- The role of polyomaviruses WU and KI in human disease and tumour development -

G. Mijnheer

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by

G. (Gerdien) Mijnheer
Oudwijkerdwarsstraat 88
3581 LH Utrecht

(s) 0470880
(m) 06 2344 3497
(e) g.mijnheer@students.uu.nl

University of Utrecht
Graduate School of Life Sciences
MSc Biomedical Sciences
Infection and Immunity

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Supervisors:

Dr. F.E. J. (Frank) Coenjaerts || *Microbiology department, University Medical Centre Utrecht*

Dr. M.E. (Maaike) Ressing || *Microbiology department, University Medical Centre Utrecht*

About the cover:
The cover shows a cartoon of 'innocent until proven guilty' (derived from www.cartoonstock.com), to illustrate the question whether the newly discovered polyomaviruses WU and KI are involved in human disease.

Abstract

After the shocking discovery of SV40 as being a polyomavirus (PyV) capable of transforming human cells more than three decades ago, the recent discovery of four human PyV WU, KI, Merkel cell carcinoma virus (MCV) and trichodysplasia spinulosa-associated polyomavirus (TSV) have regained interest in this family of PyV. Especially their (possible) role in human disease is a current and interesting research theme.

An important characteristic of all PyV is that they initially cause asymptomatic infections, but are able to cause disease particularly in immunosuppressed patients. Insight into reactivation of latent viruses or of normally 'benign' persistent viruses in immunocompromised patients has become increasingly important in de field of organ transplantations and for HIV infected patients. This rising problem and especially the discovery of three new human PyV set these viruses in a scientific spotlight. Moreover, the World Health Organization (WHO) estimated that about 20% of all cancers worldwide are associated with chronic infections. Particularly, up to 15% of human cancers are marked by a viral aetiology.

The challenges are now to characterize the prevalence, disease associations, and pathophysiology for the new viruses. Because these viruses are recent discoveries, comprehensive information is missing. For none of the four new PyV an infectious virus has been isolated. However, general features already become apparent. For WU and KI, there are few indications regarding their possible pathogenic potential. In case of MCV molecular mechanisms underlying the oncogenesis are recently uncovered.

Here we try to find mechanisms by which the newly discovered PyV WU, KI and MCV can cause disease by comparing them to other well known and disease-causing viruses such as BK and JC. Moreover, we provide an overview of the recent knowledge of hPyV with focus on the biology of WI, KU and MCV.

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1 Introduction: polyomaviruses

The polyomaviruses (PyV) are non-enveloped DNA viruses with icosahedral capsids containing small, circular, double-stranded DNA genomes, which encode 3 structural (late) proteins and 2 or 3 early proteins. Up till now, the genus contains 6 human and 16 non-human known polyomavirus species[1]. Mouse polyomavirus (mPyV) was the first polyomavirus to be discovered almost 60 years ago by Gross, Stewart and Eddy[2, 3]. The focus of these studies was on the transmission of mouse leukemias, however Ludwig Gross noticed that newborn mice inoculated with cell free extracts from leukaemia's not only developed leukaemia but also developed parotid tumours. Isolation of this specific agent was achieved by filtration; because of the smaller size of the ''parotid'' agent it could easily be distinguished from the bigger leukaemia agent by filters with a small pore size. The characteristic of the parotid agent to be capable of inducing many tumours resulted in the name polyoma which is Greek for many tumours (poly oma)[3]. A few years later simian vacuolating virus 40 (SV40) was discovered[3, 4].

SV40

Sweet and Hilleman, the discoverers of SV40, observed that an agent isolated from normal monkey kidney cells induced cytopathic effects and vacuole formation in these cells[3, 4]. At that time, the same monkey kidney cells were used to prepare Sabin poliovirus vaccine. The subsequent observation that the isolated SV40 virus was capable of transforming non-simian cells *in vitro*[5] and of inducing tumours in a hamster used as experimental animal had major consequences[6]. This discovery pointed out that an estimated 100 million people were likely exposed to a potential oncogenic virus by the inoculation with a contaminated vaccine[7]. Because of its unintentional presence in poliovirus vaccines SV40 is extensively studied in the decades after this alarming discovery. Human fibroblast cell lines were first used to further study the biology of SV40 in human cells[8]. This revealed some characteristics of polyomavirus behaviour in human cells: the cultured cells infected with SV40 have a low but constant virus output and undergo a short crisis period where after a small number of cells recover and grow out. These cells have a typical transformed phenotype[9]. After the crisis the virus production generally decreases along with the viral capsid protein production, but an increase in the production of the early gene product large T antigen (LT) is observed. LT is the most well known protein encoded on the SV40 (and other PyV) genome. This viral protein plays a dominant role in infection by regulating viral early and late gene transcription, acting as a initiation factor for viral DNA replication and maximizing virus production by the dysregulation of the cell cycle and the prevention cell apoptosis (for a detailed explanation of LT function see chapter 4)[10]. Finally, the human cell culture becomes overgrown with cells expressing a high level of LT, while other cells die reaching their passage limit[11]. The immortalization of cell lines and the display of oncogenic transformation is a characteristic of infection of PyV in non-permissive cells, in which viral DNA replication is not supported resulting in an abortive infection or cell transformation, related to the *in vivo* situation of oncogenesis. Permissive cells, however, support viral DNA replication, which leads to a lytic infection and viral amplification (thereby killing the host cell). Most human cells appeared to be semi-permissive with respect to SV40 infection because DNA replication and virus amplification is only partial effective and cell transformation also occurs[12].

