

Human Polyomaviruses: The Battle of Large and Small Tumor Antigens

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ABSTRACT: About 40 years ago, the large and small tumor antigens (LT-Ag and sT-Ag) of the polyomavirus (PyVs) simian vacuolating virus 40 have been identified and characterized. To date, it is well known that all the discovered human PyVs (HPyVs) encode these 2 multifunctional and tumorigenic proteins, expressed at viral replication early stage. The 2 T-Ags are able to transform cells both in vitro and in vivo and seem to play a distinct role in the pathogenesis of some tumors in humans. In addition, they are involved in viral DNA replication, transcription, and virion assembly. This short review focuses on the structural and functional features of the HPyVs' LT-Ag and sT-Ag, with special attention to their transforming properties.

KEYWORDS: Polyomavirus, large T antigen, small T antigen, neoplasia

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Introduction

The Polyomaviridae family has been considered capable of transforming mammals' cells in vitro. In fact, since the first polyomaviruses were discovered in the 1950s, their tumorigenic properties observed in cell culture and animal models rendered the family name: "polyoma"-multiple tumors.¹⁻⁵ Despite the transformative abilities of simian vacuolating virus 40 (SV40) and murine polyomavirus (MPyV), the human BK polyomavirus (BKPyV)⁶ and human JC polyomavirus (JCPyV)⁷ infections often are asymptomatic and mostly related to nonneoplastic diseases in immunocompromised individuals. For instance, BKPyV has been associated with nephropathy (polyomavirus-associated nephropathy) in renal transplant recipients and hemorrhagic cystitis in allogeneic hematopoietic stem cell transplantation,⁸⁻¹⁰ whereas JCPyV is the causative agent of progressive multifocal leukoencephalopathy in HIV/AIDS and oncologic patients, along with those under immunomodulatory therapies.^{11,12}

Almost 40 years have elapsed since the discovery of the first 2 human polyomaviruses (HPyVs) until the third and fourth HPyVs identification took place, in 2007. The Washington University polyomavirus (WUPyV) and the Karolinska Institute polyomavirus (KIPyV) were detected in children with acute respiratory tract illness through molecular techniques, although their pathogenicity is still controversial.^{5,13-18} In 2008, Feng et al,¹⁹ using digital transcriptome subtraction, identified a polyomavirus clonally integrated in about 80% of Merkel cell carcinoma (MCC), a rare and aggressive neuroendocrine neoplasia and hence named the virus after the cancer. Several other research groups confirmed the Merkel cell polyomavirus (MCPyV) presence in MCC, although the viral prevalence in

MCC varies around the world.²⁰⁻³⁴ This HPyV was the first and, so far, the only HPyV to be etiologically related to a neoplasia in humans. Since its discovery, the scientific curiosity on polyomaviruses, especially regarding their relationship to human cancer, has been reignited. This resulted in new researches aiming for the identification of HPyVs in different biological samples through new sequencing technologies.³⁵⁻⁴¹ Consequently, there are 14 proposed HPyV species, but only 6 of them (BKPyV, JCPyV, MCPyV, HPyV-6, HPyV-7, and the trichodysplasia spinulosa – associated HPyV [TSPyV]) have been associated with human diseases.^{39,42,43}

To accommodate the fast growing number of new PyVs, an update in the family taxonomy was published in 2016. So far, the International Committee on Taxonomy of Viruses (ICTV) acknowledges 73 polyomavirus species, 13 of which are HPyVs, classified in 4 genera, according to their genomic properties and natural host.⁴⁴ A new nomenclature was proposed, grouping HPyV in 3 genera and using the official term "*Human polyomavirus*" followed by the viral discovery order, as follows: *Alphapolyomavirus* genus: MCPyV or HPyV-5, TSPyV or HPyV-8, HPyV-9, HPyV-12, NJPyV or HPyV-13; *Betapolyomavirus* genus: BKPyV or HPyV-1, JCPyV or HPyV-2, KIPyV or HPyV-3, and WUPyV or HPyV-4; and *Gammapolyomavirus* genus: HPyV-6, HPyV-7, HPyV-10, or MWPyV and HPyV-11 or TSPyV.⁴⁴ Despite the taxonomical effort made to encompass all described polyomaviruses, 3 unclassified viruses and a recently described HPyV have not been classified yet.^{41,44}

Human PyVs have a small, nonenveloped icosahedral capsid, presenting 72 pentameric capsomers formed mainly by



VP1 associated with minor structural proteins such as VP2 and VP3.⁴⁵⁻⁵⁰ Viral genome is formed by a double-stranded, histone-associated circular DNA functionally divided into 3 main regions: a noncoding control region, an early coding region, and a late coding region. Polyomaviruses have bidirectional genomes, and therefore, the early and late coding regions are in opposite strands.^{19,51-55} Most, if not all, of the PyVs' transforming capacities are derived from the expression of the early coding region, also known as tumor antigen locus. This region codes for multiple spliced transcripts that, despite the variations observed among HPyVs, generate 2 main proteins: the large tumor antigen (LT-Ag) and small tumor antigen (sT-Ag).⁵⁶⁻⁶⁰ These 2 T-Ags are involved in the coordination of viral replication and gene expression, as well as in the cell cycle progress and malignant transformation both in vivo and in vitro.⁵⁷⁻⁶⁵ Hence, this review presents the principal interactions of HPyVs' T-Ag involved in tumorigenesis.

Human Polyomaviruses' Large Tumor Antigen

The LT-Ag is a complex, multifunctional protein with many roles in viral replication and cell cycle progression. Due to its ability to replicate in and transform cell cultures, most of the knowledge about T antigens was obtained from SV40 studies,⁶⁶ which can be extrapolated to other PyVs.⁵⁵ Indeed, genetic and protein analyses have shown that LT-Ag virtually belonging to all HPyVs have several common domains: DnaJ domain, origin-binding domain (OBD), zinc (Zn)-binding domain, and helicase/ATPase (adenosine triphosphatase) domain^{55,66-68} (Figure 1, panels A to C). For viral DNA replication, 6 LT-Ag molecules interact as a hexameric structure through the recognition of 3 out of 4 pentanucleotide G(A/G)GGC (P1, P2, and P4) motifs in viral replication origin core, which also presents a highly conserved AT-rich region. This recognition is allowed by multiple and complex interactions between OBD, pentanucleotide motifs, GpC dinucleotides, Zn-binding domain, AT-rich region, and histidine residues in the helicase/ATPase.⁶⁹⁻⁷² Two hexameric LT-Ag molecules unwind the viral genome in both directions through ATP hydrolysis, followed by the binding of the eukaryotic replication protein A at the single-stranded viral DNA. The cellular topoisomerase I then releases the torsional stress, whereas α -primase and DNA polymerase initialize the viral DNA synthesis.^{55,72-76} This replicative role of LT-Ag is frequently seen in lytic infections and can be abrogated in neoplastic tissues.

