Clinical Cancer Research

Merkel Cell Polyomavirus Infection, Large T Antigen, Retinoblastoma Protein and Outcome in Merkel Cell Carcinoma

Harri Sihto[1,2,3], Heli Kukko[4], Virve Koljonen[4], Risto Sankila[5], Tom Böhling[6], and Heikki Joensuu[1,2,3]

Abstract

Purpose: Merkel cell carcinoma (MCC) is rare skin cancer that is often associated with Merkel cell polyomavirus (MCPyV) infection. Polyomaviruses repress tumor suppressor proteins, thus influencing cell-cycle progression, but the effect of MCPyV on the key cell-cycle regulating proteins is poorly understood.

Experimental Design: We evaluated expression of the MCPyV large T-antigen (LTA), Ki-67, and the key putative tumor suppressor proteins, the retinoblastoma protein (RB and phospho-RB) and p53, and their regulatory proteins (cyclin D1, cyclin E, p16, p21, p27, and MDM2) by using immunohistochemistry from tumors of 91 MCC patients identified from a population-based nationwide cohort. Tumor MCPyV DNA was measured by using quantitative PCR, and *TP53* mutations were identified with sequencing.

Results: MCPyV LTA expression was strongly associated with presence of MCPyV DNA in tumor, and it was almost invariably associated with tumor RB expression ($P < 0.0001$ for both comparisons). Both MCC LTA and RB expression were strongly associated with favorable MCC-specific and overall survival in univariable analyses ($P \leq 0.01$ for all four analyses). Presence of MCPyV LTA was also associated with the female gender, the intermediate type of tumor histology, location of the tumor in a limb, cell proliferation rate, and absence of p53 expression. *TP53* mutations were detected only in MCPyV DNA–negative tumors.

Conclusions: MCPyV DNA–positive MCC has several clinical and molecular features that differ from MCPyV DNA-negative cancers. MCPyV-associated MCCs express RB, but may not harbor *TP53* mutations. These findings provide further support that MCPyV causes the majority of MCCs. *Clin Cancer Res; 17(14); 4806–13. ©2011 AACR.*

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer. The tumor is uncommon, but data from the United States suggest that the age-adjusted annual incidence of MCC has tripled from 1.5 in 1986 to 4.4 cases per million in 2001 (1). An increased risk of MCC has been linked with immunosuppression related to UV radiation, viral infections, organ transplantation, and chronic lymphocytic leukemia (2–6).

Authors' Affiliations: [1]Laboratory of Molecular Oncology, Biomedicum Helsinki; [2]Molecular Cancer Biology Program, University of Helsinki; [3]Department of Oncology, Helsinki University Central Hospital and University of Helsinki; [4]Department of Plastic Surgery, Helsinki University Central Hospital; [5]Finnish Cancer Registry; and [6]Department of Pathology, HUSLAB, Helsinki University Central Hospital and University of Helsinki, Helsinki, Finland

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Corresponding Author: Heikki Joensuu, Department of Oncology, Helsinki University Central Hospital, Haartmanninkatu 4, FIN-00290 Helsinki, Finland. Phone: 358-0-40-7210438; Fax: 358-0-9-47174202; E-mail: heikki.joensuu@hus.fi

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Approximately 80% of MCCs were found to harbor DNA of a novel human polyomavirus, called Merkel cell polyomavirus (MCPyV), integrated in the tumor genome (7). The MCPyV T-antigen expression is necessary for the maintenance of MCPyV-positive MCC cell lines, but not a MCPyV-negative cell line (8). UV radiation exposure of the skin increases MCPyV small T-antigen mRNA expression, suggesting a link between MCC, age, MCPyV infection, and UV irradiation (9). Patients who have MCPyV DNA–positive MCC had more favorable survival than those diagnosed with MCPyV DNA–negative MCC, when MCPyV-positivity was defined as presence of any MCPyV DNA in the tumor tissue (10). These findings lend support to the hypothesis that MCPyV is involved in the genesis of most MCCs, but the molecular mechanisms remain inadequately understood. Polyomavirus-encoded T antigens target several tumor suppressor proteins, including the retinoblastoma protein (RB) and p53 protein (11). In particular, the MCPyV large T antigen (LTA) might have an essential role in MCC tumorigenesis by inhibiting the cell-cycle regulation function of RB, whereas MCPyV T antigens lack a putative p53-binding domain because of tumor-associated T-antigen deletion mutations (12, 13).