Human polyomaviruses

Next to mPyV and SV40, a large number of additional members of this virus family were found in many other mammalian species and in birds (reviewed in Krumbholz et al. 2009[1]). In 1971, the first two human PyV were discovered: BK virus (BKV) and JC virus (JCV), named after the initials of the patients in which these viruses were found[13, 14]. BKV was isolated from the urine of a kidney transplant recipient who suffered from ureteral stenosis, whereas JCV was cultivated from the brain tissue from an immunocompromised patient with Hodgkin's lymphoma who presented progressive multifocal leukoencephalopathy (PML). Both viruses are widely spread within the human population (up to 98% seropositivity)[15]. The primary subclinical infection occurs in early childhood and normally leads to lifelong persistence, primarily in the cells of the kidney, urinary tract and central nervous system[16-19]. However, immunocompromised patients may allow reactivation of the virus from a persistent to a lytic infection resulting in virus spreading, potentially leading to severe or fatal disease. For BKV, reactivation is most common in bone marrow transplant and kidney transplant patients where the lytic infection results in hemorrhagic cystitis and polyomavirus nephropathy (a form of tubulointerstitial nephritis), respectively[20, 21]. JCV reactivation is mainly induced by immunosuppression caused by AIDS, where the virus causes PML as a result of lytic infection of oliogodendrocytes in the brain[22-24]. Although both BKV and JCV have the capability to transform human and animal cells in culture and to induce tumour formation when injected in experimental animal models, there is no certainty about their involvement in human cancers[12, 25].

WU, KI and MCV

Three human PyV have been discovered in the past few years: KI, WU and MCV[26-28]. KI and WU are identified by screening human respiratory secretions using high throughput sequencing technologies, whereas viral DNA derived from MCV was obtained by a completely different approach, namely digital transcriptome substraction of RNA from Merkel cell carcinomas (MCC). So from the moment of the discovery of MCV, the virus is associated with this relatively rare but aggressive human skin cancer of neuroendocrine origin[25]. In fact, the observation that the incidence of MCC is enhanced by immunosuppression was reason to look for a viral origin. Very recently a fourth human polyomavirus was discovered, also associated with a disease that appears under immunosuppression: trichodysplasia spinulosa-associated polyomavirus (TSV)[29]. The observation that both MCV and TSV only cause disease in immunocompromised patients indicates that these viruses circulate within the human population and that disease occurs due to unrestricted virus and host cell proliferation.

Not much is known about the pathogenic potential of KI and WU. Since WU and KI were detected in the respiratory tract samples of patients with respiratory tract infections, it has been thought that these viruses are involved in respiratory diseases. A recent study investigated the presence of WU in paediatric patients and reports a higher prevalence of WU in patients with acute respiratory tract infections compared to patients without[30]. However, other reports do not support the initial suspicion that these infections might be linked to respiratory tract disease[31-33].

Viral DNA detection and multiple serological tests show that both WU and KI are widespread within humans (reviewed in Dalianis et al. 2009[34]). Overall, the prevalence of WU has been generally higher than that of KI. Both viruses are mainly found in children with a peak of KI at 1-2 years, and for WU the peak is between 5

and 24 years of age. It seems that there is a subsequent decline in prevalence at adult age for both viruses[32, 35, 36]. Thus far, no link has been found between infections of WU and KI and human benign or malignant proliferations[25].

Aim

An important characteristic of PyV is that they initially cause asymptomatic infections, but are able to cause disease particularly in immunosuppressed patients. Insight into reactivation of latent viruses or of normally 'benign' persistent viruses in immunocompromised patients has become increasingly important in de field of organ transplantations and for HIV infected patients. This rising problem and especially the discovery of three new human PyV set these viruses in a scientific spotlight. Moreover, the World Health Organization (WHO) estimated that about 20% of all cancers worldwide are associated with chronic infections. Particularly, up to 15% of human cancers are marked by a viral aetiology[37].

The challenges are now to characterize the prevalence, disease associations, and pathophysiology for the new viruses. Because these viruses are recent discoveries, comprehensive information is missing. However, general features already become apparent and others can be anticipated by looking at the other well studied PyV.

Here we try to find mechanisms by which the newly discovered PyV WU, KI and MCV can cause disease, by reviewing the available knowledge of the well known PyV and the preliminary knowledge of the new PyV.

2 The viral life cycle: attachment and entry of polyomaviruses

Although some polyomaviruses like SV40 are extensively studied over the last decades, the complete life cycle of SV40 and other polyomaviruses in humans is poorly understood[10]. Some general aspects are known, but especially in case of the newly discovered MCV, WU and KI viruses a lot of research has to be done to gain insight into their infectivity and pathogenicity *in vivo*. Here some general aspects of PyV and new insight of MCV, KI and WU are combined to get a picture of the life cycle of these viruses.

Transmission and tissue tropism

Little is known about the transmission, tissue tropism and sites of latency of WU and KI. As mentioned above, KI and WU are detected predominantly in respiratory tract samples, suggesting that the respiratory tract may be the primary site for infection and latency by these viruses[38]. The report that KI and WU are both shown to be present in human tonsils, supports this hypothesis[39]. In immunocompromised patients, re-activation of WU and KI causes excretions of the virus in the stool of these patients which may reflect an oral-fecal transmission route[40]. The etiological role of WU and KI in respiratory disease remains debatable[31], but it could be that the primary infection occurs asymptomatic.

Although there is much uncertainty about the infection route of WU and KI, when comparing these viruses with the strongly related (see chapter 4) JCV and BKV there are some important distinctions. Whereas WU and KI are mainly detected in respiratory samples of children, BKV and JCV are much less common in paediatric samples. Moreover, BKV and JCV are detected in blood and urine but WU and KI have not been found in these body fluids[26, 27, 38, 41].

MCV is also found in respiratory tract samples but mainly in adults, in contrast to WU and KI[42, 43]. There are indications that MCV might persist in tonsils[42] and has an oral-fecal transmission route[44]. Besides this, the widespread occurrence of MCV in many tissues, even in non-tumoral tissues, might suggest that transmission happens through skin contact or saliva[45]. However, it appears that only neuroendocrine skin cells are susceptible to transformation by MCV[44].

