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IDENTIFICATION OF HCMV UL97 IN GBM CELL LINES AND A POSSIBLE ROLE FOR GANCICLOVIR

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ABSTRACT

Identification of HCMV UL97 in GBM cell lines and a possible role for Ganciclovir

By

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Glioblastoma multiforme (GBM) is the most common form of malignant glioma, comprising 80% of all malignant gliomas. Recently, active Human Cytomegalovirus (HCMV) was identified in GBM cells, and has been a topic of debate concerning its role with tumor progression. This study used three established GBM cell lines; T98, LN229, and U87 in order to identify and examine the presence of HCMV phosphotransferase protein UL97. Reverse transcriptase polymerase chain reaction identified UL97 within two of the three cell lines, T98 and LN229. Western blotting confirmed that UL97 protein was being expressed and was present in both T98, and LN229 cell lines. UL97 is a phosphotransferase that has the ability to phosphorylate guanosine analogues, creating a guanosine triphosphate that inhibits DNA elongation and replication. Ganciclovir, a guanosine analogue, was used to treat GBM cell lines and our results demonstrate that it significantly decreases cellular proliferation in UL97 expressing cells. This project identifies a new role for HCMV in GBM and suggests a possible future treatment option.

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LIST OF ABREVIATIONS

ATP-	Adenosine-5'-triphosphate
bp-	Base Pairs
CDK-	Cyclin Dependent Kinase
cDNA-	Complementary DNA
DNA-	Deoxyribonucleic acid
GBM-	Glioblastoma multiforme
GFAP-	Glial fibrillary acidic protein
GTP-	Guanosine-5'-triphosphate
HCMV-	Human cytomegalovirus
HPV-	Human papillomavirus
HSV-	Herpes Simplex virus
ie-	Immediate early gene
pRb-	Protein Retinoblastoma
RNA-	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
tk-	Thymidine Kinase

INTRODUCTION

Gliomas are primary brain tumors that are histologically characterized by their shared traits with glial cells. Glioblastoma multiforme (GBM), also known as a Grade IV glioma, comprises 80% of malignant gliomas. GBMs are identified by their diffuse projections into the brain parenchyma and thus are termed diffuse gliomas. These tumors typically involve the white matter of the cerebral hemispheres, but also have the ability to occur in gray matter, and spreading along the spinal cord[1]. GBMs are considered to be of astrocytic origin, and have an elongated and irregular shape with hyperchromatic nuclei (excess chromosomes) [1].

One of the key characteristics of a GBM is the ability to regenerate after surgical resection. Current studies have shown that malignant gliomas arise from progenitor cells, but at which stage differentiation and transformation occurs is not well known[2]. GBMs contain multipotent stem cells that are responsible for their regeneration, and these tumor stem cells may be the point of transformation for creating a tumor cell line[3].

Tumor stem cells may have many therapeutic implications; current therapies focus on removing bulk tumor contents, as opposed to the small portion of tumor progenitor cells. Cell origin and malignant gliomas remains to be a controversial topic, and the possibility of a GBM occurring from a mature cell has not yet been refuted. The occurrence of cancer is associated with cell cycle dysregulation by means of oncogenes, loss of function of tumor suppressor genes, and DNA repair gene mutations[4].

One of the major pathways controlling cell cycle progression involves the p16, cyclin dependent kinase (CDK)-4, Cyclin D, and retinoblastoma proteins (pRb). Alterations in at least one component of this pathway occur in many anaplastic astrocytomas and in a majority of GBMs[5]. The protein encoded by the RB1 gene, pRB, is important in cell-cycle arrest; the loss of pRb function in gliomas thereby removes an important brake on the cell cycle.

One upstream mediator of pRb function is the p16 product of the CDKN2A gene (also called p16INK4A) on chromosome 9p.16 15, a tumor suppressor inactivated in a number of human tumors. This inhibits the cyclin-CDK (cyclin D and CDK4/6) complex that regulates pRb[5].

The majority of glioma cell lines and two-thirds of malignant gliomas possess homozygous deletions of chromosome 9 that include this gene. It is likely that these deletions result in loss of expression of the p16 and p14ARF transcripts from CDKN2A and the p15 transcript from the nearby CDKN2B, resulting in the loss of many checkpoints and greater cell proliferation[6].

The CDK4 gene is amplified and overexpressed in 10 to 15 percent of malignant gliomas. CDK4 itself is regulated by p16 and inactivates pRb through phosphorylation. Thus, nearly all high-grade tumors have impairments of this single critical cell cycle control pathway[6].

CHAPTER 1: LITERATURE REVIEW

Introduction

In the early history of cancer biology, viruses were thought to be the primary reason for cellular transformation. Transformation is marked by loss of cell to cell contact inhibition, and ability to grow beyond a monolayer to create stacking of cells *In vitro*. The Rous sarcoma virus was identified, and had the ability to transform cells within avian species and was proved to do so by a viral gene called V-Src[7]. As time passed, it was found that viruses could cause cellular transformation and promote the formation of oncogenes from proto-oncogenes. Proto-oncogenes are genes that function to increase growth and survival of cell and are under control by various checks and balances, which include tumor suppressor genes. An oncogene is a proto-oncogene that has mutated to cause increased functional capacity without being down regulated resulting in uncontrolled growth or survival. However, the bulk of tumors rarely have constituently active oncogenes, but alternatively have altered tumor suppressor genes[7]. The possible role of tumor viruses has become more prevalent in popular media with Human Papillomavirus and recently with the identification of Human cytomegalovirus (HCMV) in brain tumor cells. Although many mutations are responsible for the cellular transformation that leads to what is known as a GBM, this project's goal is to define the if HCMV infection may play a role in GBM development.

A Brief Overview of HCMV

Cytomegalovirus (CMV) is a viral genus within the family Herpesviridae. The Herpesviridae is divided into several subfamilies based on where latency is established. CMV is a part of the Betaherpesvirinae subfamily, which establishes latency in leukocytes as opposed to neurons in the alphaherpesvirinae subfamily (i.e., herpes simplex), or B-cells in the Gammaherpesvirinae (i.e., Lymphocryptovirus) [8].

The human form is best known as human CMV (HCMV), or Human herpesvirus-5 (HHV-5). HCMV is very closely related to Herpes simplex-1, -2, and Varcella Zoster virus. The HCMV genome is 230Kb long, and codes for 70 viral proteins. HCMV infections are associated with persistent viral shedding which is secreted in the mucous[9]. Disease manifestations are associated with mononucleosis, pneumonia, hepatitis, aseptic meningitis, and immunological abnormalities[9].

Life Cycle

HCMV is a double stranded DNA virus that is covered by an icosodeltahedral shaped protein capsid. The capsid is surrounded by another protein coat known as the tegument, which is wrapped in a lipid bilayer called the envelope. Glycoprotein projecting from the envelope mediate interactions with the host cells. The viral cycle takes approximately seventy-two hours, which is where the virus is able to replicate its genome and express structural proteins to form a complete clone to leave the cell. After initial fusion with the host's bilipid membrane, the encapsulated viral particle is released into the cytosolic space, and is transited to the nucleus. By active transport mechanisms,

the virus is translocated to the nucleus through the nuclear pores, and the double-stranded DNA is deposited.

Viral gene expression occurs in three steps that are identified as immediate early genes, early genes, and late genes[10]. The immediate early genes are required for early and late gene synthesis. Early genes encode proteins that are necessary for viral replication, and late genes encode proteins for virion structural components. Nucleocapsid particles are assembled within the nucleus and exit via the nuclear pore, and the tegument forms in the cytosolic space. The tegument is comprised of various scaffolding proteins that contain the ability to evade the host immune system by inhibiting interferon signaling. The viruses exit the cell utilizing the hosts exocytosis machinery gaining a bilipid envelope enabling them to infect other cells[10].

