

MALARIA AND SOME POLYOMAVIRUSES (SV40, BK, JC, AND MERKEL CELL VIRUSES)

VOLUME 104

This publication represents the views and expert
opinions of an IARC Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon, 7-14 February 2012

LYON, FRANCE - 2014

IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS

MERKEL CELL POLYOMAVIRUS

1. Exposure Data

1.1 Cell and tissue tropism

Merkel cell polyomavirus (MCV) was first discovered clonally integrated in Merkel cell carcinoma (MCC) ([Feng et al., 2008](#)). Merkel cells reside in the basal layers of the skin and express dual epithelial/neuroendocrine phenotypic markers. In conjunction with sensory afferent neurites, Merkel cells normally function as mechanoreceptors. A comprehensive initial study by Loyo and colleagues showed that MCV DNA can be detected, in varying amounts, in specimens from a wide range of different tissues ([Loyo et al., 2010](#)). The highest amounts of MCV DNA are detected in oral samples and are shed from apparently healthy skin surfaces in the form of assembled virions ([Wieland et al., 2009](#); [Loyo et al., 2010](#); [Schowalter et al., 2010](#)). Kidney and bladder specimens carry a much lower level of MCV DNA, indicating that the urinary tract is unlikely to be a major site of productive MCV infection, as it is for BK polyomavirus (BKV) and JC polyomavirus (JCV). It appears that MCV DNA can be readily detected in environments occupied by humans ([Foulongne et al., 2011](#)). The possibility of environmental contamination should be considered a very serious concern for polymerase chain reaction (PCR)-based studies of MCV ([Cohen & Enserink, 2011](#)).

1.2 Methods for the detection of MCV

Detection of current MCV infection is based on the detection of viral DNA by PCR amplification, and current and past infection can be detected by the detection of specific antibodies. The sequence diversity of known wild-type (WT) MCV isolates is very low, with > 98.5% nucleotide identity across the viral genome.

1.2.1 Detection of MCV DNA

Viral DNA is detected by PCR, nested PCR, real-time PCR, quantitative PCR (qPCR), and rolling circle amplification (RCA), using different primers specific to the genes encoding MCV large T-antigen (LT), small T-antigen (sT), and capsid viral protein 1 (VP1) antigen (for references, see [Table 1.1](#)). Methodological differences across studies may explain some of the variations observed in MCV DNA prevalence. Samples positive for one set of primers only could be attributed to the failure of PCR to detect low levels of viral DNA, or to disruption of the virus during integration (for MCC samples). For PCR amplification from formalin-fixed, paraffin-embedded (FFPE) samples, as used in many studies, it has been reported that formalin fixation may fragment DNA and results in differences in amplification according to the size of the amplicons ([Kassem et al., 2008](#)). As reported by [Pastrana et al. \(2012\)](#), this is particularly important for

Table 1.1 Detection of MCV DNA in healthy skin or inflammatory skin lesions

Reference	Study location	Samples tested	Methods	IC patients	No. of subjects	Detection of MCV DNA n (%)
<i>PCR and nPCR</i>						
Feng et al. (2008)	USA	Skin/inflammatory skin lesions	PCR	–	18	1 (5.6%)
Dworkin et al. (2009)	USA	Normal skin adjacent (SCC patients)	PCR	–	32	9 (28.1%)
Wieland et al. (2009)	Germany	Perilesional healthy skin	nPCR	–	34	8 (23.5%)
Andres et al. (2010a)	Germany	Inflammatory skin lesions	PCR + SB	–	23	2 (8.7%)
Andres et al. (2010b)	Germany	Sun-exposed skin with seborrhoeic keratosis	PCR + SB	–	12	2 (16.6%)
Mogha et al. (2010)	France	Skin	PCR	–	20	2 (10.0%)
Mangana et al. (2010)	Switzerland	Skin	PCR	–	11	0 (0.0%)
Mertz et al. (2010a)	Switzerland	Common wart	PCR	–	30	2 (6.7%)
Mertz et al. (2010a)	Switzerland	Common wart	PCR	+	16	1 (6.3%)
<i>RT-qPCR and RCA</i>						
Garneski et al. (2009)	North America	Skin	RT-qPCR	–	15	0 (0.0%)
Foulongne et al. (2010b)	France	Skin swabs	RT-qPCR	–	25	25 (100.0%)
Schowalter et al. (2010)	USA	Skin swabs	RCA	–	35	14 (40.0%)
Faust et al. (2011)	Sweden, Austria	Benign skin lesions	RT-qPCR	–	119	22 (18.5%)
Wieland et al. (2011)	Germany	Forehead swabs	RT-qPCR	–	239	118 (49.4%)
Wieland et al. (2011)	Germany	Forehead swabs	RT-qPCR	+	210	124 (59.0%)

IC, immunocompromised; MCV, Merkel cell polyomavirus; nPCR, nested PCR; RCA, rolling circle amplification; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative PCR; SB, Southern blot hybridization; SCC, squamous cell carcinoma

samples containing very low copy numbers per cell.

Comparisons of the results of MCV DNA prevalence and viral load must be undertaken with caution.

1.2.2 Detection of MCV antibodies

Most of the studies investigating anti-MCV antibodies use VP1, or VP1 plus VP2 virus-like particles (VLPs) as antigens, and they are produced in insect cells, human embryonic kidney 293TT cells, or glutathione S-transferase (GST)-VP1 recombinant protein (capsomeres) ([Kean et al., 2009](#); [Tolstov et al., 2009](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)). Immunological tests using VLPs or capsomeres derived from strain MCC350 were found to be less sensitive than tests using capsomeres derived from strain MCC339 ([Kean et al., 2009](#)). However, the VP1 protein of strain MCC350 did not assemble into VLPs or capsomeres ([Touzé et al., 2010](#)). In addition to VLPs and capsomere enzyme-linked immunosorbent assay (ELISA)-based assays, neutralization assays using MCV pseudovirions produced in human embryonic kidney 293TT cells and Luminex-based multiplex serological assays (for further details, see Section 1 of the *Monograph* on BKV in this Volume) have been used ([Pastrana et al., 2009](#)).

Pre-incubation of serum samples with JCV, BKV, and lymphotropic papovavirus (LPV) has shown that there is limited cross-reactivity between MCV and these other human polyomaviruses ([Kean et al., 2009](#); [Tolstov et al., 2009](#); [Touzé et al., 2010](#); [Viscidi et al., 2011](#)). Pseudovirion neutralization assays also support the specificity of MCV reactivity ([Pastrana et al., 2009](#)).

Detection of antibodies against MCV LT and sT is useful to assess the presence of MCV in MCC and to monitor tumour progression in MCC patients ([Paulson et al., 2010](#)). However, they are rarely detected in the general population

and are therefore not used for detecting exposure to the virus.

1.2.3 Detection of MCV proteins

Detection of the MCV LT (using the monoclonal antibody CM2B4) is a marker commonly used to examine the presence of MCV in MCC cells. This antibody recognizes the MCV LT and 57kT isoforms but will not detect MCV sT ([Busam et al., 2009](#)).

1.3 Epidemiology of infection

1.3.1 Seroprevalence of MCV

MCV serology studies show that a high proportion of adults have been exposed to MCV and that infection is acquired early in life ([Carter et al., 2009](#); [Kean et al., 2009](#); [Pastrana et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Sadeghi et al., 2010](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Faust et al., 2011](#); [Touzé et al., 2011](#); [Viscidi et al., 2011](#); [Table 1.2](#)). The age-specific seroprevalence of MCV indicates widespread exposure early in life, with a seroprevalence of 20% in children aged 1–5 years ([Kean et al., 2009](#)) and of 35–50% in children aged < 10–15 years ([Tolstov et al., 2009](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)).

Seroprevalences of 46–87.5% have been reported in adults ([Carter et al., 2009](#); [Kean et al., 2009](#); [Pastrana et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Touzé et al., 2010, 2011](#); [Viscidi et al., 2011](#)). Among newly infected individuals, a transient increase in immunoglobulin (Ig) M antibodies that declines over 1–2 years is observed, and the majority of individuals (65%) demonstrate a robust IgG seroconversion, with stable levels over time ([Tolstov et al., 2011](#)). An increase in MCV antibodies ELISA optical density (OD) values with age has also been reported in adults ([Viscidi et al., 2011](#)), suggesting that antibody titres increase with age.

Table 1.2 Detection of MCV antibodies in healthy subjects and in patients with cancer other than MCC or in patients with skin diseases

Reference	Study location	MCV antigen used	Age (years)	No. of subjects	Anti-MCV antibodies <i>n</i> (%)
<i>Children</i>					
Kean et al. (2009)	USA	GST-VP1	1–4	112	23 (20.5%)
			5–17	517	182 (35.2%)
Tolstov et al. (2009)	USA	VP1 + VP2 VLPs	2–15	81 ^a	38 (46.9%)
Chen et al. (2011)	Finland	VP1 VLPs	1–4	298 ^b	19 (6.4%)
			5–13	26 ^c	9 (34.6%)
			1–4	158 ^d	6 (4%)
Viscidi et al. (2011)	Italy	VP1 VLPs	1–9	42	19 (45.2%)
			10–19	38	23 (60.5%)
<i>Adults</i>					
Carter et al. (2009)	USA	GST-VP1	42–86	76	40 (53%)
			24–77	451	268 (59.4%)
Kean et al. (2009)	USA	GST-VP1	18–70s	1593	734 (46.1%)
Pastrana et al. (2009)	USA	VP1 + VP2 pseudovirions	47–75	48	42 (87.5%)
Tolstov et al. (2009)	USA	VP1 + VP2 VLPs	> 18	166	107 (64.5%)
Sadeghi et al. (2010)	Finland	VP1 VLPs	18–45	462	212 (45.9%)
Touzé et al. (2010)	Belgium	VP1 VLPs	18–25	101	78 (77%)
Touzé et al. (2010)	Italy	VP1 VLPs	18–85	194	150 (77.5%)
Faust et al. (2011)	Sweden, Austria	VP1 + VP2 VLPs	50–94	434 ^e	283 (65.2%)
Tolstov et al. (2011)	USA	VP1 + VP2 VLPs	18–69	564	447 (79.3%)
Touzé et al. (2011)	France, Italy	VP1 VLPs	22–90	82	70 (85.4%)
Viscidi et al. (2011)	Italy	VP1 VLPs	20–70s	865	640 (74%)

^a The subjects were children with Langerhans cell histiocytosis (LCH).

^b The subjects were children with otitis media (*n* = 158) or acute lower respiratory infection (*n* = 140).

^c The subjects were children with acute lower respiratory infection.

^d The subjects were children with otitis media.

^e This population included subjects with squamous cell carcinoma (*n* = 67), basal cell carcinoma (*n* = 159), actinic keratoses (*n* = 785), seborrhoeic keratosis (*n* = 1), and other benign lesion (*n* = 1).

GST, glutathione S-transferase; MCV, Merkel cell polyomavirus; VLPs, virus-like particles; VP1, capsid viral protein 1; VP2, capsid viral protein 2

MCV seropositivity was not found to be associated with other chronic viral infections (HIV, hepatitis B virus, hepatitis C virus) or with sexual activity, and no specific symptoms or signs were associated with MCV seroconversion at adult age ([Tolstov et al., 2011](#)).

1.3.2 Prevalence of MCV DNA

Using PCR or nested PCR, MCV DNA has been detected in 0–28.1% of samples taken from a variety of skin samples ([Table 1.1](#); [Feng et al.,](#)

[2008](#); [Dworkin et al., 2009](#); [Andres et al., 2010a, b](#); [Mangana et al., 2010](#); [Mertz et al., 2010a, b](#); [Mogha et al., 2010](#); [Wieland et al., 2011](#)). With more sensitive techniques such as real-time PCR, qPCR, or RCA, MCV sequences have been amplified in up to 100% of skin samples examined ([Garneski et al., 2009](#); [Foulongne et al., 2010a](#), [Schowalter et al., 2010](#); [Faust et al., 2011](#); [Wieland et al., 2011](#)). Furthermore, [Wieland et al. \(2009\)](#) reported the detection of MCV DNA in 6 (30%) of 20 anal swabs and in 3 (50%) of 6 penile swabs. In addition to the detection of MCV on

the skin, high levels of MCV DNA detection have also been reported in the oral cavity, with detection rates ranging from 8.3% to as high as 39–60% ([Dworkin et al., 2009](#); [Wieland et al., 2009](#); [Loyo et al., 2010](#)). The quantity of MCV DNA has been reported to be lower in the skin than in the oral cavity mucosa ([Loyo et al., 2010](#)), although [Foulongne et al. \(2010b\)](#) observed that MCV DNA is less frequently detected in buccal mucosa swabs than in skin swabs. The variation observed in the detection of MCV may be due in part to the sampling methods, including biopsies and surface swabs.

Low levels of MCV can be amplified from many human tissues ([Feng et al., 2008](#); [Kantola et al., 2009](#); [Bergallo et al., 2010](#); [Loyo et al., 2010](#)). This low level of viral DNA may indicate that MCV is systemically distributed, but not pathogenic, in most tissues, where it may undergo low-level replication, persistence, or latency.

1.3.3 Primary infection and transmission

Infection with MCV occurs early in childhood and is widespread among adults ([Kean et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Sadeghi et al., 2010](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)). The virus is present on all skin surfaces and is part of the normal skin flora. The detection of the same DNA sequence at different skin surfaces from the same individual ([Schowalter et al., 2010](#)) and the absence of variation in prevalence of DNA detection at different skin locations ([Foulongne et al., 2010b](#)) suggest that MCV is often established as a persistent infection of the skin in healthy subjects. The MCV viral load on the skin surface varies from as little as 1 copy per 1000 cells to 1000 copies per cell ([Katano et al., 2009](#); [Loyo et al., 2010](#); [Mogha et al., 2010](#); [Faust et al., 2011](#); [Pastrana et al., 2012](#)). In addition, [Pastrana et al. \(2012\)](#) did not observe significant variation in viral load according to age or sex of subjects or between different skin locations in the same subject. Furthermore, MCV

antibody titres are highly correlated with DNA viral load on the skin ([Faust et al., 2011](#); [Pastrana et al., 2012](#)), indicating that individuals with very high anti-MCV titres are those with persistent and active skin shedding of MCV virions.

