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THE CHARACTERIZATION AND IN VITRO SILENCING OF THE ONCOGENE ZBTB7A IN MALIGNANT GLIOMA

Justin Joseph Segula

THE CHARACTERIZATION AND *IN VITRO* SILENCING OF THE ONCOGENE ZBTB7A IN MALIGNANT GLIOMA

By

Justin Joseph Segula

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This thesis by Justin Joseph Segula is recommended for approval by the student's thesis committee in the Department of Biology and by the Dean of Graduate Studies.

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Committee Chair: Dr. Robert Winn Date

_____

First Reader: Dr. Rich Rovin Date

_____

Second Reader: Dr. Alec Lindsay Date

_____

Department Head: Dr. Robert Winn Date

_____

Dean of Graduate Studies: Dr. Cynthia Prosen Date

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ABSTRACT

THE CHARACTERIZATION AND *IN VITRO* KNOCKDOWN OF THE ONCOGENE ZBTB7A IN MALIGNANT GLIOMA

By

Justin Joseph Segula

Despite advances in the understanding of cancer, the treatment for patients with high grade brain tumors has changed little in the last 10 years. ZBTB7A has been shown to be over-expressed in a majority of cancer cell types and is suggested to be an important oncogene in those cancer types. Using PCR (differential, reverse transcription and real time) the expression of ZBTB7A in malignant glioma cell lines and in surgically resected tissue was characterized. Our group has concluded that ZBTB7A is expressed in a majority of high grade gliomas and further report evidence of gene amplification in 25% of those tumors sampled. Small interfering RNA was used to silence ZBTB7A in cultured cells and the growth and survival rate of siRNA treated cells was determined. *In vitro* silencing of ZBTB7A using siRNA was successful in two of the four cell lines, but changes in proliferation and survival was not observed. The characterization of ZBTB7A in brain tumors is a novel finding and this project is among the few investigating the use of biomolecular agents (siRNA) to eventually treat patients with brain tumors.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisors Dr. Rob Winn and Dr. Rich Rovin for their tireless patience, wisdom, and trouble-shooting advice throughout the course of my graduate studies. Dr. Winn and Dr. Rovin were responsible for the concept of ZBTB7A characterization and silencing and it was my privilege to perform the following experiments under their guidance. I am truly grateful to have been a part of the Upper Michigan Brain Tumor Center and to have such great mentors. Additionally, I would like to thank Dr. Alec Lindsay for his mentorship throughout my undergraduate years. His teachings laid the foundation upon which I built my graduate education.

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In addition to those who contributed the man power, I would also like to give thanks to each of my family members (Rob, Eric, Brianne, and Katelynn), especially my father for his wisdom and guidance through tough times, and my mother for her continual support and encouragement ("Never, never, never give up." Winston Churchill).

This thesis follows the format prescribed by the journal *Cell* and Northern Michigan University's Department of Biology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS OR ABBREVIATIONS

CHAPTER 1: LITERATURE REVIEW

1.1 Malignant Gliomas

Within the mammalian central nervous system (CNS) there exist two populations of cells: neurons and glia. Astrocytes, microglia, and oligodrendocytes surround and support neurons in the CNS and thus constitute the glial cell types. Pathologists classify tumors according to the perceived cell type from which they are derived. When cells extracted from a brain tumor maintain morphological or molecular characteristics of an astrocyte—such as glial fibrillary acid protein (GFAP)—the tumor is designated an astrocytoma. Among tumors found in the CNS, the astrocytoma is the most common type accounting for 42% of primary brain tumors (Maher et al., 2001). The World Health Organization (WHO) has developed a grading scheme which classifies astrocytomas (I-IV) based upon the presence or absence of four histological criteria: 1) atypical nuclei, 2) high mitotic activity, 3) angiogenesis, and 4) necrosis. Prominent angiogenesis and necrotic tissue are essential diagnostic features of the highest grade glioma (grade IV). The grade IV astrocytoma is referred to as a glioblastoma multiforme (GBM). Due to the aggressive nature and consequent neurological destruction, GBMs are among the most deadly forms of human cancers.

1.2 Traditional Glioma Therapy

Traditional glioma treatment includes surgical resection, radiation, and chemotherapy. Among these, cytoreductive surgery is the most heavily relied upon for the treatment of accessible gliomas and is essential for relieving intracranial pressure and

focal symptoms associated with surrounding neuron destruction (Hess, 1999). However, the invasiveness of glioma cells leaves the margins of the tumor irregular and indistinct from normal brain tissue, a characteristic that makes even the most aggressive surgical resections less than a cure (Bruner, 1994).

Radiation therapy has been effectively used to treat brain tumors since the 1960's (Bouchard, 1966) and randomized studies in the late 1980's reported no significant difference in survival between those patients treated with whole brain radiation versus those who received radiation to the tumor bed and surrounding margin (Shapiro et al., 1989; Kita et al., 1989). However, given the lack of tumor recurrence in distant areas of the brain and long term neurocognitive deficit associated with whole brain radiation therapy, most radiation oncologists favor focal field radiation (Laperriere et al., 2002). Although the use of radiation has proven useful for the treatment of gliomas, its use is limited by CNS toxicity and the inability to destroy all tumor cells (Shaw et al., 2002)

An additional mainstay for the clinical management of malignant glioma is chemotherapy (Chang et al., 2005). As with radiotherapy, toxicity is also a major limiting factor of chemotherapy treatment. Unique to brain tumor chemotherapy, is the need for the agent to cross the blood brain barrier in an effective concentration. Besides local side effects such as necrotizing leukoencephalopathy after radiochemotherapy, the most important complications include myelosuppression (caused by nitrosourea based agents), or peripheral neurotoxicity (due to vinca alkaloids) (Roth and Weller, 1999).

By combining gross resection with radiation and chemotherapy the median survival for patients with GBM was extended from 4-6 months (Frankel and German, 1958) to 9-12 months (Fine et al., 1993). With the exception of temozolomide—which

increased survival rates from 12.1 months to 14.6 months (Stupp et al., 2005) — few treatment regimens have improved survival rates of individuals diagnosed with glioma in the last decade.

1.3 Brain Tumor Stem Cells

It is commonly accepted among neurophysiologist that neural tissue regenerates in the adult mammalian brain (*see reviews:* Abrous et al., 2005; Gould and Gross, 2002; Ming and Song, 2005). However, neurogenesis has only been documented to occur in discrete areas of the brain; the subventricular zone (Gage, 2000), the dentate gyrus (Luskin, 1993), and the hippocampal formation (Lois and Alvarez-Buylla, 1994). From within these discrete areas a group of multipotent cells (neural stem cells) have been isolated and are responsible for neurogenesis (Reynolds and Weiss, 1992). After isolating neural stem cells (NSCs), Lendahl and colleagues noted the expression of an intermediate filamentous protein called nestin (1990). In addition, researchers have developed a method for isolating NSCs using a transmembrane protein known as CD133, which is specific to NSCs within the CNS (Uchida et al., 2000). Using these cell markers, laboratories have isolated a multipotent fraction of cells from within gliomas (Ignatova et al., 2002), and further observed the role of these cells in tumor propagation (Hemmati et al., 2003; Singh et al., 2003, Kondo et al. 2004; Yuan et al., 2004). The presence of this multipotent cell type lends support to the monoclonal argument of tumor formation, but more importantly it provides a target for future glioma treatment. As a result, much research has been focused on the genes and molecular pathways that regulate the proliferation of progenitor and stem cells including: Akt-1 (Sinor and Lillien, 2004),

MELK (Nakano et al., 2008), and PTEN (Groszer et al., 2006). In addition to those mentioned in the current literature, POK transcription factors are of specific interest to this research.

1.4 POK Transcription Factors

Transcription factors are critical regulators of gene expression that function during embryonic development, cell differentiation and cell proliferation (Kelly and Daniels, 2006). POK is a term used to describe a family of transcriptional factors that have an amino terminal POZ domain and a Kruppel- type zinc finger at the carboxyl terminal (Kelly and Daniels, 2006). Kruppel-type zinc finger motifs are among the most common type of DNA binding domains. The tertiary structure of a Kruppel-type zinc finger domain is essential for specific DNA sequence recognition and binding. The tertiary structure of the protein is partially dictated by a central zinc atom that coordinates an interaction between two pairs of cysteine and histidine residues. Kruppel-type zinc fingers are generally 25-30 amino acids in length (Costoya, 2007). The POZ or BTB (bric-a`-brac, tramtrack, broad-complex) domain facilitates the homo and hetero-dimerization with transcriptional co-factors which modulate promoter access and thus transcription (Stogios, 2007). Included among these co-factors are SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) and NCOR1 (Nuclear Receptor Co-Repressor 1). Both SMRT and NCOR1 bind to and recruit enzymes that remodel histones called Histone DeACetylases (HDACs). HDACs remove acetyl groups from lysine residues within the nucleosome. Removal of acetyl groups from lysine residues

imparts a greater positive charge on the histone that facilitates stronger DNA-histone interaction and thus prevents the transcription of nearby genes (Melnick et al., 2000).

One of the most heavily researched POK transcription factors is PLZF. Barna and colleagues (2000) noted substantial limb and axial skeleton abnormalities during PLZF knockout mice development. Those abnormalities in the mice were the result of an increase in cellular proliferation and the loss of apoptotic mechanisms that are normally conferred by PLZF (Barna et al., 2005). In addition, homozygous deletions of PLZF in mice resulted in smaller testes and fewer spermatogonia as compared to heterozygous littermates (Costoya et al., 2004). PLZF is also expressed in myeloid progenitor cells, which is down-regulated as cells progress to more committed hematopoietic lineages (Reid et al., 1995).

Another well characterized POK transcription factor is the BCL-6 protein. The BCL-6 protein binds to and represses specific DNA sequences—called STAT sequences (Signal Transducer and Activator of Transcription)—during B cell formation of germinal centers (Chang et al., 1996). Knockout mice (BCL-6 $^{-/-}$) could more effectively class switch in response to cytokines which resulted in multi-organ inflammatory disease (Harris et al., 1999). Overall, BCL-6 acts to repress genes that are important in pathways such as apoptosis, differentiation and Ig switching during immune response (Niu et al., 2002).

