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MCV and Merkel Cell Carcinoma: A Molecular Success Story

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Abstract

Merkel cell polyomavirus (MCV), discovered in 2008, is clonally integrated in ~80% Merkel cell carcinoma (MCC). MCV is a common skin flora and initiates cancer in susceptible hosts only after it acquires a precise set of mutations that render it replication incompetent. Both MCV large and small T proteins promote cancer cell survival and proliferation. Large T targets pocket proteins regulating cell cycle transit while small T activates cap-dependent translation critical for cancer cell growth. These findings already have led to new diagnostics and clinical trials to target MCV-induced survivin and to promote antitumor immunity. In four years, the cause, diagnosis and therapy for an intractable cancer has been changed due to the molecular discovery of MCV.

Introduction

Merkel cell polyomavirus (MCV or MCPyV) is the newest member of the surprisingly small group of viruses known to cause human cancer [1]. It is also one of seven new human polyomaviruses discovered in the past five years [2–10]. In a very short time, newly identified viral markers and serologic assays for MCV infection have been developed that improve Merkel cell carcinoma (MCC) diagnosis. Discovery of MCV has already led to studies on a precise molecular-targeted therapy based on rational drug testing that may alter clinical treatment for this often-intractable disease [11]. Work on immune-based therapies to complement existing cancer treatments is being explored as well [12,13]. MCV has also helped us understand a new cancer mechanism in which mutations to a typically harmless component of our skin flora—rather than the cancer cell genome itself—contributes to tumor formation [14]. Taken together, this recent research led to the WHO International Agency for Research on Cancer (IARC) to classify MCV as a group 2A carcinogen [15]. These recent advances all stem from isolation of a small piece of RNA from a Merkel cell carcinoma tumor four years ago [6].

Polyomaviruses have formed much of the basis for our understanding of the molecular biology of cancer. Animal polyomavirus tumor (T) antigens led to discoveries of p53 and PI3K as well as other oncogene/tumor suppressor signaling pathways [16,17]. They have also contributed to uncovering fundamental cellular processes such as protein nuclear localization signals and mammalian DNA replication [16,17]. MCV, which is clonally integrated into the MCC cell genome, adds new insights into the mechanisms of polyomavirus-induced cancers. In contrast to small T (sT) protein of other polyomaviruses, MCV sT is the major transforming oncogene, and exerts its tumor promoting effects at least in part through targeting of the cap-dependent translation regulator, 4E-BP1 [18]. Similar to

other polyomavirus large T (LT) proteins, MCV LT targets cellular pocket proteins (pRB, p107 and p130) [14] but one critical consequence of this is the activation of survivin, an important mediator for cancer cell proliferation [11].

Discovery of MCV

Merkel cell carcinoma is an uncommon but aggressive primary cutaneous neoplasm having a poor prognosis once disseminated [19,20]. It arises from mechanoreceptor Merkel cells sparsely distributed in the basal layer of the epidermis [21,22]. Similar to other skin cancers, prolonged UV exposure is a risk factor for MCC, as is advanced age, and the risk for MCC increases dramatically in persons 50 years or older [23]. The risk for MCC is also strikingly associated with loss of immune competence; the risk of MCC is 13-fold higher in AIDS patients and 10-fold higher among organ transplant recipients than in the general population [24], an epidemiologic pattern reminiscent of Kaposi's sarcoma and other cancers having a viral etiology [25].

Population-based studies from the United States and Europe reveal a rising MCC incidence [20,26,27–29] and the public health burden of this cancer is generally underappreciated. Approximately 1,500 MCC cases occur annually in the US with MCC being responsible for more deaths than chronic myelogenous leukemia [30]. Other cancers, such as chronic lymphocytic leukemia, basal cell carcinoma and squamous cell carcinoma [29,31–36], occur in conjunction with MCC at unexpectedly high rates. None of these secondary cancers have been robustly linked to MCV infection and reports vary as to whether MCV might also be present in these non-MCC tumors [37–44].

A focused search for oncogenic viruses in MCC was initiated by Feng *et al.* in 2007 [6]. This approach, called digital transcriptome subtraction (DTS), uses high-throughput complementary DNA (cDNA) sequencing and in silico subtraction of human sequences from tumor transcriptome to isolate candidate viral sequences [6,45]. Two MCV transcript sequences were found in the DTS analysis of MCC tumors, one of which had high sequence homology to a primate lymphotropic polyomavirus sequence [46].