Infection with HCMV leads to specific changes in the cell cycle progression that promote viral replication. HCMV optimizes it own replication by inhibiting the endogenous cyclins D and A, while activating cyclins E and B. The virus pushes the cell through G1 by activating Cyclin E, and inhibiting the E2F protein[11]. This halts the cell cycle just before G1 transitions into the S phase. At this stage the early genes are expressed, allowing for rapid viral DNA replication[12 13](Figure 1).

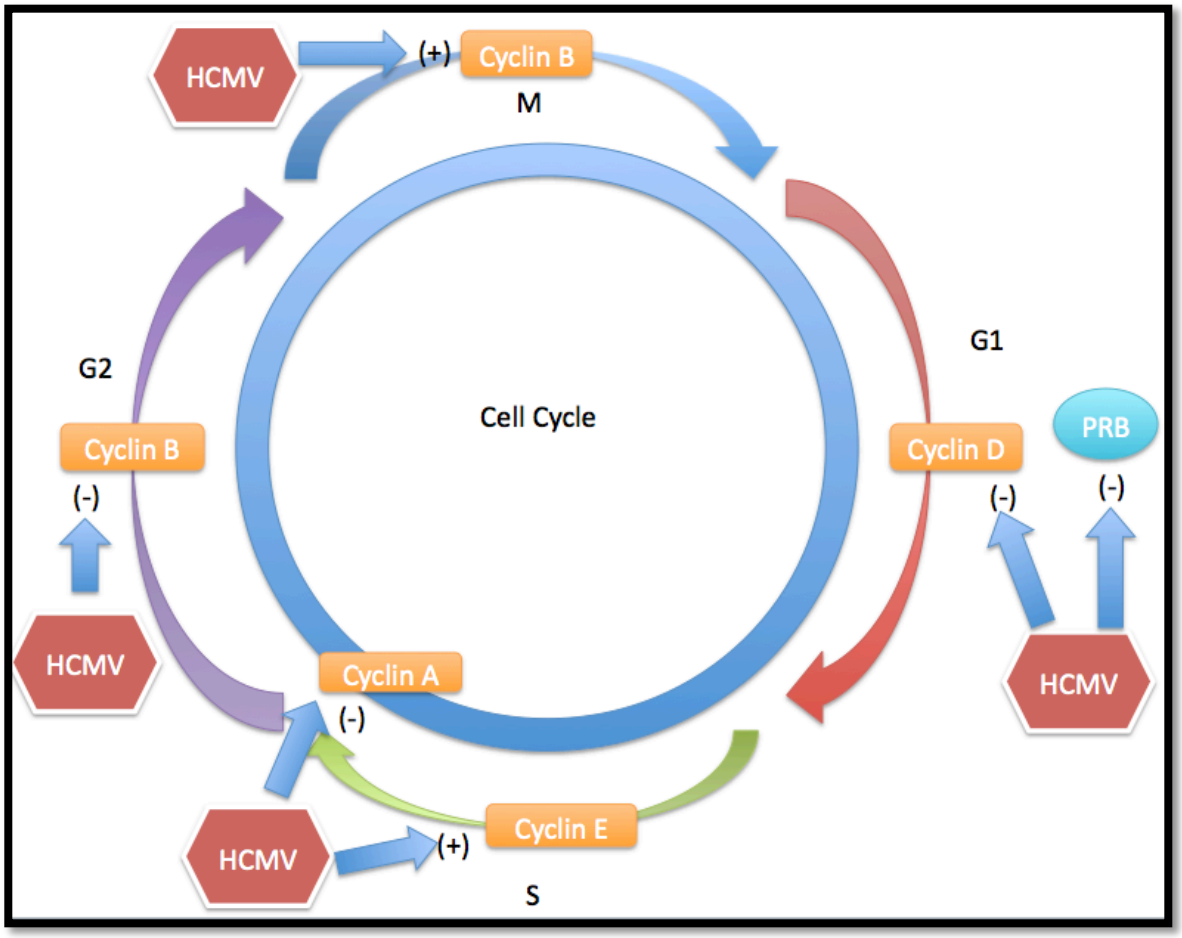


Figure 1. Alteration in cell cycle progression by HCMV
Infection with HCMV leads to specific changes in the cell cycle progression that optimize viral replication. HCMV optimizes it own replication by inhibiting cyclins D and A, while activating cyclins E and B. The virus pushes the cell through G1 by activating Cyclin E, and inhibiting the pRb protein.

UL-97 Protein As a Target

Human cytomegalovirus (HCMV) encodes a serine-threonine protein kinase (thymidine kinase) that shares homologues with most members of the Betaherpesvirinae, and alphaherpesvirinae family. UL-97 is an early gene that is first expressed five hours post infection, and is present throughout infection. Eukaryotes have two types of thymidine kinase, mitochondrial (TK2) and nuclear (TK1). UL-97 has a different tertiary structure but carries out the same primary enzymatic process as TK1 and TK2[14].

Thymidine kinases catalyze the reaction between deoxythymidine and ATP to form deoxythymidine 5'-phosphate and ADP (figure2.)[15].The deoxythymidine 5'-phosphate is then phosphorylated by thymidylate kinase to form deoxythymidine diphosphate. The diphosphate is then phosphorylated by nucleoside diphosphate kinase to form deoxythymidine triphosphate, which is incorporated into a DNA molecule by DNA polymerase[16].

The thymidine kinase enzymes are critical for DNA replication in both the eukaryote and the viral life cycle. UL-97 (HCMV TK) is necessary for the viral DNA replication, but also carries additional characteristics its homologues do not[17]. UL-97 continuously phosphorylates the pRb[18]. In quiescent cells pRb is hypophosphorylated and sequesters the protein E2F inside of its pocket[19]. When hyperphosphorylated by UL97, pRb makes a conformational change and releases E2F. E2F is a transcription factor that acts as a driving factor for DNA replication, cell cycle progression, and mitosis[18]. The papilloma viruses uses a similar mechanism for creating cell cycle deregulation by targeting pRb, and is a primary point of interest in cervical cancer[19].

Link between HCMV and Malignant Gliomas

Malignant gliomas are the most common primary tumor in human adults, and have no known etiology[1]. Survival after diagnosis and treatment ranges between 9- 14 months[1]. HCMV being part of the Betaherpesvirinae subfamily have the ability to infect supporting cells in the central nervous system, known as glial cells. It is known that HCMV can cause severe encephalitis in fetuses, as well as immunocompromised adults. HCMV virulence is promoted in astrocytes when increased inflammatory stimuli are

present. Glioblastoma multiforme is thought to be of astrocytic origin, and poses a possible link between tumor development and viral infection[20].

HCMV persistently infects 50-90% of the adult human population (presumably leukocyte and epithelial like populations), and expression of viral genes can induce dysregulation of cell cycle progression, inhibit apoptosis, and promote signals for angiogenesis[20]. In a previous study with 27 malignant gliomas, immunohistochemistry (IHC) was performed with monoclonal antibodies specific for HCMV IE1-72 protein. All 27 samples were positive for IE1-72 [20]. Comparisons were made between different types of malignant gliomas: grade III oligoastrocytomas, astrocytomas, normal brain tissue, and meningiomas. Both samples of astrocytomas and glioblastomas were positive for IE1-72, while all other tissue samples were negative[20]. These results support the role of HCMV infection in tumors with astrocytic origin. With an estimated population of 90% percent of human adults being infected with HCMV, it has been thought that these results support the presence of a latent virus [21]. Recent work by Bornali et al. demonstrated that HCMV DNA was present in 16/17 (94%) tumor specimens, and with variable concentration of DNA copies. Furthermore viral protein synthesis was identified in 94% of the tumor samples, suggesting an active viral infection within high-grade gliomas[21].