Mutations of both PLZF and BCL-6 genes have been documented in hematopoietic malignancies. In a small subset of patients with acute promyelocytic leukemia, Chen and colleagues (1993) discovered a chromosomal translocation of PLZF adjacent to the RARα gene resulting in a chimeric protein thought to be responsible for

malignancy. In addition to co-repressor interaction, the POZ domain of PLZF is necessary for homodimer formation. The role of the mutant chimeric protein in the development of leukemia is poorly understood, however, it is thought that the mutant chimeric protein interferes with normal dimerization of PLZF which essentially nullifies or interferes with the repressor during differentiation (Castoya, 2007).

Similarly, BCL-6 mutations were the result of chromosomal rearrangement. The BCL-6 gene is altered (via chromosomal translocation) in 30-40% of diffuse large cell lymphomas and 6-11% follicular lymphoma (Ye et al., 1995). Those chromosomal alterations disrupt the expression of the BCL-6 gene by placing it under the control of an overactive heterologous promoter (Ye et al., 1995).

1.5 ZBTB7A Gene and Protein Structure

ZBTB7A is one of the 60 different POK transcription factors within the human genome. Davies and colleagues (1999) mapped the gene for ZBTB7A to the 19p13.3 chromosomal locus using fluorescent *in situ* hybridization. The gene for ZBTB7A consists of three exons and three introns. Interestingly, 19p13.3 is a gene-rich region where numerous deletions, amplifications and translocations have been reported in hematopoietic malignancies (Heim and Mitelman, 1987; Mitelman et al., 2007).

The product of the ZBTB7A gene is a 584 amino acid protein that is a member of the POK family of transcriptional repressors. Similar to the other POK transcription factors, the POZ domain in ZBTB7A is known to facilitate homodimerization and oligomerization with other proteins (Bardwell and Treisman, 1994). Melnick and colleagues (2000) reported conserved residues within the POZ domain that were

necessary for corepressor (SMRT), and histone deacetylase (HDAC) recruitment. Within the carboxyl terminal domain of ZBTB7A lie four Kruppel-type zinc fingers which mediate DNA recognition and binding (Figure 1). The core of the protein takes shape by interactions of five α-helices with two small β-sheets (Schubot et al., 2006).

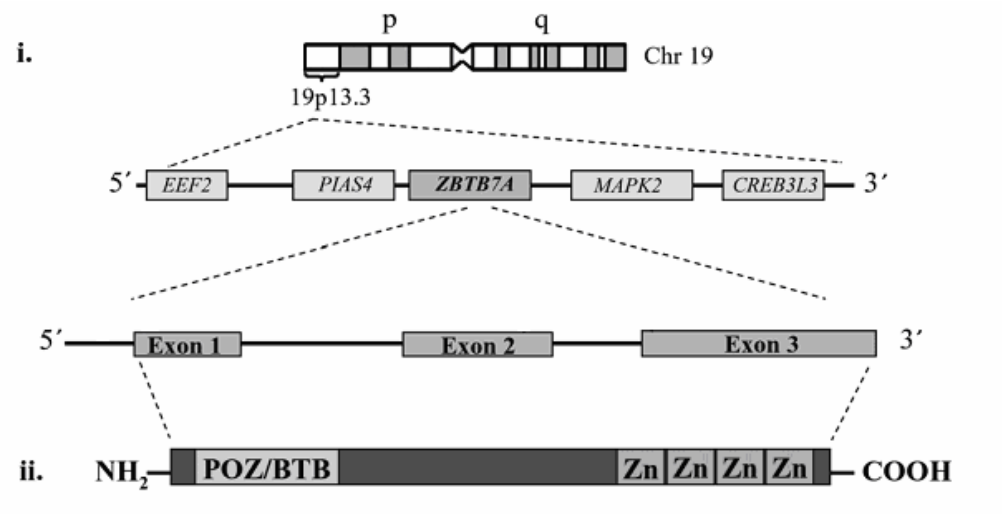


Figure 1.1: The above image is a modified figure depicting the (i) chromosomal arrangement of *19p13.3* and (ii) the ZBTB7A protein structure, including the NH2-terminus containing a POZ/BTB domain and the COOH-terminus four Kruppel-type zinc fingers as reported by Apostolopoulou and colleagues (2007).

1.6 ZBTB7A in Malignancies

In a retrospective study, Apostolopoulou and colleagues (2007) examined tissue samples of non-small cell lung carcinomas (NSCLC) for the presence of ZBTB7A mRNA and protein. Immunohistochemical analysis detected ZBTB7A in more than half of the samples (>50%) and the expression was verified using protein immunoblotting. The cause for the elevated ZBTB7A mRNA was attributed to gene amplification (2-5 fold) in 27.7% of patients. Interestingly, in the non-amplified cases ZBTB7A mRNA was found elevated in 61.5% of the patients. Apostolopoulou and colleagues (2007)

concluded that epigenetic, posttranscriptional, and posttranslational mechanisms may be the reason for ZBTB7A deregulation and further expressed the importance of using ZBTB7A for predicting clinical outcome.

With the use of monoclonal antibodies, Maeda *et al.* (2005a) observed abnormal expression of ZBTB7A in human T-cell lymphomas, diffuse large B-cell lymphomas, and follicular lymphomas. They documented BCL-6 as more oncogenic when ZBTB7A is over-expressed (Maeda et al., 2005a). In addition, Maeda *et al.* (2005a) reported that murine embryonic fibroblasts (MEF) were resistant to transformation when ZBTB7A was lacking (ZBTB7A $^{-/-}$) and also observed diminished tumor suppressor p19^{ARF} protein when ZBTB7A was over-expressed. Maeda *et al.* (2005a) also provided evidence that ZBTB7A physically binds to the promoter region for p19^{ARF} and thus acts as a potent transcriptional repressor.

1.7 ZBTB7A Action in Malignancies

ZBTB7A repression of the ARF gene is of interest in cancer because the ARF tumor suppressor indirectly mediates nuclear p53 concentrations. Critical to this process is the protein Mouse Double Minute 2 (MDM2). One of the known functions of MDM2 (as a E3 ubiquitin ligase), is to attach ubiquitin to p53 and hence mediate its proteasome degradation. In response to DNA damage, normal cellular mechanisms inhibit AKT/PKB phosphorylation of MDM2 preventing its entry into the nucleus (Mayo and Donner, 2001). Because the concentration of p53 within a cell is controlled primarily by its rate of degradation, absence of MDM2 in the nucleus allows p53 to reach functional levels and trigger DNA repair or apoptosis (Lahav et al., 2004). Besides phosphorylation, the cell

also modulates MDM2 activity via the ARF tumor suppressor. Within the nucleoli, ARF physically sequesters MDM2 thus inhibiting nuclear export and subsequent ubiquitination of p53 (Sharpless, 2005).The transcriptional repression of ARF by ZBTB7A allows nucleic levels of MDM2 to accumulate, as a result, p53 is degraded, and apoptosis is circumvented (Figure 1.2). The gene encoding the ARF protein is referred to as p^{14}ARF, and is one of two known tumor suppressor products transcribed from the *INK4a/ARF* locus found on the human chromosome band 9p21 (Gallager et al., 2006). The inactivation of apoptotic mechanisms despite DNA damage is an essential event that occurs early in the progression of cancer allowing the cell to survive and accumulate mutations.

In addition to decreasing p53 levels, excessive nuclear MDM2 also facilitates the degradation of retinoblastoma protein (pRB) (Sdek et al., 2005). pRb controls the passage of the cell through the restriction (R) point by binding or releasing the E2F factors that usher the cell from G1 to S phase (Harbour and Dean, 2000). ZBTB7A over-expression is thought to decrease ARF and, as a result, MDM2 accumulates and pRb is degraded at an accelerated rate (Figure 1.2). The early loss of cell cycle check point controls allows the cells to proliferate in an uncontrolled manner. ZBTB7 is considered an oncogene because it's over-expression promotes cancer by preventing proper cellular response to DNA damage and by promoting dysregulation of the cell cycle.

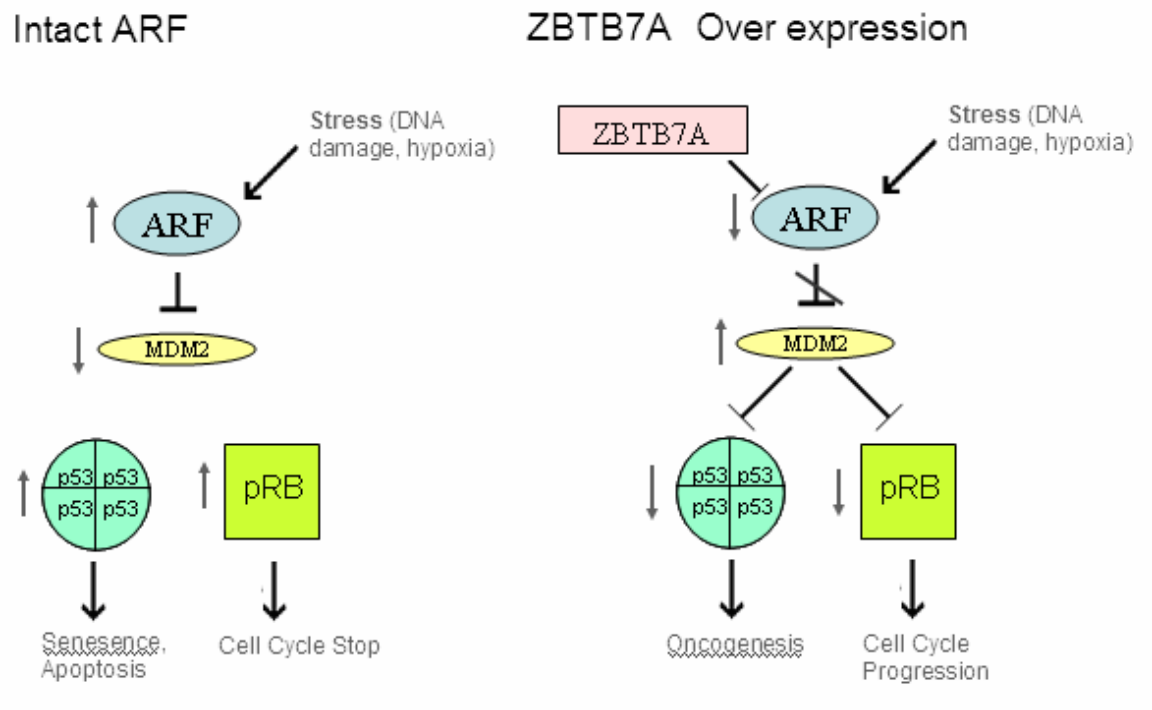


Figure 1.2: Depiction of the proposed role of ZBTB7A during oncogenesis (from Maeda *et al.* 2005). Cascade depicted on the right shows the over-expression of Zbtb7A which ultimately results in cellular transformation and oncogenesis. Normal or down-regulation of Zbtb7A depicted on the left displaying proper expression of ARF which causes senescence and or apoptosis under cellular stress.

