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Update on Human Polyomaviruses and Cancer

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Over 50 years of polyomavirus research has produced a wealth of insights into not only general biologic processes in mammalian cells, but also, how conditions can be altered and signaling systems tweaked to produce transformation phenotypes. In the past few years three new members (KIV, WUV, and MCV) have joined two previously known (JCV and BKV) human polyomaviruses. In this review, we present updated information on general virologic features of these polyomaviruses in their natural host, concentrating on the association of MCV with human Merkel cell carcinoma. We further present a discussion on advances made in SV40 as the prototypic model, which has and will continue to inform our understanding about viruses and cancer.

I. INTRODUCTION

Five human polyomaviruses have been identified to date. In 1971, the first two members, JC virus (JCV) and BK virus (BKV) were concurrently reported in the journal Lancet (Gardner *et al.*, 1971; Padgett *et al.*, 1971).

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JCV was cultured from progressive multifocal leukoencephalopathy (PML) brain tissue in a patient with Hodgkin disease and BKV was isolated from the urine of a renal transplant patient with ureteral stenosis. Both viruses manifested unusual clinical diseases in immunosuppressed patients and were named after their source patients' initials. More than 30 years intervened before two more human polyomaviruses were identified in 2007 (Allander *et al.*, 2007; Gaynor *et al.*, 2007). These two viruses, Karolinska Institute virus (KIV) and Washington University virus (WUV) were named after the institutions where their identification occurred. Both were detected from respiratory samples in symptomatic pediatric patients after DNase enrichment for encapsidated viral particles followed by library construction and mass sequencing of cloned cDNAs. The publication of the most recently identified human polyomavirus, Merkel cell polyomavirus (MCV) occurred in 2008 (Feng *et al.*, 2008). MCV was named for the uncommon, but aggressive Merkel cell carcinoma (MCC) skin cancer, from which viral transcripts were found by digital transcriptome subtraction (DTS) (Feng *et al.*, 2007). DTS involves transcriptome sequencing followed by *in silico* subtraction to exclude human from candidate viral transcripts (Feng *et al.*, 2007). In contrast to other human polyomaviruses, the discovery of MCV did not depend on the presence of replication competent, encapsidated virions.

Research on polyomaviruses began in 1953 with strong ties to cancer biology when Ludwik Gross, during the course of his investigations in transmitting mouse leukemia from cell-free filtrates, isolated an agent that induced tumors in newborn mice (Gross, 1953). This filterable agent, murine polyomavirus (MPyV) became the archetypal member of the *Polyomaviridae* family. In 1960, Sweet and Hilleman found simian vacuolating virus 40 (SV40) infecting lots of rhesus monkey kidney cells used for the production of both Sabin and Salk polio vaccines (Sweet and Hilleman, 1960). The ability of SV40 to cause tumor in experimental animals and the widespread administration of the polio vaccine raised concerns regarding xenotropic exposure in the human population to this agent. The resultant search over the past 50 years to establish a causal relationship between SV40 and human cancers has not been fruitful; however, this does not diminish the contributions that studies of SV40 have made to both viral oncogenesis and cellular biology. In this review, we will examine the biology of the human polyomaviruses concentrating on MCV and its association with human cancer. SV40, and its gene products, serve as well-established models for cancer and provide a context for these discussions due to commonalities in genome, structure, and biochemical properties with the human polyomaviruses.

II. BIOLOGY OF POLYOMAVIRUSES

A. Classification and Phylogeny

Polyomaviruses were historically categorized with the papillomaviruses under the designation of papovaviruses until their separation into two distinct families in 2000. In addition to MPyV, SV40, and the five human members, other polyomaviruses from diverse species of animals including other nonhuman primates, birds, bats, rabbits, and rodents have been found. To date, full genome sequences of over 21 polyomaviruses have been deposited in GenBank. Although variations exist with respect to phylogenetic relatedness when different genes are used for analysis, JCV, BKV, KIV, and WUV appear to group closely with SV40 (Fig. 1). Within this subgroup JCV and BKV are consistently less divergent from each other when compared to either KIV or WUV, which appear to be in a clade of their own. In contrast to the first four identified human polyomaviruses, MCV is more closely related to the archetypal murine polyomavirus (MPyV) and the African green monkey lymphotropic polyomavirus (LPV) (Fig. 1).