HCMV contains a gene for viral thymidine kinase (UL-97), which has been well studied in the past several decades. The most common antiviral drug, Ganciclovir specifically targets UL-97[22]. Ganciclovir is a synthetic analogue of 2'-deoxy-guanosine that is phosphorylated by UL-97 to create a guanosine triphosphate analogue (dGTP analogue)[17]. Ganciclovir is only phosphorylated by UL-97, and does not react with cellular thymidine kinases TK1 or TK2[22]. The dGTP analogue competitively inhibits the

incorporation dGTP by DNA polymerase, resulting in DNA elongation termination[22]. Ganciclovir is a prodrug, in that it poses no cytotoxic affect to cells unless the viral UL-97 is present to convert it to a dGTP analogue[17]. With recent evidence that HCMV is present in malignant gliomas, Ganciclovir may pose a possible treatment option for glioma patients. Unlike traditional treatments (i.e., chemotherapy and radiology), Ganciclovir would not create any bystander effect, since it would only target and kill cells with active HCMV infection [23].

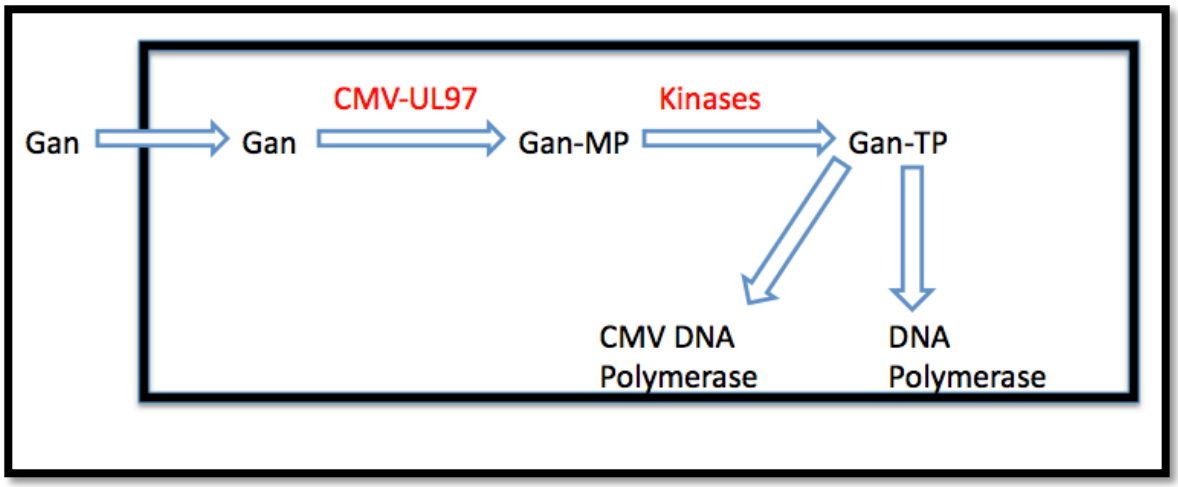


Figure 2. Mechanism of action of Ganciclovir

A novel approach to treating cancer is to introduce non-mammalian genes encoding enzymes into the tumor that react with non-cytotoxic prodrugs. For example Herpes simplex thymidine kinase (HSV-TK) expression in mammalian cells renders non-toxic nucleotide analogues into a cytotoxic form after phosphorylation[15]. The most common nucleotide analogue used is Ganciclovir. When administered after HSV-TK transfection, Ganciclovir acts as a chain terminator in DNA synthesis. Tumors do not regularly express non-mammalian herpes genes, but when experimentally introduced

with anti-viral treatment they have been shown to have a dramatic effect in reducing tumor size[15].

Wei et al.[24] demonstrated that transfecting murine melanoma cells with Herpes simplex virus type-1 thymidine kinase followed by guanosine analogue treatment reduced tumor growth. This was demonstrated by characteristic morphological changes, *In situ* DNA end labeling, flow cytometry detection of G1 DNA content, and annexin V binding. Apoptosis was shown to be associated with the cell killing ability of Ganciclovir on HSV-tk transfected melanoma[24]. Kinetic analysis showed that signs of killing were not observed until 60 hours after Ganciclovir administration at a dose of 100 uM. Ganciclovir treatment was preceded first by a rise in p53 protein level at 12 h and then by S-phase/G2-phase cell cycle arrest associated with increases in the level of cyclin B1 protein. There was no change in protein level of Bax or Cdc2. These results suggest that apoptosis occurred as a result of Ganciclovir-induced cell cycle arrest rather than direct chemical effect of HSV-tk-transfection[24]. Negative controls included B16F10 melanoma cells that were only treated with Ganciclovir, and showed no inhibition of cell growth.

The viral enzyme HSV-TK is morphologically similar to UL-97, and capable of phosphorylating Ganciclovir, though Ganciclovir is 100 times more reactive and has higher specificity[15] to UL-97. The natural presence of UL-97 in Glioblastoma multiforme creates a promising approach for treatment by Ganciclovir with no need of non-mammalian gene transfection[17].

CHAPTER 2: IDENTIFICATION OF HCMV IN HUMAN GBM CELL LINES

Introduction

Viral detection is known to be very difficult, and is not only identified by polymerase chain reaction, but must be verified by employing several primer sets. Following PCR, western blot analysis and Ganciclovir treatment supports that UL97 is present within the cell lines.

RNA Extraction

Human glioma cell lines were grown in Minimal Essential Media Eagle (MEME) and 10% fetal bovine serum. Cell lines included T98, LN 229, and U87. All three-cell lines were grown until confluent. Cells were pelleted at 300 g, and resuspended in a 1 ml solution of 10% FBS and MEME. Cells were lysed with the QIA Shredder system under manufacturer's protocol (Qiagen). RNA was isolated from the cell lysate with RNeasy Mini Kit (Qiagen) following manufacturers protocol. Total RNA was stored at -80° C until further use.

Reverse transcriptase polymerase chain reaction

In a 0.5 ml thin-walled reaction tube, reverse transcriptase polymerase chain reaction was performed by using the Access RT-PCR system (tables 1 and 2) and was performed under manufactures protocol (Promega). All reactions were conducted in triplicate, and repeated with two different primer sets per cell line. A third primer set was

used, and was composed of degenerate primers to control for the possibility that a mutation event had occurred. Primers sets 1 and 2 were designed by Göhring et al. and was checked using nucleotide basic alignment search tool (nBLAST) and primer-BLAST to ensure specificity to UL97 without any amplifying off-target mammalian homologues[25]. Primer set 3 was previously designed by Donald et al., in order to detect the broad range of Herpesviridae family members (Table 1.)[26].