MCV was initially found to be clonally-integrated into the human genome in tumors [6]. No preferential integration sites have so far been found [6,47–49]. In addition to disruption of the viral genome as a result of integration, viral sequences revealed a second peculiar feature for tumor-associated MCV. All tumor isolates possessed truncating mutations that deleted the origin-binding or helicase domains of the virus' LT protein [14]. Additionally, tumor isolates have been found possessing mutations in the noncoding origin sequence [50] and VP1 structural genes [51] that prevent replication and virion formation. This suggests that there is a strong selection pressure to eliminate MCV replication within MCC and consistent with the notion that virus-induced tumors generally do not support productive ("lytic") viral replication [1,50,51]. Active virus replication activates innate immune signaling and, in the case of MCV, unlicensed viral origin firing from the viral-human integrant will generate fragmented DNA [14], which will kill the nascent tumor cell.

MCV Virology

MCV is a non-enveloped, double-stranded DNA virus belonging to the mammalian genus *Orthopolyomavirus* [52]. MCV has been difficult to cultivate in the laboratory as a natural infection but several attempts have been made to produce infectious MCV molecular clones [53–55]. In each case, primary low-level virion production can be achieved (Figure 1) but secondary transmission to uninfected cells has not been successful. While early electron microscopy studies suggested that MCV virions might be seen in some MCC tumors [56], the weight of evidence now indicates that structural proteins required for encapsidation are

not expressed in MCC tumors and encapidated viruses seen in tumors are likely to be coincidental [57,58].

MCV Genes and Genome

The MCV genome displays features found in other polyomaviruses. It has a ~5.4 kb genome divided into early and late gene regions by a noncoding regulatory region (NCRR). The early region encodes for alternatively spliced, overlapping RNAs that generate large T (LT), small T (sT) and 57kT antigens (analogous to the SV40 17-kT antigen [59]), and share a common 78 amino acid N terminus encoded by exon1 (Figure 2) [14]. Mutations to the T antigen region that arise in tumor-derived MCV (substitutions, frameshift, missense, insertions and deletions) [14,49] truncate LT and 57kT proteins but do not affect full length sT protein translation [14]. Despite MCV's similarity to murine polyomavirus (MPyV), no middle T antigen has been identified.

MCV LT antigen retains conserved domains that are present across different polyomaviruses, such as DnaJ and LXCXE retinoblastoma (Rb) protein binding motifs [16,60], as well as the origin binding and helicase/ATPase regions needed for viral replication [14]. Tumor-specific mutations spare the LXCXE domain (aa 212–216), indicating its importance to MCC tumorigenesis [14,47–49,61]. Similar to other polyomaviruses, MCV LT interaction with RB1 requires the LXCXE domain [14,47,48,61], which is expected to deregulate E2F-related gene transcription. Direct evidence for the requirement of this motif for cell survival, has been generated by complementing T antigen knockdown experiments in MCC cell lines by Houben and colleagues [62]. One unexpected consequence of LT targeting of pocket proteins is the specific activation of survivin transcription, a finding that has been exploited in therapeutic studies [11].

MCV LT protein contains a nuclear localization signal (NLS) at aa 277–280 (RKRK) [61], resulting in a typical nuclear LT localization pattern for most cell cultures and tumors [37] (Figure 3). Signature tumor truncation mutations can disrupt this domain resulting in diffuse nuclear and cytoplasmic distribution of LT [37,54]. A novel interaction, so far only found for MCV LT, between human Vamp6 protein (Vps39) and the MCV unique region in LT adjacent to its Rb binding motif [54], causes this cytoplasmic protein to relocalize to the nucleus. The function(s) of Vam6p relocalization in MCC tumors is unknown; evidence suggests that in non-tumor MCV infections, LT targeting of Vam6p may regulate MCV replication [54].

The MCV early region mRNA also splices to produce a 57kT antigen (predicted size = 47kDa [14]) that is identical to large T protein but lacking an origin-binding domain. Similar multiply spliced T antigen isoforms occur in other polyomaviruses [14,17,59] and their functions remain poorly understood.