The restricted specificity of the PyV is reflected in the few species and cell types that allow a lytic infection. In contrast, transformation often can occur in many hosts and cell types[46]. Important determinants for this discrepancy are the presence of certain host cell specific proteins like transcription and replication factors. For example, JCV growth in tissue culture is restricted largely to primary human fetal glial cells. The expression of JCV early mRNA depends on recognition of the early enhancer/promoter elements by tissue-specific factors found in both human and rodent glial cells. Furthermore, in the presence of JCV LT, viral DNA replication requires a species-specific factor, presumably a component of DNA polymerase which is found only in primate cells[47, 48]. Hence, the interaction of the LT with the viral origin and the host cell machinery efficiently contributes to the restricted (lysis) behaviour of the virus[49, 50] (regulation of gene expression is extensively reviewed in White et al. 2009[48]). Next to the presence of these specific host cell proteins, the virus-receptor interactions are of major importance for the restricted specificity of PyV[46, 51] (e.g. capsid/VP1 structure and sialic acid residue interactions; described in more detail in 'receptor binding and endocytosis' in this chapter).

Virus structure

The polyomavirus capsid consists of 72 capsomeres, which are composed of 360 VP1 molecules. VP1 is the major capsid protein, with each capsomere being comprised of a pentameric VP1 structure. The capsomeres together assemble into a T = 7d icosahedral capsid[52]. This capsid symmetry allows the virus to have an appropriate enclosure for its DNA build from identical subunits, thereby eliminating the need to encode many different structural proteins. However, there are other capsid protein; VP2 and VP3. These proteins are not present on the outside of the capsid, but VP2 is associated with VP1 probably to stabilize the capsid structure. The function of VP3, a much smaller molecule compared to VP2 and VP1, is unknown but it might also be to stabilize the capsid[53]. The viral genome, encoding structural proteins and 2 or 3 early proteins, is enclosed inside the capsid. The DNA binds to the four cellular core histones to form a minichromosome, which resembles host chromatine[54]. Because VP1 is the only protein present on the outside of the virion, this viral protein is a strong determinant for interactions with the host cell.

Receptor binding and endocytosis

The infection of cells by polyomaviruses is initiated by the binding of the virion to a receptor on the outside of the cell membrane. Like many other viruses the PyV use various and often distinct receptors and entry mechanisms to infect their target cells. When the viral load is high enough, many of these viruses can infect cells using alternative and often poorly described mechanisms[46]. For the PyV that are known for many years, like mPyV, SV40, JC and BK, some general aspects are known about their receptors and entry mechanisms. In general, gangliosides act as receptors for PyV. These are glycolipids with a ceramide moiety spanning the cell membrane and, on the outside of the cell membrane, one or more sialic acids are linked to a sugar chain. PyV can bind to the sialic acids residues to initiate infection[46, 55]. Gangliosides are highly concentrated in lipid rafts, and participate in signal transduction, two properties that are important during polyomavirus entry into cells[53]. Each PyV has one or more types of gangliosides that are decisive for successful infection of specific PyV species (reviewed in [46, 55]). Besides the glycolipids, some other receptors are involved in infection of PyV, like MHC class I molecules for SV40[56] and α4β1 integrin for mPyV[57, 58].

Not much is known about the glycolipid receptors for WU and KI, but for MCV there are indications that ganglioside GT1b can interact with MCV particles[59].

After binding to the receptor, polyomavirus capsids undergo endocytosis. SV40, mPyV and BKV mainly use caveolae-mediated endocytosis to enter the host cell, whereas JCV behaves differently than any of the described PyV and utilizes clathrin-dependent mechanisms to infect the cell. The endocytic pathway is determined by receptor usage of the virus. For example, caveolae are membrane microdomains rich in sphingolipids and signaling molecules and SV40 exploits these domains most likely by its interaction with the ganglioside GM1. This causes the activation of signaling pathways necessary for caveolin-dependent endocytosis[46, 55] (for more detailed information about caveolae see Parton et al. 2007[60]). The endocytosed viral particles can be viewed by electron microscopy (EM), and occasionally crystalloid structures are observed resembling a cluster of PyV[61] (described in chapter 3, page 12 'Characteristics of release and spreading of polyomaviruses).

Next to glycolipids, glycoproteins can also contain sialic acid residue(s) on their surface, so PyV can also bind to these receptors. However, this binding results in internalization and degradation of the virus particles, so sialic acid residues bound to glycoproteins act as decoy receptors[62].

The VP1 protein of the viral capsid plays an essential role in the entry of all polyomaviruses because it is responsible for the binding to sialic acids. Although the receptors for WU and KI are not known, aligning the sequences of the VP1 proteins of PyV provides some information. When comparing the VP1 sequences of the PyV, the viruses can be separated into three classes: BKV–JCV–SV40, KIV–WUV and MCV. Strikingly, KI, WU and MCV are much less closely related to each other regarding VP1 than are the older known viruses[63].

Intracellular trafficking and nuclear delivery

There are small differences in internalization pathways used by all PyV (reviewed in Sapp et al. 2009[55], Neu et al. 2009[46], and Tsai et al. 2010[64]). However, the common aspect is the cross-talk between internalization pathways (caveolin- and clathrin-dependent endocytosis)[65, 66]. SV40 and BKV for example, are trafficking from caveosomes to caveolin-free vesicles which are transported in a microtubule-dependent manner to the endoplasmic reticulum (ER)[67, 68]. Most likely, the virus is bound to its ganglioside receptor throughout the transfer from the plasma membrane to the ER[69]. After clathrin-dependent endocytosis, JCV is also sorted to caveosomes via early endosomes[70-72]. From here, JCV is transported to the ER as well[66] (Fig. 1). Notably, the internalization mode of PyV is very similar to the intoxication pathway of the AB(5) bacterial toxins, such as cholera toxin, whose known receptors are gangliosides. So PyV and bacterial toxins hijack similar cellular pathways during infection[46, 64].