The effect of MCPyV on the key cell-cycle regulating proteins is poorly understood. MCPyV LTA binds RB (12) and influences its function in experimental models,

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Translational Relevance

Merkel cell carcinoma (MCC) is rare, neuroendocrine skin cancer that often contains Merkel cell polyomavirus (MCPyV) DNA. MCPyV may be a causative agent in this disease, but the clinical features of MCPyV-related MCC compared with non–MCPyV-related cancer have remained elusive. The present findings suggest that there are 2 molecularly and clinically distinct subtypes of MCC; (i) MCPyV DNA–positive and retinoblastoma protein (RB)-expressing cancer that is generally associated with favorable disease outcome, and (ii) MCPyV DNA–negative tumors that may harbor *TP53* mutations and have unfavorable outcome. Positive immunostaining for MCPyV large T antigen in tumor correlates closely with detection of MCPyV DNA by using quantitative PCR and with presence of RB in tumor. The data suggest that MCPyV infection can be detected reliably by using immunohistochemistry. Besides favorable prognosis, MCPyV-infected MCC is associated with several clinical, histologic, and molecular biological features supporting the hypothesis that MCPyV causes the majority of MCCs.

in which the LTA and RB interaction is required for sustained tumor growth (14). Therefore, the mechanisms of the cell-cycle regulation might be different in MCPyV-positive and MCPyV-negative MCCs. In this study, we investigate expression of cell-cycle regulatory proteins in MCPyV-positive and MCPyV-negative MCCs, the molecular and clinical correlates of MCPyV infection in MCC, and immunostaining for the LTA as an alternative method to PCR and DNA sequencing to detect MCPyV infection.

Materials and Methods

Patients and tumors

Patients diagnosed with MCC in Finland between January 1, 1979 and December 31, 2004 were identified from the files of Finnish Cancer Registry. Following this, the hospital and primary care center records of the 207 patients identified were reviewed, as well as the histologic diagnoses by a pathologist with a special interest in MCC (T.B.). The diagnosis of MCC was considered confirmed when tumor morphology was compatible with MCC, immunostaining for cytokeratin 20 was positive, or both synaptophysin and chromogranin A immunostainings were positive when cytokeratin 20 was not expressed ($n = 3$), and immunostaining for thyroid transcription factor-1 was negative to exclude metastatic small cell lung carcinoma. Tumor histology was classified according to the World Health Organization criteria (15) and as described elsewhere (16).

Ninety-three (44.9%) patients were excluded for following reasons: formalin-fixed paraffin-embedded archival tumor tissue was not available for review ($n = 37$), the tumor was not MCC upon histologic review ($n = 13$), the primary tumor site was unknown ($n = 8$), clinical information was not available ($n = 16$), the quality of the DNA extracted from the tumor was considered unsuitable for analysis of MCPyV DNA ($n = 19$), or representative tissue was not available for LTA expression analysis ($n = 23$). The remaining 91 patients with histologically confirmed MCC and with clinical information and representative tumor tissue available were included in the study (Fig. 1).

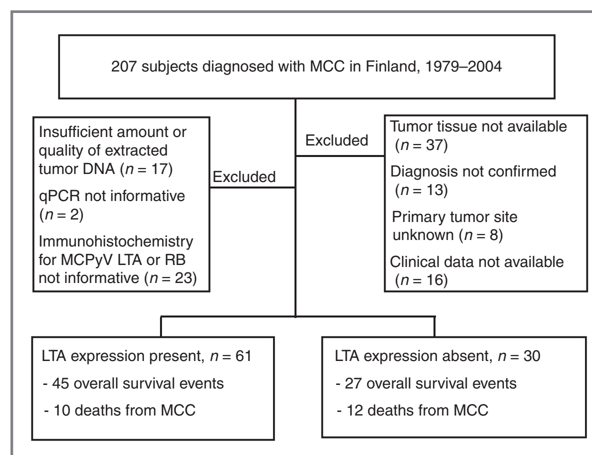

Figure 1. The Consort diagram.