Table 1. Primer sets for UL97

Primer Set	Function	Sequence (5'-3')
1	Forward primer	CTGCTGCACAACGTCAAGGT
1	Reverse primer	CCCAGCGCCGACAGCTCCGACAT
2	Forward primer	CCGCGCGTTGGAGAACGGCAAG
2	Reverse primer	CAGGCCGCGCCGGCGTGCTTAA
3	Forward primer	TCAAAGCTTGAYGGNSCNYAYGG
3	1-Reverse primer	1/2-CTCGAATTCGSRTGNCGRTC
3	2-Reverse primer	1/2-CTCGAATTCGSRTGNGCRTC

Table 2. RT-PCR Reaction Mix

Reverse Transcriptase Reaction Components
10 ul of AMV
1 ul of dNTP mix
2.5 ul of downstream primer
2.5 ul of upstream primer
2 ul of $MgSO_4$
1 ul of AMV reverse transcriptase
1 ul of DNA polymerase
2 ul of total RNA

Table 3. RT-PCR Reaction Cycles

PCR (40 Cycles)	Temperature (C)	Time (min)
First Strand Synthesis	45	45
Second Strand Synthesis (1)	94	0.5
Second Strand Synthesis (2)	60	1
Second Strand Synthesis (3)	68	2

Gel Electrophoresis.

PCR products were separated on 1% agarose gels (0.5x TBE, 0.001 ug ethidium bromide). The wells were loaded with 20 ul of PCR amplified cDNA or 15 ul of 100 bp DNA ladder (Sigma Aldrich). Ribosomal RNA served as an inter-experiment positive control. Electrophoresis was conducted for 80 minutes at 80 V, and imaged under UV light using gel documentation station (BioRad).

Western Blot

Cell lysates were prepared from the T98 and LN229 cell lines. Cells were grown to a confluent state in a 175cm^2 culture flask supplemented with MEME 10%FBS. Lysis buffer comprised of 20mM HEPES, and 1% SDS.. Cell culture media was aspirated off of each flask, and the Lysis buffer was added. Flasks were incubated for 10 minutes at 37°C, and the cell lysate was transferred to a 50 ml centrifuge tube (Sigma Aldrich, St. Louis Mo). DNA was sheared by passage through a 12-gauge needle.

Protein concentrations were determined by using the BCA protein assay (Thermo Scientific). T98 cell lysate had a concentration of 1.1 ug/ul, and LN229 had protein concentration of 1.5 ug/ul. Lysates were heated to 100°C for 10 minutes. Proteins were resolved through TGX precast gel (BioRad). Either 20 ug or 50 ug were loaded into each well. A volume of 5 ul of Precision Plus protein standards was used to identify protein size in kDa (BioRad). The gel was run at 150V for 60 minutes. Protein was transferred to nitrocellulose by sandwich method using a western transfer buffer. The transfer buffer was composed of 48 mM Tris, 39 mM glycine, and 0.04 mM SDS dissolved in 500 ml dH_20 and 200 ml of methanol. The transfer proceeded at 0.1 A for 10 hours at room

temperature. Nitrocellulose membrane was removed and washed in TBST pH 8.0 (20mM Tris, 150mM NaCl, and 0.1% tween) and blocked with 5% dehydrated milk TBST solution for 30 minutes. The membrane was soaked in 1 ml of TBST pH 8.0 with of anti-UL97 polyclonal rabbit antibody (Abcam: ab11394) in 5% milk((1:250 dilution). The procedure was repeated with polyclonal donkey anti-rabbit secondary antibody at a 1:1000 dilution (Abcam, ab98488). The polyclonal antibody was incubated with the membrane alone to show that the secondary antibody was specific to rabbit and not human proteins. Membranes were incubated overnight with parafilm cover. The polyclonal donkey anti-rabbit secondary antibody was diluted to a 1:1000 dilution in 5% milk and added to the membrane containing the primary antibody. The membrane was incubated at room temperature for one hour. Following this membrane was washed with fresh TBST (pH 8.0) for ten minutes for four consecutive washes. Protein was visualized by HRP substrate super signal (Millipore) and Kodak 1D IS440CF (Rochester, NY).

MTT Assay

The cell lines U87, T98, and LN229 were grown in MEME 10% FBS until confluent, and harvested by trypsin. Cells were counted with a hemocytometer, and diluted to 50,000 cells per ml in complete media. 5000 cells per well for each cell line per treatment were seeded into 96 well plates in triplicate for each treatment. Cells were incubated overnight at 37°C. The media was aspirated and replaced with 100 uL of 100 uM Ganciclovir in 1% DMSO and MEME[15]. Vehicle control contained 1% DMSO in complete media, and the negative control contained only complete media. Cells were incubated for 30 hours. A volume of 20 ul of 5mg/ml MTT was added to all wells

containing cells, and a single row of empty wells as a negative control (Sigma-Aldrich). The cells were incubated at 37°C for 3.5 hours[27]. Media was aspirated from each well, and replaced with 150 ul of MTT solvent (Sigma-Aldrich). The 96-well plate was covered in aluminum foil, and placed on a shaker for 15 minutes[27]. The 96 well plate was read at an absorbance of 590 nm with a reference filter of 620 nm[28]. This was repeated three times. All values were normalized to the negative control and plotted in percent absorbance. Each treatment group was compared independently to Vehicle control (DMSO) and untreated (UT) with the students T-Test with a cut off value of 0.05 for significance.

CHAPTER 3: ANALYZING UL97 EXPRESSION IN GBM CELL LINES

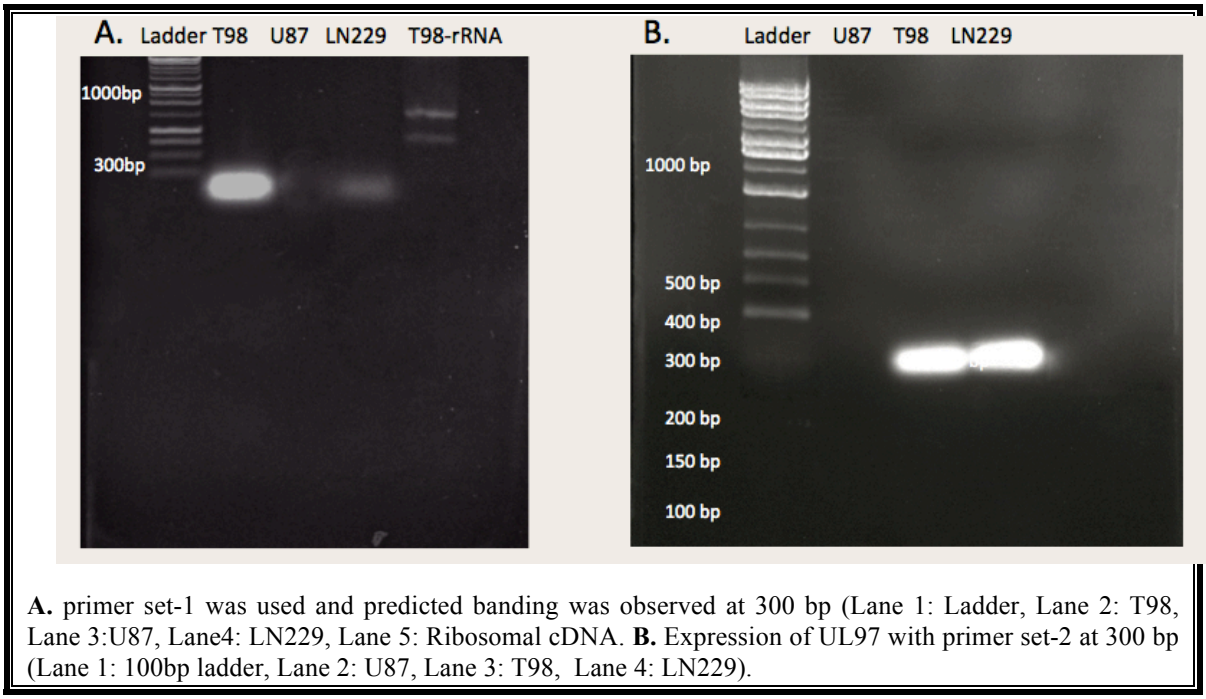
Introduction

RT-PCR and western blotting determined the presence of UL-97 transcripts. A possible role for UL-97 as a therapeutic target using guanosine analogues was investigated. UL-97 was detected in both T98 and LN229 cell lines, but was absent in U87 cell lines. This evidence suggests that HCMV may not be present within all GBMs, but may be important in screening processes for suggested treatment options, possibly involving guanosine analogues.

