2006

The Expression and Function of Wilms' Tumor 1 in Malignant Glioma

Aaron J. Clark
Virginia Commonwealth University

THE EXPRESSION AND FUNCTION OF WILMS' TUMOR 1 IN MALIGNANT GLIOMA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

AARON J. CLARK
B.S., University of California, San Diego, 1999

Director: William C. Broaddus, M.D., Ph.D.
Hord Professor
Department of Neurosurgery, Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, Virginia
December, 2006

Acknowledgement

The work presented in this dissertation was made possible with the assistance of the following people. First, my advisor Dr. William Broaddus provided me with the opportunity to pursue the MD/PhD degree under his direction and provided the initial idea behind extensively characterizing WT1 in brain tumors. He has also served as a role model exemplifying the clinician-scientist. As an expert in the field of WT1 biology and graduate committee member, Dr. Joy Ware provided ideas and guidance particularly pertaining to the animal studies. Drs. Lynne Elmore and Helen Fillmore also served on my graduate committee and provided invaluable advice and support. I am grateful to Dr. John Povlishock, chairman of the Department of Anatomy and Neurobiology and committee member, for the opportunity to study in the department and for his support.

This work would not have been possible without collaborations with Dr. Arti Pandya and laboratory (VCU Department of Human Genetics), Dr. Sumitra Deb (VCU Department of Biochemisty), Dr. Kellie Archer (VCU Department of Biostatistics), Dr. Shawn Holt and laboratory (VCU Department of Pathology), and Dr. Charles Roberts (Oregon Health Sciences University, Department of Pediatrics). This work was also made possible in part through the support of Naomi Berkowitz and the American Brain Tumor Association. The work of Dana Chan on WT1 silencing and radiosensitivity was crucial to complete the WT1/p53/radiotherapy story. Dr. Mike Chen designed and produced the shRNA used in the latter part of the study.

Dr. Jessica McCready provided a wealth of technical and scientific knowledge as well as intangible inspiration in the form of constant and far-reaching discussion and the reciprocal exchange of a variety of ideas and thoughts. I would also like to thank my MD/PhD predecessors in the laboratory, Dr. Mark Richardson and Dr. Peter Haar for their advice and encouragement having already progressed on the path that I had just begun. Dr. Wagner Dos Santos and Dr. Tim Van Meter supplied indispensable guidance in design and planning of the experiments. I would also like to acknowledge other laboratory members past and present; Dr. Yin Zhang, Berkeley Carter, Dr. Zhi Jian Chen, Dr. Heather Hamilton, Dr. Jessicca Martin, Adrian Edwards, and Virginia Sykes. I would like to thank Dr. Richard Costanzo for the initial encouragement to pursue the MD/PhD degree.

Lastly, I would like to thank my parents, Patrick and Sunny Clark, and my brother, Nathan Clark, for instilling in me a dedication to scientific curiosity and their unquestioning support.

Table of Contents

List of Tables

Page

List of Figures

Page

Abstract

THE EXPRESSION AND FUNCTION OF WILMS' TUMOR 1 IN MALIGNANT GLIOMA

By Aaron J. Clark, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: William C. Broaddus, M.D., Ph.D
Hord Professor
Department of Neurosurgery, Department of Anatomy and Neurobiology

The Wilms' tumor 1 gene is overexpressed in many types of cancer and is associated with poor prognosis and resistance to anti-cancer therapies. *In vitro* studies in non-glioma cells types have demonstrated that WT1 plays a role in increased proliferation, resistance to apoptosis, and increased cellular invasion. We aimed to thoroughly characterize the expression pattern of Wilms' tumor 1 in human malignant glioma and discern its function in this complex disease process. We screened a large sample of established human malignant glioma cell lines and glioma tissue specimens of all grades

for WT1 expression. The majority of cell lines and 80% of all glioma tissue expressed WT1 mRNA, all of which expressed WT1(+KTS) isoforms. Further screening of the glioblastoma specimens for p53 mutation followed by logistic regression analysis demonstrated a positive correlation between WT1 expression and wild-type p53 ($p = 0.04$). To determine if WT1 and p53 functionally interacted, we generated LN-229 glioblastoma cells that stably expressed WT1. As LN-229 cells harbor a p53 mutation, transient transfection with wild-type p53 induced apoptosis. However, stable WT1 expression did not protect cells from p53-mediated cell death. We then generated U87MG cells (p53 wild-type) that stably expressed WT1 to model an endogenous p53 response. It is well known that after treatment with ionizing radiation, U87MG cells readily undergo p53-mediated apoptosis. Again, WT1 expression did not protect against ionizing radiation-induced p53-mediated cell death. We next examined the effect of transient WT1 silencing on ionizing radiation induced cell death in T98G and LN-18 cells which express endogenous WT1. Combination treatment with ionizing radiation and silencing of WT1 using short interfering RNA caused a decrease in viability and clonogenic survival relative to radiation alone in both cell lines. Lastly, we studied the effect of stable WT1 silencing using short hairpin RNA on glioblastoma cell tumorigenicity. Stable transduction of U251MG and LN-18 cells with WT1 short hairpin RNA resulted in a marked decrease in proliferation. WT1 silencing in U251MG cells also caused a decrease in *in vitro* invasion. WT1 silencing in U251MG cells caused an increase in tumor latency and a decrease in tumor growth rate when cells were used to subcutaneously inoculate nude mice. Not only do these studies support an oncogenic role for WT1 in glioma biology, they provide

encouraging evidence that WT1 may be a therapeutic target for molecular treatment of glioblastoma.

CHAPTER 1.

INTRODUCTION

THE OVERLAP BETWEEN GLIOBLASTOMA AND WILMS' TUMOR 1 BIOLOGY

Abstract

Glioblastoma is the most aggressive and most common primary brain tumor which, despite maximal treatment efforts, carries a poor prognosis. Numerous molecular changes have been characterized which may partially explain the extreme resistance of glioblastoma cells to surgery, radiotherapy, and chemotherapy. In spite of these advances in knowledge, a comparable increase in patient survival has not been attained. Further genetic characterization of these tumors promises to uncover additional targets for directed therapies which have the potential for use in combination with traditional therapies. One such gene may be the Wilms tumor 1 (WT1) gene. WT1 has joined the ranks of a growing number of gene products critical for development and differentiation that become dysregulated during tumorigenesis. WT1 is expressed in the normal developing central nervous system (CNS) and may play a role in neuronal differentiation. In the adult, WT1 expression in the brain is restricted to the area postrema, but is aberrantly overexpressed in

the majority of gliomas of all grades. Several physiologic and pathologic functions of WT1 suggest possible roles in glioblastoma, including trans-activation of growth factor and growth factor receptor genes, transcriptional regulation of cell cycle regulatory genes, interaction with the p53 tumor suppressor, and modulation of cell motility and invasion. Future study of WT1 in glioblastoma may establish its oncogenic role and its potential as an efficacious therapeutic target. Here we review malignant glioma clinical behavior and molecular genetics with an emphasis on pathways that may be modulated by WT1, the molecular biology of which is then extensively described.

Brain tumor classification and grading

Neuroectodermal tumors, whether arising in the central or peripheral nervous system, are classified according to the cell of origin, as determined by morphology and immunohistochemistry of the predominant cell type comprising the tumor. This classification system is largely based on the seminal work of Harvey Cushing and his colleague Percival Bailey in the early 1900's, who were investigating methods of correlating outcome with histology[1]. The gliomas are central nervous system neuroectodermal tumors of glial origin and include astrocytomas, oligodendrogliomas, mixed gliomas, ependymomas and a variety of other glial tumor types. Gliomas can arise throughout the brain, brain stem, and spinal cord, but most commonly occur in the deep white matter of the cerebral hemispheres[1]. Because gliomas rarely metastasize outside of the central nervous system (CNS), staging of these tumors is not performed[184]. Rather, gliomas are graded I – IV according to the histological determination of malignancy described by the World Health Organization (WHO) grading system[104].

The WHO grading system is based on the presence of nuclear atypia, vascular proliferation, mitotic figures, and necrosis[1]. Grade I gliomas are benign, well-circumscribed, and often curable tumors while grades II – IV correspond to increasingly malignant and invasive tumors that prove difficult to eradicate.

Epidemiology of brain tumors

Primary brain tumors of all grades, when considered as a group, are a relatively rare type of tumor with an incidence rate of 14.8 cases per 100,000 person-years, according to the Central Brain Tumor Registry of the United States[27]. In 2005, an estimated 43,800 brain tumors were diagnosed in the United States. However, brain tumors are very prevalent in certain populations and responsible for significant morbidity. They are the most common solid tumor in children ages 0 – 19 and the second most common cancer following leukemia in this age group. They were also the leading cause of cancer death among children and young adult men ages 20 – 34 in 2000. Malignant primary brain tumors constitute a large proportion of all primary brain tumors and therefore have a high mortality rate. It is estimated that 21,690 primary brain tumors diagnosed in 2005 will be malignant causing an estimated 12,760 deaths.

Epidemiology of glioblastoma

The gliomas account for 40% of all primary brain tumors[27]. The diffuse astrocytomas comprise more than 80% of the gliomas and include grade II astrocytoma, anaplastic astrocytoma (grade III), and glioblastoma (grade IV). Anaplastic astrocytoma and glioblastoma are often collectively referred to as the malignant gliomas. In addition to being the most deadly brain tumor, glioblastoma is also the most common primary

malignant brain tumor accounting for 20.3% of all primary brain tumors and 50.7% of gliomas. The incidence of glioblastoma increases with age with a median age at diagnosis of 64. Incidence rates of glioblastoma are 1.6 times higher in males than females and 2 times higher in whites than blacks[171]. Although the cause is unknown, the incidence of glioblastoma appears to have increased between 1977 and 2000, particularly in the elderly[82].

In spite of high quality epidemiologic data, few risk factors and predisposing conditions have been definitively identified. Whole brain radiation during childhood is the only known risk factor for sporadic glioma. A large cohort of children treated for acute lymphoblastic leukemia (ALL) was retrospectively analyzed for development of secondary neoplasms[169]. Nearly half of all secondary neoplasms were malignant gliomas and all occurred in children who had received cranial radiation. These results could be interpreted to indicate an interaction between DNA damaging therapies and underlying genetic predispositions to cancer. In fact, several genetic syndromes include predisposition to glioma development and the tumor suppressor genes involved provide clues to their role in sporadic gliomagenesis[149]. The hereditary tumor syndromes neurofibromatosis types 1 and 2, tuberous sclerosis complex, Li-Fraumeni syndrome, melanoma-astrocytoma syndrome, and Turcot syndrome include predisposition to glioma formation. Neurofibromatosis type 1 and tuberous sclerosis complex predispose to optic glioma and subependymal giant cell astrocytomas, respectively, both generally benign tumors while the others confer risk of diffuse astrocytoma development including glioblastoma. The mutations underlying these syndromes and their propensity for development of glioma

demonstrates the importance of molecular pathways which will be mentioned here and discussed in more detail below (please see "Glioblastoma molecular biology"). Neurofibromatosis type 1 is caused by germ line mutation of the tumor suppressor NF1. Its tumor suppressive function derives from its ability to maintain the proto-oncogene Ras in an inactive GTP-bound state, as demonstrated by observational studies in tumor cell lines derived from NF1 tumor specimens[41]. In sporadic gliomagenesis, Ras is a critical downstream transducer of growth factor receptor mediated signaling. Li-Fraumeni syndrome is caused by mutation of the tumor suppressor p53, which is mutated or otherwise functionally inactivated in a significant proportion of sporadic gliomas[139]. P53 exerts its function by inducing cell cycle arrest and/or apoptosis in response to DNA damage, including oncogenic mutations[132]. Melanoma-astrocytoma syndrome is associated with loss of the CDKN2A locus which encodes two tumor suppressors; $p16^{INK4a}$ and $p19^{ARF}$, both of which are commonly lost in sporadic glioblastoma[9]. $P16^{INK4a}$ is responsible for maintaining the retinoblastoma (Rb) tumor suppressor in an active state, while $p19^{ARF}$ functions by preventing p53 degradation. Familial glioma syndromes have also been reported in the absence of other tumor predisposition syndromes but the causative lesion is unknown.

Clinical features, prognosis, and treatment of glioblastoma

The classically described triad of symptoms for glioblastoma on presentation are; headache, seizure, and hemiparesis. The preliminary diagnosis of glioblastoma is generally made by identification of characteristic radiographic features. Biopsy and subsequent histologic classification are required for definitive diagnosis. Diagnosis of

glioblastoma confers a poor prognosis with a median survival of approximately 1 year and a five year survival is less than 4%[27, 148]. Analysis of over 1500 patients enrolled in Radiation Therapy Oncology Group clinical trials demonstrated that prognosis within this narrow range is predicted by age, histology, Karnofsky Performance Scale (KPS) score, mental/neurologic status, extent of surgical resection, and dose of radiation[214]. Specifically, younger age, less malignant histology, higher KPS score and neurologic function, and more aggressive treatment are associated with improved prognosis. Recently, microarray analysis of a large sample of malignant gliomas followed by classification based on gene expression profiles was shown to better predict survival than histopathologic analysis[170]. A similar study found that gene expression profiling based on microarray analysis of tumor tissue extracts identified novel genes associated with prognosis that were superior to the predictive power of established glioma-associated genetic changes[199]. These findings suggest that complete molecular characterization of gliomas may improve prognosis prediction. The genetic analysis of long term survivors (those surviving greater than 3 years, LTS) is intriguing as survival past three years is associated with extended disease-free survival[21]. Comparative genomic hybridization was used to compare genetic changes in LTS tumors compared with those patients surviving less than 1.5 years. Loss of chromosome 19q was associated with LTS in this study[21]. Studies such as these highlight the importance of such analyses not only in distinguishing different prognostic groups but also by identifying changes that determine disease progression. Future treatments may feature standard therapy supplemented with targeted molecular therapies based on the results of such studies. Current standard of care

for patients with glioblastoma is surgical resection followed by radiotherapy and adjuvant chemotherapy.

