SAPIENZA
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JC Polyomavirus in the etiology of brain tumors: reality or random association?

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SUMMARY

ABBREVIATIONS

AIDS: Acquired Immunodeficiency Syndrome

AP1: activating protein

APC: adenomatous polyposis coli

ATM: ataxia-telangiectasia mutated

ATR: ATM-and Rad3-Related

CK1: casein kinase 1

CNS: Central Nervous System

CSF: cerebrospinal fluid

DDR: DNA damage response

ds: double-stranded

DSB: double-stranded breaks

EBV: Epstein-Barr virus

ER: endoplasmic reticulum

FDA: Food and Drug Administration

FFPE: formalin-fixed paraffin-embedded

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase*

GBM: glioblastoma

GCN: Granule cell neuronopathy

GSK-3β: glycogen synthase kinase-3

HIV: Human Immunodeficiency Virus

HPV: Human Papillomavirus

HPyV: Human Polyomavirus

HR: homologous recombination

IARC: International Agency for Research on Cancer

IGF-IR: insulin-like growth factor

IRS-1: insulin receptor substrate-1

JCPyV: JC Polyomavirus

LIU Cytotoxic T lymphocytes

LSTc: lactoseries tetrasaccharide c

LTAg: Large T Antigen

MAPK: mitogen-activated protein kinase

MCPyV: Merkel Cell Polyomavirus

miRNA: microRNA

NCCR: Non-Coding Control Region

ND10: nuclear domain 10

NF-1: nuclear transcription factor-1

NF2: neurofibromatosis type 2

NHEJ: non-homologous end joining

NLS: Nuclear Localization Signal

OD: optical density

ori: origin of replication

PCR: polymerase chain reaction

PHFG: human fetal glial

PP2A: protein phosphatase 2A

pRb: Retinoblastoma protein

PyV: Polyomavirus

qPCR: quantitative PCR

RPA: replication protein A

RSV: Rous Sarcoma Virus

RT: reverse-transcription

SP-1: specificity protein-1

sTAg: small T antigen

TBP: TATA-binding protein

TF: transcription factor

UV: ultraviolet

VP: Viral Protein

WHO: World Health Organization

INTRODUCTION

Cancer represents one of the main public health and economic concern of the 21st century. Worldwide, viral infections are estimated to contribute to approximately 1.4 million new cancer cases each year, accounting for nearly 8% of the global cancer burden [Xiao *et al.*, 2025].

Since the discovery of Rous Sarcoma Virus [RSV] as a causative agent of chicken tumors, the transforming properties of some viruses and their direct link with human cancers are now well established [De Martel *et al.*, 2018]. Indeed, either by themselves or in combination with other co-factors, certain viruses are able to cause cancers affecting cellular pathways involved in cell cycle progression [Ahmed *et al.*, 2023; Xiao *et al.*, 2025]. Despite the potential driving role of viruses in carcinogenesis remains extremely controversial, investigation of viral etiology is of particular interest, especially for brain tumors, whose causative factors are still unclear [Ostrom *et al.*, 2019; Gunasegaran *et al.*, 2024].

Central nervous system (CNS) tumors comprise a highly heterogeneous group of neoplasms that arise from various structures within the CNS, including the brain, cranial nerves, spinal cord, and meninges. They are classified by the World Health Organization (WHO) into grades I-IV, ranging from non-malignant (grades I-II) to malignant (grades III-IV) forms. The most prevalent malignant CNS tumors are gliomas, with glioblastoma (GBM) representing the most frequent histologic subtype. [Ostrom *et al.*, 2018].

While the molecular features of CNS tumors have been extensively investigated, its underlying etiology remains largely unclear.

Genetic factors, advancing age and lifestyle factors have been proposed [Ostrom *et al.*, 2019]; moreover, since a wide range of viral infections cross the blood-brain barrier, a viral etiology has also been suspected [Michalicová *et al.*, 2017; Gunasegaran *et al.*, 2024].

Several viruses such as Epstein-Barr virus (EBV), Human Papillomavirus (HPV) and Polyomaviruses (PyVs) have been reported in adults and pediatric brain tumors; consequently, their role in Central Nervous System (CNS) cancers has been hypothesized [Vidone *et al.,* 2014; Limam *et al.,* 2019; Egan *et al.,* 2021].

Human Polyomaviruses (HPyVs) are small, non-enveloped viruses with icosahedral capsid and a circular, double-stranded (ds) DNA genome. They belong to the *Polyomaviridae* family and are capable to infect humans where they may cause or have an association with diseases but most often establish latent/persistent infections without clinical symptoms [Moens *et al.,* 2017].

The term "Polyomavirus" (PyV) derives from the Greek roots *poly-*, which means "many", and *-oma*, which means "tumors", reflecting their oncogenic potential [Delbue *et al.,* 2017; Prado *et al.,* 2018]. To date, at least fourteen HPyVs have been identified [Ciotti *et al.,* 2019].

Among them, the only HPyV associated with tumor formation in humans is Merkel cell PyV (MCPyV), well recognized as the major etiological agent of Merkel Cell carcinoma (MCC), an aggressive skin cancer [Feng *et al.,* 2008]. However, the cell transforming potential observed for HPyV proteins *in vitro* raised the hypothesis that other members, in addition to MCPyV, may have a role in human tumorigenesis has been proposed [Prado *et al.,* 2018].

Based on experimental models, JC (JCPyV) and BK (BKPyV) PyVs have been recently identified by the International Agency for Research in Cancer (IARC) as "possible carcinogens", despite studies in humans reported inconsistent evidence for an association with cancers at various sites [Bouvard *et al.,* 2012].

1.1 JC Polyomavirus (JCPyV)

JCPyV is a small DNA virus, isolated for the first time from the brain of a patient with initial JC, affected by Hodgkin's disease, who died for progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system (CNS) [Padgett *et al.,* 1971]. So far, JCPyV is recognized as the causative agent of

PML [Ferenczy *et al.,* 2012; Pietropaolo *et al.,* 2018]. Moreover, due to its known oncogenic potential, a plausible contribution of JCPyV in human tumors has been proposed [Ahye *et al.,* 2020].

JCPyV is widely diffused among human population, with sero-positivity reaching about 70-80% in healthy adults [Kean *et al.,* 2009; Moens *et al.,* 2013].

Initial infection likely occurs during childhood via the respiratory or fecal-oral route and is typically asymptomatic [Pietropaolo *et al.,* 2018]. Following primary infection, the virus establishes persistence/latency in the host, with the kidney and the bone marrow as major sites [Ferenczy *et al.,* 2012]. Specifically, in the kidney JCPyV persistence is characterized by continuous low viral replication [Kitamura *et al.,* 1997; White *et al.,* 2013], whereas in the brain, JCPyV is probably maintained in a non-replicating transcriptionally silent state since viral DNA can be detected in normal brain tissue even in the absence of viral protein expression [Perez-Liz *et al.,* 2008].

Under severe immunosuppression such as Acquired Immunodeficiency Syndrome (AIDS), autoimmune diseases, transplantation and treatment with immunosuppressive drugs, JCPyV can reactivate, causing lytic destruction of oligodendrocytes and thus leading to the onset of PML (Figure 1) [Caldarelli-Stefano *et al.,* 1999; Pietropaolo *et al.,* 2018].

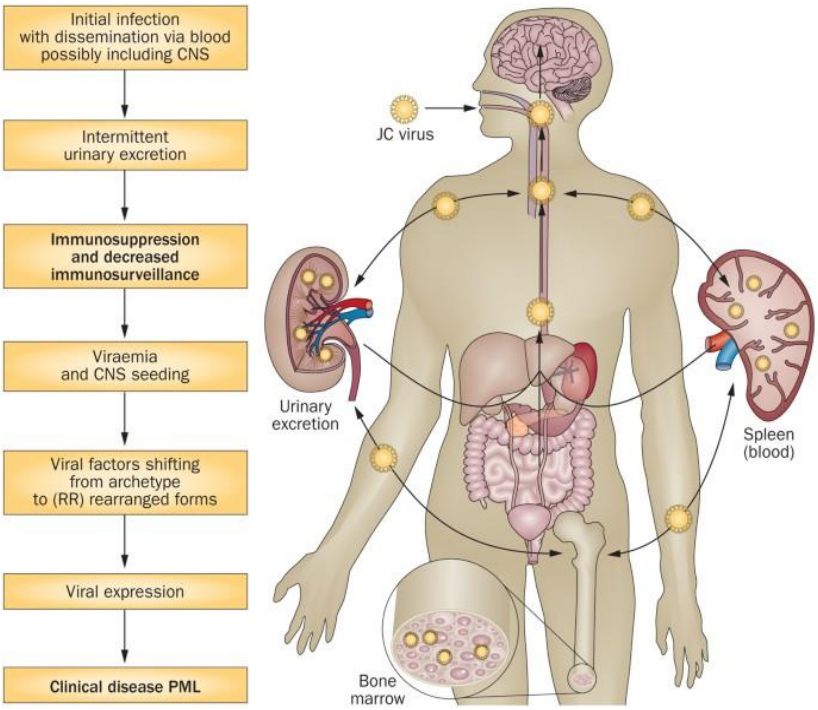

Figure 1. Physiopathology of JCPyV infection [Calabrese *et al.*, 2015]

1.2 JCPyV genome

JCPyV virion is characterized by a non-enveloped, icosahedral capsid with a diameter of 40-45 nm and a molecular weight of $3.2x10^6$ Dalton. The capsid is composed of three coat proteins, Viral Protein (VP) 1, VP2 and VP3. The VP1 represent the major structural protein, being the only one exposed on the surface, thus defining the receptor specificity [Ferenczy *et al.*, 2012]. Viral capsids are predicted to contain 360 molecules of VP1 organized in 72 pentamers, including five VP1 molecules associated to a single VP2 or VP3 [Chen *et al.*, 1998; Diotti *et al.*, 2013]. The nucleocapsid contains the genome, which consists of a circular ds-DNA molecule of tipically 5130 Kb, associated with cellular histones (H1, H2A, H2B, H3 and H4), to form the "viral minichromosome", which is structurally indistinguishable from the chromatin of the host cell [Cole, 1996; Delbue et al., 2013; Kanse *et al.*, 2023] (Figure 2).

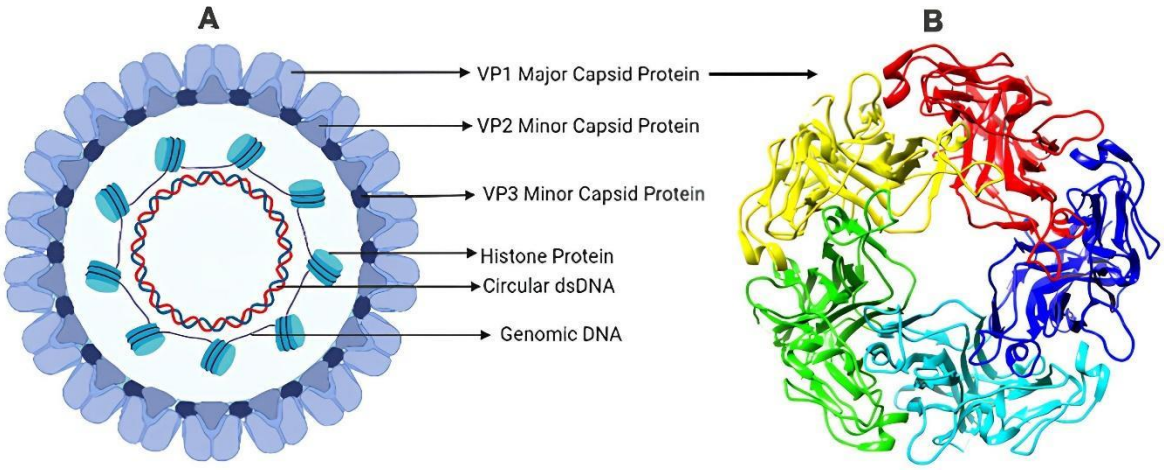

Figure 2. Structure of JCPyV virion (**A**) JCPyV ichosaedral capsid and ds-DNA genome are shown schematically, and (**B**) the major capsid protein is shown in a three-dimensional view using 3NXG.pdb through PyMol Software [Kanse *et al.*, 2023].

The JCPyV genome has a typical tripartite functional organization with early and late coding regions and a non-coding control region (NCCR) (Figure 3) [Frisque *et al.*, 1984; Morris-Love *et al.*, 2022]. The terms "early" and "late" describe the timing of the onset expression of the genes comprised within these regions, following viral infection [Prezioso *et al.*, 2023].

The early region encodes for the regulatory proteins, Large Tumor-antigen (LTAg), small T antigen (stAg) and T' proteins, which are involved in the regulation of the virus cycle and in cell transformation [Frisque *et al.*, 1984; Assetta & Atwood, 2017]. The late region encodes the structural proteins of the capsid, VP1, VP2 and VP3, the accessory agnoprotein and two microRNAs (miRNAs), miR-J1-3p and miR-J1-5p acting to control viral replication and transcription by downregulating LTAg expression [Seo *et al.*, 2008].

Interposed between the two coding regions lies the NCCR, harbouring the origin of viral replication (ori) and the regulatory elements with binding sites for host

transcription factors (TF), such as the nuclear transcription factor-1 (NF1), the activating protein 1 (AP1), and the specificity protein-1 (SP1) [Moens *et al.*, 2020; Auvinen *et al.*, 2024], able to mediate the host cell specificity, the timing of early and late genes' expression and viral genome replication [Ferenczy *et al.*, 2012; Prezioso *et al.*, 2022]. Unlike the early and late regions, the NCCR is a hypervariable region that contributes to neurotropism and neurovirulent properties of JCPyV [Pietropaolo *et al.*, 2018; Moens *et al.*, 2020].

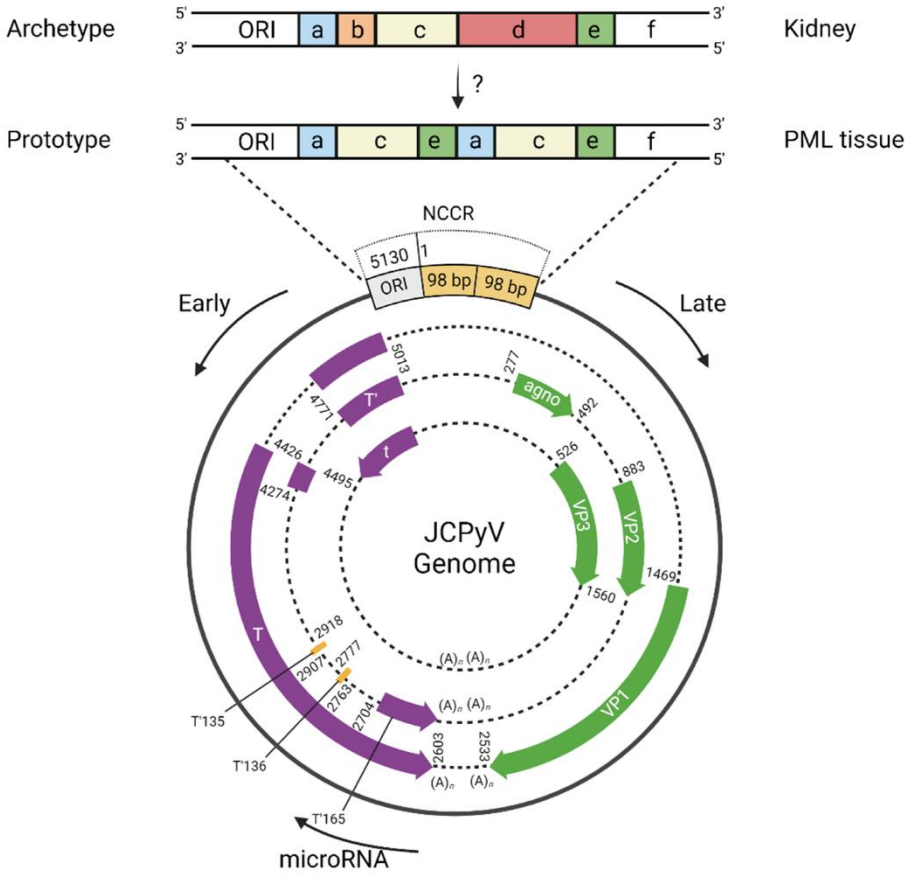

Figure 3. Schematic representation of JCPyV genome. The circular ds-DNA genome is 5,130 bp long and consists of early and late regions, separated by a NCCR that includes the origin of replication (ORI). The early and late regions are transcribed in opposite directions from complementary DNA strands. The early region (purple) encodes the regulatory proteins LTAg, sTAg and the T' proteins while the late region (green) encodes the structural proteins VP1, VP2, VP3 and the Agnoprotein. The JCPyV microRNA gene is expressed late and has a seed region complementary to LTAg. The NCCR is composed by a combination of six boxes a-f. Two different NCCRs are depicted: the archetype, usually found in in the kidney and urine, and the prototype, a variant isolated from the brain of patients with PML [Morris-Love *et al.*, 2022].

1.2.1 The Non-Coding Control Region

The NCCR contains the ori, the TATA box, to which RNA polymerase II binds during transcription, bidirectional promoters, and enhancer elements [Moens *et al.,* 2020]. The NCCR harbored several TF binding sites, including NF-1, a cell-specific regulator of promoter and enhancer activity [Chen *et al.,* 1995; Moens *et al.,* 2020], AP-1, implicated in early gene expression [Sadowska *et al.,* 2003] and SP-1, able to mediate the host cell specificity and to regulate viral replication and transcription [Romagnoli *et al.*, 2008]. Unlike the conserved coding regions, the NCCR is a hypervariable region and contains the determinants for the neurotropism and neurovirulence of JCPyV. Indeed, DNA binding sites located within NCCR can undergo rearrangement processes, such as deletions or duplications, giving rise to new viral variants with a different tropism and degree of pathogenicity [Bellizzi *et al.,* 2013; Pietropaolo *et al.*, 2018].

The diversification based on sequence variations contributed to define JCPyV NCCR as "archetypal" or "rearranged" (*rr*) [Moens *et al.,* 2020].

JCPyV carrying an archetypal NCCR is considered the transmissible form of the virus among the population [Yogo & Sugimoto, 2001] since is usually found in the urine from healthy subjects due to the periodic and subclinical reactivation in kidney [Yogo *et al.,* 1990] and in wastewater [Bahrami *et al.,* 2025], while it is rarely found in the brain of PML patients [Ferenczy *et al.,* 2012; Pietropaolo *et al.,* 2018]. The NCCR of the archetypal CY variant is divided into six regions called boxes A (36 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp) and F (69 bp) (Figure 4A) [Yogo *et al.,* 1990]. Each region contains binding sites for cellular transcription factors involved in viral transcription [Yogo & Sugimoto, 2001].

The prototype NCCR, instead, is the pathogenic variant that derives from the rearrangement of the archetype sequence and is isolated from the tissues of patients with PML. The original prototype is the Mad-1 isolate, which contains an NCCR consisting of a tandemly repeated 98 bp A-C-E sequence (A-C-E-A-C-E-F), resulting

in duplication of the TATA box and binding sites for specific cellular transcription factors, including NF-1 and Spi-B (Figure 4B) [Frisque *et al.*,1983; Ferenczy *et al.*, 2012]. Furthermore, Marshall *and colleagues* demonstrated the presence of a T→G transversion at the Spi-B binding site responsible for increased binding affinity for this transcription factor [Marshall & Major, 2010; Marshall *et al.*, 2012]. Besides the Mad-1 strain, many *rr*-NCCR sequences have been obtained from either cell-passaged virus or viral sequences obtained from healthy individuals and patients. The Mad-4 variant is a variant of the Mad-1 prototype and is often used as an experimental reference strain; it differs from the Mad-1 prototype only by a 19 bp deletion at the second TATA box in the proximal position (Figure 4C) [Padgett *et al.*, 1977]. Finally, the Mad-8 variant is the most commonly found among patients with PML; it has a structure similar to the Mad-1 prototype, with a large deletion and a large insertion, but also some minor insertions and simple base changes (Figure 4D) [Martin *et al.*, 1985].

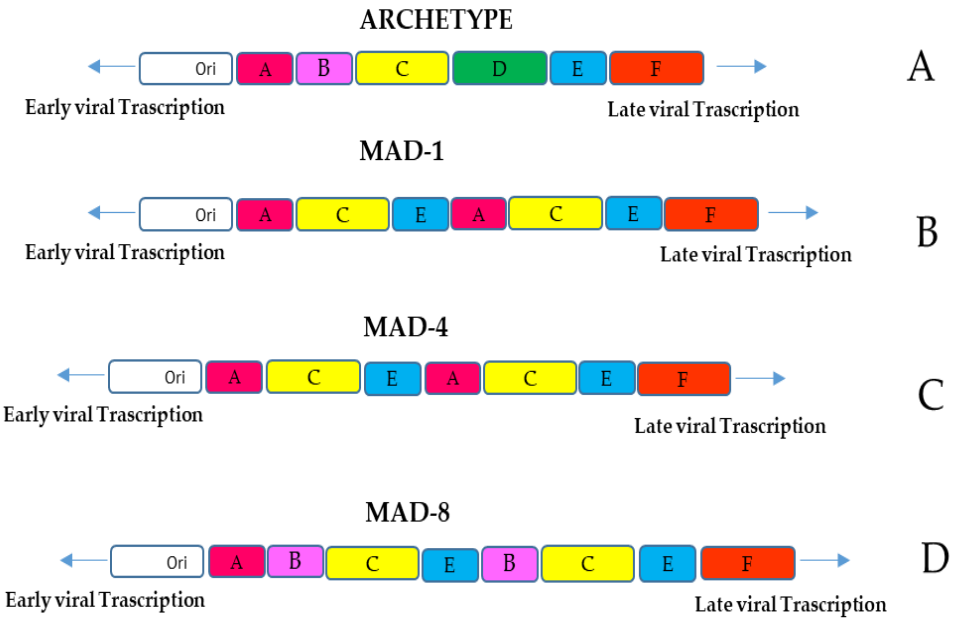

Figura 4 A-D. Schematic representation of JCPyV NCCRs. **A)** Archeype NCCR, **B)** Mad-1, **C)** Mad-4, **D)** Mad-8.