HPyV integration and LT-Ag truncation

As mentioned previously, the first HPyV associated with human cancer, MCPyV, was discovered clonally integrated in about 80% of Merkel cell carcinomas. The viral integration into the cellular genome is a well-known event that may lead to cancer through several pathways, such as functional loss or gain in cell cycle regulation genes, viral activation of human gene

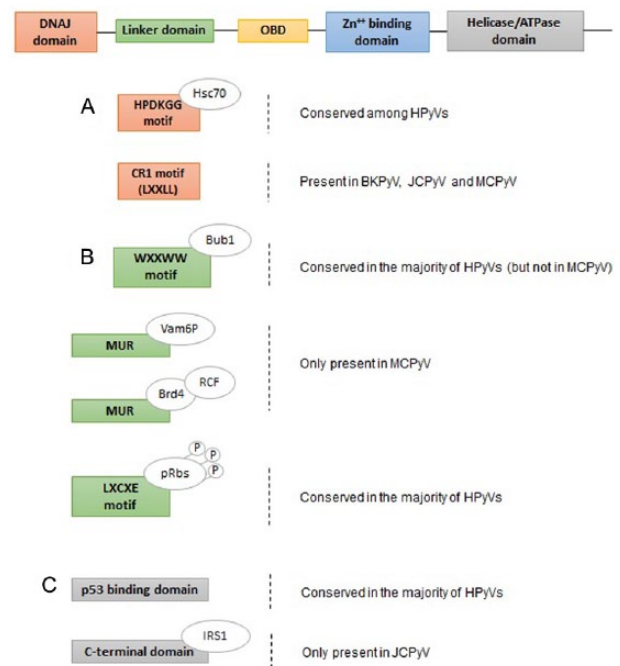


Figure 1. Scheme of the functional domains of the HPyVs' large tumor antigen (LT-Ag). The LT-Ag consists of several functional domains: DnaJ domain, linker domain, origin-binding domain (OBD), zinc (Zn^{++})-binding domain, and helicase/ATPase domain. (A) The DnaJ domain contains a HPDKGG motif, conserved among HPyVs, which is able to bind to the Hsc70, a cellular chaperone and transcriptional repressor; a CR1 motif (LXXLL), present only in BKPv, JCPyV, and MCPyV. (B) The linker domain contains WXXWW motif, conserved in most of the HPyVs but not in MCPyV, for binding Bub1, a mitotic checkpoint serine-threonine protein kinase; a unique region (MUR), only present in MCPyV, that binds the Vamp6P-protein and the Bromodomain protein 4 for the recruitment of cellular protein factor C (RFC); a LXCXE motif, conserved in most of the HPyVs, crucial for the interaction with the retinoblastoma protein (pRb) family. (C) The helicase/ATPase domain comprises a p53-binding domain, conserved in most of the HPyVs; a C-terminal domain, only present in JCPyV, that binds the insulin receptor substrate 1 (IRF1). ATPase indicates adenosine triphosphatase; BKPv, human BK polyomavirus; HPyVs, human polyomaviruses; JCPyV, human JC polyomavirus; MCPyV, Merkel cell polyomavirus; MUR, MCPyV T antigen unique region.

promoters, expression of viral oncogenes, and acquisition of mutational profile both in human and viral genes.⁷⁷ In fact, LT-Ag premature truncation in MCC samples is considered as a MCPyV tumor-specific signature because the LT-Ag from nontumoral samples does not harbor LT-Ag-truncated forms. Hence, it is suggested that after MCPyV integration into the cellular genome, the infected cells undergo selection of LT-Ag mutations to prevent viral replication and the viral Ori bidirectional unwinding, which would lead to collision with cellular replication forks.⁵⁷ In 2013, Li et al⁷⁸ showed that the intact helicase/ATPase domain activates DNA damage response and enhances p53 phosphorylation and cell cycle arrest, which corroborates the proposed explanation for the need of LT-Ag truncation for MCPyV tumorigenesis. Moreover, LT-Ag truncation mutations are unequivocally observed downstream the

retinoblastoma protein (pRb)-binding domain, occurring near to the OBD and helicase/ATPase domains. Therefore, a critical role for either pRb-binding domain or sT-Ag preservation in MCC pathogenesis has been suggested.⁶²

Although a strong causal association between other HPyVs with human tumors has not been confirmed yet, circumstantial data suggesting their transformation properties are growing. In fact, a recent meta-analysis revealed that the BKPyV prevalence was significantly higher in prostate cancer (PCa) tissues than in the control, considering as "PCa tissues" samples ranging from very early onset of PCa to well-defined tumors and, as controls, non-PCa samples from patients with PCa or benign prostate hyperplasia.⁷⁹ There are also evidence linking BKPyV to bladder carcinoma in both immunocompetent⁸⁰ and renal transplant recipients, as well as in urothelial and renal carcinomas.^{5,77,81-83}

Furthermore, BKPyV DNA has been found integrated into a limited number of brain tumor,⁸⁴ urothelial carcinoma,⁸⁵ and, more recently, a case of renal allograft tumor.⁷⁷ The BKPyV genome was found linearized and inserted into human chromosome 12 in a high-grade urothelial carcinoma from the allograft after almost 10 years of uneventful renal transplant. Viral genome sequencing revealed a new, undescribed variant of BKPyV subtype 1A, which was named Chapel Hill tumor-associated polyomavirus 1 (or CH-1).⁷⁷ Similarly, the same group reported another undescribed variant of BKPyV 1A subtype, named Chapel Hill 2 (or CH-2) integrated into human chromosome 2 in a poorly differentiated renal cell carcinoma.⁸³ Kenan et al⁸³ suggest that BKPyV integration into host genome after linearization disrupts the expression of late viral transcripts, which would impair LT-Ag downregulation and could eventually lead to tumorigenesis.