B. Genome Organization

The genomes of human polyomaviruses range between ~5.0 and 5.3 kb (JCV-5130 bp; BKV-5153 bp; KIV-5040 bp; WUV-5229 bp; MCV-5387 bp). They exist as circular dsDNA closely associated with histones, and are packaged into chromatin resembling cellular genomes (minichromosomes) within nonenveloped, 40–45 nm icosahedral capsids. The polyomavirus genome is almost evenly divided into an early and a late region encoded on opposite strands.These two regions are separated by a noncoding regulatory region (NCRR) containing the origin of replication and transcriptional control elements. The SV40 genome is depicted in Fig. 2, since we will later use it as a model for polyoma-induced neoplastic transformation. The early region is transcribed from the early promoter immediately upon entry and uncoating of the genome, while the late region is expressed from the late promoter after the onset of viral DNA replication. Early message is differentially spliced to encode at least two proteins, and up to five in some polyomaviruses. The large T (tumor) antigen (LT) and small t antigen (ST) proteins are invariantly expressed in all polyomaviruses including the five human members, although in KIV and WUV these two proteins have been predicted only by open reading frame analysis and not by experimentation (Fig. 3). These early proteins are referred to as tumor antigens, because they were originally detected using antibodies from tumor bearing animals.

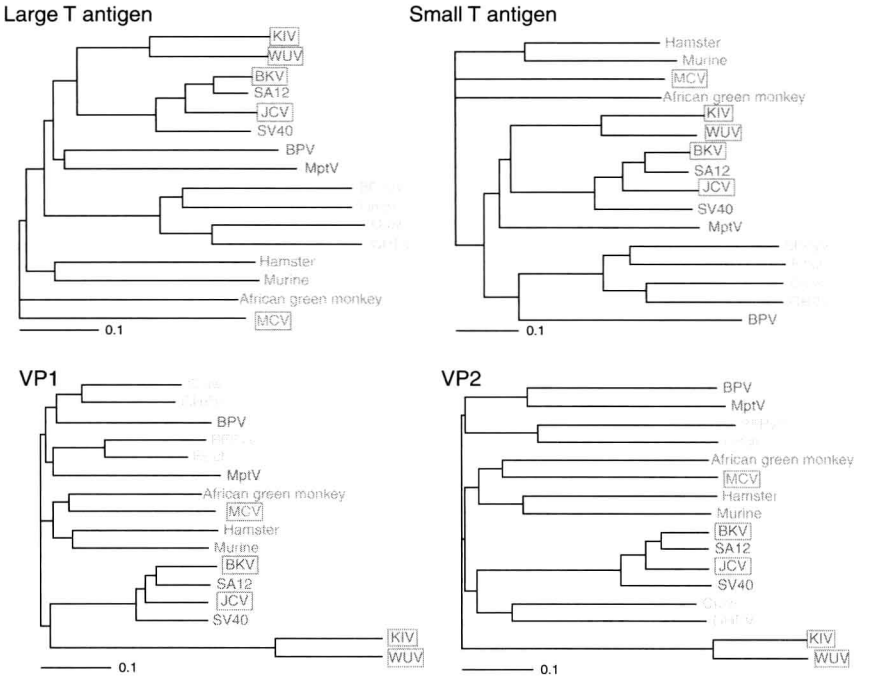

Fig. 1 *Phylogenetic analysis of polyomavirus LT, ST, VP1, and VP2 protein sequences.* The analysis includes: human polyomaviruses (BKV, JCV, KIV, WUV, and MCV, all marked with a green rectangle) as well as simian agent 12 (SA12), SV40, bovine polyomavirus (BPV), murine pneumotropic virus (MptV, also known as Kilham strain of polyomavirus), budgerigar fledgling disease polyomavirus (BFPyV), finch polyomavirus, crow polyomavirus, goose hemorrhagic polyomavirus (GHPV), hamster polyomavirus, murine polyomavirus, African green monkey polyomavirus (also known as lymphotropic polyomavirus), and MCV. The subgroup of SV40 is outlined in blue, of the murine polyomavirus in red, and of the avian polyomavirus in orange. While BKV, JCV, KIV and WUV LT and ST sequences cluster together with SV40, MCV, in contrast, clusters with the murine polyomavirus subgroup (modified from Feng *et al.*, 2008; Fig. 2B). (See Color Insert.)