1.2.2 The early region

The JCPyV early RNA is alternatively spliced to produce five transcripts that encode the regulatory proteins LTAg, sTAg and the three T antigen splice variants, known as T' proteins (135, 136 and 165) [Trowbridge & Frisque, 1995; Frisque *et al.*, 2003]. The splicing process is differentially regulated in transformed versus lytically infected cells, and at different times during a productive infection [Frisque *et al.*, 2003]. The major early proteins are the LTAg and the sTAg, involved in viral replication and transcription and in promoting cellular transformation [White *et al.*, 2005].

LTAg

The LTAg is a non-structural, multifunctional protein spanning 688 amino acids that plays a role in viral cycle regulation by modulating viral replication and transcription, as well as in cellular transformation [Del Valle *et al.*, 2008; Diotti *et al.*, 2013].

LTAg is strictly implicated in viral DNA replication due to its capability to bind viral *ori*, where, through its helicase activity, LTAg promotes the unwinding of DNA double helix thus leading to the recruitment of proteins required for DNA synthesis [White & Khalili, 2004]. To exploit these cellular proteins, JCPyV LTAg manipulate cell signaling pathway to drive quiescent cells into S-phase thus activating the host's replication and transcription apparatus (DNA polymerase α and transcription factors) in order to replicate and transcribe viral DNA. A crucial event in this process, is LTAg interaction with the Retinoblastoma protein (pRb) and p53 families [Caracciolo *et al.*, 2006]. The interaction with p53 prevents apoptosis that would normally result from improper activation of S phase and facilitates viral replication in cells that are arrested in the G2 phase. [Del Valle *et al.*, 2001; Del Valle & Khalili, 2010; Orba *et al.*, 2010]. In addition to its role in JCPyV replication and transcription, the ability of LTAg to interact with host factors such as pRb and p53, also enhances

to its capacity to transform multiple cell types [Sullivan & Pipas, 2002; Caracciolo *et al.*, 2006].

The LTAg functions have been attributed to specific structural domains and motifs within its amino acid sequence (Figure 5) [Caracciolo *et al.*, 2006]. Starting from the N-terminal to the C-terminal, JCPyV contains the following domains:

- the DNaJ domain, which binds to the chaperone protein Hsc70 and polymerase α;
- the LXCXE motif, able to bind and inhibit members of the Rb family (pRb, p107 and p130);
- the Nuclear Localization Signal (NLS) domain, necessary for LTAg translocation in the nucleus;
- a DNA-binding domain;
- a domain with helicase activity;
- a domain with ATPase activity;
- a zinc finger motif;
- a binding domain for the p53 protein.

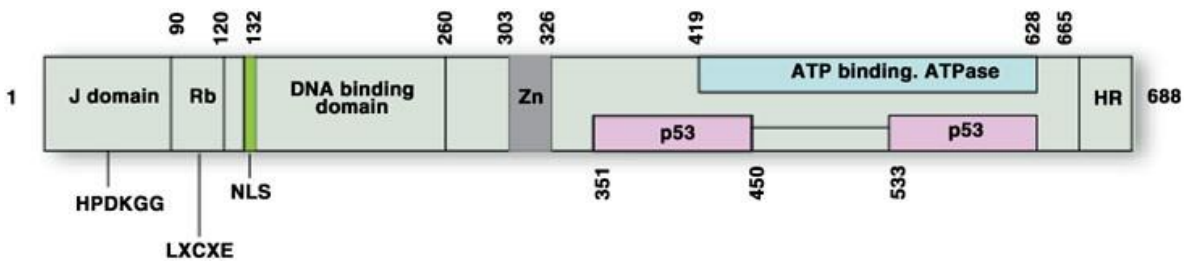

Figure 5. Structure of JCPyV LTAg. The scheme depicts the position of the DNA-binding domain, the zinc-finger motif, and the sequences required for nucleotide binding ATPase activity and p53 interaction. HPDKGG and LXCXE represent the conserved sequences for binding to heat-shock protein and pRb [Caracciolo *et al.*, 2006].

The N-terminal region comprises the LXCXE motif, and the J domain containing the HPDKGG motif [Pipas *et al.,* 1992; Nemethova *et al.,* 2004]. The LXCXE motif is responsible for binding pRb family members, thus inducing cell cycle progression and contributing to virus-mediated transformation [Pipas *et al.,* 1992; White & Khalili, 2006] whereas, the J domain interacts with the chaperone protein Hsc70, stimulating its ATPase activity [Sullivan & Pipas, 2002; Caracciolo *et al.,* 2006].

The C-terminal region, instead, contains the DNA binding domain, implicated in specific binding to viral ori, and harbours the ATP-binding and helicase activities, which are crucial for viral replication [Fanning & Knippers, 1992], as well as a zinc-finger domain also involved in replication of viral DNA [Nemethova *et al.,* 2004]. Finally, JCPyV C-terminus comprises sequences essential for p53 binding which in turn enable the virus to regulate transcription of viral and cellular genes [Kim & Woo, 2002].

sTAg and T' proteins

Like LTAg, sTAg also contributes to induction of the S phase of the cell cycle. Indeed, previous studies demonstrated that this early protein is able to interact with pRb family members, leading to their inactivation, while also binding and sequestering the protein phosphatase A (PP2A) [Bollag *et al.,* 2010]. The interaction between sTAg and this phosphatase prevents the dephosphorylation of agnoprotein, promoting JC virus replication. It has been observed that a reduction in sTAg or PP2A protein levels leads to a significant reduction in viral replication [Sariyer *et al.,* 2008].

T' proteins were discovered by Trowbridge & Frisque in 1995. First considered a product of LTAg degradation due to a sequence homology of 132 amino acids [Trowbridge & Frisque, 1995], they were then recognized as generated through an alternative splicing event of the LTAg mRNA [Prins & Frisque, 2001; Frisque *et al.,* 2003]. Like LTAg, they have J and LXCXE domains in the N-terminal region but differ at the C-terminal in the degree of phosphorylation that influences the interaction between T' proteins and members of the Rb family (pRb, p107 and p130)

and the Hsc70 chaperone. These interactions promote the release of cellular TF of the E2F family that favours the transition from to the S phase of the cell cycle [Prins & Frisque, 2001; Frisque *et al.,* 2003]. T' proteins, therefore, contribute to enhance viral replication in human cells [Prins & Frisque, 2001] and to inactivate cell cycle regulators, stimulating transformation and immortalization of cells [Bollag *et al.,* 2006; Del Valle & Piña-Oviedo, 2019].

1.2.3 The late region

The late region spans about 2300 bp and produces three structural proteins, VP1, VP2 and VP3 [Frisque *et al.,* 1984] and the accessory protein, thought to be involved in virion formation and release but whose function still remains not fully understood [Suzuki *et al.,* 2012].

VP1

The VP1 protein is the main component of the viral capsid: for each VP1 pentamer, there is a single VP2 or VP3 molecule [Sthele & Harrison, 1997; Diotti *et al.,* 2013].
Several studies demonstrated VP1 implication in promoting JCPyV attachment to host cell receptors and starting a viral infection [Nelson *et al.,* 2012; Mayberry *et al.,* 2017]. Moreover, this protein contains epitopes for antibodies induction and recognition [Weissert, 2011; Pietropaolo *et al.,* 2018].
Characterization of JCPyV species reported a single major VP1 serotype but at least eight major genotypes (Table 1) [Agostini *et al*., 2001; Ferenczy *et al*., 2012; Hirsh *et al*., 2013]. Specifically, in 1996, Agostini *and colleagues* introduced a classification of JCPyV genotypes based on the analysis of point mutations located in a 215 bp gene fragment of VP1, between nucleotides 1710-1924 [Agostini *et al.,* 1996; Hansjurgen *et al.,* 2001]. Within this classification, genotypes and subtypes are indicated by a combination of numbers and letters [Chang *et al.,* 1996]. Notably, each genotype shows distinct geographic distributions, being preferentially isolated in different parts of the world [Hirsch *et al.,* 2013].

Table 1. JCPyV genotypes/subtypes. Classification is based on the nucleotide position of polymorphic sites within the VP1-coding gene [Ferenczy *et al.*, 2012].

Type	VP1 type change(s) from consensus[b]	Predominantly associated ethnic group(s)	Identical VP1 sequence	Coding sequences required for complete type identification
1A	75R, 117S, 158L, 345K	European/European-American		
1B	74S, 117S, 126A	European/European-American		
2A1	113(L), 117(A), 126(A), 164(T)	Asian/Native American		
2A2	115L	Asian/Native American		
2B[c]	126A	Asian/Eurasian		
2D1	Consensus	Asian/South Asian	7C1, 7C2	VP2/VP3, VP2, T
2D2	126A	Asian/South Asian	2B	VP2/VP3, agnoprotein, T
2E	113L, 321I	Western Pacific populations	7A	VP2, agnoprotein, T
3A	134A, 164T, 321I, 332Q	African/African-American	3B	t, T
3B	134A, 164T, 321I, 332Q	African/African-American	3A	t, T
4[d]	134A, 164T	European/European-American		
6	164T	African		
7A	113L, 321I	Asian	2E	VP2, agnoprotein, T
7B1	113(L)	Asian		
7B2	37V, 321I	Asian		
7C1	Consensus	Asian/South Asian	2D1, 7C2	VP2/VP3, VP2, agnoprotein, T
7C2	Consensus	Asian/South Asian	2D1, 7C1	VP2/VP3, VP2, T
8A	12H, 164T	Inhabitants of Papua New Guinea	8B	VP2/3, VP2. agnoprotein, T
8B	12H, 164T	Western Pacific populations	8A	VP2/3, VP2. agnoprotein, T
PML-associated mutations	55F,[e,f] 60 M/E/N,[f] 66H,[f] 265D/T,[g] 267F/L,[g] 269F/Y/C[e,g]			

For this reason, JCPyV can be used as a marker for studies of migratory flows (Figure 6) [Stoner *et al.*, 2000; Jobes *et al.*, 2001; Hirsch *et al.*, 2013]. Specifically, genotypes 1 and 4 and their subtypes are more common in Europe and North America, while genotypes 3 and 6 are more prevalent in East Africa and Central-West Africa, respectively. Genotypes 2 and 7 are more common in Asia, while genotype 8 has been identified in Papua New Guinea and the Pacific Islands [Agostini *et al.*, 2001; Sugimoto *et al.*, 2002; Yanagihara *et al.*, 2002] (Table 1). Among these, genotype 2 has been detected with high frequency in the brain tissue and cerebrospinal fluid (CSF) of subjects affected by PML, supporting the hypothesis that different genotypes have distinct capabilities to induce virus-associated disease [Agostini *et al.*, 1997a; Ferrante *et al.*, 2001; Mischitelli *et al.*, 2005]. In particular, the Asian and Eurasian genotype 2B has been associated with a high risk of developing PML, while the European and European-American genotype 4 appears to be associated with a lower risk [Agostini *et al.*, 2000].

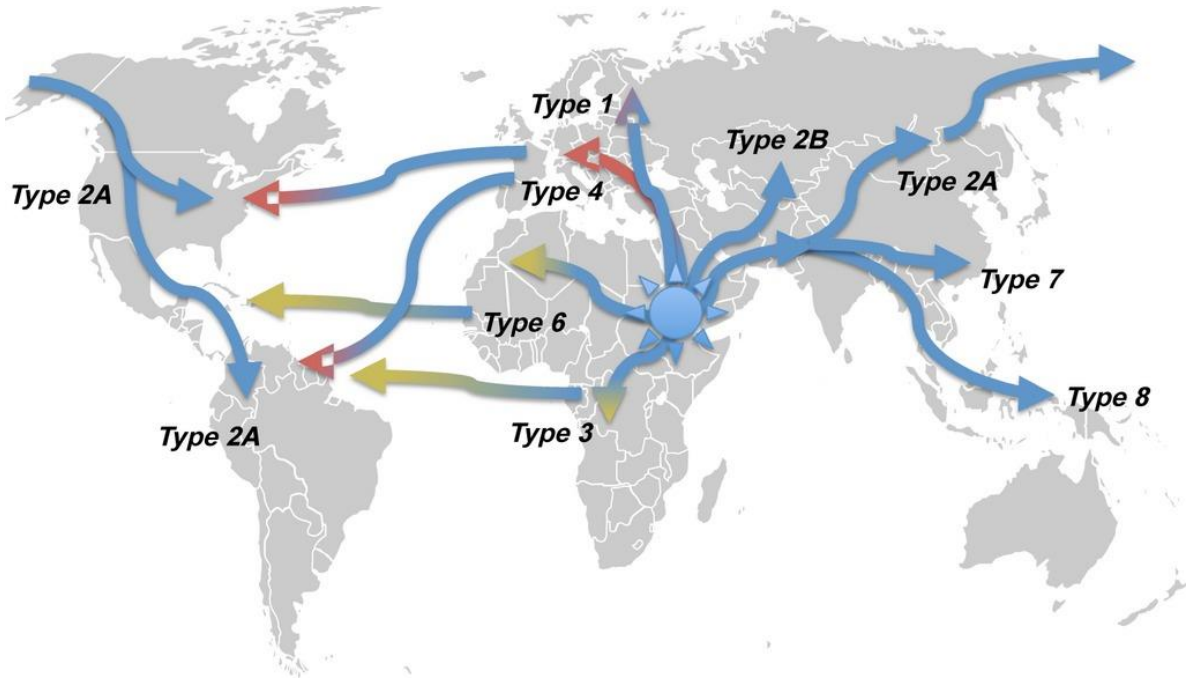

Figure 6. JCPyV genotypes and human migratory flows [Hirsch *et al.,* 2013]

Moreover, VP1 sequencing identified several mutations specific for PML patients which have never been isolated in healthy individuals. Among them, mutations at positions L55, K60, N265, S267 and S269 are located within the binding site for cellular receptors and are associated with PML progression [Gorelik *et al.,* 2011; Hirsch *et al.,* 2013]. Other specific VP1 polymorphisms have been identified at amino acid positions 74, 75, 117 and 128, which appear to correlate with a more favorable PML prognosis [Delbue *et al.,* 2009].

VP2 and VP3 proteins

The late proteins VP2 and VP3 are translated from the same mRNA by use of alternative, in frame start codons, making residues 1–119 unique for VP2, and residues 120–344 identical for VP2 and VP3 [Prezioso *et al.,* 2023]. These two minor capsid proteins contribute to capsid assembly and viral propagation [Gasparovic *et al.,* 2006; Sami Saribas *et al.,* 2014].

Indeed, previous studies demonstrated that the interaction between the VP2 and VP3 proteins, the AgT protein and the Hsp70 chaperone promotes the formation of pre-virions within the nucleus of infected cells. In particular, Hsp70 associates with the late proteins VP2 and VP3 and mediates their interaction with LTAg in the cytoplasm or in the replication centers in the nucleus. This interaction induces a conformational change in LTAg, which increases its binding affinity for the viral replication origin, initiating the process of new DNA synthesis. The newly formed DNA is immediately captured by the capsid proteins VP2 and VP3, which then initiate the virion assembly process [Gasparovic *et al.,* 2006].

The agnoprotein

The Agnoprotein (or VPx) is a protein of approximately 8 kDa that is active during the final stage of JCPyV. It is mainly located in the cytoplasm in the perinuclear region [Okada *et al.,* 2001; Safak *et al.,* 2002]. Agnoprotein interacts with LTAg and regulates its activity during the late stages of the virus's lytic cycle; indeed, by binding to this protein, it negatively regulates viral DNA replication and the transcription of late viral genes to optimize the production of mature virions [Safak *et al.,* 2001; Safak *et al.,* 2002]. Moreover, it seems to act as a viroporin, promoting virus release from infected cells [Suzuki *et al.,* 2010]. Finally, a plausible role of the agnoprotein in tumorigenesis has been proposed; indeed, it may act in synergy with LTAg contributing to alter the regulation of cellular metabolic processes, promoting the acquisition of the transformed phenotype in non-permissive cells. In particular, it would contribute to the onset of mutations at the genomic DNA level, blocking the activity of proteins involved in damaged DNA repair mechanisms [Khalili *et al.,* 2005].

1.2.4 Viral miRNAs

The late region also contains a miRNA coding sequence producing a pre-microRNA which further generates two mature miRNAs of 22 bases, recognized as miR-J1- 5p and miR-J1-3p, with the latter being identical to BKPyV miR-B1-3p [Seo *et al.,* 2008].

These two miRNAs are encoded by the late region and consequently expressed in the late phase of JCPyV infection [Seo *et al.*, 2008; Sullivan *et al.*, 2009]. However, studies on archetypal BKPyV revealed that miRNAs can also be expressed before the onset of genome replication, suggesting their potential role in regulating the replication process [Broekema & Imperiale, 2013]. Consistent with this idea, subsequent studies reported that rearranged BKPyV strains exhibit higher replication rates but reduced miRNA expression compared with the archetypal virus [Martelli *et al.*, 2018], supporting miRNAs' implications for the control of viral replication and the mechanism of viral persistence [Broekema & Imperiale, 2013; Martelli *et al.*, 2018].

MiRNAs-mediated regulation occurs through differential control of promoters' activity modulating both miRNAs and early mRNA expression in the archetype strains compared to the rearranged variants. In the archetypal virus, the NCCR shows weak early promoter activity and strong late promoter activity, which determines high levels of viral miRNA; conversely, the rearranged strain exhibits strong early promoter activity that results in reduced miRNA levels [Broekema & Imperiale, 2013].

The functions of miRNAs in viral pathogenesis and latent infection have begun to be elucidated. Due to their intrinsic property of complementarity with early regions of the viral genome, JCPyV-encoded miRNAs are able to target and downregulate LTAg mRNA levels [Seo *et al.*, 2008; Sullivan et al., 2009; Broekema & Imperiale, 2013], thus leading to a reduced immune response and facilitating the establishment of a persistent infection [Pietila *et al.*, 2015]. Moreover, among viral miRNAs, JC-miR-3p is able to target the cellular transcript of the stress induced ligand ULBP3 [Bauman *et al.*, 2011], which is specifically recognized by the killer receptor NKG2D, well known to be involved in the detection and elimination of virus-infected cells [Zingoni *et al.*, 2018]. Therefore, JCPyV miRNA-mediated ULBP3 downregulation may represent a mechanism for the virus to escape the immune system [Bauman *et al.*, 2011].

The discovery of JCPyV miRNAs circulating in biological fluids encouraged their use as diagnostic markers [Basnyat *et al.*, 2013].

Previous studies detected viral miRNAs in PML tissues [Seo *et al.*, 2008], in multiple sclerosis patients undergoing natalizumab treatment [Giovannelli *et al.*, 2015], in HIV-infected patients [Rocca *et al.*, 2015], and in cancer patients [Link *et al.*, 2015].

For JCPyV, detection of viral DNA in plasma (viremia) is rare and has been shown not to be useful for predicting PML [Zou *et al.*, 2021]. Moreover, due to the high prevalence of viral antibodies in the general population, seropositivity is not sufficient to assess risk of developing PML [Delbue *et al.*, 2015]. Therefore, the application of JCPyV miRNA as a diagnostic marker may allow early identification of patients at risk for PML and contribute to assess the progression of JCPyV-associated diseases or the development of PML [Prezioso *et al.*, 2022].

However, viral miRNAs have also been isolated with high prevalence in the blood and urine of healthy individuals [Seo *et al.*, 2008; Rocca *et al.*, 2015]. Therefore, the translational significance and clinical value of these miRNAs need additional comprehensive studies.

Since JCPyV miRNAs have been detected in healthy and disease subjects (PML patients, HIV-positive individuals, patients with multiple sclerosis under natalizumab treatment and colon-cancer patients) their use as diagnostic and prognostic markers has been investigated [Giovannelli *et al.*, 2015; Rocca *et al.*, 2015; Prezioso *et al.*, 2021].

Some authors have highlighted how an analysis of the relationship between JCPyV viral load and microRNA expression in biological fluids could help monitor possible viral reactivation and, consequently, assess the risk of PML onset [Rocca *et al.*, 2015].

Furthermore, since there are no FDA (Food and Drug Administration)-approved drugs for the treatment of diseases associated with JCPyV infection, it has been proposed that a greater understanding of the role of miRNAs in viral pathogenesis could contribute to identifying new therapeutic targets [Zou & Imperiale, 2021].

1.3 JCPyV: viral cycle

The life cycle of JCPyV begins with the attachment of the VP1 protein to host cellular receptors, containing sialic acid and negatively charged [Pietropaolo *et al.*, 2018]; therefore, the virus traffics to the nucleus, where viral transcription and genome replication occur (Figure 7) [Maginnis & Atwood, 2009; Assetta & Atwood, 2020]. JCPyV initially binds to the lacto series tetrasaccharide c (LSTc), which contains a terminal α2,6-linked sialic acid [Neu *et al.*, 2010]. In addition, JCPyV requires the $5HT_{2A}$ serotonergic receptor to infect glial cells [Elphick *et al.*, 2004]. Transient interaction with $5HT_{2A}$ serotonergic receptor facilitates internalization through clathrin-mediated endocytosis [Pho *et al.*, 2000; Querbes *et al.*, 2004]. Notably, during the cell adhesion phase, the VP2 and VP3 proteins are essential in promoting the interaction between the VP1 protein and the cellular co-receptor [Cole, 1996; Pho *et al.*, 2000]. JCPyV then switches from a Rab5+ early endosome to a caveolin-1+ late endosome before entering the endoplasmic reticulum (ER) [Assetta & Atwood, 2020]. From the ER, through a degradation process, it is released into the cytoplasm and then moves towards the nucleus. Interaction with nuclear pore complexes allows the virion to enter the nucleus, where JCPyV finally releases its DNA [Ferenczy *et al.*, 2012].

