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Merkel Cell Polyomavirus: A Causal Factor in Merkel Cell Carcinoma

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1. Introduction

The RNA viruses hepatitis C virus and human T-cell lymphotropic virus type I (or human T-cell leukaemia virus type 1), and the DNA viruses hepatitis B virus, high-risk human papillomaviruses, human herpes virus-8 (Kaposi's sarcoma-associated herpes virus) and Epstein Barr virus are acknowledged as carcinogenic to humans. Human polyomaviruses have oncogenic potentials in cell cultures and animal models, but their role in human cancer remains controversial. In 2008, a new polyomavirus member discovered in Merkel cell carcinoma tissue was concordantly named Merkel cell polyomavirus (MCPyV). Subsequent epidemiologic studies have shown that viral DNA is present in approximately 80% of Merkel cell carcinomas investigated and MCPyV positive Merkel cell carcinoma have a higher virus load than non-malignant tissues. In addition, the patients have higher viral antibody levels than controls. Moreover, the viral genome seems monoclonally integrated in the primary tumour and in metastatic cells, and expresses a truncated version of the major oncoprotein, large T-antigen. This C-terminal truncated large T-antigen retains its DnaJ and retinoblastoma binding domains, which are necessary for transformation of cells *in vitro*, but lacks its DNA helicase activity so that it cannot sustain viral DNA replication. These observations suggest that MCPyV can contribute to Merkel cell carcinoma pathogenesis and may therefore add a new virus to the list of human cancer viruses. Here we present information on the biology of polyomaviruses with special focus on MCPyV, review the role of MCPyV in Merkel cell carcinoma, and discuss the molecular mechanisms by which this virus may induce cancer.

2. Merkel cell carcinoma

Merkel cells, originally described by Friedrich Sigmund Merkel in 1875, are believed to migrate during development from the neural crest to the basal layer of the epidermal cells near the end of axons and the outer root sheet of hair follicles [Szeder et al., 2003; Calder & Smoller, 2010]. These cells are especially dense in the skin of the limbs and face, and around hair follicles, but they are also present in oral mucus membranes [Lacour et al., 1991]. Their

role remains incompletely understood, but they may function as mechanoreceptors or chemoreceptors [Mahrle & Orfanos, 1974]. This was recently supported by phenotypic observations in conditional knock out mice deficient in expressing the transcription factor Atoh1 [Van Keymeulen et al., 2009]. These mice lacked Merkel cells and *ex vivo* skin/nerve preparations of these animals demonstrated complete loss of the characteristic neurophysiologic responses normally mediated by Merkel cell-neurite complexes. Merkel cells are therefore required for the proper encoding of Merkel receptor responses, suggesting that these cells form an indispensible part of the somatosensory system [Maricich et al., 2009]. Although data suggest that Merkel cells may have a neural crest origin [reviewed in Moll et al., 2005; Boulais & Misery, 2007, The Rockville MCC group, 2009], they also express the epithelial marker cytokeratin 20 (CK20) [Southgate et al., 1999], indicating an epithelial origin. In 1972, Toker described an unusual form of skin cancer as trabecular carcinoma of the skin [Toker, 1972]. Later, these carcinomas were shown to derive from Merkel cells and this type of cancer was therefore renamed Merkel cell carcinoma (MCC) [Gould et al., 1985]. Whether MCC arises from normal Merkel cells is still controversial [The Rockville MCC group, 2009]. Currently, MCC is thought to originate from a primitive totipotent stem cell with the ability to differentiate along divergent histological phenotypes [Calder & Smoller, 2010].

MCC is a rare but aggressive skin cancer of neuroendocrine origin with propensity for local recurrence and regional lymph node metastasis. MCC is most common on the head and neck (approximately 50% of the cases) and the extremities (40% of the patients), and the remaining cases occur on the trunk and on genitalia [Pectasides et al., 2006; Lemos & Nghiem, 2007; Heymann, 2008; Becker et al., 2009; Pulitzer et al., 2009]. MCC is heterogeneous clinically, morphologically and in expression of neuroendocrine, epithelial and oncogenic markers. Histological characterization of the MCC is based on demonstration of cytoplasmic neurosecretory granules of Merkel cell by electron microscopy and positive immunohistochemical staining for cytokeratins CK20, Cam 5.2, CD56, and neuroendocrine markers such as chromogranin A, synaptophysin and neuron-specific enolase [Heymann, 2008]. Classic MCC cell lines grow as non-adherent, clustered cells, while variant MCC cells grow as adherent cells [Leonard et al., 1995]. Comparing the gene expression profile between these two distinct MCC subtypes revealed higher transcript levels of genes encoding signal transduction proteins in classic MCC, while variant MCC possessed elevated transcription of cell cycle genes [Van Gele et al., 2004].

MCC is extremely unusual before the age of 50 (~5% of the cases) and people at risk include those with fair skin, excessive UV-light exposure and immunosuppressed patients. The median age at presentation is 76 years for women and 74 years for men and there is a slight male predominance [Calder & Smoller, 2010]. The majority of the patients are white (94.9%) and chronically immunosuppressed individuals are 10 to 15 times more likely to develop MCC than age-matched controls. Moreover, this malignancy is more lethal in immunodeficient patients than in immunocompetent individuals [Engels et al., 2008; Houben et al., 2009; Becker et al., 2009; Pulitzer et al., 2009; The Rockville MCC group, 2009; Wong & Wang, 2010; Calder & Smoller, 2010]. The incidence rate is 0.44 cases per 100,000 individuals in the USA and 0.15 cases per 100,000 individuals in Japan. The number of MCC cases has tripled the last 15 years [Hodgson, 2005; Nakajima et al., 2009]. The reason for this may be ageing of the population and improved diagnosis. So far, no optimal therapeutic regime exists so that surgical excision of the primary tumour and

lymph node dissection, followed by postoperational radiation therapy is the standard practice. Although MCC is claimed to be an aggressive cancer, a retrospective study on almost 4,000 patients has shown that the 10-year relative survival rate is 57% [Albores-Saavedra et al., 2009].

The molecular mechanisms that drive the pathogenesis of MCC remain incompletely understood. Aberrant DNA methylation of the promoters of the cyclin-dependent protein kinase inhibitor $p14^{ARF}$ and the tumour suppressor RASSF1A is believed to be important in MCC tumorigenesis [Lassacher et al., 2008]. Heterozygous loss of chromosome 10q23, which contains the tumour suppressor *PTEN*, overexpression of the anti-apoptotic proteins Bcl-2 and survivin, and of the L-*myc* proto-oncogene have frequently been observed in MCC, while expression levels of the p53 family member p63 correlates with the aggressiveness of the cancer [Kennedy et al., 1996; Feinmesser et al., 1999; Van Gele et al., 2001; Asioli et al., 2007; Kim & McNiff, 2008; Paulson et al., 2009]. However, the role of these events in MCC has to be established. The recent discovery of the presence of a new polyomavirus in most MCC may have shed some light on the enigma what may be a major cause of MCC.

3. Human polyomaviruses

Members of the *Polyomaviridae* family are characterized by a double-stranded circular DNA genome surrounded by a viral protein structure, referred to as capsid. They lack, however, a host-cell derived lipid structure known as the envelope. The virus particles are about 45 nm in diameter and the viral genome is approximately 5,000 base-pairs [Imperiale & Major, 2007]. The first member of this family was isolated in from mice 1953. The name polyomavirus ("poly" for many and "oma" for cancer) refers to the virus' ability to induce different types of tumours when injected in mice [reviewed in Stoner & Hübner, 2001]. The oncogenic properties of mouse polyomavirus encouraged scientists in their search to isolate other polyomaviruses, including human polyomaviruses. In 1960, the second member of this family was isolated from monkey kidney cells used for producing poliovirus vaccine. This virus was named simian virus 40 (SV40) and could transform cell culture, including human cells and induce tumours in animal models [Vilchez & Butel, 2004]. In 1971, two independent research groups reported the isolation of the first human polyomaviruses. One virus was detected in the urine of a kidney transplant recipient with the initials B.K., while the other human polyomavirus was isolated from the brain of a Hodgkin lymphoma patient with the initials J.C. who suffered from progressive multifocal leukoencephalopathy (PML). These viruses were named as BK virus (BKV) and JC virus (JCV), respectively [Gardner et al., 1971; Padgett et al., 1971]. The International Committee on Taxonomy of Viruses recommends using the abbreviations BKPyV and JCPyV, respectively [Johne et al., 2011].