Analogous to the 17k T antigen of SV40 which is expressed from an alternatively spliced early transcript (Zerrahn *et al.*, 1993), additional T antigen isoforms have also been identified in JCV, BKV, and MCV: JCV encodes three T′ antigens, T′165, T′136, and T′135 (Trowbridge and Frisque, 1995); MCV has a 57 kDa T antigen (57kT) (Shuda *et al.*, 2008) (Fig. 3); and BKV encodes a truncated T antigen close in structure to T′136 of JCV (Abend *et al.*, 2009b). No accessory T antigens have yet been identified for KI or WU. Regardless of the number of T antigen mRNAs, exon 1 is shared in common with all alternatively spliced early mRNAs of each virus (Fig. 3). The functions of the accessory T antigen proteins are still largely unknown. Determination of their function in the viral life cycle awaits

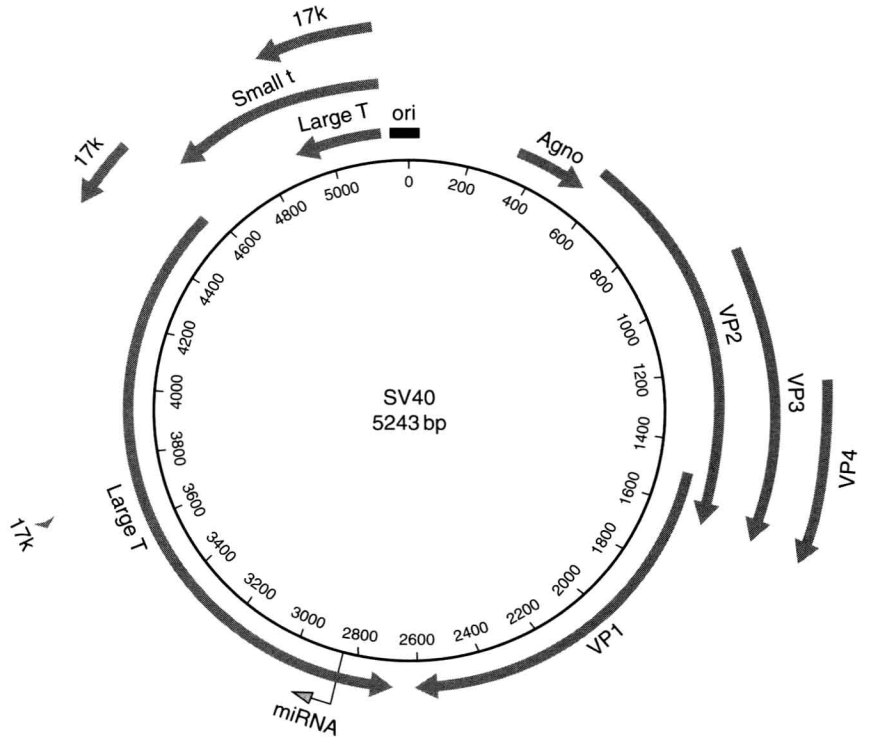

Fig. 2 *SV40 genome organization.* The early region of the viral genome (the left half) encodes LT, ST, and 17k by differential splicing. The respective open reading frames are colored blue. The late region of the viral genome (the right half) encodes agnoprotein and the structural proteins VP1, VP2, VP3, and VP4. These gene products are generated by differential splicing and internal translation. The core origin of replication (ori) is located on top adjacent to transcriptional control elements, together encompassing the NCRR. A red arrow indicates the viral miRNA that targets the early message. (See Color Insert.)

generation of mutant viruses lacking their expression. It is known that the 17k SV40 accessory T is expressed at low levels during SV40 infection, where it has been suggested to fine-tune cell-cycle regulation (Zerrahn *et al.*, 1993). It can bind pRB family members, drive cell-cycle progression, and is capable of causing minimal transformation of F111 rat cells (Boyapati *et al.*, 2003; Zerrahn *et al.*, 1993). A mutant 17k deficient in pRB binding drives normal human fibroblasts into premature senescence (Gjoerup *et al.*, 2007).