Surgery for glioblastoma

The goals of surgery for glioblastoma are relief of neurologic symptoms caused by the expanding mass, obtaining a definitive diagnosis, and cytoreduction[235]. Aggressive surgery for glioblastoma is controversial as wider margins of resection carry the risk of inducing iatrogenic neurologic deficits. However, studies have shown that open craniotomy for tumor resection yields superior survival relative to biopsy alone[113]. In addition, extent of resection improves survival suggesting that cytoreduction of malignant cells is crucial for disease control[224, 118]. Glioblastoma cell invasiveness remains the major obstacle to eliciting a surgical cure. In 1961, Matsukado *et al* reviewed 100 necropsy specimens and showed that 47/100 had invaded the corpus collosum to involve both cerebral hemispheres, demonstrating the classic "butterfly glioma" pattern[142]. Most agree that these tumors cannot be completely resected. Furthermore, with the exception of tumors located in the tip of the frontal lobe, all tumors that involved a single hemisphere had spread into adjacent lobes. Microscopically, tumor cells were present beyond the gross demarcation between tumor and normal tissue. A more recent study demonstrated that, in a significant number of glioblastoma specimens (3/15), tumor cells were detected at autopsy more than 2 cm outside the enhancing margin shown on computerized tomography (CT) scans[20]. Taken together, this study indicates that invading cells make gross total resection, even when utilizing intraoperative CT image guidance systems, difficult and, in nearly half of cases, impossible. In the future,

cytoreductive surgery combined with molecular targeting of residual invading cells may improve prognosis. The molecular biology of glioma invasion and potential therapeutic anti-invasive targets are described in more detail below (please see "Molecular biology of glioma invasion").

Chemotherapy for glioblastoma

Chemotherapy is a component of standard glioblastoma treatment and modestly improves 1 and 2 year survival by approximately 5%[227]. Alkylating agents are the most commonly used chemotherapeutic agents used in the treatment of malignant gliomas which induce DNA damage by covalently attaching an alkyl group to the O^6 position of guanine. Of these 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been the most commonly used since it was first described to improve survival when compared to patients who received no chemotherapy and no radiotherapy[252]. Alternative methods of delivery to bypass the blood-brain-barrier, such as BCNU-impregnated implantable polymers, are also employed, and in certain subgroups of patients may improve survival[262].

Temozolamide (TMZ), a second generation imidazotetrazine pro-drug which is physiologically converted to an alkylating agent, has a more favorable therapeutic profile than BCNU and has become the chemotherapy agent of choice[193]. TMZ, an oral agent, crosses the blood-brain barrier and exhibits limited systemic toxicity. Furthermore, TMZ improves progression-free survival by 13% at six months in patients with recurrent glioblastoma[269]. When combined with radiotherapy, TMZ increases median survival relative to radiotherapy alone[228]. The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) is primarily responsible for removal of alkyl groups from

DNA and, therefore, is involved in resistance to alkylating agents in glioblastoma[107]. Patients with methylation of the MGMT promoter, and subsequent silencing of the *mgmt* gene, have a significant survival benefit from TMZ combined with radiotherapy relative to those without methylation underscoring the importance of molecular characterization of glioblastoma in optimizing treatment[79].

Radiotherapy for glioblastoma

In the treatment of glioblastoma, radiotherapy is used post-operatively in combination with chemotherapy. Ionizing radiation induces DNA damage by directly damaging the DNA molecule and indirectly by generating oxygen free radicals from water molecules, which leads to apoptosis. While normal cells undergo apoptosis in response to ionizing radiation, glioblastoma cells are notoriously resistant to apoptosis by molecular mechanisms which will be described below[219]. Therefore, although escalating doses of radiotherapy increase survival, radiotherapy is not curative[253]. Nevertheless, surgery followed by radiotherapy improves survival when compared to surgery alone[252]. Current therapy consists of a total dose of 50 – 60 Gy in thirty 1.8 – 2.0 Gy fractions targeting the entire enhancing tumor mass plus a 2 cm margin[11]. In spite of numerous large clinical trials, alternative delivery schedules, hyperthermia, particle therapy, and radiosensitizers have not been shown to improve survival relative to conventional fractionated radiotherapy[120]. The majority of glioblastomas recur within 2 cm of the original tumor site, in one study even after delivery of a 14 Gy boost to this area, suggesting the presence of residual tumor cells near the resection cavity that are capable of resisting apoptosis induced by adjuvant radiotherapy[56]. Therefore studies focused on

improving the apoptotic response of residual tumor cells to radiotherapy become critical in the search for a cure. Such improvements may come from identification of molecular changes in glioma that confer a resistant phenotype.

Glioblastoma molecular biology

The ability of glioblastoma cells to resist DNA damaging therapies suggests that glioblastoma cells possess inherent resistance to DNA-damage induced cell death. Specific molecular changes may explain this extreme resistance. In addition, molecular mechanisms supporting cell invasion may allow tumor cells to disseminate widely enough to evade surgical resection. Full molecular characterization of glioblastoma promises to provide the basis for targeted molecular therapies that will complement the traditionally employed modalities described above.

Glioblastoma can be divided into two subgroups based on clinical history; primary and secondary glioblastoma. Cytogenetic and molecular examination of the two subgroups of glioblastoma provides clues to central mechanisms underlying gliomagenesis and glioblastoma behavior. Primary glioblastoma arises *de novo* without evidence of a less malignant precursor tumor, occurs in older age groups, and is rapidly progressive with a clinical history of less than three months. Secondary glioblastoma arises from a pre-existing lower grade glioma, occurs in younger patients with the progression to grade IV glioblastoma over 5 - 10 years. However, once diagnosis of glioblastoma is established, the prognosis and histology of the tumors are indistinguishable. In addition to differences in clinical history, these tumors can be distinguished by characteristic genetic aberrations[258]. Primary glioblastoma possesses amplification of the receptor tyrosine

kinase (RTK) epidermal growth factor receptor (EGFR)[258], murine double minute 2 (MDM2) amplification[211], mutation of the phosphatase PTEN[234], and deletion of $p16^{INK4a}$ [241]. Secondary glioblastoma is characterized by mutation of the p53 tumor suppressor[258], loss of heterozygosity (LOH) on chromosome 10q[119], and overexpression of the RTK platelet-derived growth factor (PDGF) receptor and its ligand PDGF[71]. Although the exact mutations are different between primary and secondary glioblastoma, the pathways affected are similar; receptor tyrosine kinase-activated pathways, and the tumor suppressor Rb- and p53-mediated pathways. As critical to both primary and secondary glioblastoma, these pathways represent targets of action of aberrantly expressed oncogenes in addition to being functional targets for novel therapeutics.

Receptor tyrosine kinases

RTKs dimerize in response to ligand binding, undergo autophosphorylation, and recruit adapter proteins which activate downstream effectors, such as the proto-oncogene Ras. EGFR is amplified in approximately 40% of primary glioblastomas leading to overexpression of the receptor[125]. Of those that overexpress EGFR, 40% are termed EGFRvIII and possess a deletion of exons 2 – 7 which encodes the extracellular ligand binding domain, thus resulting in constitutive activation[86]. Activated EGFR, either by ligand binding or activating mutations, stimulates downstream Ras/Raf/MEK/MAP kinase and PI3 kinase/AKT signal transduction pathways, which are involved in cell proliferation, survival, and invasion[97]. EGFRvIII increases the tumorigenicity of glioblastoma cells both *in vitro* and *in vivo*, by increasing proliferation rate and decreasing apoptosis[164].

EGFRvIII expression also increases the chemoresistance of glioma cells[165]. Both of these observations are associated with increases in the anti-apoptotic Bcl-2 family member, Bcl-X_L[165, 164]. Additionally, EGFRvIII increases the *in vitro* invasiveness of glioblastoma cells, possibly due to up-regulation of several invasion related genes which will be discussed in more detail below, including matrix metalloproteinases (MMPs) and extracellular matrix (ECM) molecules(please see "Molecular biology of glioma invasion")[117]. Specifically relevant to the treatment of glioblastoma, EGFR expression strongly correlates with resistance to radiotherapy in a large clinical study[10].

PDGFR and PDGF ligands are co-expressed in the same glioblastoma cells suggesting an autocrine stimulatory loop[71]. Ligand binding to PDGFR activates the same pathways as EGFR (i.e. MAPK and PI3K pathways) [218]. PDGF and PDGFR overexpression represent early changes in glioma progression and therefore may play a role in glioma development. In a large sample of low grade gliomas, PDGFR was overexpressed in 50% and expression correlated with progression to high grade glioma highlighting its role in secondary glioblastoma[247]. Mouse models provide clues as to the function of PDGF in glioma progression. Artificial expression of PDGF in mouse models induces the formation of oligodendrogliomas, however if AKT signaling is also perturbed, astrocytomas form, underscoring the complexity of signaling pathways leading to glioblastoma[36]. In addition to its role in glioma development, evidence indicates that PDGF signaling continues to play a role in glioblastoma. Treatment of several glioblastoma cell lines with specific PDGFR inhibitors decreased clonogenicity and tumor growth in nude mice[130]. PDGF signaling also contributes to glioma neovascularization,

an important component of glioblastoma tumorigenicity, by stimulating vascular endothelial growth factor (VEGF) expression in tumor endothelial cells and subsequent endothelial proliferation[72]. Therefore, PDGF not only has autocrine function, but also paracrine action on tumor associated blood vessels. Most importantly, nude mice harboring glioma xenografts respond to the orally active specific PDGFR tyrosine kinase inhibitor, Gleevec (Imatinib) which is currently being used clinically to treat other types of human cancers[101]. This has prompted a clinical trial to examine the efficacy of this inhibitor in the treatment of glioblastoma. Although clearly important in gliomagenesis, the mechanism of overexpression of PDGF is currently unknown.

Through either LOH on the long arm of chromosome 10 (secondary glioblastoma) or mutation (primary glioblastoma), the **p**hosphatase and **ten**sin homology gene (*PTEN*) is inactivated in the majority of malignant gliomas[119, 234]. PTEN is a 3' phosphoinositol phosphatase that dephosphorylates PIP3, a second messenger involved in PI3K/AKT signaling, thus acting as a negative regulator of the pathway. Loss of PTEN allows constitutive activation of AKT leading to cell proliferation, survival, and development of glioblastoma. Mutations in downstream effects of RTK represent alternative mechanisms of stimulating tumor cell growth and resistance.

Retinoblastoma (RB) pathways

The gene encoding the tumor suppressor Rb is mutated in approximately 30% of malignant gliomas[80]. Un-phosphorylated Rb binds E2F, inhibiting transcription of genes involved in cell cycle progression. In response to growth promoting stimuli, Cyclin D1 is activated which in turn activates cyclin-dependent kinase (CDK) 4 and 6, which

phosphorylate Rb. Rb releases E2F which transactivates target genes leading to mitosis and progression through the G1 checkpoint. The CDK inhibitor $p16^{INK4A}$ inhibits CDK 4 and 6, preventing phosphorylation of Rb and maintaining cells in a quiescent state. Inactivation of $p16^{INK4A}$ occurs in 50% of primary glioblastoma and represents an alternative mechanism of inactivating the Rb tumor suppressor, leading to unregulated growth[241].

p53 pathways

Mutations in the gene encoding the p53 tumor suppressor occur in 33% of human glioblastomas and are more common in secondary glioblastoma. The most common mutations in gliomas occur at codons 273, 248, and 175 in order of decreasing frequency[12]. Tetramerization is required for wild-type p53 function which is attributed to a C-terminal oligomerization domain. Therefore, mutant p53 can exert dominant negative effects by oligomerizing with wild-type p53. Its tumor suppressor function is intimately tied to its function as a transcription factor. DNA damage, including oncogenic mutations, activates p53 which directly initiates cell cycle arrest and apoptosis by transactivation of downstream target genes, most importantly the CDK inhibitor, $p21^{WAF1}$, and the pro-apoptotic Bcl-2 family member, Bax, respectively[45, 158]. More recent studies show that p53 is critical for autophagy, senescence, DNA repair, and differentiation, all of which are consistent with tumor suppression[33, 65, 271].

Normal cells possess low levels of p53 due to its short half life[5]. The ubiquitin ligase MDM2 targets p53 for proteasomal mediated degradation and is the most important mechanism of inactivation of p53[78]. As such, it is amplified in 13% of primary

glioblastomas[211]. Interestingly, the same study showed that MDM2 amplifications are mutually exclusive with p53 mutations suggesting it is one specific mechanism for inactivating wild-type p53. Given the central role of p53 in tumor suppression and sensitivity to DNA damaging therapies, glioma cells which possess wild-type p53 in the absence of MDM2 amplification must harness alternative mechanisms of evading the p53 response.