The exact mode(s) of MCV transmission, the site(s) of initial infection, and the existence of a latent phase have not yet been characterized ([Foulongne et al., 2010a, b](#); [Loyo et al., 2010](#); [Schowalter et al., 2010](#)). MCV has not been detected in fetal autopsy samples, and thus vertical transmission from mother to infant does not seem to occur ([Sadeghi et al., 2010](#)). However, this does not exclude the possibility of perinatal transmission at time of delivery.

MCV DNA was detected in 0.6–1.3% of nasopharyngeal aspirates of children and in 2.1–8.5% of adults ([Bialasiewicz et al., 2009](#); [Goh et al., 2009](#); [Kantola et al., 2009](#)). MCV was also detected in 3.5% of tonsils, 6.7% of lung tissues, and 17.2% of bronchoalveolar and bronchoaspirates ([Kantola et al., 2009](#); [Babakir-Mina et al., 2010](#); [Loyo et al., 2010](#)). The presence of MCV in the upper and lower respiratory tract thus raises questions about its potential aerodigestive transmission ([Bialasiewicz et al., 2009](#); [Goh et al., 2009](#); [Kantola et al., 2009](#); [Babakir-Mina et al., 2010](#); [Abedi Kiasari et al., 2011](#)), as suggested for other polyomaviruses.

MCV was not detected in cerebrospinal fluid, peripheral blood mononuclear cells, urine, plasma/blood, brain or prostate tissue samples, or frozen autopsy samples from both immunocompetent and immunosuppressed subjects ([Kassem et al., 2008](#); [Bluemn et al., 2009](#); [Duncavage et al., 2009a](#); [Giraud et al., 2009](#); [Katano et al., 2009](#); [Lam et al., 2010](#); [Sadeghi et al., 2010](#); [Dang et al., 2011](#)). However, others have reported detection of MCV DNA sequences in 15–25% of urine samples and in 0.1–12% of serum samples ([Kantola et al., 2009](#); [Bofill-Mas et al., 2010](#); [Husseiny et al., 2010](#)). Although observed in only a fraction of the studies, the detection of MCV in serum raised the possibility of blood transmission; however,

this route of transmission is not expected to have a subsequent role since most adults have antibodies against MCV. The low copy number of MCV in the urine could be due to contamination by skin when passing urine.

MCV is present in urban sewage and river water and is detected in 85% of environmental surface samples, suggesting that viral DNA is present on all objects in contact with humans ([Bofill-Mas et al., 2010](#); [Foulongne et al., 2011](#)). Viral MCV DNA from environmental surface samples remains detectable after DNase treatment before nucleic acid extraction ([Foulongne et al., 2011](#)), indicating the presence of encapsidated DNA belonging to potentially infectious viruses. This was confirmed by [Schowalter et al. \(2010\)](#), and thus transmission of MCV from environmental sources to humans is a possible mode of transmission.

1.4 Diseases associated with MCV

MCV is associated with MCC ([Feng et al., 2008](#)). This is discussed in detail in Sections 2 and 4 of this *Monograph*.

MCV has not been associated with any other specific primary disease or symptoms to date.

2. Cancer in Humans

Methodological considerations: case-control versus case-series study designs

Numerous studies have reported the prevalence of markers of infection by polyomaviruses in tumour tissues or blood obtained from humans with cancer. Many of these studies included specimens from individuals without cancer as “controls,” but such studies were not generally considered by the Working Group as case-control studies, given the convenience sampling strategies used or the lack of comparability of exposure measurement between comparison groups.

Specifically, convenience sampling of controls led to the possibility that the control subjects were not representative of the source population. Also, the comparison of tumour tissues in cases with normal tissues (such as blood, urine, or biopsies of normal tissues) in controls may also be biased because it is uncertain whether polyomaviruses are uniformly present in these normal tissues or can be reliably detected by the assays used. However, because these studies contribute information on cancer sites not investigated by the case-control studies, included comparisons with both normal and pre-malignant control tissues, compared tumour tissue with a convenience sample of controls, compared different tissues in cases or controls, and/or presented findings for susceptible populations (i.e. transplant patients), they are considered here as case series.

2.1 Background

There are few epidemiological studies (no cohort studies, few case-control studies) evaluating the association between MCV and human cancer. However, multiple case series describing detection of MCV DNA in a large fraction of MCC tumours are consistent and provide some evidence of the association between MCV and MCC. Furthermore, some studies also explore the joint association between MCV and other cancer sites, as a first or subsequent primary after MCC.

2.2 Case-control studies

2.2.1 *Merkel cell carcinoma*

MCC is a neuroectodermal tumour that originates from Merkel cells. MCC is a rare tumour (approximate incidence, 4 cases per million), but in some regions of the world, its incidence is on the rise ([Hodgson, 2005](#)). In the USA, there are about 1500 cases per year and the reported incidence has significantly increased between 1986

and 2001 ([Hodgson, 2005](#)). [It is unclear why there is such an increase, but better registration of MCC and changes in the tumour classification cannot be ruled out.] MCC is more common in white men, and the average age at presentation is 70 years ([Agelli & Clegg, 2003](#)).

MCC tends to recur locally and gives rise to regional as well as distant metastases. It is an aggressive skin tumour with a disease-specific survival rate of 60–70% at 5 years after diagnosis ([Reichgelt & Visser, 2011](#)). MCC occurs more often among immunosuppressed subjects, such as organ transplant recipients or HIV-positive persons, suggesting a possible infectious etiology. It has been postulated that exposure to ultraviolet (UV) radiation could also play a role. MCC is often diagnosed in sun-exposed areas of the skin ([Mogha et al., 2010](#)). [However, Merkel cells are unevenly distributed in skin ([Lacour et al., 1991](#)), and it is unclear whether this has an impact on tumour site distribution.] Special stains are used to distinguish MCC from other forms of cancer, such as small cell lung cancer (SCLC), lymphoma, and small cell melanoma ([Smith & Patterson, 2001](#)).

Few case–control studies have been published analysing the association between MCV and MCC, and these are described in [Table 2.1](#) and in the following text.

[Carter et al. \(2009\)](#) and [Paulson et al. \(2010\)](#) carried out a case–control study with 205 MCC cases and 530 population controls. Cases included 139 subjects matched on age and sex to controls from the repository at the Fred Hutchinson Cancer Research Center and 66 individuals who were unmatched. Case subjects were identified from January 1, 2008, to May 1, 2010. Multiplex serology antibody was used to specifically recognize antibodies against VP1, LT, and sT antigens of MCV and BKV. MCC cases were more likely than control subjects to have antibody reactivity to MCV capsid (odds ratio [OR], 5.5; 95% confidence interval [CI]: 2.9–11.2). However, the association with antibodies to MCV sT was the

strongest (OR, 63.2; 95% CI, 24.4–164.0). The association with antibodies to MCV LT was also strong (OR, 16.9; 95% CI, 7.8–36.7). These associations were not observed for antibodies against BKV. [The study populations of [Carter et al. \(2009\)](#) and [Paulson et al. \(2010\)](#) overlap, although the extent of overlap is not clear.]

[Viscidi et al. \(2011\)](#) from the USA evaluated MCV capsid antibodies in 33 MCC cases that had been diagnosed at Moffitt Cancer Center in Tampa, Florida, between 2006 and 2008 and compared them with 37 controls who were patients undergoing skin cancer screening and were free of skin cancer. Seroprevalence of antibodies to MCV VP1 was 90.9% (30/33) in the cases and 67.6% (25/36) in the controls [OR, 4.4; 95% CI, 0.99–26.7], and mean antibody levels were higher in the cases than in the controls (1876.0 vs 1521.5; $P_{\text{age-adjusted}} = 0.04$).

2.2.2 Other cancers

The role of MCV has been reported so far in only one case–control study each for squamous cell carcinoma (SCC) of the skin, transitional cell carcinoma of the bladder, and oesophageal cancer ([Table 2.2](#)).

[Rollison et al. \(2012\)](#) compared seroresponse against MCV capsid in 173 SCC cases and in 300 controls screened negative for skin cancer. Seroresponse was evaluated through fluorescence-based multiplex serology. MCV DNA was evaluated in the cases through multiplex PCR targeting a conserved region of the LT gene. MCV DNA was present in 55 (38%) of 145 cases. MCV antibodies were significantly higher in SCC cases compared with levels observed in controls. Overall seropositivity was higher in SCC cases (OR, 1.58; 95% CI, 0.96–2.6). MCV titres were significantly higher among MCV DNA-positive cases (OR, 2.49; 95% CI, 1.03–6.04), with increasing risk with increasing antibody levels (fourth quartile vs first quartile, OR, 3.93; 95% CI, 1.43–10.76). When transplant recipients ($n = 5$) were excluded,

Table 2.1 Case–control studies of Merkel cell carcinoma and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Covariates	Comments
Carter et al. (2009) , USA	41	Group 1 (n = 76), matched; group 2 (n = 451), women	Hospital-based (group 1); population-based (group 2)	Multiplex antibody-binding VP1-GST fusion proteins; Seropositivity: MFI > 5000 units for strains MCVw162 and MCC350; qPCR	MCC	Antibodies to: MCVw162 VP1	36 (88%)	6.6 (2.3–18.8)	Age, sex	No cross-reactivity between MCVw162 VP1 and VP1 of other polyomaviruses (BKV, JCV, WUV, or KIV); MCV DNA detected in 24 (77%) of the 31 MCC tumours available, with 22 (92%) of these 24 patients also carrying antibodies against MCV.
Paulson et al. (2010) , USA, Germany	205	530	Random-digit dialling, frequency matched by age and gender	MCC multiplex antibody binding to antibodies against VP1, LT, and sT of MCV and BKV; Seropositivity: MFI > 5000 units;	MCC	Antibodies to: MCV sT MCV LT MCV VP1	51 31	63.2 (24.4–164.0) 16.9 (7.8–36.7) 5.5 (2.9–11.2)		This study overlaps with the study by Carter et al. (2009) . Cases included 139 subjects, matched on age and sex to controls and 66 individuals not matched.
Viscidi et al. (2011) , USA	33	37	Cancer-free	VLP-based ELISA	MCC	Overall positivity; mean antibody	30	[4.4 (0.99–26.7)]	Age	

BKV, BK polyomavirus; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; JCV, JC polyomavirus; KIV, KI polyomavirus; LT, large T-antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus; MFI, median fluorescence intensity; qPCR, quantitative polymerase chain reaction; sT, small T-antigen; VLP, virus-like particles; VP1, capsid viral protein; WUV, WU polyomavirus.

Table 2.2 Case–control studies of other cancers and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Covariates	Comments
Rollison et al. (2012) USA	173	300	Screened negative for skin cancer	Multiplex serology to MCV capsid; multiplex PCR	Skin SCC	MCV seropositive cases			Age, sex	All analyses with JCV were negative for an association.
						All	140	1.58 (0.96–2.60)		
						MCV DNA-positive	48	2.49 (1.03–6.04)		
						Low	6	1.0 (ref)		
						High quartile	26	3.93 (1.43–10.76)		
						High quartile in DNA-positive vs DNA-negative	26	5.76 (1.82–18.28)		
								$P_{\text{trend}} < 0.01$ $P_{\text{trend}} = 0.0004$		
Polesel et al. (2012) Italy	114	140	Hospital	Multiplex PCR in urine	Bladder TCC	MCV DNA	3	0.51 (0.12–2.13)	Age, sex, education, tobacco smoking, alcohol drinking	
Sitas et al. (2012) Australia, China, South Africa, eastern Europe, Islamic Republic of Iran, and Brazil	1561	2502	Mixed hospital- and population-based	Multiplex serology to MCV VP1	Oesophageal cancer	MCV VP1	1261	0.88 (0.73–1.06)	Full adjustment based on individual case–control analysis	

CI, confidence interval; JCV, JC polyomavirus; MCV, Merkel cell polyomavirus; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma, VP1, capsid viral protein 1

the risk persisted (OR, 3.45; 95% CI, 1.25–9.52). The case–case comparison for the presence of high titres of antibodies to MCV comparing DNA-positive with DNA-negative cases showed that the odds ratio was 5.76 when subjects in the fourth quartile were compared with those in the first quartile (95% CI, 1.82–18.28; $P_{\text{trend}} = 0.0004$). Among MCV DNA-negative subjects, no association was observed with antibody response. No association was observed between JCV seroresponse and SCC irrespective of MCV status.

[Polesel *et al.* \(2012\)](#) from Italy reported on 114 cases with transitional cell carcinoma of the bladder and 140 hospital controls. Urine samples were evaluated for the presence of MCV DNA using multiplex PCR to detect LT sequences. The presence of MCV was detected in 3 (2.7%) cases and 8 (5.8%) controls (adjusted OR, 0.51; 95% CI, 0.12–2.13). Lower detection of polyomavirus was observed among women compared with men and among current or former smokers compared with never-smokers.

[Sitas *et al.* \(2012\)](#) explored the presence of antibodies to MCV in 1561 oesophageal cancer cases and 2502 controls originated from 6 case–control studies in Australia, China, South Africa, central and eastern Europe, Brazil, and the Islamic Republic of Iran. Using a multiplex approach to measure seroresponse to MCV, VP1 antibodies were detected in 80.8% of cases and in 83.4% of controls (OR, 0.88; 95% CI, 0.73–1.06).

2.3 Case series

[Table 2.3](#) describes some of the case series of MCC in which MCV was evaluated. Reports including fewer than 5 cases were excluded.

2.3.1 Merkel cell carcinoma

It was in 2008 that [Feng *et al.* \(2008\)](#) studied MCC samples by digital transcriptome subtraction and detected a fusion transcript between an undescribed LT and a human receptor tyrosine

phosphatase. This finding, together with subsequent investigations, led to the identification of MCV. Since that initial study, multiple case series have been published using different approaches to detect MCV using either formalin-fixed paraffin-embedded (FFPE) tissue or fresh tissue, with variation also in time periods of data collection but with a consistent detection of MCV DNA of > 50%.