The late message, by differential splicing and internal translation, produces three to four capsid proteins VP1, VP2, VP3, and VP4. VP3, and VP4 when present, is generated by internal translation of VP2. VP4 has so far only been detected in SV40, where it promotes lysis of the cell and egress of

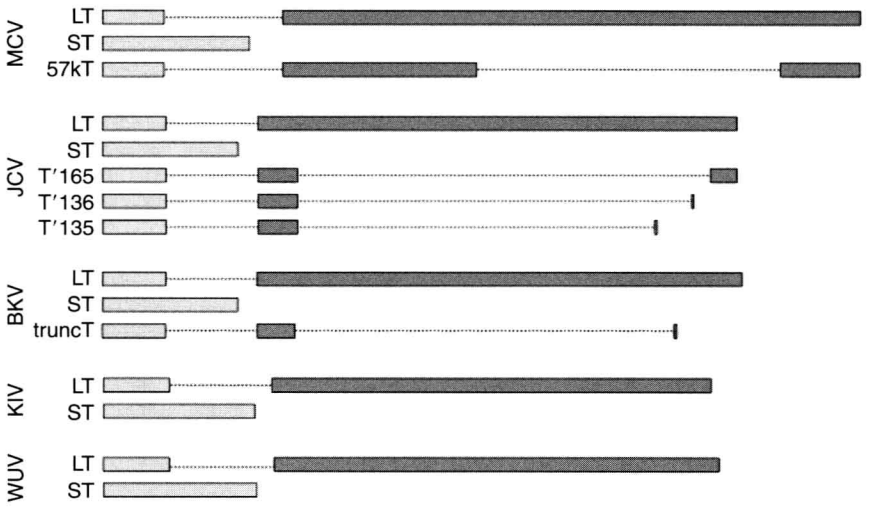

Fig. 3 *Splicing arrangement for the human polyomavirus T antigens.* Colored rectangles indicate coding sequences, whereas the broken line depicts intron sequences. Different color rectangles refer to distinct reading frames after splicing events. All T antigens of each polyomavirus share the sequence encoded within exon 1. The accessory T antigens in addition share fragments of their respective LT sequences. (See Color Insert.)

the virus (Daniels *et al.*, 2007). The viral capsid is composed of 72 pentamers of the major capsid protein VP1 that contacts 72 copies of the minor capsid proteins VP2/3. In BKV and JCV, the leader region of the late message additionally encodes the agnoprotein, which may be involved in virion maturation (Khalili *et al.*, 2005; Ng *et al.*, 1985). KIV, WUV, and MCV are without known agnoproteins. Only murine and hamster polyomaviruses are known to encode a middle T antigen, which is their principal transforming protein (Cheng *et al.*, 2009).

Several polyomaviruses have been found to express miRNAs derived from the late transcript. SV40, MPyV, JCV, and BKV each has a single pre-miRNA from which two miRNAs of opposing orientations are processed (Seo *et al.*, 2008; Sullivan *et al.*, 2005, 2009). The JCV miRNAs have been detected in PML lesions (Seo *et al.*, 2008). These miRNAs are predicted to autoregulate early gene expression at late times in infection. For SV40, mutants that cannot produce miRNA show increased expression of viral T antigens and are more susceptible than wild-type virus to lysis by cytotoxic T cells (Sullivan *et al.*, 2005). In contrast, no *in vivo* differences in antiviral CD8 T cell responses can be discerned between infections with wild-type MPyV and MPyV lacking miRNA (Sullivan *et al.*, 2009).

C. Viral Life Cycle

The polyomaviruses generally have a narrow host range and limited cell type tropism. They are able to infect cells of their natural hosts, giving rise to a productive life cycle that results in cell lysis. In addition, these viruses are also able to establish a persistent infection rarely associated with disease except when immunodeficiency is induced. The full, infectious viral life cycle has only formally been studied in JCV and BKV as no infectious system has yet been devised for KIV, WUV, or MCV. However, it is generally thought that polyomaviruses are internalized by the interaction of VP1 with specific cellular receptors and co-receptors. It is known that BKV uses gangliosides GD1b and GT1b (Low *et al.*, 2006); JCV uses GT1b and the serotonin receptor, 5HT2AR (Elphick *et al.*, 2004); and GT1b has been proposed as a putative host cell receptor for MCV (Erickson *et al.*, 2009). Virus then traffics through caveolae and the endoplasmic reticulum to the nucleus, where it is uncoated and the early message is transcribed. In the case of JCV, clathrin-dependent endocytosis precedes localization in caveosomes (Eash *et al.*, 2006). After translation, LT then initiates DNA replication of the viral genome from the origin in the NCRR. The shift to late viral expression is not fully elucidated but likely involves LT transcriptional activation of the late and repression of the early promoter. VP1 is expressed and assembled together with VP2 and VP3 and then imported into the nucleus where encapsidation of viral genomes takes place.