Moreover, JCPyV genome has been found integrated into various brain tumors in animal models such as mice and owl monkeys.^{11,86,87} Viral integration might also happen in human neoplasia, especially in colorectal cancer,^{88,89} in which JCPyV is considered a cofactor for chromosomal instability.^{90,91} Interestingly, most of the BKPyV and JCPyV integration studies did not report a truncated LT-Ag. Instead, the viral genome was disrupted at the VP1 region, although replicative impairment has also been observed.^{77,82,83} Nonetheless, unregulated LT-Ag expression, loss of late protein expression, and replicative arrest occurred following viral integration.⁸³

Although the role of BKPyV integration in human oncogenesis is still hazy, MCPyV integration into host genomes and its involvement at least in MCC carcinogenesis have been better characterized. Still, the low frequency of MCPyV integration in non-MCC tumor has raised attention. Pantulu et al⁹² identified LT-Ag truncation mutations in 4 chronic lymphocytic leukemia cases, which might indirectly testify the viral integration. In addition, the deletion of a 90-pb fragment in the MCPyV VP1 gene in nonmelanoma skin cancer may also indicate incomplete viral integration.⁹³ Thus, the real role of

low-frequency HPyVs' integration into human cancers is yet to be established.

HPyV LT-Ag domains for cellular protein interaction

Large tumor antigen contains several interaction domains with cellular proteins involved in cell cycle regulation. Some of these motifs are well conserved and most of them facilitate viral replication through activation of cellular proliferation and phase S entry, resulting in upregulation of enzymes involved in cellular DNA replication, DNA damage response, and accessory replicative enzymes.^{55,65,66} Indeed, LT-Ag expression itself induces viral replication and cellular transformation in animal models and in cell cultures, under specific conditions.^{62,64,66,79,91-96} The first exon of LT-Ag (approximately 1-70 amino acids, shared with sT-Ag) contains the DnaJ domain, which has a HPDKGG (His-Pro-Asp-Lys-Gly-Gly) motif conserved among HPyV. The DnaJ domain is able to bind to the Hsc70, a cellular chaperone and transcriptional repressor. Human PyVs' ability to replicate viral DNA in vivo and to promote cell transformation depends on the intact DnaJ domain, as well as capsid assembling in vivo.^{55,68,97-99} In fact, the MCPyV LT-Ag interaction between its DnaJ domain and Hsc70 has been shown to be necessary for viral replication in vitro.^{65,68} Moreover, DnaJ domain binding to Hsc70 promotes the chaperone ATPase activity, generating enough energy to dissociate pRb/E2F.^{55,100-102} The HPyVs, BKPyV, JCPyV, and MCPyV, also contain a CR1 motif, formed by the pentapeptide LXXLL (Leu-X-X-Leu-Leu), which apparently has an auxiliary role in pRb/E2F disruption and cell proliferation.^{100,103}

The LT-Ag linker domain is located downstream to the DnaJ domain. Almost all HPyVs (JCPyV, BKPyV, WUPyV, KIPyV, TSPyV, HPyV-6, HPyV-7, HPyV-9), but not MCPyV, present a conserved motif WXXWW (Trp-X-X-Trp-Trp) that can bind to the Bub1, as demonstrated for SV40 LT-Ag.^{66,104} Bub1 is a mitotic checkpoint serine-threonine protein kinase that, when functionally impaired, may result in chromosomal instability, as observed in cells expressing SV40 LT-Ag.¹⁰⁵ Although MCPyV is oncogenic, it does not present the Bub1-binding motif. Thus, it is not conclusive whether Bub1:LT-Ag interaction is relevant for HPyVs' transformation in vivo. Instead, at the nucleotide position, where other HPyVs encode Bub1-binding domain, MCPyV has a sequence with little similarity with other PyVs, denominated unique region (MUR [MCPyV T antigen unique region]).^{65,103} The MUR contains a minimal fragment (171-218 nucleotides), the Vam6P-binding domain, that sequesters this cytoplasmic protein to a nuclear location, resulting in lysosome clustering impairment, without disruption of transforming growth factor β or mTOR (mammalian target of rapamycin) signaling pathways.¹⁰³ The LT-Ag interaction with Vam6P seems to be regulatory, as its loss enhances viral

replication; consequently, it is considered a mechanism for persistent infection establishment.^{65,94,95}

Another interesting MUR interaction was observed for other known oncoviruses, such as the bovine papillomaviruses (BPVs). Wang et al¹⁰⁶ investigated the interaction between LT-Ag and bromodomain protein 4 (Brd4), a member of bromodomain and extra-terminal family involved in cellular growth control, cell cycle progression, and cancer development. The Brd4 interacts with BPV's early protein 2 (E2) promoting the rightful partition of viral episomes to daughter cells during mitosis.^{106,107} During the MCPyV infection, a similar interaction between LT-Ag and Brd4 is observed, promoting the MCPyV DNA replication through the cellular protein factor C (RFC) recruitment to viral Ori.¹⁰⁶ In addition, recent studies have shown that the human papillomavirus (HPV) are able to interact with Brd4, which is implicated in viral replication and E2 transcriptional activation function as the use of Brd4 inhibitor reduces HPV transcription.^{108,109} Likewise, the use of a specific peptide, named Brd4 410 to 730, which is homologous to the same region of the Brd4 but without other domains, successfully inhibited MCPyV replication in vitro as it disrupts Brd4:LT-Ag interaction.¹⁰⁶