SV40 and lymphotropic polyomavirus (LPyV), another monkey virus also seem to infect the human population, but with a much lower incidence than BKPyV and JCPyV. Whereas up to 90%, respectively 60% of the adults have antibodies against BKPyV, respectively JCPyV. SV40 and LPyV infections are less common with only 2% of the human population showing specific anti-SV40 antibodies and 15% possessing anti-LPyV antibodies [Knowles, 2006; Carter et al., 2009; Kean et al., 2009]. The latter number may be overestimated because recently a new human polyomavirus (HPyV9) that is closely related to LPyV has been isolated so that the seropositivity to LPyV may be partially explained by cross-reactivity

with HPyV9 [Scuda et al., 2011]. Human polyomaviruses establish a life-long latent infection in immunocompetent hosts, while immunocompromised individuals such as autoimmune patients and AIDS patients or immunosuppressive treatment such as in bone marrow and renal transplant patients may lead to reactivation of the viruses. Reactivation of BKPyV can lead to nephropathy or hemorrhagic cystitis, while JCPyV is the causative agent of progressive multifocal leukoencephalopathy [Jiang et al., 2009].

For many years, occurrence of the known human polyomaviruses in individuals was monitored mostly by PCR and serological methods. In recent years, mainly by use of novel methodology, several new polyomaviruses have been isolated. They include KIPyV, WUPyV, HPyV6, HPyV7, trichodysplasia spinulosa-associated polyomavirus (TSPyV), and HPyV9 [Allander et al., 2007; Gaynor et al., 2007; Schowalter et al., 2010; van der Meijden et al., 2010; Scuda et al., 2011; reviewed in Moens et al., 2010 and Moens et al., 2011]. Their role in human pathologies remains unknown, except for TSPyV which may be the etiological factor in trichodysplasia spinulosa, a rare skin disease exclusively found in immunocompromised patients and characterized by the development of follicular papules and keratin spines known as spicules [van der Meijden et al., 2010; Matthews et al., 2011].

Another new polyomavirus was isolated in Merkel cell carcinoma tissue, hence its name Merkel cell polyomavirus or MCPyV [Feng et al., 2008]. Whereas, the role of human polyomaviruses in cancer remains a matter of dispute despite their oncogenic properties in cell culture and in animal models, as well as the presence of viral nucleic acid sequences and proteins in cancer tissue, MCPyV is considered as an etiological factor in MCC [Abend et al., 2009; Maginnis & Atwood, 2009; Gjoerup & Chang, 2010]. This virus and its role in MCC is the focus of this review.

4. Molecular biology of human polyomaviruses

The viral genome of human polyomaviruses can be divided into three functional regions: the early region encoding large T-antigen (LT-ag) and small t-antigen (st-ag), the late region encoding the capsid proteins VP1, VP2 and VP3, and the non-coding control region encompassing the origin of replication and promoter sequences that control transcription of the viral genes (Figure 1). LT-ag is required for viral DNA replication and regulates the expression of the viral genes, while st-ag has an auxiliary role for LT-ag. The early region of some human polyomaviruses encodes alternative proteins whose functions are incompletely understood. The late regions of BKPyV, JCPyV and SV40 also contain an ORF for the agnoprotein that is required for efficient virus propagation in cell culture [Carswell & Alwine, 1986; Khalili et al., 2005; Sariyer et al., 2006; Johannessen et al., 2008; Suzuki et al., 2010]. No corresponding ORF seems to be present in the other human polyomaviruses [Yoshiike & Takemoto, 1986; Allander et al., 2007; Gaynor et al., 2007; Feng et al., 2008; Schowalter et al., 2010; van der Meijden et al., 2010; Scuda et al., 2011]. The reason why agnoprotein seems to be abundant in the human polyomaviruses LPyV, KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV and HPyV, but not in SV40, BKPyV and JCPyV remains mysterious, but it may be because of different life cycle strategies or host cell tropism. SV40 encodes a fifth late protein, referred to as VP4. This protein enhances lysis of the host cell and facilitates release of mature virus particles [Daniels et al., 2007]. KIPyV, WUPyV, MCPyV, TSPyV, HPyV6, HPyV7, and HPyV9 all lack a VP4-like open reading frame (ORF).

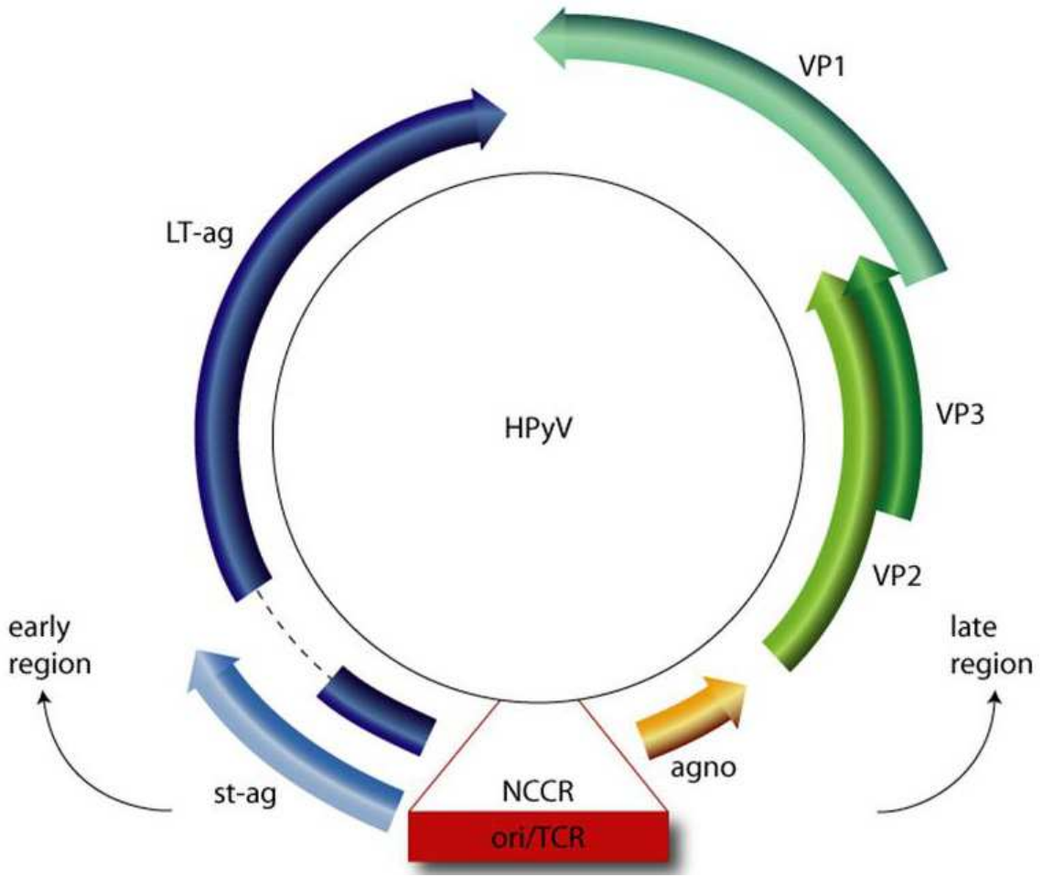


Fig. 1. Genomic organization of human polyomaviruses. The open reading frame encoding agnoprotein (agno) is not present in all human polyomaviruses. See text for details.

BKPyV and JCPyV contain a putative VP4 ORF with the N-terminal MVRQVAxREG amino acid sequence (x= E in BKPyV and Q in JCPyV), which is reminiscent of SV40 VP4 (MVRQVANREG), while LPyV has a putative ORF with the N-terminal motif MRQA at the corresponding site. However, VP4 expression by BKPyV, JCPyV and LPyV has not been confirmed.

The genomes of SV40, BKPyV, JCPyV and MCPyV encode a miRNA that down-regulates LT-ag expression levels [Sullivan et al., 2005; Seo et al., 2008]. Cells expressing SV40 miRNA are less susceptible to cytotoxic T cells and trigger less cytokine expression than cells infected with an SV40 mutant lacking miRNA. Hence, miRNA-mediated downregulation of LT-ag levels may allow the virus to escape the immune system [Sullivan et al., 2005]. BKPyV and JCPyV miRNA can also reduce the protein levels of LT-ag by preventing mRNA translation or targeting it for destruction. Whether this also affects cytotoxic T cells responses remains unknown. Recent findings by Bauman and colleagues shed light on the molecular mechanism by which these viruses can evade the immune system and remain latent. They demonstrated that JCPyV- and BKPyV-encoded miRNA target the stress-induced ligand ULBP3, which is recognized by the natural killer cell receptor NKG2D. Consequently, viral miRNA downregulation results in reduced

NKG2D-destruction of BKPyV/JCPyV infected cells by natural killer cells [Bauman et al., 2011]. This miRNA-mediated evasion of the immune system may allow the viruses to establish a latent infection. MCPyV also encodes a miRNA that is antisense to LT-ag mRNA and consequently can reduce the levels of LT-ag [Seo et al., 2009].

5. Mechanisms by which human polyomaviruses cause cellular transformation

Infection of cultured cell lines, including human, with SV40, BKPyV, JCPyV and LPyV leads to transformation, while transgenic animals expressing early gene products or animals infected with these viruses can develop tumours [Moens et al., 2007b; Gjoerup and Chang, 2010]. The oncogenic properties of these viruses are primarily attributed to the action of LT-ag, but also st-ag and agnoprotein possess oncogenic potentials.