MCV sT is encoded by a read-through of the exon1-intron1 splice donor site [14]. In tissue sections of tumors, MCV sT is more commonly expressed than MCV LT antigen (Figure 3) [18]. Knockdown studies, however, reveal that both MCV sT and LT antigens are independently required for MCC tumor cell survival and proliferation [18,63] and both are likely to contribute to tumorigenesis.

In polyomaviruses, LT primarily target tumor suppressor pathways and sT activates Akt-mTOR signaling by binding to protein phosphatase 2A (PP2A) [64], a pathway which has been found to be critical for tumor cell survival in many types of genetic cancers [65]. For SV40, LT is a potent *in vitro* transforming oncoprotein while sT plays a supporting role and is not transforming alone [16,64,66]. In contrast, MCV sT is the primary transforming

oncoprotein *in vitro* while MCV LT has no effect in focus formation and soft agar assays [18].

SV40 sT acts to inhibit Akt dephosphorylation by binding the cellular protein phosphatase 2A (PP2A) A and C subunits while displacing its B subunit [64,67,68]. MCV sT similarly binds PP2A, but this interaction is dispensable for MCV sT-induced transformation [18]. MCV sT instead promotes hyperphosphorylation of 4E-BP1, a downstream target of mTORC1 kinase through interaction with unidentified cellular partner protein(s). MCV sT thus may be a useful tool to dissect cap-dependent regulation of 4E-BP1 in cancer signaling.

The MCV late region encodes 3 capsid proteins (VP1, VP2 and VP3), expressed after the onset of viral DNA replication. These structural proteins, when expressed in uninfected cells, self-assemble into a ~55-nm diameter icosahedral viral particles that can be harvested as antigen for serological assays [57,69]. MCV does not encode an agnoprotein [70,71] or VP4 [72] found in some polyomaviruses. Formally, little is known about the kinetics and regulation of MCV late gene expression because virus replication studies have been limited. Comparison of late gene expression for the MCV-HF molecular clone to a replication defective mutant clone suggests that MCV late gene expression depends on active DNA replication of the viral genome, analogous to late gene expression among large DNA viruses (e.g., herpesviruses) [54]. MCV encodes an miRNA, MCV-mir-M-5p that is generated from long RNAs transcribed late in infection [73,74]. It is antisense to early transcripts (regions 1217–1238) and may behave similar to the SV40 miRNA in negatively regulating early gene expression during late phases of virion encapsidation [75].

The NCRR region of MCV separates early and late gene regions and contains a core 71-bp origin sufficient to initiate DNA replication. This core sequence is comprised of an AT-rich tract involved in DNA melting and a region containing 8 GAGGC pentanucleotide sequences (PS) that are bound by the MCV LT origin-binding domain at the initiation of replication [14,50,76]. Four of these PS sites are absolutely required for virus replication [50], including a core of three PS that form an interacting helicase complex seen in crystallization studies [76]. Unlike SV40, but similar to JCV, MCV origin replication is highly activated by coexpression of MCV sT proteins [50,54]. Early evidence suggested that this may be due to sT sequestration of PP2A, but this has subsequently been shown to be PP2A-independent. The NCRR also contains bidirectional transcriptional promoters and regulatory elements for early and late viral gene expression.

MCV Epidemiology

Similar to most of the human polyomaviruses, MCV is a near-ubliquitous infection of adults. MCV seroassays based on late structural capsid protein VP1 reveal MCV prevalence of 60–80% in adults [69,77–79]. Both VLP-based EIA and neutralization tests demonstrate that conformational epitopes are important for the immunodominant antibody response after infection [57,69], and that assembled particles are generally a more sensitive serologic reagent than purified VP1 recombinant protein. Seroconversion to MCV IgG positivity is generally stable and antibodies can be detected for decades after primary infection [80]. Among persons with MCC, antibody titers to MCV VLP are significantly elevated giving evidence that an episode of viremia probably precedes tumor development [57,69].

Primary MCV infection, at least among adults, is generally asymptomatic [80]. MCV antibodies are detected in children with the prevalence of infection increasing with age [69,77,78,81]. In contrast to VLP, healthy adults do not generally have antibody responses to MCV T antigens [69,82], although T antigen antibodies can also develop in a subset of MCC patients and have been used to monitor tumor recurrence or dissemination [69,82].