The JCPyV LT-Ag is also able to bind the cellular β -catenin, a cellular protein belonging to the Wnt pathway, important for tissue development, polarity, differentiation, and cell cycle control through cell-cell contact. When hypophosphorylated, β -catenin complexes with LEF-1/TCF-4 transcription factors and migrates to the nucleus, where it promotes cell cycle progression through c-myc and cyclin D1 expression. Phosphorylated β -catenin undergoes degradation via ubiquitin-dependent proteasome through the activation of a complex formed mainly by the glycogen synthase kinase-3 (GSK-3), Axin scaffold proteins, and adenomatous polyposis coli (APC) protein, which phosphorylates β -catenin.^{91,110-112} In this context, JCPyV promotes β -catenin stabilization through an LT-Ag central domain, which comprehends amino acid residues from 82 to 628. Besides, this interaction increases β -catenin levels and promotes its nucleic localization, with subsequent enhancement of c-myc expression.^{61,113} Although the JCPyV LT-Ag: β -catenin interaction was also described in mouse medulloblastoma¹¹⁴ and in glioblastoma cell lines,¹¹⁵ it has been recently described in human colorectal carcinoma (CRC), in which β -catenin and Wnt pathway are frequently increased (Figure 2, panel C). Co-localization of JCPyV LT-Ag and β -catenin into the tumor cell nucleus, as well as c-myc and cyclin D1 activation in a subset of JCPyV-positive CRC has been described. This suggests a role of JCPyV in CRC pathogenesis, although JCPyV detection is frequent in both normal colorectal and CRC tissues.^{91,116} The JCPyV LT-Ag also interacts with the insulinlike growth factor/insulin receptor substrate 1 (IGF/IRS1) pathway, which has been proposed as a mechanism for malign transformation in medulloblastoma. It has been shown that JCPyV LT-Ag binds IRS1 through its

C-terminal domain.¹¹⁷ The LT-Ag:IRS1 promotes the IRS1 nuclear translocation, followed by IRS1 interaction with Rad51, which indirectly and eventually prevents DNA damage response⁹⁰ (Figure 1, panels A to C).

Despite its recent discovery, it is already known that MCPyV is involved in different molecular regulatory mechanisms. For example, MCPyV detection in non-small-cell lung cancer (NSCLC) was found to deregulate BRAF and Bcl2: the first is involved in cell cycle progression through mitogen-activated protein kinase (MAPK) signaling pathway and was upregulated in MCPyV-positive samples; the second is an antiapoptotic mitochondrial protein that was downregulated in MCPyV infection. These findings were suggestive of MCPyV-mediated deregulation in NSCLC, mainly in smoker patients.¹¹⁸ Furthermore, another NSCLC study reported a significant association between MCPyV DNA detection and LT-Ag expression with mutation on epithelial growth factor receptor mainly in nonsmoker patients, supporting the hypothesis of MCPyV's participation in a subgroup of NSCLC oncogenesis.¹¹⁹

A similar study investigating PIK3's mutational profile showed a higher frequency of PIK3 mutations in MCPyV-positive MCC than in MCPyV-negative MCC, although the Akt/mTOR expression was higher in MCPyV-negative tumor, supporting the employment of different mechanisms for MCC oncogenesis in distinct MCPyV backgrounds.¹²⁰ Furthermore, the expression of tumor-derived MCPyV T-Ag in mice-stratified epithelium promotes gross epithelial phenotypes, consistent with neoplastic progression comparable with those observed for high-grade HPV-16 E6 and E7 oncoproteins. Further molecular studies demonstrated the LT-Ag interaction with BIRC5 survivin and increased E2F target gene expression, being both the pathways mediated by LT-Ag LXCXE motif.¹²¹

Finally, BKPyV LT-Ag and other HPyV LT-Ag have been recently found in the upregulation of the DNA cytosine deaminase APOBEC3, an important innate immune system enzyme responsible for suppressing viral replication and cancer. Evidence suggest that APOBEC3 may be correlated with cytosine-based mutation patterns and in coincident sites of DNA rearrangement. As APOBEC3B was also upregulated in HPV-related cancers, it is suggested that HPyV LT-Ag upregulation may also contribute to carcinogenesis through host genomic mutations.¹²²

HPyV LT-Ag binding with pRb

Indubitably, one of the most important LT-Ag interactions is that with pRb family^{55,66} (Figure 2, panel A). This tumor suppressor family comprises 3 main proteins, pRb, p107, and p130, encoded by the RB1, RBL1, and RBL2 genes, respectively. Although the pRb can regulate the cell cycle through many pathways, its interaction with the E2F transcription factors family is one of the most important, as its loss potentiates