LT-ag is a multifunctional protein that is mandatory for viral DNA replication. It contains several functional domains performing different roles (Figure 2). LT-ag binds as an hexamer to G(G/A)GGC motifs at the viral origin of replication and forms a complex with the cellular replication proteins DNA polymerase α/primase, replication protein A, and topoisomerase. LT-ag has also DNA helicase activity that helps unwinding the double-stranded DNA during replication [reviewed in Simmons et al., 2004]. It can bind the tumour suppressors p53 and the retinoblastoma (pRb) family members, thereby forcing the cell into the S phase. This is pivotal for the virus because the virus depends completely on the host cell's DNA replication machinery. LT-ag contains a so-called pRb pocket with the consensus motif LXCXE that is required for interaction with pRb family members. The pRb family members control S phase progression by controlling the expression of E2F-responsive genes. E2F is a family of transcription factors that transcribes genes controlling cell cycle progression and DNA replication such as cyclins, cyclin-dependent kinases, dihydrofolate reductase, thymidine kinase, DNA polymerase I, c-Myc, c-Myb, but also genes whose products are involved in DNA repair, differentiation and apoptosis. Another LT-ag domain that plays a crucial role in regulating the activity of pRb is the DnaJ domain. This N-terminal located region binds Hsc70, a chaperone with weak intrinsic ATPase activity. The DnaJ domain stimulates Hsc70's ATPase activity and this DnaJ/Hsc70 interaction influences viral DNA replication, transactivation of the viral promoters and viral assembly. Additionally, it is critical for oncogenic transformation via functional inactivation of the pRb family members. The energy generated by DnaJ domain-mediated hydrolysis of ATP is used to split pRb/E2F complexes [Moens et al., 2007a; Moens et al., 2007b; Gjoerup and Chang, 2010]. The C-terminal domain of LT-ag is involved in binding the tumour suppressor p53. The p53 protein represses cell cycle proliferation and angiogenesis, stimulates apoptosis and is involved in controlling DNA repair [Vogelstein et al., 2000; Vousden et al., 2009]. Several other properties of LT-ag can contribute to neoplastic transformation. It has been shown that LT-ag stimulates telomerase activity, inhibits apoptosis, activates signalling pathways triggering cell cycle progression, interferes with DNA repair and chromosome fidelity, promotes angiogenesis, perturbs protein turn-over, and affects gene expression by interfering with transcription, DNA methylation, chromatine structure. Hence, prolonged LT-ag expression may lead to uncontrolled cell cycle progression, inhibition of angiogenesis and apoptosis, and accumulation of DNA damage [reviewed in Moens et al., 2007a; Moens et al., 2007b; Gjoerup and Chang, 2010].

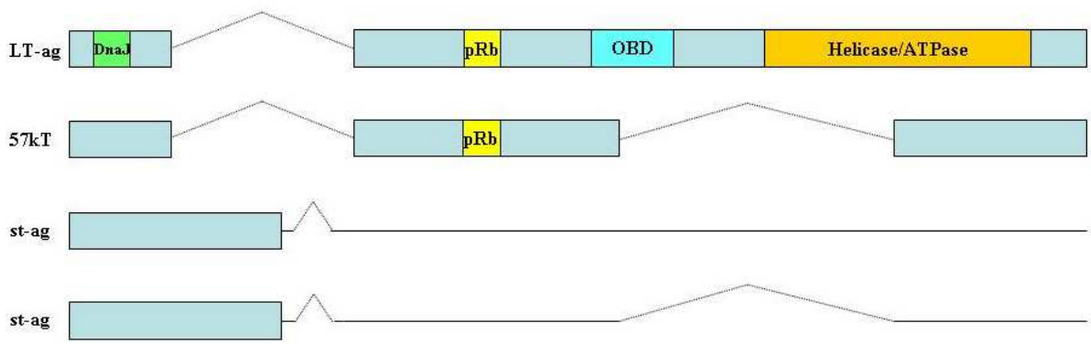


Fig. 2. Functional domains of the human polyomavirus LT-ag and the different splicing products generated from transcription of the early region of MCPyV. These transcripts encode LT-ag, the truncated variant 57kT and two different mRNAs for st-ag. See text for details.

St-ag seems to exert an auxiliary function in LT-ag-mediated transformation. St-ag shares the N-terminal ~80 amino acids with LT-ag and therefore contains the DnaJ domain, but unlike LT-ag, it lacks the pRb pocket. The major contribution of st-ag in the oncogenic process is through inactivation of protein phosphatase 2A (PP2A). The unique C-terminal part of st-ag binds to the A-subunit of the trimeric PP2A holoenzyme, leading to inhibition of the catalytic activity. St-ag mediated inactivation of PP2A can activate signalling pathways, alter gene expression, inhibit apoptosis/prolongation of cell survival, change protein stability, stimulate telomerase activity, disrupt the cytoskeleton and induce chromosome instability [Moens et al., 2007b].

The agnoprotein of JCPyV can arrest cells in G2/M phase probably through upregulated expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ [Darbinyan et al., 2002]. JCPyV agnoprotein can interact with the Ku70, which is a component of the non-homologous end joining (NHEJ) DNA repair complex, leading to inhibition of NHEJ DNA repair [Darbinyan et al., 2004]. Thus, agnoprotein may contribute to transformation through interference with the cell cycle and DNA repair.

Similar studies with the newly discovered human polyomaviruses are lacking, but as these viruses also encode LT-ag and st-ag, it is assumed that they may possess similar oncogenic properties.

6. Merkel cell polyomavirus (MCPyV): Molecular biology

In 2008, Feng and colleagues detected two sequences with highest homology to the LT-ags of LPyV and BKPyV in MCC transcriptomes consisting of >380,000 cDNA sequences obtained by pyrosequencing [Feng et al., 2008]. The authors succeeded in cloning and sequencing the entire genome of two variants of this virus, which turned out to be a novel polyomavirus. This new polyomavirus was commonly found in MCC samples and accordingly named Merkel cell polyomavirus (MCPyV). The MCPyV variant 350 (accession number EU375803) and variant 339 (accession number EU375804) diverge by 191 base-pairs inserted in the LT-ag of MCPyV339, 5 base-pairs inserted in the MCPyV 339 non-coding region, and additional 41 nucleotides substitutions dispersed throughout their genomes

[Feng et al., 2008]. The genetic organization of MCPyV (Figure 3) resembles that of other polyomaviruses, except for the absence of an open reading frame encoding agnoprotein. The early region of MCPyV encodes three different proteins that are translated from differently spliced transcripts. The LT-ag and st-ag share the N-terminal 79 amino acids, but diverge in their C-terminal domain. The 57kT is identical to LT-ag except for a deletion in the C-terminal region of the protein (Figure 2). The late region encodes the capsid proteins VP1 and VP2, but the VP2 ORF lacks the conserved Met-Ala-Leu motif that forms the start of VP3 in other human polyomaviruses. This may suggest that MCPyV may not encode a functional VP3 [Pastrana et al., 2009]. However, an open reading frame encoding a putative VP3 starting with Met-Thr-Ile is present in both MCC and non-MCC isolates suggesting that a functional VP3 is expressed [Feng et al., 2008; Carter et al., 2009; Schowalter et al., 2010; Touzé et al., 2011]. MCPyV also encodes a viral miRNA that can downregulate the levels of LT-ag [Seo et al., 2009].

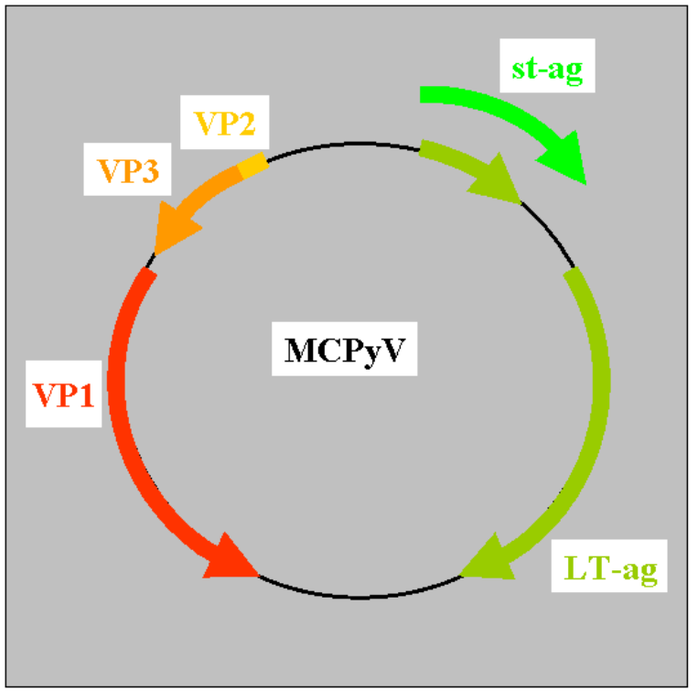


Fig. 3. Genomic organization of MCPyV. The early region encodes for LT-ag, st-ag and 57kT (not shown), while the late region encodes the capsid proteins VP1, VP2 and VP3.