[Pastrana *et al.* \(2009\)](#) from the USA studied 21 histologically confirmed MCC cases, all of which harboured MCV DNA. A control series of 48 was retrieved from a paid plasma donor bank. Samples were analysed using a reported vector-based neutralization assay to quantify MCV-specific serum antibody response. All cases displayed substantial MCV-specific antibody responses. Although 88% (42/48) of controls without MCC were MCV seropositive, the geometric mean titre (GMT) of the control group was 59-fold lower than that of the MCC patient group (GMT of 21 500 in controls vs 222 000 in cases; $P = 0.0001$). Only 4% (2/48) of control subjects displayed neutralizing titres greater than the mean titre of the MCV-positive MCC patient population. MCC tumours were found not to express detectable amounts of MCV VP1 [suggesting that the strong humoral responses observed in MCC patients could be explained by an unusually immunogenic MCV infection, and not by viral antigen expressed by the MCC tumour itself.] Specific evaluation of antibodies against BKV did not correlate with that observed for MCV [suggesting that the immune response associated with MCV was unlikely to be explained by an immunodeficient status of cancer patients.]

[Tolstov *et al.* \(2009\)](#) from Pittsburgh, USA, evaluated MCV through VLP enzyme immunoassay (EIA) in 21 MCV DNA-positive MCC cases and in 6 MCV DNA-negative MCC cases. Controls included 166 blood donors, 100 commercially available blood donors, and 50 adults with systemic lupus erythematosus

Table 2.3 Case series of Merkel cell carcinoma and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Feng et al. (2008) USA	10	59	Case–case study	Digital transcriptome subtraction	Presence of vDNA	8 of 10 (80%)	6 of the 8 MCV-positive MCC had integration. No mention of integration in the control samples.
Pastrana et al. (2009) USA	21	48	Plasma donor bank	Neutralizing antibodies	Positivity GMT	100% 222000	All cases were MCV DNA-positive.
Tolstov et al. (2009) USA	27	316	Blood donors (166), commercially available blood donors (100), and patients with systemic lupus erythematosus (50)	VLP EIA	MCV antibody positivity	100% of vDNA-positive cases; 50% of vDNA-negative cases	
Garneski et al. (2009) USA, Australia	37	0	NA	PCR to detect MCV LT and sT	Presence of vDNA	62% of primary MCCs; 23% of MCC recurrences; 46.7% of nodal metastases; 100% of distant metastases	PCR modified from Feng et al. (2008) .
Paulson et al. (2009) USA	28	0	NA	PCR to detect MCV LT and sT	Presence of vDNA	59%	PCR based on Garneski et al. (2009)
Sastre-Garau et al. (2009) France	10	1241	Wide range of other human tumours	PCR and RT-PCR to detect MCV LT and sT	Presence of vDNA vDNA integration LT and sT mRNA	100% 100% 100%	Very high specificity
Shuda et al. (2009) USA, Spain, Israel	36	325 + 104	Tissue arrays available from commercial sources of haematological malignancies and subjects with HIV status known	IHC for LT, MCV DNA, and qPCR	Presence of vDNA Expression of LT protein in tumour cells Viral genome copies per cell	70% of 10 58.30% 5.2 copies/cell	2% of CLLs were DNA-positive; HIV-positive subjects had low MCV copy number per cell (range, $2.8\text{--}8.8 \times 10^{-3}$). None of the 6 CK20-negative MCC tumours had MCV DNA.

Table 2.3 (continued)

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Sihto et al. (2009) Finland	207	22	Randomly selected FFPE tissue samples from university archives, including other cancers and normal tissue	qPCR using the LT1, LT3, and VP1 primer pairs	Presence of vDNA LT1 LT3 VP1	79.80% 29.60% 49.50% 11.10%	PCR based on Feng et al. (2008) . Detailed information retrieved from Sihto et al. (2011)
Touzé et al. (2009) France	32	9	NETs of the lung, intestine, and cervix	Nested PCR with LT1 and VP1 primer pairs	Presence of vDNA in FFPE ($n = 20$) In fresh tissue ($n = 12$)	45% 100%	PCR based on Feng et al. (2008)
Nakamura et al. (2010) Japan	19	Not reported	Various normal tissues	PCR and RT-PCR, viral integration, IHC	Presence of vDNA Expression of LT in MCV DNA – positive cases	57.90% 70%	
Foulongne et al. (2010a) France	18	24	Other skin diseases ($n = 18$); healthy subjects ($n = 6$)	PCR and real-time PCR	Presence of vDNA in tumour samples In distant non-tumour samples	82% 71%	PCR based on Feng et al. (2008)
Werling et al. (2011) Germany	32	Not reported	Glabrous skin, human colon, heart tissues, and pancreatic carcinomas	Real-time PCR using primers for LT and VP1	Presence of vDNA	84%	PCR based on Becker et al. (2009)
Schrama et al. (2011) Australia, Germany	174			PCR for LT gene and viral copy	Presence of vDNA Viral copies/cell > 10	86.8% 20%	PCR based on Becker et al. (2009)
Sihto et al. (2011) Finland	93			qPCR	Presence of vDNA	96.7% MCV LT expression strongly associated with presence of MCV DNA in the tumour ($P < 0.0001$)	The cases had also been reported in Sihto et al. (2009)

Table 2.3 (continued)

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Touzé <i>et al.</i> (2011) France	68	82	Sera from 23 patients with skin cancer or other skin disease; 36 Italian blood donors; 23 patients with infectious diseases	qPCR/VLP-based ELISA	Antibody positivity GMT High antibody titres > 10 000	Cases:100% Controls: 85% Cases: 21701 Controls: 1608 Cases: 64.7% Controls 7.3%	PCR based on Laude <i>et al.</i> (2010) ; better progression-free survival was associated with high antibody titres (HR, 4.6; 95% CI: 1.7–12.2)

CI, confidence interval; CLL, chronic lymphocytic leukaemia; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed, paraffin-embedded; GMT, geometric mean titre; HR, hazard ratio; IHC, immunohistochemistry; LT, large T-antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus; mRNA, messenger RNA; NA, not applicable; NETs, neuroendocrine tumours; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT-PCR, reverse transcriptase PCR; sT, small T-antigen; vDNA, viral DNA; VLP, virus-like particle, VP1, capsid viral protein 1

(SLE). Whereas 100% of the MCV DNA-positive tumours had antibodies to MCV, only half of the MCV DNA-negative MCC tumours had detectable antibodies. Among controls, sero-response was detected in 64% of blood donors, in 63% of commercial donors, and in 74% of SLE patients, showing evidence for prior MCV exposure. [The estimated odds ratio using all cases and all controls was 1.84. A limitation of the study is the use of samples from a blood bank; the demographics of the control population are unknown, and there is no information on potential confounding factors.]

[Garneski et al. \(2009\)](#) and [Paulson et al. \(2009\)](#) are two overlapping reports from the USA studying MCV in 28 MCC specimens from 25 patients using PCR and FFPE tumours and fresh tissue. MCV was detected in 13 (59%) of 22 patients.

[Sastre-Garau et al. \(2009\)](#) investigated DNA, RNA, and viral integration from 10 cases of MCC and 1241 specimens of a wide range of human tumours from France. All 10 cases of MCC (100%) were MCV DNA-positive, whereas none of the 1241 specimens of other tumour types harboured MCV. Both sT and LT viral DNA sequences were found to be significantly expressed in all MCCs. MCV DNA was integrated in all the MCC cases in a single integration site in each case. [The authors interpreted this observation as indicative that integration takes place before clonal expansion.] Low copy numbers of viral genomes were present per cell, but there was always expression of both LT and sT genes. MCV LT transcripts were found to harbour mutations in the 3' part of the LT protein in the majority of cases. [This mutation is relevant as it may affect the replicative properties of the virus. The high specificity of the study is surprising in view of other case series.]

[Shuda et al. \(2009\)](#) explored MCV DNA in 36 MCC cases and in 325 haematological malignancies from the USA, Spain, and Israel. The study included an MCC tissue core microarray

for 36 cases and a tissue microarray for lymphoid malignancies available from commercial sources. Further additional haematolymphoid tissues were obtained from tissue banks. Samples were evaluated using the CM2B4 monoclonal antibody that recognizes endogenous and transfected MCV LT antigen. The study showed expression of MCV LT protein localized to nuclei of tumour cells in MCC cases. The average number of LT DNA sequence copies per cell was 5.2 (range, 0.8–14), and 21 (58.3%) of 36 cases were CM2B4-positive. The study also included peripheral blood mononuclear cells from 83 subjects undergoing genetic testing for Factor V Leiden deficiency and 21 HIV-positive subjects with no MCC. qPCR was used in these samples to detect and measure MCV LT or VP2 DNA sequences. Among all of these subjects, only 3 (14.3%) of the HIV-positive subjects were positive for MCV DNA at low copy number per cell (range, $2.8\text{--}8.8 \times 10^{-3}$ for either LT or VP2 sequences). [Results on haematological malignancies are shown in Section 2.3.2.]

[Sihto et al. \(2009\)](#) analysed FFPE MCC tissue samples from 114 of 207 patients diagnosed in Finland from the period 1979–2004 for the presence of MCV DNA. qPCR and DNA sequencing was performed following the methods and using the primers of [Feng et al. \(2008\)](#), with an additional probe. MCV DNA was present in 91 (79.8%) of the MCC cases.

[Touzé et al. \(2009\)](#) investigated the presence of MCV in 32 patients with MCC (20 FFPE and 12 fresh tissue samples) and 9 neuroendocrine tumours (NETs) from lung ($n = 5$), intestine ($n = 3$), and cervix ($n = 1$). MCV was detected by nested PCR using the LT1 and VP1 primers published by [Feng et al. \(2008\)](#); 20 (45%) of the FFPE MCC samples and all of the 12 fresh tissue samples were MCV-positive, and none of the NETs were MCV-positive.

[Wieland et al. \(2009\)](#) from Germany explored MCV DNA from a range of 355 skin lesions, including MCC, eyebrow hairs, anogenital and oral specimens, and blood samples, by means of

PCR as described by [Feng et al. \(2008\)](#) and by sequencing. MCV DNA was detected in 88% of 19 MCCs, in contrast to 16% of other skin tumours. MCV was also found in anogenital and oral samples (31%) and eyebrow hairs (50%) of HIV-positive men and in forehead swabs (62%) of healthy controls. Of 13 blood samples examined, 1 was positive for MCV DNA and corresponded to an MCC patient.

[Nakamura et al. \(2010\)](#) from Japan explored 19 MCC cases, all CK20-positive, for MCV by means of real-time PCR using the LT 77 bp primer (as in [Katano et al., 2009](#)); 11 cases (57.9%) were MCV DNA-positive. Immunohistochemical (IHC) analysis using a polyclonal antibody detected LT antigen in 7 (70%) of 10 MCV DNA-positive cases. Antibody staining was explored in several other organs, including 10 normal skin samples, with negative results.

[Foulongne et al. \(2010a\)](#) from France studied a series of patients with MCC ($n = 18$), patients with other skin diseases ($n = 18$), and healthy subjects ($n = 6$). Exposure to MCV was evaluated through detection of MCV DNA using the methods of [Feng et al. \(2008\)](#), real-time PCR, and sequencing of LT gene. MCV DNA was identified in 78% of MCC cases, in 28% of skin lesions ($P = 0.007$), and in 17% of healthy skin samples. Sequencing analysis did not identify signature mutations.

[Werling et al. \(2011\)](#) conducted a study using paraffin-embedded samples of MCCs ($n = 52$) that had been removed surgically from 32 different patients (26 primary MCC tumours, 9 recurrences, 15 lymph node metastases, 1 metastasis of the thoracic wall, 1 parotid gland metastasis) between 1995 and 2008 at the Department of Pathology of the University Medical Centre Mannheim, Germany. Samples of human glabrous skin, colon, heart tissues, and pancreatic carcinomas to be used as positive and negative controls were obtained from the University Medical Centre Mannheim and the University Hospital Heidelberg. Real-time PCR was used

to detect MCV LT and VP1 genes, following [Becker et al. \(2009\)](#). Among all cases, 84% were MCV-positive, with fully concordant results for all samples derived from the same patient.

[Schrama et al. \(2011\)](#) reported on 174 FFPE samples of MCC cases from Australia and Germany using PCR and sequencing of LT gene as described by [Becker et al. \(2009\)](#). A total of 116 (85.3%) of 136 European MCC cases and 33 (86.8%) of 38 Australian cases were both MCV-positive and MCV LT-positive. A substantial number of cases (30/149) had > 10 copies of viral genome per cell. Samples derived from the same patient were concordant in relation to MCV status. MCV-positive cases were significantly more likely to be females, with tumours located on the extremities, and less likely to report a previous history of skin cancer.

[Sihto et al. \(2011\)](#) from Finland identified 207 incident MCC cases in a population-based nationwide cohort from the period 1979–2004. Of those, 91 MCC cases with histologically confirmed MCC and with clinical information and representative tumour tissue available were included in the study. MCV DNA was detected using PCR, qPCR, and DNA sequencing. MCV LT expression was strongly associated with the presence of MCV DNA in the tumour ($P < 0.0001$).

[Touzé et al. \(2011\)](#) from France investigated MCV DNA, VP1 protein expression in tumour cells, and antibodies to MCV using ELISA in 68 MCC patients and compared them with 82 controls (patients with skin lesions, including cancer; patients with infectious diseases; and blood donors). High antibody titres $> 10\ 000$ were observed to be significantly more common in cases compared with controls. The GMT was also 14 times higher in cases than controls. VP1 expression was not observed in MCV DNA-positive tumours or in surrounding normal skin, or in MCV DNA-negative cases.

2.3.2 Chronic lymphocytic leukaemia and other lymphomas

CLL is the most common leukaemia in developed countries and is characterized by the accumulation of monoclonal mature B-cells aberrantly expressing CD5. Several reports have investigated the potential role of MCV in its etiology, mainly based on the increased incidence of CLL among MCC patients observed in some reports.

Several case reports are available, with a wide range of positivity from 0% to 33%. We describe the largest cases series.