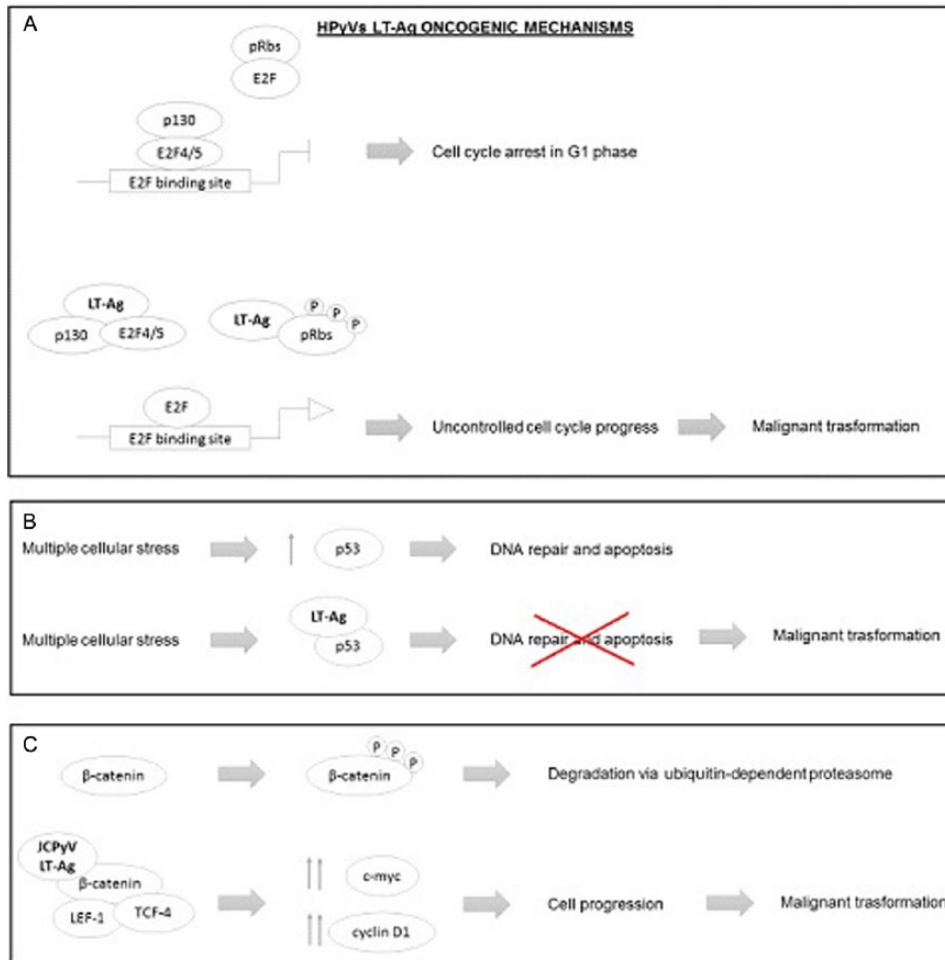


Figure 2. HPyVs' large tumor antigen (LT-Ag) oncogenic mechanisms. (A) In physiological conditions, the retinoblastoma proteins (pRbs) are in a hypophosphorylated state, which allows them to bind and inhibit the E2F transcription factors, preventing the E2F-mediated gene expression and consequently the transition from G₁ to S phase. HPyVs' LT-Ag is able to bind the pRbs promoting their hyperphosphorylation, thus pRb is unable to bind E2F, leading to its transcriptional activity; at the same time, the hyperphosphorylation of p130 disrupts the transcriptional repressor complex (p130-E2F4/5), leading to uncontrolled cell cycle progression and sometimes to malignant transformation. (B) Multiple cellular stress, normally, raises the levels of p53, which promotes the DNA repair and cell cycle arrest. HPyVs' LT-Ag is able to bind and block the activity of p53 protein, preventing apoptosis and cell cycle arrest induced by DNA damage. (C) In physiological conditions, the phosphorylated β -catenin undergoes degradation via ubiquitin-dependent proteasome. JCPyVs' LT-Ag binds the β -catenin protein promoting its hypophosphorylation, thus β -catenin complexes with LEF-1/TCF-4 transcription factors, promoting the cell cycle progression by c-myc and cyclin D1 expression. HPyVs indicate human polyomaviruses; JCPyV, human JC polyomavirus.

tumorigenesis.^{123,124} In a hypophosphorylated state, pRb binds the E2F transcription factor, preventing the E2F-mediated gene expression and thus promoting cell cycle arrest. During normal cell cycle progress, activation of cyclin-dependent kinases (CDKs) leads to hyperphosphorylation of serine and threonine residues at pRb phosphorylation-specific sites resulting in the release of the E2F transcriptional factors; their transcriptional activity promotes the cell cycle progress from G₁ to S phase.¹²⁵ The components of the pRb family are also known as “pocket proteins” because of a conserved “pocket” domain that interacts with viral oncoproteins, notoriously the SV40 LT-Ag and HPV E7.¹²⁶

The HPyV LT-Ag, as observed in SV40 LT-Ag prototype, has a highly conserved pRb-binding domain in its linker region. The LXCXE (Leu-X-Cis-X-Glu) motif is responsible for the LT-Ag:pRb family interaction. It has been

hypothesized that the energy provided by the LT-Ag DnaJ domain binding to Hsc70 is used to disassociate pRb/E2F through pRb phosphorylation, as corroborated by studies showing the DnaJ domain requirement for the SV40 LT-Ag growth activities.¹²⁷⁻¹²⁹ Large tumor antigen is also capable of disrupting the transcriptional repressor complex formed by p130 and the repressive E2F4/5 proteins through p130 dephosphorylation in a DnaJ domain-dependent pathway.^{66,91,130} The overall pRb:E2F disruption leads to uncontrolled cell cycle progress and, eventually, to malignant transformation. In fact, the pRb pathway deregulation is frequently observed in many tumors.^{127,128,131}

The SV40 LT-Ag interactions with pRb, p107, and p130 cause well-known transformative effects in cell culture and animal models and have been recently reviewed.⁶⁶ However, HPyV LT-Ag also mediated cell transformation through pRb

family binding. For instance, JCPyV LT-Ag binds the hypophosphorylated pRb in hamster glioblastoma cells and this interaction successfully disables cell transformation, although JCPyV LT-Ag might be less efficient in cell transformation than SV40 LT-Ag.¹³² Moreover, JCPyV LT-Ag might alter pRb expression pattern and cellular distribution in mouse primitive neuroectodermal tumors (PNET) and human medulloblastoma (HMB) cell lines, suggesting a cell-type-specific LT-Ag-mediated tumorigenesis.¹³³

The BKPyV LT-Ag can significantly reduce pRb, p107, and p130 levels and increase transcriptionally active E2F, requiring intact LT-Ag DnaJ and pRb-binding domains to induce cell cycle deregulation.^{101,134} Furthermore, BKPyV LT-Ag interaction with pRb and consequent E2F release leads to DNA methyltransferase 1 (DNMT1) gene expression, one of the E2F target genes.¹³⁵ This enzyme adds methyl residues on cytosine located 5' to guanosine and is involved in cellular expression control, as methylation significantly reduces gene transcription.¹³⁵ Thus, DNMT1 expression promoted by BKPyV LT-Ag probably induces the inheritable silencing of tumor suppressor genes and might contribute to carcinogenesis.¹³⁵

Likewise, MCPyV LT-Ag contains LXCXE motif and interacts with pRb family proteins.⁵⁷ In fact, this natural interaction is the decisive evidence for LT-Ag tumorigenesis pathway through pRb family, as pRb-binding domain is systematically maintained in MCPyV-positive MCC while the LT-Ag C-terminal is truncated.⁵⁷ Moreover, truncated LT-Ag is more efficient in transforming cells than full-length LT-Ag, mainly due to its LXCXE motif and to the disruption of the C-terminal region inhibitory activity on cell growth.⁹⁴ The MCPyV LT-Ag C-terminal inhibitory activity on cell growth might be mediated by the phosphorylation of an LT-Ag serine residue at the position 816, stimulated by the ATM pathway activation. This phosphorylation site might represent a negative selection mechanism to eliminate functional and growth-inhibitory C-terminal during tumorigenesis, which also preserves pRb domain.¹³⁶ In vitro studies showed that the ablation of MCPyV LT-Ag:pRb would lead to tumor regression, suggesting that the LT-Ag:pRb interaction is critical for cell proliferation and sustained tumor growth.¹³⁷ Also, a new interaction for MCPyV LT-Ag was observed when Arora et al.¹³⁸ found that the cellular oncoprotein survivin BIRC5a, an antiapoptotic protein, was 7-fold more expressed in MCPyV-positive MCC than in MCPyV-negative MCC. This study also identified the need for an intact pRb-binding domain for the direct LT-Ag binding to BIRC5a gene promoter and hence proposed the survivin as a therapeutic target for MCC.¹³⁷ However, it was already known that the HPyV LT-Ag:survivin interaction might promote cell survival, as observed in oligodendrocyte and astrocyte cell lines infected in vitro and expressing LT-Ag.⁹¹