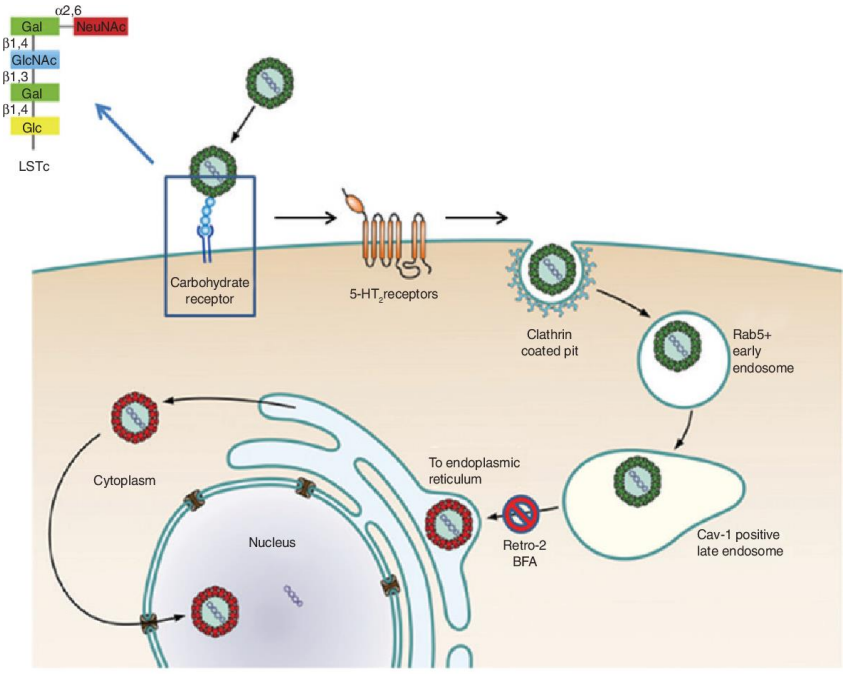

Figure 7. The JCPyV life cycle. JCPyV attaches to host cells through interactions between its VP1 capsid protein and the receptors LSTc and 5-HT_{2A}. The virus is then internalized via clathrin-mediated endocytosis and transported to Rab5-positive early endosomes, followed by Cav-1–positive late endosomes. JCPyV traffics to the ER, it is translocated into the cytosol and subsequently delivered to the nucleus [Assetta & Atwood, 2016].

Replication of the JCPyV genome is initiated when LTAg binds to the viral *ori*, triggering bidirectional DNA synthesis, which requires the cell DNA polymerase, the replication protein A (RPA) and host enzymes and cofactors, expressed in the S-phase of the cell cycle [Delbue *et al.*, 2013]. This process is further modulated by host cellular factors such as NF-1 [Monaco *et al.*, 2001; Major, 2010]. Viral DNA replication and capsid assembly take place within specialized nuclear substructures known as nuclear domain 10 (ND10) bodies, which serve as scaffolds for genome replication and virion assembly [Shishido-Hara *et al.*, 2008]. Concurrently with DNA replication, late viral mRNAs, encoding the capsid proteins and the agnoprotein, are transcribed and subsequently translated. Expression of these late genes depends on LTAg interactions with components of the transcription machinery, including the TATA-binding protein (TBP), its associated factors, and transcription factors such as Sp1 [Kim *et al.*, 2000]. The structural proteins of JCPyV are expressed from the 16S and 19S mRNAs, with the 16S encoding the major capsid protein VP1, and the 19S encoding the minor capsid proteins VP2 and VP3, as well as the agnoprotein.
These proteins are then transported to the nucleus, where virion assembly takes place. Mature virions are released from host cells primarily through cell lysis, typically occurring 24-48 hours after the onset of viral replication. However, electron microscopy studies also revealed the release of viral particles through an intact plasma membrane [Sariyer *et al.*, 2006]. The relative contribution of these two mechanisms still remains unclear.

1.4 JCPyV infection: latency, persistence and reactivation

JCPyV is a neurotropic virus widely diffused among the general population. Blood samples from healthy adults show that 50-90% of individuals have been exposed to

JCPyV with approximately 20% exhibiting urinary viral shedding [Wollebo *et al.,* 2015]. Although the precise mode of transmission is still unclear, the detection of JCV DNA in stromal cells of the tonsils and oropharynx suggests that the tonsils may serve as an initial site of infection [Kato *et al.,* 2004]. The virus is thought to enter through the mouth or nose, potentially via close interpersonal contact or contaminated surfaces, before spreading hematogenously from the primary site to secondary sites such as the kidneys, bone marrow, lymphoid tissues, and brain, where it can establish latent or persistent infections [Pietropaolo *et al.,* 2018]

Plausible routes of transmission include oral-fecal, transplacental, and via blood transfusion [Boland *et al.,* 2005; Berger *et al.,* 2006] and parent-to-child transmission is common [Kunitake *et al.,* 1995]. Furthermore, the isolation of archetype JCPyV from sewage samples in various regions suggests that contaminated food or water may also play a role in transmission [White *et al.,* 2011]. Finally, the recent detection of viral DNA in semen and in vaginal secretions suggests JCPyV possible transmission through sexual contact [Rotondo *et al.,* 2017; Oliveira *et al.,* 2025].

Primary infection generally occurs without specific symptoms during early childhood and results in primary viremia.

Since humoral immunity against JCPyV does not prevent the onset PML, as evidenced by the presence of high anti-JCPyV antibody titers in patients during advanced stages of the disease, it is believed that cellular immunity plays the primary role in controlling JCPyV infection. Cytotoxic T lymphocytes (CTLs) target viral protein epitopes presented on class I HLA molecules, thereby restricting viral spread [Pietropaolo *et al.,* 2018]. Although this immune response limits viral replication, it does not completely eliminate the virus, which instead persists in a latent state within the body [Khalili *et al.,* 2006; Elia *et al.,* 2017]. The dissemination of JCPyV to various tissues, where it subsequently becomes latent, is thought to occur during primary viremia. While the exact characteristics of this phase remain unclear, current hypotheses suggest that the virus may circulate either as free virions or associated with white blood cells [White *et al.,* 2011].

A wide variety of tissues have been identified as potential reservoirs for latent/persisten JCPyV infection. In the kidney, the archetypal JCPyV may persist, with low levels of viral replication and occasionally in tubular epithelial cells [Yogo *et al.,* 1990; Wollebo *et al*., 2015]. The tonsils and peripheral blood leukocytes, particularly B lymphocytes, are also considered among the major sites of viral persistence [Kato et al., 2004].

Under severe immunocompromised conditions such as AIDS, organ transplantation or treatment with certain immunomodulatory therapies, may induce JCPyV reactivation which consists of the migration of the virus through the bloodstream to the CNS where, lytic infection of oligodendrocytes results in PML development [Delbue *et al.*, 2013; Calabrese *et al.,* 2015; Pietropaolo *et al*., 2018].

1.4.1 JCPyV and PML

PML is a fatal, rare neurological syndrome characterized by lytic infection of oligodendrocytes, glial cells that are responsible for myelin production and maintenance of neuronal axons and astrocytes, which play crucial roles in CNS [Wollebo *et al.,* 2015; Schweitzer *et al.,* 2023]. First described in 1958 by Astrom *and colleagues* in 1958 [Astrom *et al.,* 1958], was originally recognized as a rare complication of hematological malignancies or systemic inflammatory disorders. The actual causes remained unclear until 1965, when viral particles were identified within the characteristic brain lesions of PML [Zu Rhein & Chou, 1965]. Subsequently, in 1971, Padgett *and colleagues* successfully isolated the virus in human fetal brain cell cultures [Padgett *et al.,* 1971].

PML occurs only exceptionally in immunocompetent hosts, indicating that sustained impairment of cellular immunity is a key predisposing factor for JCPyV reactivation. In particular, $CD8^+$ T cell reactivity in particular is associated with prevention and resolution of disease [Cortese *et al.,* 2021]. JCPyV reactivation occurs most frequently in patients with $CD4^+$ T cell deficiencies, such as those with HIV infection, and $CD4^+$ T cell counts are closely correlated with PML prognosis

[Ferenczy *et al.,* 2012]. Even in patients without PML, JCV reactivation and viraemia may develop when cellular immune responses are compromised, for example during immunosuppressive therapy. This viral reactivation is often associated with increased variability and rearrangements within the NCCR, reflecting the accumulation of replication-driven mutational events [Cortese *et al.,* 2021].

While the viral etiology of the PML is now well recognized, the mechanisms underlying JCPyV reactivation and consequent PML development remain poorly understood.

To date, a three-stage model of PML pathogenesis has been proposed (Figure 8). In stage 1, the non-pathogenic archetype JCPyV strain establishes a persistent infection in the kidney and possibly a latent infection at other secondary sites in immunocompetent individuals; in most cases, the infection remains subclinical at this stage. Stage 2 typically arises in the setting of severe immunosuppression, leading to viral reactivation and the accumulation of genetic rearrangements within the NCCR. These changes give rise to neuropathogenic JCPyV variants. The virus may access the CNS during either stage 1 or stage 2. Indeed, since viral DNA has been detected in the brains of healthy individuals, it is still controversial whether PML arises from the entry of *rr*-JCPyV strains into the brain through the hematogenous route or from the reactivation of latent JCPyV already present in the CNS [Doerries *et al.,* 2003; Chapagain & Nerurkar 2010; Cortese *et al.,* 2021]. Finally, stage 3 corresponds to the clinical manifestation associated with PML.

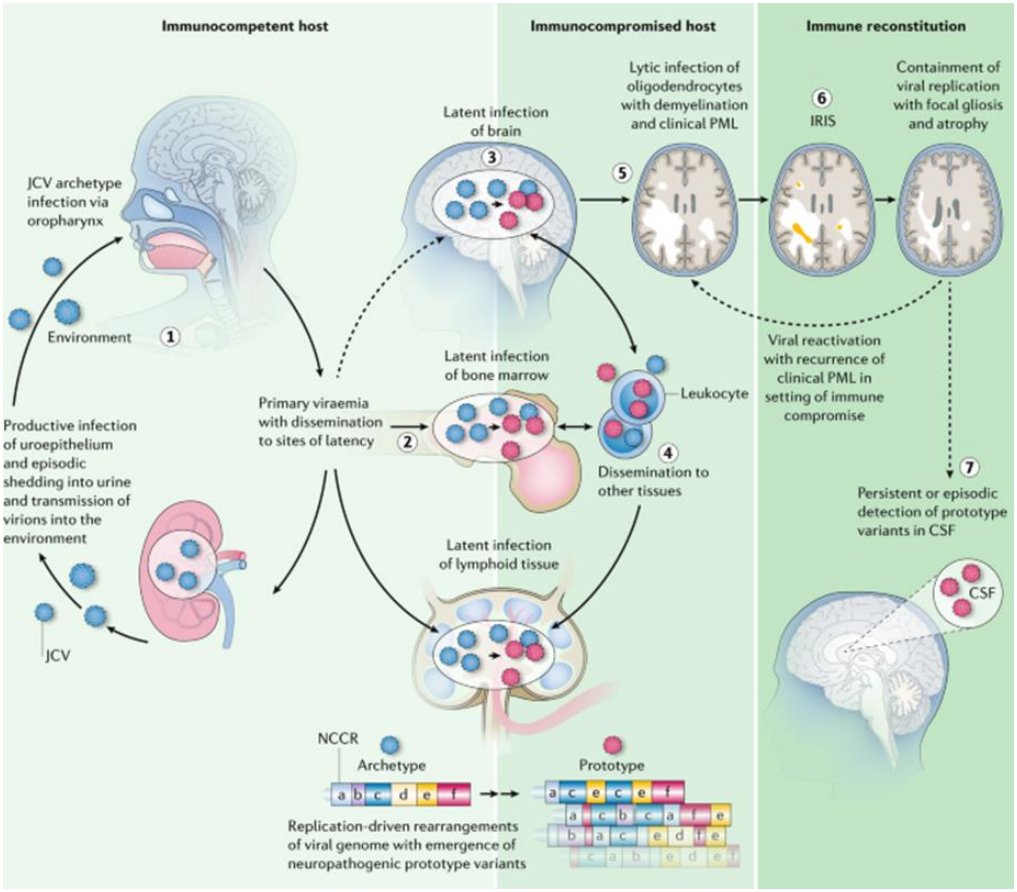

Figure 8. The three stage model proposed for PML [Cortese *et al.*, 2021]

The pathological viral strain isolated from PML patients are characterized by *rr*-NCCR, including deletions, duplications and point mutations within TF-binding sites. These NCCR structure are usually referred as PML-type. Among them Mad-1 was the first pathological variant to be isolated and Mad-4 is the most frequent *rr*-NCCR among PML patients [Pietropaolo *et al.*, 2018; Moens *et al.*, 2020].

As a consequence of JCPyV infection, oligodendrocytes undergo cytolytic destruction that leads to progressive loss of myelin, resulting in the formation of demyelinating foci that are initially asymmetrically distributed, and tend to enlarge as the disease advances [Diotti *et al.*, 2013]. Although less frequently, astrocytes can also be infected, enlarged with irregular, bizarre-shaped nuclei, and they resemble transformed cells in malignant glioblastoma tumors [Astrom *et al.*, 1958; Richardson

1961]. However, unlike oligodendrocytes, astrocytes do not undergo lysis [Prezioso *et al.,* 2023].

The neurological manifestation of PML is usually related to the location and extent of the demyelinating lesions [Bernal-Cano *et al.,* 2007; Takeda *et al.,* 2009]. The signs and symptoms are diverse and complex, which makes the PML diagnosis difficult and challenging, especially at an early stage of the disease [Brooks & Walker, 1984; Schweitzer *et al.,* 2023]. The three hallmark symptoms of PML at onset during disease progression are (i) visual deficits (35-45%), (ii) motor impairment (25%), and (iii) cognitive or behavioral changes in one third of patients [Diotti *et al.,* 2013]. Visual disturbances are the most frequent initial symptom, followed by motor weakness and changes in mentation, including personality changes, memory difficulties, emotional lability, and dementia. Other common symptoms include weakness, headache, parkinsonism, sensory deficits, aphasia and neglect syndrome. Epileptic seizures are common in lesions located in more cortical areas and in patients with radiological signs of inflammation due to local immune responses [Berger, 2011; Diotti *et al.,* 2013; Hirsch *et al.,* 2013; Schweitzer *et al.,* 2023]. According to current diagnostic criteria, a definite diagnosis of PML requires either direct demonstration of JCPyV in brain tissue or detection of viral DNA in the CSF in combination with characteristic MRI findings and clinical presentation.

The prognosis of PML is generally poor, with an average survival of approximately six months following diagnosis; however, long-term survival and even remission have been reported in up to 10% of cases [Marzocchetti *et al.,* 2009; Santa Cruz *et al.,* 2016].

1.4.2. Other JCV-related CNS Manifestations

The clinical manifestations of JCV were attributed solely to its productive infection of oligodendrocytes, which underlies PML. However, it is now evident that JCV can infect a broader range of CNS cell types, giving rise to atypical clinical syndromes

such as cerebellar degeneration, encephalopathy, and meningitis [Cortese *et al.,* 2021].

Granule cell neuropathy (GCN) results from lytic infection of cerebellar granule neurons, leading to focal loss of the internal granule cell layer, progressive cerebellar atrophy, and corresponding neurological decline [Koralnik *et al.,* 2005] Initially described in HIV-associated PML [Du Pasquier *et al.,* 2003], GCN has since been reported in several immunosuppressive contexts [Wutrich *et al.,* 2009]. Both archetype and prototype JCV have been detected in the CSF of affected patients. MRI typically shows progressive cerebellar atrophy, and mixed presentations of GCN with classic supratentorial or infratentorial PML lesions are relatively common [Du Pasquier *et al.,* 2003].

JCPyV-associated encephalopathy arises from lytic infection of cortical pyramidal neurons, presenting as fulminant encephalopathy with or without focal parenchymal lesions. Reported cases involved archetypal strains; moreover, deletions in the agnoprotein gene seemed to contribute to the atypical presentations in the reported cases [Dang *et al.,* 2012].

Meningitis associated with JCPyV has been described in patients with detectable viral DNA in the CSF but without the white matter lesions characteristic of PML [Blake *et al.,* 1992; Viallard *et al.,* 2005]. Whether these cases represent primary infection or viral reactivation remains uncertain. Post-mortem findings from one case revealed productive infection of the leptomeninges and choroid plexus by archetype JCPyV, suggesting that the virus may be an under-recognized cause of aseptic meningitis [Cortese *et al.,* 2021]. Although these atypical presentations are categorized as distinct syndromes, their specific histopathological features have also been observed in classic PML.

1.5 JCPyV cultivation *in vitro*

The study of JCPyV biology has long been hampered by the absence of suitable *in vitro* culture systems capable of supporting efficient viral replication and enabling investigation of variations within the NCCR. Unlike other PyVs such as BKPyV and SV40, JCPyV propagation in cell culture presents significant difficulties due to the virus's restricted host range. This limitation is mainly attributed to the specific interaction between the viral LTAg and human DNA polymerase [Cole, 1996]. Although JCPyV is capable of infecting several human cell types, including tonsillar stromal cells, hematopoietic progenitor cells, renal epithelial cells and B lymphocytes, efficient viral replication was initially achieved only in primary cultures of human fetal glial (PHFG) cells [Jiang *et al.*, 2010; Ferenczy *et al.*, 2012]. The first successful isolation of the virus occurred in PHFG cultures and later in PHFG cultures containing numerous spongioblasts [Padgett *et al.*, 1977]. Subsequent studies revealed that other glial cell types, such as human embryonic astroglial cells, can also sustain a complete lytic replication cycle of JCPyV, although with variable efficiency [Frye *et al.*, 1977; Major et al., 1985]. However, the difficulty of maintaining them in long-term culture prompted the development of immortalized cell lines to facilitate biochemical and molecular analyses of the virus [Major *et al.*, 1985; Ferenczy *et al.*, 2012; Hirsch *et al.*, 2013]. Among the immortalized lines, the most extensively used are the human astroglial SVG cells [Gluzman, 1981; Prezioso *et al.*, 2022] and the renal Cos-7 cell lines, derived from African green monkey kidney tissue [Hara *et al.*, 1998; Prezioso *et al.*, 2018; Prezioso *et al.*, 2022]. Both were generated through transfection with a replication-defective SV40 mutant carrying a deletion in its *ori*, which prevents SV40 replication but enables constitutive expression of the oncogenic LTAg [Gluzman, 1981; Major *et al.*, 1985]. The presence of the viral protein allows the expression of JCPyV early genes in SVG cells, which also possess a specific viral receptor (JCV-TCR) [Kenney *et al.*, 1984].

Replication of JCPyV has additionally been observed in human tonsillar stromal cells, though these cells appear only moderately permissive to infection [Khalili *et al.,* 2001].

Viral transcription tends to be more active in glial cells, while DNA replication can also occur in several other human cell types (Shinohara et al., 1997; Khalili et al., 2001].

The U87-MG human glioblastoma cell line has also been employed in studies investigating virus-host interaction, with particular focus on the Wnt/β-catenin pathway [Gan *et al.,* 2001; Enam *et al.,* 2002].

Despite these advances, *in vitro* cultivation of JCPyV remains technically challenging and often results in the production of defective virions lacking viral nucleic acid.

1.5 JCPyV Oncogenic Potential

A productive infection of JCPyV occurs when early gene expression is followed by viral DNA replication and late gene expression, as observed in oligodendrocytes, glial cells permissive to lytic infection, which are destroyed during infection, leading to PML [Pietropaolo et al., 2018]. In contrast, in cells that are susceptible but not permissive, such as astrocytes, only early genes are expressed since the cells cannot support viral genome replication or late gene expression. In this scenario, LTAg can inactivate tumor suppressors, disrupt signaling pathways, or induce genomic instability, potentially contributing to cellular transformation (Figure 9) [Khalili et al., 2003a; Del Valle & Piña-Oviedo, 2019]. Factors that may influence the permissiveness of a cell type include, for example, the tissue of origin or the state of differentiation.