As expected based on its role in response to DNA damage, p53 plays a key role in response to radiotherapy. Adenoviral transduction of p53 mutant glioma cell lines with wild-type p53 induces virtually 100% apoptosis within 4 – 8 days[68]. Stable transfection of p53 into p53 mutant glioma cells decreases clonogenic survival after radiotherapy[66]. *In vivo* adenoviral transduction of mutant p53 gliosarcoma cells with wild-type p53 causes reduction in tumor size and increased survival when combined with radiotherapy[8]. Pro-apoptotic downstream targets of p53 are critical to a successful apoptotic response to radiotherapy. Wild-type p53 glioma cell resistance to radiotherapy demonstrated by lack of ionizing radiation-induced apoptosis correlates with lack of Bax induction[219]. Adenoviral transduction of p53 mutant glioma cell lines with Bax increases sensitivity to fractionated radiotherapy by inducing both *in vitro* apoptosis and *in vivo* tumor regression in nude mice[6]. These results indicate that functional inactivation of wild-type p53 may lead to a defect in Bax activation and therefore may explain glioma radioresistance.

In the response to radiotherapy, functional inactivation of p53 in glioma cells may be achieved by decreased expression of Ataxia Telangiectasia Mutated (ATM), which phosphorylates and activates p53 in response to DNA damage. In a study of a small

sample of primary glioblastoma cultures, lower levels of ATM expression correlated with increased clonogenic survival after irradiation[237]. Our laboratory has shown that adenoviral transduction of glioma cells that possess wild-type p53 with additional wild-type p53 can decrease radioresistance suggesting the supraphysiologic levels of p53 can overcome functional inactivation[14]. A complete understanding of the mechanisms of functional inactivation of p53 in glioblastoma promises to uncover novel therapeutic targets to increase apoptosis and decrease resistance to radiotherapy.

Molecular biology of glioma invasion

In addition to increased proliferation and resistance to therapy, glioma cell invasion is also a key component of tumorigenicity. The most provocative illustration of the extreme invasiveness of glioma cells is the observation that these tumors recur even after radical resections such as hemispherectomy of the affected cerebral hemisphere. This underscores the concept that, even after wide margin resection of a glioblastoma, tumor cells invading grossly normal appearing brain can cause recurrence and death[221]. It has long been recognized that glioma cells preferentially invade along vascular basement membranes in Virchow-Robins spaces, white matter tracts, and the subependymal zone suggesting that factors, such as ECM components, present in defined areas of the brain promote tumor cell migration[142]. Glioma cell invasion requires a complex interaction between the ECM and the cell. In point of fact, the first step in glioma invasion involves adhesion of the cell to ECM components. Exposure of glioma cells to the ECM components fibronectin, collagen IV, tenascin-C, vitronectin, and laminin stimulates migration[135, 62]. In addition, glioma cells can modulate the ECM by secreting ECM

components which can lead to increased adhesion ultimately enhancing invasion[63]. The major cellular mediator of adhesion is the intergrin family which is comprised of heterodimeric glycoproteins formed by various combinations of different α and β chains. Up-regulation of β1 integrin in C6 glioma cells causes diffuse invasion when implanted in the brains of nude mice[186]. Conversely, *in vitro* invasion of glioma cells is blocked after treatment with anti-β1 antibodies[63]. The second step is cell movement which involves a reorganization of the actin cytoskeleton resulting in a polarized cell with lamellopodia in the invading front end and a retracting rear end[259].

The third step is invasion into an intercellular space created by the secretion of ECM degrading proteases, in particular the MMPs. The MMPs are a multigene family subdivided into groups based on their substrate specificity. Membrane type 1-MMP (MT1-MMP), MMP-1, MMP-2, and MMP-9 are overexpressed in gliomas and are the most well studied of the family[245, 146, 50, 167]. Levels of MMP activity increases with increasing grade, being highest in glioblastoma, and may play a role in aggressiveness[167]. They are normally tightly regulated to maintain activity only when needed for functions such as wound healing, angiogenesis, and axonal growth cone extension. The MMPs are regulated at the transcriptional level and are often up-regulated in response to growth factor stimulation[201]. Results from our laboratory show that a single nucleotide polymorphism (SNP) in the MMP-1 promoter which creates a binding site for ETS transcription factor family members leads to increased MMP-1 expression in glioblastoma[146]. The MMPs are secreted as inactive zymogens and require cleavage for activation, thus introducing a second layer of regulation. Our laboratory has shown that

EGF stimulation can cause up-regulation of MT1-MMP, which activates MMP-2 and subsequently increases *in vitro* invasiveness[244]. MMP-2 and MT1-MMP are often expressed in the same cell providing a mechanism by which the cell can directly activate its secreted pro-MMP-2 by MT1-MMP in the cell membrane[50]. MMP-9 is expressed by endothelial cells at the invading edge of glioblastoma and may be involved in the ingrowth of oxygen supplying tumor vasculature into the rapidly growing tumor[50]. Third, naturally occurring **t**issue **i**nhibitors of **MMP**s (TIMPs) inhibit the enzymatic activity. TIMP-1 expression is under-represented in glioblastoma suggesting a mechanism of increased invasion[167]. Transfection of a highly invasive glioma cell line that expresses low endogenous levels of TIMPs with TIMP-1 causes a decrease in invasion[143].

The concept behind adjuvant therapies following surgery such as radiotherapy and chemotherapy impregnated wafers deposited in the tumor resection cavity is to target residual invading tumor cells. Recent evidence suggests that certain characteristics of invading cells may confer resistance to such therapies. DNA damaging therapies, such as ionizing radiation and chemotherapy, target rapidly proliferating cells. However, glioma cells selected for migration ability proliferate more slowly when plated on glioma-derived ECM[147]. Decreased proliferation of invasive cells may be due to decreases in expression of cell cycle regulatory proteins such as Cyclin A and B after exposure to ECM proteins[141]. Therefore, more slowly dividing invading cells may resist DNA damaging therapies leading to regrowth and recurrence. Furthermore, glioma cells stimulated to migrate by ECM components down-regulate pro-apoptotic genes while up-regulating anti-apoptotic genes[141]. Also relevant to choice of therapy is the surprising observation that

inhibition of tumor angiogenesis with a monoclonal antibody against the VEGF receptor decreased tumor growth but caused dramatically increased invasion of glioma cells along host blood vessels eventually leading to leptomeningeal spread[112]. These results demonstrate the interrelatedness of glioma molecular pathways and the importance of understanding and targeting several pathways when treating malignant gliomas.

Wilms' tumor 1 and glioblastoma

In search of novel genes involved in glioma biology, our laboratory performed exploratory studies examining the expression of Wilms' tumor 1 (WT1) in glioblastoma cell lines. We reported that 4/7 established glioma cells lines overexpressed WT1[191]. Subsequently, we and several other groups demonstrated WT1 overexpression in larger samples of glioma cells lines and glioblastoma tissue specimens[152, 43, 178, 168, 30]. The function of WT1 in these tumors is currently unknown. Evidence based on the known physiologic and pathologic function of WT1 which will be extensively discussed below suggests many potential mechanisms of WT1 function in glioblastoma.

Wilms' tumor

In 1899, Dr. Carl Max Wilms, a renowned German surgeon, comprehensively reviewed childhood renal cancer in his book *Die Mishgeschewülste der Niere*. Due to his thorough description of the disease and the correct assertion as to the cell of origin of the tumor, the malignancy that he described, nephroblastoma, is now known as Wilms' tumor[32]. Wilms' tumor, for which WT1 is named, is the most common pediatric kidney cancer with a median age at diagnosis of 3.5 years[13]. Overall, it accounts for 6% of pediatric malignancies[73]. The majority of Wilms' tumors are solitary, however 6% are

bilateral and 12% are multifocal. Wilms' tumors metastasize to the lungs most commonly, followed by the lymph nodes and liver, rarely to the bone and brain. Patients typically present with an asymptomatic abdominal mass that is palpable on physical examination. Current standard of care in North America involves nephrectomy followed by chemotherapy with or without radiotherapy depending on the stage of disease[70]. In contrast to GBM, survival has improved from 30% in the 1930's to greater than 85% in the present day. Studies now focus on tailoring therapies to cure the disease while sparing the patient from the side effects of systemic therapy.

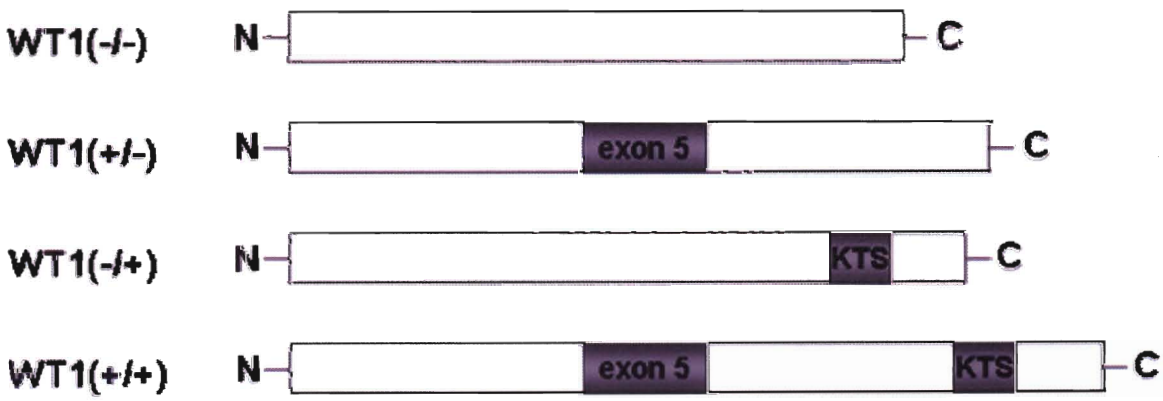
Wilms' tumor 1 (WT1) structure and function

Knudson and Strong reviewed a large number of case studies and proposed a two-hit model for Wilms' tumorigenesis, similar to that of retinoblastoma in which inactivation of both alleles of a tumor suppressor gene were necessary for tumor formation[106]. Cytogenetic analysis of individuals with WAGR syndrome (**W**ilms' tumor predisposition, **A**niridia, **G**enitourinary malformations, mental **R**etardation) implicated mutation in chromosome 11p13 in the development of Wilms' tumor[53, 67]. The region was also observed to be mutated in sporadic Wilms' tumors, thus potentially harboring a tumor suppressor[108]. Subsequent examination of Wilms' tumor cell lines and embryonic tissue led to the isolation of a transcript from this region[23, 59]. This gene was shown to be mutated in approximately 6% of sporadic Wilms' tumors and was named Wilms' tumor 1 (WT1)[59, 246, 126]. As confirmatory evidence, WT1 was found to be mutated in patients with Denys-Drash syndrome, which consists of renal failure, pseudohermaphroditism, and Wilms' tumor[187]. To date, WT1 is the sole gene demonstrated to be involved in the

development of Wilms' tumor. Several characteristics of WT1 biology suggest that it may be important in glioblastoma behavior.

The *wt1* gene is composed of 10 exons approximately 50 kb in length and encodes a 3.1 kb transcript[58, 23]. Alternative splicing generates four major isoforms depending on the inclusion or exclusion of 51 nucleotides encoded by exon 5 and nine nucleotides in exon 9, known as KTS[75]. WT1(+/+) contains both exon 5 and KTS, WT1(+/-) contains exon 5 but lacks KTS, WT1(-/+) lacks exon 5 but contains KTS, and WT1(-/-) lacks both exon 5 and KTS (Figure 1-1).

Figure 1-1. Schematic representation of the four major protein isoforms of WT1 with corresponding nomenclature. Gray boxes denote the two alternatively spliced exons; exon 5 and KTS in exon 9.


WT1(-/-)
N
C
WT1(+/-)
N
exon 5
C
WT1(-/+)
N
KTS
C
WT1(+/+)
N
exon 5
KTS
C

The WT1 protein has a molecular mass of 52 – 54 kDa, depending on the isoform[162]. RNA editing converts uridine at the 839 position to cytosine, resulting in a change in codon 280 from leucine to proline[216]. Translation from an in-frame CUG upstream of the major WT1 start site generates novel 60 – 62 kDa WT1 isoforms[18]. Translation initiated at a downstream AUG generates N-terminally truncated WT1 isoforms of 36 – 38 kDa[208]. Both larger and smaller WT1 isoforms are expressed in human tissue and retain transcriptional activity. Thus, the number of WT1 isoforms characterized is 24. Both larger and smaller isoforms are expressed at lower levels and are less prevalent than the 4 major WT1 isoforms and are, therefore, less well studied[18, 208]. Furthermore, studies examining WT1 isoforms generated from the upstream CUG demonstrate that inclusion of the extended N-terminus does not modify the function relative to the corresponding major isoform[18].

WT1 is a transcription factor that contains an N-terminal proline and glutamine rich region and four C-terminal Cys_2-His_2 zinc fingers[23, 59]. The proline-glutamine-rich region mediates homodimerization and transcriptional activation[195, 257]. Zinc fingers 2 - 4 share 61% homology with the early growth response-1 (EGR-1) transcription factor and all four are involved in DNA binding[23, 192]. The zinc fingers also contain two nuclear localization signals required for trafficking to the nucleus[17]. As expected based on the structural similarities, WT1 binds to the GC-rich EGR-1 consensus sequence[192]. In addition, WT1 binds to unique promoter sequences; TCC repeat motifs and a high affinity binding site referred to as WTE[256, 166]. As would be expected of a transcription factor involved in cancer development, WT1 transcriptionally regulates the expression of several

growth regulatory genes including the CDK inhibitor $p21^{WAF1}$ and *cyclin E*[49, 129]. WT1 also up-regulates the expression of PDGF-A[255], which is of particular interest to glioblastoma biology for reasons which have been outlined above. WT1 also represses transcription of the anti-angiogenic factor, Thrombospondin-1 (TSP-1) in FR3T3 immortalized rat embryonic fibroblast cells[42]. In a separate study, antisense induced WT1 knock-down caused decreased clonogenicity which was associated with up-regulation of TSP-1 in HL-60 leukemia cells[198]. This also is potentially relevant to glioblastoma because TSP-1 expression in glioblastoma xenografts decreases growth rate and vascularity[109]. At the transcriptional level, WT1 may play a role in glioma biology by modulating the cell cycle, growth factor-induced proliferation and survival, and angiogenesis.