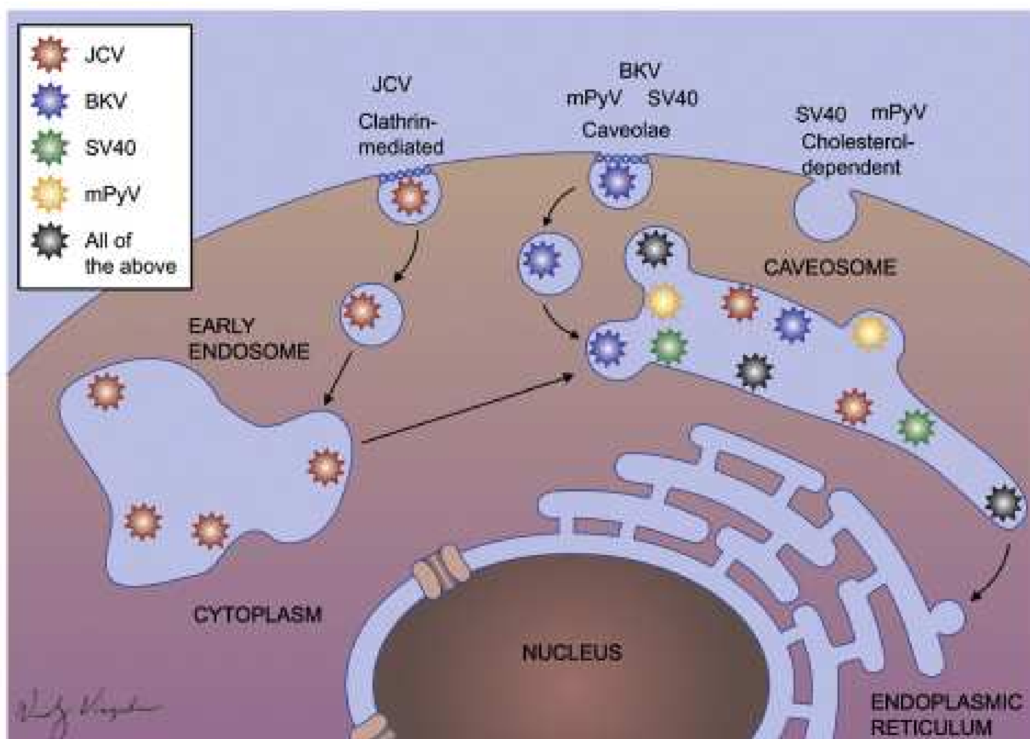

Figure 1. General model of infectious entry pathways utilized by polyomaviruses.
'JCV is unique in that it initially enters cells by clathrin dependent endocytosis. The virus then traffics to early endosomes and then to caveosomes. BKV, SV40, and mPyV are reported to use caveolae dependent mechanisms of entry that traffic these viruses to caveosomes. SV40 and mPyV can also use non-caveolar but cholesterol dependent mechanisms of entry to access the caveosome and the mechanism used depends on cell type. Once in the caveosome SV40 and mPyV traffic to the endoplasmic reticulum in tubular structures that bud off the ER membrane. It is presumed that JCV and BKV use the same pathway but data on this is lacking.'
Adapted from Neu et al. 2009[46]

It is now appreciated that most if not all polyomaviruses are trafficking to the ER where uncoating occurs or at least begins. This process has been studies extensively in case of SV40 and mPyV. ER associated factors that recognize the virion play a critical role in probably all PyV disassembly and release from the ER[73]. One example of such an ER protein is a member of the protein disulfide isomerase family, ERp29[74]. This protein is responsible for inducing a conformational change in mPyV VP1 which results in destabilisation of the virus particle by causing a dissociaton of the capsid proteins VP1, VP2 and VP3. Whereas VP1 contains the major determinants for cell attachment and entry, VP2 and VP3 (sharing a DNA binding domain, a nuclear localization signal, and a VP1-interacting domain) contribute to the escape out of the ER by interaction with VP1 and mediate nuclear entry[75-78]. Essentially, the destabilized virion is recognized as a misfolded protein and is translocated out of the ER. The way the destabilized virion enters the nucleus is not completely clear. One plausible explanation would be that in the cytosol the virion encounters a low calcium environment which contributes to further destabilization of the virion. Exposure of the nuclear localization signals on VP2 and VP3 proteins, bound to the mini-chromosome, then transport the mini-chromosome across the nuclear pore[46, 78, 79].

Genome integration

When the viral DNA is delivered into the nucleus to get access to the host cell's DNA machinery, it can become integrated into the chromosomal DNA of the cell or stay present in the nucleus in an episomal form[80]. This occurs primarily upon nonpermissive infection. For SV40, genome integration has been described[81], but this is also the case for human PyV like JCV[82], BKV[83] and MCV[28]. Remarkably, transformation of cells and tumour development are usually preceded by integration of the viral DNA into the chromosomal DNA of the host cell[1, 25, 84]. When comparing between individual MCC patients the tumours show distinct integration patterns so, at least for MCV, integration occurs at random in terms of the site in the host genome. Also the point in the viral DNA where recombination occurs is randomly chosen[12, 28, 85]. This non-specific integration pattern implicates that the insertion of the viral genome itself is not an oncogenic event. Up till now, genome integration of WU or KI has not been described.

3 The viral life cycle: assembly and release of polyomaviruses

There are roughly three outcomes when a cell is infected with a polyomavirus: latency, host-cell lysis or host cell transformation. The first two mainly occur in permissive cells whereas the latter can occur in non-permissive cells. Here the late stages of the infection cycle in permissive and non-permissive cells is generally described, and in the next chapter the details about host cell transformation are described.

Assembly and release

The initial event when viral DNA arrives in the nucleus is early and late gene transcription followed by viral DNA replication. The genomes of PyV can be divided into three regions: a non-coding control region (NCCR), an early and a late region (Fig. 2). The NCCR contains the viral promoters and origin of replication, the early region encodes the LT and the small t antigen (st), and the late region encodes the structural proteins together with the agnoprotein (except for WU, KI and MCV[26-28]) and VP4 (only in case of SV40, not shown in figure 2). It is generally understood that PyV, like most small dsDNA viruses, assembles the progeny within the nucleus[86, 87].