The LT-Ag:pRb interaction has also been studied in the newly discovered HPyVs. The TSPyV LT-Ag mediates cells

cycle progression in trichodysplasia spinulosa cases through pRb interaction. Kassem et al.¹³⁹ showed evidence of TSPyV LT-Ag clusters with phosphorylated pRb by histologic immunofluorescence and concluded that LT-Ag:pRb may induce cell proliferation as a potential driver of papule and spicule formation. In contrast, the MWPyV LT-Ag is less stable and has not been yet associated with transforming abilities in vitro, although it is able to bind to pRb, p107, and p130.^{139,140} Likely, HPyV-7 LT-Ag expression in thymic epithelial tumors does not correlate with pRb expression,¹⁴¹ although HPyV detection and expression in thymomas were relatively frequent (62%).¹⁴²

HPyV LT-Ag binding with p53

Finally, another important LT-Ag interaction in HPyV-mediated tumorigenesis is that with p53, a tumor-suppressing protein that regulates the gene expression in response to stressful events such as DNA damage, leading to cell apoptosis, cell cycle arrest, or senescence and is usually deregulated in cancer.¹⁴³ Large tumor antigen of the PyVs contains a p53-binding site on the helicase/ATPase domain. During SV40 carcinogenesis, LT-Ag binds and blocks p53 activity, preventing apoptosis and cell cycle arrest, induced by DNA damage, derived from pRb:E2F disruption. Instead of inducing p53 degradation through ubiquitinase, as seen for HPV E6 protein, SV40 LT-Ag stabilizes p53, which is sufficient for the abrogation of its transcriptional activities⁶⁶ (Figure 2, panel B).

The BKPyV LT-Ag also complexes with p53 and, in BKPyV-positive renourinary tumors, such association is considered an important oncogenic mechanism. The complex formation also promotes p53 kidnapping to cytoplasm, which is observed in both infected and transformed cell lines. Even if the sequestration is not a requirement for p53 inhibition, the p53 cytoplasmic accumulation correlates with an increased cellular mutational profile in LT-Ag-expressing cells and is considered the main hallmark of BKPyV involvement in PCa development.¹⁴³ In addition, the preservation of the LT-Ag:p53-binding domain observed in tumors with BKPyV integration is suggestive for the LT-Ag:p53-mediated oncogenesis.

In the same way, as observed for the p53 cytoplasmic sequestration mediated by BKPyV LT-Ag, JCPyV LT-Ag is able to alter p53 expression and its cellular distribution in PNET and HMB cell lines.¹³³ A recent study conducted on paraffin-embedded specimens reported a dramatic decrease in p53 expression in JCPyV-positive glioblastomas when compared with JCPyV-negative glioblastomas, suggesting a potential role of JCPyV LT-Ag in p53 expression modulation and tumorigenesis.¹⁴⁴

Nevertheless, MCPyV-mediated MCC tumorigenesis does not require p53 inactivation. In fact, p53 disruption in MCC is MCPyV LT-Ag independent, as demonstrated by Houben et al.¹⁴⁵ This experimental study corroborates with other

previously published papers, showing that the C-terminal region of MCPyV LT-Ag mediates cell cycle progress inhibition,⁹⁴ and that the truncated LT-Ag has a higher transformation potential compared with the full-length LT-Ag.⁶² Finally, these findings are consistent with the MCPyV tumor-specific signature in MCC, where the truncated LT-Ag lacks the helicase/ATPase and p53-binding domain.⁵⁷

Human Polyomaviruses' Small Tumor Antigen

The sT-Ag is another PyV protein with cell transformation properties. Although sT-Ag expression is not always a condition for viral replication or transformation, it is required for PyVs' optimal functioning.^{68,146} The SV40 sT-Ag is functionally comparable with HPV E7, in such a way that E7 is capable of successfully replacing the functions of sT-Ag in vitro.¹⁴⁷ The PyV sT-Ags are encoded in the early region of PyVs' genomes, superposed to the LT-Ag coding sequence. Produced by an alternative splicing of this region, sT-Ag shares about 80 amino acids in its N-terminus with LT-Ag.⁵⁵ Therefore, the sT-Ag presents the DnaJ domain with a conserved HPDKGG motif among all HPyVs, which potentially affects viral replication, cell proliferation, and transformation, although the functional importance of this domain for sT-Ag activity is unknown.^{65,148-150} The BKPyV, JCPyV, and SV40 sT-Ag share high amino acid similarities with each other, especially on the N-terminus, although the sT-Ag middle region is more divergent among HPyVs,¹⁵¹ and its hydrophobicity and flexibility are important for sT-Ag functionality.¹⁵²

The sT-Ag transcript also contains a unique region, removed from LT-Ag during the intronic processing, which may have distinct features among HPyVs. For instance, JCPyV sT-Ag contains a LYCKE and a LHCWE motif and BKPyV has only one LYCKE motif. Thus, these LXCXE motifs are potentially able to interact with pRb family proteins, whereas MCPyV sT-Ag does not present any LXCXE motif, as well as the other HPyVs.^{55,153} Regardless, all HPyVs' sT-Ags have 2 conserved domains at their unique C-terminus region: 2 zinc-binding domains (Cys-X-Cys-X-X-Cys motif), which provide structural and functional stabilities for sT-Ag, and 2 domains for phosphatase 2A (PP2A), rich in cysteine and proline residues responsible for sT-Ag:PP2A interaction, that require the sT-Ag DnaJ domain and the second zinc-binding motif^{55,151} (Figure 3, panels A to C).