Results

Using two primer sets, two different portions of the UL-97 gene were amplified by RT-PCR to determine the presence UL-97 in the cell lines. Previous research has identified several immediate early genes, but identification of UL-97 has not been performed previously in these cell lines. In order to ensure that the primers used amplified the correct transcript, two different sets of primers were used. Primer set 1 showed banding at 300 bp for both T98 and LN 229, but did not show any banding for U87 (figure 3:A). Banding in figure 3:A migrated beyond the smallest ladder band of 300 bp and may have been the result of primer dimers. PCR was repeated for T98 with a ladder extending to 100 bp to exclude the possibility of primer-dimers (Figure 3:B). Banding appeared at 300 bp when using the first set of primers, excluding the possibility of primer dimers. Reverse transcriptase PCR was repeated with the second set of primers,

results supported the presence of UL-97 transcript in T98 and LN229 cells but absent in U87 cells.



A. primer set-1 was used and predicted banding was observed at 300 bp (Lane 1: Ladder, Lane 2: T98, Lane 3:U87, Lane4: LN229, Lane 5: Ribosomal cDNA. **B.** Expression of UL97 with primer set-2 at 300 bp (Lane 1: 100bp ladder, Lane 2: U87, Lane 3: T98, Lane 4: LN229).

Figure 3. Identification of UL97 gene transcription by RT-PCR in LN229 and T998 cell lines

Western Blot Analysis

Western blot analysis showed that UL97 was present in both LN229 and T98 cell lines. In the nature state, UL97 is known to be 78 kDa, but is also a homodimer in its functional form. Intensity of band brightness indicates that the dimerized form was more prevalent in the presence of SDS. To test non-specific binding by the secondary anti-rabbit donkey polyclonal antibody, a separate protein transfer was prepared and incubated overnight at a 1:1000 dilution. When imaged, no signal was identified, indicating that no non-specific binding was present for polyclonal donkey antibody (figure 4: B).

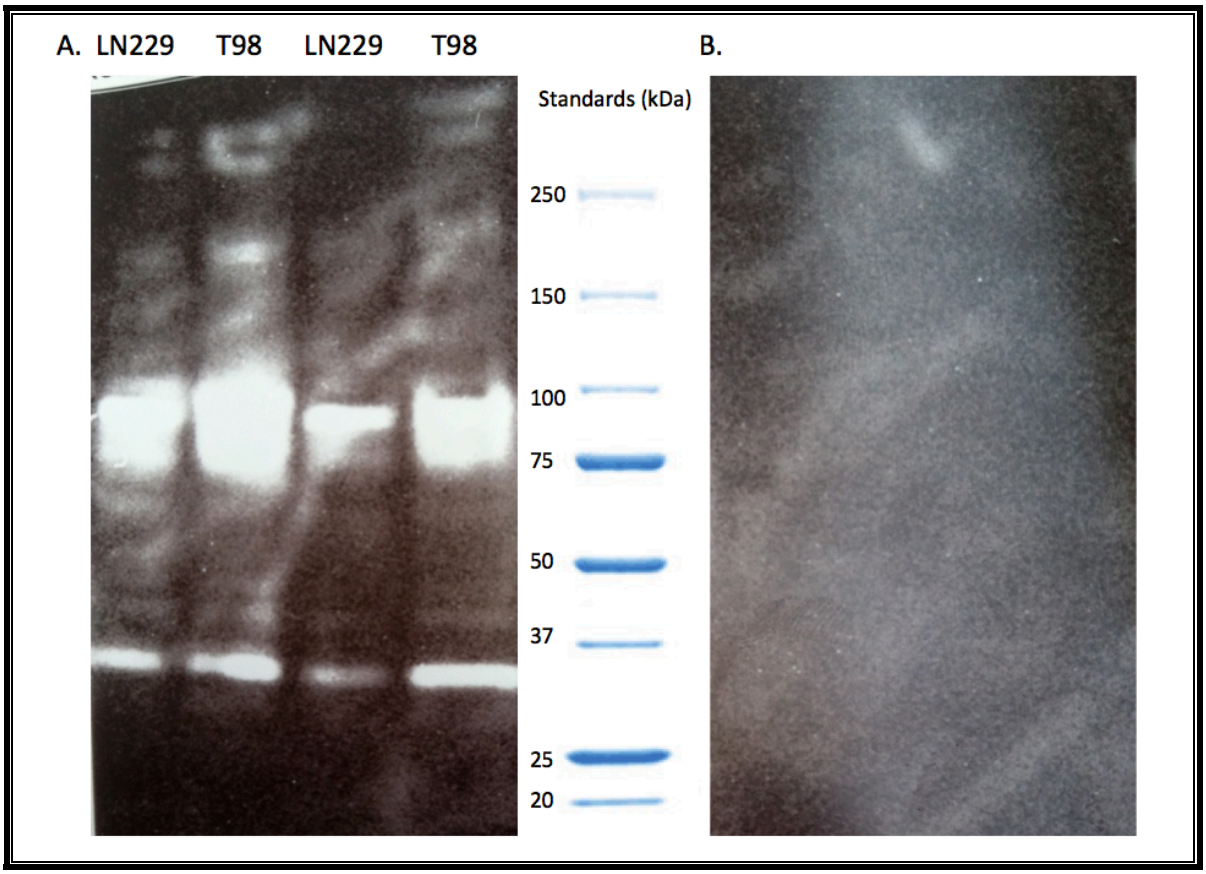


Figure 4. UL97 Western Blot

A. Western blotting showed UL97 at 78kDa. Lane 1 was loaded with 20 ug of Ln229 cell lysate, lane 2 with 50 ug of T98 cell lysate, Lane 3 with 20ul of LN229 cell lysate, and lane 4 with 50u ug of T98 cell Lysate. B. Complete absence of signal indicating that no non-specific binding was made by the anti-rabbit polyclonal donkey antibody.

Ganciclovir Treatment

Proliferation assays were performed in order to validate that the UL97 protein identified by rtPCR and Western blotting is functional and a valid target for Ganciclovir. Cell proliferation was determined by MTT assay. Each cell line was treated with 100 uM Ganciclovir in 1% DMSO, 1% DMSO, or standard media. The MTT assay is a colorimetric assay in which the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to its insoluble formazan producing a purple color. The production of the purple formazan is determined by measuring absorbance at 590 nm. The first proliferation trial was conducted in a nontransparent 96 well plate, which required that the well contents be transferred to a transparent 96 well plate to be able to measure absorbance. In this first trial cell growth variation was drastic and could possibly be due to the transfer from one 96 well plate to another (Figure 5). Ganciclovir treated T98 cells had significantly reduced absorption than either control group. Ganciclovir treated LN229 cells did not differ from the untreated controls.

The MTT proliferation assay was repeated two more times as described except a transparent 96 well plate was used for each assay. Ganciclovir treated cells had significantly less cell growth compared to the vehicle and untreated controls (Figure 6). The third MTT proliferation assay was similar to the second trial. Ganciclovir treated cells had significantly less cell proliferation than either the DMSO vehicle controls or the untreated control (Figure 7).