[Shuda et al. \(2009\)](#) explored MCV DNA in 36 MCC cases and in 325 haematological malignancies from the USA, Spain, and Israel. [This study is also described in Section 2.3.1.] The study included haematolymphoid tissues obtained from tissue banks. Among the 325 haematological malignancies, 2.2% showed evidence for MCV DNA by PCR. None were positive at high viral copy numbers, and none of 173 lymphoid malignancies examined on tissue microarrays expressed MCV LT protein. Only 1 of 33 CLL cases was MCV-positive. All of the 11 post-transplant patients with a lymphoproliferative disorder were negative for MCV LT protein in tumour cells.

[Toracchio et al. \(2010\)](#) from the USA and Canada analysed 353 specimens, including 152 non-Hodgkin lymphomas (NHL), 44 Hodgkin lymphomas, 110 benign lymph nodes, 27 lymph nodes from patients with metastasis, and 20 extranodal tissue samples. MCV DNA was detected by qPCR in 13 (6.6%) of 196 lymphomas, including 5 (20.8%) of 24 CLL specimens, and in 11 (10%) of 110 benign lymph nodes, including 8 (13.1%) of 61 samples of reactive hyperplasia and 3 (10.3%) of 29 normal lymph nodes. Other samples were MCV-negative. Sequence analysis of 9 virus-positive samples confirmed the presence of MCV; three viral strains were represented. IHC testing showed that among 17 lymphomas,

7 of which were positive for MCV DNA, only 1 T-cell lymphoma expressed MCV T-antigen.

[Pantulu et al. \(2010\)](#) from Germany studied 70 CLL cases and investigated the presence of MCV DNA in blood. PCR to detect MCV was performed using primers for LT3, M1/M2, and VP1. PCR products were sequenced. MCV DNA was detected in 19 patients (27.1%) and in only 13.4% of the healthy blood donors. Mutations (246 bp LT antigen deletion) were observed in 6 of 19 MCV-positive cases. [No information was provided for the control group apart from noting that they were healthy blood donors and were younger compared with the CLL patients.]

[Andres et al. \(2010a\)](#) from Germany explored a series of 19 subjects with cutaneous lymphomas, 12 cases of pseudolymphomas, and 23 subjects with inflammatory skin disease. MCV was evaluated through PCR, followed by Southern blotting. MCV was detected in 17% of the lymphoma cases, in 15.4% of the pseudolymphoma cases, and in 8% of the inflammatory skin disease cases.

[Tolstov et al. \(2010\)](#) evaluated 18 CLL and 18 acute lymphoblastic leukaemia (ALL) patients for antibody response to MCV and showed no statistically significant differences in seroresponse between CLL and ALL patients (median values, 0.645 vs 0.521 OD units, respectively).

[Teman et al. \(2011\)](#) from the USA examined 18 consecutive CLL cases and 17 low-grade follicular lymphomas as a control group from the period 2002–10. FFPE samples were used to look for MCV DNA using PCR and real-time PCR. When PCR was used, all subjects tested negative, but when real-time PCR was used, 33% of CLL cases showed a low level of MCV amplification. All control subjects were negative for MCV.

2.3.3 Merkel cell carcinoma and other cancers

There exist in the literature many case reports of concomitant MCC cases and CLL. Pooling the available data, there are about 50 patients with

MCC and CLL; in 12 cases MCC was the primary tumour [for a review of case reports, see [Tadmor et al., 2011](#)].

[Tolstov et al. \(2010\)](#) reported on 2 cases with concurrent CLL and MCC from the USA. The IHC evaluation of tumour cells with CM2B4 and CD20 showed MCV LT protein expression in metastatic MCC cells but not in the CD20-positive CLL cells.

Several large studies had observed a stronger than expected association between MCC and other cancers using record linkage data. [Howard et al. \(2006\)](#) from the USA explored incident primary cancers in 1306 patients with MCC from 11 population-based cancer registries from the Surveillance, Epidemiology and End Results (SEER) programme. Patients with first primary MCC were at significantly increased risk of developing a subsequent cancer (standardized incidence ratio [SIR], 1.22; 95% CI, 1.01–1.45; $n = 122$), with significant excesses restricted to the first year after diagnosis (SIR, 1.71; 95% CI, 1.21–2.33; $n = 39$). Significantly elevated site-specific risks were observed for salivary gland cancers (SIR, 11.55; 95% CI, 2.32–33.76; $n = 3$), biliary sites other than liver and gallbladder (SIR, 7.24; 95% CI, 1.46–21.16; $n = 3$), and NHL (SIR, 2.56; 95% CI, 1.23–4.71; $n = 10$). Non-significantly increased risks of 2-fold or higher were seen for CLL and cancers of the small intestine and brain. A SIR of 1.36 (95% CI, 1.19–1.55; $n = 221$) for MCC as a second primary malignancy was observed among patients with all other first primary cancers taken together. In particular, significant 3–7-fold excesses of MCC followed CLL (SIR, 6.89; 95% CI, 3.77–11.57; $n = 14$), multiple myeloma (SIR, 3.70; 95% CI, 1.01–9.47; $n = 4$), NHL (SIR, 3.37; 95% CI, 1.93–5.47; $n = 16$), and malignant melanoma (SIR, 3.05; 95% CI, 1.74–4.95; $n = 16$). [The lack of specificity of the associations including several distinctive haematological malignancies argues against, but does not exclude, that MCV could play an etiological role.]

[Koljonen et al. \(2009, 2010\)](#) identified in a retrospective study 4164 CLL patients and 172 MCC cases based on the Finnish Cancer Registry from 1979 to 2006. In [Koljonen et al. \(2009\)](#), 6 cases had both CLL and MCC and were selected for MCV analysis. FFPE tissue was available in 5 cases, in which morphological and IHC diagnosis was confirmed for MCC. MCV DNA was tested by means of real-time PCR (LT3 region; [Sihto et al., 2009](#)). In the 5 cases (100%) MCV DNA was identified, with a median copy number of 5.49 relative to the control gene (*PTPRG*) DNA copy number. [A limitation of this study was that CLL samples were not tested, and therefore this study was not informative.]

Of the 172 MCC patients identified by [Koljonen et al. \(2010\)](#), a total of 34 second primary cancers were detected in 30 individuals after the diagnosis of MCC. Female MCC patients were diagnosed with 25 subsequent cancers (SIR, 2.35; 95% CI, 1.52–3.47; $P < 0.001$) and male patients with 9 cancers (SIR, 2.32, 95% CI, 1.06–4.40; $P < 0.05$). The MCC patients had an increased risk for a subsequent cancer (any site) compared with age-, gender- and calendar period-matched members of the general population (SIR, 2.34; 95% CI, 1.62–3.27). The risks for basal cell carcinoma (BCC) of the skin ($n = 11$; SIR, 3.48; 95% CI, 1.74–6.22) and CLL ($n = 2$; SIR, 17.9; 95% CI, 2.16–64.6) were significantly elevated.

[Kaae et al. \(2010\)](#) used data from the Danish national health and population registers on MCC diagnoses, deaths, and population counts during the study period (1978–2006) to calculate MCC incidence rates, cumulative risks of MCC at age 100 years, and MCC mortality rates by tumour stage. A total of 185 persons were diagnosed with MCC. MCC incidence between 1995 and 2006 was 2.2 cases per million person-years. MCC incidence was statistically significantly increased > 1 year after a diagnosis of SCC of the skin (SIR, 14.6; 95% CI, 8.4–25.6), BCC (SIR, 4.3; 95% CI, 2.7–6.6), malignant melanoma (SIR, 3.3; 95% CI, 1.1–10.3), Hodgkin lymphoma (SIR, 17.6; 95%

CI, 2.5–126), CLL (SIR, 12.0; 95% CI, 3.8–37.8), and NHL (SIR, 5.6; 95% CI, 1.4–22.4). [The Working Group considered that these high SIRs could be affected by an increased surveillance of cancer patients.]

[Bzhalava et al. \(2011\)](#) investigated the risk of secondary cancers after the diagnosis of MCC using the national cancer registries in Denmark, Norway, and Sweden. The overall cancer incidence was increased among patients diagnosed with MCC compared with the general population in these countries (79 secondary cancers total, SIR, 1.38; 95% CI, 1.10–1.72; 49 secondary cancer in females, SIR, 1.7; 95% CI, 1.29–2.25; 30 secondary cancers in males, SIR, 1.05; 95% CI, 0.73–1.5). There were significantly increased incidence ratios for non-melanoma skin cancers (NMSCs) (34 secondary cancers, SIR 8.35; 95% CI, 5.97–11.68), melanoma of skin (6 secondary cancers, SIR, 4.29; 95% CI, 1.93–9.56), and laryngeal cancer (2 secondary cancers, SIR, 9.51; 95% CI, 2.38–38). The SIRs for these three cancer sites were also elevated on restricting the follow-up to cancers occurring ≥ 1 year after MCC diagnosis. [The Working Group considered that these high SIRs could be affected by an increased surveillance of cancer patients.]

2.3.4 Other cancers of the skin

Several reports based on collection of skin cancers other than MCC have been published exploring the presence of MCV in tumour lesions or skin lesions. The range of MCV DNA detection varied across the series, with a range from 0% to 19.2% for both SCC and BCC.

[Wieland et al. \(2009\)](#) from Germany explored MCV DNA from a range of 355 skin lesions, including MCC, eyebrow hairs, anogenital and oral specimens, and blood samples, by means of PCR as described by [Feng et al. \(2008\)](#) and by sequencing [see Section 2.3.1]. MCV DNA was detected in 16% of skin tumours other than MCC.

[Kassem et al. \(2010\)](#) explored the presence of MCV in Germany with PCR using the LT3 and VP1 primer pairs as described by [Feng et al. \(2008\)](#). Included were 26 BCC samples from 6 patients with naevoid basal cell carcinoma syndrome (NBCCS) and 42 trichoblastoma samples from 41 patients. Of 26 BCC samples, 5 (19.2%) were MCV-positive with LT3 primers, 1 of them was also positive using VP1 primers. No PCR amplification was obtained, using any of the two MCV primer pairs LT3 and VP1, for the 3 BCC samples from 1 of the 6 NBCCS patients. Of 42 trichoblastoma samples, 12 samples (from 11 subjects) were positive using MCV LT3. None of them was positive for MCV VP1 DNA. MCV viral loads in BCC and in trichoblastoma samples were 2–10-fold lower compared with MCC positive controls.

[Reisinger et al. \(2010\)](#) from the USA evaluated the expression of MCV LT protein through detection with CM2B4 in 20 patients with MCC with and without secondary SCC or BCC tumours. MCV LT was detected in 15 (75%) of 20 MCC tumours, including 11 MCC tumours from patients with secondary SCC or BCC. In contrast to MCC, none of these secondary BCC or SCC was positive for MCV LT.

2.3.5 Other cancer sites

A few studies have explored the presence of MCV in a variety of cancer sites. MCV was not detected in melanoma ([Giraud et al., 2008](#); [Koburger et al., 2011](#)), in colon cancer ([Militello et al., 2009](#); [Campello et al., 2011](#)), in mesothelioma ([Bhatia et al., 2010a](#)), in prostate cancer ([Bluemn et al., 2009](#)), in lung cancer ([Andres et al., 2009a](#); [Joh et al., 2010](#); [Stebbing et al., 2010](#)), in central nervous system tumours ([Giraud et al., 2009](#)), in NETs ([Duncavage et al., 2009b](#); [Chernock et al., 2011](#)), and in fibroxanthoma ([Andres et al., 2010c](#)).

2.4 Susceptible populations

Few studies are available exploring specifically the association between MCV and human cancer in immunosuppressed populations. AIDS patients had a 13-fold increased age- and sex-adjusted risk of developing MCC compared with the general population ([Engels et al., 2002](#)). Likewise, there was a 16-fold over-representation of MCC in chronically immunosuppressed patients ([Heath et al., 2008](#)). [Ridd et al. \(2009\)](#) examined the presence of polyomavirus DNA in 156 NMSCs among organ transplant recipients from the USA, which included 85 SCCs, 37 keratoacanthomas, 28 Bowen disease (BD), and 6 actinic keratoses. MCV was detected using the approach of [Feng et al. \(2008\)](#). Polyomavirus sequences were detected in only 1 case of actinic keratoses (2.7%), with fainter bands compared with those seen for a control series of MCC (7 of 13 MCC cases were positive).

[Kassem et al. \(2009\)](#) from Germany explored the presence of MCV in a group of 56 NMSCs from 11 immunosuppressed patients, and 147 NMSC lesions from 125 immunocompetent patients were tested for MCV by PCR, targeting the LT and VP1 DNA sequences. NMSC included SCC, BCC, and BD. In addition, normal skin and 89 colorectal cancers were tested. MCV-specific sequences were found significantly more frequently in NMSC of immunosuppressed patients compared with immunocompetent patients (positivity to LT3 or VP1, 62.5% vs 32%, respectively, $P < 0.001$). In particular, BD and BCC revealed a significant increased association with MCV in immunosuppressed patients (positivity to LT3 or VP1, 69% in BD and 72.3% in BCC in immunosuppressed patients and 17.4% in BD and 37.5% in BCC in immunocompetent patients; all comparisons were statistically significant with $P < 0.01$). No MCV was detected within normal skin, and only 3 of 89 additionally tested colorectal cancers were MCV-positive.

3. Cancer in Experimental Animals

No data were available to the Working Group.

4. Mechanistic and Other Relevant Data

4.1 Transforming capacity of MCV

Only limited experimental data on the mechanisms related to a direct transforming capacity of MCV are available. However, current findings suggest unique features for this virus, which could distinguish it from other known human polyomaviruses. For example, expression of the sT, but not the LT, induces transformation of rodent fibroblasts and serum-independent growth of human fibroblasts, independent of PP2A and Hsc70 binding ([Shuda et al., 2011](#)).

4.2 Relevant biological properties of MCV-encoded viral proteins

The MCV genome encompasses a non-coding region and two major transcription units with early and late genes. The early region encodes LT, sT, and a 57kT protein, all of which represent alternatively spliced variants that share a 78 amino acid N-terminal ([Shuda et al., 2008](#)). The MCV T-antigens have several functions in common with the simian virus 40 (SV40)-related viruses. The shared leader sequence of sT and LT encompasses a functional DnaJ domain that binds Hsp70 and is conserved among all MCV T-antigen isoforms ([Kwun et al., 2009](#)). The late region, encoding the viral structural proteins VP1, VP2, and VP3, is expressed after the onset of viral DNA replication. In addition, MCV encodes a microRNA that is expressed from the late region and is oriented antisense to the early transcripts ([Seo et al., 2009](#); [Lee et al., 2011](#)).