D. Natural Infection, Reactivation, and Clinical Disease

All five human polyomaviruses have high prevalence in the human population and infections start in childhood; however, variability has been reported in the age patterns of polyomavirus acquisition. A study of 2435 sera from English and Welsh individuals ranging from 1 to 69 years showed an overall seroprevalence rate of 81% for BKV with seroconversion occurring very early in childhood, peaking at 91% in the 5–9 age range, and dampening in elderly individuals. For JCV, the overall seroprevalence was 35% with a steady increase of 11% from children below 5 years up to 50% in the 60–69 age group (Knowles *et al.*, 2003). These findings were largely replicated in a study examining 400 consecutive healthy blood donors from Basel, Switzerland. Egli and colleagues found an overall IgG seroprevalence of 82% for BKV and 58% for JCV in this cohort (Egli *et al.*, 2009). The first large serosurvey of KIV and WUV in 1501 adults using GST-VP1 capture ELISA showed rates of 55% and 69%, respectively (Kean *et al.*, 2009). Age-specific prevalence studies have not been performed for KIV and WUV;

however, the detection of viral DNA in respiratory specimens from children suggests that initial exposure occurs at a relatively early age. Similar findings are emerging for MCV. Using wild-type MCV strain sequences in VLP-based ELISA, Tolstov and colleagues reported an age associated increase in MCV prevalence from 50% among children 15 years or younger up to 80% among persons older than 50 years (Tolstov *et al.*, 2009). Pastrana and colleagues found 88% MCV seropositivity in adults without MCC. These studies additionally demonstrate that MCC patient sera showed markedly elevated MCV IgG responses with the geometric mean titers in controls 59-fold lower than in the MCC patient group (Pastrana *et al.*, 2009; Tolstov *et al.*, 2009). Carter and colleagues reported the following seroprevalence results in 451 general population adults: 92% for BKV, 45% for JCV, 90% for KIV, 98% for WUV, and 59% for MCV (Carter *et al.*, 2009). Although rates of infection for JCV and BKV found in various studies are in general agreement, additional studies on KIV, WUV, and MCV will more precisely define overall and age-specific prevalence rates.

Fecal–oral, oral, and respiratory routes of transmission have been proposed for different human polyomaviruses. For JCV and BKV, studies of urban sewage samples and rivers show significant numbers of stable virus particles from divergent geographical areas suggesting the possibility of virus acquisition through fecally contaminated water, food, and fomites (Bofill-Mas and Girones, 2003; Bofill-Mas *et al.*, 2000; McQuaig *et al.*, 2006). Initial studies of KIV, WUV, and MCV have also shown viral DNA in stool samples (Allander *et al.*, 2007; Babakir-Mina *et al.*, 2009a; Loyo *et al.*, 2009). The detection of salivary shedding of BKV and productive infection of salivary cell lines implicates oral transmission as another possibility for this virus (Jeffers *et al.*, 2009). MCV is also detected at high level in saliva (Loyo *et al.*, 2009). In contrast to JCV and BKV, which are only rarely detected from respiratory sources, KIV and WUV can be isolated from respiratory secretions of children worldwide. Curiously, these viruses cannot be detected in the respiratory specimens of adults except in the setting of immunosuppression (Bialasiewicz *et al.*, 2009; Loyo *et al.*, 2009; Norja *et al.*, 2007; Ren *et al.*, 2008). MCV DNA can also be detected in respiratory specimens from symptomatic patients and occurs in nasal swabs and nasopharyngeal aspirates at a frequency similar to or even higher than that of KIV and WUV (Bialasiewicz *et al.*, 2009; Kantola *et al.*, 2009). Significantly more adults than children are positive for respiratory MCV in contrast to KIV and WUV (Goh *et al.*, 2009).

