA status and the clinical variables and tumor histopathologic types were examined by using the  $\chi^2$  test or Fisher exact test. Distributions of age, tumor size, and viral DNA to control DNA ratio between MCPyV DNA–positive and MCPyV DNA–negative cancers were compared with the Mann–Whitney *U* test. The cumulative survival rates of subjects with MCPyV DNA–positive and MCPyV DNA–negative carcinomas were estimated by the Kaplan–Meier method and compared by using the Mantel–Cox log-rank test. Overall survival was calculated from the date of diagnosis to the date of death, with censoring of subjects who were alive on August 1, 2008, when data

collection for the study was closed. Merkel cell carcinoma–specific survival was calculated from the date of diagnosis to the date of death due to Merkel cell carcinoma, with censoring of subjects who, on the date of data collection closure, were still alive as well as of subjects who had died from another cause as determined by clinical or autopsy evidence or from an unknown cause. Subjects who had died with advanced Merkel cell carcinoma were considered to have died from Merkel cell carcinoma. Multivariable survival analysis was done by using a stepwise Cox proportional hazards model that used forward covariate entry to the model. Deviations from the proportional hazards assumption were assessed using qualitative graphical methods, which confirmed the proportional hazards assumption. All P values are two-sided, and P values less than .05 were considered statistically significant.

## Results

A total of 91 (79.8%) of the 114 carcinomas had MCPyV DNA as detected by quantitative PCR. In all of these 91 cancers, the presence of MCPyV DNA was confirmed by DNA sequencing of the quantitative PCR products. The MCPyV DNA to control (PTPRG) DNA ratio among the 91 carcinomas classified as MCPyV DNA positive ranged from 0.0003 to 4224, whereas 23 carcinomas (20.2%) were entirely negative for MCPyV DNA (the MCPyV DNA to control DNA ratio was 0). Three tumors (2.6%) had a very small MCPyV DNA to control DNA ratio (ie, >0 but <0.01; range = 0.0003-0.008), six tumors (5.3%) had ratios that ranged from 0.01 to 0.10, 25 tumors (21.9%) had ratios that ranged from 0.11 to 0.99, and 57 tumors (50.0%) had ratios that ranged from 1.0 to 4224. Quantitative PCR analysis of all 22 control tissue samples was negative for MCPyV DNA, suggesting that presence of viral DNA is restricted to Merkel cell carcinomas and that there was no crosscontamination between the samples.

Standard PCR performed with any of the three viral primer pairs (ie, LT1, LT3, or VP1) was less sensitive for the detection of MCPyV DNA than quantitative PCR with the LT3 primer pair (which was designed to amplify a short fragment). The LT3 primer set detected MCPyV DNA in 54 cancers (49.5%), whereas the VP1 and LT1 primer sets detected MCPyV DNA in only 12 (11.1%) and 32 (29.6%) cancers, respectively (Table 1). The LT1 and VP1 primer pairs tended to detect MCPyV infection when the tumor contained a large amount of MCPyV DNA relative to the control DNA (ie, when the median MCPyV DNA to control DNA ratio determined by quantitative PCR was >4.9). Regardless of the primer pair used for viral DNA amplification, the tumors deemed MCPyV DNA positive by standard PCR had a higher MCPyV DNA to control DNA ratio in a quantitative PCR analysis than those classified as MCPyV DNA negative by standard PCR (P < .001 for all three viral primer pairs; Mann–Whitney U test), indicating that standard PCR is not sensitive enough for MCPyV DNA detection.

MCPyV DNA–positive cancers were located in either the upper or the lower limb more often than MCPyV DNA–negative cancers (40.7% vs 8.7%, respectively; P = .015) (Figure 2, Table 2). Most of the MCPyV DNA–positive cancers (71%) had intermediate morphology; the remainder had trabecular or small cell morphology. Only six (6.6%) of the 91 subjects with MCPyV DNA–positive cancer had regional nodal metastases at diagnosis compared with five (21.7%) of the 23 subjects with MCPyV DNA–negative cancer (P = .043). There was no statistically significant difference between subjects with MCPyV DNA–positive tumors and those with MCPyV DNA–negative tumors with regard to sex, median age at diagnosis, tumor histological subtype, or the primary tumor size.