Serologic and molecular studies indicate that MCV is a persistent and life-long infection [7]. MCV DNA is predominantly found in skin [7,61,83–85] but can be detected in a variety of tissues including, respiratory tract samples and nasopharyngeal aspirates [86–89], saliva [84], gut [27,90], lymphoid tissue [27,37], urine [91–94] and whole blood from healthy donors [44,90,93,95,96]. For this reason, PCR-based studies identifying MCV in tumors or other diseases require confirmation-using techniques less prone to experimental false positivity than PCR (e.g., immunohistochemistry, Southern blotting). Transmission is through a form of casual contact but the precise mode is not known.

MCV – a new human carcinogen

In Feng *et al.*'s original description of MCV, 8 of 10 tumors harbored MCV infection [6] and this has been confirmed through multiple studies worldwide. Of 2354 MCC tumors examined in various settings, 1743 (74.2%) were positive for MCV (Supplementary Table 1). Little is known about the cause of MCV-negative MCC—although low MCV VLP antibody levels in these patients makes a hit-and-run event by MCV seem unlikely. Further, careful examination of MCV-negative MCC often reveals differences in immunophenotype (e.g., CK20) and miRNA profiles (unpublished results) from MCV-positive tumors, making it likely that MCV-positive and MCV-negative MCC have different histogeneses.

Evidence is now abundant that MCV is a component of healthy skin flora that only rarely initiates tumorigenesis. What are the factors that promote transformation of this harmless agent into a cancer virus?

Immunity

Similar to other human tumor viruses, cell-mediated immune (CMI) surveillance is critical in suppressing Merkel cell carcinoma formation and AIDS, post-transplant and other immune-deficient populations are at increased risk for MCC [19,24]. The elevated risk among the elderly is also consistent with age-related loss of immune surveillance having a critical role in promoting MCC [32]. Tumor infiltrating lymphocytes are a common feature of MCV-positive tumors [97] and virus-specific CD8+ and CD4+ T cells have been isolated from MCC [98]. The immune defect contributing to MCC may be subtle, however, Iyer *et al.* have shown virus-reactive T cell responses for both MCC patients and healthy volunteers [98]. Reports of spontaneous MCC remission may reflect reconstitution of CMI against tumor antigens [99] and provides hope for adoptive immunotherapies in the treatment of this cancer.

Persistence and Loss of MCV Replication

MCV, when present, is nearly uniformly integrated into MCC genomes [6,49]. Whether integration occurs spontaneously or requires exogenous mutagenesis, such as UV exposure, is unknown. One possibility is that loss of immune surveillance allows active MCV replication, leading to nonhomologous recombination of genome replication fragments that generate the integrated virus in the proto-tumor cell. This is an appealing explanation for why MCV-positive MCC patients have high capsid antibody titers, but no direct evidence for this is currently exists.

Regardless of how viral integration occurs, expression of T antigen will lead to unlicensed viral DNA replication from a viral origin fused into the human genome—a potential catastrophe for the nascent tumor cell. Precise and independent mutations eliminating the T antigen replication capacity, without disturbing oncogenic domains, are also required for MCC cell survival. Each successive step in this evolutionary process—loss of immune surveillance, virus integration and T antigen mutation—are required for MCC formation but

are uncommon. Thus, rare tumors can emerge from infection with this common skin infection.

MCV Oncoprotein Expression

Knockdown experiments show that MCV LT and sT oncoproteins are needed for MCV-positive tumor cell survival and replication once MCV integrates. These experiments provide critical support for MCV being the causative agent for MCV positive MCC [18,62,63]. Research on how these proteins contribute to tumorigenesis has progressed rapidly because of the existing knowledge base gained from other polyomaviruses.

The importance of understanding the molecular causes for MCC is not limited to basic science. New MCV diagnostics help distinguish MCC from other closely related neuroendocrine cancers and may help predict the severity of the cancer when it does occur [27,37]. Even more importantly, these studies have prompted the search for fundamental changes in therapy for this difficult-to-treat tumor. Interferons are being explored to harness innate immune responses to this viral tumor [13]. Examination of cellular genes activated by MCV identified the *BIRC5* gene encoding survivin oncoprotein as being highly upregulated by MCV LT sequestration of RB. This in turn led to examination of a small molecule survivin inhibitor (YM155) as a potential therapy for MCV-MCC [11]. YM155 inhibits MCV-positive MCC growth at nanomolar concentrations whereas a screen of over other 1300 drugs, including those in the NCI Oncology Drug Set, revealed only one compound (bortezomib) having similar potency. Early MCC xenograft studies (Figure 4) reveal that YM155 prolongs survival of mice bearing MCC tumors [11]. An Eastern Cooperative Oncology Group trial is slated to open in late 2012 to test efficacy of survivin inhibition in MCC. Thus, MCC has progressed from being a cancer with no known etiology and “More deaths but still no pathway to blame” [30] to having rationally-targeted molecular therapeutic trials based on its viral etiology, in just four years.