Alternative splicing of exon 9 inserts three amino acids, lysine-threonine-serine (KTS), in the linker sequence between zinc fingers 3 and 4[75]. Nuclear magnetic resonance analysis indicates that inclusion of KTS decreases the affinity of WT1 for DNA[115]. This is explained by studies which show the increased length of the linker caused by inclusion of KTS increases the flexibility between zinc fingers 3 and 4 that prevents binding of zinc finger 4 to DNA[116]. However, these results were obtained using only the EGR1 consensus sequence to determine the stability of the WT1/DNA complex[115, 116]. Evidence suggests that WT1(+KTS) recognizes unique motifs in gene promoters[83].

In addition to functioning as a transcription factor, WT1(+KTS) isoforms may have a unique function in post-transcriptional processing. Careful analysis of WT1 localization

in WT1 expressing mouse kidney cell lines and tissue demonstrate that WT1(+KTS) co-localizes in nuclear "speckles" with splicing factors, a pattern which is disrupted by treatment with RNase A[121]. In the same study, WT1 co-immunoprecipitates with antigens common to small nuclear ribonucleoprotein particles (snRNPs) which are involved in mRNA splicing. More specifically, WT1(+KTS) is incorporated into spliceosomes and physically interacts with the ubiquitous splicing factor U2AF65, which is known to be present in nuclear speckles[100]. WT1 also co-purifies with the poly(A)+ fraction of nuclear extracts from cell lines and fetal kidney tissue along with U2AF65 and p116, another splicing factor[114]. Three-dimensional structural modeling comparing WT1 with U1A, a snRNP with known RNA recognition motif (RRM), identified a putative RRM in the N-terminal region of WT1, however a functional significance of this model has not been confirmed[100]. Recently, oligo(dT) chromatography to isolate RNPs followed by Western and SD gel analysis identified WT1 in RNPs in two human leukemia cell lines, for the first time suggesting that WT1 may function in cancer by interacting with both DNA as a transcription factor, and RNA at the post-transcriptional level[163]. The mechanisms are currently unknown. To date, one RNA target of WT1 has been identified; gel mobility shift assays demonstrate that WT1 binds to exon 2 of IGF-2 RNA in an interaction which requires the zinc finger region of WT1[24]. WT1 may therefore be one of a limited number of genes that affect gene expression at the DNA and RNA level.

Regulation of WT1

Transcriptional regulation

Evidence suggests that the regulation of the WT1 gene is at the transcriptional level. During nephrogenesis, WT1 mRNA and protein are expressed at the same time. In human cancers, there are rarely mutations in the *wt1* gene that could lead to its upregulation (Table 1). While many studies have focused on the role of WT1 in tumorigenesis and organ development, relatively few have examined the regulation of the WT1 gene. However, six transcription factors have been described that may be directly involved in WT1 gene expression. PAX2, a transcription factor expressed at the same time as WT1 during nephrogenesis, binds to the WT1 promoter as shown by EMSA and DNaseI footprinting. Co-transfection of PAX2 caused upregulation of a WT1 promoter reporter construct[145]. Furthermore, in breast cancer and acute myeloid leukemia (AML), PAX2 expression positively correlated with WT1 expression [222, 220]. Another paired box transcription factor, PAX8, both endogenous and exogenous, can regulate the WT1 promoter as demonstrated by transient transfection experiments. Binding was demonstrated by EMSA[52]. GATA-1, a transcription factor involved in hematopoeisis, binds to an enhancer region in the WT1 gene and activates WT1 expression, shown by EMSA and co-transfection with enhancer/promoter-CAT reporter constructs, respectively[263]. Furthermore, WT1 and GATA-1 mRNA are both expressed in hematopoeitic cell lines and mouse spleens. Microarray analysis of MDA-MB-468 cells transfected with a dominant negative Pea3 construct identified WT1 as a potential target gene of the ETS transcription factor family member. Transfection with Pea3 increased

transcription of a reporter gene driven by the WT1 promoter and increased WT1 protein expression. Recombinant Pea3 is also able to bind to ETS binding sites in the WT1 promoter as shown by EMSA[46]. Our laboratory has shown that several glioma cell lines express Pea3 (McCready *et al.*, unpublished data). Deletion studies of the WT1 promoter identified a putative Sp1 binding site as being involved in WT1 gene activation which was confirmed by stable transfection experiments, EMSA, and Southwestern analysis. In the same study, immunohistochemistry of the developing and adult rat kidney showed that Sp1 is expressed in structures that express WT1[31]. Interestingly, hypoxia inducible factor-1 α (HIF-1α) trans-activates the WT1 promoter in cells exposed to hypoxia which leads to increased survival[250]. This is potentially interesting for brain tumor research, in that HIF-1α expression and activity, particularly in response to intra-tumoral hypoxia, appear to play a prominent role in the biology of high-grade gliomas[94]. However, our laboratory has observed *in vitro* a decrease in WT1 mRNA expression in two WT1 expressing glioblastoma cells in response to 24 hours of hypoxia (Appendix 1).

Transient co-transfection studies in 293 transformed human fetal kidney cells show that WT1(+/+) strongly represses the WT1 promoter, while WT1(-/+) is the weakest repressor. WT1 is one of the first genes to be shown to negatively autoregulate its own expression by direct interaction of the protein with its promoter, as demonstrated by EMSA[203]. Studies with 293 cells stably expressing an inducible WT1 (+/-) expression vector confirmed this result[138]. Hypermethylation of CpG islands is another mechanism of inhibiting the transcription of genes. The 5' promoter and intron 1 region as well as a 3' enhancer region of the *wt1* gene contain several sites of methylation, and are methylated in

several disease states including breast cancer and Wilms' tumor[105, 123, 140]. In ovarian cancer, WT1 expression significantly correlates with methylation status[98]. Furthermore, in ovarian and breast cancer cells, treatment with a demethylating agent restores WT1 expression[98, 123, 128]. However, the clinical relevance of WT1 gene methylation is questionable, as in breast cancer WT1 is highly expressed regardless of methylation[128]. In fact, in a large sample of human astrocytomas, WT1 was found to be hypermethylated, while other studies have found WT1 to be overexpressed in astrocytic tumors[267].

Post-transcriptional regulation

Very little is known about the post-transcriptional regulation of WT1. WT1 can be phosphorylated by protein kinase A and C (PKA, PKC) at Serine residues 365 and 393, which are located in the zinc finger 2 and 3 region of the protein[205, 265]. Phosphorylation of WT1 inhibits its ability to bind DNA and its ability to repress the PDGF-A promoter[205]. In addition, phosphorylation prevents WT1 binding to RNA and leads to cytoplasmic retention of the protein[265]. The significance of phosphorylation in human disease is unknown, however aberrant cytoplasmic localization of WT1 has been observed in cancer, including glioblastoma[178].

WT1 and development

Wilms' tumor arises from pluripotent embryonic precursor cells. The tumor is composed of the three cell types derived from these precursor cells which are normally present in kidney development; blastemal, stromal, and epithelial, and is therefore classically described as "triphasic"[154]. In addition, the observation that WT1 mutations not only lead to Wilms' tumor development, but also to urogenital abnormalities supports a

critical role for wild-type WT1 in normal development. The study of WT1 dysregulation in Wilms' tumor has provided unique insight into the relationship between normal development and tumorigenesis. During development, WT1 is expressed in two types of tissues. One type is that undergoing mesenchymal to epithelial transition; the developing kidney, spleen, adrenal glands, the mesothelial lining of the peritoneal, pleural, and pericardial cavities, and the epithelial cells of the gonads[7, 19]. The second is that of ectodermal origin, namely defined regions of the brain and spinal cord[7]. In the adult, WT1 expression is restricted to the glomerular podocytes of the kidney and area postrema in the brain[217].

Homozygous WT1 mutant mice die early in gestation, possibly due to abnormal formation of the mesothelial lining of the heart, lung, and abdominal organs[110, 160]. Examination of the embryos revealed failure of kidney and gonad development which correlated with an increase in apoptotic cells in the tissue[110]. Lack of spleen development in *wt1*$^{-/-}$ mice also demonstrated enhanced apoptosis in embryonic spleen cells[81]. This suggests that WT1 may function in early development as a survival factor. One potential mechanism for this effect could be by affecting the intrinsic apoptotic pathway via Bcl-2, which contains a WTE site in its promoter. WT1 transactivates the anti-apoptotic gene encoding Bcl-2 which can protect cells against apoptosis-inducing stimuli[144]. This may also be relevant to the function of WT1 in cancer, as the same study demonstrates that WT1 levels correlate with Bcl-2 levels in a small sample of Wilms' tumors. Bcl-2 is overexpressed in 33 – 70% of glioblastoma however no studies to date have examined a correlation between WT1 and Bcl-2.

In addition to the pro-survival function, WT1 may mediate epithelial branching in the developing kidney. WT1 trans-activates the *amphiregulin* gene, which encodes a ligand for EGFR that is expressed concurrently with WT1 in the developing kidney and that stimulates ureteric bud branching in mouse embryo kidney organ cultures[124]. Amphiregulin expression has not been studied in glioblastoma, however it would be highly relevant, given the established importance of EGFR signaling in the tumorigenicity of glioblastoma. This is underscored by the observation that Amphiregulin is overexpressed in hepatocellular carcinoma and contributes to the increased growth rate and resistance to chemotherapy of these cancer cells[26].

Although the exact role of WT1 in the development of the central nervous system has not been studied, several studies suggest a role for WT1 in neuronal differentiation. Low levels of WT1 are expressed in the developing eye, specifically the retina[7, 249]. WT1 homozygous mutant mice have smaller eyes, thinner neuroretinas, and defective optic nerves, associated with decreased proliferation and increased apoptosis of retinal ganglion progenitor cells[249]. In vitro models of neuronal differentiation suggest potential mechanistic roles for WT1 in this process. Retinoblastoma cells, which are malignantly transformed pluripotent retinal progenitor cells, can be induced to differentiate into neurons, during which WT1 is downregulated[251]. Furthermore, inhibition of WT1 expression in undifferentiated retinoblastoma cells partially blocks neuronal differentiation, suggesting that WT1 plays an important role in the early stages of neuronal differentiation. Likewise, pheochromocytoma cells can be induced to differentiate into neurons by treating with nerve growth factor (NGF), which is known to downregulate

EGFR. This appears to be dependent on WT1 transcriptional regulation of the TCC repeat-containing EGFR promoter, as WT1 expression is decreased with NGF treatment and parallels the decrease in EGFR expression[127]. The ability of WT1 to up-regulate EGFR is interesting, given the central importance of EGFR signaling to glioblastoma behavior. In support of a role for WT1 in neuronal differentiation, our laboratory has shown that WT1 expression is downregulated in human NTera2 teratocarcinoma cells during retinoic acid-induced differentiation into neurons (Appendix B).

WT1 and cancer

Controversy exists surrounding the function of WT1 as a tumor suppressor or an oncogene in tumorigenesis[151]. The expression of WT1 in human cancer specimens has been extensively studied (Table 1-1).