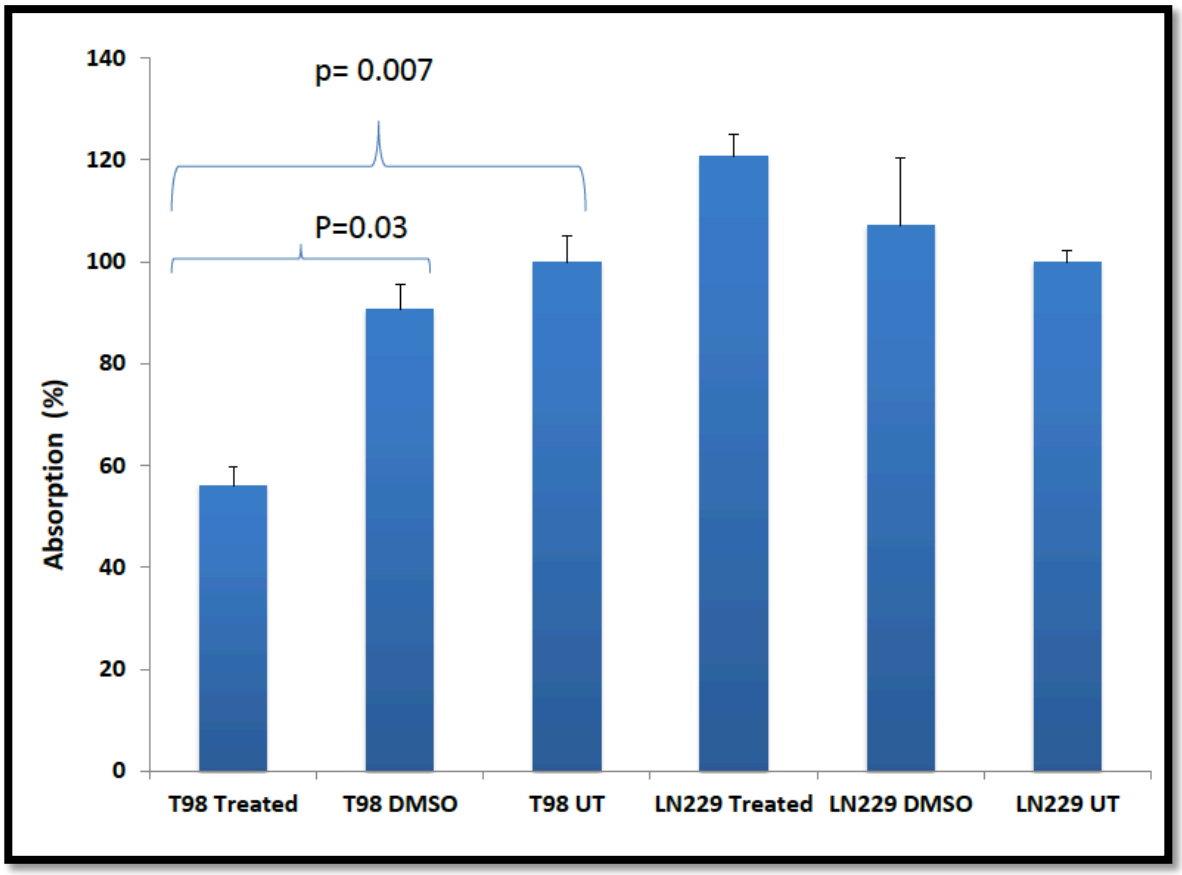


Figure 5. MTT Assay for T98 and LN229 (I)

T98 cells grown in 100 uM Ganciclovir (treated) had significantly reduced absorbance when compared to untreated (UT) and Vehicle controls (DMSO). LN229 did not have any significant change in absorbance when grown in Ganciclovir when compared to controls.

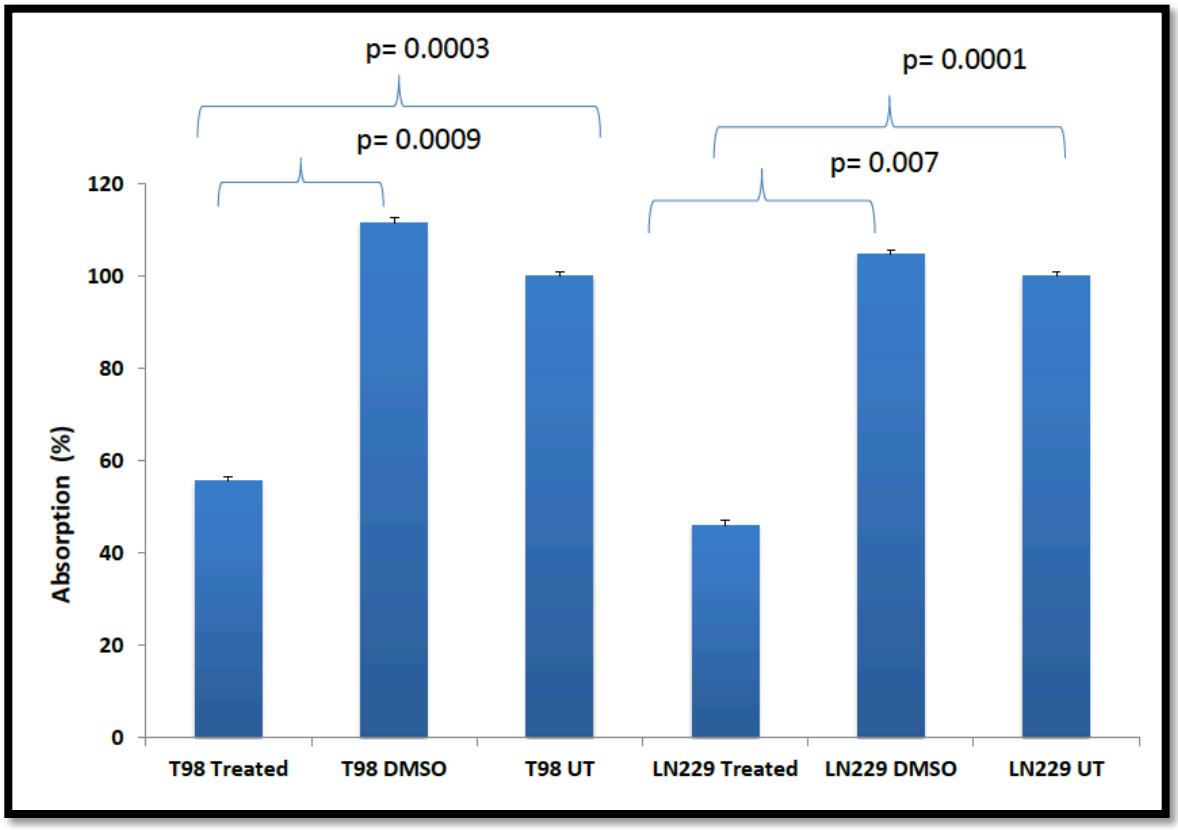


Figure 6. MTT Assay for T98 and LN229 (II).
T98 and LN229 cells grown in 100 uM Ganciclovir (treated) had significantly reduced absorbance when compared to untreated (UT) and Vehicle controls (DMSO).