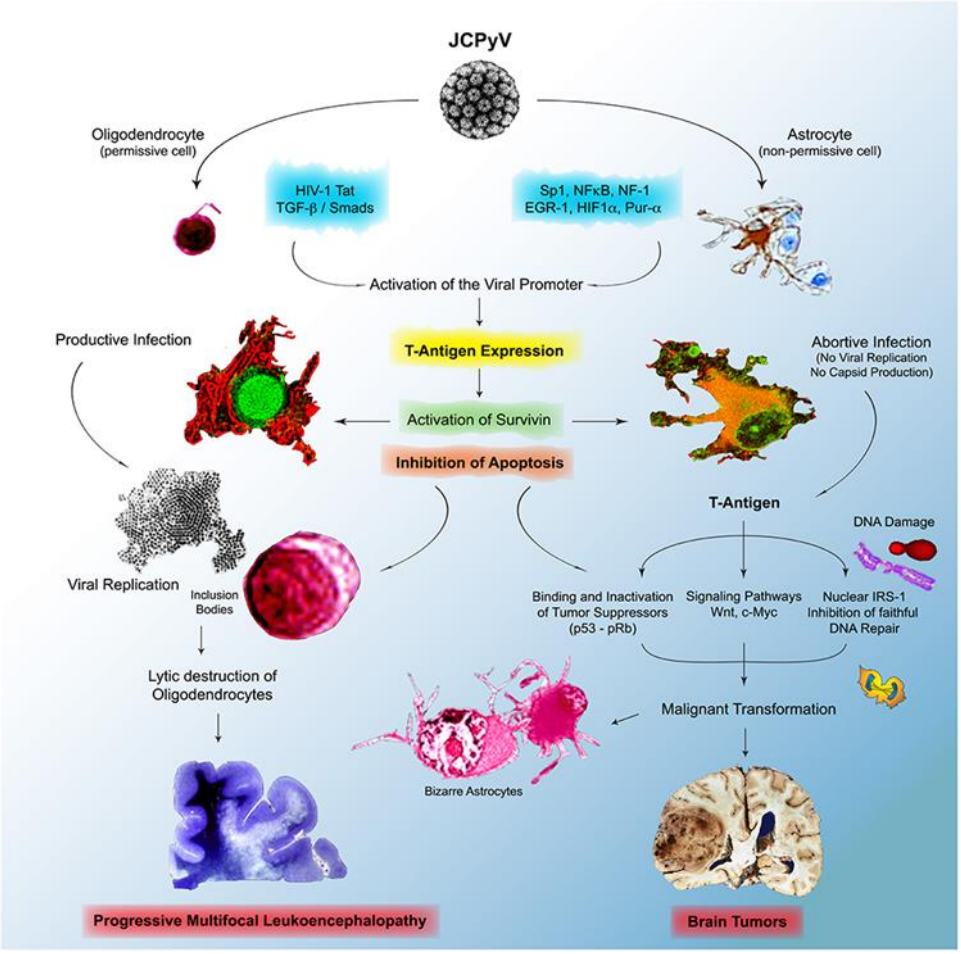

Figure 9. JCPyV Oncogenic Potential in the brain [Del Valle & Piña-Oviedo, 2019]

1.5.1 The oncogenic properties of JCPyV LTAg

The principal effector of JCPyV-mediated transformation and tumorigenesis is the LTAg, an early multifunctional protein able to bind and inhibit key cellular regulators, thus driving the cell cycle from G1 to S-phase (Figure 10) [Del Valle & Piña-Oviedo, 2019; Moens *et al.*, 2022]. In permissive cells, these interactions support viral replication and spread, whereas in non-permissive cells they contribute to cellular transformation [Delbue *et al.*, 2017].

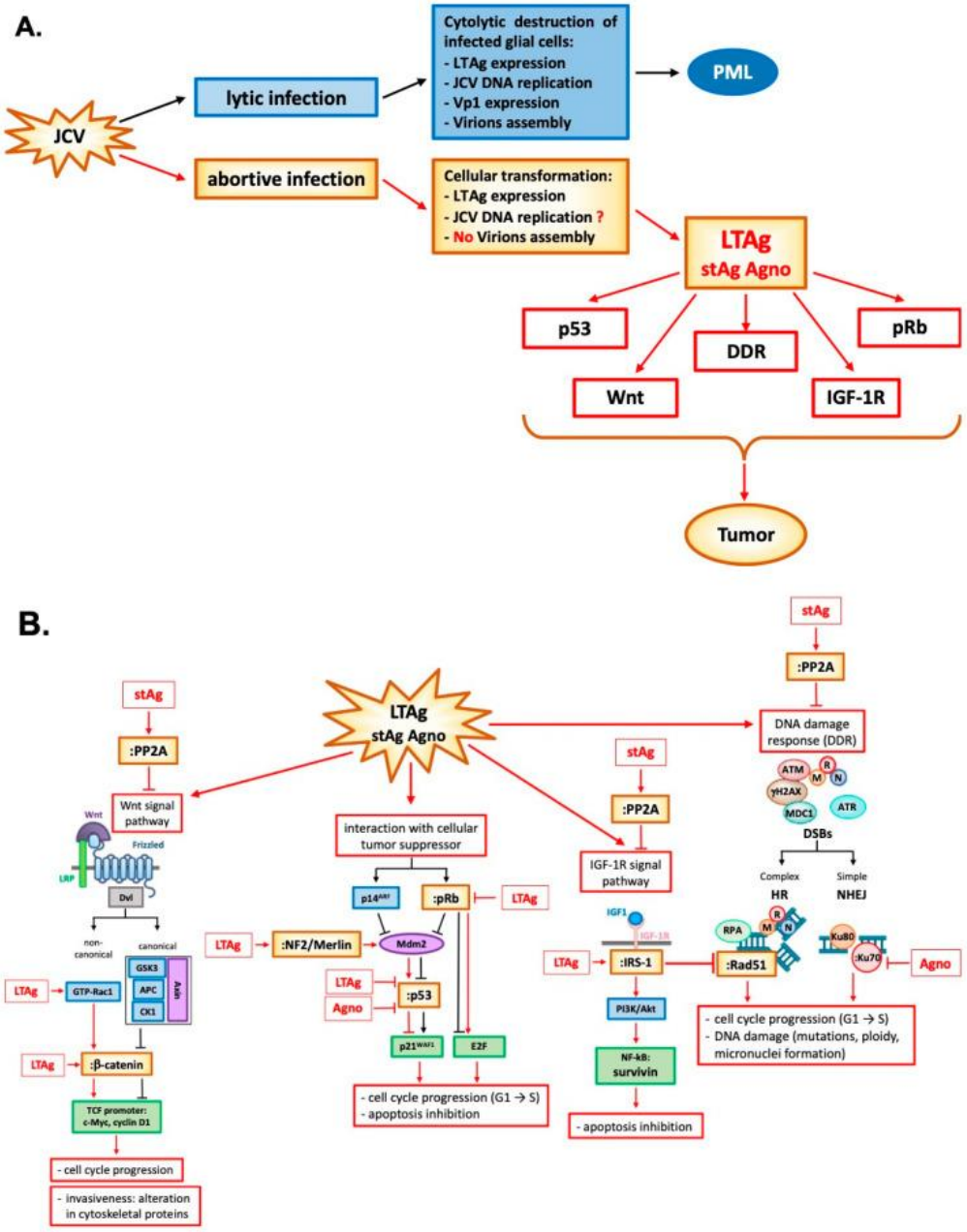

Figure 10. Schematic representation of the interactions between JCPyV proteins and host cellular proteins. (**A**) In cells permissive to JCPyV infection, these interactions are essential for initiation and progression of the lytic cycle, whereas (**B**) non-permissive cells, JCPyV proteins interactions with host factors they contribute to cellular transformation [Ahye *et al.*, 2020].

A critical step in this process is the interaction between the N-terminal LTAg LXCXE motif and the tumor suppressor Rb; normally, Rb sequesters the E2F transcription factor and prevents cell cycle progression from G1 to S phase.

Sequestration of pRb by LTAg, releases E2F and promotes cellular proliferation [Cress *et al.,* 1996; Bollag *et al.,* 2000].

Moreover, LTAg is able to bind and inactivate p53 [Sharma & Kumar, 1991], preventing growth arrest and apoptosis [Moens *et al.,* 2022].

Beyond, pRb and p53, LTAg interacts with several other host factors involved in signaling and survival, including the insulin receptor substrate-1 (IRS-1) and the insulin-like growth factor (IGF-IR) [Lassak *et al.,* 2002; Khalili *et al.,* 2003], the anti-apoptotic protein survivin [Piña-Oviedo *et al.,* 2007] and DNA damage response factors [Orba *et al.,* 2010].

The association between LTAg and the NF2 gene product has also been demonstrated [Shollar *et al.,* 2004], though its functional consequences remain not fully understood [Beltrami *et al.,* 2013].

LTAg binds IRS-1, a membrane-associated tyrosine kinase, promoting its nuclear translocation and disrupting the DNA double-stranded breaks (DSBs) by homologous recombination (HR). In normal conditions, the IRS-1 pathway supports HRR via cytoplasmic binding of IRS-1 to Rad51. Upon LTAg–mediated nuclear translocation, IRS-1 binds Rad51 at DNA damage sites, impairing repair and increasing mutation rates, which may underlie malignant transformation [Lassak *et al.,* 2002; Trojanek *et al.,* 2006].

LTAg also interacts with IGF-1R, resulting in increased levels of survivin, an anti-apoptotic protein that safeguards infected cells from programmed cell death and promotes disruption of cellular homeostasis and oncogenic transformation [Khalili *et al.,* 2003]. Activation of the surviving promoter has also been demonstrated in primary oligodendrocyte and astrocyte cultures expressing the viral protein, where it supports viral replication and may drive the transformation of neural progenitor cells [Piña-Oviedo *et al.,* 2007].

Moreover, several studies reported that LTAg closely interact and manipulate DNA damage response (DDR) machinery, including the ataxia-telangiectasia mutated (ATM) and ATM-and Rad3-Related (ATR) kinases [Orba *et al.,* 2010; White *et al.,* 2017]. The interaction with DDR components creates a cellular environment that favors viral replication but compromises genomic stability, potentially contributing to tumorigenesis [Tahseen *et al.,* 2020].

LTAg and Wnt/ β-catenin pathway

Another essential LTAg interaction in JCPyV-mediated oncogenesis seems to be that with β-catenin [Gan *et al.,* 2001; Gan & Khalili 2004; Ripple *et al.,* 2014].

This cellular factor is part of the Wnt signaling pathway, that is involved in cell proliferation, survival and transcription processes [Yang *et al.,* 2016; Liu *et al.,* 2022].

In the absence of Wnt signaling, β-catenin is in a complex with axin, tumor suppressor adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3β), CK1 (casein kinase 1), and the E3 ubiquitin ligase β-TrCP. and gets phosphorylated and targeted for degradation within the proteasome (Figure 11) [Moon & Gough, 2016; Liu *et al.,* 2022].

In the presence of Wnt signaling, β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where it binds Lef/Tcf transcription factors, thus activating target genes, including c-myc and cyclin D1 [Liu *et al.,* 2022], as well as Fibroblast Growth Factor 20 (Fgf20) [Chamorro *et al.,* 2005]. Several mutations in the proteins belonging to this pathway have been associated with several type of cancers [Reya & Clevers, 2005].

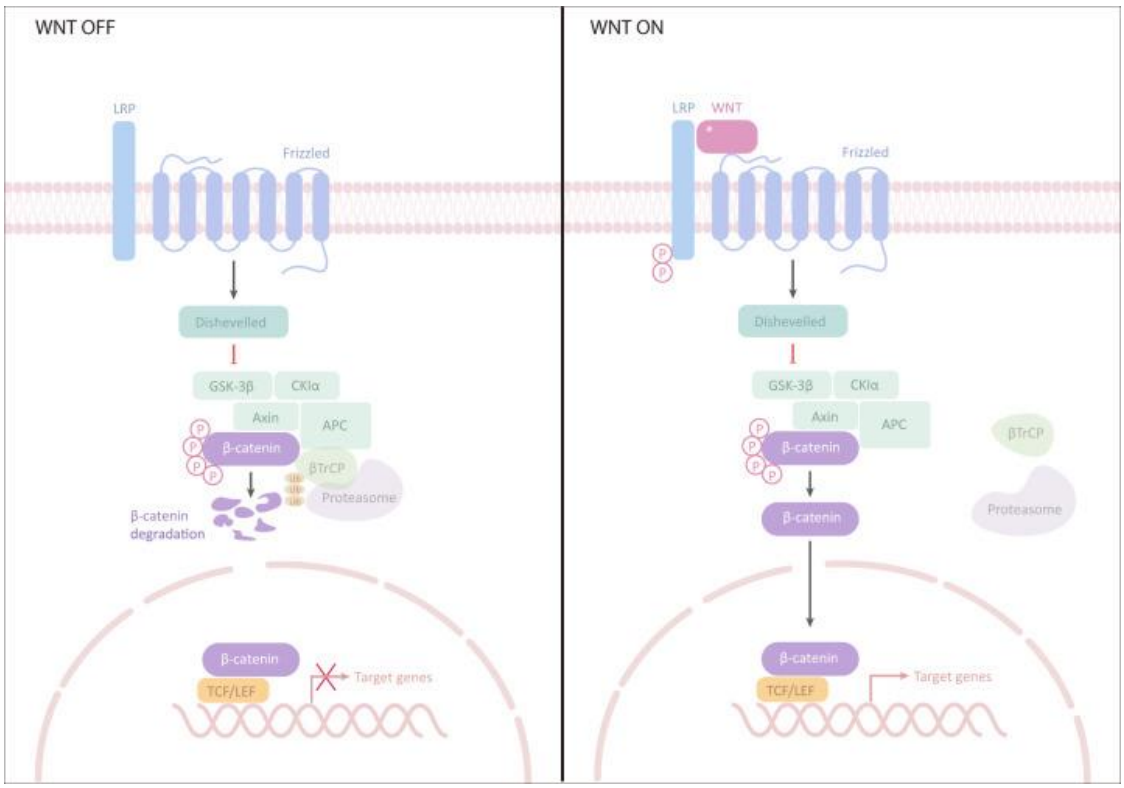

Figure 11. Wnt/β-catenin signaling pathway. **Left (inactive Wnt signaling):** In the absence of Wnt signals, β-catenin is degraded by a multiprotein destruction complex composed of AXIN, APC GSK-3, CK1 and the E3 ubiquitin ligase β-TrCP. This degradation prevents β-catenin from accumulating in the cytoplasm and entering the nucleus. **Right (active Wnt signaling):** When Wnt ligands bind to their cell surface receptors, the Frizzled/LRP complex is activated, leading to the recruitment of AXIN to phosphorylated LRP. This disrupts the β-catenin destruction complex, preventing its degradation. Stabilized β-catenin accumulates in the cytoplasm, translocates to the nucleus, and interacts with TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factors to regulate the expression of Wnt target genes.

The central domain of LTAg interacts with the C-terminal region of β-catenin, promoting its translocation into the nucleus, where it enhances transcription of β-catenin downstream targets such as c- myc and cyclin D1, well recognized to be involved in cell proliferation. c-myc has a number of putative targets, including genes involved in cell cycle control and apoptosis [Dunn & Cowling, 2015]; cyclin D1 is responsible for cell cycle progression in the transition from G0/G1 to S phase and is over-expressed in various cancers [Montalto & De Amicis, 2020].

In addition, other β-catenin responsive genes encoding proteins involved in anti-apoptosis [e.g., survivin] and angiogenesis [e.g., vascular endothelial growth factor] seem to be upregulated by LTAg [Moens & Macdonald, 2019]. The mechanism by which LTAg interferes with the β-catenin cascade is not known, but it seems to stabilize this cellular protein by inhibiting its proteasomal degradation. Alternatively, LTAg may stimulate nuclear translocation of β-catenin, which is supported by the observation that nuclear localization of β-catenin is more frequent in JCPyV LTAg-positive cancers compared with LTAg-negative ones [Ripple *et al.,* 2014; Moens & Macdonald, 2019].

LTAg can also enhance β-catenin stability via a non-canonical Wnt signaling pathway by recruiting the GTPase Rac1. Rac1 stabilizes β-catenin, preventing its degradation through the ubiquitin-proteasome system and facilitating its translocation into the nucleus [Bhattacharyya *et al.,* 2007].

Therefore, it is clear that JCPyV LTAg's ability to interfere with β-catenin may contribute to the oncogenic potential of this virus.

1.5.2 The oncogenic properties of sTAg and Agnoprotein

sTAg is another early protein implicated in viral replication and in cellular transformation [Bollag *et al.,* 2010; Ahye *et al.,* 2020]. sTAg is able to bind and inhibit the serine/threonine phosphatase PP2A, thus activating a number of signaling involved in cell proliferation such as the mitogen-activated protein kinase (MAPK) pathway [Bollag *et al*., 2010].

Finally, oncogenic properties have also been attributed to the Agnoprotein, a small accessory regulatory protein encoded by the late region [Okada et al., 2001; Del Valle *et al.,* 2008]. It can bind directly to p53, leading to cell cycle arrest at the G2/M phase through activation of the p21/WAF-1 promoter [Darbinyan *et al.,*2002]. Additionally, its interaction with Ku70 inhibits the non-homologous end joining (NHEJ) DNA repair pathway, contributing to genomic instability during infection [Darbinya *et al.,* 2004]. Agnoprotein is normally phosphorylated, but when PP2A is sequestered by

sTAg, it cannot dephosphorylate Agnoprotein, resulting in reduced viral replication and concurrent activation of MAPK signaling [Sariyer *et al.,* 2008]. Together, these mechanisms underscore the crucial role of this late protein in promoting cell transformation.

1.6 JCPyV Oncogenicity: *In Vitro* and Experimental Animal Studies

JCPyV has the capability to transform certain cell types in vitro, including human fetal glial cells and primary hamster brain cells. These transformed cells display a classic "transformed phenotype," characterized by growth in soft agar, serum independence, altered morphology, multinucleation, production of plasminogen activator, and increased glucose uptake (Del Valle *et al.,* 2001). The virus' transforming capacity appears to be restricted to cells of neural origin, likely because transcriptional regulation of the viral promoters, located within the NCCR, relies on cell type–specific transcription factors [Del Valle *et al.,* 2008].

Beyond *in vitro* studies, JCPyV tumorigenic potential has been well documented in animal models [Walker *et al.,* 1973; London *et al.,* 1978; Miller *et al.,* 1984; Del Valle *et al.,* 2008]. The first evidence came from studies in newborn Golden Syrian hamsters, which developed a variety of brain tumors when inoculated with JCPyV [Walker *et al.,* 1973]. The majority of these tumors were of glial origin, including medullo-blastomas [Padgett *et al.,* 1977; Zu Rhein & Varakis, 1979], gliomas [Walker *et al.,* 1973] and neuro-blastoma [Varakis *et al.,* 1978].

A wide variety of gliomas and neuro-blastoma also developed following intracerebral inoculation of the virus in non-human primates such as owl and squirrel monkeys [London *et al.,* 1978; Del Valle *et al.,* 2001]. Notably, analysis of the tissues taken from hamsters and monkeys revealed the high levels of LTAg protein but no detectable structural proteins or infectious virions, supporting that animal cells are not permissive for JCPyV replication [London *et al.,* 1978; London *et al.,* 1983; Major *et al.,* 1992].

These findings offer important insights in JCPyV-mediated tumorigenesis, highlighting the virus's ability to transform non-permissive cells. Consequently, it has been hypothesized that JCPyV might also trigger transformation in non-permissive cells in humans [Del Valle *et al.,* 2008; Delbue *et al.,* 2017].

Further evidences about the JCPyV oncogenicity come from studies on transgenic mice, generated to carry only the entire LTAg coding sequence under the control of the natural JCPyV promoter [Small *et al.,* 1986; Krinska *et al.,* 1999; Gordon *et al.,* 2000; Del Valle *et al*., 2021]. In these models, the expression of the early protein mainly induced the development of adrenal neuroblastomas [Small *et al.,* 1986], pituitary adenomas [Gordon *et al.,* 2000], malignant peripheral nerve sheath tumors, and medullo-blastomas [Del Valle *et al.,* 2021].

The types of tumors that develop appear to be influenced by both the structure of the JCPyV NCCR and the mouse strain used [Del Valle *et al.,* 2001; White & Khalili, 2004].

Notably, neoplastic cells in all tumor types expressed the early protein LTAg, which co-localized with the tumor suppressor p53 in the nucleus. In contrast, normal tissues showed no LTAg expression, suggesting that expression of this early protein is specifically associated with oncogenic transformation.

1.7 JCPyV and Human Cancer

Despite the IARC classification consider JCPyV as a possible human carcinogen (Group B) [IARC, 2014], the direct association between JCPyV and human tumors is still a topic of debate.

To date, an increasing number of studies detected JCPyV genomic sequences and the expression of LTAg protein in tissues from a wide variety of human tumors.

JCPyV DNA and early proteins have been isolated from gastrointestinal tumors, including esophageal carcinoma [Del Valle *et al.,* 2005], gastric carcinoma [Ksiaa *et al.,* 2010], sporadic adenomatous polyps [Jung *et al.,* 2008], and colorectal adenocarcinomas [Lin et al., 2008; Link *et al.,* 2009; Ripple *et al.,* 2014; Ksia *et al.,* 2015],

but also in normal tissues and in adjacent non-cancerous tissue from the gastrointestinal tract [Chen *et al.,* 2015].

In the context of colorectal cancer, JCPyV seems to be a cofactor for the induction of the chromosomal instability [Niv *et al.,* 2005], but it also interacts with the β-catenin protein, with the consequent enhanced activation of Wnt pathway target genes, such as c-Myc and Cyclin D1 [Ripple *et al.,* 2014]. Both c-Myc and Cyclin D1 are involved in cell cycle control and progression, and their enhanced activation, mainly due to LTAg-mediated β-catenin nuclear translocation, could result in unchecked cell cycle progression, high proliferation rate, and ultimately a more malignant phenotype [Enam *et al.,* 2002; Ripple *et al*., 2014].

JCPyV was identified also in respiratory cancers, with higher levels of LTAg in lung tumor compared to normal tissues [Abdel-Aziz *et al.,* 2007; Zheng *et al.,* 2007]. Lung tumors with higher JCPyV copy numbers display high proliferation and down-regulation of cell adhesion mediated by β-catenin–mediated cell adhesion [Zheng *et al.,* 2007].

In urinary tract neoplasms, JCPyV has been identified in urothelial carcinoma and renal cell carcinoma [Knoll *et al.,* 2003]. Moreover, viral sequences and proteins have been detected in prostate cancer, with JCPyV-positive tumors showing elevated prostate-specific antigen levels and higher Gleason scores [Anzivino *et al.,* 2015].

Higher positivity rates and LTAg expression have also been observed in breast and pancreatic cancers compared to non-malignant tissues, as well as in cervical carcinoma compared to normal cervical epithelium [Zheng *et al.,* 2022]

Taken together, these data strongly highlight the prevalence across a wide range of malignancies, strongly supporting a potential role for JCPyV in human tumorigenesis.