The median age of the patients at diagnosis was 79 (range = 35–100) and 28 (30.8%) were male. Staging was done as suggested by Lemos and colleagues (17). The longest tumor diameter was measured from the hematoxylin-eosin–stained slides whenever feasible, and the diameters of large tumors were extracted from the case records. The patients were treated with excision of the primary tumor. Postoperative external beam radiation therapy was administered to 13 (14.2%) patients, and none received systemic adjuvant chemotherapy. The median follow-up time of the patients alive at the time of the analysis was 9.8 years (range = 5.5–18.8 years).

An Institutional Review Board of the Helsinki University Central Hospital approved the study. The Ministry of Social Affairs and Health, Finland, granted a permission to collect clinical data and tumor tissue for the study.

MCPyV DNA and *TP53* analysis

Genomic DNA was extracted from deparaffinized tissue sections by using QIAamp DNA Mini kit (Qiagen, GmbH). The tissue sections used for DNA extraction were representative and consisted mostly of tumor cells. MCPyV DNA-to-reference gene (protein tyrosine phosphatase gamma receptor gene, *PTPRG*) ratio in tumor tissue was investigated by using quantitative PCR (qPCR) with hydrolysis probes and a LightCycler 480 instrument (Roche Diagnostics GmbH; ref. 10). Viral origin of each PCR product was verified by sequencing by using BigDye v.3.1 termination chemistry (Applied Biosystems) and an ABI 3730xl DNA Analyzer (Applied Biosystems).

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Aberrations of *TP53* exons 4 to 9 were studied by using PCR and DNA sequencing from tissue of 20 tumor samples that did not contain any MCPyV DNA and in 20 randomly selected MCPyV DNA–positive tumors. DNA was amplified in a 20-μL reaction with a FastStart Taq DNA Polymerase dNTPack kit (Roche Diagnostics GmbH) following the manufacturer's recommendations (the primer sequences are provided in Supplementary Table S1 at http://research.med.helsinki.fi/cancerbio/joensuu/supplementarytables.htm). The PCR cycling conditions consisted of an initial denaturation step at 95°C for 4 minutes, followed by 40 cycles with denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 45 seconds, and the final elongation at 72°C for 7 minutes. The PCR products were sequenced. Mutation analysis was considered successful when at least 5 of the 6 exons could be amplified and sequenced.

Immunohistochemistry

Tissue microarrays (TMA) were constructed (18), and 5 μm were cut on SuperFrost+ slides (Menzel-Gläser). The sections were deparaffinized in xylene (2 × 5 minutes) and rehydrated through decreasing alcohol gradient. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Antigen retrieval was carried out in sodium citrate (10 mmol/L, pH = 6.0) in an autoclave (at 120°C for 2 minutes) or in water bath for the LTA (at 98°C for 20 minutes). LTA expression was detected by using mouse monoclonal antibody (CM2B4, sc-136172; Santa Cruz Biotechnology Inc.; dilution 1:100; ref. 19). The antibodies used and their dilutions are provided in Supplementary Table S2. All antibodies were diluted in a PowerVision preantibody blocking solution and incubated either overnight at 4°C (Ki-67, p16, p53, phospho-RB, MDM2, and LTA), for 30 minutes (cyclin D1, cyclin E, and RB), or for 1 hour (p21, p27) at room temperature. Binding of the primary antibody was detected by using a PowerVision+ Poly-HRP Histostaining Kit (Immunovision Technologies Co.) following the manufacturer's recommendations.

A soft tissue sarcoma TMA served as positive control for the MDM2 expression, and a breast cancer TMA for other immunostainings. When classifying immunostainings, the proportion of positive tumor cell nuclei was counted, and the tumors were divided into 5 categories on the basis of the percentage of tumor cell nuclei that stained positively out of all tumor cell nuclei (0%, 1%–10%, 11%–19%, 20%–50%, or >50%). The tumors were further divided into 4 categories on the basis of the staining intensity (negative, faint, intermediate, or strong), but this was not done for immunostainings of cyclin E, Ki-67, p16, p21, and RB, because in these assays, the staining intensity was uniformly strong with little variation. Nuclear LTA expression was classified either negative (no staining) or positive [either faint (+), moderate (++), or strong (+++) staining intensity present in 50% or more of cancer cells]. Nuclear MDM2 expression was graded only by the staining intensity because of the presence of uniformly faint staining in the few positive cases, and p53 staining was graded on the basis of the percentage of positive nuclei. Phospho-RB intensity varied from faint to strong within the TMA core samples, and this staining was, therefore, best categorized on the basis of the frequency of positive tumor cell nuclei. In further analyses, samples with 20% or more positive tumor cell nuclei were considered positive.