1.8 RNA Interference

In the early 90's plant physiologists were inserting genes ("transgenes") into petunias expecting to deepen their color. The researchers were surprised by the variegated pigmentation of the petunias, with some even lacking pigment all together (Jorgensen, 1990; van der Krol et al., 1990). Jorgensen referred to the repression of endogenous genes by inserted DNA fragments as "co-suppression". Further studies confirmed that co-suppression or gene silencing was a process that was evolutionarily conserved across taxa: including *Caenorhabditis elegans* (Fire et al., 1991), *Neurospora* (Ramano and Macino, 1992) and *Drosophila melanogaster* (Pal-Bhadra et al., 1997). Ultimately, the puzzling nature of gene silencing via dsRNA was elucidated by Fire and

Mello who were later awarded the 2006 Nobel Prize in physiology or medicine (Fire et al., 1998).

RNA interference (RNAi) begins in the cytoplasm of the cell when foreign dsRNA is cleaved by the endogenous ezyme Dicer (Bernstein et al., 2001). Dicer produces 20-25 nucleotide sequences—called small interferening RNAs (siRNAs) (Hamilton and Baulcombe, 1999)—that have two unpaired nucleotides on the 3' strand ends (Elbashir et al., 2001). Following ATP-dependent unwinding of the dsRNA, the antisense strand of the siRNA binds to a nuclease known as RNA-induced silencing complex (RISC) (Hammond et al., 2000). The unwound siRNA directs RISC to bind and ablate cognate mRNAs.

When researchers realized that RNAi machinery could be exploited to silence any number of genes in mammalian cells, they utilized its potential to degrade transcripts responsible for chronic inflammation, metabolic disease, apoptosis (associated with liver disease), and cancer (Uprichard, 2005). A large amount of siRNA research has been directed against glioma cell adhesion/motility, EGFR sensitivity, angiogenesis, metabolism, hypoxia inducible factors, immunoactivity, and oncogene over-expression (*see review:* Mathupala et al., 2006).

1.9 Clinical Relevance

The ideal cancer treatment would target tumor cells without destroying healthy cells. Current treatments for individuals diagnosed with brain tumors destroy both tumor and healthy cells and do little to change clinical outcome (Maher et al., 2001). The insufficiencies of current malignant glioma treatment call for the development of

alternate treatment options. One such option is the use of RNA interference to target oncogenes in tumor cells. Targeting oncogenes is a treatment that naturally distinguishes between healthy and tumorigenic cells and to date is the focus of much research (*see reviews*: Morris, 2008; Bruserud, 2007). In the future RNAi may provide additional treatment options by silencing oncogenes such as ZBTB7A. Silencing oncogenes has the potential to restore cell cycle control and DNA repair mechanisms that may lead to apoptosis of the cancer cells. The hypotheses to be tested in this study are the following: 1) Compared to β-actin, ZBTB7A mRNA is elevated in glioma tissues. 2) *In vitro* application of ZBTB7A siRNA will reduce ZBTB7A mRNA in glioma cell lines. 3) In vitro ZBTB7A siRNA will reduce glioma cell line proliferation and or survival.

Investigation in this area will provide clarification of intracellular events that lead to glioma development; aid in the development of techniques that will bridge the gap between advancing biochemical knowledge and cancer treatment; and will add to a compilation of data that emphasizes the necessity for developing molecular and genetic testing during patient assessment and treatment.

CHAPTER 2: QUANTIFICATION OF ZBTB7A IN SURGICAL GLIOMA SPECIMENS AND GLIOMA CELL LINES

2.1 Introduction

ZBTB7A has been found to be over-expressed in several human malignancies, including: breast, bladder, colon, and lung carcinomas as well as diffuse large B cell lymphomas (Apostolopoulou et al., 2007; Maeda et al., 2005b). An exhaustive literature search has yet to reveal any proposed association between ZBTB7A and glioma development and or progression. The purpose of this study was to determine the relative quantities of ZBTB7A mRNA in 18 formalin-fixed paraffin-embedded (FFPE) glioma samples and 4 glioma cell lines using endpoint RT-PCR.

2.2 Materials and Methods

This analysis was performed on formalin-fixed paraffin-embedded tissues gathered from 18 operative glioma specimens and 4 glioma cell lines (Table 2.1). According to the World Health Organization criteria and graded by the pathologist at Marquette General Hospital, one sample was given the grade of pilocytic astrocytoma (PA), two were WHO grade III astrocytomas and the other 15 samples were GBMs. One specimen was extracted from a patient who received prior chemotherapy, the other 17 samples received no chemo-, radio- or immunotherapy prior to surgical resection. The Institutional Review Boards of Marquette General Hospital and Northern Michigan University approved the use of the tissue samples prior to analysis.

Table 2.1: Summary of surgical specimen characteristics (n=18) and glioma cell line characteristics (n=4).

Surgical Cases FFPE Specimens		
Median age (years)		52.9
Sex		
	males	8
	females	10
Classification		
	GBM	15
	WHO (III)	2
	PA	1

Glioma Cell Specimens		
Median age (years)		53.5
Sex		
	males	2
	females	2
Classification		
	GBM	3
	WHO (III)	1

Cell Culture: The malignant glioma cell lines designated T98G, U-138 MG, U-87 MG, LN-229, obtained from American Type Culture Collection (ATCC) were originally seeded at 10-15 cells/µl in 25 cm^2 flasks containing 90% EMEM or DMEM growth mediums (ATCC Manassas, VA) and 10 % fetal bovine serum. Flasks were kept in a humidified incubator at 37°C and 5% CO_2 (Figure 2.1).

RNA extraction from glioma cell lines: Approximately $1x10^6$ cells growing in a monolayer were detached from the culture flasks using 2% trypsin ethylene diamine tetraacetic acid (EDTA). Cells were lysed with buffer RLT and homogenized using a QIAshredder (Qiagen Valencia, CA). RNA was isolated using the RNeasy Mini Kit without deviation from the manufacturer's protocol (Qiagen Valencia, CA).

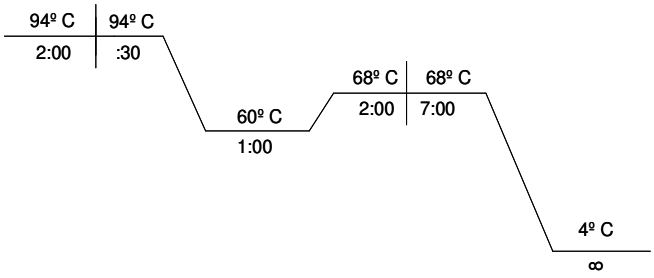
FFPE Nucleic acid extraction: The RNA was extracted from three, 20-micron sections of formalin fixed paraffin embedded tissue from each of the 18 glioma samples according to the manufacturer's protocol for the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Foster City, CA). Following de-paraffinization and protease digestion each sample was split into separate aliquots for isolation of total RNA and genomic DNA.

ZBTB7A mRNA expression: Extracted RNA was reverse transcribed using the one step AccessQuick RT-PCR kit (Promega, Madison, WI). Table 2.2 lists the primers used

to amplify the ZBTB7A and β-actin mRNA (Rovin and Winn, 2005). Reactions were run for 40 cycles (thermal profile depicted in table 2.2) in an Eppendorf Master Gradient thermal cycler. Messenger RNA from the housekeeping gene β-actin served as a control for each reaction. PCR products were electrophoresed using the FlashGel system. Ultraviolet light was used to visualize products and photographs were taken using a Kodak DC 290 camera and Kodak 1D software. GelEval 1.10 software (FrogDance Software) was used to determine the band intensities and thus provide ZBTB7A:β-actin mRNA ratios (Figure 2.2).

Table 2.2: Primers sequences and annealing temperatures for the ZBTB7A and the β-actin locus. In the lower section of the table the thermal profile that was used in the RT-PCR reaction is listed. Each reaction was run for 40 cycles.

Locus	Primers	Sequence	Product (bp)	Annealing temp. (ºC)
ZBTB7A	RT-PCR			
	Fw	5-CAG CAG CGA CAT CCT GAG TG-3	188	57
	Rv	5-AGT CGA TCT CGT ACA CGT TCT-3		
β actin	RT-PCR			
	Fw	5-TCG ACA ACG GCT CCG GCA-3	250	60
	Rv	5-AAG GTG TGG TGC CAG ATT TTC-3		



2.3 Results

Four glioma cell lines (T98G, U-138 MG, U-87 MG, LN-229), 15 GBM specimens, 2 WHO grade III astrocytomas and one pilocytic astrocytoma from Marquette General Hospital were evaluated. Using RT-PCR, ZBTB7A mRNA was detected in 13 of

15 GBM specimens, both WHO grade III astrocytomas, the pilocytic astrocytoma specimen. ZBTB7A was also detected in each of the four cell lines (T98G, U-138 MG, U-87 MG, LN-229). Figure 2.1 is a gel image showing PCR products for ZBTB7A and β-actin from each cell line (188 bp product representing ZBTB7 right and 250 bp β-actin product left). Figure 2.2 is a graph displaying ZBTB7:β-actin band intensity ratios. Eleven of eighteen gliomas displayed ratios greater than 1 and 2 of the 4 cell lines also displayed elevated levels of ZBTB7A. The U-138 MG and the U-87 MG cell lines displayed the highest level of ZBTB7A expression, with ratios of 1.4 and 1.76 respectively.