Eight subjects were considered to be immunocompromised because they had undergone a kidney transplant (three subjects), had chronic lymphocytic leukemia (two subjects), or used corticosteroid medication for rheumatoid arthritis (three subjects). MCPyV DNA was detected by quantitative PCR in the tumors of five (62.5%) of these eight subjects. The rate of viral DNA positivity of the cancers that were diagnosed during the 1980s or 1990s (n = 62) was similar to that of the cancers that were diagnosed after 1999 (n = 52; 82.2% vs 76.9%, P = .48).

The median follow-up time after Merkel cell carcinoma diagnosis for subjects who were alive was 9.9 years (range = 4.9-21.9years). A total of 87 subjects (76.3%) died during follow-up; of those, 25 (28.7%) were considered to have died from Merkel cell carcinoma, 45 (51.7%) died from other causes, and for 17 (19.5%) subjects the cause of death was not specified or was unknown. Subjects with MCPyV DNA-positive cancer had better overall

able 1. Initiation of the Ferrite industry and the associated hazard for death							
PCR primers or method	PCR product length (bp)	No. of MCPyV DNA–positive tumors/Total no. of tumors (%)†	Median MCPyV DNA to control DNA ratio in qPCR (range)‡	HR for death (95% CI)§			
LT1	440	32/108 (29.6)	7.1 (0-4224)	0.66 (0.40 to 1.08)			
LT3	309	54/109 (49.5)	2.0 (0-4224)	0.75 (0.49 to 1.16)			
VP1	352	12/108 (11.1)	4.9 (0-4224)	0.65 (0.30 to 1.42)			
qPCR	74	91/114 (79.8)	1.6 (0.0003-4224)	0.39 (0.24 to 0.65)			

Table 1	Influence	of the P	CR method	on the	detection	rate o	f MCPvV	and the	associated	hazard	for c	leath
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\* PCR = polymerase chain reaction; MCPyV = Merkel cell polyomavirus; qPCR = quantitative PCR; HR = hazard ratio; CI = confidence interval; LT1 = large T antigen region 1; LT3 = large T antigen region 3; VP1 = viral capsid protein 1.

+ Analysis was not successful in six cancers using primers for LT1 or VP1 and in five cancers using primers for LT3 for the 114 cancers that were successfully analyzed with qPCR.

+ Among tumors positive for MCPyV DNA by qPCR. The control DNA was a sequence in exon 19 of the protein tyrosine phosphatase gamma receptor gene.

§ For MCPyV DNA positive vs MCPyV DNA negative. Calculated using univariate Cox proportional hazards regression analysis, with presence of MCPyV DNA as the covariate.



Figure 2. Tumor sites and presence of Merkel cell polyomavirus DNA in tumor. Filled circle = viral DNA positive; open circle = viral DNA negative.

survival than those with MCPyV DNA–negative cancer (5-year survival: 45.0% vs 13.0%, P < .001). Subjects with MCPyV DNA–positive cancer also had better Merkel cell carcinoma–specific survival than those with MCPyV DNA–negative cancer (5-year survival: 75.9% vs 41.1%; P = .022) (Figure 3).

Because some cancers contained a small amount of MCPyV DNA, in a sensitivity analysis, we repeated the survival analysis by using MCPvV DNA to control DNA ratios that were greater than 0 as cutoff values for tumor MCPyV DNA positivity. When we used an MCPyV DNA to control DNA ratio of 0.01 as the cutoff for MCPyV DNA positivity instead of the presence of any MCPyV DNA in the sample, subjects with MCPvV DNA-positive cancer (n = 88) had better overall survival than those with MCPyV DNA-negative cancer (n = 26; 5-year survival: 45% vs 15%, P = .001). However, there was no statistically significant difference between these groups in terms of Merkel cell cancer-specific survival (P = .09). When we used an MCPvV DNA to control DNA ratio of 0.1 as the cutoff, subjects with MCPvV DNA-positive cancer (n = 82) had better overall survival and Merkel cell cancerspecific survival than those with MCPyV DNA-negative cancer (P < .001 and P = .029, respectively). There were no statistically significant differences in the distributions of sex, median age at diagnosis, or survival between the 37 patients who were excluded from the study because of lack of tumor tissue for testing and the subjects who were included in the study.