Table 1-1. Major studies examining WT1 expression in cancer

Cancer type	Detection method	Findings	References
Wilms' tumor	PCR, PCR-SSCP	8/130 WT1 mutations	Little *et al.*, 1992[126], Varanasi *et al.*, 1994[246]
Breast cancer	RT-PCR, Western blot, sequencing	7/7 express WT1 mRNA, 27/31 express WT1 protein, 0/36 mutations	Loeb *et al.*, 2001[128], Oji *et al.*, 2004[175]
Leukemia	Northern blot, RT-PCR, immuno-fluorescence	151/220 acute leukemias and chronic leukemias in blast crisis express WT1 mRNA, 12/20 express WT1 protein	Miwa *et al.*, 1992[157], Menssen *et al.*, 1995[153]
Pancreatic adeno	IHC	30/40 express WT1 protein	Oji *et al.*, 2004[177]
Colorectal adenoc	Real-time RT-PCR, IHC, sequencing	20/28 express WT1 mRNA, 41/46 express WT1 protein, 0/5 mutations	Oji *et al.*, 2003[179]
Thyroid cancer	Real-time RT-PCR, IHC, sequencing	33/34 express WT1 mRNA, 20/21 express WT1 protein, 0/9 mutations	Oji *et al.*, 2003[176]
Bone and soft tissue sarcoma	Real-time RT-PCR, IHC, sequencing	28/36 express WT1 mRNA, 4/4 express WT1 protein, 0/8 mutations	Ueda *et al.*, 2003[240]
Head and neck, squamous	Real-time RT-PCR, IHC, sequencing	42/56 express WT1 mRNA, 6/6 express WT1 protein, 0/5 mutations	Oji *et al.*, 2003[173]
Lung cancer	Real-time RT-PCR, IHC, sequencing	54/56 express WT1 mRNA, 5/6 express WT1 protein, 0/7 mutations	Oji *et al.*, 2002[174]
Ovarian cancer	Northern blot, PCR-SSCP	15/20 express WT1 mRNA, 0/20 mutations	Bruening *et al.*, 1993[16]
Esophageal cancer	Real-time RT-PCR, IHC	12/12 express WT1 mRNA, 36/38 express WT1 protein	Oji *et al.*, 2004[180]
Malignant mesothelioma	PCR-SSCP, IHC	No mutations, 54/56 express WT1 protein, 1/33 WT1 mutation	Kumar-Singh *et al.*, 1997[111], Park *et al.*, 1993[183]
Desmoplastic small round cell tumor	RT-PCR, sequencing	4/6 rearrangement with fusion of EWS and WT1 genes	Gerald *et al.*, 1995[57]
Retinoblastoma	RT-PCR, IHC	6/6 express WT1 mRNA and protein	Wagner *et al.*, 2002[251]
Malignant glioma	RT-PCR, real-time RT-PCR, IHC	35/46 express WT1 mRNA, 71/76 express WT1 protein	Menssen *et al.*, 2000[152], Dennis *et al.*, 2002[43], Oji *et al.*, 2004[178], Nakahara *et al.*, 2004[168]

Abbrev., RT-PCR, reverse-transcriptase polymerase chain reaction; SSCP, single strand comformational polymorphism; IHC, immunohistochemistry

Although originally discovered as mutated in Wilms' tumor and classified as a tumor suppressor, WT1 mutations are relatively rare, occurring in a small percentage of Wilms' tumors, leukemia, mesothelioma, and the rare desmoplastic small round cell tumor[126, 102, 183]. In contrast, wild-type WT1 is overexpressed in breast cancer, acute leukemia, colorectal cancer, lung cancer, ovarian cancer, head and neck squamous cell carcinoma, and bone and soft tissue sarcomas[128, 157, 179, 174, 16, 173, 240]. As shown in Chapter 2 below, we have confirmed that overexpression of wild-type WT1 is present in approximately 80% of glioblastomas, as well as 80% of grade II and III gliomas[30].

Multiple studies have examined the role of WT1 in leukemia. Wild-type WT1 is overexpressed in the majority of acute leukemia relative to normal bone marrow and peripheral blood cells, indicating that WT1 is a reliable leukemia marker[157, 153, 88]. WT1 is also expressed in chronic leukemia in blast crisis, but is absent in chronic leukemia in the chronic phase[157, 153]. WT1 is mutated in approximately 10-15% of leukemia, primarily acute myelogenous leukemia[102]. High WT1 expression correlates with worse prognosis in acute leukemia and can be used to predict the progression of myelodysplastic syndrome to acute leukemia[90, 231]. Furthermore, WT1 levels in bone marrow and peripheral blood after treatment are useful in the detection of minimal residual disease and can help tailor additional therapies[90, 89]. Clues to the function of WT1 in leukemia come from overexpression and inhibition studies. Transient transfection of wild-type WT1 into normal myeloid progenitor cells inhibits the differentiation of the cells, which is significant in that differentiation arrest is characteristic of leukemia[91]. Overexpression of WT1 in a variety of human leukemia cell lines similarly causes a block in

differentiation, thereby providing a potential role for WT1 in leukemogenesis[229, 44, 25]. The capacity of WT1 to block differentiation is potentially clinically relevant, as high WT1 expression correlates with less differentiated samples in a large group of human AML[185]. WT1 expression can also protect leukemia cells from chemotherapeutic agents by decreasing activity of the intrinsic apoptotic pathway which is associated with an increase in anti-apoptotic Bak expression[93]. Simpson *et al.*, provide further evidence for a protective role of WT1 in leukemia cell behavior by demonstrating that the anti-apoptotic Bcl-2 family member A1/BFL1 is directly trans-activated by WT1[225]. When leukemia cells expressing endogenous WT1 are induced to differentiate, WT1 is down-regulated which is associated with a decrease in c-myc expression[215]. From a therapeutic standpoint, WT1 downregulation by antisense oligonucleotides causes a decrease in proliferation of leukemia cells which is accompanied by a decrease in viability and an increase in apoptosis[3]. Additional studies show that apoptosis induced by WT1 silencing is associated with an increase in pro-apoptotic Bax expression[93]. WT1 antisense oligonucleotide treatment does not effect the viability of normal cells, suggesting the potential for WT1 as an attractive target for therapy[264].

Wild-type WT1 is overexpressed in the majority of breast cancer specimens relative to normal breast tissue[128]. WT1 protein is highly expressed in estrogen receptor negative tumors which are more aggressive[223]. In a large clinical sample, real-time RT-PCR analysis revealed that WT1 mRNA level is a significant prognostic factor as high WT1 expression correlated with a significantly lower 5 year disease free survival[159]. WT1 overexpression may be in part due to the RTK HER2/neu which is overexpressed in

aggressive breast cancers, as overexpression of HER2/neu in breast cancer cells causes upregulation of WT1 expression and HER2/neu inhibition decreases WT1 expression[239]. The same study also indicates the involvement of AKT which is activated by HER2/neu. WT1 expression increases in breast cancer cells when treated with growth stimulatory agents and, more importantly, antisense oligonucleotide-induced WT1 downregulation inhibits proliferation by inducing G1 arrest[239, 270]. The decrease in proliferation is associated with decreased expression of cyclin D1, but the exact mechanism is not known. All four WT1 isoforms also trans-activate the c-myc proto-oncogene in breast cancer cells by directly stimulating the c-myc promoter[76]. Activation of c-myc is known to stimulate proliferation, and therefore may provide an explanation for the decrease in cancer cell proliferation after WT1 downregulation. However proliferation was not determined after WT1 induced c-myc upregulation[76]. Nevertheless, this may be relevant to glioblastoma biology as c-myc is overexpressed in tissue samples[236]. Decreased WT1 expression also induces apoptosis in breast cancer cells which parallels a decrease in the anti-apoptotic WT1 target gene, Bcl-2[239]. The observation that WT1 represses the insulin-like growth factor-1 receptor (IGF-1R), which acts as a survival factor in cancer, adds complexity to the putative oncogenic function of WT1[197]. The mechanism involves WT1 interaction with the estrogen receptor. High levels of WT1 expression in breast cancer are associated with estrogen receptor negative tumors[223], so the physiologic relevance of this observation is not known. Nevertheless, the exact role of WT1 in human cancer is exceedingly complex and to date not fully understood.

WT1 and invasion

The relationship between WT1 and cell invasion has not been extensively studied. A recent study, however, suggests that WT1 may play a role in migration and invasion[95]. WT1 overexpression in TYK ovarian cancer cells caused the cells to adopt a smaller size and "fibroblast-like" morphology which was associated with decreased expression of α-actinin 1 and cofilin, two cytoskeletal proteins. The expression of gelsolin, which is involved in actin filament assembly, is upregulated in response to WT1 expression. WT1 overexpression also decreased focal adhesions, while increasing migration and invasion. The phenotype could be reverted by forced expression of α-actinin 1 and cofilin, or gelsolin silencing, suggesting that WT1 may affect invasion by modulating cytoskeletal structures. As described above, α-actinin 1 plays an important role in glioma invasion by linking integrins to the actin cytoskeleton. Furthermore, the change in cell morphology induced by WT1 expression is similar to that adopted by the invading glioma cell. Morphologically, glioma cells prone to invasion are small anaplastic cells[96, 60]. In a separate study, WT1 was found to regulate the E-cadherin promoter, a cell adhesion molecule involved in cancer cell invasion[85]. Given the characteristic invasiveness of glioblastoma, the study of WT1 and glioma cell invasion is warranted.

WT1 and p53

As the debate rages over the function of WT1 as a tumor suppressor or oncogene, a player of particular interest to glioma biology has arisen as a potential explanation: the p53 tumor suppressor. In osteosarcoma cells and immortalized kidney cells, WT1 and p53 co-immunoprecipitate demonstrating direct binding[137]. Arguing for an oncogenic

function of WT1, WT1 inhibits p53 mediated apoptosis in addition to stabilizing the p53 protein[136]. In large samples of female genital tract tumors and a small sample of Wilms' tumors, WT1 expression strongly correlates with p53 expression[2, 136]. In endometrial carcinoma, WT1 and p53 double positivity is associated with worse prognosis[47]. Unfortunately, none of these studies screened the *p53* gene for mutations, which is crucial for interpretation because of the stabilizing effect of WT1 on p53. However, our laboratory has shown that in a large sample of glioblastoma specimens, WT1 expression correlates with wild-type p53 (please see Chapter 2 and ref[30]). This may explain, in part, the observation that 30% of glioblastomas overexpress p53 in the absence of stabilizing mutations[202]. Furthermore, it may provide a partial mechanism for functional inactivation of wild-type *p53* in glioblastoma. It would be interesting to determine if WT1 expression and MDM2 amplification are mutually exclusive in glioblastoma, which would suggest two pathways of functionally inactivating p53, however this study has yet to be undertaken.

The reciprocal effect of the WT1/p53 interaction is exemplified by the dual regulation of the insulin-like growth factor-I receptor (IGF-IR) promoter. The IGF-IR is overexpressed in cancer and may function in malignant transformation by inhibiting apoptosis[54]. WT1 represses the IGF-IR promoter due to multiple WT1 binding sites in embryonic kidney cells and prostate cancer cells[261, 37]. The decrease in IGF-IR expression leads to decreased proliferation. Wild-type p53 also represses transcription of the IGF-IR however this is not a consequence of direct binding to the promoter[260]. As another consequence of their physical interaction discussed above, p53 inhibits the

transcriptional repression activity of WT1 at the IGF-IR[87]. This mechanism may be more generalizable as reciprocal effects on transcriptional regulation due to WT1/p53 interaction have been documented in other studies[137, 136]. An interesting side note is that p53 increased the expression of WT1, a finding which requires follow-up[87]. It is possible that one component of the tumor suppressive function of p53 is to act as a switch which converts WT1 to a repressor of oncogenes involved in transformation, such as the IGF-IR. Potentially, in certain contexts, such as wild-type p53, WT1 acts as a tumor suppressor, while in others, such as the absence of p53, WT1 acts as an oncogene.

Conclusion

As a gene involved in the development and differentiation of the central nervous system, in addition to its function as a regulator of several key glioma associated molecules, WT1 is in a unique position to be involved in both normal development and gliomagenesis, when aberrantly re-activated. The ability of WT1 to modulate receptor tyrosine kinase pathways, anti-apoptotic proteins, components of cell cycle regulation, cell invasion, and the tumor suppressor p53 are of particular interest to glioma biology. More extensive studies of the function of WT1 in glioblastoma may prove WT1 to be critical to gliomagenesis. Furthermore, WT1 targeting strategies may decrease the tumorigenicity of glioma cells and potentially enhance other anti-glioma therapies, proving it to be a gene of substantial clinical significance in neuro-oncology.

CHAPTER 2.

WILMS' TUMOR 1 EXPRESSION IN MALIGNANT GLIOMAS AND CORRELATION OF +KTS ISOFORMS WITH P53 STATUS

Aaron J Clark, Wagner G Dos Santos, Jessica McCready, Mike Y Chen, Timothy E Van Meter, Joy L Ware, Sharon B Wolber, Helen Fillmore, William C Broaddus

Department of Neurosurgery, Department of Anatomy and Neurobiology, Department of Pathology, Department of Human Genetics, Virginia Commonwealth University, School of Medicine, Richmond, Virginia, USA

Abstract

Object. Wilms' tumor 1 (WT1) is overexpressed in many types of human cancer. Studies demonstrate that WT1 promotes tumor cell proliferation and survival in some cell lines by inhibiting p53-mediated apoptosis, however this relationship has not been studied in gliomas.

Methods. We screened nine malignant glioma cell lines, fifty glioblastoma (GBM) samples and sixteen lower grade glial tumors for WT1 expression. 5/9 cell lines, 44/50 GBM samples and 13/16 lower grade gliomas expressed WT1 mRNA by reverse

transcriptase polymerase chain reaction (RT-PCR) analysis. WT1 expression was not detected in normal astrocytes. Two WT1 isoforms, +/+ and -/+, were expressed by the majority of these samples. Real-time PCR analysis of the GBM cell lines revealed that the level of WT1 mRNA ranged from 6.33 – 214.70 ng per ng 18S RNA. We screened the GBM samples for p53 mutation by PCR and single-stranded conformational polymorphism (PCR-SSCP) analysis and demonstrated an association between WT1 expression and p53 status. Tumors that contained wild-type p53 were significantly more likely to express WT1 than tumors that contained mutant p53.

Conclusions. The presence of WT1 in glioma cell lines and the majority of primary tumor samples, and its absence in normal astrocytes, suggests that WT1 expression is important in glioma biology.

Introduction

Glioblastoma multiforme (GBM) is a WHO grade IV glial tumor that carries a universally poor prognosis. Extremely resistant to both radiotherapy and chemotherapy, the median survival is only one year. Unfortunately, it is also the most common primary brain tumor[27]. Decades of intense research have determined that alterations in several growth factors such as PDGF, EGF and their receptors, and tumor suppressors such as p53, Rb, and PTEN are important to glioma biology[134]. Despite these advances, a significant clinical improvement has not been realized. Therefore, a more complete characterization of the molecular changes in gliomagenesis is necessary to uncover potential markers and targets for novel therapies. Previous studies examined the expression of the Wilms' tumor 1 (WT1) gene in human brain tumors, including gliomas, and found detectable expression

in tumor cells, while normal cells expressed none[43, 152, 168, 178]. To date, no studies have examined the WT1 isoforms expressed by these tumors or the contribution of WT1 to prognosis and other clinicopathologic characteristics.