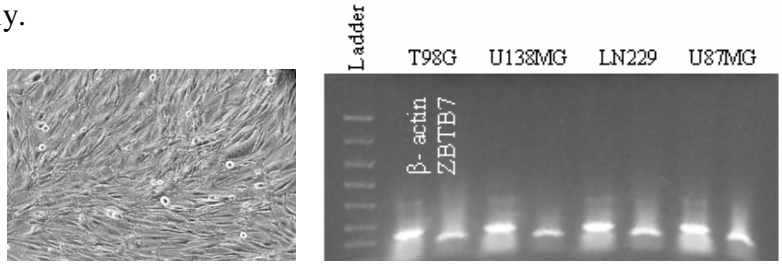


Figure 2.1: Depicted left is a photomicrograph (40x) of confluent U-138 MG cells. Right is a representative gel image of RT- PCR products from each cell line. The right band is ZBTB7A (188 bp) product and the left band is β-actin (250 bp) product.

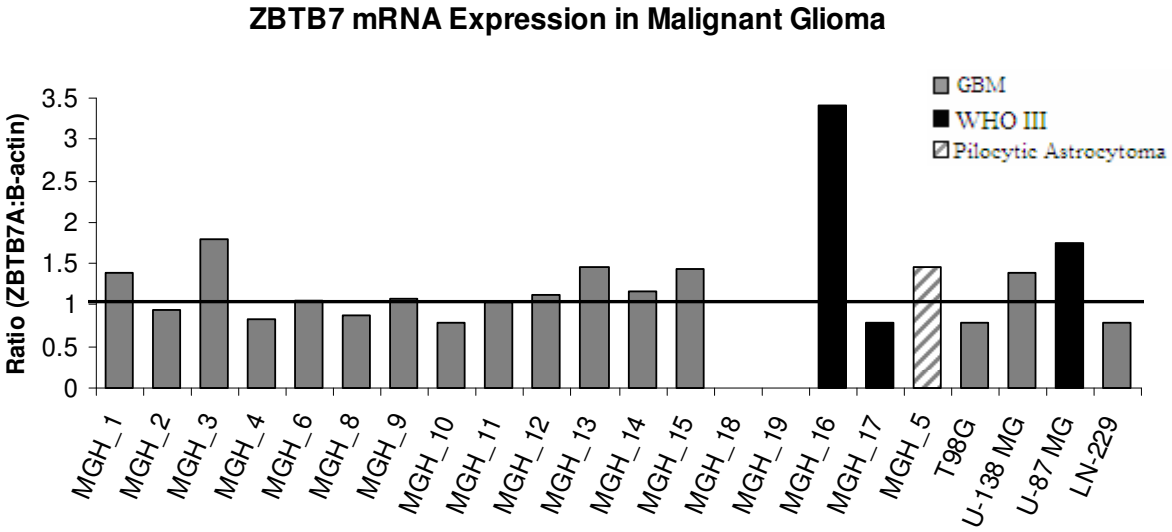


Figure 2.2: A ratio of the band intensities averaged among triplicates and displayed in graphical form above. Note a ratio of 1 was used as baseline to compare the expression level across each specimen. Including the four cell lines, 18 GBMs were analyzed, 3 WHO grade III, and 1 pilocytic astrocytoma. Eleven of sixteen specimens displayed ZBTB7: β-actin ratios greater than 1.

2.4 Discussion

The expression of ZBTB7A in healthy total brain extracts is slightly less than the median expression of ZBTB7A throughout tissues in the human body (Su et al., 2004). In light of this study, and because it was not ethically feasible to obtain healthy brain tissue from patients, the expression of ZBTB7A was compared to the expression of the housekeeping gene β-actin. A ratio of greater than 1 was considered over-expression. RNA was extracted from glioma tissue extracted from surgical patients at Marquette General Hospital.

ZBTB7A has been described as an oncogene in previous research because it specifically represses the transcription of p14ARF tumor suppressor (Maeda et al., 2005a). ARF-p53-pRb malfunction is common within malignant gliomas (Nakamura et al., 1998) and has been reported as a prognostic indicator in diffuse non-small cell lung carcinomas (Apostolopoulou et al., 2007). To our knowledge, the finding of ZBTB7A over-expression in gliomas is novel. The elevated levels of ZBTB7A suggest oncogene activity which may contribute to the malignant transformation that takes place in gliomas. The manner by which ZBTB7A becomes oncogenic (over-expressed) was the subject of further study.

CHAPTER 3: AN INVESTIGATION OF ZBTB7A GENE AMPLIFICATION IN GLIOMA TISSUES

3.1 Introduction

The activation of proto-oncogenes can occur by genetic changes that alter protein expression or structure. However, the type of oncogene activation of interest to this study is gene amplification. Gene amplification refers to mutations that produce multiple copies of a gene and thus cause a proportionate increase in protein product (Alitalo and Schwab, 1986).

Previously, our group provided evidence that ZBTB7A is over expressed in the majority of glioma tissue samples tested and in two of four glioma cell lines tested. The aim of this study was to determine if the over-expression of ZBTB7A was the result of gene amplification. To this end, differential PCR was used determine the number of ZBTB7A gene copies in 18 formalin-fixed paraffin-embedded glioma samples and 4 glioma cell lines.

3.2 Materials and Methods

This analysis used the same formalin-fixed paraffin-embedded tissue gathered from the 18 glioma operative specimens and 4 glioma cell lines (see table 2.1 for characteristics). The Institutional Review Boards of Marquette General Hospital and Northern Michigan University approved the use of all tissue samples prior to analysis.

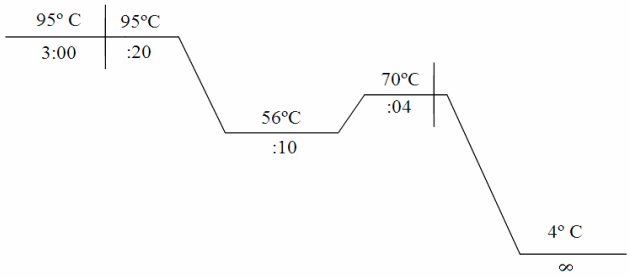
FFPE Nucleic acid extraction: During RNA extraction using the RecoverAll Total Nucleic Acid Isolation Kit for formalin fixed paraffin embedded tissues, three, 20

micron sections from each of the 18 tumor samples were deparaffinzed followed by a protease digestion. Each sample was then split into separate aliquots and glass-fiber filter purification was used to isolate total RNA and genomic DNA.

Differential PCR: The DNA primers used to amplify the ZBTB7A and IFN-γ genes are reported in Table 3.1 (Apostolopoulou *et al.* 2007). Fifty microliter reactions contained; 1.5 millimolar $MgSO_4$, 200 micromolar dNTPs, 0.3 micromolar ZBTB7A forward and reverse primer, 0.3 micromolar IFN-γ forward primer and reverse primer, 5 microlitre KOD polymerase buffer, 1 unit KOD Hot Start DNA polymerase (Novagen, Madison, WI). Reactions with the thermal profile depicted in table 3.1 were run for 40 cycles in an Eppendorf Master Gradient thermal cycler. PCR products were electrophoresed using the FlashGel system (Cambrex, Rockland, ME). Products were visualized under ultraviolet light and photographs were taken using a Kodak DC 290 camera and Kodak 1D software. The band intensity and ratio of ZBTB7A:IFN-γ was determined using GelEval 1.10 software.

Table 3.1: Primer sequences and annealing temperatures for the differential PCR reaction. The lower section of the table depicts the thermal profile that was used in the PCR reaction. Each reaction was run for 40 cycles. Fw, forward; Rv, reverse; IFN-γ, Interferon-γ; D-PCR, differential polymerase chain reaction.

Locus	Primers	Sequence	Product (bp)	Annealing temp. (ºC)
ZBTB7A	D-PCR			
	Fw	5-GAA CGA GGG TTT AGT GCA-3	299	59
	Rv	5-CGA GCT GTT CTG GAG AGA-3		
IFN-γ	D-PCR			
	Fw	5-CTC TTT TCT TTC CCG ATA GGT-3	151	56
	Rv	5-CTG GGA TGC TCT TCG ACC TCG-3		



3.3 Results

Differential PCR was used to determine if the over-expression of ZBTB7A in malignant gliomas was the result of gene amplification. By comparing the PCR products ratios ZBTB7A:IFN-γ (Figure 3.1), it was observed that 4 of 16 (25%) samples displayed gene amplification (ratios > 2) (Figure 3.2).

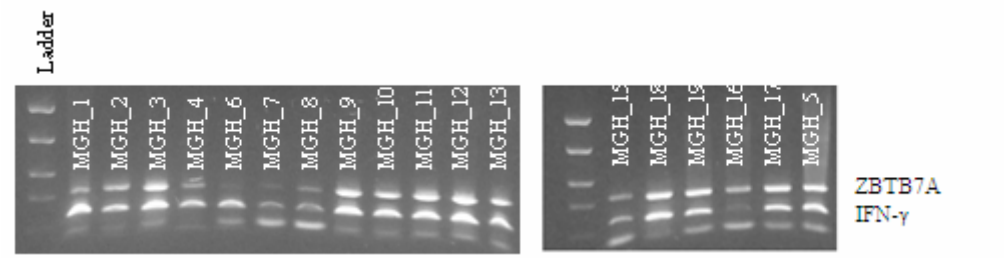


Figure 3.1: Depicted above is a representative gel image of differential PCR products from samples 1-17 (excluding 14). The top band in each lane represents the product from the amplification of ZBTB7A (299 bp), the bottom band represents IFN-γ (151 bp) reaction product.