To examine whether the presence of MCPyV DNA in the tumors was independently associated with survival, we entered this parameter as a covariable into a Cox multivariable proportional hazards model together with the two factors that were most strongly associated with outcome in the univariate survival analyses, that is, presence of regional lymph node metastases and sex (four patients with distant metastases at the time of the diagnosis were excluded from this analysis, leaving 110 patients with 83 survival events). The presence of regional nodal metastases (hazard ratio [HR] of death = 3.10, 95% confidence interval [CI] = 1.54 to 6.24, P = .002) and male sex (HR of death = 1.67, 95% CI = 1.04 to 2.68, P = .032) were independently associated with worse overall survival, whereas the presence of MCPyV DNA in the cancer was associated with better overall survival in the multivariable analysis (HR of death for MCPyV DNA positive vs MCPyV DNA negative = 0.42, 95% CI = 0.25 to 0.71, P = .001) (Table 3).

# Discussion

We analyzed 114 Merkel cell carcinomas and found that the MCPyV DNA-positive cancers tended to be located in a limb, have less frequent nodal metastases at the time of the diagnosis, and were associated with better Merkel cell carcinoma-specific and overall survival compared with MCPyV DNA-negative cancers. Importantly, we observed differences in Merkel cell carcinoma-specific and overall survival between the MCPyV DNA-positive and MCPyV DNA-negative groups in a cohort that had a median age at diagnosis of 78 years, even though such differences can be challenging to detect in an elderly study population that is likely to have a large number of competing causes of death.

MCPyV is one of five human polyomaviruses that have been identified thus far (the others are BK virus, JC virus, WU virus, and KI virus) (13). Although viral genomes of JC virus and BK virus have been detected in many human cancers, neither virus has been shown to be causally associated with human malignant Table 2. Subject and tumor characteristics according to tumor MCPyV DNA status\*

	Tumor MCPyV DNA status			
Characteristic	Negative (n = 23)	Positive (n = 91)	Р	
Sex, No. (%)			.109†	
Male	13 (56.5)	67 (73.6)		
Female	10 (43.5)	24 (26.4)		
Median age at diagnosis (range), y	79.0 (40–90)	78.0 (35–100)	.878‡	
Primary tumor site, No. (%)			.015†	
Head or neck	16 (69.6)	42 (46.2)		
Trunk	5 (21.7)	12 (13.2)		
Limb	2 (8.7)	37 (40.7)		
Tumor morphology, No. (%)			.327†	
Intermediate	14 (60.9)	65 (71.4)		
Trabecular or small cell	9 (39.1)	26 (28.6)		
Median diameter of primary tumor (range), mm	18 (6–40)	16 (3–85)	.438‡	
Regional nodal metastases at diagnosis, No. (%)			.043§	
No	18 (78.3)	85 (93.4)		
Yes	5 (21.7)	6 (6.6)		
Distant metastases at diagnosis, No. (%)			>.999§	
No	22 (95.7)	88 (96.7)		
Yes	1 (4.3)	3 (3.3)		
Any metastases at diagnosis, No. (%)			.154§	
No	18 (78.3)	82 (90.1)		
Yes	5 (21.7)	9 (9.9)		

\* MCPyV = Merkel cell polyomavirus.

† Two-sided χ<sup>2</sup> test.

‡ Two-sided Mann–Whitney U test.

§ Two-sided Fisher exact test.

tumors. However, several lines of evidence suggest that MCPyV infection is causally related to Merkel cell carcinogenesis. The MCPyV genome is frequently and clonally integrated in Merkel cell carcinoma, and there is evidence that MCPyV infection and integration may occur before clonal expansion of tumor cells (12). In addition, viral sequences derived from Merkel cell carcinoma tumor genomic DNA have been shown to harbor mutations that prematurely truncate the MCPyV large T antigen helicase, which eliminates viral DNA replication capacity and might enhance integration of the viral DNA into the host cell genome (23). In this study, we identified clinical factors associated with the presence of MCPyV DNA, lending support to the hypothesis that MCPyV has a role in the pathogenesis of Merkel cell carcinoma. The molecular mechanisms for how MCPyV might contribute to pathogenesis of Merkel cell carcinoma remain speculative, and it is not known when or how MCPyV infection occurs. Of note, we found that Merkel cell carcinomas located on the limbs contained MCPyV DNA statistically significantly more often than tumors located on the trunk. This finding suggests that MCPyV might be transmitted by physical contact.