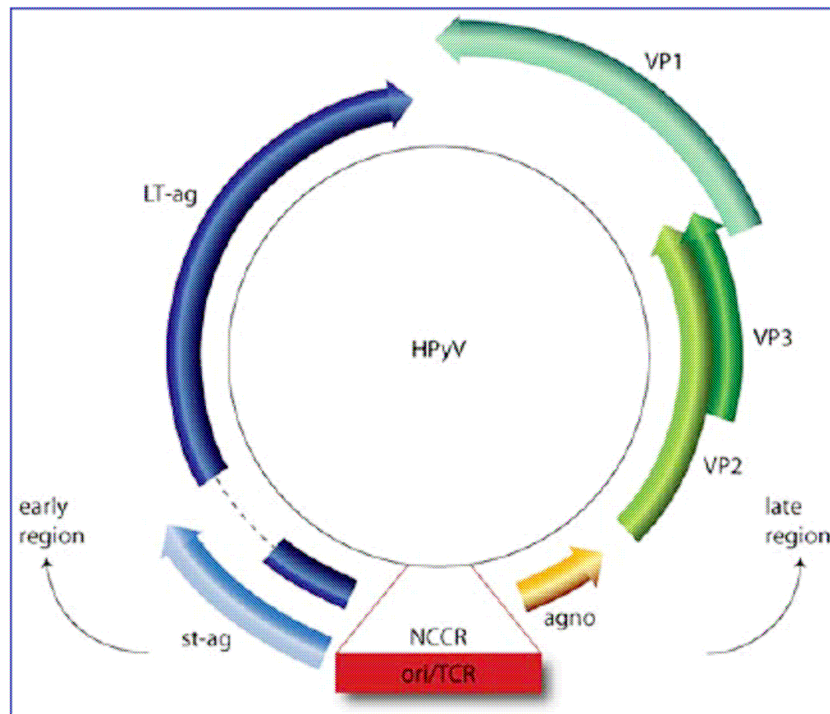

Figure 2. Schematic presentation showing the genome organization of human PyV.
The gene products encoded by the early region (LT and st) and the late region (agnoprotein, capsid proteins VP1, VP2, and VP3) are indicated. KI, MCV, and WU lack an open reading frame corresponding to the agnoprotein. The non-coding control region (NCCR), consisting of the origin of replication (ori) and the transcription control region (TCR), is interspersed between the early and late region. The NCCR controls viral DNA replication and transcription of the early and late genes. *Derived from Moens et al. 2008*[109]

The agnoprotein facilitates the assembly of virus particles and the release out of the nucleus[88-90] (molecular mechanism reviewed in [91] and[92]). PyV leave their host cells by cell lysis, which involves breakdown of the cell membrane and is associated with cell death. Here, the agnoprotein acts as a viroporin, regulating the membrane permeability and cell lysis, thereby promoting the release of progeny virions[90]. Next to the agnoprotein, VP4 is also implicated in host cell lysis[93].

Both the agnoprotein and VP4 are dispensable for assembly and release to occur. Since WU, KI and MCV neither have the agnoprotein and VP4, there must be other mechanisms by which PyV progeny is assembled and released. The structural proteins of PyV are very likely the major determinants for appropriate caspid formation. VP1 has a DNA-binding domain which plays a major role in particle assembly[94]. Using site-directed mutagenesis it was found that VP3 but not VP2 is involved in SV40 capsid formation[95]. However, other studies showed that VP2 does have a role, it promotes VP1 assembly into particles[96] and the myristylation site on Vp2 is essential for the viral life cycle, including the proper packaging of the viral genome[97].
Alternative ways by which WU, KI and MCV are released from the host cell are not discovered yet. During primary infection, however, not all cells die because of virus-induced cell lysis. Primary infection is almost always followed by a subclinical lifelong persistence, so the virus can stay dormant within the host cell for many years.

Characteristics of release and spreading of polyomaviruses

When a cell is infected with a PyV some general features regarding the histopathologcial aspects become apparent. The discovery of the most recent PyV, TSV[29], was based on EM pictures of a trichodysplasia lesion[25, 98]. This picture showed PyV-like particles between 30 and 50 nanometers in diameter in a typical crystalloid structure, which most likely represents the densely packed progeny virions surrounded by chromatin within the nucleus. Using light microscopy, this phenomenon is observed as intranuclear viral inclusion bodies[61]. This is the final phase of intranuclear replication and hereafter host cell lysis will occur. All steps of the viral life cycle of BKV are followed and shown using EM[87].
In case of BKV, viral activation, potentially after latency, is marked by the presence of decoy cells in urine. These are intranuclear viral inclusion bearing cells, present in the urine, that are easily detectable by light microscopy and thus provide a measurable clinical indication. Another often observed cytopathic change is that cells with viral-induced changes are enlarged and have polymorphic nuclei. The shedding of decoy cells or free viral particles does not provide a diagnostic confirmation for BKV activation so other approaches are necessary. More clarity can be obtained by EM pictures of the urine samples to search for ‘Haufen’, three-dimensional viral aggregates, also typical for BKV infection. Moreover, biopsy sample containing both pieces of the cortex and medulla of the kidney can be analysed with microscopy for intranuclear viral inclusion bodies and virally induced cell injury, but ruling out a viral infection of other viruses than PyV can only be achieved by immunohistochemistry. An antibody that detects the LT is used to confirm that the intranuclear viral inclusion bodies are indeed caused by infection by a PyV. However, the LT antigen is only abundantly expressed in the early stages of intranuclear viral replication, so some of the cells representing late phases of the viral life cycle can be LT negative. On the other hand, LT expression can precede the formation of intranuclear viral inclusion bodies when the viral life cycle is in its first stage. Consequently, a positive staining may be detected in histologically normal nuclei[61, 99-101].
For JCV, it is much more difficult to reach the affected tissue, the brain. After reactivation, JCV very likely replicates in promyelocytic leukemia nuclear bodies (PML-NBs) within oligodendrocytes, which are structures with important nuclear functions such as cell cycle regulation, DNA replication and repair. Although JCV can infect many cells throughout the body, only an efficient lytic infection is established in the oliogodendrocytes. Cell death probably occurs because of disruption or dysfunction of PML-NBs, like observed in other human

diseases (e.g. cancers and neurodegenerative disorders). Lysis leads to the destruction of myelin sheaths. Also for JCV, pathological examination of a biopsy or autopsy sample is required for a final diagnosis[102].
For both JCV and BKV infection, PCR techniques to demonstrate viral DNA can be used in the diagnosis as well but a drawback of this method is the high degree of uncertainty about the outcomes, so no clear conclusions can be drawn[100, 102]. Furthermore, according to a small group of researchers the presence of viral RNA indicates active viral replication[100, 103]. This is not generally assumed, however, and PCR techniques to detect RNA are not yet fully established.