The MCV LT antigen is a nuclear protein with a predicted mass of 92 kDa (Shuda *et al.*, 2008, 2009; Nakamura *et al.*, 2010). LT retains most of the features of the SV40 LT protein, including a DnaJ domain, pRb interaction domain, origin-binding domain (OBD), and helicase/ATPase domain (Feng *et al.*, 2008; Shuda *et al.*, 2008). No evidence for a direct interaction between MCV LT and p53 has thus far been found. Thus, like SV40 LT, MCV LT has dual capacities, i.e. to target at least one major class of tumour suppressor protein and to initiate viral DNA replication.

MCV LT exon 2 encodes pRb binding (Shuda *et al.*, 2008) and viral origin replication (Kwun *et al.*, 2009) functions. pRb binding is mediated by a classic LXCXE (LFCDE) motif common to tumorigenic adenoviruses, polyomaviruses, and papillomaviruses (Chemes *et al.*, 2011). It is unknown which of the proteins of the pRb family (pRb, p107, p130) are the principal targets for the virus in infected cells, but preliminary evidence suggests that MCV LT binds all of these proteins (Shuda *et al.*, 2008). As with SV40 LT binding to pRb, MCV LT binding to pRb may inhibit E2F repression and activate transcription of E2F-responsive genes active at the G1/S cell-cycle transition. It is unknown whether the DnaJ domain of LT participates in this inhibition, as has been seen with SV40 LT (Ahuja *et al.*, 2005).

The 5' portion of LT exon 2 encodes a 200 amino acid domain, termed the Merkel unique region (MUR) due to its relative lack of homology with the LT proteins of other polyomaviruses (Liu *et al.*, 2011). The MUR extends to the LXCXE motif and contains a binding site for the cytoplasmic vacuolar sorting protein Vam6p, which is then translocated to the nucleus with LT (Liu *et al.*, 2011). While the function of this interaction remains obscure, MCV LT antagonizes lysosomal clustering induced by Vam6p overexpression (Liu *et al.*, 2011) and, surprisingly, mutation of the interaction site enhances the replication of MCV genomic DNA in otherwise

poorly MCV-permissive cultured cell lines (Feng *et al.*, 2011).

Intact LT is also required for MCV genome replication and is found in replicating virus from asymptomatic skin infections (Schowalter *et al.*, 2010). The OBD has been crystallized partially bound to MCV origin DNA sequences (Harrison *et al.*, 2011), revealing similar features to those of SV40 LT. Replication can be initiated *in vitro* with a ≥ 71 bp central region of the origin (Kwun *et al.*, 2009).

sT antigen is expressed from an unspliced open reading frame that encompasses LT exon 1 and most of the LT intron. The sT open reading frame encodes a protein with a predicted size of 22 kDa. Unlike SV40, MCV sT is sufficient to induce serum-independent growth of immortalized human cells and to fully transform immortalized rodent fibroblasts (NIH3T3 and Rat-1) into independently growing cancer cell lines; this is not enhanced by co-expression of either wild-type (WT) or tumour-derived forms of MCV LT. MCV sT antigen binds to the serine/threonine protein phosphatase 2 (PP2A) A and C subunits. However, this does not appear to be critical for *in vitro* transformation of rodent cells and for serum-independent growth of human cells induced by MCV sT antigen. Mutations in the MCV sT PP2A interaction domain abolish its ability to enhance LT-mediated origin replication, yet do not have major effects on MCV sT as an oncoprotein, suggesting that PP2A targeting is not the primary function of the protein during cell transformation (Shuda *et al.*, 2011). Instead, MCV sT activates cap-dependent translation by maintaining hyperphosphorylation of the 4E-BP1 regulatory protein in a PP2A-independent manner (Shuda *et al.*, 2011). 4E-BP1 is a downstream target of the Akt-mTOR signalling pathway. When 4E-BP1 is hyperphosphorylated, it releases the protein translation initiation factor eIF4E, which then binds to 7-methylguanosine-capped mRNA, thereby recruiting the ribosomal machinery required for

translation. Accordingly, enhancement of cell transformation may result from dysregulated cap-dependent translation promoted via hyperphosphorylation of 4E-BP1 by sT ([Dowling et al., 2010](#); [Shuda et al., 2011](#)).

The MCV 57kT transcript is similar to LT but has a second intronic splice that deletes portions of the OBD and the helicase function. 57kT currently has no known biological activity. Under *in vitro* replication conditions, the LT cDNA alone is not sufficient for maximum efficiency of virus replication, but co-expression of LT with sT promotes replication of the viral genome with an efficiency that is comparable to that of the WT virus. Co-expression of LT with 57kT does not increase replication over that seen with LT alone, and neither MCV sT nor 57kT individually have replication capacity ([Kwun et al., 2009](#); [Feng et al., 2011](#)). Knockdown of all T-antigen transcripts or the sT transcript by small interfering RNA (siRNA) results in inhibition of origin replication, confirming the functional importance of sT expression to MCV origin replication ([Kwun et al., 2009](#)).

4.3 *In vivo* and *in vitro* evidence for a role of MCV in human malignancies

4.3.1 *Effects of ectopically expressed MCV genes on cell growth in vitro*

WT LT protein does not initiate transformation of immortalized rodent fibroblasts (NIH3T3 and Rat-1), as measured by colony formation in soft agar, focus formation, or cell growth in low-serum conditions ([Shuda et al., 2011](#)). Still, tumour-derived truncated MCV LT proteins induce tenuous multicellular aggregates in soft agar that do not develop into full colonies, suggesting that truncated LT proteins do not transform cells but have an increased cell proliferation capacity compared with the WT

protein ([Shuda et al., 2011](#)). It remains to be seen whether this is due to increased protein stability or acquisition of particular features. However, these findings do not imply that MCV LT does not play a role in MCV-induced tumorigenesis. Nearly all tumour mutations found so far preserve the pRb-binding domain, i.e. a function that may contribute to the development of MCC. Consistent with this hypothesis, survival of tumour cells from established MCC-derived cell lines was found to depend on a functional pRb interaction domain ([Houben et al., 2010a](#)).

Unlike MCV LT, sT acts as a transforming oncoprotein that is sufficient to fully transform rodent fibroblasts (NIH3T3 and Rat-1) into independently growing cancer cell lines characterized by loss of contact inhibition, anchorage independence, and serum-independent growth ([Shuda et al., 2011](#)). sT also accelerates the proliferation of human immortalized fibroblasts. This characteristic appears not to be shared with the SV40 sT antigen. Cell transformation by sT is not enhanced by co-expression of either WT or tumour-derived forms of MCV LT. MCV sT-induced transformation is not dependent on its interaction with PP2A or with heat shock protein, and mutated sT recombinant proteins that fail to bind PP2A or Hsc70 still retain the capacity to transform rodent cells as efficiently as does the WT protein. However, expression of a constitutively active 4E-BP1 protein, which cannot be phosphorylated, antagonizes sT-induced Rat-1 cell transformation, implying that sT maintenance of hyperphosphorylated 4E-BP1 is required for sT antigen-mediated cell transformation. In addition, MCV sT increases the steady-state phosphorylation of other mTOR targets, including pp70 S6K and 4E-BP2, but the significance of these functions is unknown ([Shuda et al., 2011](#)).

4.3.2 MCV in Merkel cell carcinoma

(a) MCV genome copies

The detection of MCV genome by PCR in most MCC tumours reported in several case series suggests a close association between MCV and this rare cancer ([Table 4.1](#); see also Section 2.3.1). A review of > 1000 tumours from MCC patients that were reported in several studies conducted through May 2011 revealed that about 75% of tumours are positive for MCV DNA (see [Table 4.1](#)).

In the studies for which quantitative analysis has been done, > 90% of the MCV-positive MCCs bear > 0.6 genome copies per cell. [Shuda et al. \(2009\)](#) first reported an average of at least 1 copy (range, 0.8–14.3 copies) of the MCV genome per tumour cell in the 8 MCV-positive MCCs analysed ([Shuda et al., 2009](#)). Other groups reported numbers of genome copies varying between 3×10^{-4} and 4×10^3 per MCC tumour cell, and between 0.02 and 10 for MCC cell lines ([Katano et al., 2009](#); [Koljonen et al., 2009](#); [Sastre-Garau et al., 2009](#); [Sihto et al., 2009](#); [Bhatia et al., 2010b](#); [Fischer et al., 2010](#); [Foulongne et al., 2010a](#); [Foulongne et al., 2010b](#); [Houben et al., 2010a](#); [Laude et al., 2010](#); [Waltari et al., 2011](#)).

Infection with MCV is common in the general population, and most adults shed MCV from the surface of their skin (see Section 1). This is why MCV is also found in non-MCC skin biopsies and in skin swabs, but usually in much smaller copy numbers. Despite extensive variation in virus abundance, which could be due to the detection method used, to differences in tissue quality and the proportion of tumour versus non-tumour cells, and to other technical differences between studies, non-MCC tissues almost always contain fewer genome copies per cell than do MCC samples in the same study. MCV DNA has been detected in cutaneous swabs from the face, trunk, and upper and lower limbs. MCV DNA levels were higher in swabs obtained from patients with MCC (median, 861 copies/ng DNA,

corresponding to 5 copies/cell) than in those from patients with other skin diseases (median, 45 copies/ng DNA, corresponding to 0.27 copies/cell; $P < 0.001$) or from clinically healthy volunteers (median, 43 copies/ng DNA, corresponding to 0.26 copies/cell; $P < 0.001$) ([Foulongne et al., 2010b](#)). In another study, MCV DNA was found in 14 (40%) of 34 swabs drawn across the skin of the forehead ([Schowalter et al., 2010](#)). However, certain non-MCC skin samples were reported to have > 1 copy of MCV per cell ([Faust et al., 2011](#)), and skin-swab specimens from some subjects were found to contain thousands of copies of MCV per copy of β -globin ([Pastrana et al., 2012](#)). A possible explanation for this discrepancy is that MCV is produced in higher levels in the outermost layers of the skin, while wiping of the skin with ethanol or iodine solution, for example when skin punch biopsies are taken, may remove potential viral DNA from the epidermis. Thus, cutaneous swabbing may produce a more thorough sample for testing of skin viruses ([Forslund et al., 2004](#)).

MCV DNA was also detected in low copy numbers in several other human tissues. The relative viral copy numbers in different tissues were estimated by arbitrarily assuming that MCC had an average of 10 MCV genome copies per cell (range, 0.05–173). Based on this assumption, saliva had an average of 0.13 genome copies per cell (range, 0.01–5); oral cavity, liver, and skin samples had an average of 0.026, 0.015, and 0.007 copies, respectively; and lung, kidney, bladder, and prostate cancer had < 0.001 genome copies per cell ([Loyo et al., 2010](#)). The copy numbers in lymphoid cells from different groups of patients without MCC were found to range between 0.001 and 0.01 genome copies per cell ([Shuda et al., 2009](#); [Pancaldi et al., 2011](#)).

(b) Antibody response to MCV

Human seroresponsiveness against MCV LT and sT was specifically associated with MCC. LT- or sT-specific antibodies were present in only

Table 4.1 Detection of MCV DNA in biopsies from MCC patients

Total patients (%)	Male patients (%)	Female patients (%)	Study location	Reference
8/10 (80%)			Pennsylvania, USA	Feng et al. (2008)
8/9 (88.9%)			France	Foulongne et al. (2008)
30/39 (77%)	13/20 (65%)	17/19 (89%)	Germany	Kassem et al. (2008)
21/31 (68%)	8/13 (61.5%)	13/18 (72%)	Germany	Andres et al. (2009b)
45/53 (84.9%)			Germany	Becker et al. (2009)
15/17 (88%)	12/12 (100%)	3/5 (60%)	New York, USA	Busam et al. (2009)
22/29 (76%)			Missouri, USA	Duncavage et al. (2009a)
16/37 (43%)			North America, Australia	Garneski et al. (2009)
90/98 (92%)			Germany	Helmbold et al. (2009)
11/14 (78.6%)	4/5 (80%)	7/9 (77.7%)	Japan	Nakajima et al. (2009)
10/10 (100%)			France	Sastre-Garau et al. (2009)
91/114 (79.8%)	67/80 (83.7%)	24/34 (70.5%)	Finland	Sihto et al. (2009)
21/32 (65.6%)			France	Touzé et al. (2009)
7/8 (87.5%)			Hungary	Varga et al. (2009)
30/34 (88%)			Germany	Wieland et al. (2009)
21/33 (64%)	8/14 (57%)	14/19 (73.6%)	Germany	Andres et al. (2010b)
60/70 (86%)			Italy	Asioli et al. (2011)
17/23 (74%)	13/17 (76%)	4/6 (75%)	Ohio, USA	Bhatia et al. (2010b)
14/18 (78%)			France	Foulongne et al. (2010a)
34/59 (57.6%)	15/28 (53.5%)	19/31 (61.3%)	Germany	Handschel et al. (2010)
31/33 (94%)			France	Laude et al. (2010)
20/30 (66.7%)			Switzerland	Mangana et al. (2010)
67/87 (77%)			Finland	Waltari et al. (2011)
20/26 (77%)			Japan	Kuwamoto et al. (2011)
19/104 (18.3%)	9/58 (15.5%)	10/46 (21.7%)	Australia	Paik et al. (2011)
149/174 (85.6%)			Australia, Germany	Schrama et al. (2011)
Total				
877/1192 (73.6%)	149/247 (60.3%)	11/187 (59.3%)		

Compiled by the Working Group from case series conducted through May 2011.
MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus

0.9% of 530 control serum samples tested, and in 40.5% of 205 samples from MCC cases ([Paulson et al., 2010](#)). In addition, the GMT of 2900 (95% CI, 1500–5800) for antibodies recognizing LT in the 56 initial MCC patients was > 10-fold greater than that in the 36 matched controls: 200 (95% CI, 100–300). The difference in sT reactivity was even greater: GMTs of 2100 (95% CI, 800–1500) for MCC cases versus 5 (95% CI, 1–30) for controls. Furthermore, these authors found that titres of LT and sT antibodies dropped rapidly (about 8-fold per year) in 8 of 10 treated patients whose cancer did not progress, whereas they increased in 6 of 6 cases with progressive disease. The results suggest that ongoing expression of T-antigens within the MCC tumour is sometimes reflected by humoral responses. A similar trend to high antibody titres, albeit with VP1 VLPs as an antigen, was reported by [Touzé et al. \(2011\)](#), who detected antibodies against MCV in all patients with MCC and in 85% of controls. High antibody titres were rarely observed in controls (7.3%), but they were detected in 64.7% of patients with MCC.