The pace of MCV and MCC research has been rapid and is only growing faster. Speed records for research on virus discovery, viral oncogene studies, and “bench-to-bedside” research have been broken in the MCV field but it is still at a very early stage. Ever since the discovery of Epstein-Barr virus in 1964, discovery of each new human tumor virus has led to new and fundamental insights into carcinogenesis. MCV and related human polyomaviruses hold open the promise to continue this scientific tradition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MCV was discovered in 2008 by digital transcriptome subtraction and is one of seven new human polyomaviruses described in the past five years.
- Merkel cell polyomavirus (MCV), a new human polyomavirus, is clonally integrated in 70–80% of Merkel cell carcinoma (MCC) tumors.
- MCV is part of the normal, healthy skin flora but causes cancer after viral genome mutations eliminate its replication capacity.
- While similar to known polyomaviruses, MCV oncogenes act in new ways, such as activation of the survivin oncoprotein and PP2A-independent targeting of cap-dependent translation.
- In four years, the diagnosis and treatment potential for an intractable and enigmatic cancer has dramatically changed through discovery of the viral cause of MCC.

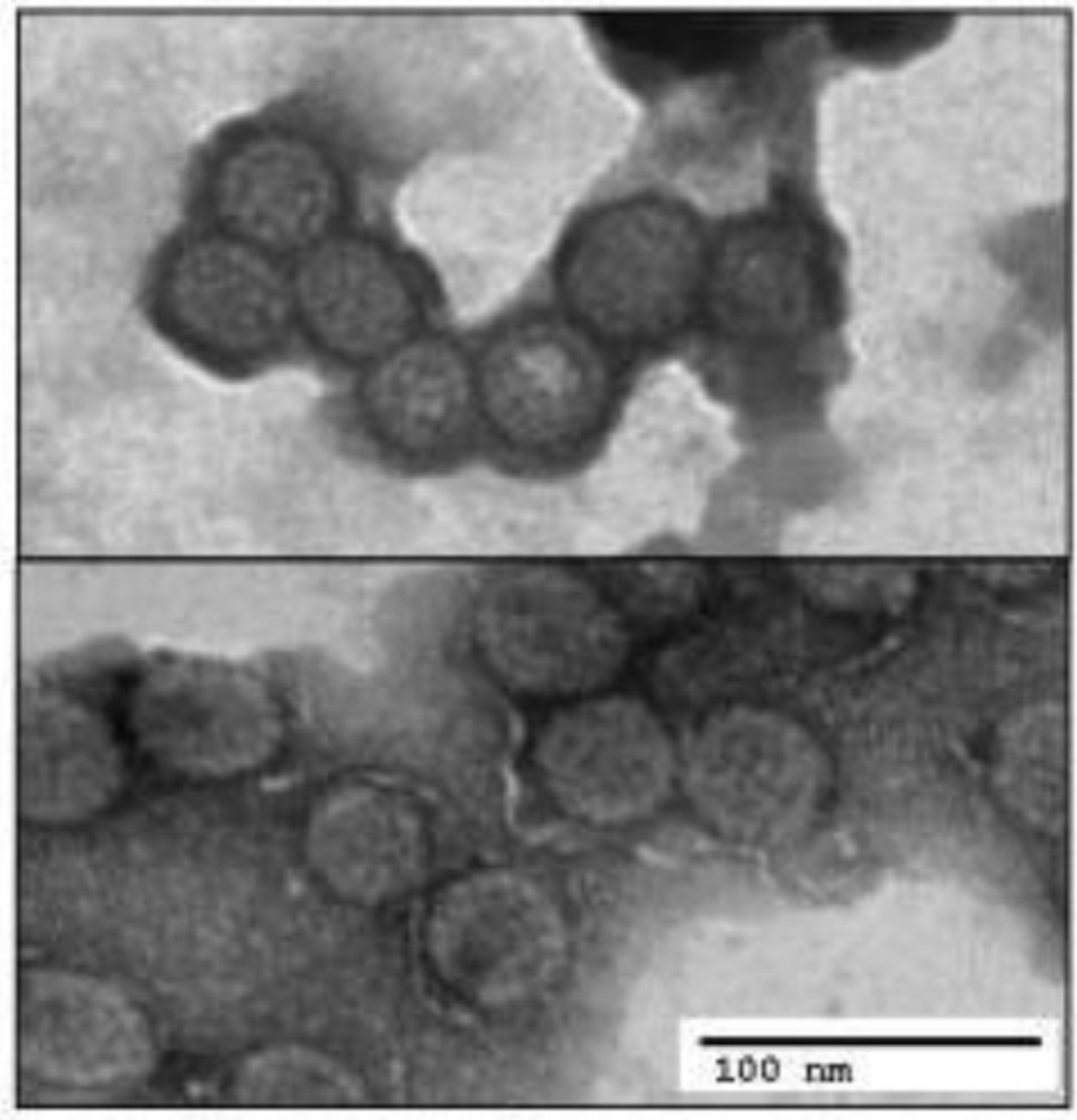

Figure 1. Merkel cell polyomavirus virions

Top panel shows typical Merkel cell polyomavirus particles produced by transfection of whole genome in 293 cells. In comparison, lower panel reveals assembled MCV virus-like particles (VLP), generated by expression of VP1 and VP2 genes alone, that can be used in serologic assays (Modfied from Feng *et al.*, PLoS one, 2011).

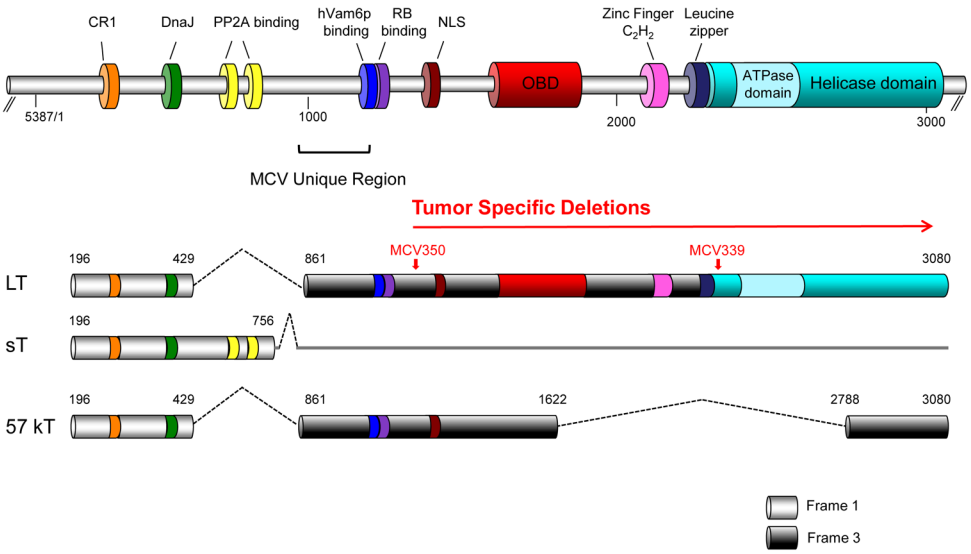

Figure 2. MCV T antigen locus

Three MCV T antigen isoforms are generated by alternative splicing of the T antigen gene: Large T (LT), small T (sT) and 57kT (amino acid positions, shown). Exon1 is common to all three T antigen proteins. Major conserved MCV T antigen motifs (top, base pair positions, shown) are in color (CR1, conserved region 1; DnaJ, Hsp70-binding conserved region; RB, retinoblastoma-binding; PP2A, protein phosphatase 2A-binding; NLS, nuclear localization signal; OBD, origin-binding domain). LT and 57kT encode a MCV-unique region (MUR) that includes the Vam6p/Vps39-binding motif. Tumor specific mutations (red arrow) occur C-terminal to the RB-binding domain and disrupt the helicase activity of LT but do not eliminate tumor suppressor binding domains. Locations for mutations for two MCV tumor strains (MCV350 and MCV339) are shown.