Although originally isolated from MCC, MCPyV infection is probably not restricted to Merkel cells in the skin because these cells make up <1% of the cells in the epidermis, yet MCPyV virions are being shed from the skin at high numbers [Schowalter et al., 2010]. These findings strongly suggest that more common cells in the epidermis like keratinocytes and/or melanocytes may be involved in the production of virions. The presence of MCPyV in keratinocytes has not been investigated, while MCPyV DNA was not detected in melanocytes [Giraud et al., 2008; Mogha et al., 2010]. Hence, a role for these two cell types as reservoirs for MCPyV remains unclear. Other cell types in the skin may function as reservoir

because MCPyV DNA is present in eyebrow hairs, skin lesions and warts from immunocompetent and immunosuppressed patients [Wieland et al., 2009; Schowalter et al., 2010; van der Meijden et al., 2010; Mertz et al., 2010a; Foulongne et al., 2010a].

7. MCPyV: Route of infection, cell tropism and transmission

The route of infection of MCPyV is not know, but viral DNA is found in nasal swabs, oral cavity, mouth wash, tonsil biopsies, and oesophagus from healthy individuals, signifying an oral route of infection [Bialasiewicz et al., 2009; Dworkin et al., 2009; Goh et al., 2009; Kantola et al., 2009; Sharp et al., 2009; Loyo et al., 2010; Babakir-Mina et al., 2010]. The primary target cells and the host cells that can function as reservoir are not known, but the virus seems to be common in healthy skin, skin lesions, eyebrow hairs, and warts, indicating a dermatotropism of this virus [Wieland et al., 2009; van der Meijden et al., 2010; Mertz et al., 2010a; Faust et al., 2011; Wieland et al., 2011]. MCPyV DNA is also found in blood, gall bladder, appendix, liver, lung, lymphoid, and intestine tissue [Feng et al., 2008; Sharp et al., 2009; Goh et al., 2009; Shuda et al., 2009; Foulongne et al., 2010a; Husseiny et al., 2010; Loyo et al., 2010; Toracchio et al., 2010; Campello et al., 2011; Pancaldi et al., 2011; reviewed in Moens et al., 2010 and Gjoerup and Chang, 2010]. Another study has suggested that MCPyV may persist in and spread through inflammatory monocytes [Mertz et al., 2010b].

To expound the cell tropism, Erickson and colleagues identified the MCPyV receptor by using purified VP1 pentamers and liposomes. They found that MCPyV pentamers bound strongly ganglioside GT1b, very weakly GD1b (i.e. the receptor for BKPyV), and not GM1 (i.e. the receptor for SV40) and GD1a (i.e. the mouse polyomavirus receptor). This indicates that GT1b is a putative receptor for MCPyV and that MCPyV has different host cell targets than the other HPyV [Erickson et al., 2009]. The binding of MCPyV pentamers to its target receptor may be more complicated. GT1b is abundant in neuronal cell membranes, yet MCPyV DNA seems absent in cerebrospinal fluid and brain and central nervous system tumours [Feng et al., 2008; Barzon et al., 2009; Busam et al., 2009; Giraud et al., 2009]. Moreover, MCPyV pentamers could bind to HeLa (human cervical cancer carcinoma cells) and HEK29T (adenovirus transformed human embryonal kidney cells that express SV40 LT-ag) cells, which may not be genuine target cells as no MCPyV has been detected in cervical tissue nor in the kidneys so far. Moreover, MCPyV viruria is rare, indicating that the kidneys may not be an *in vivo* reservoir [Feng et al., 2008; Giraud et al., 2008; Duncavage et al., 2009a; Katano et al., 2009; Sastre-Garau et al., 2009]. The use of pentamers that may acquire a different conformation than intact virus particles and the requirement of a co-receptor may explain infection of none bona fide host cells.

The way of transmission of MCPyV is also unknown, but the faecal-oral route has been suggested because MCPyV DNA can be detected in samples from the gastrointestinal tract and in urban sewage [Feng et al., 2008; Bofill-Mas et al., 2010; Loyo et al., 2010; Campello et al., 2011]. Moreover, the average viral DNA load in aero-digestive tract is 100 fold higher than the respiratory and genitourinary tract [Loyo et al., 2010]. The results should be interpreted with caution because a limited number of patients were tested. Other studies failed to detect MCPyV DNA in colon adenocarcinoma and in stool samples, jeopardizing a faecal-oral route of transmission [Kantola et al., 2009; Sastre-Garau et al., 2009]. The virus may also spread through respiratory droplets or blood as viral sequences are detected in nasopharyngeal aspirates, nose swabs and blood, but proof for this way of transmission is

lacking. Moreover, the prevalence of MCPyV in these samples is lower than in MCC [Dworkin et al., 2009; Helmbold et al., 2009; Kantola et al., 2009; Sharp et al., 2009; Shuda et al., 2009; Tolstov et el., 2009; Mertz et al., 2010b; Babakir-Mina et al., 2010; Loyo et al., 2010; Pancaldi et al., 2011]. Direct contact is most probably the mode of transmission because MCPyV DNA is frequently detected in benign skin, healthy individuals are chronically infected with MCPyV, and the virus seems to be continuously shed from the skin [Feng et al., 2008; Dworkin et al., 2009; Helmbold et al., 2009; Wieland et al., 2009; Mertz et al., 2010b; Foulongne et al., 2010b; Loyo et al., 2010; Schowalter et al., 2010]. Schowalter and collaborators collected skin swab samples with approximately 3 months interval from 16 volunteers. All samples tested MCPyV DNA positive and contained ~$2x10^6$ genome equivalents/ml of gradient material. [Schowalter et al., 2010]. Efficient horizontal spreading of MCPyV through skin-to-skin contact may also explain the high seroprevalence in the human population (see next section). The absence of MCPyV antibodies in children less than 1 year old argues against vertical transmission [Tolstov et al., 2009].

8. MCPyV seroprevalence

PCR-based studies have demonstrated that MCPyV infection is common in the normal population and that different tissues can harbour viral DNA [reviewed in Moens et al., 2011]. Serological studies demonstrate that MCPyV infection is widespread in man (Table 1). Of 41 MCC patients (age 42-86 years; 27 men and 14 women) tested, 36 (88%) had MCPyV VP1 antibodies. Tumour material of 31 MCC patients was available for MCPyV DNA analysis and viral DNA was detected in 24 specimens. Of these 24 patients, 22 (92%) had MCPyV VP1 antibodies [Carter et al., 2009]. The authors tested 76 age-and sex-matched controls and found that 40 (53%) were seropositive, showing that there was a significant difference ($p<0.001$) between the MCC patients and the 76 controls for the presence of MCPyV antibodies. The same group analyzed another 451 randomly selected women whose age varied between younger than 30 years and >70 years and found that 271 (60%) were seropositive. No significant differences were observed with increasing age. The seroprevalences for the age groups <30, 30-39, 40-49, 50-59, 60-69 and <70 were 60%, 54%, 66%, 61%, 66% and 58%, respectively. This indicates that most viral infection must have occurred before the age of 30 and that the prevalence was stable at older ages [Carter et al., 2009]. Although this second control group existed exclusively of women, there was no significant difference with the first control group. Thus, the presence of MCPyV VP1 antibodies does not seem to be gender-dependent. Carter and his colleagues found that antibodies in serum samples reacted strongly with VP1 from MCPyV strain w162, while little or no reactivity was found against VP1 from strain 350, although these VP1s differ only by four amino acids [Carter et al., 2009]. Hence, caution must be taken when performing serological studies because the amino acid composition of VP1 in the assay may affect the reactivity of the circulating MCPyV VP1 antibodies in the serum of the patient and therefore the outcome of the test. Kean and co-workers also reported a differential seroreactivity to MCPyV isolates 350 and 339 [Kean et al., 2009]. In their study, 1501 adult healthy blood donors (age >21 yrs) were tested and 379 individuals (25%) had antibodies that reacted with MCPyV strain 350 recombinant VP1, while 692 blood donors (46%) possessed antibodies that recognized strain 339 recombinant VP1. The difference was less in a paediatric cohort <21years of age. Of 721 children examined, 164 (23%) were seropositive for MCPyV strain 350 and 247 (34%) for strain 339. The authors did not detect

differences in seroprevalence in respect to gender, nor was there a significant difference in seropositivity between children and adults. This indicates that primary infection occurs during early childhood. However, for MCPyV strains, there was a tendency to increased seropositivity in elderly individuals.