WT1 was originally discovered as mutated in nephroblastoma, a common pediatric kidney cancer also known as Wilms' tumor[23]. It was subsequently found that the *wt1* gene, located at chromosome locus 11p13, encodes a 52-55 kD polypeptide containing four C-terminal zinc fingers and an N-terminal transactivation domain[75]. The structure is consistent with its known function as a transcription factor, and a large number of target genes have been identified[151]. Furthermore, the message is alternatively spliced to generate four main isoforms, designated +/+, -/+, +/-, -/-, depending on the inclusion or exclusion of exon 5 and nine nucleotides in exon 9 known as KTS, respectively, which may modulate the transcriptional activity of the protein[75]. WT1 plays a major role in normal development, most notably in the urogenital system, and is expressed in the developing central nervous system[77, 7].

Although originally classified as a tumor suppressor, WT1 does not adhere to the normal characteristics of such a gene. It is not ubiquitously expressed throughout the adult body, nor is it commonly mutated in cancers[19]. On the contrary, it is overexpressed in many types of cancer, including breast carcinoma and acute leukemia. In both cases, high levels of WT1 expression detected by real-time PCR correlate with a worse prognosis relative to those that express low levels of WT1[159, 90]. Furthermore, inhibition of WT1 expression in human breast cancer and leukemia cell lines using antisense modalities leads to differentiation, decreased proliferation, and increased sensitivity to treatment[270, 229].

Inactivation of the tumor suppressor p53 is a common aberration in human malignant gliomas. p53 has well established functions in cell-cycle arrest and apoptosis induced by DNA damage, as well as in DNA repair, senescence, and differentiation[248]. The *p53* gene is mutated in approximately 30% of GBM with the vast majority of these mutations being point mutations located in exons 5-8[12]. Many treatments for glioma, such as radio- and chemotherapy, cause DNA damage, and thus are likely to rely on p53 function for a therapeutic response[133]. Alterations in other genes, such as *mdm2,* which targets p53 for degradation, represent alternative means of inactivating p53 in gliomas[196]. In some cell types, WT1 inhibits p53-mediated apoptosis[136].

In this study, we assessed a large sample of human glioma cell lines and glioma specimens for WT1 isoform expression and p53 status, to determine if a correlation exists between WT1 expression and prognosis, or p53 status. The majority of glioma cell lines and specimens expressed the +KTS isoforms of WT1 which correlated with the presence of wild-type p53. These results suggest a critical role for WT1+KTS isoforms in glioma biology, possibly through interaction with the tumor suppressor p53.

Clinical Materials and Methods

Cell culture and tissue preparation

Human glioma cell lines (U87-MG, T98G, U373, U118, LN18, LN229) were obtained from American Type Culture Collection and were grown and passaged in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, glutamine, non-essential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. LN-Z308 glioma cells, a kind gift from Dr. Erwin Van Meir, were maintained in the

same conditions. VC95G and VC95T were derived in our laboratory from human glioblastoma tissue and were maintained in the same conditions. Normal human astrocytes (NHA) were obtained commercially from Clonetics (Cambrex Biosciences). PC3 prostate carcinoma cells were used as a positive control for WT1 expression. For RNA extraction, the cell lines were plated at a concentration of 1 X 10^6 cells/100 mm tissue culture dish and allowed to attach overnight. Fresh frozen glioma specimens were selected from the VCU Neuro-Oncology Tissue and Cell Culture Facility. The specimens were obtained in the operating room, dissected from adventitial material, immediately snap frozen in liquid nitrogen, and stored at -80° C in accordance with a protocol approved by the VCU Institutional Review Board. Before RNA, DNA, or protein extraction, tissue was homogenized using a -80° C mortar and pestle. Total RNA was isolated from tissue and cell line samples using the Trizol extraction protocol (Invitrogen). DNA was isolated from tissue using the QIAamp DNA Mini kit protocol (Qiagen). The concentration of RNA and DNA was determined by spectrophotometry. Protein was extracted from cell lines using SDS buffer (50 mM Tris-Cl, 1% SDS, 10% glycerol) supplemented with protease inhibitors. Protein was extracted from tissue samples using the same extraction buffer followed by sonication. The concentration of protein was determined by DC protein assay (BioRad).

RT-PCR and sequencing

Reverse transcription was performed using the Superscript II RT-PCR system (Invitrogen). A 10 µl sample of the RT reaction mixture was added to 40 µl PCR mixture containing PCR buffer, dNTP mixture, Platinum Taq DNA polymerase, primers, and

$MgCl_2$ to a final concentration of 1.82 mM. The first set of amplifications utilized a 21-mer 5'-GTT-ACA-GCA-CGG-TCA-CCT-TCG-3' located in exon 2 and a 22-mer 5'-CCT-GAA-TGC-CTC-TGA-AGA-CAC-C-3' located in exon 6 (primer pair 1, Figure 2-1a). A separate set of amplifications was performed with a 22-mer 5'-GAC-GTG-TGC-CTG-GAG-TAG-CCC-C-3', a primer located in exon 7 and a 21-mer 5'-GCT-GCC-TGG-GAC-ACT-GAA-CGG-3', a primer located 17 bp upstream of the stop codon (primer pair 2, Figure 2-1a). In a separate reaction, 2 μl of RT reaction mixture was added to 48 μl of a similar PCR mixture to amplify a segment of GAPDH mRNA as a control. PCR products were electrophoresed on ethidium bromide-stained 1% agarose gels. For sequencing, the PCR product was extracted from gels using the QIAquick Gel Extraction Kit (Qiagen). The extract was then sent to the Molecular Core of the Virginia Commonwealth University-Massey Cancer Center Nucleic Acids Research Facilities for direct sequencing to determine the KTS status of the mRNA.

Real-time PCR analysis

The experiments were performed in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems) using the TaqMan® One Step PCR Master Mix Reagents Kit (P/N: 4309169). All the samples were tested in triplicate under the conditions recommended by the fabricant. The cycling conditions were: 48°C/30min; 95°C/10min; and 40 cycles of 95°C/15sec and 60°C/1min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The probes and primers were designed using the software Primer Express® 2.0. The probes were labeled at the 5' end with FAM (6-carboxyfluorescein) and at the 3' end with TAMRA (6-

carboxytetramethylrhodamine). Ribosomal RNA (18S rRNA) from the Pre-developed TaqMan®Assay Reagents (P/N: 4310893E) was used as endogenous control. The reactions and the synthesis of the probes and primers were performed in the VCU Nucleic Acid Research Facilities.

Western blot analysis

For WT1 protein analysis, 100 μg of total protein lysate was separated by SDS–PAGE, transferred to nitrocellulose membrane, and the membrane blocked with 5% nonfat milk solution for 1 hour at room temperature. Mouse anti-WT1 monoclonal antibody (Dako) was diluted 1:200 in blocking buffer. The membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed six times in Tris buffered saline containing 0.05% Tween-20 before and after a one hour incubation at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody (Rockland, Inc.) at a dilution of 1:1000. Blots were developed using the ECL detection System (Amersham Biosciences). Anti-Cyclophilin A monoclonal antibody (Upstate Biotechnology) at a dilution of 1:1000 was used as a control for protein loading.

PCR-Single strand conformational polymorphism analysis

100 ng of genomic DNA was used as the template for PCR as described above. Exons 5-8 of the human p53 gene were amplified from tumor DNA in 4 separate reactions using primer pairs described previously[188]. DNA extracted from blood samples obtained from five normal controls was used as SSCP controls. The PCR products were electrophoresed on a 7% acrylamide gel and visualized by silver staining. Bands which differed from those in normal control blood were considered mutations.

Statistical Analysis

WT1 expression was dichotomized as tumors expressing full-length WT1 versus non-full-length, which included tumors not expressing WT1 and those expressing potentially truncated WT1. Similarly, p53 was dichotomized as wild-type versus mutant. Thereafter, the relationship between WT1 and the categorical clinical variables (e.g., sex, tumor location, and p53 status) of the GBM patients was first analyzed using Fisher's exact test. For any variable found univariately to be significantly associated with WT1, a logistic regression model was fit to estimate the odds ratio and associated 95% confidence interval. The relationship between WT1 expression and the continuous variable age was analyzed using a t-test. Survival was calculated from the date of diagnosis of GBM until the date of death, censoring for patients still alive. Kaplan-Meier survival curves were estimated for the WT1 full-length and non-full-length groups and were compared using the log-rank test.

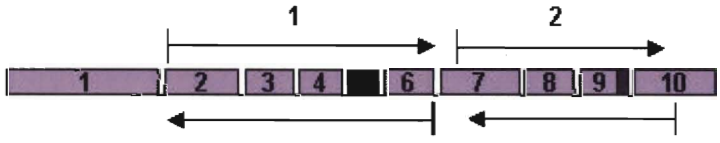
Results

Malignant glioma cell lines expressed WT1. We screened nine human malignant glioma cell lines for WT1 expression by RT-PCR, first using primer pair 1 that spans the exon 5 splice site (Figure 2-1b). Five glioma cell lines expressed detectable WT1 mRNA. RT-PCR using primer pair 2 spanning the KTS splice site in exon 9 confirmed WT1 mRNA expression in the five cell lines and a lack of expression in normal human astrocytes (Figure 2-1c). Sequencing of the PCR products revealed that the cell lines expressed the +/+ and -/+ isoforms of WT1, with the exception of VC95G, which

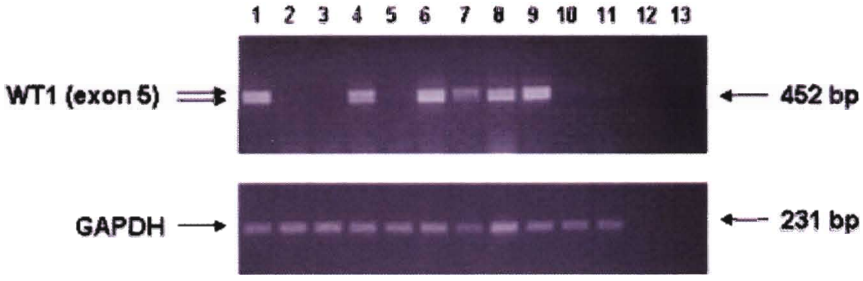
expressed the +/- and -/- isoforms. We quantified the level of WT1 mRNA, relative to 18S RNA, being expressed by the cell lines using real-time PCR (Figure 2-1d).

Figure 2-1. Human glioma cell lines express WT1 mRNA. A. Schematic representation of the WT1 cDNA. Each exon is depicted as a separate numbered box. Alternative splices are depicted as darkly shaded boxes. Primers and products are depicted as arrows. Primer pair 1 spans the splice site in exon 5. The alternative splice in exon 9 is designated KTS and is spanned by primer pair 2. B. Glioma cell line RNA extract was analyzed by RT-PCR using primer pair 1. Numbered lanes correspond to the following cell lines; (1) PC3 prostate carcinoma positive control, (2) NHA normal human astrocytes, (3) U87MG, (4) VC95G, (5) VC95T, (6) T98G, (7) U373, (8) U118, (9) LN18, (10) LN229, (11) LN-Z308, (12) no RNA template control, (13) no cDNA template control. In a separate reaction, detection of GAPDH was included as a control for RNA loading. C. Glioma cell line RNA extract was analyzed by RT-PCR using primer pair 2. D. Levels of WT1 mRNA expression were determined by real-time PCR. WT1 levels are shown as ratios of ng WT1: ng18S mRNA levels.

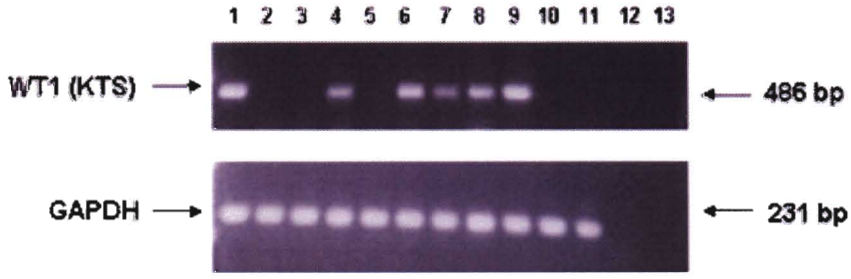
A



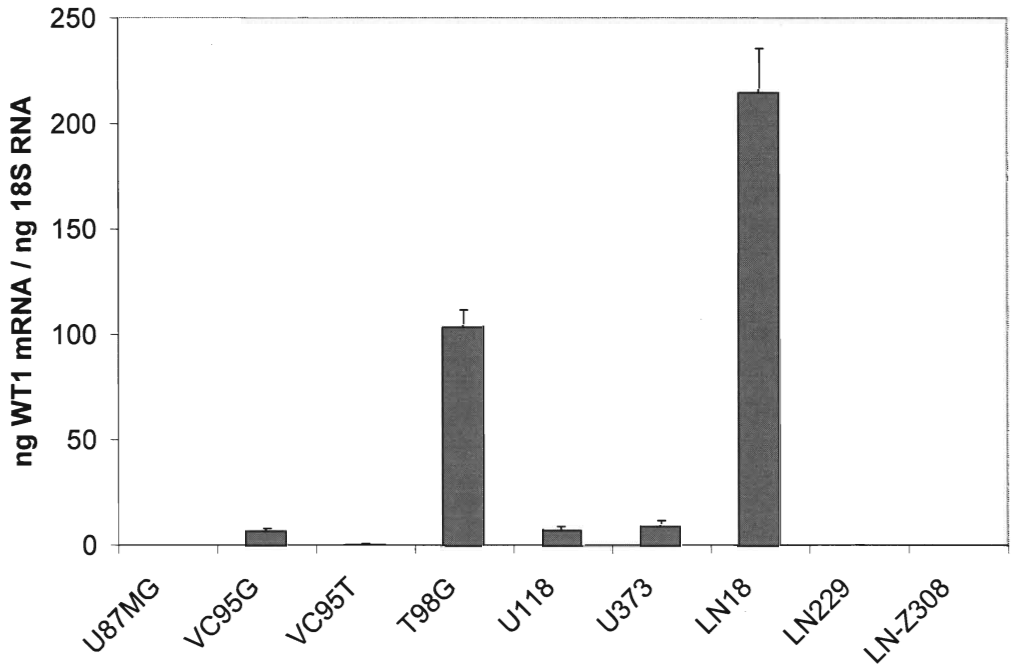
B


1 2 3 4 5 6 7 8 9 10 11 12 13
WT1 (exon 5)
452 bp
GAPDH
231 bp

C


1 2 3 4 5 6 7 8 9 10 11 12 13
WT1 (KTS)
486 bp
GAPDH
231 bp

D


ng WT1 mRNA / ng 18S RNA
250
200
150
100
50
0
U87MG
VC95G
VC95T
T98G
U118
U373
LN18
LN229
LN-Z308

The real-time PCR confirmed the qualitative RT-PCR data, detecting WT1 mRNA in the same lines by both assays. LN18 and T98G cell lines expressed relatively high levels of WT1 mRNA, while VC95G, U118, and U373 expressed low levels. Sequencing and real-time PCR data are summarized in Table 2-1.