1.7.1 JCPyV and human CNS tumors

JCPyV transforming abilities in vitro and its oncogenicity in animal models are well recognized [Del Valle et al., 2008; Ahye et al., 2020]. As previously discussed, viral

injection into the brain of rodents and non-human primates, led to the development of several CNS cancers [Walker et al., 1973; London et al., 1983]. Other proofs about JCPyV oncogenicity come from studies with transgenic mice where the expression of the only LTAg results in the development neuro-ectodermal tumors [Krynska et al., 1999; Del Valle et al., 2021].

The molecular mechanisms described appear crucial to JCPyV-induced neural oncogenesis, largely mediated by the LTAg and its interaction with host factors, including Rb and p53 [Del Valle et al., 2008; Ahye et al., 2020]. Specifically, Rb binding promotes cell cycle progression, whereas the LTAg-p53 complex leads to the inhibition of apoptosis [Moens et al., 2022]

Moreover, interactions between LTAg and β-catenin play a crucial role in malignant transformation, particularly in pediatric medullo-blastoma [Gan et al., 2001].

The first evidence speculating a plausible association between JCPyV to human brain tumors was reported in a patient with concurrent chronic lymphocytic leukaemia with PML [Richardson, 1961]. Following the identification of JCPyV as the etiologic agent of PML, further investigations explored its potential role in brain tumors, with a number of cases documenting the simultaneous occurrence of CNS neoplasm and PML *[*White & Khalili, 2005; Brassesco *et al., 2013].*

Detection of JCPyV sequences and/or protein expression has also been reported in primary CNS malignancies in both immunocompetent and immunosuppressed patients without PML. These studies encompass a wide spectrum of CNS tumors, including gangliocytoma, choroid plexus papilloma, pilocytic astrocytoma, subependymoma, pleomorphic xanthoastrocytoma, oligodendroglioma, all astrocytoma subtypes, ependymoma, oligoastrocytoma, glioblastoma multiforme, medullo-blastoma, pineoblastoma, gliosarcoma, and primitive neuroectodermal tumors [Delbue et al., 2017; Ahye et al., 2020].

The percentage of JCPyV-positive CNS tumor tissues is highly variable, ranging from 20% to 75% for detection of the viral genome and from 20% to 68% for viral protein expression [Delbue et al., 2017]. Notably, studies focusing on protein expression consistently detected the early viral protein LTAg in the nuclei and Agnoprotein in the perinuclear region of tumor cells but never the late VP1 protein. This pattern of protein expression is consistent with the observation that most of the CNS are non-permissive for JCPyV replication, and that the transforming activity of LTAg appears largely restricted to neural tissue [Del Valle et al., 2008; Delbue et al., 2018].

Despite increasing evidence for an association between JCPyV and brain carcinogenesis, a number of studies failed in detecting viral genome and protein expression in CNS tumors. Several studies have failed to detect either viral genome or protein in tumors such as meningioma [Weggen et al., 2000], oligodendroglioma, astrocytoma [Herbarth et al., 1998], glioblastoma multiforme [Arthur et al., 1994], glioma, and medulloblastoma [Muñoz-Mármol et al., 2006]. These discrepancies may be attributed to the differences in sample types and detection methods [Del Valle et al., 2008]. DNA from formalin-fixed, paraffin-embedded (FFPE) tissues is typically of lower quality than DNA from fresh/frozen specimens, potentially leading to false-negative results. Similarly, the sensitivity of commonly used amplification techniques (PCR, nested PCR, quantitative PCR, Southern blot hybridization) can significantly affect detection rates [Del Valle et al., 2008; Delbue et al., 2017].

Moreover, due to its ubiquitous nature, JCPyV was detected also in the brain of healthy subjects. This notable observation raises the question of whether JCPyV may have a direct role in tumor pathogenesis or merely resides in the brain as a latent virus [Perez-Liz et al., 2008; Delbue et al., 2008].

In this framework a plausible model to explain these inconsistences has been proposed. After primary infection, JCPyV establishes latency in the brain without replicating or expressing viral proteins. Under severe immunosuppression, the virus can infect permissive cells, such as oligodendrocytes, triggering a lytic cycle, cell destruction, and the development of PML. In contrast, transient physiological changes in healthy individuals may allow limited expression of LTAg, leading to its accumulation in neural cells. This oncogenic protein can then interact with host cell cycle regulators, promoting uncontrolled proliferation and potentially contributing to tumor formation [Perez-Liz et al., 2008; Del Valle et al., 2008].

2. AIMS

JCPyV is well recognized as the causative agent of PML but its plausible contribution to brain carcinogenesis is disputed [Prado *et al.,* 2018; Ahye *et al.,* 2020]. Several studies described the isolation of viral sequences and proteins from CNS tumors, as well as the effects of JCPyV on regulatory pathways [Khalili, 2001; White & Khalili, 2004; Delbue *et al.*, 2005; Passerini *et al.,* 2023]. The mechanisms through which JCPyV contributes to cellular transformation involve the capability of LTAg to disrupts signaling cascade implicated in cell cycle control [Del Valle *et al.,* 2008; An *et al.,* 2012]. One of the major pathways influenced by JCPyV is the canonical Wnt/β-catenin signaling pathway [Gan *et al.,* 2001; Gan & Khalili, 2004; Ripple *et al.,* 2014]. Dysregulation of this pathway is frequently observed in cancer, including glioblastoma, where it contributes to tumor aggressiveness, therapeutic resistance, and maintenance of glioma stem-like cells [Alkailani et al., 2022; Manfreda et al., 2023]. Previous studies showed that JCPyV LT protein can interact with β-catenin and co-localize in the nuclei, where it enhances transcription of β-catenin downstream targets such as c-myc and cyclin D1 [Gan *et al.,* 2001; Gan & Khalili, 2004]. These findings are supported by the observation of β-catenin nuclear localization in LT-positive tumors [Ripple *et al.,* 2014]. Moreover, other β-catenin responsive genes involved in cellular differentiation, angiogenesis and apoptosis seem to be regulated by LT. However, despite increasing evidence of JCPyV ability to activate the Wnt signaling cascade, especially in the context of brain tumors, the downstream consequences of this activation remain largely unknown.

Altogether, the evidence discussed above highlights the need for further molecular and epidemiological investigations to elucidate the mechanisms underlying JCPyV-mediated tumorigenesis in the human brain.

In light of these considerations, the aim of this PhD project was to elucidate the plausible contribution of JCPyV in brain tumor formation and progression, focusing on the impact of JCPyV infection on Wnt signaling pathway.

Notably, the study was conducted both *ex vivo*, in a cohort of pediatric patients affected by glioma and *in vitro*, employing a glioblastoma cell line. The specific aims are outlined below.

The objectives of the *ex vivo* studies was:

- Investigate JCPyV viral load and DNA sequences mutations
- Examine early (LTAg) and late (VP1) genes' expression, as well as viral miRNAs
- Analyze the expression of mRNA levels of β-catenin and its downstream effectors

Regarding the *in vitro* studies the aims were:

- Examine JCPyV replication and transcription in a glioblastoma cell line, investigating viral load, early and late genes, and miRNAs expression
- Examine the expression profile of β-catenin and Wnt-responsive oncogenes following JCPyV infection

3. MATERIALS AND METHODS

EX VIVO STUDIES

3.1 Study population

To evaluate the prevalence of JCPyV in brain tumors, formalin-fixed paraffin-embedded (FFPE) tissue sections from glioma biopsies were collected from 101 pediatric patients (57 males and 44 females, mean age 12.8±11.4) as part of the Italian National Program of Centralization of Pediatric Brain Tumor.

Specimens were chosen based on the accessibility of complete clinical data and the availability of FFPE tissues for analysis. To ensure adequate tumor content, hematoxylin and eosin (H & E) slides were reviewed after the initial cut of each FFPE block for DNA extraction.

This research study was conducted retrospectively from data obtained for clinical purposes. Moreover, the study was performed according to local Ethical and Institutional Review Board approval (Protocol Number: 1337/20), and informed consent was obtained in accordance with the Declaration of Helsinki.

The demographic and clinical characteristics of patients are listed in Table 2.

Table 2. Demographic and clinical characteristics of patients

Features	**Population**	
Patients	101	
Sex, n (%)	M 57 (56.4%)	F 44 (43.6%)
Mean age, years (SD)	12.8 (±11.4)	
Tumor type, n		
Adamantinomatous craniopharyngioma (gr. I)	1	
Anaplastic astrocytoma (gr. II)	1	
Anaplastic astrocytoma (gr. III)	13	
Pilocytic astrocytomas (gr. I)	13	
Pilocytic astrocytomas (gr. III)	1	
Anaplastic medulloblastoma (gr. IV)	2	
Anaplastic pleomorphic xanthoastrocytoma (PXA) (gr. III)	1	

Central neurocytoma (gr.II)	1
Medulloblastoma (gr. IV)	4
Diffuse astrocytoma (gr. II)	7
Ependymoma (gr. II)	6
Anaplastic ependymoma (gr. III)	3
Ganglioglioma (gr. I)	1
Anaplastic medulloblastoma (gr. IV)	2
Glioblastoma (gr. IV)	27
Gliosarcoma (gr. IV)	1
Anaplastic Xanthoastrocytoma (gr. III)	1
Pleomorphic Xanthoastrocytoma (gr. II)	3
Emangioblastoma (gr. I)	1
Neuroblastoma	1
High-grade glioma	8
Low-grade glioma	3

3.2 DNA extraction from brain tissues

After deparaffinization with Xylene, total DNA extraction was performed using Quick-DNA FFPE Miniprep (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The extracted nucleic acid was eluted in a final volume of 50 μl, and DNA was evaluated for its PCR suitability by amplifying the *β-globin* sequences [Hashida *et al.*, 2013].

3.3 RNA extraction from brain tissues

Following deparaffinization with Xylene, total RNA, including miRNAs, was extracted from the FFPE tissue sections, using the Total RNA purification kit (Norgen, Thorold, ON, Canada).

3.4 Nucleic Acids Quantification

Nucleic acids have nitrogenous bases that absorb ultraviolet light between 230 and 320 nm, with a peak absorbance at 260 nm. This property allows nucleic acids to be quantified by spectrophotometry. According to Lambert-Beer's law, the light absorbed by a medium is proportional to the concentration of the medium traversed. An OD260 of 1 corresponds to a concentration of approximately 50 ng/μl of ds-DNA

and approximately 40 ng/µl of single-stranded RNA. To assess the purity of the sample, an absorbance reading was taken at 280 nm, which corresponds to the absorbance peak of proteins, one of the main contaminants of the extract, and the ratio between the absorbance at 260 nm (DNA) and 280 nm (proteins) was calculated. A DNA sample with an A260/A280 ratio of approximately 1.8 can be considered pure, while for RNA this ratio must be approximately 2. If this ratio is lower, there is a high probability that the sample is contaminated with proteins.
To measure the purity and concentrations of DNA and RNA extracted from FFPE samples, a NanoDrop spectrophotometer 2000 (Thermo Scientific) was used.

3.5 Detection of JCPyV DNA by Real Time Polymerase Chain Reaction (qPCR)

The presence and quantity of viral DNA in FFPE sections were evaluated by a TaqMan quantitative polymerase chain reaction (qPCR), using primers and probe targeting a 54 bp amplicon in JCPyV LTAg region [Delbue *et al.*, 2008; Prezioso *et al.*, 2022] (Table 3). The standard curve was obtained from serial dilutions (range: 10^5–10^2 copies/ml) of a plasmid containing the entire JCPyV genome. The lower detection limit of the qPCR system was 10 DNA copies of the target gene per amplification reaction.
The qPCR was performed with a 7300 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), using the following thermocycling conditions: 50 °C for 2 min, 95 °C for 10 s, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Primers, probe and template DNA were added to the SensiMix™ II Probe Hi-ROX Kit (Bioline USA Inc., Taunton, MA, USA), in a final reaction volume of 20 µL. Specifically, 3 µL template DNA were added to 10 µL of master mix, 1 µL of forward primer (0,5 µM), 1 µL of reverse primer (0,5 µM), 0.5 µL of probe (0,25 µM) and 4.5 µL of DNase/RNase-free water. Each sample was analyzed in triplicate, and JCPyV DNA loads, given as the mean of at least three positive reactions, were expressed as copies/cell. Specifically, a standard curve of *β-globin* was used for cell quantification, and JCPyV copies were

normalized by calculating the ratio of viral copy numbers to half of *β-globin* gene copy numbers. Precautions to prevent contamination were followed, and a negative control was included in each qPCR session.

The sequence of each primer and probe is shown in Table 3.

Table 3. Sequences of primers employed for viral DNA analysis.

PCR assay	Target gene	Sequence (5′→3′)	Reference
Real Time PCR	LTAg	F: 5′-GAGTGTTGGGATCCTGTGTTTTC-3′ R: 5′-GAGAAGTGGGATGAAGACCTGTTT-3′ probe: 5'-FAM-TCATCACTGGCAAAC ATTTCTTCATGGC-MGB-3'	[Delbue *et al.*, 2008]
Standard PCR	LTAg N-terminal	F: AGTCTTTAGGGTCTTCTACC R: GGTGCCAACCTATGGAACAG	[Krinska *et al.*,1999]
	LTAg C-terminal	F: AACCAGCTTTACTTAACAGTTG R: CTCTAGGAAAGTCAAGAATGGG	
	VP1	5′-ACAGTGTGGCCAGAATTCCACTAC-3′ 5′-TAAAGCCTCCCCCCCAACAGAAA-3′	[Pagani *et al.*, 2003]
Nested PCR	NCCR	I N: 5′- AAGGTCCATGAGCTCCATGGATTCTTCC -3′ I N: 5′-CATGGTCCCCCAAAAGTGCTAGAGCAGC-3′ II N: 5′-CCTCCACGCCCTTACTACTTCTGAG-3′ II N: 5′-AGCCTGGTGACAAGCCAAAACAGCTCT-3′	[Flægstad *et al.*,1991]

3.6 Standard PCR for LTAg N-terminal and C-terminal regions and VP1

JCPyV-positive samples were further analyzed using standard PCR for LTAg N-terminal and C-terminal regions and for VP1, employing previously described primers [Krinska *et al.*, 1999; Pagani *et al.*, 2003]. Specifically, PCRs were carried out using a 9700 GeneAmp® PCR System (Perkin-Elmer Cetus, Emeryville, CA, USA), in a final volume of 50 μl consisting of 0.3 μM of each primer, 0.2 μM of dNTP and 1.25 U of DreamTaq Polymerase in a suitable reaction buffer (DreamTaq™ Buffer (10X), Thermo Fisher Scientific, Waltham, MA). The PCR products were analysed on a 2% agarose gel and were visualized using SafeView reagent (Applied Biological

Materials, Vancouver, BC, Canada) under UV light. The employed primers are listed in Table 3.

3.7 Nested PCR for NCCR

Virus-positive specimens were also subjected to a nested PCR for NCCR region, using two set of primers (Table 3) [Flægstad *et al.*,1991; Prezioso *et al.,* 2018]. In the first amplification reaction, 5 μl of DNA were added to the PCR mix, while in the second reaction, 5 μl of template DNA obtained from the first amplification were added. The final amplification product is a 308 bp fragment [Markowitz *et al.,* 1993]. PCR products were analyzed on a 2% agarose gel and visualized under UV light.

3.8 Sequencing of NCCR and VP1 regions

In order to asses NCCR and VP1 variations, the PCR products were purified using the miPCR purification kit (Metabion, Plannegg, Germany) and sequenced in a dedicated facility (Bio-Fab research, Rome, Italy). Obtained sequences were compared to the reference strain (GenBank: AB038249). Sequence alignment was performed using ClustalW2 on the European Molecular Biology Laboratory–European Bioinformatics Institute (EMBL–EBI) website using default parameters. Moreover, JCPyV genotypes were classified based on single-nucleotide polymorphisms (SNPs) found within the amplified VP1 region [Jobes *et al.,* 2001; Pagani *et al.,* 2003].

3.9 Reverse-transcription

Reverse-transcription was performed on 100 ng of purified RNA using the SensiFast cDNA Synthesis kit (Meridian). Briefly, a 20 μL was prepared containing 10 μL RNAse/DNase free water, 4 μL 10x buffer, 1 μl of reverse-transcriptase per sample. The RTase mixture was combined with the extracted RNA and processed in a thermocycler employing the following step's reaction profile: 25 °C for 10 min (primer annealing), 42 °C for 15 min (reverse transcription), 85 °C for 5 min (inactivation).

3.10 Detection of LTAg and VP1 transcripts

An aliquot (3 µl) of the RT reaction mixture was employed for the subsequent PCR amplifications targeting *LTAg* and *VP1*, as previously described [Passerini *et al.*, 2023]. The primer sequences used are listed in Table 4. The *GAPDH* gene was also amplified to confirm the presence of PCR-amplifiable cDNA.

Table 4. Sequences of primers employed for viral RNA analysis.

PCR assay	Target gene	Sequence (5′→3′)	Reference
RT-PCR and RT-qPCR	LTAg	F: AGTCTTTAGGGTCTTCTACC R: GGTGCCAACCTATGGAACAG	Passerini *et al.*, 2023
	VP1	5′-ACAGTGTGGCCAGAATTCCACTAC-3′ 5′-TAAAGCCTCCCCCCCAACAGAAA-3′	
RT-PCR	miR-5p	F: 5′-GCAGTTATTTTGGGGGAGGGG-3′ R: 5′-GAGACCCATTCTTGACTTTCC-3′)	Giovannelli *et al.*, 2015

3.11 Detection of viral miRNAs

Viral miRNAs detection was performed using the specific Applied Biosystems™ TaqMan™ MicroRNA Assays (Thermo Fisher Scientific, Waltham, MA, USA). Specifically, the pre-designed TaqMan microRNA assay for JCPyV-miR-J1-5p (ID 007464) was employed. Moreover, the human miRNA RNU6B (ID001093) was included as an endogenous control for extraction and reverse-transcription efficacy.

3.12 Sequencing of viral miRNAs

In order to evaluate plausible variations in JCPyV miRNAs, PCR-amplification was carried out employing primers specific for the miRNAs expressing region (Giovannelli *et al.*, 2015). The PCR products were purified using the miPCR purification kit (Metabion, Plannegg, Germany) and sequenced in service (Bio-Fab research, Rome, Italy). The obtained sequences were compared to the reference (miRbase MI0009980) using ClustalW algorithm, applying default parameters.

3.13 Analysis of *β-catenin*, *c-myc* and *cyclin D1* mRNA levels

RT-qPCR was performed for *β-catenin*, *c-myc* and *cyclin D1* with a StepOne Real-Time PCR system (Applied Biosystem) using previously described primers [Sareddy *et al.*, 2009; Di Magno *et al.*, 2020].

The template cDNA and specific primers for the target genes were added to the SYBR™ Green Universal Master Mix (Applied Biosystems), containing SYBR Green I dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer. Specifically, 5 μL template cDNA was added to 10 μL of master mix, 0.5 μL of forward primer (0,5 μM), 0.5 μL of reverse primer (0,5 μM), and 4 μL of DNase/RNase-free water. Each sample was analyzed in triplicate and negative controls were included in each session. The qPCR was carried out with the following thermos-cycling conditions: 95 C for 10 minutes, 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 1 second for a total of 40 cycles. The housekeeping *GAPDH* gene was used as an internal quantitative control to normalize the obtained mRNA levels, and the relative expression of the genes was expressed as $2^{-\Delta Ct}$. The primers employed for the analysis are listed in Table 5.

Table 5. Sequences of primers used for RT-qPCR analysis.

PCR assay	Target gene	Sequence (5′→3′)	Reference
RT-qPCR	β-catenin	5′-CACAAGCAGAGTGCTGAAGGTGC-3′ 5′-AAGGAGGCCTTCCATCCCTTC-3′	[Sareddy *et al.*, 2009]
	c-myc	F: 5′-CACCACCAGCAGCGACTCT-3′ R: 5′-TTCCACAGAAACAACATCGATTTC-3′	
	Cyclin D1	F: 5′- GTGGCCTCTAAGATGAAGGAG -3′ R: 5′-GAACTTCACATCTGTGGCACAG-3′	
	Fgf20	5′-TAGAGGTGTGGACAGTGGTCTC-3′ 5′-CTTCAAACTGCTCCCTAAAGATGC-3′	[Di Magno *et al.*, 2020]
	Sall4	F: 5'-TGCAGCAGTTGGTGGAGAAC-3'	
		R: 5'-TCGGTGGCAAATGAGACATTC-3'	

3.14 Statistical analysis

For statistical analysis, data were expressed as the mean ± standard deviation or as median and interquartile range (IQR). Viral loads and gene expression were tested using the Shapiro–Wilk test to assess normal distribution. Viral loads were compared by Mann-Whitney U for unmatched data, whereas mean values of Wnt target genes were compared by Student's t-test. $p < 0.05$ was considered statistically significant.

IN VITRO STUDY

3.15 Cell line

U-87 MG is a cell line with epithelial morphology, isolated from malignant gliomas from a male patient, likely with GBM.

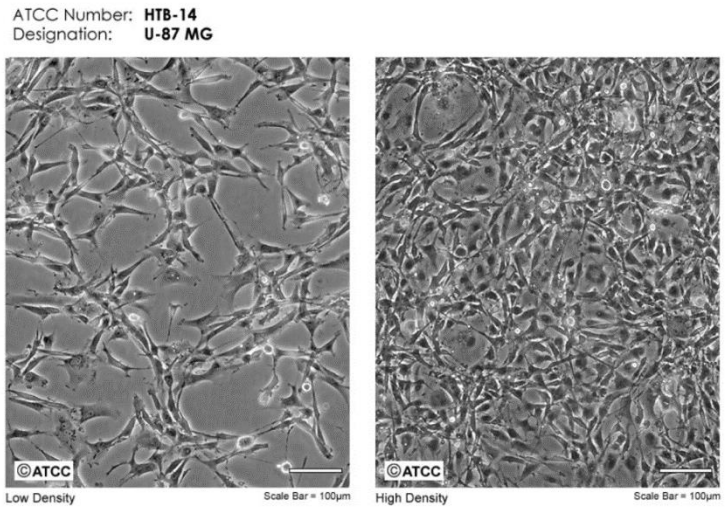

Figure 12. Morphological characteristics of U87-MG cells [https://www.atcc.org/].