Although a few exceptions have been identified, a strong positive correlation between the overall viral load and the average serological titre has been reported ([Faust et al., 2011](#); [Pastrana et al., 2012](#)). Given the correlation between MCV DNA loads and antibody titre, together with the higher titres of MCV-specific antibodies in MCC patients, it is likely that MCC patients have increased MCV viral load.

(c) *Viral gene expression in Merkel cell carcinoma*

If MCV is a causal agent of MCC, it is likely that MCC cells will express MCV proteins to initiate and maintain the tumorigenic phenotype. Yet a transient expression of potentially oncogenic viral proteins could act as a carcinogen that promotes the accumulation of genetic lesions and the formation of cancer. In this scenario, MCV may only be necessary for

tumour initiation, whereas a gradual loss of MCV may take place during tumour progression and perhaps explain the MCCs with low viral load and even with no MCV. This situation has been recently supported by T-antigen knockdown experiments that showed a lack of T-antigen dependence of an MCV-infected MCC cell line (LoKe) for cell growth ([Houben et al., 2012b](#)). However, this finding has been reported in one cell line, while incomplete knockdown of T-antigens may account for the sustained growth of these cells under the given experimental conditions. Finally, MCV-negative tumours may represent cells that have never been infected by the virus and therefore may have a distinct nature.

Transcripts corresponding to LT, sT, and 57kT mRNAs have been detected by Northern blot hybridization and mapped from four MCC tumours ([Shuda et al., 2008](#)).

LT protein expression in MCC was demonstrated using a monoclonal antibody (CM2B4) to a conserved peptide epitope in exon 2 of LT that can detect a truncated LT in protein extracts from MKL-1 cells, which is consistent with premature termination ([Shuda et al., 2009](#)). As expected, a diffuse nuclear and granular localization was evident from staining cells that ectopically express LT with this antibody. A similar pattern of strong reactivity among most tumour cells, but not in surrounding tissue, was evident from analysis by IHC of MCV DNA-positive MCC biopsies. Of 30 MCCs, 21 (70%) were positive for LT protein, and this finding was concordant with DNA detection. Nevertheless, one MCV DNA-positive MCC scored negative for LT expression ([Shuda et al., 2009](#)). A similar trend of positive staining in the majority of MCV PCR-positive MCCs was reported subsequently ([Busam et al., 2009](#), 37/51, 72.5%; [Reisinger et al., 2010](#), 15/20, 75%; [Wieland et al., 2012](#), 3/3, 100%; [Erovic et al., 2013](#), 29/30, 97%). CM2B4 did not show immunoreactivity with other skin tumours (see Section 4.3.3). Similar results were obtained

with polyclonal rabbit antibody against MCV LT, which detected 8 of 10 MCV PCR-positive MCC cases by IHC analysis but did not produce any signal in various organs, including lymph nodes, liver, spleen, brain, salivary glands, lung, pancreas, muscle, heart, and uterus ([Nakamura et al., 2010](#)).

In addition to LT, sT protein expression is detected in MCV-infected cell lines and in MCCs ([Shuda et al., 2011](#)). MCV sT is expressed more commonly in MCCs than is MCV LT ([Shuda et al., 2011](#)). Increased sensitivity of PCR-based DNA detection compared with IHC detection of a protein could account for the discrepancy of MCV detection obtained for certain MCCs. Yet some MCV DNA-positive MCCs lack LT expression but express robust sT, thus suggesting that in certain cases MCV sT plays a critical tumorigenic role ([Shuda et al., 2011](#)).

By using IHC staining, Pastrana and co-workers failed to detect expression of the VP1 capsid protein in each of 10 MCV LT-positive MCCs, suggesting that the virus does not replicate in MCCs ([Pastrana et al., 2009](#)).

Low-level expression of MCV microRNA has been reported in 19 (50%) of 38 MCV-positive MCCs. The amount of viral DNA positivity correlates with copies of MCV microRNA. This virus-encoded microRNA is predicted to target LT but also to target genes that may play a role in promoting immune evasion and regulating viral DNA replication ([Lee et al., 2011](#)).

(d) *Mutations in MCV LT and other viral genes*

MCV is clonally integrated in the tumour cell DNA, while mutations at the C-terminal end of the LT antigen that abrogate its OBD and helicase activity have been documented ([Feng et al., 2008](#)). Therefore, the heterogeneity of the viral genome present in tumours could result in loss of function but may also generate new distinctive features.

Nearly all MCV genomes analysed so far from tumours are truncated either through

point mutations or via integration into the human chromosome ([Feng et al., 2008](#); [Shuda et al., 2008](#); [Sastre-Garau et al., 2009](#); [Laude et al., 2010](#)). These tumour-specific mutations are C-terminal to the LXCXE motif and do not affect pRb binding but disrupt the replicative functions (OBD or helicase). The sequence and expression of the sT protein are unaffected by these mutations as well. The tumour-specific LT mutations prevent virus DNA replication and are incompatible with a transmissible agent. This presumably has the effect of preventing DNA replication from integrated viral origins that would generate onion-skinning and DNA fragmentation. Due to truncating mutations, the molecular size of the MCV LT protein varies among cell lines and tumours.

Mutations or deletions were identified that prematurely truncate exon 2 encoding the LT helicase in all sequenced LT genes from 9 MCV-positive MCCs ([Shuda et al., 2008](#)). In line with this, most LT mutations reported in another study ([Laude et al., 2010](#)) were located downstream of the pRB-binding domain, while LT exon 2 interruption resulting from genomic integration has also been found. Mutations with a stop codon were detected downstream of the LT nuclear localization signal (NLS), within the upstream OBD and the helicase domain, of 5 of 11 LT gene sequences from MCV-positive MCCs. In addition, the conservation of the LT NLS motif in all 11 MCV-positive MCCs analysed suggested the necessity for tumorigenesis of nuclear targeting of the mutated LT ([Nakamura et al., 2010](#)); yet a few sequences of MCV LT had a stop-codon mutation before or within the NLS, while a few others encoded a non-truncated protein ([Shuda et al., 2008](#); [Laude et al., 2010](#)). The latter cases could potentially display mutations at other sites critical for MCV replication. It is likely that a truncating mutation that prevents expression of both the OBD and the helicase domain is found in the classic MCV-infected cell line MKL-1 ([Shuda et al., 2008](#)). In contrast,

full-length LT coding sequences with no truncating mutations were obtained in control tissues and skin swabs from non-MCC patients who were found to harbour MCV ([Shuda et al., 2008](#); [Mogha et al., 2010](#); [Schowalter et al., 2010](#)). Only one truncation of the C-terminal 15 amino acids of the sT protein was identified in one skin swab of a healthy volunteer ([Schowalter et al., 2010](#)). Substitution, deletion, and insertion mutations were identified in 7 of 10 DNA sequences of buffy coats. These mutations could have an effect on the function of sT, but they are not expected to alter LT function as they are situated on its intronic sequence ([Pancaldi et al., 2011](#)). Since high mutation rates have not generally been observed in cellular cDNA from MCCs ([Shuda et al., 2008](#)), a mutator phenotype was ruled out, whereas a selection for certain LT mutations in the tumours has been suggested.

Unlike WT MCV LT, two different mutated LTs derived from MCC failed to support replication from the MCV origin, as evidenced by replication assays via two experimental approaches: a plasmid containing the MCV replication origin and the tumour cell line MKL-1 ([Shuda et al., 2008](#)). When these findings are taken together, it appears that the LT-truncating mutations result from selective pressure to avoid virus DNA replication and/or completion of the viral life cycle, and are in line with the perception that polyomavirus transformation does not require free replicating virus.

Despite the expression of replication-deficient LT in MCC, viral particles were detected in the cell cytoplasm and the nucleus of one primary MCC by electron microscopy, suggesting the expression of a functional LT and the occurrence of active viral replication. Although these findings may suggest that not all MCV-positive MCCs merely express truncated LT, they require careful consideration as they lack PCR examination for the presence of other cutaneous polyomaviruses, while the nature of the observed virus

particles and the virus-producing cells has not been evaluated ([Wetzels et al., 2009](#)).

Limited polymorphisms in both sT and LT sequences, without signature mutations, have been detected in some MCC biopsies ([Foulongne et al., 2010a](#); [Laude et al., 2010](#)). Furthermore, coexistence of integrated LT-truncated viral sequences and integrated concatemers or episomes has been documented in different body compartments ([Sastre-Garau et al., 2009](#); [Laude et al., 2010](#)). This situation might explain the case studied by [Wetzels et al. \(2009\)](#), hence explaining the presence of viral particles in MCV-positive MCC cells. Yet another group reported sequences encoding full-length LT in MCCs, casting doubt on the role of truncated LT in the development of MCC ([Schrama et al., 2010](#)). In conclusion, MCV-positive MCCs may express truncated LT and/or full-length LT from an integrated MCV and/or episomal genome. Of note, the discrepancy in the findings of viral sequences encoding truncated LT or full-length LT may be due to limitations of the PCR method that selectively amplifies the most abundant template. It may also stem from a mixed population of infected cells. Hence, further studies are required to resolve the significance of LT mutations in MCC.

In addition to LT truncation, 90 bp and 200 bp deletions of the VP1 gene that alter the open reading frame have been reported in MCC ([Kassem et al., 2008](#); [Feng et al., 2011](#)). The extent to which this alteration applies to the function of VP1 has not been evaluated. However, point mutations in the VP1 gene of at least one MCC-derived isolate, MCV350 (also termed MCC350), have been shown to exhibit defective assembly ([Pastrana et al., 2009](#)). A possible implication of this finding is that ongoing expression of assembly-competent VP1 might be detrimental to the developing MCC.

Another viral mutation has been identified during sequencing of the MCV replication origin from one non-tumour tissue, eight MCCs, and two cell lines: a single point mutation in a

tumour-derived MCV strain at a critical pentanucleotide sequence outside of the canonical LT-binding region. This natural mutation abrogates MCV origin replication ([Kwun et al., 2009](#)).

(e) *Genomic integration of MCV*

Like high-risk human papillomaviruses (HPVs), tumour-derived MCV genomes are integrated into the MCC cellular genome. The integration pattern of MCV DNA is clonal, and the primary tumours as well as metastatic cells carry the same integration signature. Southern blot hybridization and PCR-based analyses of MCC and cell lines are consistent with the virus integrating at a single insertion site, sometimes as head-to-tail concatemer, in each particular tumour ([Feng et al., 2008](#); [Sastre-Garau et al., 2009](#); [Fischer et al., 2010](#); [Laude et al., 2010](#)). The monoclonal pattern of MCV integration into MCC suggests that virus integration precedes tumour-cell expansion and thus argues for a causative role of MCV infection in MCC development. The clonal finding fulfils Hill's criterion of temporal relationship, which is an important epidemiological causality criterion. Viral DNA could be episomal in certain MCV-positive cells, but this may represent coexistent episomally replicating virus ([Sastre-Garau et al., 2009](#); [Fischer et al., 2010](#)).

Integration of MCV on chromosome 3p14.2, disrupting the receptor tyrosine phosphatase type G (*PTPRG*) gene, was identified during the initial discovery of the virus ([Feng et al., 2008](#)). *PTPRG* has been identified as a putative tumour suppressor gene that is hypermethylated in certain malignancies. Sastre-Garau and co-workers mapped random integrations of MCV DNA at distinct chromosomal loci, including those in the vicinity of *MYC* and *IL20RA* ([Sastre-Garau et al., 2009](#)). In another study, integration sites were found close to the *PARVA* and *DENND1A* genes, involved in cell junction and in formation of clathrin-coated vesicles or cell adhesion and cytoskeleton, respectively ([Laude et al., 2010](#)). An

additional integration site was mapped adjacent to the transcriptional activator TEAD1 encoding gene and another, which led to a complex rearrangement, within intronic sequences of the GDP-mannose 4,6-dehydratase (*GMD5*) gene.

Duncavage and colleagues used next-generation sequencing to reveal unique integration sites of MCV in four MCCs, with the exception of a paired primary tumour and metastasis samples, which showed identical sites ([Duncavage et al., 2011](#)). Alteration of the expression of cellular genes located in the vicinity of the integration sites could also participate in the induction of the tumorigenic phenotype, yet no evidence of deregulation of putative adjacent cellular genes has been detected, favouring an alternative mechanism of tumorigenesis ([Sastre-Garau et al., 2009](#)).

(f) *Merkel cell carcinoma cell lines*

Fully transformed and tumorigenic MCV-positive and MCV-negative MCC lines have been established ([Shuda et al., 2008](#); [Fischer et al., 2010](#); [Houben et al., 2010b](#)). Based on neuroendocrine markers and the characteristics of their tumour-derived cell lines, MCC can be categorized into classic and variant types that form suspension or adherent cell cultures, respectively ([Leonard et al., 1993](#)). Three variant lines and one classic MCC cell line were tested for the presence of MCV DNA; the virus, in a clonal concatemeric integration form, was found only in the classic variant MKL-1 cell line, which expresses both LT and sT mRNAs and lacks the 57kT mRNA due to the elimination of a putative splice-donor junction. A truncating mutation (stop codon at nucleotide 1452) that prevents expression of the OBD and the helicase domain has been documented in this cell line as well ([Shuda et al., 2008](#)).