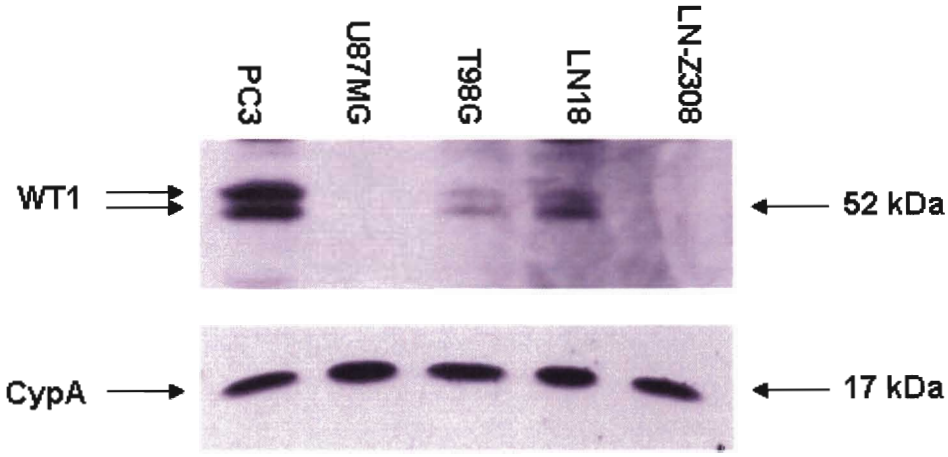
Table 2-1. Sequencing data demonstrating the specific isoforms and levels of WT1 mRNA expressed by glioma cell lines per ng 18S RNA

Cell line	WT1 isoform*	ng WT1 / ng 18S
U87MG	Nd	0.0 ± 0.0
VC95G	+/-, -/-	6.33 ± 1.2
VC95T	Nd	0.0 ± 0.0
T98G	+/+, -/+	103.61 ± 7.9
U373	+/+, -/+	7.34 ± 1.7
U118	+/+, -/+	9.04 ± 2.4
LN18	+/+, -/+	214.70 ± 20.9
LN229	Nd	0.0 ± 0.0
LN-Z308	Nd	0.0 ± 0.0

*WT1 isoforms are designated (exon 5)/(KTS) or nd, not detected

To confirm WT1 gene expression at the protein level, we performed western blotting on a representative subset of the cell lines; U87-MG, T98G, LN18, and LN-Z308. Consistent with the RT-PCR data, T98G and LN18 cells expressed WT1 protein, while U87-MG and LN-Z308 cells did not (Figure 2-2).

Figure 2-2. Western blot analysis of WT1 protein expression. 100 μg of protein extracted from PC3 prostate carcinoma positive control, U87MG, T98G, LN-18, and LN-Z308 cell lines were analyzed by Western blotting using mouse anti-WT1 monoclonal antibody (Dako). Blots were also probed with anti-cyclophilin A to demonstrate equal protein loading. CypA, cyclophilin A.


PC3
U87MG
T98G
LN18
LN-Z308
WT1
52 kDa
CypA
17 kDa

Glioblastoma multiforme specimens expressed WT1. Previous studies of small samples of GBM tissue suggest that WT1 expression is not clinically relevant and is only a phenomenon caused by extended culture of glioma cells[43, 152]. We found that WT1 mRNA expression did not change with increasing passage number in established glioma cell lines (data not shown). To confirm that a significant proportion of human malignant gliomas expressed WT1, we screened fifty GBM specimens. The specimens contained > 80% tumor tissue relative to normal brain tissue as determined by pathologic analysis. Twenty three (46%) of the samples were from female patients and the remaining twenty seven (54%) were from male patients. The average age of the patients was 57.3 years. Nine (18%) of the GBM samples were located in the frontal lobe, 11 (22%) in the parietal lobe, 20 (40%) in the temporal lobe, 9 (18%) in the occipital lobe, and one (2%) in the insular lobe. The patient population survived a median of 45 weeks after the diagnosis of GBM. By RT-PCR, 44/50 (88%) of the specimens expressed WT1 mRNA (Table 2-2).

Table 2-2. Summary table depicting the WT1 expression and p53 mutaion status of glioblastoma multiforme specimens and respective clinical information.

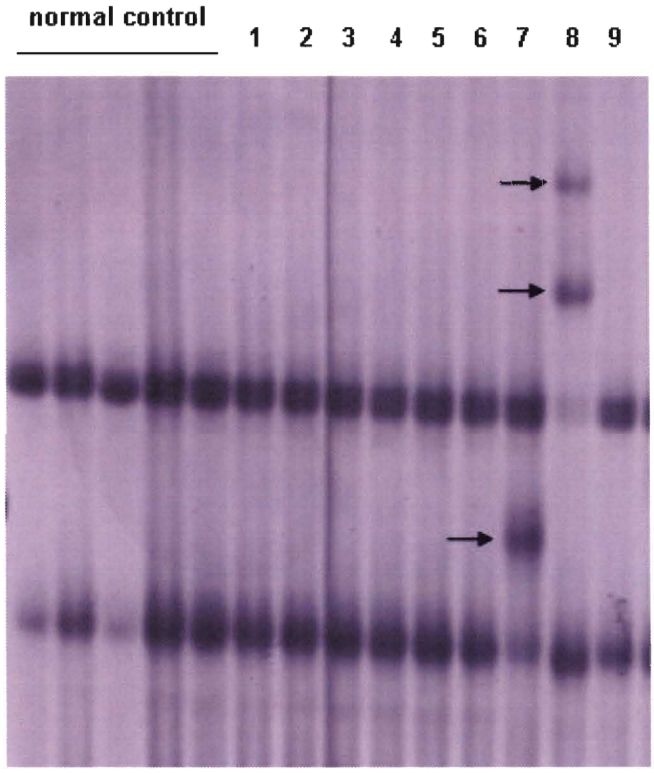
Pt #	Sex	Age	Location	WT1(exon 5)	WT1(KTS)	P53	Survival (w)
1	F	39	Frontal	nd	nd	wild-type	living
2	F	79	Occipital	+,-exon 5	+KTS	wild-type	33
3	M	80	Temporal	+,-exon 5	+KTS	wild-type	11
4	M	68	Occipital	+,-exon 5	nd	wild-type	19
5	M	75	Temporal	+,-exon 5	+KTS	wild-type	15
6	F	66	Occipital	+,-exon 5	+KTS	wild-type	48
7	M	19	Frontal	+,-exon 5	nd	mutant	64
8	M	45	Occipital	+,-exon 5	nd	mutant	19
9	F	55	Insula	+,-exon 5	+KTS	wild-type	39
10	F	60	Parietal	nd	nd	mutant	82
11	F	49	Parietal	+,-exon 5	+KTS	mutant	67
12	F	58	Parietal	+,-exon 5	+KTS	wild-type	36
13	M	64	Occipital	+,-exon 5	+KTS	wild-type	184
14	M	49	Temporal	nd	nd	wild-type	77
15	F	77	Parietal	+,-exon 5	+KTS	wild-type	14
16	F	43	Temporal	+,-exon 5	+KTS	wild-type	106
17	M	61	Temporal	+,-exon 5	+KTS	wild-type	124
18	M	64	Frontal	+,-exon 5	+KTS	wild-type	27
19	M	62	Frontal	+,-exon 5	+KTS	wild-type	39
20	F	60	Temporal	+,-exon 5	+KTS	wild-type	4
21	F	51	Occipital	+,-exon 5	+KTS	wild-type	190
22	F	20	Temporal	+,-exon 5	+KTS	wild-type	107
23	F	54	Temporal	+,-exon 5	+KTS	wild-type	49
24	F	67	Occipital	+,-exon 5	+KTS	mutant	84
25	M	54	Temporal	+,-exon 5	+KTS	wild-type	67
26	M	33	Frontal	+,-exon 5	+KTS	wild-type	55
27	M	60	Temporal	+,-exon 5	nd	mutant	28
28	F	47	Parietal	+,-exon 5	+KTS	wild-type	66
29	M	64	Parietal	+,-exon 5	+KTS	wild-type	14
30	F	44	Frontal	+,-exon 5	+KTS	wild-type	48
31	F	58	Parietal	+,-exon 5	+KTS	mutant	61
32	M	51	Parietal	+,-exon 5	+KTS	wild-type	44
33	M	65	Occipital	+,-exon 5	+KTS	mutant	ltf/u
34	M	78	Parietal	+,-exon 5	+KTS	wild-type	8
35	M	80	Temporal	+,-exon 5	+KTS	wild-type	29
36	F	64	Temporal	+,-exon 5	+KTS	wild-type	88
37	F	68	Frontal	nd	nd	mutant	15
38	M	76	Parietal	nd	nd	wild-type	5
39	F	51	Frontal	+,-exon 5	+KTS	mutant	20
40	M	37	Temporal	+,-exon 5	+KTS	wild-type	10
41	F	49	Occipital	+,-exon 5	+KTS	wild-type	102
42	F	33	Parietal	+,-exon 5	+KTS	wild-type	45
43	M	74	Temporal	nd	nd	wild-type	52
44	M	63	Temporal	+,-exon 5	+KTS	mutant	45
45	M	72	Temporal	+,-exon 5	+KTS	wild-type	25
46	M	50	Frontal	+,-exon 5	+KTS	wild-type	13
47	M	62	Temporal	+,-exon 5	+KTS	wild-type	77
48	M	37	Temporal	+,-exon 5	+KTS	mutant	29
49	F	59	Temporal	+,-exon 5	+KTS	wild-type	85
50	M	71	Temporal	+,-exon 5	+KTS	wild-type	63

Pt #, patient number, nd, not detected, ltf/u, lost to follow-up, -,+exon 5 denotes presence of both – and +exon 5 isoforms.

Forty samples expressed WT1 detectable by both primer pairs. Similar to the cell line isoform profile, all 40 expressed both the +/+ and -/+ isoforms of WT1. However, samples # 4, 7, 8, and 27 expressed WT1 detectable only by primer pair 1. There was no significant correlation between WT1 expression and either sex ($p = 0.31$), age ($p = 0.72$), or location ($p = 0.81$). Patients with tumors that expressed WT1 survived for a shorter time than those that did not express WT1 (45 weeks versus 52 weeks) but that trend did not approach statistical significance ($p = 0.87$). To confirm that the samples that expressed WT1 mRNA expressed WT1 protein, western blot analysis was performed on samples # 13, 18, 21, 28, 31, and 39, all of which expressed WT1 mRNA. All of the samples tested expressed detectable WT1 protein (data not shown).

WT1 expression was associated with p53 status. As WT1 interacts with p53 and in certain types of cancer correlates with p53 expression, we screened the GBM samples for p53 status[137, 2, 47]. Using PCR-SSCP, we screened all 50 GBM samples for p53 mutations in exons 5-8 (Figure 2-3).

Figure 2-3. Representative SSCP analysis of exon 5 of *p53* gene. Normal control lanes contain DNA extracted from 5 different patients without brain tumors. Lanes 1-9 contain DNA extracted from GBM samples from patients 1-9. Mutations in samples 7 and 8 are demonstrated as extra fractions compared to normal controls and are indicated by arrows.


normal control
1
2
3
4
5
6
7
8
9

We detected mutations in 12 samples (Table 2-2). The expression of wild-type p53 was significantly associated with WT1 expression ($p < 0.05$, Table 2-3).

Table 2-3. WT1 expression is associated with p53 mutation status as determined by PCR-SSCP in human GBM specimens

		WT1		
		Full-length	**Non full-length***	**Total**
p53	**Wild-type**	33**	5	38
	Mutant	7	5	12
	Total	40	10	50

*Non-full length includes putative truncated mutant WT1 and lack of WT1 expression.