Cells, obtained from the American Type Culture Collection (Figure 12) (ATCC-HTB-14, ATCC, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with supplemented with 100 U of penicillin and 100 µL of streptomycin per mL, respectively (Sigma-Aldrich S.r.l., Milano, Italia), and fetal bovine serum (FBS) (10%). The cells were incubated at 37 °C in the presence of 5% CO_2 and propagated at a ratio of 1:4. The cells were then plated in 12-well

plates at a density of $1x10^5$ cells/milliliter (ml) and grown for 24 hours in complete growth medium to reach 50% confluence on the day of transfection.

3.16 Transfection and infection experiments

The pCY/cl1 plasmid, which carries the archetype JCPyV genome sequence, was purchased from ATCC (ATCC® VRMC-1™). The JCPyV DNA was recovered for BamHI-digested pCY/cl1 plasmid and the linear DNA was gel-extracted and purified using a GenepHlow ™ DNA Cleanup Maxi Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan). The DNA was then quantified, and 2.5 μg were used for transfection experiments.

U87-MG cells were transfected with 2.5 μg of JCPyV CY strain DNA using the Xfect TM Transfection Reagent kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The cells were incubated at 37 °C for 4 h with the transfection mixture. Therefore, after two washes with phosphate-buffered saline (PBS), cells were incubated with complete medium for the time-course experiment. Supernatants and cellular fractions were harvested and stored at 2, 7 and 12 days' post transfection (d.p.t.). Supernatants harvested from transfection experiments were subjected to 6 cycles of freezing and thawing, followed by centrifugation at 2000 rpm for 5 min and then analyzed by qPCR for JCPyV LTAg, as previously described [Delbue *et al.*, 2008; Prezioso *et al.*, 2022]. Supernatants corresponding to $1x10^3$ copies/ml were then used to infect freshly seeded U87-MG cells. After a 3 h adsorption phase, the cells were washed 2 times with PBS and incubated with fresh medium. Supernatants and cell fractions were harvested at 2, 7 and 12 days' post infection (d.p.i.).

3.17 DNA extraction from U87-MG cells

Total DNA was extracted from $1x10^5$ cells using the Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA) and stored at -20°C until use. Before being used for analysis, the extracted DNA was examined for its PCR suitability by amplifying the GAPDH gene sequences [Sareddy *et al.*, 2009], whereas supernatants were subjected to 6

cycles of freezing and thawing, followed by centrifugation at 2000 rpm for 5 min and then directly used in qPCR assays.

3.18 Real Time PCR for JCPyV LTAg

The presence and quantity of JCPyV DNA was assessed by a TaqMan qPCR targeting LTAg sequence [Delbue *et al.,* 2008; Prezioso *et al.,* 2022]. Specifically, each sample was examined in triplicate and a negative control was included in each run. Viral loads were determined using a standard curve obtained from serial dilutions of a plasmid containing the entire JCPyV genome (range 10^5-10^2 copies/ml). The amount of cellular DNA was calculated using a standard curve of *β-globin* and JCPyV copies were normalized by calculating the ratio of viral copy numbers to half of *β-globin* gene copy numbers. For U87-MG cells the DNA content was expressed as copies of viral DNA per cell (copies/cell), and as copies of viral DNA per milliliter (copies/mL) for supernatants.

3.19 Total RNA isolation and RT-qPCR

Total RNA, including miRNAs, was extracted from $1x10^5$ cells using the Total RNA purification kit (Norgen, Thorold, ON, Canada). Following quality and quantity assessment by A230/A260 ratios, RNA was reverse-transcribed by SensiFAST cDNA Synthesis kit (Meridian Bioscience, Cincinnati, OH, USA). An aliquot of cDNA was used in a qPCR in order to investigate JCPyV *LTAg* and *VP1* genes' expression during time course experiment [Passerini *et al.,* 2023]. Relative mRNA levels were normalized to *GAPDH* [Sareddy *et al.,* 2009].

3.20 Quantification of viral miRNAs

Viral miRNAs expression was examined using the Applied Biosystems™ TaqMan™ MicroRNA Assays (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Specifically, a pre-designed TaqMan miRNA assays for JCPyV-miR-J1-5p (ID 007464) was used for viral miRNA detection whereas RNU6B (ID 001093) was used as an endogenous control for normalization of miRNA

expression. Relative miRNAs expression levels were calculated by the comparative ΔCt method and reported as $2^{-\Delta Ct}$.

3.21 Sequencing of viral miRNAs

To identify potential mutations within miRNA-coding regions, PCR amplification was performed using primers specific to the miRNA-expressing regions, as previously described [Giovannelli *et al.*, 2015]. The resulting PCR products were purified (miPCR Purification Kit, Metabion, Planegg, Germany) and sequenced (Bio-Fab Research, Rome, Italy). The obtained sequences were analyzed using the ClustalW algorithm.

3.22 Analysis of Wnt genes expression

To further confirm the activation of Wnt/β-catenin signaling following JCPyV infection, mRNA expression levels of *β-catenin*, *c-myc*, *cyclin D1*, *Fibroblast Growth factor 20* (Fgf20) and *Sall4* were examined in pellets from infected and control cells collected at 2, 7 and 12 days.

Sybr-Green-based RT-qPCR were carried out using the StepOne Real-Time PCR system (Applied Biosystem) with the following thermo-cycling conditions: 95°C for 10 minutes, 95 °C for 15 seconds, 60 °C for 15 seconds and 72°C for 10 seconds for a total of 40 cycles. Specific primers targeting each gene and cDNA template were combined to the SYBR™ Green Universal Master Mix (Applied Biosystems) for a final reaction volume of 20 μL. Specifically, 3 μL of template cDNA was added to 10 μL of Master Mix, 0.5 μL of Forward Primer (0.5 μM), 0.5 μL of Reverse Primer (0.5 μM) and 7 μL of DNase/RNase-Free water.

All reactions were performed in triplicate. The house keeping *GAPDH* gene was used as an internal quantitative control to normalize the obtained mRNA levels Therefore, the relative expression of the genes was expressed as $2^{-\Delta\Delta Ct}$.

The primers used are listed in Table 5.

3.23 Cell Treatment for β-Catenin Pathway Activation Controls

To assess whether viral infection specifically induced activation of the Wnt/β-catenin pathway, a control treatment was performed to experimentally modulate Wnt signaling. Cells were cultured under starvation medium, consisting of Opti-MEM (Thermo Fisher Scientific) without serum supplementation.

This treatment provides reference conditions to confirm whether β-catenin translocation observed in virus-infected cells reflected genuine activation of the Wnt/β-catenin pathway. Cell fractions were collected at 2, 7 and 12 days; therefore, following RNA extraction, Wnt target genes were analyzed as described.

3.24 Fragmentation nucleus-cytoplasm

Cytoplasmic and nuclear proteins were extracted with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockland, IL) following the manufacturer's instructions.

Therefore, protein concentrations were determined using the Bio-Rad Protein Assay kit (BioRad Laboratories, Inc.). Equal amounts of proteins were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter membrane which was blocked with 5% nonfat milk in TBS with 0.05% Tween 20 for 1 h at room temperature. The membrane was then incubated overnight with the murine monoclonal antibody anti- β-catenin (1:500 dilution, clone E5, Santa Cruz Biotechnology, Santa Cruz, CA, and with the anti-mouse HRP-conjugated antibody (Bio-Rad). Lamin B1 and Vinculin were used as nuclear and cytoplasmic marker, respectively (anti-lamin B1 antibody, 1:100 dilution, sc-374015; anti-vinculin antibody, 1:1000 dilution, sc-73614, Santa Cruz Biotechnology). The signals were detected with enhanced chemiluminescence reagents (Bio-rad).

3.25 Statistical analysis

Statistical analysis was performed using Graphpad Prism Software. The Shapiro-Wilk test was used to assess whether the variables were normally distributed. Mean values of Wnt target genes in infected cells and controls were compared by Student's *t*-test whereas one-way ANOVA followed by Student's t-test with Bonferroni correction was used to compare mRNA levels among uninfected, infected and starvation-treated cells. The statistical significance was set at $p<0.05$.

4. RESULTS

In order to investigate JCPyV prevalence in brain tumors and to elucidate its plausible role in brain tumor development, two different approaches were followed: *in vivo* studies on clinical samples from gliomas and *in vitro* studies on a glioblastoma cell line.

Ex vivo results

4.1 Prevalence of JCPyV DNA in brain tumor tissues

JCPyV DNA was found in 31/101 (30.7%) tumor biopsies with a median viral load of 3.2 (IQR: 0.9-14) copies/cell. JCPyV was isolated at a higher rate (n=21/31, 67.7%) in high-grade tumors. However, when comparing viral loads in high and low-grade tumors, no significant differences were observed ($p>0.05$) (Table 7; Figure 10).

Table 7. JCPyV viral load and molecular state in JCPyV-positive tumors.

Case n°	Diagnosis	Viral load (copies/cell)	Viral Sequences		Viral Transcripts		
			NCCR	VP1	LT	VP1	miR-5p
5	Anaplastic Xanthoastrocytoma (gr. III)	247	archetype	1A	P	N	N
6	Pilocytic astrocytoma (gr. I)	1.09	archetype	1A	P	P	P
7	Anaplastic ependimoma (gr. III)	11	archetype	1B	P	N	N
8	Ganglioglioma (gr. I)	41.7	archetype	1A	P	N	N
9	Central neurocytoma (gr. II)	19.3	archetype	1B	P	N	N
10	Glioblastoma (gr. IV)	0.7	archetype	1A	P	P	P
11	Anaplastic astrocytoma (gr. III)	3.2	archetype	1B	P	P	P
12	Glioblastoma (gr. IV)	1.7	archetype	1A	P	P	P
13	Glioblastoma (gr. IV)	0.3	archetype	1B	P	P	P
14	Glioblastoma (gr. IV)	3.2	archetype	1A	P	P	P
15	High-grade glioma	0.8	archetype	1A	P	P	P
16	Gliosarcoma (gr. IV)	0.9	archetype	1A	P	P	P
20	Diffuse astrocytoma (gr. II)	0.7	archetype	1B	P	P	P
21	Glioblastoma (gr. IV)	2.35	archetype	1B	P	P	P
22	Anaplastic astrocytoma (gr. III)	7.97	archetype	1A	P	P	P
23	Diffuse astrocytoma (gr. II)	53.4	archetype	1B	P	N	N
28	Diffuse astrocytoma (gr. II)	3.4	archetype	1A	P	P	P
29	High-grade glioma	0.9	archetype	1B	P	P	P
57	High-grade glioma	3.1	archetype	1B	P	P	P
59	Glioblastoma (gr. IV)	2.1	archetype	1A	P	P	P
60	Glioblastoma (gr. IV)	14	archetype	1A	P	P	P

62	Glioblastoma (gr. IV)	9.6	archetype	1B	P	P	P
63	Glioblastoma (gr. IV)	3.3	archetype	1B	P	P	P
74	Low-grade glioma	16.6	archetype	1A	P	P	P
82	Glioblastoma (gr. IV)	59.9	archetype	1B	P	P	P
85	Pilocytic astrocytoma (gr. III)	1.84	archetype	1A	P	P	P
93	Glioblastoma (gr. IV)	93.3	archetype	1B	P	P	P
95	Ependimoma (gr. II)	0.65	archetype	1A	P	P	P
96	Pilocytic astrocytoma (gr. I)	4.6	archetype	1A	P	P	P
98	Pilocytic astrocytoma (gr. I)	0.6	archetype	1B	P	P	P
99	High-grade glioma	3.25	archetype	1A	P	P	P

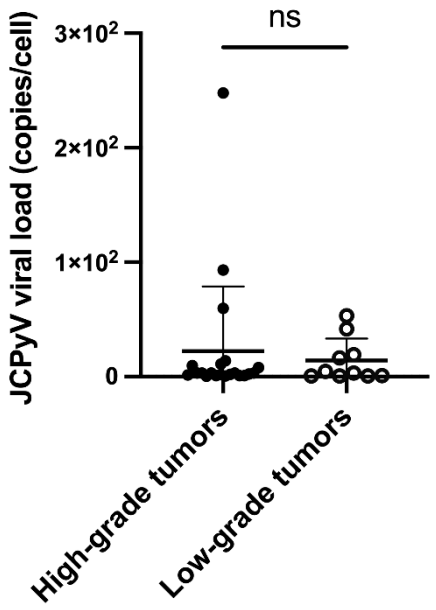

Figure 10. JCPyV viral loads according to tumor grading.
Grades I and II were considered low-grade tumors, whereas grades III and IV were considered high-grade tumors. Viral loads in high-grade and low-grade tumors were compared by the Mann-Whitney U test. ns = $p > 0.05$

4.2. Analysis of JCPyV NCCR and VP1 structure

LTAg N-terminal and C-terminal region, as well as NCCR and VP1 sequences were detected in all JCPyV-positive samples. Moreover, in order to explore sequence variations, the amplified JCPyV NCCRs were aligned to the reference strain, revealing an archetype structure in all samples tested positive for JCPyV DNA detection (Table 7, Figure 13). VP1 also showed a high degree of homology with the

reference strain. Specifically, when investigating the viral genotype, the European genotype 1A was found in 17/31 (54.8%) and the 1B in 14/31 (45.2%) virus-positive samples (Table 7).

Figure 13 Archetypal structure of JCPyV NCCR. All virus-positive samples display an identical archetypal NCCR organization (blocks A–F). *Created by BioRender.com*

4.3 Expression of transcripts from LTAg and VP1 genes

JCPyV-positive tumors were further subjected to transcript analysis of early and late genes by RT-PCR. All samples showed the expression of *LTAg*, whereas *VP1* was detected in 26/31 (83.9%) JCPyV-positive tissues (Table 7).

4.4 Viral miRNAs detection and sequencing

Among JCPyV DNA-positive samples, JCPyV-miR-J1-5p was found in 26/31 (83.9%) samples. More specifically, viral miRNAs were detected in the same 26 tissues tested positive for *VP1* gene expression (Table 7). In addition, when sequencing JCPyV miRNA coding region, all virus-positive samples displayed a canonical sequence, identical to the reference (JCV-miR-J1, miRbase) (Figure 14)

Figure 14. JCPyV miRNA-coding region in brain tumors. The mature miR-J1-5p and miRNA-J1-3p are indicated in red and green, respectively. All virus-positive samples (n=26, 100%) reported canonical sequences, identical to the reference JCV-miR-J1 from miRBase [Lagatie *et al.*, 2013].

4.5 Relative expression of Wnt target genes

Expression levels of genes encoding *β-catenin* and its downstream targets *c-myc*, *cyclin D1*, *Fgf20* and *Sall4*, were measured in tumor tissues. A significant over-expression of mRNA levels was observed in JCPyV-positive samples compared to negative ones, for all the examined genes (p<0.05) (Figure 15).

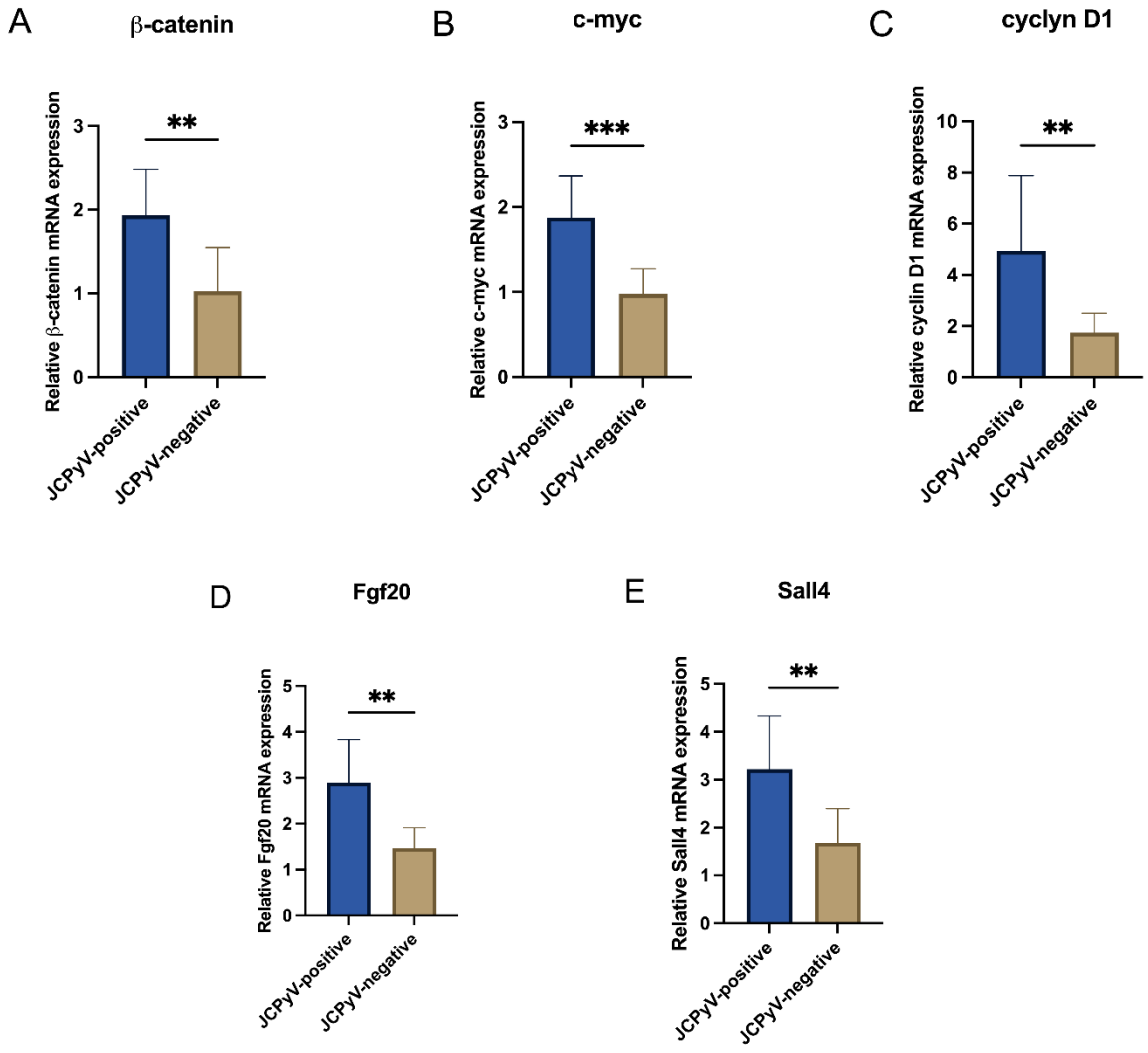

Figure 15. Relative expression of genes encoding *β-catenin*, *c-myc*, *cyclin D1*, *Fgf20* and *Sall4* in JCPyV-positive and JCPyV-negative brain tumors. Relative mRNA levels were calculated and expressed as $2^{-\Delta Ct}$. Mean values of examined genes (*β-catenin*, Panel A; *c-myc*, Panel B; *cyclin D1*, Panel C; *Fgf20*, Panel D; *Sall4*, Panel E) in JCPyV-positive and negative groups were compared by independent T-test. *p < 0.05; **p < 0.01; ***p ≤ 0.001.

In vitro results

4.6 JCPyV replication in U87-MG cell line

In order to examine the efficiency of JCPyV replication, U87-MG cells were transfected with JCPyV DNA. Results from qPCR experiments revealed that JCPyV efficiently replicates in this glioblastoma cell line. Indeed, JCPyV LTAg DNA was detected during sampling period. Specifically, an increase in viral replication was observed from 2 to 7 d.p.t., which then slightly decreased at 12 d.p.t. (Figure 16A). Therefore, in order to confirm that viral particles were produced during transfection, supernatants corresponding to $1x10^3$ copies/ml were used to infect freshly seeded U87-MG cells. An efficient viral replication was observed even during infection experiments. Indeed, at 2 d.p.i., a viral load of $3.9x10^3$ was reported in cell fractions, which then peaks at 7 d.p.i. reaching $7.25x10^3$ copies/cell and then declines at $6.4x10^3$ at 12 d.p.i. In parallel, JCPyV replication was examined by measuring LTAg DNA in supernatants by qPCR. Comparing intracellular JCPyV replication (copies/cell) with the one reported in supernatants (copies/ml), a similar trend was observed. The amount of viral DNA increased during the first 7 days and then decrease at 12 d.p.i. (Figure 16B).