Statistical analysis

Frequency tables were analyzed with the χ^2 test or Fisher's exact test. Continuous variables between groups were analyzed by using Mann–Whitney's *U* test. Cumulative survival was estimated with the Kaplan–Meyer method, and survival between groups, HRs, and their 95% CIs were computed by using the Cox proportional hazards model. Multivariate survival analyses were done with the Cox proportional hazards model, excluding patients who had distant metastases at diagnosis ($n = 4$). The assumption of proportional hazards was ascertained by assessing log minus log survival plots. Overall survival was calculated from the date of the diagnosis to death censoring subjects alive on August 1, 2008. MCC-specific survival was calculated from the date of the diagnosis to death considered to be due to MCC. *P* values are 2-sided. Statistical analyses were done by using a PASW Statistics 18 software (SPSS Inc.).

Results

MCPyV DNA, LTA, and RB

Viral LTA expression was easily detectable in immunohistochemistry (Fig. 2). It was expressed in 61 (67.0%) of the 91 MCCs [faint expression in 4, moderate (++) in 5, and strong (+++) in 52; expression was present in all tumor cell nuclei except in 1 case in which approximately 50% of cancer cell nuclei were stained]. LTA expression was strongly associated with presence of MCPyV DNA in tumors when any copy number of MCPyV DNA in the tumor sample was considered positive ($P < 0.0001$), but 12 (40.0%) of the 30 MCCs that did not express viral LTA in immunohistochemistry still had detectable MCPyV DNA in a qPCR analysis (Table 1). However, 5 of these 12 LTA-negative tumors contained very small copy numbers of MCPyV DNA, only from 0.0003 to 0.02 copies per one reference gene (*PTPRG*) and thus were likely not truly MCPyV-infected tumors. We, therefore, selected a MCPyV DNA copy number 0.1 or greater per one reference gene as the cutoff for MCPyV DNA positivity in subsequent analyses.

Tumor RB expression had a very strong positive association with tumor viral LTA expression (Table 1). All LTA-positive tumors expressed RB, whereas 26 (86.7%) of the 30 LTA-negative tumors were also RB-negative ($P < 0.0001$). The MCPyV DNA copy numbers assessed with qPCR were higher in RB-positive MCCs compared with RB-negative tumors (median, 2.03; range = 0–4,224 vs. median, 0; range = 0–1,149, respectively; $P < 0.0001$). The associations between presence of MCPyV DNA, viral LTA,

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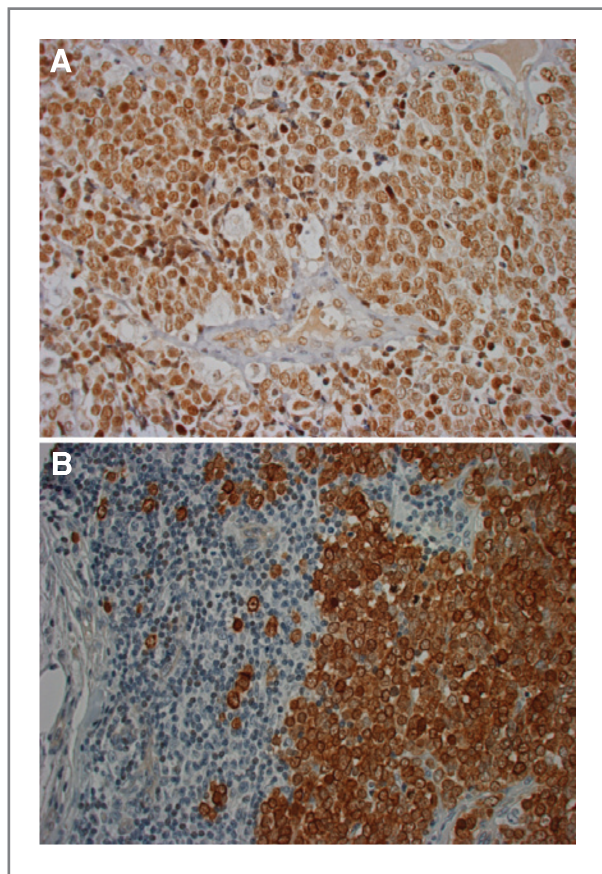