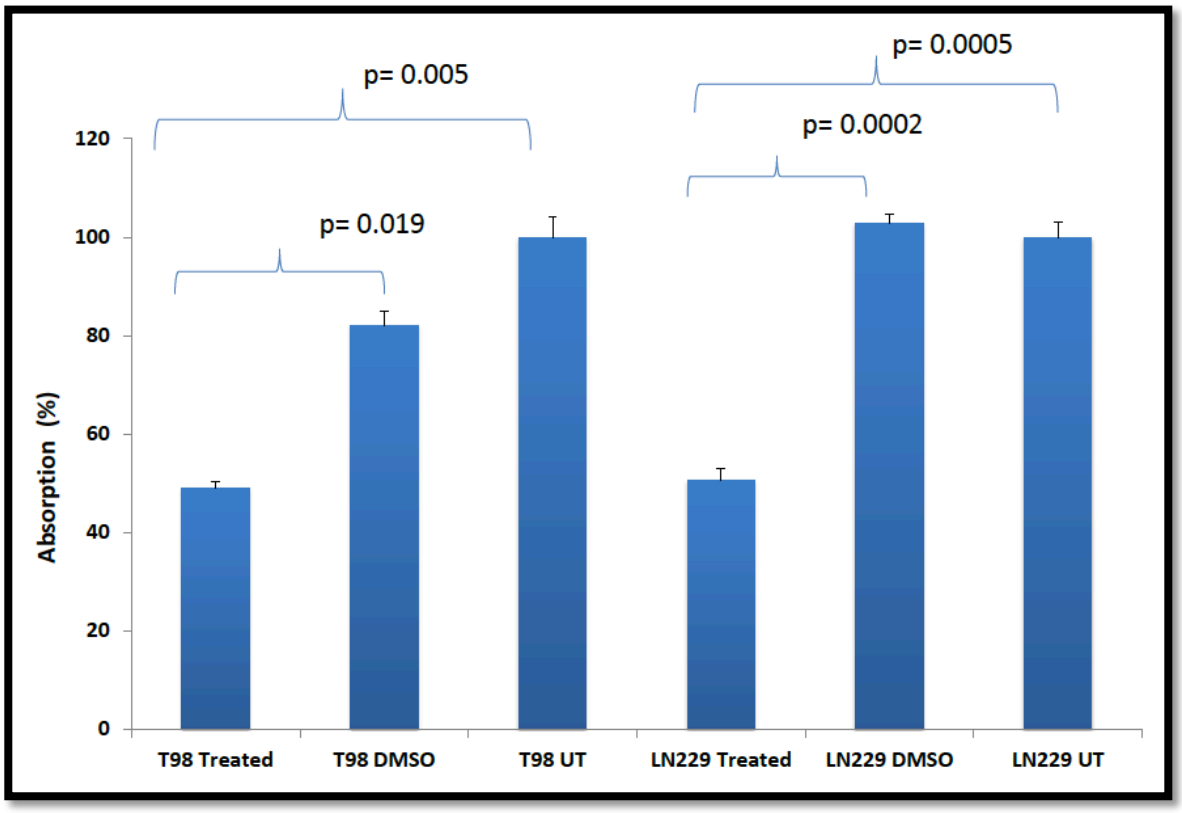


Figure 7. MTT Assay for T98 and LN229 (III).
T98 and LN229 cells grown in 100 uM Ganciclovir (treated) had significantly reduced absorbance when compared to untreated (UT) and Vehicle controls (DMSO).

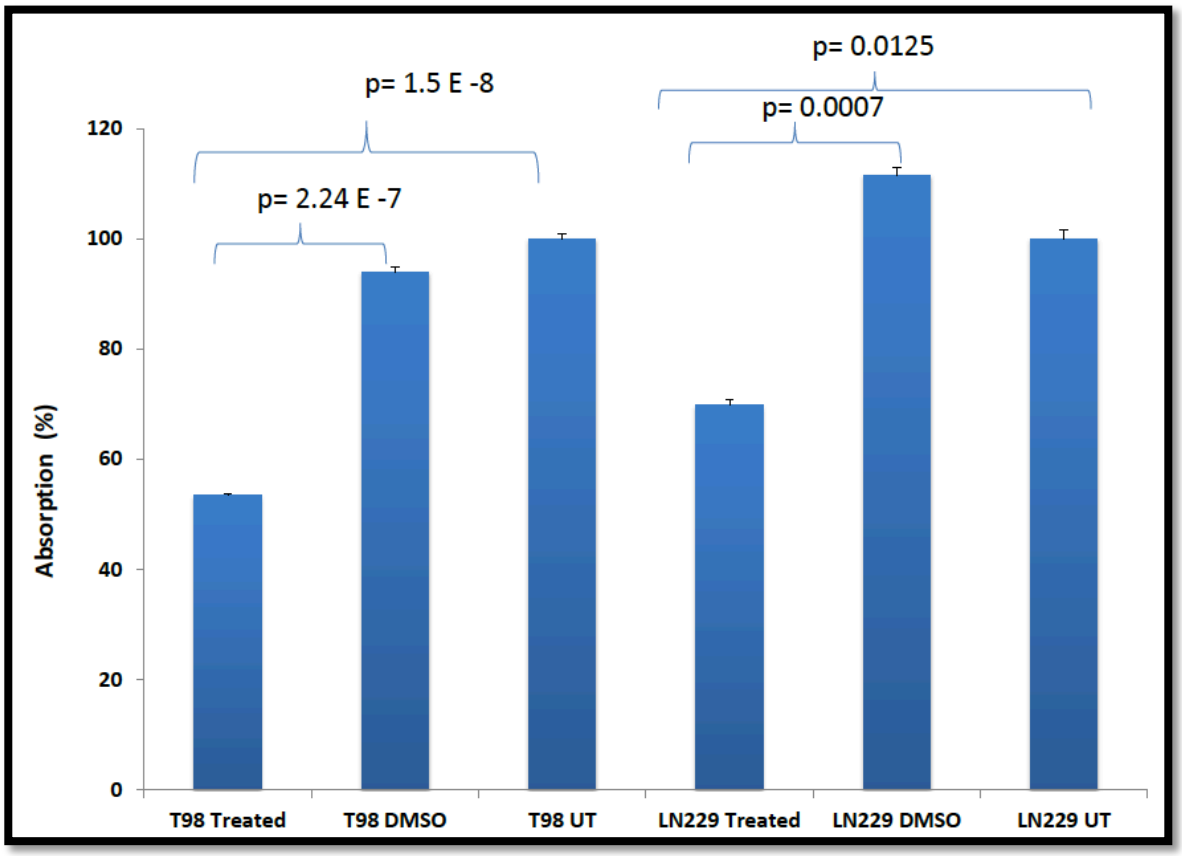


Figure 8. MTT Assay for T98 and LN229 (IV).
T98 and LN229 cells grown in 100 uM Ganciclovir (treated) had significantly reduced absorbance when compared to untreated (UT) and Vehicle controls (DMSO).

Proliferation data was averaged for all three proliferation assays and Ganciclovir treated cells had significantly less cell proliferation than either of the controls (Figure 8). However Ganciclovir showed to have no effect on absorbance in U87 cells (Figure 9.). In conclusion, Ganciclovir was shown to significantly reduce MTT absorbance to nearly half that of the controls which contain UL-97 transcripts (T98 and LN229 cell lines).