MCPyV positive MCC	Non MCC subjects	ag§	reference
88% (n=41; age 42-86 yrs)	53% (n=76; age <75 yrs) 60% (n=451; age 24-77 yrs)	VP1	Carter et al., 1999
	46% (n=1501; age >21 yrs) 34% (n=721; age < 21 yrs)	VP1	Kean et al., 1999
100% (n=21; age 14-95 yrs)	88% (n=48; age 47-75 yrs)	VP1	Pastrana et al., 2009
100% (n=21; age 14-95 yrs)*	64% (n=116; age >18 yrs) 63% (n=100; age > 47 yrs) 74% (n=50; age nm#)	VP1	Tolstov et al., 2009
not tested	11% (n=217; age 0-13 years) 37% (n=71; age 6-11 years)	VP1	Chen et al., 2011
91% (n=139; age 31-91 yrs)	66% (n=530; age-matched)	VP1	Paulson et al., 2010
100% (n=51; age 37-91 yrs)	85% (n=82; age 22-83 years)	VP1	Touzé et al..,2011
not tested	65% (n=434; age 29-97 years)	VP1	Faust et al., 2011
average VP1 seropositivity: 95% (n=252)	**average VP1 seropositivity: 57% (n=4397)**		
26% (n=205; age 31-96 yrs)	2% (n=530; age-matched)	LT-ag	Paulson et al., 2010
41% (n=205; age 31-96 yrs)	1% (n=530; age-matched)	st-ag	Paulson et al., 2010

§antigen; *same patients as in Pastrana et al., 2009; #nm= not mentioned.

Table 1. Seropositivity against MCPyV VP1, LT-ag and st-ag in MCC and non MCC subjects.

The average seropositivity for strain 350 in individuals <70 years is 23%, while 40% of persons older then 70 had antibodies. Similarly, 38% of the tested persons younger than 70 were seropositive for MCPyV strain 339 VP1, while 61% of those older than 70 years were seropositive [Kean et al., 2009]. In another study, the presence of MCPyV neutralizing antibodies in sera was determined. All (n=21) MCC patients (age 14-95 years) whose tumours were MCPyV DNA positive had specific MCPyV antibodies with a geometric mean titre of 160,000 in the assay used. While 42/48 (88%) sera of blood donors (age 47-75 years) also contain MCPyV specific antibodies, the mean titre was ~60 times lower. Similarly, low titres were found in 3 out of 6 MCC patients negative for MCPyV, while the other two patients had no detectable levels of MCPyV antibodies [Pastrana et al., 2009]. This was confirmed by the work of two other groups. Tolstov and collaborators found that IgG levels against the capsid proteins of MCPyV were higher in MCPyV positive MCC patients than in MCPyV negative patients, blood donors and systemic lupus erythematosus (SLE) patients

[Tolstov et al., 2009]. All of their 21 MCPyV positive MCC patients had significantly higher MCPyV specific IgG antibodies than antibody levels found in 3/6 (50%) MCPyV negative MCC patients, in 107/166 (64%) blood donors, in 63/100 (63%) commercial donors and in 37/50 (74%) SLE patients. The IgM levels in all patient groups were comparable [Tolstov et al., 2009]. French MCPyV positive MCC patients had on average 15-fold higher anti-VP1 antibody titres than controls [Touzé et al., 2011]. One obvious explanation for the higher MCPyV-specific antibody titres in MCPyV positive MCC patients is that VP1 is expressed on the cell surface of the tumour cells. However, immunohistochemical staining of 10 MCC tumour cells that had previously shown to express MCPyV LT-ag was negative for VP1, suggesting that VP1 is not expressed in MCC [Pastrana et al., 2009]. Touzé and colleagues were also unable to detect VP1 in 21 MCPyV positive MCC samples obtained from 16 patients [Touzé et al., 2011]. This indicates that tumour cells do not produce VP1 (due to integrational interruption of the late region) or do so at levels undetectable by immunohistochemical staining. Another reason for the higher antibody levels in MCPyV positive MCC patients could be their immunocompromised condition such that a delayed immune response upon primary infection may have resulted in much higher virus load inducing higher titres. Another possibility is that MCPyV may establish a persistent infection in other cell types than Merkel cells in these patients or that unusually high immunogenic MCPyV strains are circulating in these patients [Pastrana et al., 2009]. The fact that a substantial number of individuals of healthy controls and other patient groups displayed MCPyV VP1 antibody titres in the same range as MCPyV positive MCC patients argues against this [Pastrana et al., 2009; Tolstov et al., 2009]. This also implies that not everybody with high MCPyV specific antibody titres develops MCC.

Pastrana and colleagues observed a gradual age-related decline in MCPyV antibody titres for the MCPyV positive MCC patients, but the number in each age category was limited [Pastrana et al., 2009]. This was in contrast with the findings of Tolstov and co-workers in 150 Langerhans cell histiocytosis patients whose age ranged from 1 month to 72 years old. The authors measured an age-dependent increase of MCPyV-specific IgG antibodies with approximately 40% of the patients younger than 50 years old having antibodies; this number increased to 80% in patients older than 50 years [Tolstov et al., 2009]. The reason for the opposite results by the two groups remains unknown, but may be due to different assays, different patient groups, or the limited number of individuals examined. Interestingly, none of the six Langerhans cell histiocytosis patients who were less then 1 year old had MCPyV antibodies, arguing against vertical transmission of the virus [Tolstov et al., 2009]. A serological study with 217 Finnish children suffering from acute lower respiratory tract infection confirmed that MCPyV infection probably occurs after the age of one [Chen et al., 2011]. IgG antibodies immunoreactive with VP1 strain 339 were present in sera from 2 out of 51 (4%) children less than 1 year old, while 9% (n=140) of children between 1 and 4 years of age were seropositive. Seroprevalence increased to 35% (n=26) in the age category 4-13 years. The overall seroprevalence was 11% (24/217). Serum samples obtained 5-8 years later were available for 72 of these 217 children. The initial (at age 0-3 years) MCPyV VP1 IgG positivity rate of 6% in these children rose to 37% (26/71) during the follow-up. Notably, of the 67 children whose first sample was seronegative, 22 (33%) were seropositive for their follow-up sample. The same authors also examined 158 children age 1-4 years who had inflammation of the middle ear. Six of them (4%) were seropositive, while 3 years later, 29 (18%) had VP1 antibodies. While in the group of children with acute lower respiratory tract

infection no significant gender differences were observed, seroprevalence, seroconversion and antibody titres were higher (p=0.08, p=0.08 and p=0.1, respectively) [Chen et al., 2011].
Another study confirmed the higher prevalence and higher titres of VP1 antibodies in sera from MCC patients compared to control subjects. While 91% (n=134) of the MCC patients were seropositive, 66% (n=530) of the controls had anti-VP1 antibodies [Paulson et al., 2010]. The authors included an additional 66 sera from MCC patients and tested for the presence of antibodies against LT-ag and st-ag. While 2% (respectively 1%) control subjects had antibodies against LT-ag (respectively st-ag), 26% (respectively 41%) of the MCC patients were seropositive for LT-ag (respectively st-ag). Interestingly, higher MCPyV DNA load in MCC tumours is associated with higher LT-/st-ag antibody titres and patients with advanced disease had significantly higher titres. These results suggest that LT-ag/st-ag antibodies may serve as a clinically useful MCC disease marker [Paulson et al., 2010].
In conclusion, seroprevalence and antibody titres in MCPyV positive MCC patients are higher than in control subjects, but MCPyV infection is very common in the adult population. Results from independent groups revealed that 46-88% of the non MCC adults (approximately 4,400 subjects) had MCPyV antibodies [Carter et al., 2009; Kean et al., 2009; Pastrana et al., 2009; Tolstov et al., 2009; Paulson et al., 2010; Touzé et al., 2011; Faust et al., 2011]. Serological studies also indicate that primary infection is acquired early in life and that seropositivity inclines with age. MCPyV DNA was amplified in a nasopharyngeal aspirate from a ~2 month old baby [Bialasiewicz et al., 2009], and the prevalence of MCPyV DNA in these samples increase from 0.6% in children <15 years (n=340) to 8.5% in adults ≥15 years of age [Goh et al., 2009]. These findings are in agreement with the age-related occurrence of MCPyV antibodies.

9. MCPyV and MCC: Genoprevalence

After the original isolation of MCPyV in MCC, a large number of studies have been performed world-wide to look for viral DNA in MCC. Results from these studies show that approximately 80% of the examined MCC samples contain MCPyV DNA, hence strongly suggesting MCPyV as a candidate etiological agent in the development of MCC (Table 2). Although MCC occurs most commonly in the skin, it is also detected in sun-protected sites such as the oral and nasal mucosa, vulva, and penis. In a case report, MCPyV DNA and LT-ag expression were demonstrated in a patient with nasopharyngeal MCC, both in the primary tumour and in a lymph node metastasis, showing that MCPyV may also be an etiological factor in these types of MCC [Wu et al., 2010]. MCPyV seems to be distributed globally, but the rates of MCPyV DNA positive MCCs in European (80%; n=865), North America (72%; n=254) and Asia (76%; n=58) are higher than in Australia (24%; n=21; **Table 2**). However, only a limited number of cases were examined in Asia and in Australian [Garenski et al., 2009; Katano et al., 2009; Nakajima et al., 2009; Woo et al., 2010]. Studies that tested 10 or more subjects, gave a prevalence that varies between 54-88% for North-America, 64-100% for Europe, 55-79% for Asia, and 24% for Australia.
Recently, an immuno-histochemistry study confirmed that only 19 of 104 (18%) MCC samples from Australian patients had detectable LT-ag expression [Paik et al., 2011]. It is speculated that owing to increased sun exposure in Australia, viral pathogenesis could be a less important cofactor in MCC. Sun exposure may drive MCC through an alternative, virus-independent oncogenic pathway [Garenski et al., 2009; Paik et al., 2011].