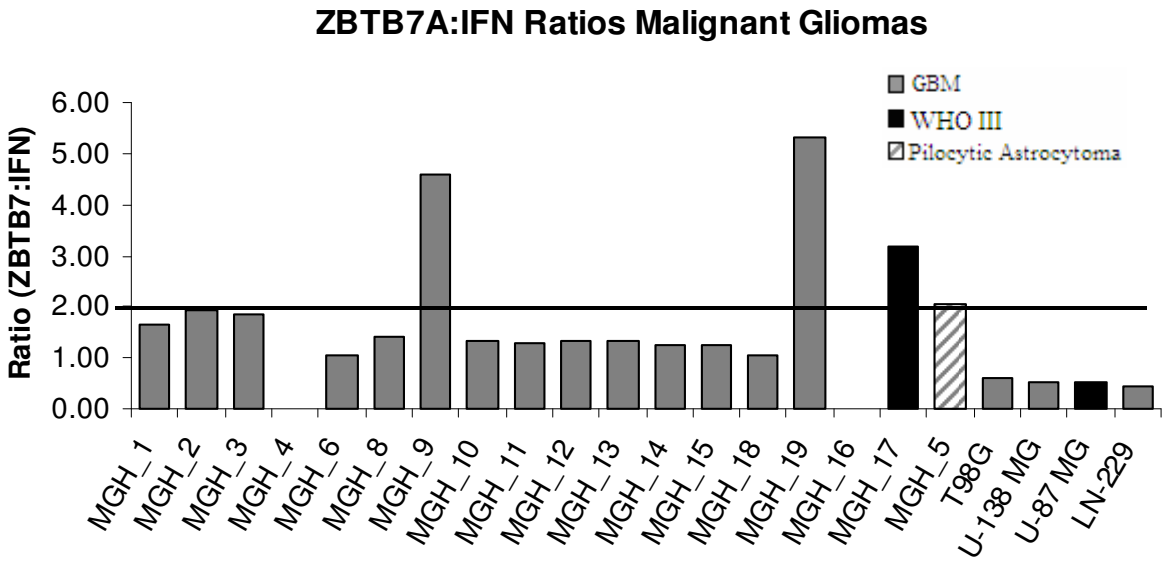


Figure 3.2: Above graph displays the band intensity ratios that were averaged among triplicates. Note a ratio of 2 was used as baseline for gene amplification. Three GBMs and a WHO III astrocytoma (4 of 16) displayed gene amplification (ratios 2 or greater).

3.4 Discussion

In an effort to determine if gene amplification was the mechanism leading to ZBTB7A mRNA over-expression (Chapter 2), differential PCR was used. These data indicate 4 of the 16 FFPE (25%) samples had ZBTB7A:IFN-γ band intensity ratios greater than 2. Interestingly the four samples (MGH_19, MGH_9, MGH_5, and MGH_2) that displayed the highest ZBTB7A:IFN-γ ratios were not samples previously characterized as over-expressing ZBTB7A mRNA (chapter 2). From these data it is clear that gene amplification does not necessarily correlate with gene expression. Apostolopoulou and colleagues (2007) reported a ZBTB7A gene amplification rate of 27.7% in patients with non small cell lung carcinomas which is strikingly similar to the 25% rate of gene amplification found in this study. In addition, Apostolopoulou found a substantial number of samples that demonstrated ZBTB7A mRNA over-expression yet lacked gene amplification.

Reports of malignancies associated with specific gene amplifications date back to the mid 1980's. Among those, Slamon and colleagues (1987) reported longer disease free survival in patients with no amplification of HER-2/neu. Additionally, Lengauer and colleagues (1998) reported less than 10 copies of N-myc was a positive prognostic indicator in children diagnosed with neuroblastoma.

Several researchers investigating gliomas have yet to find gene amplification associated with patient outcome (Brandes et al., 2008; Galanis et al., 1998; Hunter et al., 1995). Our findings support the lack of predictability between gene amplification and oncogene activation. Further analysis on a larger group of glioma patients will need to be done in order to determine whether ZBTB7A gene amplification can be used as prognostic indicator for future clinical reports.

New technology has spurred genome-wide searches in attempt to identify the somatic changes that are responsible for human cancers. The genes known to be associated with the differentiation of glial progenitor cells are of special interest to researchers attempting to elucidate gliomagenesis. The finding of ZBTB7A gene amplification in malignant gliomas suggests the possibility of a genetic alteration that causes progression of gliomas. However, the lack of correlation between mRNA levels and gene copies imply that there are other mechanisms that are responsible for causing the activation of the oncogene ZBTB7A. Regardless of the means for activation, preventing the expression of ZBTB7A in malignant gliomas may be a viable means for clinically treating gliomas in the future and was therefore examined in the next study.

CHAPTER 4: MEAUREMENT OF ZBTB7A MRNA IN MALIGNANT GLIOMA CELL LINES BEFORE AND AFTER *IN VITRO* SIRNA TREATMENT

4.1 Introduction

Maeda *et al.* (2005a) proposed ZBTB7A as a critical mediator of oncogenic transformation in hematopoietic malignancies and further reported its over-expression in several other types of malignancies. In a retrospective study, we observed the over-expression of ZBTB7A in large percentage of the glioma tissues examined (chapter 2) and have proposed a possible role of ZBTB7A in glioma formation and or development.

Previous reports documented the use of siRNA as an effective means for reducing the expression of oncogenes in glioma cells (Jiang et al., 2005; Zang et al., 1999). The objectives here were to use real-time PCR to determine baseline ZBTB7A gene expression in four glioma cell lines and to determine if siRNA treatment can be used to effectively reduce ZBTB7A mRNA transcripts *in vitro.*

4.2 Materials and Methods

Cell Culture: Each of four cell lines designated LN-229, U-87MG, U-138MG, and T98G (ATCC, Manassas, VA.) were originally seeded at 10-15 cells/µl in 25 cm^2 flasks. LN-229 cells were cultured in 95% DMEM (ATCC, Manassas, VA) with 5% fetal bovine serum. The cell lines U-87MG, U-138MG, and T98G were grown in 90% EMEM (ATCC, Manassas, VA) and 10 % fetal bovine serum. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2.

RNA extraction from glioma cell lines: Cells growing in a monolayer were detached from the culture flasks using 1x trypsin EDTA (Gibco). Using the RNeasy mini kit, approximately $1x10^6$ cells were lysed with buffer RLT and homogenized using a QIAshredder (Qiagen Valencia, CA). RNA was isolated without deviation from the manufacturer's protocol (Qiagen Valencia, CA).

Real-time PCR: In order to quantify baseline mRNA for ZBTB7A, RNA isolated from each of four cell lines were subjected to real-time PCR using pre-designed TaqMan® Assays for ZBTB7A (Applied Biosystems; #hCG2004107) and an internal control GAPDH (Applied Biosystems; #4333764-0612018). The manufacturer's protocol for the TaqMan One-Step RT-PCR Master Mix (Applied Biosytems) was followed. The parameters for the real-time reaction were as follows: The reaction was held at 50º C for 2 minutes, then ramped to 95º C for 10 minutes. After the 95º C step, the first of 40 cycles began, which consisted of 15 seconds at 95º C then 60 º C for 1 minute. A 48-well StepOne™ System (Applied Biosystems, Foster City, CA) was used to determine relative quantities (RQ) of PCR product and to compute comparative C_T values. The software used ratios of ZBTB7A to GAPDH and then compared those ratios across each sample (Livak and Schmittgen, 2001).

siRNA Assays: Cell lines designated LN-229, U-87MG, U-138MG, and T98G were seeded at a density of 5,000 cells diluted in 100 uL of complete media in each well of a 96 well plate. Twenty-four hours after plating cells were treated with predesigned *Silencer*® siRNAs (sense: 5'-CCUUGUAGAUCAAAUUGAUtt, antisense: 5'-AUCAAUUUGAUCUACAAGGtc-3'). To transport the siRNA into the cells siPORT *NeoFX* Transfection agent was used (Ambion, Foster City, CA). Each assay was prepared

in triplicate. Additionally negative control siRNA—which Ambion verified as having no known homology to human mRNA (Negative Control #1 from Ambion Biosystems; #AM4611)— and untreated controls were prepared in triplicate. After seventy-two hours at 37°C with 5% CO_2, both RNA and protein were collected using a PARIS extraction kit (Ambion, Foster City, CA).

4.3 Results

Baseline Gene Expression: Real-time PCR was used to determine ZBTB7A mRNA in each cell line. The point at which the product signal reached the threshold determined the expression level (Figure 4.1). Threshold for GAPDH was set to 0.2100 and the threshold set for ZBTB7A was 0.2416. The U-87 MG cell line displayed the highest level of ZBTB7A expression and was given a value of 1. The U-138 MG cell line expressed 25% less ZBTB7A mRNA, followed by the T98G cell line which expressed 59% the level that U-87 MG cells expressed. The LN-229 cell line expressed the least with only 38% the level ZBTB7A mRNA compared to U-87 MG expression (Figure 4.2).

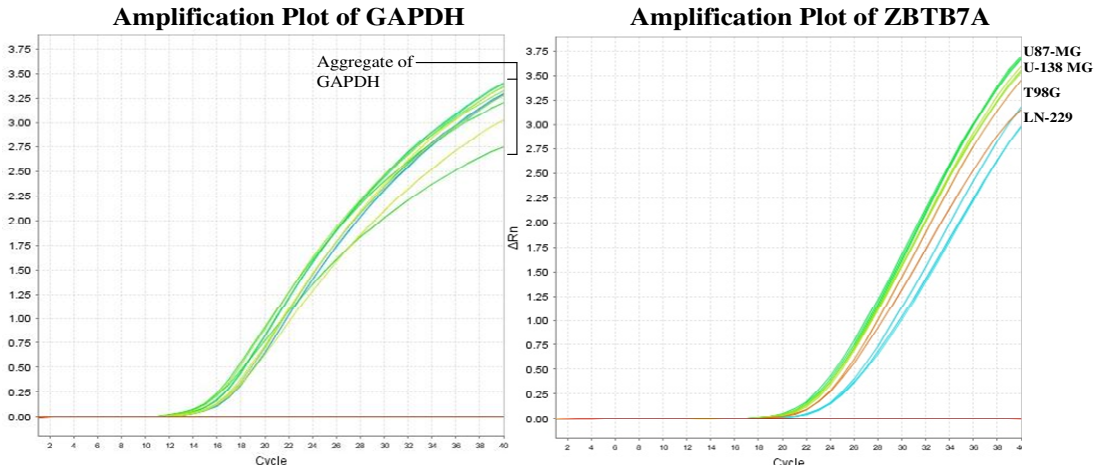


Figure 4.1: To avoid confusion, GAPDH and ZBTB7A are represented separately. Note left amplification plot displays on aggregate of GAPDH curves with little variation between cell lines. Right is a representative amplification showing clear variation between cell lines (Top to bottom U87-MG, U183-MG, T98G, and LN-229).