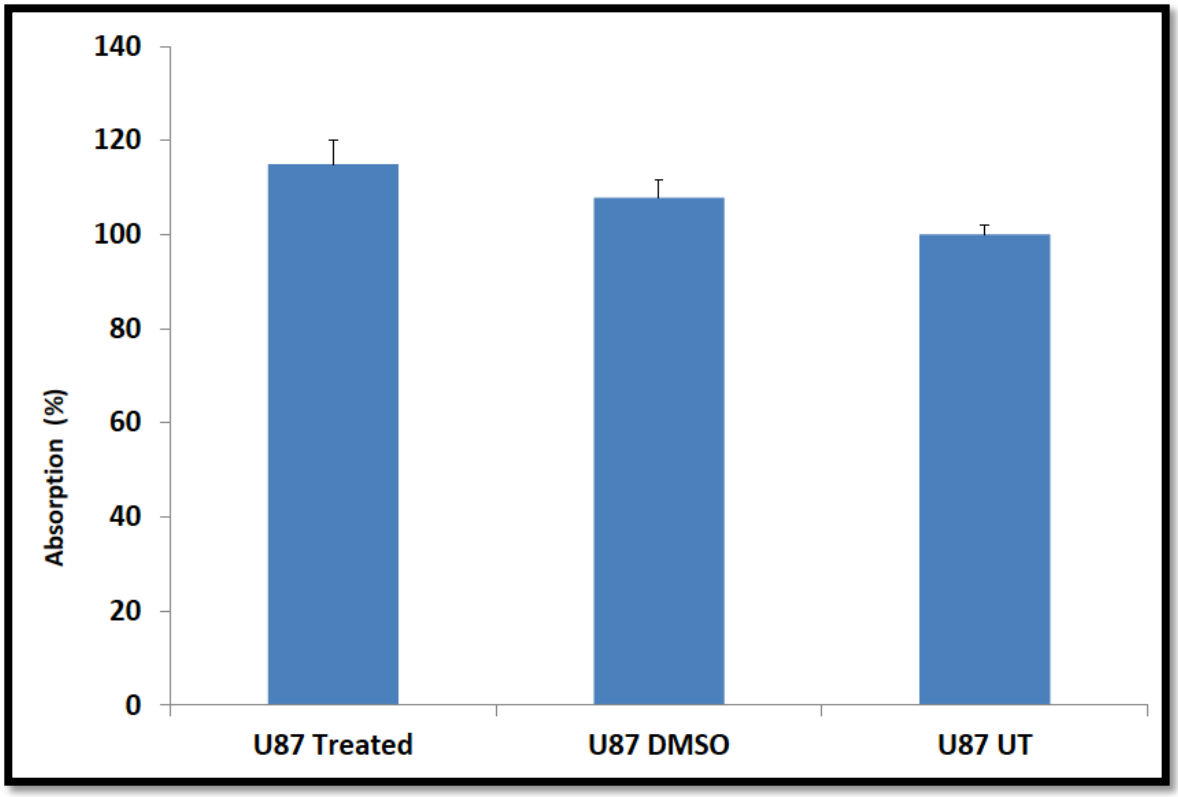


Figure 9. MTT Assay for U87 cells (V).
U87 cells grown in 100 uM Ganciclovir (treated) had no significant reduced absorbance when compared to untreated (UT) and Vehicle controls (DMSO).

CHAPTER 4:DISCUSSION

Glioblastoma multiforme is the most common and aggressive primary malignant brain tumor in adults and is derived from supporting tissue composed of glial cells[1]. HCMV, a part of the Betaherpesvirinae subfamily primarily infects leukocytes and supporting tissue cells as opposed to neurons as seen in the alphaherpesvirinae subfamily. HCMV has the ability to remain in a dormant stage called latency. Normally, immediate early genes and early genes within HCMV genome are not expressed without the progression of complete viral infection. An active infection has no benefit to the life cycle of a cancer cell as HCMV causes a lytic infection that would destroy the host cell. The fact that few genes are being expressed, specifically immediate early genes and early genes, without viral replication indicate that viral genome insertion may have been erroneous based upon our evidence. Viral infection is not as precise as eukaryote replication, causing increased mutation rates within the viral genome, and promoter fusion to proto-oncogenes [29].

Both early and late genes are needed to halt cell cycle progression at the early point in S-phase. HCMV inhibits cyclins D and A, while activating cyclins E and B. The result is a push from G_0 /G_1, onto the S phase. The inhibition of cyclin A prevents the cell from entering G_2. If complete expression of HCMV early genes occur, the infection would deter cancerous growth. Recent data has shown that only a few of the immediate early genes and even fewer HCMV late genes are expressed[24]. This results in a partially

infected cell has committed to replicating, and is doing so at a faster pace, and is not expressing a lytic virus; thus causing cellular transformation[30].

By using reverse transcriptase polymerase chain reaction (rt-PCR). The early gene UL97 was demonstrated. Viral replication is known to be fairly unconserved, creating very different variants within virus families throughout history. Two different primer sets were used to amplify two different portions of the UL97 gene. The multiple primer set method supports the presence of UL97 transcription in T98 and LN229 cell lines although not in U87. A total of eight additional rt-PCR reactions were performed on U87 cDNA and an additional set of degenerate primers was used to identify if a mutant form of UL97 may be present in the U87 cell line. All PCR reactions for U87 cells were negative suggesting UL-97 is not present.

Results from the rt-PCR reactions support the theory that transcripts of UL-97 was present, but could not identify transcription quantity or the presence of U-L97 protein. Western blot analysis was used to identify protein content. Both LN229 and T98 cell lines showed the presence of UL-97 in cell extracts. Banding appeared at both 78 and 37-kDa. The 37-kDa bands may be due to degradation of whole transcript or a nonspecific endogenous protein. To identify if non-specific binding was occurring with the secondary antibody, a separate protein transfer was prepared and was incubated with the anti-rabbit donkey antibody. Banding was absent, indicating that UL-97 protein was present in both LN229 and T98 cell lines and was not due to non-specific binding.

Identification of UL-97 supports the role of HCMV as a target to inhibit GBM proliferation. To test if UL-97 is functioning within the GBM cell lines, three

proliferation assays were prepared to identify if the pro-drug Ganciclovir has the ability to reduce cellular proliferation. The first proliferation data set was more variable than the other two and the variability may be due to the additional pipetting step required to put the assay in the correct 96 well plate. However, taken as a whole both T98 and LN229 cell lines demonstrated that Ganciclovir was effective in significantly reducing cell proliferation compared to either the DMSO or media control. It is important to note U87 cells did not express UL-97 transcripts by rt-PCR analysis; this then was confirmed by Ganciclovir treatment having no effect on cell viability in the MTT assay with U87 cells (figure 9).

In general LN229 cells tend to proliferate more rapidly than the T98 cell line. Therefore it was thought that Ganciclovir might not have as great of an effect on reducing cell growth. However these proliferation assays demonstrated that Ganciclovir has been able to reduce cell proliferation in the LN229 and T98 cells.

This project identified UL-97 within GBM cell lines, and showed an alternate form of treatment. Only three cell lines were used, and of them, two were positive for UL-97. Indicating that HCMV may not be a direct causal factor for cancer but may play a role in co-modulating cell cycle progression. This role may be due to hyperphosphorylation of pRb, up regulating cyclin E, and inhibiting cyclin D. The mechanisms by which HCMV increases cell growth needs to be explored further. Importantly the presence of UL-97 in the cell lines shows promise as a target for the drug Ganciclovir. Ganciclovir was shown, in this study, to significantly reduce cellular proliferation in treated cells. Most recently Ganciclovir is being used in a clinical trial for

tumors, which have been transduced with human herpes simplex thymidine kinase. This project supports the suggestion that HCMV may play an important role in oncomoduclation, and could be the target of many guanosine analogues already presently used for Herpes infections.

Further investigations will be needed to explore expression patterns in primary tumor samples. Tumor cells are known to show severe changes in protein expression patterns after three passes in culture. The Upper Michigan Brain Tumor Center has several GBM tumors frozen in liquid nitrogen, and the experiments provided above would be suitable for identifying UL-97 in the tumor samples. Furthermore, identification of Ganciclovir-triphosphate should be identified; this could be identified by high-pressure liquid chromatography in collaboration with Northern Michigan University's chemistry department. This project has created a new approach to understanding the role of HCMV in GBMs, and has created a strong basis for future projects.

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