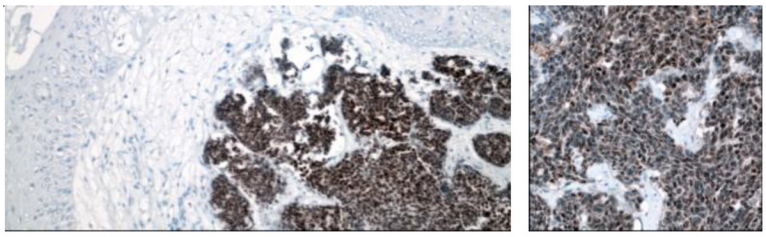

Figure 3. Merkel cell polyomavirus large T (left) and small T (right) antigen expression in MCC tumors

MCV large T antigen usually shows distinct nuclear expression in MCC cells (dependent on an intact nuclear localization signal that can be deleted in some tumors), while MCV small T antigen displays both nuclear and cytoplasmic staining patterns. Only tumor cells show strong positivity with antibody staining, and not the surrounding non-tumor tissues.

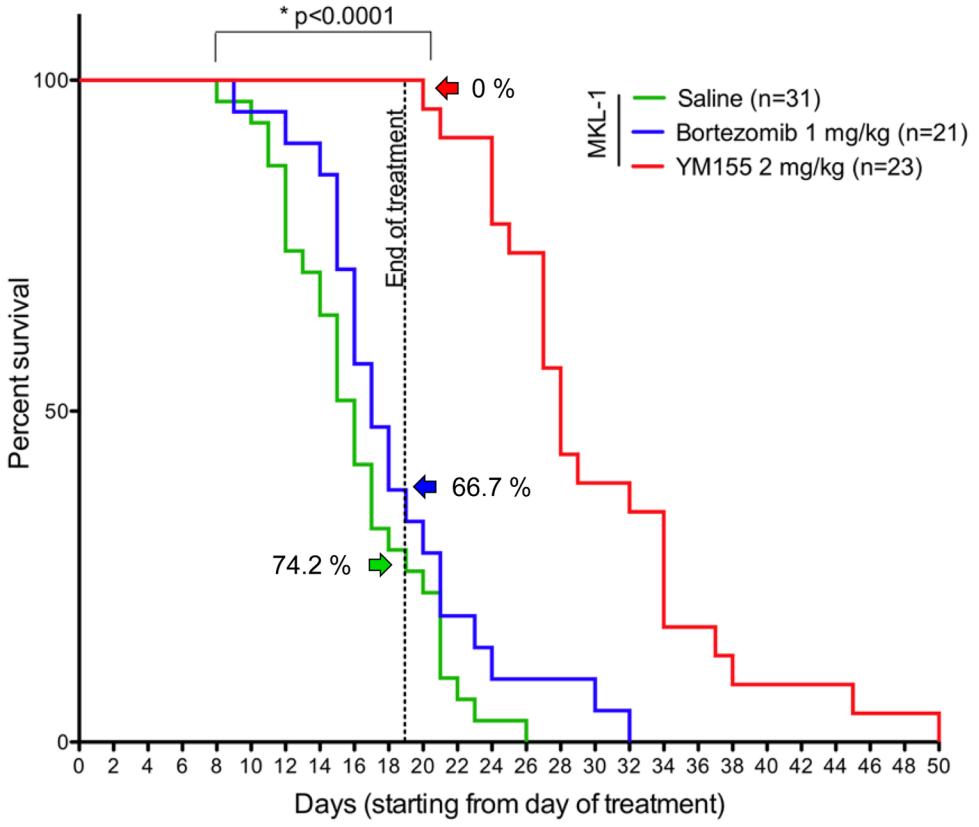

Figure 4. Survivin inhibition improves survival of mice bearing human MCC xenografts

Survival curves are shown for mice with MCC xenografts and treated with YM155 (red line), bortezomib (blue line) or saline (green line) for three weeks. MCV positive MKL-1 cells were injected into immune deficient mice and the three-week treatment was given once tumors became palpable. Only 26–33% of bortezomib/saline-treated mice survived three weeks after appearance of tumors while 100% of YM155-treated mice survived the treatment period. Tumors resumed growth once YM155 was discontinued indicating a cytostatic rather than cytocidal effect for YM155 with short-term treatment. (Modified from Arora *et al.*, STM, 2012)