Figure 2. Examples of immunohistochemical stainings of RB (A) and MCPyV large T antigen (B). Original magnification ×400.

and tumor RB expression were close, because as many as 80 (87.9%) of the 91 tumors were either positive or negative for all 3 factors and in only 11 (12.1%) cases, a discrepant finding was found in these 3 assays (Fig. 3). There was no significant difference in phospho-RB expression between LTA-positive and LTA-negative tumors (Table 1).

p53 expression and *TP53* mutations

Unlike RB, p53 was only infrequently expressed in LTA-positive MCCs, whereas its expression was common in LTA-negative cancers [4 (7.0%) of 57 vs. 13 (43.3%) of 30, respectively; $P < 0.0001$; Table 1]. MCPyV DNA copy numbers were lower in p53-positive MCCs compared with p53-negative ones (median, 0.0; range = 0–35.8 vs. median, 1.09; range = 0–4,224, respectively; $P = 0.001$). MDM2, an inhibitor of p53, was expressed infrequently regardless of tumor LTA expression (Table 1).

TP53 exons 5 to 9 were successfully sequenced in 39 (97.5%) of the 40 MCCs selected for mutation analysis. All mutations were either deletions or single-base substitutions (data not shown). As expected, presence of *TP53* mutation was associated with tumor p53 protein expression; 3 (11.5%) of the 26 p53-negative cancers harbored *TP53* mutation compared with 8 (61.5%) of the 13 p53-positive MCCs ($P = 0.002$). Two of the 3 mutations detected in p53-negative tumors were single-base substitutions causing a premature stop codon in the *TP53*-reading frame, and the third tumor expressed p53 only in the cytoplasm and was, therefore, classified as p53 negative.

TP53 mutations were exclusively present in LTA-negative cancers (Table 1). *TP53* mutation–negative MCCs contained higher MCPyV DNA copy numbers compared with *TP53* mutation–positive MCCs (median, 0.365; range = 0–651 vs. median, 0; range = 0–0.286, $P = 0.018$). None of the 18 studied RB-positive MCCs contained *TP53* mutation compared with 11 (52.4%) of the 21 RB-negative tumors ($P = 0.0003$).

Other cell-cycle regulatory proteins

MCCs were highly proliferating tumors regardless of the tumor MCPyV LTA status and most (97.8%) expressed Ki-67 (Table 1; Supplementary Table S3). The median percentage of Ki-67–positive tumor cell nuclei was higher in LTA-negative compared with LTA-positive MCCs (61.4% vs. 52.3%, $P = 0.004$). Interestingly, cyclin D1 was expressed only in 2 (2.4%) tumors, whereas strong expression of p16, an inhibitor of the cyclin D–CDK4/6 complex, was present in almost all tumors [86 (97.7%) of 88 tumors available for analysis]. Unlike cyclin D1, cyclin E was frequently expressed; 78 (94.0%) of 83 tumors expressed it despite that approximately one-half of the tumors expressed p21 and p27 that are inhibitors of the cyclin E–CDK2 complex activity (Table 1). LTA-positive MCCs expressed cyclin E more often compared with LTA-negative tumors (98.2% vs. 85.2%, $P = 0.037$).

We carried out a sensitivity analysis choosing 10% as the cutoff value instead of 20% for the immunostainings listed in Table 1. The results remained essentially the same except for cyclin E expression that was no longer significantly associated with MCPyV LTA expression when the 10% cutoff value was used ($P = 0.246$).

Clinical correlations

Presence of viral LTA in MCC was associated with the tumor site in a limb, absence of regional nodal or distant metastases at the time of the diagnosis (stage 1 or 2), female gender, and intermediate type of tumor histology but not with age at diagnosis (Table 1).

Presence of viral LTA and RB expression in tumor were strongly associated with both favorable MCC-specific survival (HR = 0.22; 95% CI: 0.09–0.52; $P = 0.0005$; and HR = 0.63; 95% CI: 0.47–0.84; $P = 0.0016$, respectively) and overall survival (HR = 0.40; 95% CI: 0.24–0.65; $P = 0.0002$; and HR = 0.36; 95% CI: 0.21–0.60; $P < 0.0001$, respectively; Fig. 4). Presence of MCPyV DNA by qPCR using the cutoff of 0.1 copies per one copy of the reference gene was also a favorable prognostic factor, both for MCC-specific survival (HR = 0.42, 95% CI: 0.18–1.01, $P = 0.054$) and overall survival (HR = 0.49; 95% CI: 0.30–0.82, $P = 0.007$).