The process by which polyomaviruses gain access to and establish persistent infections in distal body compartments is not well established. However, DNA of all human polyomaviruses has been detected in tonsillar tissue, a possible point of entry (Babakir-Mina *et al.*, 2009b; Kantola *et al.*, 2009; Monaco *et al.*, 1998a). For JCV, virus has been localized to tonsillar stromal

cells and B lymphocytes with the latter cell type implicated in circulatory dissemination to other anatomic sites (Monaco *et al.*, 1998a,b). Since all viruses can be detected at increased frequencies in blood and lymphoid tissues during host immunosuppression (Sharp *et al.*, 2009), it is likely that hematolymphoid cells can carry or harbor polyomaviruses. Recently, Mertz and colleagues detected MCV in (CD14+/CD16−) inflammatory monocytes but not in lymphocytes or granulocytes (Mertz *et al.*, 2009).

JCV and BKV establish persistent infections in renal tissue and virus is shed into the urine. In a recent study examining 400 consecutive healthy blood donors from Basel, Switzerland, Egli and colleagues found urinary shedding of BKV in 7% of these individuals compared to 19% for JCV (Egli *et al.*, 2009). Reactivation of JCV and BKV, as reflected by increased viruria, occurs during immunosuppression, but only BKV levels correlate with the degree of immunosuppression (Behzad-Behbahani *et al.*, 2004). The bone marrow is another possible site of persistent infection for JCV and may be the source of virus positive cells detected in the circulation (Tan *et al.*, 2009). JCV can additionally gain access to glial cells of the central nervous system where reactivation or new infection can result in PML and virus can be detected in the cerebral spinal fluid (Drews *et al.*, 2000; Vago *et al.*, 1996).

PML is an acquired demyelinating disease pathologically characterized by a triad of findings: 1) oligodendrocytes having enlarged nuclei with viral inclusion bodies, 2) bizarre, atypical astrocytes, and 3) loss of myelin with accompanying phagocytic infiltrate. PML underscores how critical the host cell environment is in determining the outcome of a viral infection. Although both oligodendroglial and astroglial cells of the central nervous system are infected, only the oligodendrocytic myelin forming cells support full lytic replication of the virus causing the hallmark loss of myelin seen in PML. By contrast, infection of the astroglial population results predominantly in changes in nuclear morphology, size, and ploidy. The bizarre, atypical astrocytes which are visually indistinguishable from tumor cells in high-grade glial neoplasia may represent an infection by JCV of a cell type that is unable to support a fully lytic viral life cycle. Viral DNA and LT protein expression is detectable in astrocytes but at a much lower frequency and quantity. Whether the JCV infected astroglial population is transiently transformed is an intriguing conjecture. There are scattered, but convincing case reports in the literature demonstrating expression of JCV T antigen in tumors of the central nervous system (Krynska *et al.*, 1999; Pina-Oviedo *et al.*, 2006); however, as a group, the glial neoplasms have not been associated in a consistent way with JCV infection. Uncommon cases and case series of JCV association with other human neoplasia have also been published (see review Maginnis and Atwood, 2009). PML has recently been diagnosed in patients taking natalizumab (Tysabri) (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould *et al.*, 2005; Van Assche *et al.*, 2005), causing its temporary

withdrawal from the market (Major, 2010). Several mechanisms have been proposed for the effect of natalizumab on JCV including restriction of leukocyte trafficking across the blood–brain barrier or direct inhibition of T cell reaction against JCV (Chen *et al.*, 2009).

For BKV, important clinical diseases occur in the posttransplant setting and relate to type of tissue transplanted. Hemorrhagic cystitis, hematuria, and renal impairment are seen with hematopoetic stem cell transplantation (HSCT) (O'Donnell *et al.*, 2009); and BK-associated nephropathy occurs in 2–5% of renal transplant patients with graft loss in nearly half of these cases (Bonvoisin *et al.*, 2008). Viral reactivation during these complications is robust and can be monitored by DNA-based techniques in blood and urine (Bonvoisin *et al.*, 2008; Cimbaluk *et al.*, 2009; O'Donnell *et al.*, 2009).