Seven MCC cell lines, including four classic ones, one variant, and two intermediate phenotype subtypes, were analysed for the presence of MCV. The virus was detected in two classic and

two intermediate phenotype lines. Of note, levels of MCV genome varied, with three cell lines strongly positive for MCV DNA, whereas two contained minute amounts of the virus, detectable only by means of nested PCR, suggesting that only a minority of these cells contain the virus ([Fischer et al., 2010](#)). Like [Shuda et al. \(2008\)](#), this group did not identify the virus in the variant subtype. Therefore, as not all classic-type cultures were MCV-positive, no clear correlation could be drawn between the phenotypic characteristic of the cells and the presence of the virus. Also in accordance with [Shuda et al. \(2008\)](#) were the findings of clonal integration of head-to-tail concatemers of the virus and premature stop-codon mutations that truncate LT while preserving the pRB-binding domain ([Fischer et al., 2010](#)). Taken together, these findings suggest the importance of MCV infection in a subtype of MCC cell lines and show that neutralizing pRb activity by LT, along with pausing of virus replication, is important for the malignant process.

Knockdown of the viral LT locus (pan T-antigen) with short-hairpin RNA (shRNA) interferes with the proliferation and survival of most established MCC cell lines harbouring the MCV genome ([Houben et al., 2010b, 2012a](#)). Yet since one MCV-infected MCC cell line did not show dependence on T-antigen expression for survival, it seems that there may be a stage where expression of the viral proteins is not essential ([Houben et al., 2012b](#)). [However, it cannot be completely excluded that the LT knockdown in this experiment might not be different enough.] Neither caspase activation nor alterations in the expression levels of several pro- and anti-apoptotic proteins, including p53, have been associated with the decreased survival of the MCV-infected cells after LT repression. As predicted, MCV-negative cell lines are unaffected by MCV LT knockdown ([Houben et al., 2010b](#)).

Interestingly, knockdown of sT alone retarded MCV-positive cell growth to a similar

extent as did shRNA targeting pan T-antigen, but did not induce MCC cell death. Knockdown of pan T-antigen was more efficient in inhibiting cell-cycle entry, measured by BrdU incorporation, than was sT knockdown alone. Even though no cooperation has been observed in rodent cell transformation assays between MCV sT and LT proteins, there is evidence to suggest that this process does occur. Expression of MCV sT does not rescue MCC cells in which the T-antigen locus has been repressed, indicating that other T-antigen isoforms also play a role in MCV-induced oncogenesis ([Shuda et al., 2011](#)).

The requirement of the T-antigen locus for tumorigenesis has been substantiated by means of an inducible shRNA system that produced regression of MCC in NOD/SCID mice upon knockdown of LT and sT. The growth-inhibitory effect of LT shRNA was almost completely restored by ectopic expression of shRNA-resistant LT, whereas mutant LT protein that does not interact with pRb failed to restore knockdown of LT ([Houben et al., 2010b](#)).

Taken together, these studies suggest that LT and sT are important for the survival of MCV-infected cells, and that the virus acts as a key factor in signalling events that maintain the tumour phenotype in MCV-positive cells.

(g) *Alteration of specific proto-oncogenes and chromosomal abnormalities*

Little is known about relevant genetic alterations in MCC and the different pathways involved in the pathogenesis of MCV-negative and MCV-positive MCCs. *Katano et al.* reported that MCV-positive cases showed round and vesicular nuclei with a fine granular chromatin and small nucleoli, whereas MCV-negative cases showed polygonal nuclei with diffusely distributed chromatin ([Katano et al., 2009](#)). In line with this, multiobserver morphometric analyses to determine differences in tumour-cell morphology between MCV-positive and MCV-negative MCCs identified more irregular nuclear shapes and

abundant cytoplasm in MCV-negative tumours, which may reflect biological differences between these subgroups ([Kuwamoto et al., 2011](#)).

Several studies published before the discovery of MCV examined the expression and genomic organization of different cellular genes in MCC. For example, mutations of *p53* and the related tumour protein 73 (TP73) have been reported ([Van Gele et al., 2000](#)). Deletion of the fragile histidine triad (*FHIT*) gene at 3p14.2 and its aberrant expression have also been reported in MCC ([Leonard et al., 1996](#); [Sozzi et al., 1996](#); [Popp et al., 2002](#)). Furthermore, hypermethylation of the cellular genes *p14*, cyclin-dependent kinase inhibitor 2A (*CDKN2a*) and Ras association domain family 1A (*RASSF1A*) has been reported in MCC ([Lassacher et al., 2008](#); [Helmbold et al., 2009](#)). However, since not all MCCs are MCV-positive, both virus-dependent and virus-independent oncogenic pathways may contribute to the development of MCC.

Discrepancies in the molecular and potentially oncogenic pathways between MCV-positive and MCV-negative MCCs have been analysed in several recent studies. By using comparative genomic hybridization (CGH), Paulson and co-workers identified several regions of relatively frequent genomic alterations in MCC, including a deletion of 5q12-21, a deletion of 13q14-21, which contains the gene of the tumour suppressor pRB, and focal amplification at 1p34 that was centred on the *L-MYC* (*MYCL1*) gene. When virus-positive MCC was compared with MCC without detectable virus, there was a trend, which was not significant, towards more genomic aberrations in virus-negative tumours, with a notable exception (of unknown significance) of a deletion of chromosome arm 19q in the majority of virus-positive MCC but rarely in virus-negative MCC ([Paulson et al., 2009](#)). Interestingly, no significant correlation between *RASSF1A* methylation and MCV infection was identified when MCCs were analysed for the combined presence of hypermethylation and virus infection, suggesting that

virus infection does not contribute to this type of hypermethylation ([Helmbold et al., 2009](#)). CGH analysis of 10 MCV-positive MCCs did not show any imbalance in 3 tumours, while recurrent imbalances were gains of 1q (2 cases), 6p (3 cases), and 11 (2 cases), and loss of 17p (2 cases) ([Sastre-Garau et al., 2009](#)).

IHC was used to show that MCV-positive MCC with relatively high MCV DNA abundance (i.e. 0.06–1.2 viral copies/cell) had significantly higher pRb and terminal deoxynucleotide transferase ([Bhatia et al., 2010b, c](#)). A similar correlation between MCV DNA positivity, LT expression, and pRb detection in MCC has been reported by [Sihto et al. \(2011\)](#). In contrast, Houben and co-workers did not find a correlation between virus abundance in MCC tissues and pRb expression ([Houben et al., 2010a](#)). The function of p53-regulated pathways in MCC has not yet been evaluated. Significantly higher p53 protein levels correlated with low viral copy numbers (i.e. 0.0005–0.0035 genome copies/cell) or with the absence of MCV ([Bhatia et al., 2010b](#)). In line with this, p53-positive MCCs were reported to contain a lower copy number of MCV than p53-negative tumours, with the mean number of MCV DNA copies decreasing as the percentage of p53-positive nuclei in the sample increased. This group also found a similar trend of inverse association for the receptor tyrosine kinase KIT ([Waltari et al., 2011](#)). This may indicate that MCC arises through sequential multiple oncogenic pathways and that accumulation of mutations in host genes, enhanced by virus infection, is important for the onset of MCC. In accord with this suggestion is the notion that there is a long time interval between the primary exposure to MCV and the development of MCC.

(h) *Intermediate working model of MCV tumorigenesis*

The LT-truncating mutations as well as the common and exclusive integration of the MCV genome in MCC render it less likely that MCV

is simply a coincidental passenger infection in these tumours. This notion is sustained by the recent finding that expression of the T-antigens is vital for the survival and growth of MCV-positive MCC cell lines and xenograft tumours ([Houben et al., 2010a](#)). Because virus integration and LT mutation are presumed to be uncommon, the requirement for both events may explain why MCC is rare, despite widespread infection with MCV.

4.3.3 MCV in other tumours

Several studies described in Section 2 reported weak associations of MCV DNA positivity for tumours other than MCC. With one exception ([Pantulu et al., 2010](#)), reporting on CLL and discussed below, none of the studies showed viral loads approaching 1 viral genome copy per cell or evidence for biological activity of the virus in the tumour tissues.

(a) Non-MCC skin tumours

MCV DNA was detected significantly more often in MCC than in biopsy samples of non-MCC skin tumours, in samples of the perilesional area and in histologically healthy skin samples from immunocompetent patients. Since MCV DNA has been detected only by nested PCR in non-MCC samples, relatively smaller viral loads in these samples have been suggested ([Wieland et al., 2009](#)). Similarly, Becker and colleagues found MCV DNA in 12.5% of BCC samples, and the viral load was 4 orders of magnitude lower than in MCC ([Becker et al., 2009](#)). Likewise, the viral load in NMSC samples was reported to be 3–4 orders of magnitude lower than that in MCV-positive MCCs ([Kassem et al., 2009](#)). MCV DNA was detected in 12 (28.6%) of 42 keratoacanthoma and in 14 (26.9%) of 52 SCC samples, in which MCV DNA loads were > 2 orders of magnitude lower than those in MCC ([Wieland et al., 2011](#)).

(b) Chronic lymphocytic leukaemia

A link between CLL and MCC has been suggested, and various studies have reported the presence of the MCV genome in CLL ([Quaglino et al., 1997](#); [Brenner et al., 2001](#); [Vlad & Woodlock, 2003](#); [Agnew et al., 2004](#); [Howard et al., 2006](#); see also Section 2.3.2). The physiological relevance of these findings is supported by the detection of MCV DNA by PCR in lymphocyte and inflammatory monocyte populations from MCC patients and in blood samples from healthy individuals, demonstrating that MCV can be lymphotropic ([Shuda et al., 2009](#); [Laude et al., 2010](#); [Mertz et al., 2010b](#); [Pancaldi et al., 2011](#)). Of note, circulating Merkel cells have also been detected in the peripheral blood of an elderly man with MCC and a history of CLL and multiple myeloma ([Hartley et al., 2010](#)). Patients with MCV-positive MCC tumours have an elevated risk of CLL ([Koljonen et al., 2009](#)), but direct examination of CLL tumours does not always reveal MCV genome positivity in most of the samples examined, and when detected, MCV DNA is only present at very low levels (see also Section 2.3.2). [Teman et al. \(2011\)](#), for example, reported MCV DNA in 6 (33%) of 18 CLL cases, and only 1 case presented positive MCV LT immunostaining with CM2B4 antibody. In contrast, a study of two patients with concurrent MCC and CLL demonstrated that the MCV LT protein is expressed only in the MCC-affected cells and not in the CLL-affected cells ([Tolstov et al., 2010](#)). Comparison between MCV antibody positivity in CLL patients and in a leukaemia control group also did not reveal differences in the rates of MCV prevalence and in antibody titres ([Tolstov et al., 2010](#)).

Striking, however, is the detection of MCV genome in 4 of 6 cases of CLL by fluorescence in situ hybridization (FISH), revealing patterns compatible with viral integration ([Haugg et al., 2011](#)). In this study, MCV genome and protein expression was identified from a subset of CLL tumours, and the viral genome presented a

246 bp deletion within the LT helicase domain, which is reminiscent of MCV infection in MCC; however, since all mutations were identical in the different patients, conclusions should be drawn with caution. In addition, extremely low viral copy numbers were observed, corresponding to < 1 viral genome copy per 1000 cells (3–4 orders of magnitude lower than in MCC tissues) ([Pantulu et al., 2010](#)).

4.4 Interaction between MCV and other potential cofactors

MCC is a relatively rare disease. The high prevalence of MCV infection in the general population and the low rate of MCC indicate that environmental and host genetic factors may influence the progression from infection to cancer. To evaluate the cofactors of MCV, it is important to note the complex and multifactorial origin of cancer. To date, evidence for cofactors of MCV comes from case–control studies in which exposure is determined after the diagnosis of disease, while many potential cofactors have not been evaluated rigorously in epidemiological studies. Assuming that ~80% of MCCs involve MCV infection, one may gain some information on potential cofactors from the literature published before the discovery of MCV. Obviously, these studies did not measure MCV, which is a potential confounder.

Old age, immune deficiency related to viral infections, organ transplantation, CLL (see Section 4.5), and exposure to UV radiation have been proposed as predisposing factors to MCC ([Rockville Merkel Cell Carcinoma Group, 2009](#)).

The importance of sunlight in the development of MCC is suggested by the fact that these tumours occur almost exclusively on sites exposed to the sun. The risk of MCC is possibly increased by exposure to UV radiation. Furthermore, MCC develops more frequently in individuals with light-coloured skin who live at more equatorial

latitudes and is rare in individuals with darker skin ([Miller & Rabkin, 1999](#)). In a study of 1380 psoriasis patients receiving UVA phototherapy, 3 patients developed MCC, a much higher rate than expected from the incidence rate of the general population ([Lunder & Stern, 1998](#)). Exposure to sunlight may induce DNA mutations in the MCV genome. Besides this direct role, UV exposure may also contribute to MCC development through immunosuppression. To understand the mechanism underlying this observation, MCV transcripts were examined after exposure of healthy volunteers to normal solar radiation; an increase in the expression of sT mRNA was observed ([Mogha et al., 2010](#)). *In vitro* studies confirmed induction of the sT transcript, which was attributed to activation of the early MCV promoter shortly after UV irradiation ([Mogha et al., 2010](#)).

4.5 Susceptible populations

Primary evidence to support the concept that MCC might be caused by an infectious agent came from reports of increased risk of MCC in immunocompromised subjects. Immunosuppression was recognized as a risk factor for MCC in organ transplant recipients ([Formica et al., 1994](#); [Douds et al., 1995](#)) and in AIDS patients ([Catlett et al., 1992](#)). (See also more detailed studies in Section 2.4.) Furthermore, MCC incidence sharply increases in elderly populations, and MCC is extremely uncommon before the age of 50. Together with the increased risk among the elderly, which may reflect an age-related decline in cellular immune surveillance, these clinical findings are consistent with MCC having an infectious etiology.

Immunosuppression related to lymphohaematopoietic cancers may also increase the risk of MCV-associated MCC. Statistically significant increased risks of MCC occurring 1 year after NHL, multiple myeloma, and CLL were reported, with SIR of 3.28, 4.91, and 7.43, respectively

(Howard *et al.*, 2006). A stronger inter-relationship was reported between MCV-positive MCC and CLL, with highly elevated SIR, 15.7 (95% CI, 3.2–46.0; $P < 0.01$) and also for CLL diagnosed after MCC, with SIR of 17.9 (95% CI, 2.2–64.6; $P < 0.001$) (Koljonen *et al.*, 2009).