HPyV sT-Ag binding with PP2A

The PP2A is a serine-threonine phosphatase that regulates, among many other important cellular processes, the cell cycle progression and apoptosis by dephosphorylating protein targets such as Akt, p53, c-Myc, and β -catenin and hence is considered a tumor-suppressing protein. Phosphatase 2A is composed of catalytic (PP2Ac), scaffold (PP2AA), and regulatory subunits (PP2AB) that interact to form an active enzymatic complex.¹⁵⁴ The SV40 sT-Ag prototype has a

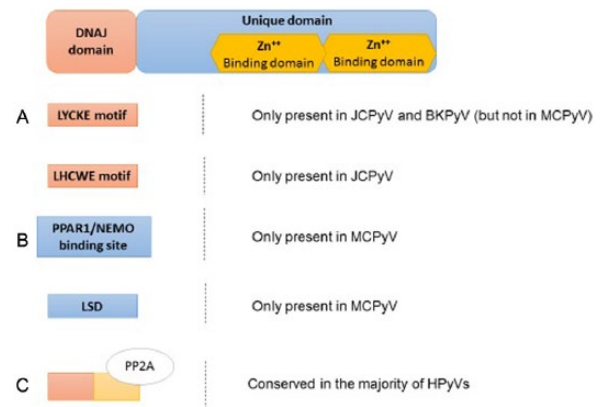


Figure 3. Scheme of the functional domains of the HPyVs' small tumor antigen (sT-Ag). The sT-Ag presents a DnaJ domain, followed by a unique domain, that is removed from LT-Ag during the splicing process. (A) The DnaJ domain contains a LYCKE motif (JCPyV and BKPyV) and a LHCWE motif (only JCPyV), able to interact with pRb family proteins. (B) The unique domains contain 2 Zn⁺⁺-binding sites and, additionally and only in MCPyV, a PPAR1/NEMO-binding site and a large T stabilization domain (LSD) that are involved in the oncogenesis process. (C) The binding site for the PP2A is conserved in most of the HPyVs and triggers several pathways related to cellular transformation. BKPyV indicates human BK polyomavirus; HPyVs, human polyomaviruses; JCPyV, human JC polyomavirus; MCPyV, Merkel cell polyomavirus; NEMO, NF- κ B essential modulator.

cysteine-proline-rich and conserved domain (Cys-X-X-X-Pro-X-Cys) that interacts with PP2A, mainly through the sT-Ag cysteine residues.^{55,151} In fact, the ablation of only one cysteine from CXXXPXC motif dramatically reduces sT-Ag:PP2A-binding rate.¹⁵⁵ The sT-Ag interacts with the PP2AA (scaffold) subunit by the HEAT repeats 3 to 7, displacing the PP2AB (regulatory) subunit that also binds HEAT repeats 3 to 7, and thus inhibiting PP2AAc phosphatase activity¹⁵² (Figure 4, panels A and B).

The sT-Ag:PP2A interaction triggers several pathways related to cellular transformation. The entry on cell cycle S phase may be stimulated by sT-Ag:PP2A, as PP2A no longer dephosphorylates the CDK inhibitor p27, leading to its degradation. In addition, sT-Ag:PP2A also induces the S phase entry by promoting cyclin A/CDK2 and cyclin E/CDK2 expression through a cell cycle-regulated E2F site.^{156,157} Furthermore, sT-Ag:PP2A has been linked to human fibroblast transformation by CDK2 activation and DNA synthesis induction.¹⁵⁸

The SV40 sT-Ag:PP2A interaction might induce oncogenesis in vitro through the activation of the MAPK cascade.¹⁵⁹ The MAPKs are involved in cell proliferation via the ERK1 and ERK2 (extracellular signal-related kinases 1 and 2) upregulation of cyclin D1, leading to phosphorylation of pRb and the subsequent releasing of the E2F transcription factor. The MAPK pathway activation has been linked to increased BKPyV replication in cell cultures, although induced by LT-Ag.¹⁶⁰ However, Wu et al¹⁶¹ demonstrated that TSPyV sT-Ag overexpression activates the MAPK pathway by

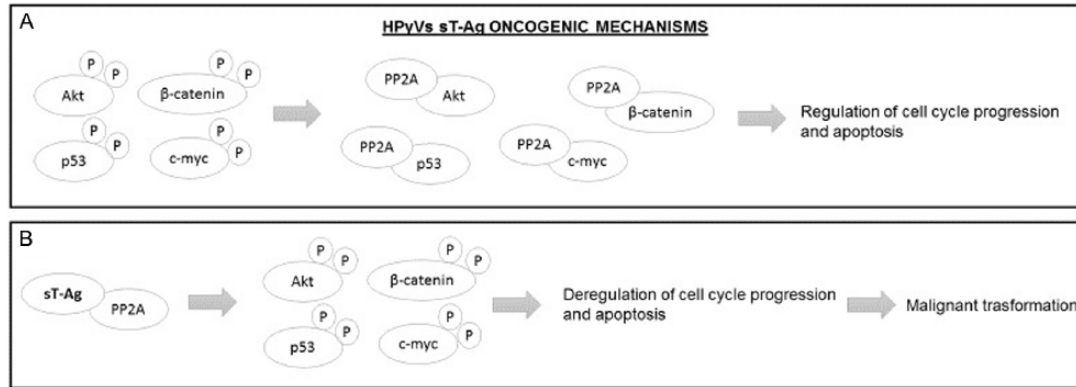


Figure 4. HPyVs' small tumor antigen (sT-Ag) oncogenic mechanisms. (A) In physiological conditions, the Akt, p53, c-Myc, and β -catenin proteins are in a phosphorylated state; the subsequent dephosphorylation due to the PP2A serine-threonine phosphatase regulates the cell cycle progression and the apoptosis process. (B) The binding between sT-Ag and PP2A avoids the dephosphorylation of Akt, p53, c-Myc, and β -catenin proteins, and the subsequent deregulation of the cell cycle progression and apoptosis process drives the cell to a malignant transformation. HPyVs indicate human polyomaviruses; PP2A, phosphatase 2A.

enhancing the MEK/ERK phosphorylation. Once more, it is suggested that sT-Ag:PP2A interaction prevents the PP2A dephosphorylation activity and ultimately the suppression of the MAPK cascade.