4 Pathological and oncogenic potential of polyomaviruses

SV40 as an oncogenic virus

When a PyV infects a non-permissive cell this will result in an abortive viral replication cycle, which eventually leads to cell transformation. There are complex molecular mechanisms underlying this process, but basically it involves 1) the integration of the viral DNA into the host genome to ensure a stable and continues viral replication and 2) the action of viral proteins like LT. SV40 is most extensively studied and the oncogenic mechanisms of this virus are well known. The early region encoding viral protein LT plays a major role in successful viral infection of permissive cells but moreover, it has a dominant role in the oncogenic transformation of (non-permissive) cells. LT is a multifunctional protein which interferes with key cellular targets involved in cell cycle regulation and apoptosis (reviewed in [12, 104, 105]). It is essential for viral DNA replication because of its helicase function, so LT can unwind the DNA and recruit the required proteins for DNA synthesis. The two main targets of LT are the tumor suppressors retinoblastoma (Rb) and p53. By binding to Rb, the inhibition of E2F family of transcription factors is released and consequently this allows the cells to enter the S phase where host DNA replication proteins are produced.

Mutations of the p53 gene are associated with a broad range of cancers. LT also hijacks this cell regulation protein by binding and thereby inactivating it so p53 cannot cause apoptosis or inhibit the cell cycle progression. Furthermore, LT can interfere with signalling pathways such as the insulin-growht factor type I pathway resulting in more cell activation[1, 12]. It has been shown that expression of LT alone is already sufficient to transform a variety of cells.

Next to LT, st also has transforming capacities: it binds protein phosphatase 2A, resulting in activation of cell growth signal transduction pathways [106]. Also, the late region encoding agnoprotein has several strategies to promote transformation and some are similar to those used by LT. Agnoprotein can also bind p53, for example, and influences the cell cycle[107]. Furthermore, there is a growing body of evidence for a role of microRNAs in transforming cells by regulating genes that control processes such as the cell cycle, apoptosis, and cell differentiation[108]. These viral microRNAs do so by interfering with cellular microRNAs and thus affecting host cell gene expression.

Overall, the above mentioned proteins affect many different cellular regulatory proteins, potentially causing tumor formation. The oncogenic mechanisms of all mentioned proteins and microRNAs and their cellular targets are listed here: hijacking the cell cycle regulating proteins (p53, pRb, cyclin dependent kinases, cyclins, signalling pathways), deregulating cellular gene expression (transcription factors, DNA methylation, microRNAs), affecting cell survival (anti-apoptosis, telomerase activity), modulating cellular protein turn-over, chromosome instability and stimulation angiogenesis (list derived from table 2 in Moens et al. 2008[109]).

JCV and BKV

Like SV40, both BKV and JCV encode the LT, st and agnoprotein. So, theoretically, both viruses have the potential to be oncogenic. There are slight differences, however, between SV40 LT and BKV/JCV LT. This involves the Rb binding domain of LT, possibly explaining the lower affinity for Rb of JCV and BKV compared to SV40[12, 110, 111]. Still, both BKV and JCV are capable of immortalizing human cell lines and inoculation of either into newborn experimental animals can induce a variety of malignant tumours. In humans, associations of these viruses with tumour (tissue) are reported on a large scale; BKV is suggested to be associated with

among others brain tumours, prostate cancers and bladder carcinomas, whereas JCV is thought to be related with mesotheliomas, brain tumours and lymphomas (reviewed in [112-114]). However, there is no clearity about a causative role of BKV or JCV in tumour formation; the fact that they are ubiquitously present in the human population and detected in multiple tissues make it difficult to specifically link these PyV to cancer[25]. Further investigations are needed to determine the relation between JCV/BKV and cancer; for now they are 'innocent until proven guilty'[115].

Structural similarities of KI, WU and MCV with other known PyV

The transforming properties of KI, WU and MCV are not known because an infectious virus is not isolated yet, so they cannot be tested in *in vitro* studies and experimental animal models. However, the structural differences and similarities of these newly discoverd PyV with other known PyV may give some insight into their pathogenic and/or oncogenic potential.

Regarding genome organization and amino acid sequence (i.e. viral proteins), KI and WU are most similar to BKV and JCV[26, 27]. On the other hand, MCV shares the most homology with the African green monkey lymphotropic polyomavirus (LPV)[28](phylogenetics of PyV reviewed in Krumbholz et al. 2009[1], Fig. 3).

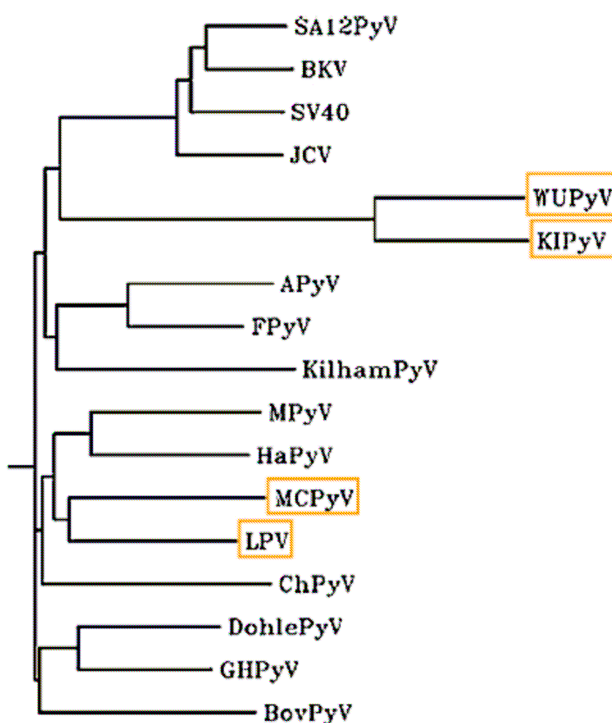

Figure 3. Phylogenetic analysis of 17 PyV.