When tumor postsurgical stage (stage 1 or 2 vs. 3) and the MCPyV DNA status (positive vs. negative) were

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Table 1. Associations of MCPyV large T-antigen expression with clinicopathologic factors in MCC

Variable	MCPyV LTA expression		*P*
	Absent (*n* = 30) No. (%)	Present (*n* = 61) No. (%)	
RB			
<20% positive	26 (86.7)	0 (0)	<0.0001
≥20% positive	4 (13.3)	61 (100)	
MCPyV DNA			
Absent (<0.1 copies)	23 (76.7)	2 (3.3)	<0.0001
Present (≥0.1 copies)	7 (23.3)	59 (96.7)	
MCPyV DNA			
Absent (none detectable)	18 (60.0)	2 (3.3)	<0.0001
Present (any)	12 (40.0)	59 (96.7)	
p53			
<20% positive	17 (56.7)	53 (93.0)	<0.0001
≥20% positive	13 (43.3)	4 (7.0)	
N.A.	0	4	
Tumor site			
Head or neck	18 (60.0)	23 (37.7)	0.0005
Trunk	8 (26.7)	5 (8.2)	
Limb	4 (13.3)	33 (54.1)	
TP53 mutation[a]			
Absent	12 (47.6)	16 (100)	0.001
Present	11 (52.4)	0 (0)	
Ki-67			
Median % (range)	61.4 (0.5–99)	52.3 (0–95)	0.004
N.A.	0	1	
Stage			
1	14 (46.7)	30 (49.2)	0.006
2	7 (23.3)	27 (44.3)	
3 or 4	9 (30.0)	4 (6.5)	
Gender			
Female	16 (53.3)	47 (77.0)	0.021
Male	14 (46.7)	14 (23.0)	
Tumor histology			
Intermediate	17 (56.7)	53 (86.9)	0.023
Trabecular or small	13 (43.3)	8 (13.1)	
Cyclin E			
<20% positive	4 (14.8)	1 (1.8)	0.037
≥20% positive	23 (85.2)	55 (98.2)	
N.A.	3	5	
p27			
<20% positive	13 (43.3)	14 (23.3)	0.051
≥20% positive	17 (56.7)	46 (76.7)	
N.A.	0	1	
Cyclin D1			
<20% positive	27 (93.1)	55 (100)	0.117
≥20% positive	2 (6.9)	0 (0)	
N.A.	1	6	
Age at diagnosis, y			
Median (range)	79.5 (40–100)	78 (35–95)	0.460
p21			
<20% positive	13 (44.8)	30 (51.7)	0.544
≥20% positive	16 (55.2)	28 (48.3)	
N.A.	1	3	

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Table 1. Associations of MCPyV large T-antigen expression with clinicopathologic factors in MCC (Cont'd)

Variable	MCPyV LTA expression		*P*
	Absent ($n = 30$) No. (%)	Present ($n = 61$) No. (%)	
Phospho-RB			
<20% positive	10 (34.5)	24 (40.7)	0.575
≥20% positive	19 (65.5)	35 (59.3)	
N.A.	1	2	
Tumor diameter, mm			
Median (range)	18.0 (8–50)	18.0 (6–85)	0.852
p16			
<20% positive	1 (3.3)	1 (1.7)	>0.999
≥20% positive	29 (96.7)	57 (98.3)	
N.A.	0	3	
MDM2			
Negative	27 (93.1)	53 (94.6)	>0.999
Faint	2 (6.9)	3 (5.4)	
N.A.	1	5	

N.A., not available.
[a] *TP53* mutation analysis was carried out in 40 cases of which RB expression was available in 39 cases.

entered as covariables into a Cox multivariate model, both factors were independently associated with overall survival (HR = 0.33; 95% CI: 0.16–068; $P = 0.003$; and HR = 0.52, 95% CI: 0.31–0.89; $P = 0.016$; respectively). Similarly, LTA expression was associated with favorable survival when entered as a covariable together with the postsurgical stage in a multivariate model (HR = 0.49; 95% CI: 0.29–0.83; $P = 0.008$; and HR = 0.41; 95% CI: 0.19–0.88, $P = 0.023$; respectively), as was RB expression (HR = 0.42; 95% CI: 0.24–0.74; $P = 0.024$; and HR = 0.45; 95% CI: 0.21–0.92; $P = 0.042$, respectively). However, presence of MCPyV DNA and LTA expression lost their independent prognostic significance when included in a multivariable model together with tumor RB expression and stage indicating strong interaction between presence of MCPyV DNA in tumor, and tumor LTA and RB expression.