Epidermodysplasia verruciformis (EV) is a very rare, autosomal recessive inherited skin condition associated with diminished cell-mediated immunity and high susceptibility to infection with specific HPVs. It is characterized by the development of multiple skin lesions, in particular BCC and SCC, predominantly at sites that are exposed to the sun. A case report describing a Brazilian EV patient who developed MCV-associated MCC could suggest similar host genetic susceptibility factors for HPV and MCV (Oliveira *et al.*, 2010). EV patients appear to have higher MCV viral loads, and MCV DNA was detected in carcinoma in situ, in invasive SCC, and in common warts of all 6 EV patients analysed (Mertz *et al.*, 2011). In addition, another case report described the occurrence of MCC in a young woman with no apparent risk factors but with a family history for a variant of EV, suggesting an incomplete EV with a distinct phenotype characterized by immunological abnormalities, further highlighting an association between immune disruption and susceptibility to MCC (Halvorson *et al.*, 2011).

4.6 Transgenic models for cancers associated with MCV infection

No studies with transgenic mice carrying MCV-derived genes and resulting in tumour formation have been reported.

4.7 Mechanisms of carcinogenesis

There is strong evidence that cell transformation of MCV in human MCC follows a mechanism generally similar to that used by other

transforming polyomaviruses in experimental systems and by oncogenic HPV. This mechanism is characterized by the persistent presence of at least one biologically active viral genome in each transformed cell. Viral persistence can be mediated through integration or through maintenance as a viral episome. The presence of the same integration site per tumour present in primary tumours, in recurrent tumours, and in tumour metastases is a marker of clonal tumour expansion and indicates that viral integration occurred before tumour expansion. Viral genes encoding regulatory proteins, i.e. the T-antigens in polyomaviruses and E6 and E7 in HPV, are consistently expressed. Apart from their other functions, viral oncoproteins interact directly or indirectly with cellular tumour suppressor proteins such as pRb and p53, leading to deregulation of the cell cycle and apoptosis. Presence of viral oncoproteins is necessary to maintain the transformed phenotype, which can lead – in the tumour-bearing animal or in the human patient – to the induction of antibodies to the viral oncoproteins. In the absence of tumours, such antibodies are rarely induced during the natural course of infection.

4.7.1 Studies in Merkel cell carcinoma tissues

According to the current literature (see Section 4.3 for references), with a total of > 1000 MCC cases analysed by many different research groups, about 75% of tumours were found to contain MCV DNA. MCV DNA has also been detected in recurrent tumours as well as in nodal and distant metastases (Feng *et al.*, 2008; Garneski *et al.*, 2009; Sastre-Garau *et al.*, 2009).

However, the detection of viral DNA alone in tumour tissues is not sufficient proof of a causal association. MCV is an ubiquitous virus, and MCV DNA can be detected, in varying amounts, in specimens from a wide range of different tissues (Loyo *et al.*, 2010) and even in the human environment (Foulongne *et al.*, 2011), raising the

possibility of contamination from neighbouring non-tumour tissues. Therefore, environmental contamination should be considered a very serious concern with PCR-based studies ([Cohen & Enserink, 2011](#)).

MCV integration at a single site is frequently present in MCV DNA-positive primary MCCs and metastases. There appears to be no specificity with respect to the cellular integration sites.

Full-length intact MCV LT is required for MCV genome replication and is encoded in replicating virus from asymptomatic skin infections ([Schowalter et al., 2010](#)). MCV LT binds to probably all pRb family proteins ([Shuda et al., 2008](#)), whereas no evidence for a direct interaction between MCV LT and the tumour suppressor protein p53 is available.

In contrast, most LT sequences isolated from MCC tissues are truncated as a result of point mutation or have deleted C-terminal LT sequences downstream of the pRb-binding domains ([Feng et al., 2008](#); [Shuda et al., 2008](#); [Sastre-Garau et al., 2009](#); [Foulongne et al., 2010a](#); [Laude et al., 2010](#)). These tumour-specific LT alterations result in loss of replication function ([Shuda et al., 2008](#)) and thus may contribute to the stability of the integrated viral genomes that still contain the viral origin of DNA replication.

In MCC, the MCV T-antigen gene region is transcribed, giving rise to sT, LT, and 57kT. The 57kT transcript is similar to LT but has a second intronic splice site that deletes portions of the OBD and the helicase function ([Feng et al., 2008](#)). No specific function has been attributed to 57kT. [Sastre-Garau et al. \(2009\)](#) found both sT and LT viral sequences to be significantly expressed in MCCs that all had > 0.6 viral genome copies per cell.

Monoclonal antibody CM2B4 targets an epitope on LT and 57kT upstream of the pRb-binding domain and can selectively stain nuclei of MCV DNA-positive MCC tissues ([Busam et al., 2009](#); [Shuda et al., 2009](#); [Reisinger et al., 2010](#); [Wieland et al., 2012](#); [Erovic et al.,](#)

[2013](#)). Using this antibody, [Busam et al. \(2009\)](#) reported that 27 (75%) of 36 MCCs stained positive for MCV LT. In the majority of the tumours, the staining of tumour cell nuclei was strong and homogeneous, labelling > 75% and up to 100% of the tumour cell population. In a subgroup for which data on viral load were available, 5 of 7 tumours with > 0.8 viral genome copies per cell and none of 3 tumours with < 0.001 viral genome copies per cell stained positive with CM2B4 ([Shuda et al., 2009](#)).

Using the monoclonal antibody CM5E1, which recognizes an epitope specific to MCV sT, [Shuda et al. \(2011\)](#) detected sT expression in 47 (92%) of 51 MCC tumours versus only 38 (75%) positive for MCV LT. Interestingly, 10 tumours were sT-positive only, versus 1 tumour staining for LT only.

In HPV-induced carcinogenesis, pRb is targeted for degradation by the viral oncoprotein E7, resulting in concomitant upregulation of cyclin kinase inhibitor p16^{Ink4a}, while the viral oncoprotein E6 inactivates p53 through ubiquitination and degradation, and the TP53 sequence is not mutated ([IARC, 2007, 2012](#)). [Sihto et al. \(2011\)](#) found 61 (67%) of 91 MCC tissues to express MCV LT. LT expression was strongly associated with pRb expression, and viral load was > 0.1 copies per cell. LT expression was also associated with downregulation of p53 and absolute absence of TP53 mutations, whereas TP53 mutations were found in 52% of the LT-negative MCCs. However, p16^{Ink4a} was upregulated irrespective of the MCV LT status. Similar observations were made in a smaller MCC series by [Bhatia et al. \(2010b\)](#).

[Paulson et al. \(2010\)](#) demonstrated that antibodies to MCV sT and LT are strongly associated with MCC. Among 530 population control subjects, these antibodies were present in only 0.9% and were of low titre. In contrast, among 205 MCC cases, 40.5% had serum IgG antibodies that recognize a portion of T-antigen shared between LT and sT. After tumour removal, titres of T-antigen antibodies fell (> 8-fold per year)

in patients whose cancer did not recur, whereas they increased rapidly in those with progressive and metastatic disease preceding clinical detection of disease spread. These results suggest that antibodies recognizing T-antigen are induced in response to T-antigen expression in the tumour, reflect tumour burden, and could serve as surrogate markers for MCV-induced carcinogenesis.

[Touzé *et al.* \(2011\)](#) used VLP ELISA in MCC patients and found 14-fold higher GMTs of serum antibodies to MCV major capsid protein VP1 compared with controls. VP1 expression was absent in MCV DNA-positive tumours. In MCV antibody-positive MCC patients, [Pastrana *et al.* \(2009\)](#) found GMTs of MCV-neutralizing antibodies to be about 60-fold higher than those in MCV antibody-positive controls. The higher MCV capsid antibody levels may indicate higher viral load in MCC patients before tumour diagnosis.

4.7.2 Studies in animals and in cell lines

[Shuda *et al.* \(2008\)](#) demonstrated the presence of MCV DNA in one of four MCC cell lines. The MCV genome was integrated and encoded a truncated T-antigen. [Fischer *et al.* \(2010\)](#) analysed 7 other MCC cell lines and found 5 to be MCV DNA-positive, but only 3 with > 1 viral genome copy per cell, and demonstrated MCV integration for one of these 3 lines.

shRNAi targeting exon 1 shared by all three MCV T-antigen proteins knocked down all T-antigen expression and triggered growth arrest and/or cell death in three MCV-positive MCC cell lines, but not in one MCV-negative cell line ([Houben *et al.*, 2010b](#)), demonstrating that T-antigen expression is necessary for maintenance of the transformed phenotype.

Rescue experiments in a xeno-transplantation model expressing various forms of LT during lentivirus-mediated drug-inducible (sh) RNAi T-antigen knockdown demonstrated that LT expression is also essential for tumour growth

in vivo. LT with a mutated pRb-binding domain failed to rescue targeting of pRb family proteins, suggesting that pRb binding by LT is essential for proliferation of MCC cells ([Houben *et al.*, 2012a](#)).

Lentiviral shRNA targeting the intron 1 sequence can selectively knock down sT and inhibit growth of the MCC cell line MKL-1 to a similar extent as shRNA targeting all T-antigen isoforms, but it did not cause cell death, suggesting that both sT and LT separately contribute to MCV-associated tumorigenesis ([Shuda *et al.*, 2011](#)). Furthermore, sT but not LT results in rodent fibroblast transformation and human fibroblast serum-independent growth, independent of PP2A and Hsc70 binding. sT appears to act downstream in the mTOR signalling pathway to preserve eukaryotic translation initiation factor 4E-BP1 hyperphosphorylation, resulting in dysregulated cap-dependent translation ([Shuda *et al.*, 2011](#)). These data suggest an oncoprotein function for sT.

Taken together, there is strong evidence generated by biochemical and biological studies performed in animal cells, in human MCC tissues, and in MCC-derived cell lines that MCV encodes oncoproteins that directly contribute to the development and maintenance of MCC.

4.7.3 MCV in chronic lymphocytic leukaemia

[Pantulu *et al.* \(2010\)](#) detected the presence of MCV DNA in highly purified leukaemic cells from 27% of 70 CLL patients. Viral copy numbers were 3–4 orders of magnitude lower than those in MCC tissues (corresponding to < 1 viral genome copy per 1000 cells). In 6 of 19 MCV DNA-positive CLL samples, the MCV LT sequences contained the same novel 246 bp deletion in the C-terminal helicase domain. This deletion was absent in MCV DNA-positive CLL samples. In addition, FISH with MCV probes revealed hybridization patterns compatible with viral integration in 4 of the 6 CLL samples ([Haugg *et al.*, 2011](#)). In two bone marrow samples containing nodular CLL

infiltrates, a faint staining with CM2B4 indicated LT expression in a significant number of CLL cells. The detection of MCV, including LT deletions and LT expression in CLL cells, could argue for a potential role of MCV in a significant subset of CLL cases. However, the extremely low viral copy number observed is not compatible with the requirement for viral persistence and viral oncogene expression in each tumour cell. Although the sequences adjacent to the deletions revealed some sequence heterogeneity, the deletion boundaries in all 6 patients were identical at the nucleotide level. This is in contrast to the observations with MCC, in which no identical deletions were found in different patients.

5. Summary of Data Reported

5.1 Exposure data

Merkel cell virus (MCV) was first discovered in human Merkel cell carcinoma (MCC), a rare and highly aggressive skin cancer. MCV infection in humans has been investigated by a variety of methods, including serology and DNA detection. Collectively, these studies show that a high proportion of adults are infected with MCV and that infection is acquired early in life and is evident in all age groups. Detection of MCV in the skin of most adults suggests that MCV is common in human skin. MCV has not been associated with any human disease other than MCC. The mode of transmission, route of dissemination, cellular tropism, and latency characteristics remain to be elucidated. There is consistent evidence that MCV infects humans.

5.2 Human carcinogenicity data

5.2.1 Merkel cell carcinoma

An etiological role of MCV in MCC is supported by a few case-control studies and several case series. There are no prospective cohort studies. Case-control studies show odds ratios ranging from 4.4 to 6.6 for serological markers of MCV infection and from 16.9 to 63.2 for serological markers of viral early gene expression. Little is known about other risk factors for MCV infection and their role in MCC, and therefore potential confounding could not be ruled out. However, there is consistency across a large number of case series in different populations and regions, in which MCV DNA is detected in tumour tissue of most MCC cases (prevalence ranges from 59% to 100%).

5.2.2 Other cancers

Overall, there is no clear association between MCV and other cancers.

5.3 Animal carcinogenicity data

No data were available to the Working Group.

5.4 Mechanistic and other relevant data

There is strong mechanistic evidence that MCV can directly contribute to the development of a large proportion of MCCs. There is weak and inconsistent evidence for a direct contribution of MCV to other human cancers.

- Multiple independent laboratories worldwide have reported the presence of MCV DNA in about 75% of > 1000 MCCs that have been analysed by PCR.
- In most MCV DNA-positive MCCs for which the viral DNA load has been analysed, MCV

genome copy numbers close to or more than 1 copy per cell have been demonstrated.

- In the majority of MCV-positive MCCs that have been studied for clonality, viral DNA is clonally integrated in the cellular genome. Integration appears to precede clonal expansion.
- In the majority of MCV-positive MCCs that have been analysed for the status of the MCV early genome region, the LT gene sequences contained truncating mutations at the C terminus that were associated with replication deficiency and possibly other functions, while the sT gene sequences were not mutated.
- Expression of the sT and LT antigens has been repeatedly demonstrated at the transcriptional and translational levels in cells of MCC.
- MCC patients have measurable antibodies to MCV-specific sT and LT, but MCV-infected individuals without MCC rarely do.
- Expression of the T-antigens is essential for the survival of MCV-positive MCC-derived cell lines and for the propagation of MCC engrafted into NOD/SCID mice.
- The intact sT and LT antigens of MCV interact with key cellular regulatory proteins to subvert cellular control pathways.
- sT of MCV transforms immortalized rodent fibroblasts and converts immortalized human fibroblasts to serum-independent growth.

6. Evaluation

6.1 Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of MCV. A positive association has been observed with MCC.

6.2 Cancer in experimental animals

There is *inadequate evidence* in experimental animals for the carcinogenicity of MCV.

6.3 Overall evaluation

MCV is *probably carcinogenic to humans* (Group 2A).

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