Pylogenetic analysis based on the VP1 primary aminco acid sequence for 17 PyV: Simian agent 12 (SA12PyV), BKV, SV40, JCV, WU polyomavirus (WUPyV), KI polyomavirus (also known as murine pneumotropic virus) (KIPyV), avian polyomavirus (APyV), ferret polyomavirus (FPyV), murine polyomavirus kilham strain (KilhamPyV), murine polyomavirus (MPyV), hamster polyomavirus (HaPyV), merkel cell polyomavirus (MCPyV), lymphotropic polyomavirus (LPV), chimpanzee polyomavirus (ChPyV), crow polyomavirus (DohlePyV), goose hemorrhagic polyomavirus (GHPyV), and bovine polyomavirus. (BovPyV).

Derived from Dalianis et al. 2009[34]

The LT of KI and WU has about 50 % identity with SV40, BKV, and JCV. Furthermore, important regions responsible for host cell transformation are conserved in the new PyV, like the Rb binding sequence (LXCXE) and the ATPase-p53 binding domains (Fig. 4). However, all three viruses lack a domain at the carboxyterminus of LT, named the 'host-range' domain[34]. Regarding the st, there is a homology of 40 % of KI and WU compared to SV40, BKV, and JCV[109].

The most striking difference of the three new PyV compared to other PyV is the lack of an agnoprotein. KI, WU and MCV also do not have an middle T antigen (Mt), which some of the PyV like mPyV do have[34,63,90]. As mentioned before, the agnoprotein has a variety of functions and among others it is partially responsible for the transforming properties of SV40, BKV and JCV. Next to this, it facilitates the nuclear export of progeny virions and the viral spreading[88, 89].

When looking at the differences between KI and WU, they are clearly phylogenetically related, but they differ substantially in amino acid sequences for the late proteins VP1 and VP2, also compared to other PyV[34]. Although the similarity is between 58 and 84 %, they are more closely related to each other than to SV40, BK and JC viruses[25-27].

At the origin of all known PyV there are specific LT binding elements, where LT can bind to help transcription with its helicase capacity (Fig. 4.). All newly discovered PyV have a distinct configuration of these elements and it is not known yet whether LT can bind to these altered elements. Also on the level of microRNAs there are differences. SV40, JCV and BKV (and some other PyV) make microRNAs during lytic infection. For MCV, it its shown that it encodes a similar microRNA, but for WU and KI no microRNAs are detected yet[34]. More information about structural similarities and differences are reviewed in Johnson et al[63].

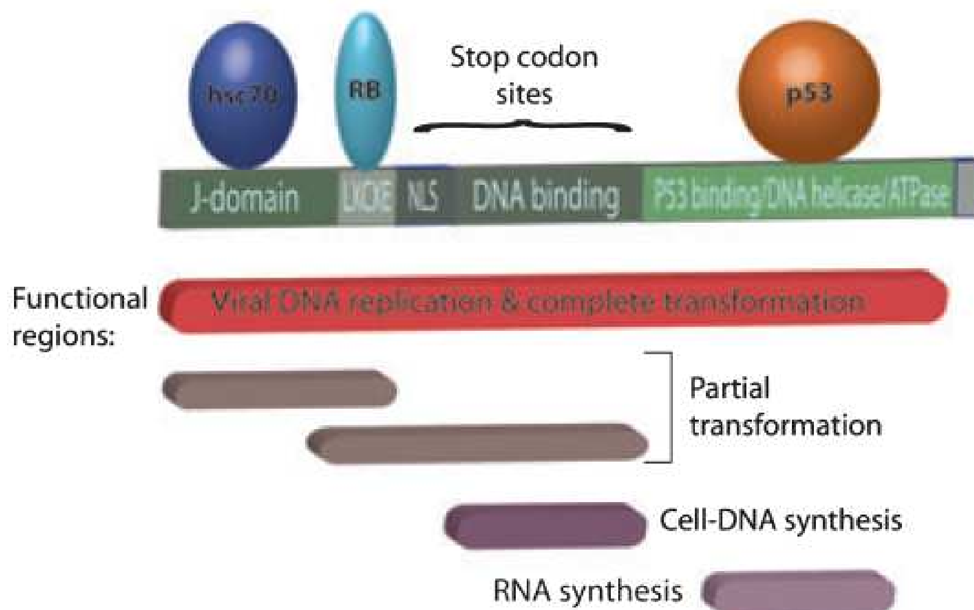

Figure 4. The domains located on the LT, and the stop codons in MCV LT.
The conserved domains that are present on the LT, and the location of MCC-associated mutations prematurely truncating the MCV LT. To date nine MCC-derived MCV sequences have been published. All, in contrast to MCV from other sources, contain one or more stop codons in the region indicated. In all cases the RB binding domain is conserved whereas parts of the protein necessary for replication of the virus as well as the supposed p53 binding domain are absent.
Derived from Houben et al. 2009[118]

Pathogenic potential of MCV

Merkel cell carcinoma (MCC) is a rare skin cancer which affects elderly and individuals who are under conditions of immunosuppression. Especially patients with AIDS are susceptible to get MCC. These features pointed this disease out as being characteristic for involvement of an infectious agent. The discovery of MCV in this setting make this PyV an exception compared to all other discovered PyV[28]. The initial report of Feng et