N#	prevalence	region	country	reference
10	8 (80%)	LT, VP1	USA	Feng et al., 2008
13	7 (54%)	LT, VP1	USA	Ridd et al., 2008
53	45 (85%)	LT	Germany	Becker et al., 2009
5	2 (40%)	LT	the Netherlands	Wetzels et al., 2009
31	24 (77%)	LT	USA	Carter et al., 2009
17	15 (88%)	LT	USA	Busam et al., 2009
23	17 (74%)	st	USA	Bhatai et al., 2009
9	8 (89%)	LT, VP1	France	Foulongne et al., 2008
50	43 (86%)	LT	Germany	Houben et al., 2010
91	84 (92%)	LT	Germany	Helmbold et al., 2009
14	11 (79%)	LT, VP1	Japan	Nakajima et al., 2009
7	6 (86%)	LT, VP1	USA	Loyo et al., 2010
1	1 (100%)	LT, VP1	Switzerland	Mertz et al., 2010
39	30 (77%)	LT, VP1	Germany	Kassem et al., 2008
34	30 (88%)	LT	Germany	Wieland et al., 2009
5	5 (100%)	LT	Germany	Wieland et al., 2009
1	1 (100%)	LT, VP1	Switzerland	Mertz et al., 2010
114	91 (80%)	LT, VP1	Finland	Sihto et al., 2009
10	7 (70%)	LT, VP2,	USA	Shuda et al., 2009
1	1 (100%)	LT	USA	Duncavage et al., 2009
29	22 (76%)	LT	USA	Duncavage et al., 2009
5	5 (100%)	LT	Finland	Koljonen et al., 2009
16	11 (69%)	LT	North America	Garenski et al., 2009
21	5 (24%)	LT	Australia	Garenski et al., 2009
33	21 (64%)	LT, st	Germany	Andres et al., 2009+2010
11	6 (55%)	LT, st, VP1-3	Japan	Katano et al., 2009
22	13 (59%)	LT	USA	Paulson et al., 2009
27	19 (70%)	LT, VP1	USA	Tolstov et al., 2009
32	21 (66%)	LT, VP, NCCR	France	Touzé et al., 2009
10	10 (100%)	LT, st, VP1	France	Sastre-Garau et al., 2009
7	5 (89%)	LT, VP1	Hungary	Varga et al., 2009
2	2 (100%)	LT1, LT3	USA	Wu et al., 2010
9	8 (89%)	LT1, LT3, VP1	France	Foulongne et al., 2008
33	31 (94%)	LT3, VP1	France	Laude et al., 2010
68	51 (75%)	VP1	France	Touzé et al.,2011
27	19 (70%)	LT	USA	Paulson et al., 2011
32	24 (75%)	LT and VP1	Germany	Werling et al., 2011
9	8 (89%)	LT1,VP1	Italy	Paolini et al., 2011
26	20 (77%)	LT, VP1	Japan	Kuwamoto et al., 2011
87	67 (77%)	LT	Finland	Waltari et al., 2010
44	29 (66%)	LT, VP1	Germany	Handschel et al., 2010
6	4 (67%)	LT	USA	Lewis et al., 2010
13	7 (54%)	LT, VP1	USA	Ridd et al., 2009
7	7 (100%)	LT	South Korea	Woo, 2010
3	2 (67%)	LT	Germany	Schmitt et al., 2011
91	71 (78%)	LT	Finland	Sihto et al., 2011
Σ=1198	**924 (77%)**			

Table 2. Prevalence of MCPyV DNA in MCC.#N=number of patients.

There is a slighter overall incidence of MCC among women than among men, while other studies found the opposite [Kaae et al., 2010; Calder & Smoller, 2010]. Not all studies that have examined the presence of MCPyV DNA in MCC have specified the distribution of positive and negative samples among male and female patients. A compilation of published data reveals that the rate of MCPyV DNA positive MCCs in men is 78% (n=130), which is similar to women (75%; n=83). However, the investigation of only a limited number of patients urges the caution interpretation of the results [Becker et al., 2009; Wetzels et al., 2009; Bhatai et al., 2010; Foulongne et al, 2008; Nakajima et al., 2009; Mertz et al., 2010 a; Sihto et al., 2009; Koljonen et al., 2009; Katano et al., 2009; Paulson et al., 2009; Varga et al., 2009; Wu et al., 2010; Woo et al., 2010]. Only Sihto and colleagues screened a relative large cohort (80 men and 34 women) and they found that 84% of their male MCC patients were positive for MCPyV DNA against 71% for the female MCC patients [Sihto et al., 2009]. A recent study by the same group on a Finnish cohort showed the opposite tendency [Sihto et al., 2011]. They found that the presence of LT-ag was associated with the female gender, but 70% (63/91) of their patients were female.

10. Mechanisms for MCPyV-induced MCC

10.1 Expression of truncated LT-ag

One of the criteria for a causal role of a virus in cancer is the requirement of expression of part of the viral genome to maintain the transformed state of the cell. This was originally proven by studies with Rous sarcoma virus carrying a temperature-sensitive mutation in the v-*src* gene and later confirmed for SV40 in a transgenic mouse model that allowed timely regulated doxycycline-dependent expression of LT-ag [Kawai & Hanafusa; 1971; Friis et al., 1980; Ewald et al., 1996]. At the non-permissive temperature, the mutant Rous sarcoma virus was unable to induce or maintain morphological transformation of chick embryo cells, while cell transformation appeared when cultures were shifted to a permissive temperature [Kawai & Hanafusa; 1971; Friis et al., 1980]. Turning on expression of SV40 LT-ag at birth, induced cellular transformation by 4 months of age. Silencing LT-ag for 3 weeks reversed the hyperplasia state. However, turning off LT-ag 7 months after birth did not abolish the hyperplasia, indicating that LT-ag was no longer required to maintain the transformed state [Ewald et al., 1996]. The observations that MCPyV positive MCC express LT-ag and that MCPyV positive MCC patients have much higher LT-ag seropositivity than non-MCC patients may indicate that the presence of this oncoprotein is involved in MCC [Shuda et al., 2009; Busam et al., 2009; Paulson et al., 2010; Houben et al, 2011; see Table 1]. This assumption is supported by results from knockdown studies in MCPyV positive MCC cell lines and a xeno-transplantation model, which illustrated that continuous expression of LT-ag and st-ag is required for cell proliferation and tumour progression [Houben et al., 2010; Houben et al., 2011].

Unexpectedly, MCPyV positive tumours express truncated LT-ag due to nonsense mutations or frame shift mutations generating premature stop codons (Figure 4; [Feng et al., 2008; Shuda et al., 2009; Duncavage et al., 2009a; Laude et al., 2010]). The mutation does not affect st-ag, but truncated LT-ag and 57kT become indistinguishable. The truncated LT-ag and 57kT proteins retain the DnaJ region and pRb-binding pocket, but lack the origin of replication- and the p53-binding domains, and the domains with helicase and ATPase activity. The fact that truncated LT-ag in MCC still can bind pRb and that loss of pRb is not a frequent occurrence in MCC suggests that inactivation of pRb through LT-ag binding is

essential for MCPyV-mediated MCC tumorigenesis [Houben et al., 2009]. This prompted Houben and co-workers to examine the importance of LT-ag:pRb interaction in MCC. Specific knock down of LT-ag resulted in a marked growth inhibition and accumulation of MCPyC positive MCC cells in G1 phase of the cell cycle. The transformed phenotype was rescued by ectopic expression of LT-ag with an intact pRb motif, but not by LT-ag with a mutated pRb binding site [Houben et al., 2011]. These observations suggest that the LT-ag:pRb interaction is essential for tumour development.