We detected MCPyV DNA in 79.8% of the tumors in this study, which is slightly higher than the proportion of MCPyV DNA-positive tumors in five earlier studies combined, in which 107 (72.3%) of 148 tumors were MCPyV DNA positive (12–16). We found that the LT3 primer set detected MCPyV more efficiently than the LT1 or VP1 sets, as have other investigators (13,14), possibly because the LT3 primer set generates a shorter DNA fragment than the other two primer sets (Table 1). However, not all strains of MCPyV may be detectable with any one of these primer sets. We found that quantitative PCR, which included the use of fluorescein-labeled probes and had a short amplicon length, was the most sensitive method for detecting MCPyV DNA in this study, in which we used archival paraffin-embedded tissue, which likely contained substantially fragmented DNA, as the starting material.

Although we detected MCPyV DNA in a large proportion of Merkel cell carcinomas, the proportion of MCPvV DNA-positive tumors might vary among populations, and the demographic features of individuals diagnosed with Merkel cell carcinoma may also differ by country. For example, one study (16) found that 11 (69%) of 16 Merkel cell carcinoma specimens from North American patients were positive for MCPyV DNA vs only five (24%) of 21 Merkel cell carcinoma specimens from Australian patients. In general, Merkel cell carcinoma is more common in men than in women (1,3). However, the majority of the subjects in this series were female. The large proportion of female patients in this series may be due, in part, to the sharp increase in Merkel cell carcinoma incidence after age 70 years (2) and the fact that the life span of men in Finland is approximately 7 years shorter than that of women(http://www.who.int/whosis/mort/profiles/mort\_euro\_fin\_ finland.pdf).

A limitation of this study is possible selection bias. Although we based our analysis on a nationwide population-based series, we had to exclude approximately 45% of the subjects identified for various reasons, including one erroneous diagnosis, which might have resulted in a bias. The most common reason for patient exclusion from the study was the unavailability of tumor tissue for testing. However, the median age at diagnosis, distribution of sex, and



**Figure 3.** Kaplan–Meier analysis of survival among patients whose tumors contained (+) or lacked (-) Merkel cell polyomavirus (MCPyV) DNA. **A**) Merkel cell carcinoma (MCC)–specific survival. **B**) Overall survival. Subjects whose tumor contained MCPyV DNA had better MCC-specific survival (hazard ratio [HR] of death = 0.037, 95% confidence interval [CI] = 0.15 to 0.89, P = .022) and overall survival (HR of death = 0.39, 95% CI = 0.24 to 0.65, P < .001) than those whose tumor lacked MCPyV DNA. The P values are two-sided (log-rank test). Five- and 10-year survival data are shown. **Error bars** correspond to 95% CIs.

survival of the excluded patients did not differ statistically significantly from those of the subjects included in the study, which suggests that exclusion of these patients did not create a major bias.

We conclude that MCPyV DNA is frequently present at detectable levels in Merkel cell carcinomas and that MCPyV infection is associated with clinical factors. These findings are compatible with a potential role of MCPyV in the molecular pathogenesis of many Merkel cell carcinomas. Confirmation of MCPyV as a contributing factor to the pathogenesis of Merkel cell carcinoma might provide novel options for future therapeutic strategies.

 Table 3. Multivariable Cox proportional hazards analysis of overall survival\*

β (SE)	HR of death (95% CI)	Р
1.13 (0.358)	3.10 (1.54 to 6.24)	.002
0.52 (0.241)	1.67 (1.04 to 2.68)	.032
-0.87 (0.266)	0.42 (0.25 to 0.71)	.001
	<b>β (SE)</b> 1.13 (0.358) 0.52 (0.241) -0.87 (0.266)	β (SE)         HR of death (95% Cl)           1.13 (0.358)         3.10 (1.54 to 6.24)           0.52 (0.241)         1.67 (1.04 to 2.68)           -0.87 (0.266)         0.42 (0.25 to 0.71)

 Four patients with distant metastases at the time of the diagnosis were not included in the analysis. β = regression coefficient of hazard function;
 HR = hazard ratio; CI = confidence interval; MCPyV = Merkel cell polyomavirus.

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