al. showed the presence of MCV in 80% of MCC cases. This finding is confirmed by others, but the frequencies ranged from about 70 to 85 %, and the copy numbers per cell ranged from less than 1 per 300 till at least 1 per cell[63, 116-118]. However, this means that in 15 to 30 % of the cases, MCV is not detected in the tumours. This may implicate that MCV is not directly involved in tumour formation. Alternatively, strain variation, LT expression below the limits of detection, paracrine mechanisms (for example, cells expressing LT secrete a growth factor like insulin-like growth factor type I[119]), or hit and run strategies of MCV may be an explanation for the relatively low prevalence of MCV DNA in MCC [10, 120].
Furthermore, there are indications that ultraviolet (UV) radiation contributes to the etiology of MCC. The majority of the tumours are expressed on the face and head, and a substantial group of MCC patients have other, sun associated skin cancers next to MCC[121, 122]. Another interesting observation is the increasing incidence of MCC along with an increase in age. This can be explained by the theory that during life, more and more oncogenic mutations occur and so the changes of getting cancer are getting higher with increasing age. Moreover, next to skin cancers MCC patients are also at higher risk for other malignancies like multiple myeloma, non-Hodgkin's lymphoma, but especially chronic lymphocytic leukaemia[121, 123].
So far, an etiologic role of MCV in the emergence of MCC has not been clearly established. There are some strong indications, however, for a mechanistic role of MCV in the disease. First of all, Feng et al. have shown that in tumours of MCC patients the integration of MCV most likely takes place prior to tumour development. The authors discovered a clonal pattern of integration by analyzing both primary and metastatic tumour tissue derived from one patient[28]. The distinct integration patterns between tumours of different patients imply that the integration of the virus occurs in different locations within the host genome[118].
Another important indication for a causal relationship between MCV and MCC is the mutated or truncated LT antigen of MCV, present in MCC tumours[124]. This means that the viral genome detected in all MCC tumours tissues has mutations that prematurely truncate the LT protein (Fig. 4). These specific mutations are only present in MCV derived from tumour tissue and not in MCV isolated from non-tumour tissue, and therefore these MCC LT mutations are called signature mutations[80, 124]. So this signature seems to be a prerequisite for tumour development. Several reasons can be thought of why the truncation of LT is a necessary event for MCC to occur. Viral DNA fragmentation can occur due to deregulated viral DNA replication by head-to-tail replication fork collision, for example[118, 125]. But moreover, full-length LT will eventually lead to host cell lysis. The truncated variants all had a disrupted helicase domain, thereby preventing MCV to replicate. Importantly, the Rb binding domain remained intact in all tumours, thus enabling the virus to have oncogenic potential but disabling it to further infect the host. Decades ago, a similar observation has been made that the transforming activity of SV40 LT is greatly enhanced by mutations that disrupt its helicase and replication ability[126, 127].
Taking all of this information in count, Shuda et al. have proposed a model for MCC disease development, in which UV light causes the mutation in the helicase domain of LT[124]. But for malignancy to occur, there are probably additional factors involved, possibly of host cell (mutated) genes[80].

Pathogenic potential of KI and WU

The strong homology of KI and WU to JCV and BKV implies that KI and WU are likely to be pathogenic in humans. Although there are differences, like the lack of an agnoprotein, the similarities are striking both on structural and epidemiological levels[63]. Despite the lack of clear associations between a disease and infection

with one of these viruses, there are some indications for the pathogenic potential of WU and KI. In has been shown for example, that under conditions of immunosuppression, WU and KI reactivate in lymphoid tissues just like JCV and BKV but unlike the more distantly related MCV[128]. Furthermore, WU and KI display a hallmark that is commonly observed in case of BKV and JCV, namely mutations in the transcriptional control region (TCR) of the viral genome[128]. The clinical implications of these mutations are not complete known yet, but this shows that WU and KI use mechanisms similar to JCV and BKV. It is very likely that lytic infections, presumably during reactivation in specific immunosuppressive conditions, can cause disease like JVC and BKV do. The involvement of WU and KI in oncogenesis by causing cell transformation *in vivo* remains uncertain, and the ubiquitous presence of both these viruses will make this association not easy to determine. Further suggestions about the pathogenic potential are difficult to make because no infectious virus is isolated for either of the viruses. Until then, hypothetical theories can be made by observations from patient derived viruses and comparing the presence, behaviour, genome and cell types with other known PyV.

5 Conclusion

After the shocking discovery of SV40 as being a PyV capable of transforming human cells, the recent discovery of the three human PyV WU, KI and MCV (and very recently TSV) have regained interest in this family of PyV. Especially their (possible) role in human disease is a current and interesting research theme. The general biology of viruses can be explained by the strategies they use on a molecular and cellular level during their viral life cycle. The knowledge of other earlier discovered PyV, especially SV40, gives insight into how the newly discovered viruses may behave in humans. The mechanism of entry, using particularly sialic acids residues attached on glycolipids; the intracellular trafficking in which multiple host cell transport machineries are used; the integration in the genome or presence as episomal DNA; the release out of the nucleus and subsequent host cell lysis; or the persistence within a cell and reactivation under conditions of immunosuppression; these are all general features of PyV.

The PyV WU and KI share homology with JCV and BKV and probably also share biology. The similarities like a benign initial infection during childhood, the persistence throughout life in a specific tissue and the presumably manifestation of pathology in immunocompromised patients are conspicuous.

MCV is an exception to all other human PyV, because of its direct relation with cancer. MCV is a rare disease and mainly occurs in elderly. Upon integration into the host genome, the virus can obtain specific mutations in the LT making it impossible to replicate and thus to cause host cell death. Also, a clonal pattern of integration has been observed in the MCC tumours. The rarity of this disease implicates that viral integration and subsequent host cell transformation is an unusual happening that likely is never attained given normal cell-mediated immunity. Despite the molecular indications for a causal role in MCC, it is difficult to make a causal relation between the virus and the oncogenic envents on a serological level because of the ubiquitous prevalence of MCV.

Until a cell culture system is developed, including an infectious virus of KI, WU and MCV and susceptible cell lines, many biological questions about the newly discovered PyV will await further investigation[34].

Important questions that remain to be answered are for example: are WU and KI involved in human disease? Do they participate in respiratory disease? Or even more, are they involved in tumour formation? Also about the site of latency of WU and KI there are uncertainties. Does primary infection happen in the respiratory tract? And how about the life cycle of the newly discovered PyV; what are the receptors, which endocytotic pathway(s) do they use, and how do they enter the nucleus? Furthermore, the lack of an agnoprotein implies that WU, KI and MCV have other strategies for assembly and release of progeny virions. In the future, perhaps diagnostic tools need to be developed for the new WU, KI and MCV. But the most important and challenging question is: do these viruses have a role in human cancers?

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