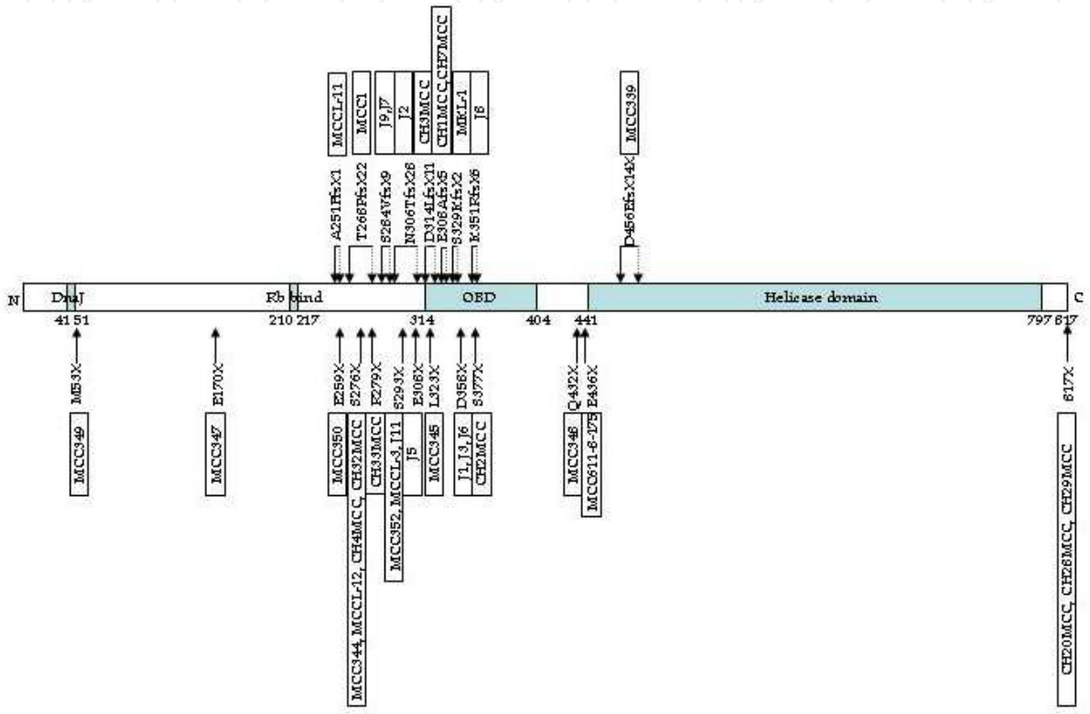


Fig. 4. Mutations causing truncation of LT-ag that have been reported in MCC [Feng et al., 2008; Fisher et al., 2010; Laude et al., 2010; Nakamura et al., 2010; Schrama et al., 2010 and MCC611-8-175 Genbank ACJ05659.1]. The arrows in the lower part of the figure represent the sites where nonsense mutations have occurred. The mutation of the amino acid into the corresponding nonsense codon (indicated by X) is given. The double arrows at the top part of the figure symbolize the site of the mutation that causes the frame shift (first arrow) and the introduction of a stop codon due to this frame shift (second arrow). The X followed by a number indicates how many codons downstream of the frameshift a stopcodon is introduced. The functional DnaJ, pRb binding, origin binding domain (ODB) and helicase domains are indicated and the numbers beneath the domains represent the amino acid residues of the beginning and the end of the respective domains. The name of the reported strain is encompassed in a rectangle.

Skin MCPyV isolates obtained from 14 different healthy subjects encoded full-length open reading frames for predicted viral proteins, including LT-ag [Schowalter et al., 2010]. Thus, in contrast to MCC, normal skin tissue seems to be infected with MCPyV encoding full-length LT-ag. Sequencing a full-length MCPyV genome from a Kaposi's sarcoma case revealed that the LT-ag gene would produce a truncated form of the protein [Katano et al.,

2009]. This example illustrates that expression of truncated LT-ag is not restricted to MCC, but can occur in other malignancies. Whether MCPyV infection is a risk for Kaposi's sarcoma remains elusive.

The truncated LT-ag mutant proteins identified in MCC are deprived of their DNA helicase activity and consequently unable to sustain viral DNA replication [Shuda et al., 2009]. This may prevent virus production and eventually lysis of the infected cells and is thus incompatible with expansion of the virus-infected cell [Houben et al., 2009]. Replication deficient MCPyV may persist in the host cell without causing destruction of the infected cell. The truncated LT-ag, however, retains its oncogenic potentials and this may lead to transformation of the infected cell. This finding is reminiscent with early observations that revealed that replication-deficient SV40 or SV40 encoding LT-ag defective in origin-binding displayed elevated transforming potentials compared to wild-type virus [reviewed in zur Hausen, 2008b]. Furthermore, studies with transgenic mice expressing SV40 LT-ag showed that a C-terminal truncated LT-ag variant that still can bind pRb, but lacks its helicase domain retains its transforming property [Rathi et al., 2009]. Hence, expression of a SV40 truncated LT-ag mutant with similar functions as MCPyV LT-ag variants described in MCC induced hyperplasia and eventually dysplasia. This observation suggests that a similar mechanism may be operational in MCPyV positive MCC.

10.2 Mutations in st-ag in MCC

So far, no mutations in st-ag expressed in MCC have been reporter. One normal skin isolate (9b) had a single base-pair insertion in the C-terminal part of st-ag causing a premature stop codon resulting in truncation of the last 15 amino acids, but the functional implication for this mutation has not been examined [Schowalter et al., 2010]. The corresponding residues in SV40 st-ag are not necessary for the interaction with PP2A, suggesting that this MCPyV st-ag mutant still can interact with PP2A [Mateer et al., 1998]. Interestingly, the transcript levels of st-ag, but not LT-ag or VP1 in the skin of a healthy woman containing episomal MCPyV DNA increased following solar UV-irradiation administration [Mogha et al., 2010]. Because the LT-ag and st-ag transcripts are generated from the same unspliced precursor, a post-transcriptional mechanism may be operational such as at the splicing level to generate different amounts of LT- and st-ag transcripts. Alternatively UV-light may activate pathways that control the decay of LT-ag and st-ag mRNA differently [Bollig et al., 2002].

10.3 Polymorphism in the non-coding control region (NCCR)

Previous studies with the human polyomaviruses BKPyV and JCPyV revealed that variation in the NCCR affects their transforming properties [Moens et al., 1995; Ricciardiello et al., 2001]. Thus, increased promoter activity of MCPyV variants may result in enhanced levels of the oncoproteins LT-ag, 57kT and st-ag or/and stimulate the expression of cellular genes in the vicinity of integrated viral genome. Mutations that increase the intrinsic activity of the MCPyV NCCR may affect the oncogenic potential of the virus and therefore be a hallmark for MCC-derived virus isolates. So far, the genomes of tumour-derived MCPyV and isolates from non MCC patients display little variation in their NCCR [Feng et al., 2008; Touzé et al., 2009; Kwun et al., 2009; Pastrana et al., 2009]. The tumour-derived MCPyV isolate MCC350 contains the 5 base-pair deletion GAGTT in its regulatory region compared to the MCC339 strain [Feng et al., 2008]. This deletion was also detected in MCPyV isolates from 5 out of 7 French MCC patients [Touzé et al., 2009], in the MCVw156 strain and the TKS MCPyV DNA

isolated from a from Kaposi's sarcoma. The latter had a 25 base pair tandem repeat in its NCCR duplicating a motif that stimulates transcription and *in vitro* replication of SV40 and JCPyV [Martin et al., 1985; Katano et al., 2009]. However, an isolate (isolate 16b) obtained from skin swabs of a healthy person contained the same duplication, showing that this feature is not restricted to MCPyV present in malignant tissue [Schowalter et al., 2010]. So far, there is no indication that a specific NCCR is associated with MCPyV in MCC.

10.4 Integrational inactivation

Chromosomal integration is a characteristic of viruses causing cancer in humans [Matsuoka & Jeang, 2007; zur Hausen, 2009; Tsai & Chung, 2010]. Integration may cause disruption of genes, creating viral-cellular fusion proteins or altered expression of cellular proto-oncogenes or tumour suppressor genes. These events might be oncogenic. MCPyV genomes are clonally integrated into the DNA of the primary MCC tumour cells and their metastatic cells, suggesting that integration occurred before clonal expansion [Feng et al., 2008; Duncavage et al., 2009a]. The viral DNA seems to be integrated at different locations within the cellular genome in different MCC tumours and MCC cell lines examined [Feng et., 2008; Sastre-Garau et al., 2009; Laude et al., 2010]. The first report on MCPyV in MCC showed integration on chromosome 3p14.2, in intron 1 of the receptor tyrosine phosphatase type G (*PTPRG*) gene, a gene identified as a putative tumour suppressor gene [Feng et al., 2008]. Table 3 summarizes other identified integration sites. The functional implication in tumorigenesis is not known because no altered gene expression related to MCPyV insertion was monitored [Sastre-Garau et al., 2009]. The virus host junction can occur in VP1, NCCR, and in the 3' part of LT-ag [Feng et al., 2008; Sastre-Garau et al., 2009; Laude et al., 2010].

insertion	putative target gene	link to cancer	reference
2q32.3	not determined		Sastre-Garau et al., 2009
3p14.2	receptor tyrosine phosphatase type G	tumour suppressor	Feng et al., 2008
3q26.33	ATP1-1B		Sastre-Garau et al., 2009
4q13.1	SRD5A2L2		Sastre-Garau et al., 2009
5q35.1	TLX3		Sastre-Garau et al., 2009
6q23.3	IL20RA	lung carcinogenesis	Sastre-Garau et al., 2009
6p24	GDP-mannose 4,6 dehydratase	fucosylation in colon cancer	Laude et al., 2010
8q24.21	MYC	Proto-oncogene	Sastre-Garau et al., 2009
9q33	DENN/MADD domain containing 1A isoform 1		Laude et al., 2010
11p15	Parvin-α,	adhesion and motility	Laude et al., 2010
11p15	TEF-1/TEAD1		Laude et al., 2010
12q23.1	AX747640		Sastre-Garau et al., 2009
15q14	ATP binding domain 4 isoform 2		Laude et al., 2010
20q11.21	SNTA1		Sastre-Garau et al., 2009
Yq12	not determined		Sastre-Garau et al., 2009

Table 3. Chromosomal integration sites of MCPyV.