III. MERKEL CELL POLYOMAVIRUS AND HUMAN CANCER

A. Human Polyomaviruses and Cancer

Of the five human polyomaviruses, only MCV demonstrates a robust correlation with human cancer. Studies showing MCV T antigen interactions with cellular proteins such as pRB, Hsc70, and PP2A have been performed, but demonstration of the biochemical effects of these interactions have not been published. As noted above, JCV and BKV are associated with important nonneoplastic clinical diseases that have significant morbidity and mortality in immunocompromised individuals; but despite their potential to act as transforming viruses in rodent and *in vitro* cell culture models, no consistent association with human cancers have been found. The reader is referred to excellent reviews on the potential role of these viruses in human cancers (see Abend *et al.*, 2009a; Maginnis and Atwood, 2009; White *et al.*, 2005). KIV and WUV have not yet been found to be associated with human disease and although both of these viruses were originally detected in the respiratory samples of symptomatic children, attributing a causal role for them in respiratory diseases is difficult because of the significant rates of co-detection with other respiratory pathogens (Bialasiewicz *et al.*, 2008; Norja *et al.*, 2007).

B. Merkel Cell Polyomavirus

MCC is an uncommon cancer with an overall age adjusted incidence of 0.24 per 100,000 person-years (Agelli and Clegg, 2003). Overall incidence of MCC has increased three fold from 1986–2001 (Hodgson *et al.*, 2005).

There is a slight male predominance and a strong association with whites/fair-skinned individuals, advanced age, and sun exposure. The 5-year relative survival is 75%, 59%, and 25% for localized, regional, and distant MCC, respectively (Agelli and Clegg, 2003). Unfortunately, most cases of primary MCC are diagnosed when the disease is no longer localized.

MCC is derived from resident Merkel cells of the skin, which along with associated terminal sensory neuritis, comprise the epidermal mechanoreceptors that allow touch discrimination of fine surface textures (Maricich *et al.*, 2009). Historically, Merkel cells have been thought to be of neuroendocrine derivation due to their elaboration of various neurosecretory markers; however they also express the low molecular weight cytokeratin (CK) 20 suggestive of epithelial origin. MCC typically affects the elderly, but studies have shown that it may occur in younger ages and at an increased frequency in immunosuppressed individuals. Engels *et al.* noted that MCC was increased in both AIDS and posttransplantation populations (Engels *et al.*, 2002), a striking epidemiologic feature that suggests strong immunologic surveillance in controlling MCC development. In 2008, Feng and colleagues specifically sought for an infectious agent in MCC using the DTS technique (Feng *et al.*, 2008). Pooling cDNA libraries made from four MCC lesions, a DTS candidate was detected that showed a significant degree of similarity to the LPV LT. The 5387 bp polyoma genome (MCC350 strain) was sequenced by PCR walking using primers designed from the DTS viral transcript.

Initial studies of MCC lesions from 10 patients showed that although 80% contained Southern blot detectable MCV DNA, there exists a minority subset of MCC cases which do not contain MCV. This estimate has held up in subsequent studies from various laboratories and has been generalized to geographically diverse populations (Becker *et al.*, 2009; Duncavage *et al.*, 2009b; Foulongne *et al.*, 2009; Kassem *et al.*, 2008; Sihto *et al.*, 2009; Touze *et al.*, 2009; Varga *et al.*, 2009). An exception is the finding of only 24% correlation between virus genome and MCC in Australian populations (Garneski *et al.*, 2009). These findings suggest that MCC is a heterogenous disease having at least two pathoetiologies. Pathological and immunophenotypic studies of virus positive MCC cases compared to virus negative cases demonstrate no other markers that can distinguish between these two types of MCC (Shuda *et al.*, 2009). However, data are beginning to accumulate on clinical correlates for MCV positive MCC compared to virus negative cases. Patients with MCV positive MCC tumors had better overall survival than those with MCV negative tumors (5-year survival: 45% vs. 13%, respectively) (Andres *et al.*, 2009b; Sihto *et al.*, 2009). These findings are preliminary since the uncommon nature of these tumors precludes easy assessment of clinical outcomes and treatment responses.

Determination of viral load in MCC lesions by quantitative PCR shows the following average copy number per tumor cell: 5.2 (range 0.8–14.3)