It has been demonstrated that PyV sT-Ag are capable of upregulating and stabilizing the myelocytomatosis transcription factor (Myc), which requires intact DnaJ and PP2AsT-Ag domains. Studies have demonstrated that sT-Ag:c-Myc upregulation is related to later increase in human telomerase and cyclin D1, suggestive for Myc-targeted expression.¹⁶² The c-Myc and cyclin D1 promoter activity was also increased by JCPyV sT-Ag and β -catenin, both separated and associated, the latter found to potentiate the effect.¹¹⁶ Moreover, MCPyV sT-Ag is capable of increasing c-Myc activity. Normally, the Fbw7 ubiquitin ligase protein complex promotes the degradation of proto-oncogene products such as cyclin E, c-Myc, c-Jun, mTOR, and nuclear factor κ B (NF- κ B) by a phosphorylation-dependent mechanism. Recent findings support the idea that the sT-Ag can inhibit the LT-Ag, c-Myc, and cyclin E proteasomal degradation through their stabilization, thus regulating several cell cycle proliferation pathways.¹⁶³

Furthermore, human cells expressing SV40 sT-Ag have shown upregulation of antiapoptotic targets of NF- κ B,¹⁶⁴ despite most viral proteic interactions with NF- κ B pathway have been shown to have inhibitory effects. For instance, MCPyV sT-Ag downregulates NF- κ B by targeting the NF- κ B essential modulator (NEMO) adaptor protein and, thus, disrupting the inflammatory pathway.¹⁶⁵ Moreover, NF- κ B regulation pathway by MCPyV sT-Ag involves the regulatory subunit 1 of the protein phosphatase 4 (PP4R1), which is required for NEMO adaptor protein interaction.¹⁶⁶ Nuclear factor κ B has also been described as a promoter of JCPyV replication during DNA damage response induced by JCPyV itself.¹⁶⁷ These sT-Ag inhibitory activities on NF- κ B pathway, responsible for innate immune response, might be

related to host immune response evasion, whose impact on tumorigenesis still remains unclear.

sT-Ag/PP2A-independent pathways

Despite the principal PyVs' sT-Ag oncogenic pathway is associated with the PP2A deregulation, MCPyV sT-Ag may be able to induce cell transformation in a PP2A-independent way. For instance, it has been shown that MCPyV sT-Ag may induce cell proliferation depending on the Akt-mTOR signaling without PP2A deregulation.¹⁶⁸ The mTOR is a serine-threonine kinase that controls cellular functions such as transcription and translation. When activated, mTOR phosphorylates some translational control proteins, such as the initiation factor 4E-binding protein 1 (4E-BP1), preventing its inhibitory activity and releasing the initiation factor 4 (eIF4E), which may then promote translation and further cell cycle progression.¹⁵¹ The PP2A domain of MCPyV sT-Ag domain is not required for epithelial transformation in transgenic mice.¹⁶⁹ Furthermore, it has been demonstrated that MCPyV sT-Ag is able to dislocate a restricted number of PP2AB subunits, which lead to a sT-Ag:PP2A interaction insufficient to promote tumorigenesis in vitro. Instead, MCPyV sT-Ag induces oncogenesis through the so-called large T stabilization domain (LSD).^{163,170} The LSD is located at residues 91 to 95 and inhibits MCPyV LT-Ag proteasomal degradation because LT-Ag is the target for the Fbw7 E3 ubiquitin ligase. Mutations at LSD disrupt LT-Ag stabilization, prevent sT-Ag cell transformation and viral replication, as well as reduce sT-Ag induction of cellular oncoprotein, still in a PP2A-independent manner.^{65,170}

Another MCPyV sT-Ag interaction related to in vitro tumorigenesis is that with c-Jun, a transcription factor that regulates cellular differentiation and proliferation. When hyperphosphorylated, c-Jun is capable of inducing tumorigenesis, especially in keratinocytes.¹⁷¹ A recent study¹⁷² observed

that c-Jun hyperphosphorylation increased after MCPyV sT-Ag overexpression in HEK293 cell line. In addition, although c-Jun phosphorylation status in in vivo MCC is currently unknown, its activation by MCPyV sT-Ag may contribute to the MCC aggressive pattern, thus demanding further investigations.¹⁷² Finally, it has also been demonstrated that TSPyV sT-Ag overexpression is associated with c-Jun phosphorylation and activation, indicating a role for TSPyV sT-Ag in the trichodysplasia spinulosa (TS) pathogenesis.¹⁶¹

Conclusions

Indubitably, among the proteins encoded by the HPyVs, the principal agent involved in cell transformation and tumor development is the LT-Ag, which is also the most studied. Interestingly, the structure and function of this protein are quite conserved among the different HPyVs, testifying that it is indispensable for both viral replication and interaction with the host cells. However, this review underlined also the strategic role of the other early proteins encoded by the HPyVs, the sT-Ag. In addition, it should be taken into account that some of the classic and newly discovered HPyVs are also able to produce agnoprotein, which may have transforming activities itself.⁹¹ Most probably, these proteins act synergically, orchestrated by LT-Ag, fighting a battle against the infected host, trying to evade from the immune system, and targeting multiple cellular pathways. To this particular regard, the evasion of the innate immune system by HPyV has been so far studied for MCPyV. Both MCPyV LT-Ag and sT-Ag, and also BKPyV LT-Ag, are able to interact with the toll-like receptor 9 (TLR9) and to inhibit it, causing the subsequent lack of transcription of C/EBP β . This last transcription factor plays several roles in the suppression activity of the tumor proliferation, ie, it regulates interleukin (IL)-6, IL-8, tumor necrosis factor α , and E2F expressions. Consequently, the suppression of C/EBP β expression by the HPyVs' T-Ag induces modification in the immune system reactions against the viruses and also triggers the cell proliferation.¹⁷³⁻¹⁷⁶

Not all of the involved mechanisms, neither the interactions among the viral proteins have been fully understood, and the continuous discovery of new HPyVs might favor the understanding of cell transformation mediated by the HPyVs.

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Author Contributions

CFB wrote the first draft of the manuscript. RBV, SV, and SD contributed to the writing of the manuscript. CFB, RBV, SV, and SD agree with manuscript results and conclusions; jointly developed the structure and arguments for the paper; and made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

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