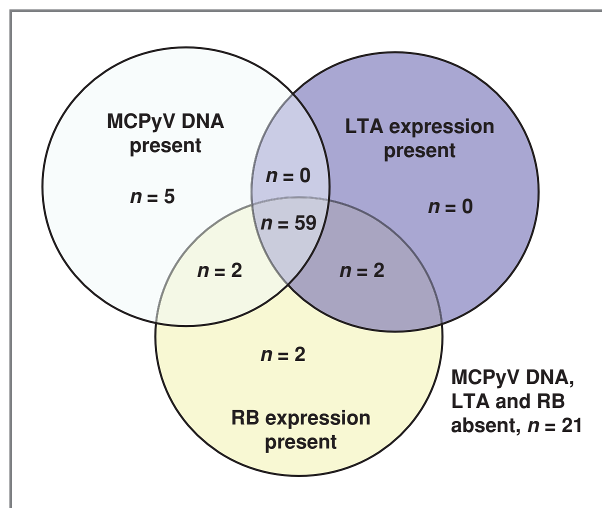

Figure 3. Venn diagram showing association of presence of MCPyV DNA, MCPyV LTA expression, and RB expression in MCC.

Discussion

Expression of MCPyV LTA in immunohistochemistry was strongly associated with presence of MCPyV DNA in tumors as detected by qPCR and DNA sequencing. Tumor MCPyV DNA, viral LTA, and RB expression were often found in the same tumors, and all 3 factors were associated with both MCC-specific and overall survival. Immunohistochemical analysis of MCPyV LTA thus offers a practical method to identify MCPyV-infected MCCs, and it is a novel tool for assessing prognosis of MCC.

RB is a key regulator of the G_1- to S-phase transition in cell-cycle progression. Unphosphorylated RB inhibits function of the E2F transcription factor, which regulates expression of genes required in DNA replication. Phosphorylation of RB by the cyclin/cyclin-dependent kinase complexes (C/CDKs) causes RB–E2F complex dissociation and cell-cycle entry, which is opposed by several inhibitor proteins such as p16, p21, and p27 (20). Polyomaviruses induce an alternative mechanism to enhance cell-cycle progression, because the LTA of polyomaviruses binds RB and thus reduces RB–E2F complex formation (11, 12).

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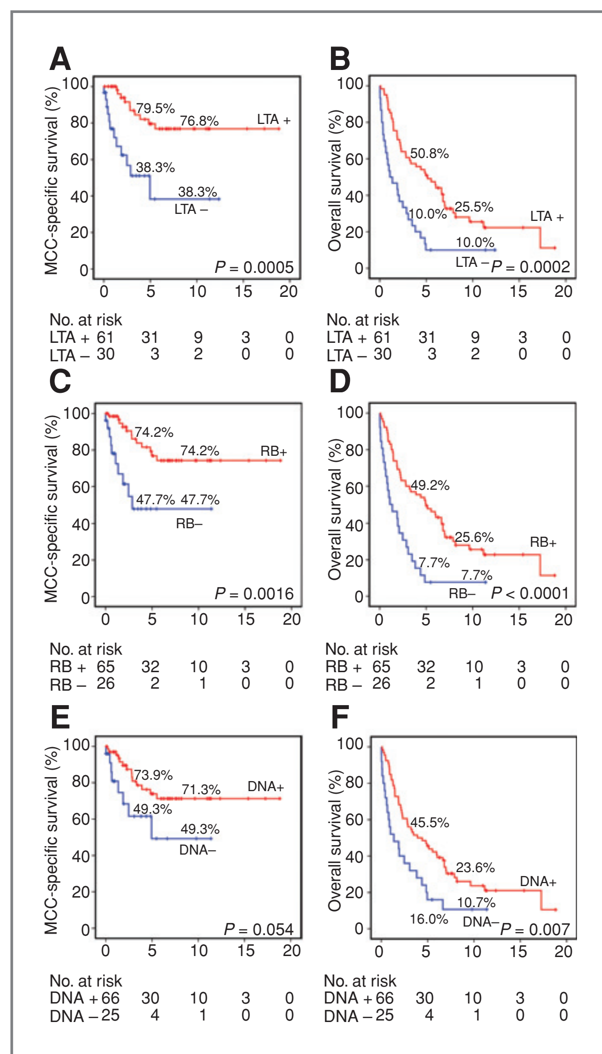

Figure 4. MCC–specific survival and overall survival stratified by MCPyV LTA expression (A and B), RB expression (C and D), and MCPyV DNA (E and F), respectively.