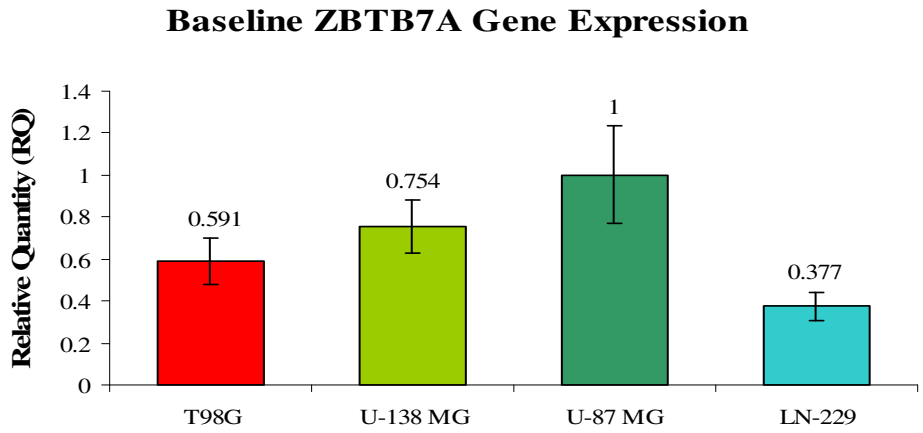


Figure 4.2: Above graph displays median real time PCR quantification (with standard error) of ZBTB7A gene expression (3 replicates) for each of the glioma cell lines T98G, U183-MG, U87-MG and LN-229.

siRNA Assays: Seventy-two hours post transfection, RNA was extracted and real time PCR was used to detect ZBTB7A and GAPDH mRNA in each of the four cell lines (Figure 4.3). Compared to untreated and negative controls, the siRNA treated groups of both cell lines T98G and LN-229 displayed no reduction of ZBTB7A mRNA. In contrast, the cell line U-87 MG decreased nearly 20% in ZBTB7A mRNA expression and the cell line U-138 MG displayed a marked reduction of over 58% (Figure 4.4).

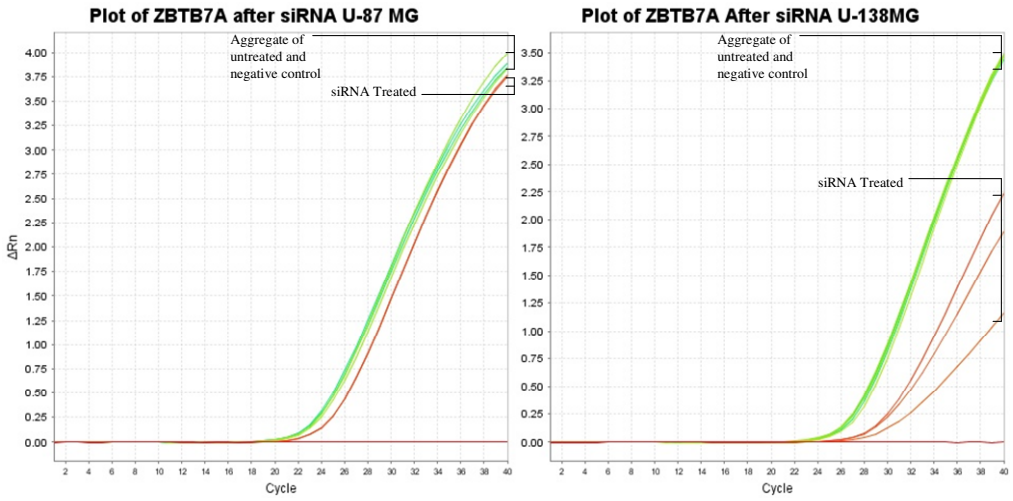


Figure 4.3: Depicted above are two representative real time PCR experimental results. The left graph shows the amplification curves for U-87 MG cells when untreated, treated with negative control siRNA and when treated with ZBTB7A siRNA. Similarly, the three experimental groups were amplified for the U-138 MG cells.

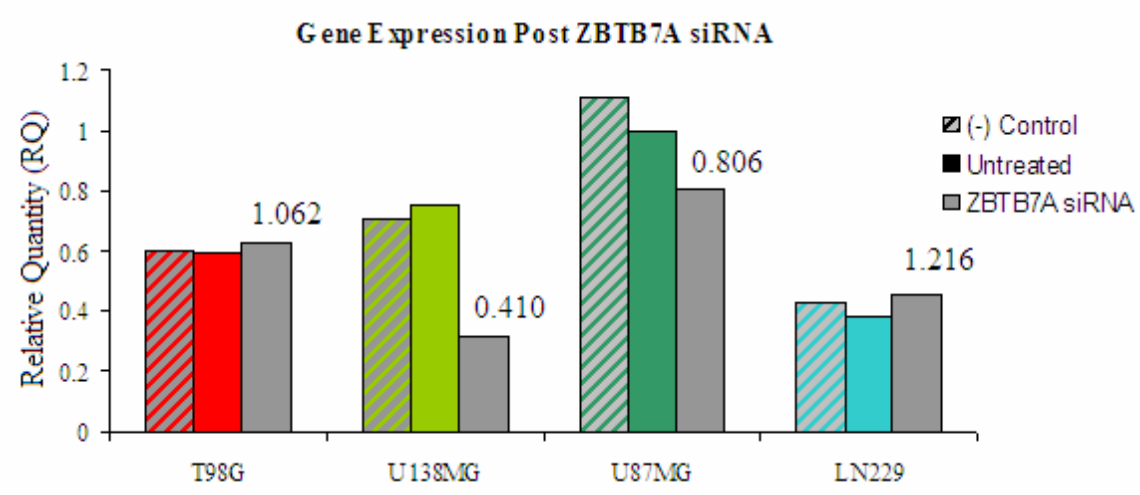


Figure 4.4: Real time PCR relative quantification of ZBTB7A gene expression in the 3 experimental groups for each cell line (untreated control, (-) control, and ZBTB7A siRNA treated group). Note, cells lines T98G and LN-229 displayed no response to the siRNA treatment, while cell lines U-87 MG and U-138 MG showed a reduction of ZBTB7A mRNA in the siRNA treated groups.

4.4 Discussion

With the use of real-time PCR, the relative expression of ZBTB7A was established in four glioma cell lines (U-87 MG > U-138 MG > T98G > LN-229). Similar to result of previous studies (chapter 2), the U-87 MG cell line displayed the highest level of ZBTB7A mRNA followed closely by the U-138 MG cell line (75%). The similarity in gene expression between these independent studies reconfirms that U-87MG cells produce the greatest level of ZBTB7A mRNA. Unlike the previous study using endpoint PCR (chapter 2), where we detected nearly identical ZBTB7A:β-actin ratios in the LN-229 and the T98G cell lines, real-time PCR demonstrated about 20% more ZBTB7A mRNA in the T98G cell line. The reason for this discrepancy could be related to the extreme sensitivity of the real-time assay. However, given the instability of the cancer cell genome, mutations could have occurred that altered expression of ZBTB7A (Li et al., 2008). Genetic instability may pose problems with establishing baseline gene expression, but would not influence the outcome of siRNA treatment due to concomitant untreated controls.

Small interfering RNA was applied to each of the glioma cell lines and ZBTB7A mRNA was measured with real-time PCR. No decrease in ZBTB7A mRNA was detected in either of the cell lines T98G or LN-229. The reason for this is unknown; however, point mutations within the ZBTB7A exons may facilitate a poor mRNA-siRNA interaction and thus prevent the normal downstream silencing mechanism. Additionally, any mutations among the many genes coding for the silencing machinery (RISC) could disable the effects of siRNA altogether. Lastly, an adequate amount of siRNA may not have been transported into the cell to silence the low levels of mRNA present in the cells.

After siRNA treatment, the cell line U-87MG showed diminished ZBTB7A expression by nearly 20%, as compared to untreated and negative controls. The U-138 MG cell line showed the greatest response to siRNA; ZBTB7A mRNA was reduced by nearly 60%. These results indicate siRNA can be used to effectively reduce the expression of the transcriptional repressor ZBTB7A in two (U-138 MG, U-87 MG) of four glioma cell lines treated.

With the recent advent of RNAi, a number of studies have been undertaken to reduce the expression of the genes responsible for human diseases. RNA interference has previously been used to treat brain tumors both *in vitro* (Jiang et al., 2005) and *in vivo* (Partridge et al., 2004). This research has reaffirmed the possibility of using siRNA to decrease gene expression in tumor cells. Further experiments were performed to determine if the reduction of ZBTB7A has any effect on glioma cell survival.

CHAPTER 5: MEASURING THE EFFECT OF ZBTB7A SIRNA ON GLIOMA CELL SURVIVAL

5.1 Introduction

Eliminating the oncogene ZBTB7A should restore p14ARF expression leading to MDM2 sequestration and stabilization of p53 and pRb. Functional p53 and pRb should prevent cell cycle progression and possibly trigger apoptosis. RNA interference targeting ZBTB7A may be a means for achieving the restoration of p53 and pRb.

As described in Chapter 4, real-time PCR analysis provided evidence that siRNA was effective at knocking down ZBTB7A in the glioma cell lines U-87 MG and U-138 MG. The objective of this study was to observe the effect of siRNA on cell survival in the glioma cell lines that had the greatest response to siRNA treatment (U-87 MG and U-138 MG).