Several groups reported that MCPyV DNA is not exclusively integrated in MCC, but may also exist episomal. Wetzels and her colleagues could demonstrate the presence of viral particles in one of their MCC samples by electron microscopy, suggesting non-integrated viral DNA. Wetzels did not examine whether integrated viral DNA was present in the MCC sample that contains virus particles [Wetzels et al., 2009]. Sastre-Garau and co-workers characterized one MCC case where the tumour cells contained both integrated and episomal MCPyV DNA [Sastre-Garau et al., 2009]. Laude and co-workers also found MCC containing MCPyV encoding full-length LT-ag. They reported the coexistence of integrated concatemers or latent episomes of MCPyV genome and truncated integrated viral sequences in five MCC cases [Laude et al., 2010]. The presence of virions indicates active viral replication and therefore a LT-ag that can sustain viral DNA replication, i.e. LT-ag that possesses helicase activity. Because integration and truncated LT-ag expression are hallmarks of MCPyV-positive MCC, it is possible that in MCC containing only episomal viral DNA, that the virus was merely a fortuitous co-inhabitant of the tumour cells.

10.5 MyCPyV and cellular miRNA expression

Viral infections can cause altered expression patterns of miRNA in the host cell because viral-encoded miRNA or/and viral proteins can target cellular miRNA/targetome pathways, thereby contributing to the oncogenic properties of viruses [Skalsky & Cullen, 2010; Lin & Flemington, 2011]. MiRNA are generated by RNA polymerase II and HPyV LT-ag and st-ag have been shown to affect the expression of many genes, including RNA polymerase II transcribed genes [Moens et al., 1997; Klein et al., 2005; Deeb et al., 2007]. It is not known whether MCPyV infection or expression of its LT-ag or/and st-ag results in altered expression of cellular miRNA, but SV40 st-ag enhances expression of miR-27a in a PP2A-dependent manner and contributes to the malignant transformation of human bronchial epithelial cells. The transcript encoding the cell-cycle progression protein Fbxw7 was a target for miR-27a. Thus, promotion of cell growth through the suppression of Fbxw7 by st-ag-induced miR-27a overexpression may play a role in malignant transformation [Wang et al., 2011]. Profiling miRNA in MCPyV positive and negative MCC may indicate whether altered cellular miRNA expression is a co-mechanism in MCC pathogenesis.

11. Therapy

Currently, there is no specific treatment for MCC. Local excision of the tumour combined with radiation therapy and chemotherapy is the most common protocol [Tai, 2008; Ramahi et al., 2011]. Because of the high association rate of MCPyV with MCC, antiviral-specific therapy may form an attractive therapy for MCC. Although a few clinical trials with MCC were started or are in progress [ClinicalTrials.gov], none of them are specifically aimed at targeting MCPyV. Vaccination is an encouraging possibility in light of the success with the oncoviruses such as hepatitis B virus and high-risk human papillomaviruses. To begin to explore the idea of a MCPyV vaccine, Pastrana and her collaborators immunized mice with virus-like particles (VLP) composed of MCPyV VP1 and VP2. All vaccinated animals generated strong neutralizing antibodies. In fact the titres were comparable to the titres of animals challenged with human papillomavirus VLP and higher than animals injected with JCPyV VLP. Thus, MCPyV VLP appears to be a strong immunogen and thus a promising vaccine candidate [Pastrana et al., 2009]. One case report describes that subcutaneous administration of 3,000,000 IU of interferon-β (IFN-β) per day to a Japanese 62-year-old

woman with MCPyV positive MCC resulted into a beneficial response after 3 weeks with complete remission that continued for more than 8 years [Nakajima et al., 2009]. IFN-α could induce apoptosis of MCC-1 cell lines *in vitro*, but its effect on patients was not tested [Krasagakis et al., 2008]. These results suggest that interferons may be useful in MCC therapy. Other feasible approaches are disruptor molecules, antiviral drugs and RNA interference. Continuous expression of LT-ag with an intact pRb binding site is required to maintain the transformed phenotype of MCC. Molecules disrupting pRb:LT-ag interactions may possess low off-target effects and therefore be an attractive strategy for future MCC therapy [Houben et al., 2011]. Similarly, disruptors of the Dna J domain may be applied. Antiviral drugs that interfere with the different stages of the life cycle of MCPyV may prevent virus spread after primary infection, but no such drugs are currently available and it is very unrealistic to establish the time point of primary infection in individuals. SiRNA-mediated knock down of MCPyV LT-ag expression may be another therapeutic approach. Eliminating LT-ag expression in MCCs may therefore be sufficient to reverse the malignant cell to a more benign cell or trigger cell death as demonstrated in MCC cell lines [Houben et al., 2010]. It was shown that JCPyV- and BKPyV-encoded miRNA target ULBP3, a ligand for the natural killer cell receptor NKG2D, thereby protecting BKPyV/JCPyV infected cells from destruction [Bauman et al., 2011]. It is not known whether MCPyV miRNA exerts the same function, but if so, anti-miRNA against the MCPyV-encoded miRNA will maintain the ULBP3 expression levels in viral infected cells and will stimulate their elimination by natural killer cells.

12. Conclusions and future perspectives

Several findings underscore a causal role for MCPyV in MCC. The viral genome load and prevalence of MCPyV DNA are significantly higher in MCC than in other MCPyV infected tissues, in contrast to non-MCC tissue, the viral genome in MCC is integration and expresses truncated LT-ag that cannot sustain viral replication, but still interacts with pRb. In accordance with other tumour viruses, there seems to be a very long incubation time between viral infection and development of the tumour because primary infection can occur in early childhood, but MCC is extremely unusual before the age of 50. These observations suggest that additional events (e.g. skin exposure to UV-light, gender, perturbation of the immune system, co-infection other viruses, ethnicity, mutations in cellular genome) are required for MCC-induced malignancy. The absence of MCPyV in about 20% of MCC indicates the existence of MCPyV-independent pathogenic pathways in this malignancy. This is supported by the finding that MCPyV seems to be associated with classic, but not variant MCC cell lines and that MCC display chromosomal abnormalities with loss of heterozygocity of chromosome 1p being the most common [Shuda et al., 2008; Leonard et al., 2000]. Alternatively, MCPyV may initiate neoplastic events, but the continuous presence of the virus is not required explaining why no traces of the virus can be detected in some of the MCC ('hit-and-run' hypothesis). The fact that MCPyV DNA is detected in many different tissues both in healthy individuals and in different patient groups raises the question whether MCPyV may be involved in other malignancies or diseases. The connection of MCPyV DNA in other tissues is not as strong as MCPyV and MCC and the state of the viral genome (episomal or integrated) and the mutational analysis of LT-ag have not been meticulously examined [reviewed in Moens et al., 2010]. World-wide studies are warranted to determine the possible association of MCPyV with other malignant and non-

malignant diseases. Other important information that should be acquired includes determining the integration sites and the consequences on viral and cellular gene expression, and does miRNA expression pattern in MCPyV positive versus MCPyV negative MCC differ and if so, are these differences provoked by the virus and are they contributing to MCC pathogenesis? Besides these cancer-related issues, other fundamental issues such as route of infection, cell tropism and transmission need to be studied.

13. References

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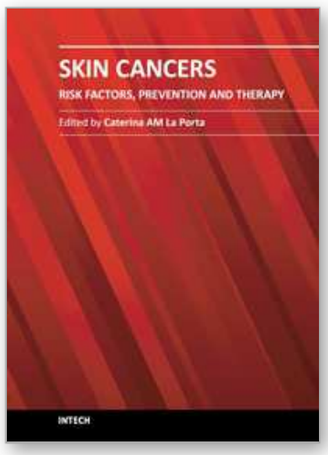
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Skin cancers are the fastest growing type of cancer in the United States and represent the most commonly diagnosed malignancy, surpassing lung, breast, colorectal and prostate cancer. In Europe, the British Isles have been the highest rates of skin cancer in children and adolescents. The overall idea of this book is to provide the reader with up to date information on the possible tools to use for prevention, diagnosis and treatment of skin cancer. Three main issues are discussed: risk factors, new diagnostic tools for prevention and strategies for prevention and treatment of skin cancer using natural compounds or nano-particle drug delivery and photodynamic therapy.

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