In the current series, RB and MCPyV LTA expression were closely associated, suggesting importance of RB inhibition as a mechanism for MCPyV-induced tumorigenesis. A study based on a xenotransplantation model found that MCPyV-positive MCCs depend critically on T-antigen expression and that the LTA interacts directly with the RB, which was required for sustained tumor growth (14). In line with these findings, almost all simian virus 40 (SV40, a polyomavirus) LTA-mediated gene expression regulation in mouse enterocytes depends upon binding and inactivation of the RB family proteins, and in this model truncated SV40 LTA interferes with the RB pathway inducing intestinal hyperplasia (21). In this study, LTA expression was not associated with expression of phosphorylated RB, suggesting that the influence of MCPyV infection on cell-cycle progression may not depend on RB phosphorylation. Tumor LTA expression was not significantly associated with tumor p21, p16, or cyclin D1 levels, whereas cyclin E and p27 levels tended to be elevated in LTA-positive MCCs.

Besides RB, polyomaviruses also target p53, an important tumor suppressor protein that regulates the cell cycle, growth arrest, cell senescence, and apoptosis (11, 22). The MCPyV genome encodes a p53-binding large T domain, which is often truncated due to MCPyV genome mutations (12, 13). Of note, *TP53* mutations were exclusively found in LTA- or RB-negative tumors suggesting p53 involvement in tumorigenesis of MCCs that are not related with MCPyV infection. Our results do not rule out a possibility that MCPyV-associated MCC tumorigenesis might involve p53 at some stage of tumorigenesis or that MCPyV-positive tumor cells might contain untruncated episomal viral genomes that produce p53-binding LTAs.

Research on prognostic influence of MCPyV infection in MCC has been hampered by having only small series of this rare tumor available for analysis. Yet, in line with the current findings, Bhatia and colleagues found in a series consisting of 23 MCCs that high MCPyV copy numbers per cell tend to be associated with absence of p53 expression, high RB expression, and possibly with favorable outcome (23), and suggested that LTA expression might also be associated with favorable survival (24). We identified a small subgroup ($n = 5$, 5.5%) of LTA-negative MCCs with very low viral DNA copy numbers (<0.1 copies per one copy of the reference gene) that did not express LTA in immunohistochemistry. In survival analyses, this subgroup resembled viral DNA-negative tumors and none of them expressed RB, suggesting that the small amount of viral DNA detected by qPCR was passenger MCPyV DNA that was not involved in tumor pathogenesis.

The risk of a selection bias might be relatively small in the current study, because the patients were identified from a nationwide cancer registry with coverage close to 100% (25). Yet, selection bias cannot be excluded, because we had to exclude 56.0% of the cases for various reasons, most commonly because of unavailability of tissue material. The patient population in the series was elderly with a median age of 79 years and a high rate of competing causes of death. Despite the confounding effect of intercurrent deaths, presence of MCPyV DNA and expression of LTA were associated with overall survival, suggesting that they are strong prognostic factors in MCC. Many MCC series have male preponderance (1, 26). Most patients in the current series were female, which is likely explained by a rapid increase of MCC incidence with age and the approximately 7 years longer life span of women than of men in Finland (http://www.who.int/whosis/mort/profiles/mort_euro_fin_finland.pdf).

We conclude that immunostaining for the viral LTA is a quick and reliable method to show the presence of MCPyV infection and that LTA expression is associated with favorable survival. MCPyV infection is associated with distinct molecular and clinical features, which lends further

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support that MCPyV is a causative agent of MCC. Presence of MCPyV DNA and the LTA in the tumor are strongly associated with RB expression, suggesting that RB is involved in MCPyV-induced cell-cycle regulation aberrations, whereas *TP53* mutations occur in MCPyV-negative MCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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