5.2 Materials and Methods

Cell Culture: The cell lines designated U-87MG and U-138MG (ATCC, Manassas, VA) were seeded at 10-15 cells/µl in 25 cm^2 culture flasks. The cells were grown in EMEM (ATCC, Manassas, VA) with 10 % fetal bovine serum and maintained in a humidified incubator at 37°C with 5% CO_2.

Cell viability assay: Cells from both cell lines (U-87MG and U-138MG) growing in a monolayer were detached from the culture flasks using 1x trypsin EDTA and were counted using a hemocytometer. Cells were seeded at a density of 5,000 cells/well in 96 well plates. Twenty-four hours after plating, cells were treated with the same predesigned

Silencer® siRNAs (sense: 5'-CCUUGUAGAUCAAAUUGAUtt, antisense: 5'-AUCAAUUUGAUCUACAAGGtc-3') that were used in previous studies (chapter 4). Similarly, the siRNA were transported into the cells using siPORT *NeoFX* Transfection agent (Ambion, Foster City, CA). Each assay was prepared in triplicate with negative control siRNA—which Ambion verified as having no known homology to human mRNA(Negative Control #1 from Ambion Biosystems; #AM4611)— and untreated controls. In addition, standard curves were prepared with each assay using serial dilutions of the original 5000 cells/well. Seventy-two hours after incubation at 37°C with 5% CO_2, cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI). The luminescence results of the CellTiter-Glo assay were collected using a GloRunner™ Microplate Luminometer (Turner Biosystems, Sunnyvale CA). Using relative light units from each of the three experimental conditions (siRNA treated, negative control siRNA, untreated control) and the standard curve, the number of viable cells was determined. Each experiment was performed in triplicate.

5.3 Results

The relative light units (RLU) from each well reading were used in the equation generated by the standard curve for each cell line (Figure 5.1). The standard curve generated for the cell line U-87 MG displayed an R^2 value of 0.9921. The ZBTB7A siRNA treated wells had slightly fewer (4,039) cells compared to untreated control (4,182). A single factor ANOVA was used to determine that there was no significant difference among the three groups: Untreated cells, negative control, and the siRNA for ZBTB7A (Figure 5.2).

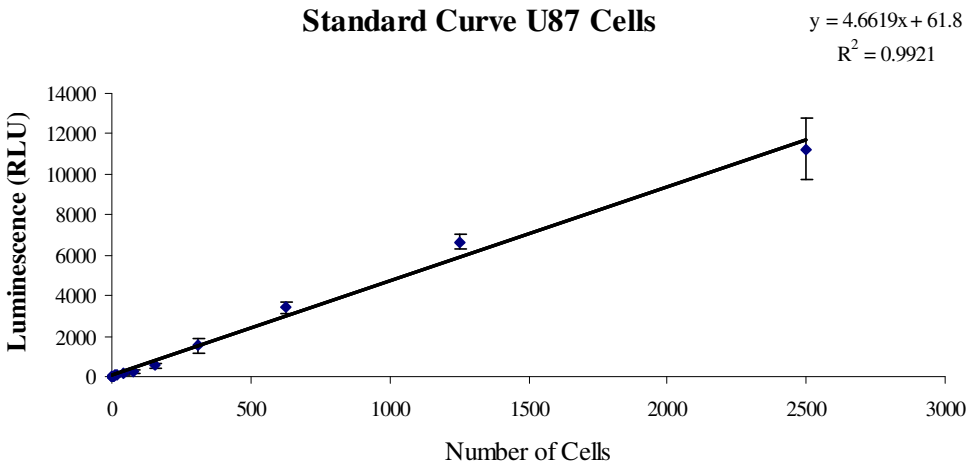


Figure 5.1: Above graph displays the standard curve generated from serial dilutions of U-87 MG cells prepared during each assay. Each point represents an average of three values with standard error. Note the equation in the top right corner that used to determine the number of cells in each well.

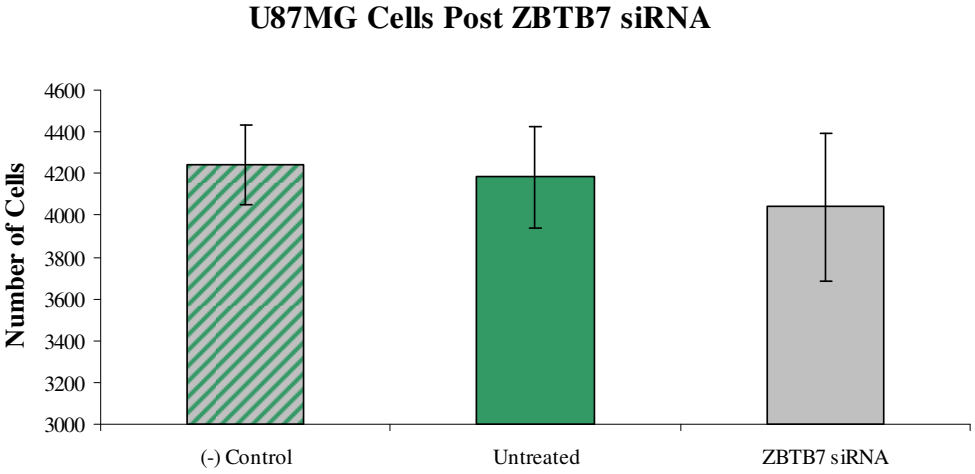


Figure 5.2: Depicted above is a graphical representation of the average cell number in each of the three experimental groups with standard error. ZBTB7A siRNA treated cells showed a slightly depressed average cell number, however, also had the highest standard error.

The standard curve generated for the U-138 MG cell line displayed an R^2 value of 0.9997 (Figure 5.3). Using the equation from the standard curve the ZBTB7A siRNA treated wells had fewer (4,099) cells compared to the untreated control (4,370). Again a

single factor ANOVA was used to determine that there was no significant difference among the three groups (Figure 5.4).

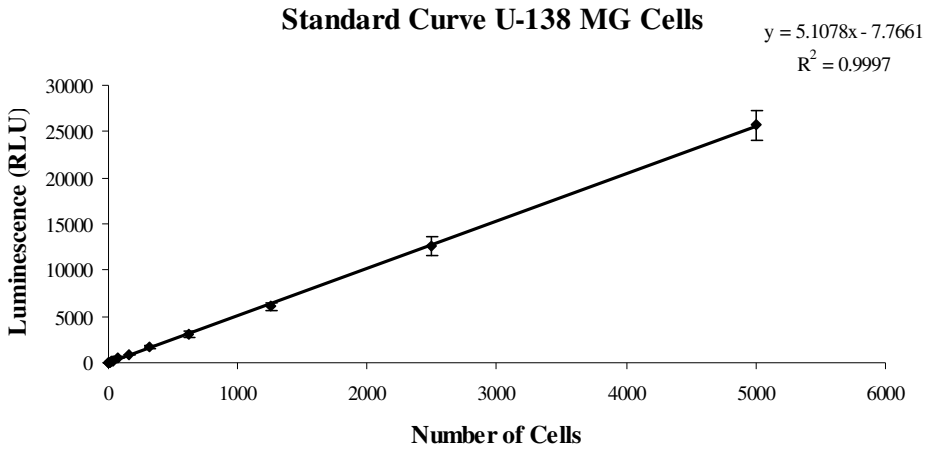


Figure 5.3: The line graph above represents the curve generated when a serial dilution of U-138 MG cells were prepared during each assay. Note the equation in the top right corner was used to determine the number of cells in each well of the experimental groups.

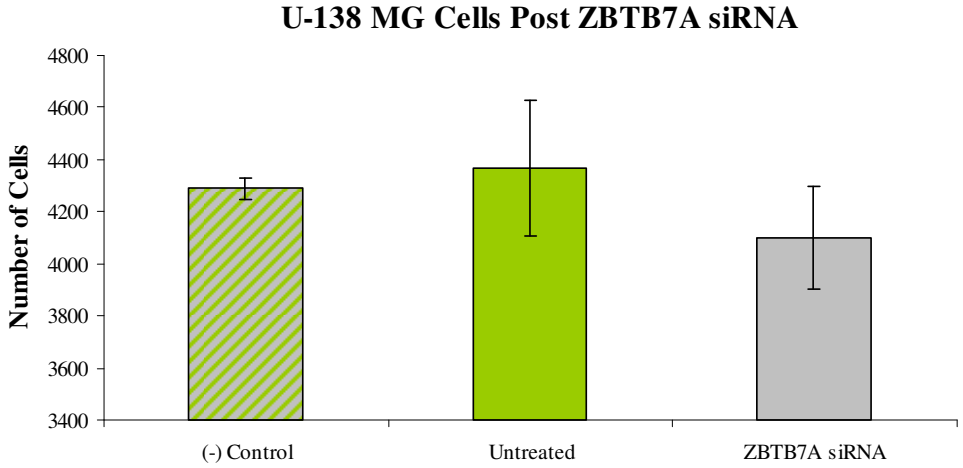


Figure 5.4: The bar graph above displays the average cell (U-138 MG) number in each of the three experimental groups. ZBTB7A siRNA treated cells showed a slightly depressed average cell number, however, also had a large amount of standard deviation.

5.4 Discussion

The aim of this study was to observe whether ZBTB7A siRNA influenced cell proliferation. ZBTB7A siRNA treatment insignificantly decreased cell numbers in both

glioma cell lines (U-87 MG, U-138 MG). Experimental error is likely the cause for the large standard deviation; however, given the repeatability of decreased cell numbers in the ZBTB7A siRNA treated group, the change in cellular proliferation is unlikely to be coincidental.

ZBTB7A is a proto-oncogene because it represses the expression of the tumor suppressor p14ARF (Maeda et al., 2005). Ishii and colleagues (1999) found homozygous deletions of the p14ARF tumor suppressor in both of the cell lines used here (U-87 MG, U-138 MG). Because p14ARF is absent, loss of ZBTB7A would not be expected to restore p14ARF levels and therefore should not have an impact on cell survival (Quelle et al., 1997). Further experiments should be undertaken on cell lines with intact downstream mechanisms (p14ARF, p53, pRB), before ZBTB7A can be discarded as having no value as a target for future glioma treatment.