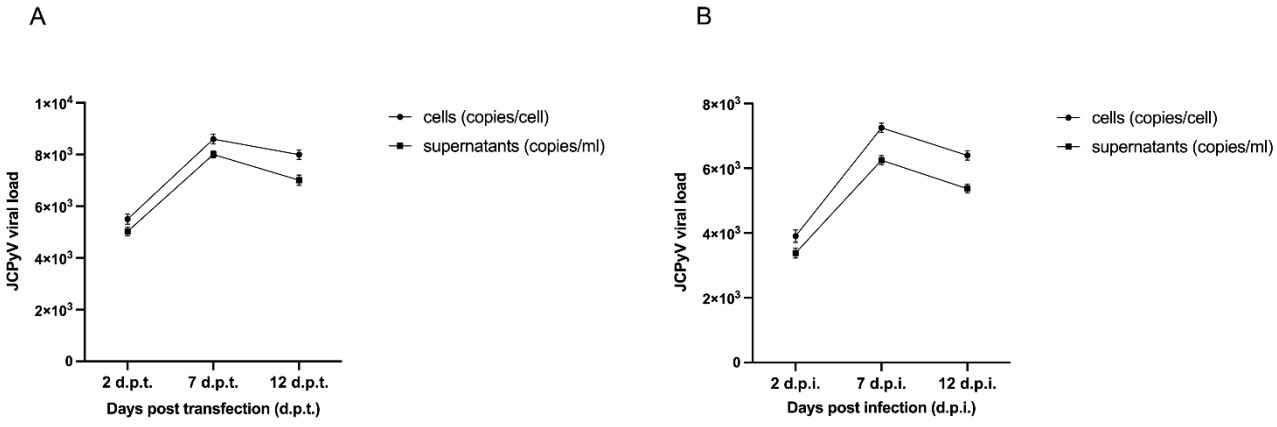

Figure 16. Replication studies of archetype JCPyV in U87-MG cells and supernatants during tranfection (**A**) and infection (**B**) experiments. JCPyV replication was assessed at selected sampling times, from 2 to 12 days. JCPyV DNA was quantified by qPCR and expressed as copies/cell for cells and as copies/ml for supernatants. Data are expressed as the mean of 3 independent experiments and error bars indicate standard deviations.

4.7 Early and late genes transcription

The efficiency of viral replication was further assessed by evaluating JCPyV early (*LTAg*) and late (*VP1*) genes expression. Transcription analysis revealed that both JCPyV *LTAg* and *VP1* transcripts were readily detectable at 2 d.p.i. Although different amounts of early and late transcripts were reported, LTAg and VP1 expression revealed a similar trend during the sampling period (Figure 17).

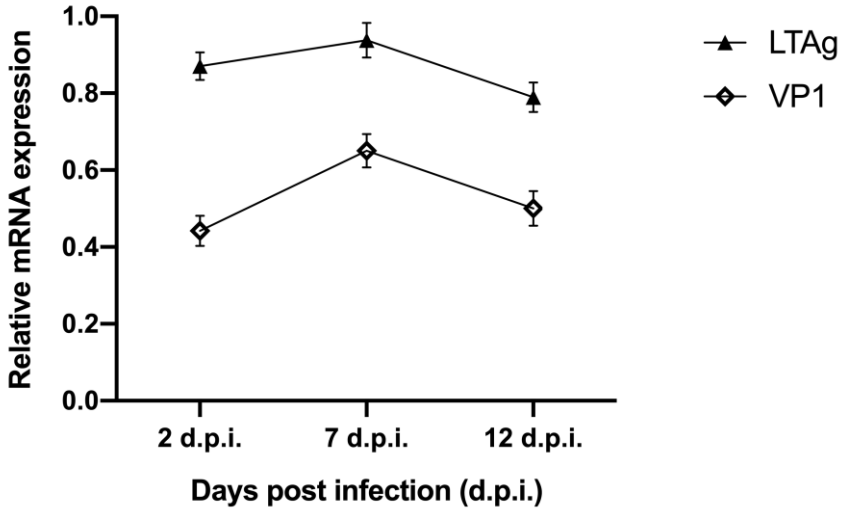

Figure 17. Relative expression of JCPyV *LTAg* and *VP1* genes in infected cells at selected sampling times, from 2 to 12 d.p.i. Following RNA extraction and reverse transcription, *LTAg* and *VP1* genes expression was measured by qPCR. Relative mRNA levels were normalized to *GAPDH* and expressed as $2^{-\Delta Ct}$. Data are reported as the mean of three independent experiments and error bars represent standard deviations.

4.8 Viral miRNAs quantification and sequencing

To better characterize JCPyV infection, viral miRNAs were investigated. Analyzing JCPyV-miR-J1-5p relative expression in U87-MG cells during time course experiment, a decrease was observed from 2 to 7 d.p.i., followed by a slight increase from 7 to 12 d.p.i. The same analysis was performed even in supernatants which revealed a similar trend in miRNAs levels (Figure 18).

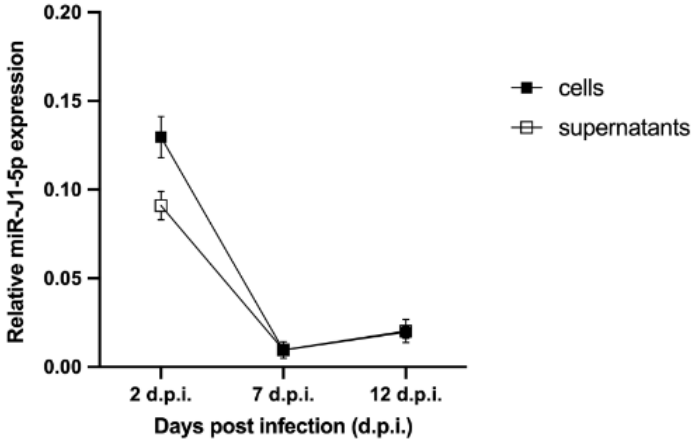

Figure 18. Relative expression of miR-J1-5p in U87-MG cells and supernatants at selected sampling times, from 2 to 12 d.p.i. A pre-designed TaqMan miRNA assay for JCPyV-miR-J1-5p was used for viral miRNA detection and RNU6B was used for normalization. Relative miRNAs expression levels were calculated by the comparative ΔCt method and reported as $2^{-\Delta Ct}$. The values are expressed as the mean of 3 independent experiments, and error bars indicate standard deviations.

Moreover, when comparing viral miRNAs relative expression with JCPyV LTAg DNA, an inverse trend was reported both in cell fractions (Figure 17A) and supernatants (Figure 17B); indeed, when viral replication increased, a decrease in miR-J1-5p relative levels was observed. Instead, from 7 to 12 d.p.i, viral miRNA expression rose and the replication rate declined (Figure 19).

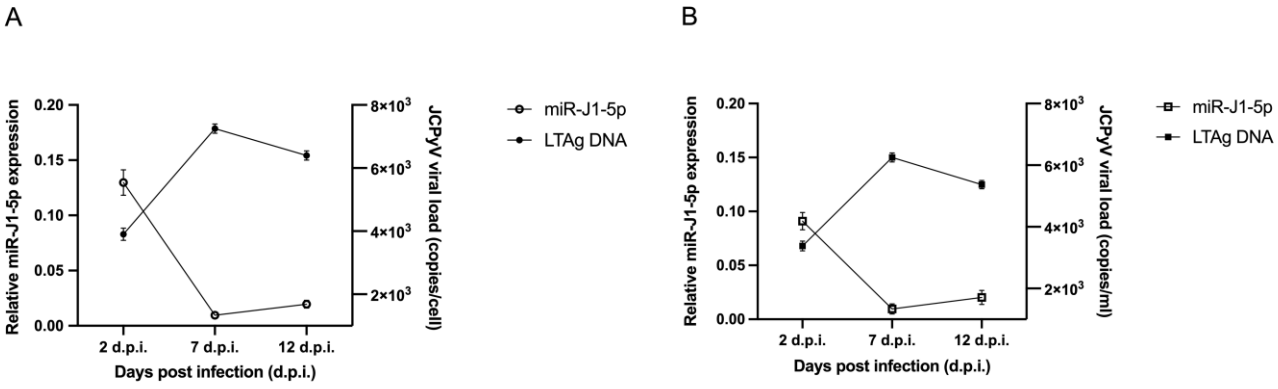

Figure 19. JCPyV viral load and relative expression of miR-J1-5p in U87-MG cells (**A**) and supernatants (**B**) at selected sampling times, from 2 to 12 d.p.i. JCPyV DNA was quantified by qPCR and expressed as copies/cell for cells and as copies/ml for supernatants. A pre-

designed TaqMan miRNA assay for JCPyV-miR-J1-5p was used for viral miRNA detection and RNU6B was used for normalization. Relative miRNAs expression levels were calculated by the comparative ΔCt method and reported as $2^{-\Delta Ct.}$ The values are expressed as the mean of 3 independent experiments and error bars indicate standard deviations.

A similar trend was observed when comparing JCPyV miRNA with LTAg mRNA relative levels. Indeed, from 2 to 7 d.p.i., when viral miRNAs decreased, an increase in LTAg transcription was reported; from 7 to 12 d.p.i., instead, miR-5p levels rose whereas LTAg gene's expression decreased (Figure 20).

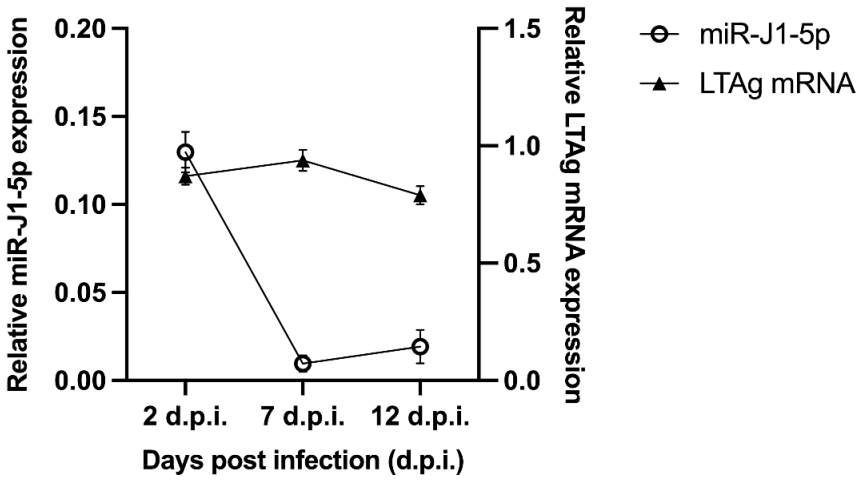

Figure 20. Relative expression of miR-J1-5p and LTAg mRNA in infected cells at selected sampling times, from 2 to 12 d.p.i. A pre-designed TaqMan miRNA assay for JCPyV-miR-J1-5p was used for viral miRNA detection and *RNU6B* was used for normalization. *LTAg* gene's expression was measured by RT-qPCR. Relative miRNAs and LTAg mRNA expression levels were calculated by the comparative ΔCt method and reported as 2^−ΔCt. Data are expressed as the mean of 3 independent experiments and error bars indicate standard deviations.

Finally, miRNA variability was assessed by sequencing JCPyV-miRNA coding region. At 2 and 7 d.p.i., sequences isolated from cell fractions were identical to the reference miR-J1 (miRbase). However, at 12 d.p.i. a variant was detected, characterized by two point mutations, one located at the 5′ miRNA whereas the other in the loop region between the 5′ and 3′ miRNAs (Figure 21).

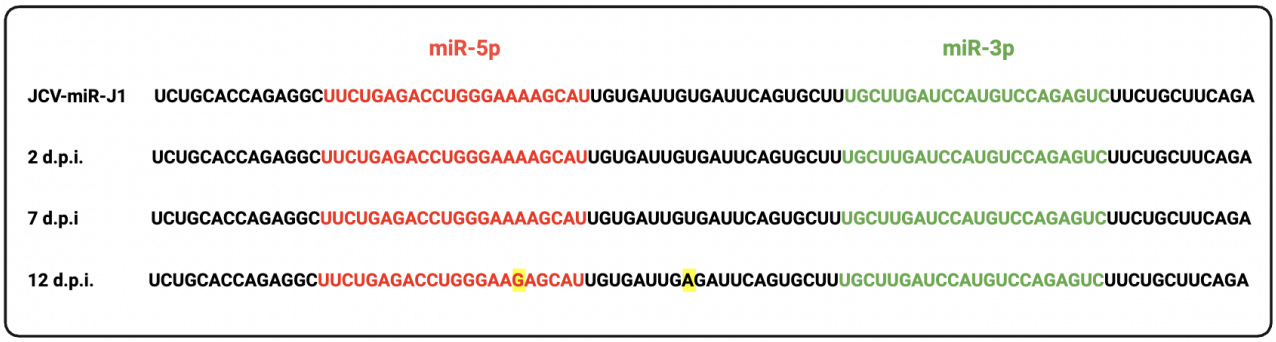

Figure 21. JCPyV miRNA-coding region in U87-MG cells during time course experiment. The mature miR-J1-5p and miRNA-J1-3p are indicated in red and green, respectively. Sequences were compared to the reference JCV-miR-J1 from miRBase [Lagatie *et al.*, 2013].

4.9 mRNA levels of Wnt target genes

After assessing that the U87-MG cell line was permissive to the virus and thus able to support viral replication, the Wnt pathway was examined. To define whether JCPyV infection may affect this signaling pathway, the RNA expression of *β-catenin* and Wnt target genes, including *c-myc*, *cyclin D1*, *Fgf20* and *Sall4* was examined in JCPyV-infected cells and controls during time course experiment.

When comparing infected U87-MG cells with non-infected ones, higher mRNA levels of the studied genes was detected in JCPyV-positive cell fractions. More specifically, except for *Sall4* at 12 d.p.i., a significant over-expression ($p<0.05$) was observed for all the examined cellular genes during sampling period (Figure 22).

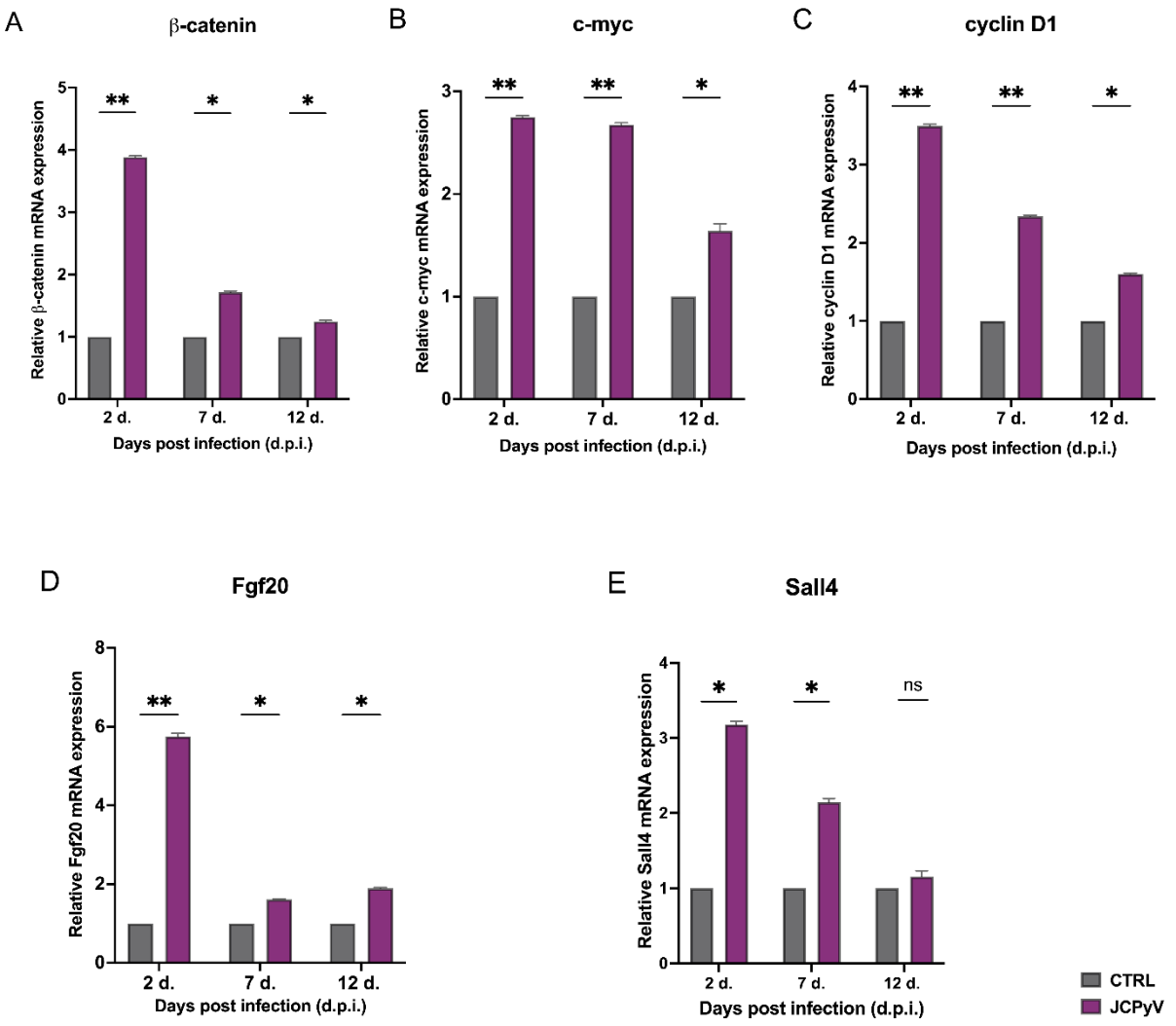

Figure 22 Relative expression of Wnt pathway genes (*β-catenin*, Panel A; *c-myc*, Panel B; *cyclin D1*, Panel C; *Fgf20*, Panel D; *Sall4*, Panel E) in controls and JCPyV-infected cells at selected sampling times, from 2 to 12 days. Genes' expression was measured by RT-qPCR. Relative mRNAs levels were normalized on the housekeeping gene *GAPDH* and expressed as $2^{-\Delta\Delta Ct}$. Data are shown as means from three independent experiments and error bars represent standard deviations. Genes' expression values were compared by Student's t-test. * $p<0.05$; ** $p<0.01$.

Moreover, in order to determine whether the observed modulation was specific to viral infection or merely a response to cellular stress, the expression levels of the selected genes were analyzed after treating the cells with a starvation medium.

The obtained results revealed a significant upregulation of Wnt target genes in infected cells compared to cells treated with strarvation (Figure 23).

Except for some time-points (2 d.p.i.) treatment with starvation medium resulted in a modest or no significant change in gene expression, suggesting that the observed pathway activation is specifically induced by viral infection rather than by general cellular stress.

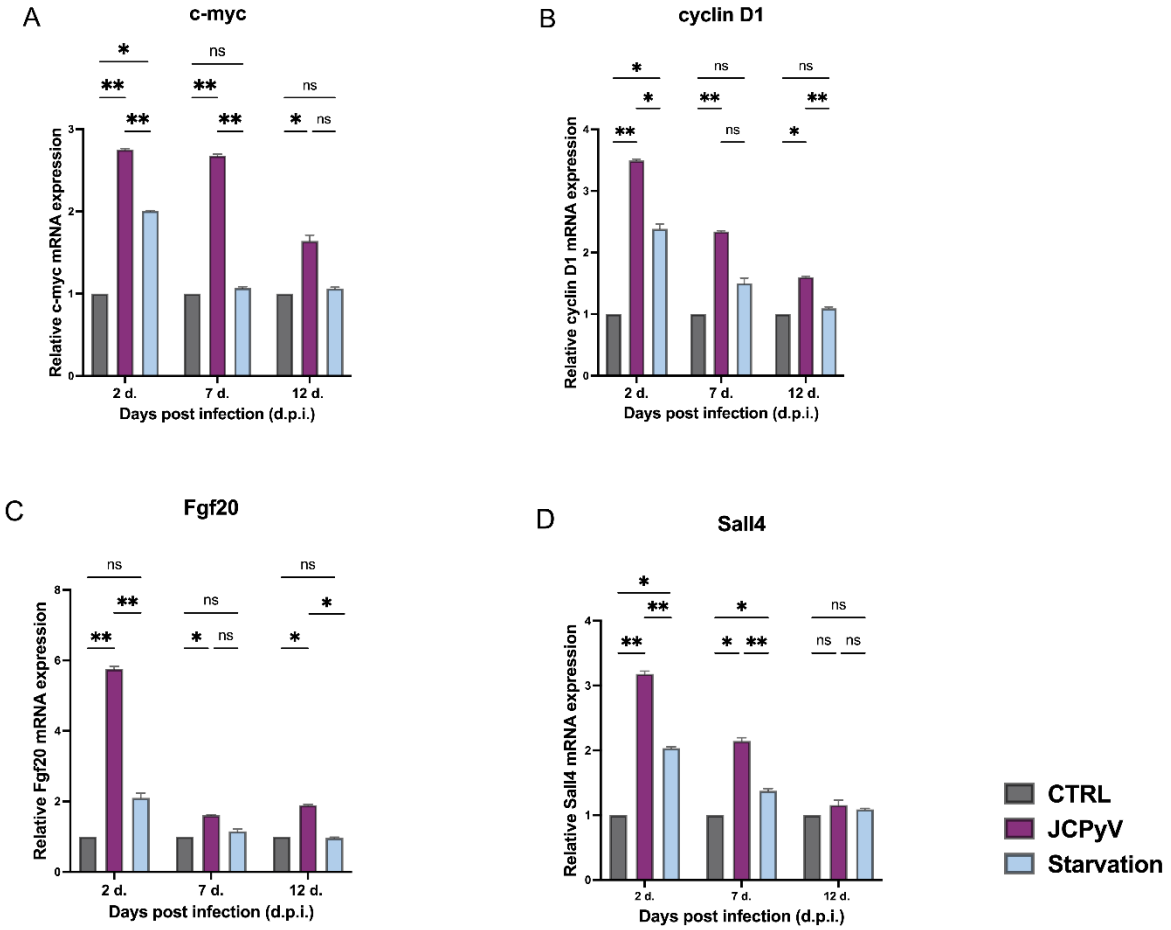

Figure 23. Relative expression of Wnt pathway genes (*c-myc*, Panel A; *cyclin D1*, Panel B; *Fgf20*, Panel C; *Sall4*, Panel D) in controls and JCPyV-infected cells at selected sampling times, from 2 to 12 days. Genes' expression was measured by RT-qPCR. Relative mRNAs levels were normalized on the housekeeping gene *GAPDH* and expressed as $2^{-\Delta\Delta Ct}$. Data are shown as means from three independent experiments, and error bars represent standard deviations. p-values were calculated using one-way ANOVA test for comparisons between three groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

4.10 Subcellular Distribution of β-Catenin: Nuclear vs. Cytoplasmic Expression

In order to determine whether JCPyV stimulate β-Catenin translocation in the nucleus, the subcellular localization of the protein was investigated at 2 d.p.i.