CHAPTER 6: SYNOPSIS AND CONCLUSIONS

Despite a vast amount of research, malignant gliomas remain difficult to clinically manage. As a result clinicians are beginning to treat malignant gliomas as a chronic disorder, administering treatment regimens in a step-wise fashion to manage rather than cure the disease. Unfortunately, the highest grade gliomas progress rapidly and few treatments alter the patient outcome after one year.

The recent discovery of brain tumor stem cells has spurred efforts to develop more efficient treatments that target this presumed source of the tumor (Hemmati et al., 2003; Singh et al., 2003, Kondo et al. 2004; Yuan et al., 2004). This concept envisions treatment of brain tumor stem cells with agents that target the molecular mechanisms known to control the proliferation of normal neural stem cells (Sinor and Lillien, 2004; Nakano et al., 2008; Groszer et al., 2006). Those factors that control healthy neural stem cells include the transcription factors.

Transcription factors are known to be especially important contributors to stem cell differentiation and proliferation and as a result have attracted much attention as possible targets of cancer treatments (Nakano et al., 2008). The transcription factor of interest in this study was ZBTB7A. ZBTB7A is a POK transcription factor known to be up-regulated during embryonic development (Bardwell and Treisman, 1994) and is expressed at lower than average (across body) levels in whole brain extracts (Su et al., 2004). POK transcription factors have a POZ domain that is essential for co-repressor recruitment and Kruppel-type zinc finger that recognize and bind specific regions within the DNA (Stogios, 2007). The four Kruppel-type zinc fingers found on ZBTB7A's

carboxy terminus are known to specifically recognize the promoter region within the p14ARF gene (Maeda et al., 2005a). Repression of p14ARF transcription leads to an accumulation of MDM2. MDM2 accumulation enhances the degradation of p53 and pRb which prevents the induction of apoptosis and disables mechanisms that regulate critical checkpoints in the cell cycle (Quelle et al., 1997).

Using endpoint PCR, 11 of 18 formalin fixed paraffin embedded (FFPE) glioma tissues and two glioma cell lines (U-138 MG and U-87 MG) were shown to over-express ZBTB7A when compared to the housekeeping gene β-actin. The finding of ZBTB7A over-expression in gliomas is novel and may suggest oncogenic activity similar to other types of malignancies (Apostolopoulo et al., 2007; Maeda et al., 2005a).

Differential PCR was used to determine if gene amplification was the cause of ZBTB7A over-expression in the 18 FFPE glioma tissues and 4 glioma cell lines. Four of sixteen FFPE glioma samples, that had adequate DNA for analysis, showed evidence of multiple ZBTB7A gene copies, while none of the cell lines displayed elevated ZBTB7A mRNA. Similar percentages of GBM samples (25%) and non-small cell lung carcinoma samples (27.7%) were found to have gene amplification of ZBTB7A (Apostolopoulou et al., 2007). Interestingly, ZBTB7A gene amplification did not correlate with ZBTB7A mRNA over-expression.

After finding ZBTB7A over expressed in glioma tissue, attempts were made to reduce its expression *in vitro.* Before attempts to reduce gene expression were undertaken, baseline gene expression in four glioma cell lines were determined using real-time PCR (U-87 MG was greater than U-138 MG, followed by T98G, and lastly LN-229). With the exception of the cell line LN-229, the real-time PCR data showed a

similar ZBTB7A mRNA expression trend as did our earlier reports using end point PCR. After siRNA treatment, the cell line U-87MG showed a 20% decrease in ZBTB7A, as compared to untreated and negative controls. The mRNA for ZBTB7A was decreased by nearly 60% in U-138 MG, and the cell lines T98G and LN-229 recorded no measureable change in ZBTB7A expression. These results substantiate the potential siRNA has to reduce the expression of an oncogene in glioma tissues.

ZBTB7A siRNA decreased cell survival in both of the glioma cell lines (U-87 MG, U-138 MG) tested, although not significantly. Experimental error during these procedures was the likely cause for the large variation between samples, but a similar depression in cell number was observed in each siRNA treatment. The minimal response of these cell lines to ZBTB7A siRNA was likely due to deletions of downstream players p14ARF and p53 (Quelle et al., 1997). Further studies on glioma cell lines (perhaps a glioma stem cell line) known to have intact downstream mechanisms (p14ARF, p53, pRB) should be done before ZBTB7A siRNA can be discarded as having a lack of clinical value. However, this study clearly demonstrates that silencing ZBTB7A is certainly not a magic bullet that alone cures glioma cells spontaneously.

Silencing the oncogene ZBTB7A should induce a change that restores the apoptotic response in glioma cells, however without viral vectors, the change will be transient. A potential downfall of siRNA using a lipid soluble vector is the transient nature of the treatment. The treatment will be less than completely effective if every cancer cell (including those that migrate great distances away from tumor bed) is not exposed to the siRNAs during the treatment regimen. However, the transient nature of the siRNA prevents later gene expression interference—that may occur with viral vectors—

after the tumor is eradicated. The transient nature of siRNA has both pros and cons, only after further *in vivo* research will additional factors be identified and addressed.

Treating gliomas is not only challenging because of the rapid cell growth and migration but also because the brain (including the tumor) is protected by the blood brain barrier (BBB). The BBB indeed prevents larger molecular weight drugs (such as siRNAs) from penetrating the cerebral circulation. As a result, osmotic pumps and time release wafers have been used to circumvent the drug delivery problems posed by the BBB and will be needed for clinical siRNA treatment in the future.

In the future, sequencing the ZBTB7A gene in the cell lines could be useful for finding common single nucleotide polymorphisms (SNPs). Identifying common SNPs may help to design siRNAs that would more effectively bind to and reduce the expression of ZBTB7A in those cell lines that were unaffected by the treatment (T98G, LN-229). It would also be interesting to obtain cell lines, without mutations in any of the p53/p14ARF genes, and do similar studies. It may also be interesting to perform a study using a human GBM stem cell line: First determine if it expresses ZBTB7A and to what extent. Then, if over-expressed, determine whether there was any gene amplification, and then test the effects of siRNA on GBM stem cell proliferation and or differentiation.

In conclusion, ZBTB7A is over-expressed in many human GBMs but gene amplification does not seem to be the prevailing mechanism for activation. RNA interference can be used to knockdown ZBTB7A expression in GBM cell lines. The lack of ZBTB7A knockdown in T98G and LN-229 might be due to SNPs that diminish the affinity of the siRNA, poor delivery of the siRNA to the cytoplasm, or alterations in the cellular machinery necessary for gene silencing.

This research is one of many early steps that will help to bridge the gap between advancing bio-technology and the clinical treatment of malignant gliomas.

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APPENDIX A

LETTERS OF APPROVAL FOR THE USE OF HUMAN SUBJECT RESEARCH FROM THE INSTITUTIONAL REVIEW BOARD OF MARQUETTE GENERAL HOSPITAL

MARQUETTE GENERAL HEALTH SYSTEM
580 W. College Avenue • Marquette, Michigan 49855 • 906-228-9440 • Toll free 1-800-562-9753 • Website: www.mgh.org

March 26, 2005

Richard Rovin, MD
Northern Neurosurgery
580 W. College
Marquette, MI 49855

RE: Genetic Profiling of Brain Tumors.

Dear Dr. Rovin:

Review of the above-referenced project by Marquette General Hospital's Institutional Review Board has now been completed. I am pleased to advise you that the rights and welfare of the human subjects appear to be adequately protected and the Board, therefore, unanimously approved the scientific content (protocol) and the consent form at the meeting of Apri 13, 2005.

You are reminded that the next Board approval for this study has been set for one calendar year or upon completely of the study - whichever comes first. Only Board approved consent form(s) and Board approved study material may be used. If you plan to continue this project beyond one year, please make provisions for obtaining appropriate IRB approval prior to April 1, 2006. If the study is completed before one year, notification of completion and survey results need to be sent to the IRB for review. Any changes in procedures involving human subjects must be reviewed by the IRB prior to initiation of the change. The IRB must also be notified promptly of any problems (unexpected side effects, complaints, etc.) involving human subjects during the course of the work.

Enclosed for your use and review are copies of IRB procedures, which are relative to the initial review that was conducted by the IRB and procedures for continuing review by the Board.

Thank you for bringing this project to our attention. If we can be of future help, please do not hesitate to let us know.

Sincerely,

Daniel J. Arnold, Chairperson
Institutional Review Board

DA/gm

Enclosure: IRB Procedures #1 & #2.

MARQUETTE GENERAL HEALTH SYSTEM
580 W. College Avenue • Marquette, Michigan 49855 • 906-228-9440 • Toll free 1-800-562-9753 • Website: www.mgh.org

March 23, 2006

Richard Rovin, MD
Northern Neurosurgery
580 West College
Marquette, MI 49855

RE: Genetic Profiling of Brain Tumors

Dear Dr. Rovin:

This is to inform you, as principal investigator of the above study, that on March 8, 2006, the Institutional Review Board approved the continuation of the study and data analysis for one year.

Please continue to inform the IRB of any changes to this protocol.

Sincerely,

Daniel J. Arnold, MD, Chairperson
Institutional Review Board

DA/gm

APPENDIX B

LETTERS OF APPROVAL FOR THE USE OF HUMAN SUBJECT RESEARCH FROM NORTHERN MICHIGAN UNIVERSITY'S DEAN OF GRADUATE STUDIES

Northern Michigan University

College of Graduate Studies
1401 Presque Isle Avenue
Marquette, MI 49855-5322
906-227-2300
FAX: 906-227-2315
Web site: www.nmu.edu

August 24, 2005

TO: **Robert Winn**
Biology

FROM: **Cynthia A. Prosen, Ph.D.** Cindy Prosen 2H
Dean of Graduate Studies & Research

RE: **Human Subjects Proposal # HS05-041**
Genetic Profiling of Brain Tumors

Exempt status has been granted under 46.101(1) of 45 CFR 46 for the above mentioned Human Subjects Application. Please include your proposal number on all research materials and on any correspondence regarding this project.

Any changes or revisions to your approved research plan must be approved by the IRB prior to implementation.

ljh