Western blot analysis revealed that β-catenin levels were higher in the nuclear fractions of JCPyV-infected cells compared to their cytoplasmic fractions.

In control cells, instead, β-catenin was more abundant in the cytoplasmic fraction than in the nucleus (Figure 24).

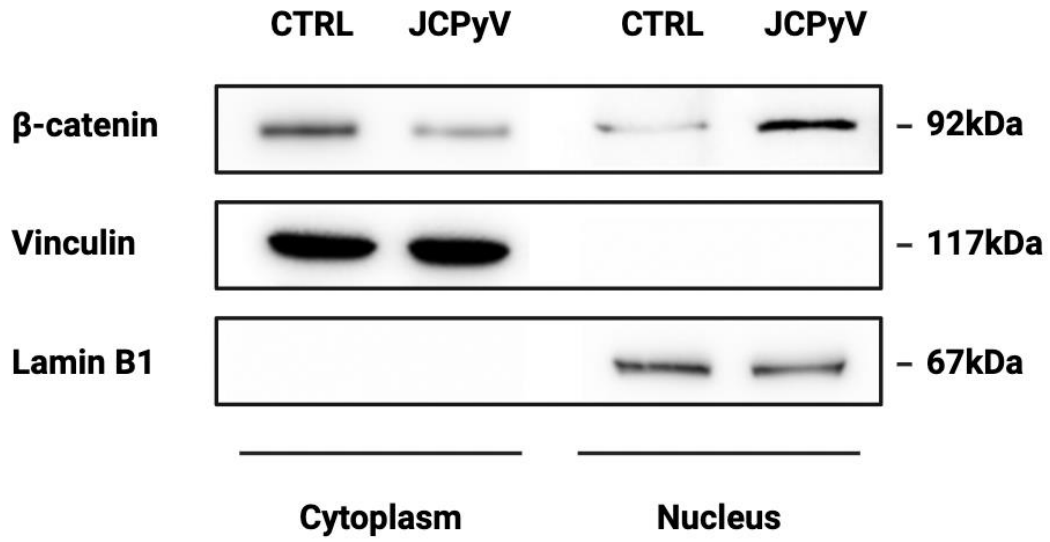

Figure 24. Subcellular localization of β-catenin following JCPyV infection.
Western Blot analysis was performed to assess of β-catenin distribution in nuclear and cytoplasmic fractions from uninfected (CTRL) and infected (JCPyV) cells collected at 2 d.p.i. Vinculin was used as a cytoplasmic marker, whereas Lamin B was used as a nuclear marker.

5. DISCUSSION

CNS tumors are highly heterogeneous neoplasms with poor understood etiology, with both genetic and environmental factors contributing to their pathogenesis [Ostrom *et al.,* 2019]. To date, many primary brain tumors remain incurable, even after multimodality treatment, combining surgery, radiotherapy and chemotherapy [Sharma *et al.,* 2023]. This highlights an urgent need to better understand the mechanism underlying tumor initiation and progression, as well as treatment resistance, with the ultimate goal of identifying novel and more effective therapeutic strategies.

Epidemiological studies reported a wide number of risk factors for CNS tumorigenesis, including infection with oncogenic viruses [Gunasegaran *et al*., 2024]. Indeed, it is well recognized that some viruses can contribute to human carcinogenesis by utilizing their oncogenic proteins for inhibiting tumor suppressor, activating proto-oncogenes or deregulating cell protein expression [Moore *et al.,* 2024; Xiao *et al.,* 2025]. Beyond the seven viruses that are currently established as oncogenic agents [De Martel *et al.,* 2018; Moore *et al.,* 2024], the IARC has also evaluated and classified additional viruses as having potential carcinogenic capacity (https://monographs.iarc.who.int/list-of-classifications/). Among them, JCPyV has gained particular interest due to its strong neurotropism, raising interest in its possible involvement in the development of CNS tumors [Ahye *et al.,* 2020].

JCPyV is well established as the causative agent of PML, a fatal demyelinating disease of the CNS, for PML but current evidences about JCPyV oncogenic potential in animal models, alongside the detection of viral sequences and proteins in human brain tumors, suggest its plausible involvement in CNS malignancies [White *et al.* 2005; Del Valle *et al.,* 2008; Ahye *et al.,* 2020].

The viral protein LTAg is considered the key player in cellular transformation due to its capability to interact with several tumor suppressor proteins such as pRb and p53, thus interfering with their cell growth inhibitory functions [Sharma *et al.,* 1991;

Caracciolo *et al.*, 2006], and with cell signaling proteins as β-catenin [Gan & Khalili, 2004]. On the latter, enhanced expression of β-catenin and transcriptional activation of its target genes has been reported in LTAg-positive cells, thus leading to consider Wnt pathway as possibly implicated in JCPyV-mediated oncogenesis in the brain [Gan *et al.*, 2001; Gan & Khalili, 2004].

Despite growing evidences about JCPyV prevalence in CNS tumors, a number of studies failed to detect JCPyV in brain tumor specimens, resulting in conflicting evidence regarding a causal relationship between viral infection and tumorigenesis [Delbue *et al.*, 2008; Perez-Liz et al., 2008].

Based on these considerations, the aim of this PhD project was to elucidate JCPyV rol in brain carcinogenesis following two complementary approaches: *ex vivo* analysis of a cohort of pediatric patients with gliomas, and the development of an *in vitro* cellular model to study viral effects on a glioblastoma cell line.

To improve our understanding about JCPyV prevalence among CNS neoplasms, 101 tissues were collected from pediatric patients with diagnosis of glioma. The distribution of different tumor types reflects the epidemiological state of the Italian population, in which GBM represents the most frequent type of brain tumor [Frosina *et al.*, 2024].

JCPyV DNA was isolated from about 31% of the analyzed samples, supporting the prevalence of the virus in CNS tumors [Ahye *et al.*, 2020]. A higher positivity rate of JCPyV was observed in high-grade tumors, likely due to the over-representation of this tumor type among the analyzed specimens. Additionally, when comparing viral loads between high-grade and low-grade cancers, no significant differences were observed.

Moreover, in order to explore the plausible correlation between virus-positivity and brain malignancies, viral sequences were examined, and transcripts analysis was performed. Mutations in the NCCR sequence have been previously associated with brain malignancies; specifically, oncogenic properties have been recognized for Mad-4 rearrangements [Gordon *et al.*, 2000; Delbue *et al.*, 2005]. Interestingly,

archetype NCCR structures were identified from JCPyV-positive tumors, suggesting that mutations within NCCR architecture may not be directly associated with virus-mediated oncogenesis [Passerini *et al.,* 2023]. Moreover, molecular analysis of the VP1 region revealed highly conserved sequences; specifically, genotype 1 was isolated from JCPyV-positive tumors, confirming it as predominant overall Italian population [Pagani *et al*., 2003; Muñoz-Mármol *et al.,* 2006].

Transcripts analysis revealed the expression of both early and late genes in the majority of the cases. In 5 virus-positive tumors, instead, only *LTAg* is expressed with no detection of *VP1* transcripts. A similar pattern of viral gene expression is observed in MCPyV-associated MCC, where hampering of viral replication is considered a hallmark for MCPyV-related oncogenesis [Zur Hausen *et al.,* 2008; Passerini *et al.,* 2023]. Based on these observations, it is rational to propose that, as for MCPyV, loss of viral replication, supported by the absence of *VP1* detection at RNA level, may contribute to JCPyV-mediated brain carcinogenesis [Del Valle *et al.,* 2008].

In addition to early and late genes transcription, viral miRNAs expression was tested. Previous studies proposed viral miRNAs as plausible biomarkers for JCPyV infection, reducing LTAg expression during viral persistence [Lagatie *et al.,* 2013; Prezioso *et al*., 2022]. In this study, viral miRNAs were identified in the majority of virus-positive tumors (n=26, 83.9%), with all sequences showing complete identity to the reference [Lagatie *et al., 2013*]. Based on these findings, it is possible to speculate that the detection of miR-5p among the majority of virus-positive tumors (n=26, 83.9%) alongside the detection of an archetype NCCR and both *LTAg* and *VP1* genes contributes to a persistent infection rather than viral reactivation. Moreover, the high degree of sequence conservation, may suggest that viral miRNAs preserve their functional integrity within the tumor microenvironment to maintain viral persistence and modulating host cell functions.

Despite JCPyV miR-5p is known to have a key role in silencing LTAg expression [Lagatie *et al.* 2013; Martelli *et al.* 2018], its concomitant detection with both early and

late transcripts in JCPyV-positive tumors, may reflect a fine-tuning of viral persistence in which miRNA-mediated suppression modulates but does not completely silence viral transcription. This suggests a complex interplay between viral gene expression and persistence, plausibly influenced by cellular microenvironment or infection stage.

Notably, viral miRNAs were undetectable in the 5 brain tissues negative for *VP1*, supporting a hampering of late region transcription.

On the light of these results, it could be hypothesized that the persistent presence of JCPyV in brain tissues may lead to the occurrence of mutations and chromosomal instability as an earlier phase for tumorigenesis [Okamoto *et al.,* 2005].

Although the biological significance of JCPyV infection in the genesis of CNS cancer still remains unclear, the detection of viral DNA and RNA in tumor tissues corroborates the plausible involvement of JCPyV in brain carcinogenesis. However, the isolation of the virus, while indicative of possible involvement of the virus in brain tumors, is not sufficient to define the actual contribution of the virus to neoplasm progression.

Therefore, once assessed JCPyV prevalence and molecular state in CNS tumors, another aim of this project was to evaluate the impact of JCPyV infection on Wnt/*β-catenin* pathway components, in order to further understand the contribution of the virus in the context of brain cancer. Dysfunction of the canonical Wnt pathway is recognized as a trigger factor for carcinogenesis in the brain [Sareddy *et al.,* 2009; Su & Choi, 2024], and JCPyV has been found as capable to modulate this cellular signaling [Gan *et al.,* 2001; Gan & Khalili, 2004; Ripple *et al.,* 2014].

Earlier studies indicated that JCPyV LTAg disrupts Wnt signaling in medullo-blastoma; specifically, enhanced *β-catenin* transcription has been observed following LTAg DNA transfection [Gan *et al.,* 2001]. Moreover, the nuclear translocation of β-catenin has been reported in JCPyV-positive tumors [Enam *et al.,* 2002; Ripple et al., 2014].

The results obtained from this study revealed an over-expression of *β-catenin* mRNA in JCPyV-positive tissues.

As the core component of the Wnt canonical signaling pathway, *β-catenin* plays a crucial role in cell proliferation, cell-cell adhesion, tumor cell migration and invasion [Liu *et al.,* 2022]. Therefore, its over-expression in virus-positive tumors support that JCPyV may induce the transcriptional activation of *β-catenin* thus contributing to perturb Wnt signaling pathway.

To further examine the effect of JCPyV-positivity on Wnt pathway, the relative levels of Wnt target genes, *c-myc*, *cyclin D1*, *Fgf20* and *Sall4* were assessed. Similarly, an enforced expression of these factors was observed in virus-positive tissues, endorsing that JCPyV may contribute to their transcriptional activation [Gan *et al*., 2001; Ripple *et al*., 2014]. Cyclin D1 and c-myc are well-established regulators of cell cycle progression, frequently over-expressed in malignancies [Suh *et al.,* 2022]. Similarly, Fgf20 is known to be a critical factor in oncogenesis induced by the Wnt/ *β-catenin* signaling. Fgf20 belongs to a class of genes, the *FGF*s, encoding secretory proteins with potent mitogenic and angiogenic roles in tumor development [Powers *et al.,* 2000]. Therefore, their over-expression in JCPyV-positive tumors may result in uncontrolled proliferation, accelerated tumor growth, and a more aggressive phenotype [Ripple *et al.,* 2014]. Moreover, the enhanced expression of Sall4, a transcription factor associated with stemless and cellular plasticity, may further support the maintenance of a progenitor-like state within tumor cells, potentially enhancing their malignancy and resistance to therapy.

However, as mRNA analysis, despite highly sensitive, does not directly confirm the activation of the Wnt signaling pathway by JCPyV, future studies exploring the interactions between JCPyV LTAg and β-catenin in CNS tumors will strongly contribute to clarify JCPyV relevance in brain carcinogenesis. Moreover, the absence of a control group represents a limitation of the study that prevent a comprehensive assessment of the association between JCPyV infection and brain carcinogenesis.

However, collecting tissues from pediatric patients without brain tumor poses major challenges due to ethical concerns.

To validate and strengthen the findings obtained from clinical sample, additional functional experiments and mechanistic analyses are essential.

For this reason, the current project also aimed to develop an *in vitro* model to further elucidate JCPyV contribution to brain tumor development, focusing on the downstream effects of viral infection in the dysregulation of the Wnt signaling pathway.

To achieve this goal, the glioblastoma cell line, U87-MG was used since applied in a number of previous studies about JCPyV DNA replication [Gan & Khalili, 2004; Enam *et al.,* 2006].

JCPyV successfully replicates in glial tumor cells, as proved by viral DNA isolation from cell fractions and supernatants during transfection experiments. Further confirmation of JCPyV replication was given by the detection of viral DNA following infection experiments. Specifically, the amount of viral DNA reported from cell fractions was slightly higher than in supernatants, probably due to a low release of viral particles in the culture medium, which, alongside the isolation of an archetype NCCR during sampling period, may support the establishment of a persistent infection [Imperiale *et al.,* 2016]. The efficient JCPyV replication in U87-MG cells was further confirmed by the detection of early and late transcripts, strongly supporting the possibility of using this glioblastoma cell line as a model to examine the biological bases and the mechanism of JCPyV infection in the context of CNS tumors.

In addition to *LTAg* and *VP1* transcription, viral miRNA expression was reported. As previously observed in other cell lines, miR-J1-5p was inversely correlated with JCPyV replication and transcription trend, suggesting that viral miRNAs may play a role in regulating JCPyV replication and transcription to maintain persistence [Lagatie *et al.,* 2013; Prezioso *et al.,* 2022]. Moreover, when investigating miRNA variability, no mutations were observed from 2 to 7 d.p.i. indicating high

conservation during the early stages of infection, whereas two polymorphisms were detected at 12 d.p.i., suggesting that as infection progresses, sequence variability may arise, potentially reflecting viral adaptation or host-virus interaction dynamics. As previously suggested, in this scenario, the presence of distinct miRNA variants may reflect functional diversification aimed at targeting additional, yet unidentified, sets of targetomes [Giovannelli *et al.*, 2015]. These sequence variations could fine-tune the regulation of early gene expression, optimizing the levels of early transcripts and proteins during infection. Alternatively, these changes may contribute to downregulate both early viral transcripts and specific host mRNAs, thereby supporting viral long-term persistence within infected cells [Chen *et al.*, 2013]. It is possible to hypothesize that some of these host targets may include factors involved in oncogenic pathways. Hence, mutations in viral miRNAs could impact the virus's capacity to modulate the expression of host genes associated with tumorigenesis. Future research on this topic is needed to validate this hypothesis, which currently remains speculative.

Once the capability of U87-MG cells to support viral replication was assessed, a key aim of this study was to investigate the effect of JCPyV infection on the Wnt signaling pathway.

Unlike previous studies, where the Wnt pathway was analyzed following transfection experiments with DNA fragments from the JCPyV early genome and β-catenin [Gan et al., 2001; Gan & Khalili, 2004], in the current one, the regulation of Wnt signaling was investigated following viral infection, representing a novel approach that proves a more physiologically relevant model of virus–host interactions.

Higher levels of β-catenin mRNA were detected in JCPyV-infected cells, suggesting β-catenin transcriptional activation by viral infection. The role of β-catenin is central to the Wnt pathway, where it functions as a key effector molecule. Indeed, when over-expressed or stabilized, it can translocate to the nucleus, where it activates the transcription of genes involved in cell cycle regulation and survival [Liu *et al.*, 2021].

Its over-expression in infected cells suggests that JCPyV may actively promote β-catenin stabilization therefore contributing altering the Wnt signaling pathway [Gan *et al.,* 2001; Gan & Khalili, 2004; Ripple *et al.,* 2014]. To assess whether higher levels of β-catenin mRNA correspond to an activation of Wnt/β-catenin axis, the expression of Wnt target genes including *c-myc*, *cyclin D1* and *Fgf20* was examined. As downstream target of β-catenin, monitoring their transcripts levels may reflect the functional activation of the pathway.

Infected cells showed an enhanced expression of these factors, strongly supporting a role of the virus in activating Wnt/ β-catenin axis, therefore facilitating β-catenin translocation in the nucleus [Gan *et al.,* 2001]. In the context of JCPyV infection, the high levels of Wnt target genes may be a result of viral-driven activation of Wnt/β-catenin signaling, thus supporting the role of the virus in exploiting cell machinery, thereby promoting tumorigenesis and/or contributing to cancer progression.

To assess whether this modulation was specific to viral infection or represented a general response to stress, we cultured cells under nutrient-deprivation (starvation) conditions. Under these conditions, gene expression levels showed only modest or non-significant changes compared with controls, indicating that the activation observed during infection is not simply due to cellular stress.

Finally, to further prove the direct role of the virus in perturbing Wnt pathway, the subcellular localization of β-catenin was assessed following JCPyV infection. Consistent with previous results [Ripple *et al.,* 2014], β-catenin was predominantly localized in the nuclei of infected cells, whereas in uninfected cells it remained largely cytoplasmic. This nuclear translocation provides direct evidence of Wnt/β-catenin pathway activation induced by the virus. The observed redistribution of β-catenin likely promotes the transcription of target genes involved in cell survival, proliferation, and immune responses, reflecting a functional modulation of host cellular processes.

Together, these findings support the notion that JCPyV actively modulates the β-catenin pathway, potentially contributing to the regulation of host cell functions that favor viral persistence or replication.

Notably, the Wnt pathway and its downstream target genes are recognized as implicated in oncogenesis [Reya *et al.,* 2005; Yang et al., 2016; Suh *et al.,* 2024]. Specifically, dysregulation Wnt signaling is frequently observed in cancer, including glioblastoma, where it contributes to tumor aggressiveness, therapeutic resistance, and maintenance of glioma stem-like cells [Alkailani *et al.,* 2022; Manfreda *et al.,* 2023].

Cyclin D1 and c-myc drive cell proliferation promoting G1/S cell cycle progression. Similarly, *Fgf20* contributes to tumor growth [Powers *et al.,* 2000], whereas *Sall4* is crucial for maintaining stemless. Their overexpression in JCPyV-infected cells suggests that the virus may favor a proliferative and undifferentiated, stem-like phenotype, which is often associated with poor prognosis.

Notably, while JCPyV likely exploits the Wnt/β-catenin pathway primarily to enhance its replication, persistent infection may result in continuous activation of this signaling cascade. This sustained pathway activation could accidentally contribute to oncogenic processes by promoting uncontrolled cell proliferation, impairing differentiation, and reinforcing stem-like cellular features. This is consistent with our observations of low viral particle release, archetype NCCR isolation, and detection of viral miRNAs during the time-course experiment, indicating a persistent infection rather than a reactivation.

Taken together, these findings highlight a dual role for JCPyV: initially, the virus may manipulate Wnt/β-catenin signaling to optimize its replication, but over time, persistent activation of this pathway may drive cellular changes characteristic of tumor development. The overexpression of c-myc and cyclin D1 in infected cells mirrors patterns observed in CNS tumors, suggesting that JCPyV could contribute to tumorigenesis by altering normal cell cycle regulation and apoptosis controls. Overall, these results support a model in which viral persistence and sustained

Wnt/β-catenin activation act synergistically to favor proliferation and malignant transformation.

In conclusion, this PhD project provides novel insights into the potential role of JCPyV in brain carcinogenesis by combining both *ex vivo* and *in vitro* approaches. The detection of JCPyV in CNS tumors supports its plausible involvement in tumor development and underscores the relevance of studying the virus directly in patient-derived specimens. In parallel, the *in vitro* model here developed, provides a platform to investigate viral replication and its impact on host cells, and reports, for the first time a focused investigation of Wnt signaling deregulation induced by JCPyV infection.

The enhanced transcriptional activation of key Wnt pathway components observed in both JCPyV-positive tumors and infected cells suggests that the virus may exploit this signaling pathway to manipulate cellular processes, promoting viral replication while driving uncontrolled cell cycle progression, increased proliferation, and acquisition of a more malignant phenotype. These findings provide a mechanistic framework to better understand how JCPyV could contribute to CNS tumorigenesis. From a public health perspective, the implications of these results are particularly significant. Gliomas remain tumors with poorly understood etiology, and JCPyV is a ubiquitous virus well known as the causative agent PML, a highly aggressive and often fatal CNS disease, associated with weakened immunity. Together, these factors highlight the importance of monitoring JCPyV within the CNS and investigating its potential role in brain tumors, particularly in high-risk populations. While this study advances our understanding of JCPyV-mediated oncogenesis, it is not without limitations. The complexity of CNS tumor biology and the multifactorial nature of glioma genesis underscore the need for additional studies to elucidate the interplay between viral infection, host genetics, and cellular signaling pathways. In this context, the *in vitro* model described here represents a powerful tool for future

research, enabling the dissection of viral-host interactions, the identification of critical molecular players, and the testing of potential therapeutic interventions. Notably, future studies could extend the application of this experimental protocol to primary neural cultures. to compare the effects of JCPyV in tumoral versus non-malignant contexts.

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