**Tumors with wild-type p53 are significantly more likely to express full-length WT1 than those with mutant p53 (odds ratio 4.714; 95% CI 1.069, 20.789)

Logistic regression showed that tumors with wild-type p53 were significantly more likely to express full-length WT1 than those with mutant p53 (odds ratio 4.714; 95% confidence interval 1.069, 20.789).

Lower grade glial tumors expressed WT1. To assess the WT1 expression pattern in relation to glioma progression, we screened sixteen lower grade gliomas for expression of WT1. 4/5 pilocytic astrocytomas, 1/1 low grade glioma, 4/4 oligodendrogliomas, 2/4 anaplastic astrocytomas, and 2/2 anaplastic oligodendrogliomas expressed WT1 by RT-PCR analysis (Table 2-4).

Table 2-4. Summary table depicting the WT1 expression status of lower grade glioma specimens

Pt #	Dx	Sex	Age	WT1(exon 5)	WT1(KTS)
51	PA	M	6	+,-exon 5	+KTS
52	PA	F	12	+,-exon 5	+KTS
53	PA	F	6	+exon 5	-KTS
54	PA	F	11	nd	nd
55	PA	F	1	+,-exon 5	+KTS
56	AII	M	5	+exon 5	+KTS
57	OII	M	35	+,-exon 5	+KTS
58	OII	M	31	+,-exon 5	+KTS
59	OII	F	29	+,-exon 5	+KTS
60	OII	M	47	+,-exon 5	+KTS
61	AAIII	M	63	+,-exon 5	+KTS
62	AAIII	F	13	nd	nd
63	AAIII	F	52	nd	nd
64	AAIII	M	47	+,-exon 5	+KTS
65	AOIII	M	50	+,-exon 5	+KTS
66	AOIII	M	36	+,-exon 5	+KTS

Pt#, patient number, Dx, diagnosis, PA, pilocytic astrocytoma, AII, astrocytoma, OII, oligodendroglioma, AAIII, anaplastic astrocytoma, AOIII, anaplastic astrocytoma, nd, not detected, -,+exon 5 denotes presence of both – and + exon 5 isoforms

With the exception of one pilocytic astrocytoma (#53) which expressed the +/- isoform and one low grade glioma which expressed the +/+ isoform only, all low grade tumors analyzed expressed both +/+ and -/+ isoforms of WT1.

Discussion

Of the nine human malignant glioma cell lines and the large sample of human GBM specimens we characterized, we demonstrated that the majority expressed WT1. All of the tumor samples that expressed the +KTS isoforms, which are the most prevalent in nature[75]. Four out of five cell lines that expressed WT1 expressed the (+KTS) isoforms. One cell line, VC95G, expressed only the (-KTS) isoforms of WT1, raising the possibility of a change due to growth conditions in culture. Nevertheless, the fact that the isoforms expressed by the majority of our glioma cell lines mirror those expressed in human glioblastoma provides the potential for *in vitro* studies to examine the function of WT1 in this disease process. We also screened 16 lower grade glial tumors for WT1 mRNA expression of which 13 expressed WT1. Since a subset of GBM, the secondary GBM, is thought to arise from preexisting lower grade gliomas[258], this finding suggests that aberrant expression of WT1 may be an early change in human gliomagenesis. This is further supported by the observation that, with the exception of one pilocytic astrocytoma specimen, the isoform expression profile of the lower grade gliomas mirrored that of GBM. Normal astrocytes did not express WT1, a finding which was to be expected as WT1, although expressed in restricted areas of the developing CNS, is not expressed in the adult brain[7].

WT1 binds to the tumor suppressor p53, which inhibits p53-mediated apoptosis[136, 137]. More recent studies of WT1/p53 interactions reveal that the presence of either wild-type or mutant p53 can modulate the transcriptional activity of WT1 on the insulin-like growth factor-1 receptor promoter. This finding led the authors to propose that the behavior of WT1 as an oncogene or tumor suppressor may be dictated by the cellular p53 status[87]. We detected mutations in 24% of the samples using PCR-SSCP, similar to the p53 mutation rate described in the brain tumor literature[12]. Previous studies show that PCR-SSCP is both a sensitive and specific method of detecting p53 mutations[10, 232]. However, this method does not detect the small proportion of mutations that exist outside of the exon 5-8 "hot spot" as well as the few mutations that do not affect DNA conformation[12]. We demonstrated an association between WT1 expression and p53 mutation status in GBM samples. Tumors that possess wild-type p53 are more likely to express full-length WT1 than tumors possessing mutant p53. It is therefore possible that WT1 is interacting with wild-type p53, contributing to oncogenic activity by preventing the cell cycle arrest and pro-apoptotic effects of p53. We have observed that silencing the WT1 gene can decrease glioma cell proliferation and improve response to chemotherapy and radiotherapy, results which are consistent with an oncogenic function of WT1(unpublished data).

The majority of the tumor samples appeared to express wild-type WT1. Sequencing of all 10 exons of WT1 would be necessary to definitively conclude that mutations are absent. The detection of WT1 mRNA by primer pair 1 and not primer pair 2 in 4 GBM samples may indicate the expression of a truncated form of WT1. This

phenomenon was not observed in any of the cell lines. Several such truncated forms of WT1 have been characterized in human disease, including cancers such as prostate cancer and Wilms' tumor[40, 212]. Previous studies of GBM did not detect any mutations in the WT1 gene. This is not surprising considering the frequency of mutation that we report here is low (4/50) and could have been overlooked in the studies which performed mutational analyses[43]. Furthermore, we detected the putative mutation by reverse transcriptase PCR using two primer pairs. Other studies have used either immunohistochemistry or real-time PCR to detect WT1, which would not detect such mutations[168, 178]. If confirmed to be expressed in these and other GBM samples, the structure and function of such putative truncated forms of WT1 will need to be examined in future studies.

WT1 expression did not correlate with any of the examined clinicopathologic characteristics of the patients, including survival. Patients with tumors that expressed WT1 appeared to have a lower median survival than those that did not express WT1. However, this was not statistically significant. The fact that the majority of lower grade tumors also expressed WT1 would further suggest that WT1 expression is not a marker for tumor grade. Survival after diagnosis of GBM is currently best predicted by clinical markers of the patient, such as age, Karnofsky performance status, and extent of resection[118, 99] . In fact, recursive partitioning analyses (RPA) identified several risk groups based on these markers to predict prognosis[118, 214]. Unfortunately, the analysis of WT1 correlation with RPA groups would require a larger sample size than that included in this study. To date, no genetic markers clearly predict survival. GBM is a genetically

heterogeneous tumor, with many overlapping pathways apparently leading to a similar clinical outcome[156, 131]. Therefore, molecular changes such as EGFR overexpression/amplification and p53 mutation, while having established functional importance in glioma biology, do not correlate with prognosis[199]. Nevertheless, our study demonstrates that anti-WT1 therapy may be a promising prospect for future glioma therapies. WT1 is expressed by over 80% of GBM specimens and does not appear to be expressed by normal cells. When expressed by GBM cells, WT1 is restricted to two isoforms and, therefore, provides an attractive target. In glioma progression, WT1 appears to be expressed early and may therefore be a target for early treatment before the development of GBM. In vitro and in vivo studies show that anti-WT1 immunotherapy can cause lysis of WT1 expressing tumor cells and rejection of tumor challenge while leaving normal WT1 expressing cells and tissue undamaged[172, 182]. Recently a phase I clinical study demonstrated that anti-WT1 immunotherapy is well tolerated and efficacious in patients with leukemia and solid tumors[181]. GBM is a tumor for which there is no effective treatment. Surgery combined with radiotherapy can improve survival by months, while chemotherapy adds a modest benefit[227]. Full characterization of WT1 expression in tumors such as GBM may establish WT1 as a target for novel therapies.

Conclusions

The results of this study are consistent with an oncogenic function for WT1 in human cancer, possibly by interacting with the p53 tumor suppressor. We believe that WT1 expression is an aberration of normal astrocyte gene expression and is critical to gliomagenesis. Furthermore, the presence of WT1 expression in a high percentage of

gliomas and its absence in normal astrocytes suggests that WT1 may be a valuable marker for gliomas as well as a potential molecular target for this currently incurable disease. This study further argues for continuing efforts to determine the role of WT1 in gliomagenesis. Future studies will focus on the function of WT1+KTS isoforms in human gliomas, specifically in relation to p53.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support of Dr. Nitya Ghatak, the neuropathologist who examined the tumor samples for confirmation of the diagnosis of GBM. We would like to thank Mark Dreusicke for assistance with the collection and compilation of the survival data. We would also like to thank Dr. Erwin Van Meir for the LN-Z308 cell line. The laboratory of Dr. Arti Pandya provided excellent technical assistance and materials for the PCR-SSCP analysis. Dr. Kellie Archer provided indispensable assistance with the statistical analysis of the data. The research presented in this paper was supported in part by a Medical Student Summer Fellowship from the American Brain Tumor Association and by the Hord and Cullather funds of the MCV Foundation.

CHAPTER 3.

DOWN-REGULATION OF WILMS' TUMOR 1 EXPRESSION IN GLIOBLASTOMA CELLS INCREASES RADIOSENSITIVITY INDEPENDENTLY OF P53

Aaron J Clark, Dana C Chan, Mike Y Chen, Helen Fillmore, Wagner G Dos Santos, Timothy E Van Meter, Martin R Graf, William C Broaddus

Department of Neurosurgery, Department of Anatomy and Neurobiology, Virginia Commonwealth Univeristy, School of Medicine, Richmond, Virginia, USA

Abstract

The Wilms' tumor 1 gene is overexpressed in human glioblastoma and correlates with wild-type p53 status. In other cell types, WT1 inhibits p53-mediated apoptosis in response to DNA damaging agents. However, neither this interaction nor the relationship between WT1 and radiosensitivity has been studied in glioblastoma. To study this interaction, we generated LN-229 glioma cell lines (p53 mutant) stably expressing WT1 isoforms and induced apoptosis by transfecting with different doses of wild-type p53 plasmid expression vector. Constitutive expression of WT1 did not protect against exogenous p53-mediated apoptosis. Likewise, WT1 expression did not protect against

endogenous p53-mediated cell death induced by radiotherapy in U87MG cells, which contain functional wild-type p53. We then tested the efficacy of WT1 siRNA in inhibiting WT1 expression and its effect on radiosensitivity. In T98G and LN-18 glioma cells, which possess p53 mutations, WT1 siRNA decreased WT1 protein to almost undetectable levels by 96 hours post-transfection. Furthermore, WT1 siRNA transfection caused a significantly larger decrease in viability following irradiation than was seen in untransfected cells in both cell lines after treatment with ED_{50} of ionizing radiation. In conclusion, WT1 overexpression did not protect against p53-mediated apoptosis or ionizing radiation induced cell death. WT1 siRNA increased the radiosensitivity of two human glioma cell lines independently of p53. Anti-WT1 strategies may, therefore, prove useful in improving the response of glioblastoma to radiotherapy, thus potentially improving patient survival.

Introduction

Glioblastoma multiforme (GBM) is a WHO grade IV glial tumor that carries a universally poor prognosis with a median survival of approximately one year after diagnosis[27]. Following surgery or biopsy, radiotherapy, often in conjunction with adjuvant chemotherapy, modestly improves survival, however glioma cell resistance to radiotherapy remains a major obstacle [253, 224, 252]. Decades of intense research have identified several molecular mechanisms of glioma cell radioresistance[74, 28]. Despite these advances, a significant clinical improvement has not been realized. Therefore, a more complete characterization of the molecular mechanisms of resistance is necessary to uncover targets for novel therapies. Previous studies by our laboratory and others have

examined the expression of the Wilms' tumor 1 (WT1) gene in human brain tumors, including gliomas of all grades, and have found detectable expression in tumor cells but not in normal cells, suggesting an important role for WT1 in glioma biology[30, 152, 43, 168, 178]. However no studies to date demonstrate a relationship between WT1 and resistance to commonly administered therapies in gliomas.

WT1 was originally discovered as mutated in nephroblastoma, a common pediatric kidney cancer also known as Wilms' tumor[23]. It was subsequently found that the *wt1* gene, located at chromosome locus 11p13, encodes a 52-55 kD polypeptide, the structural homology and function of which designate it as a zinc finger transcription factor[75]. Furthermore, the messenger RNA transcript is alternatively spliced to generate four main isoforms, designated +/+, -/+, +/-, -/-, depending on the inclusion or exclusion of exon 5 and nine nucleotides in exon 9 known as KTS, respectively[75]. WT1(+KTS) isoforms, which gliomas express[30], may be involved in RNA processing in addition to having both overlapping and unique transcriptional properties relative to WT1(-KTS) isoforms[38, 83]. The functional significance of exon 5 is less well understood, but may affect its interaction with other proteins[200]. Although originally classified as a tumor suppressor, WT1 was later found to be overexpressed in many types of cancer, including breast carcinoma and acute leukemia, suggesting an oncogenic function for the gene[90, 159]. In fact, inhibition of WT1 expression in human breast cancer and leukemia cell lines using antisense modalities leads to differentiation, decreased proliferation, and increased sensitivity to treatment[229, 270].

One possible mechanism by which WT1 could exert a tumorigenic effect in glioma cells is through an interaction with p53. Inactivation of the tumor suppressor p53 is a common aberration in human glioma progression[12]. p53 causes cell-cycle arrest and apoptosis in response to DNA damage[248]. Many treatments for glioma, such as radio- and chemotherapy, cause DNA damage, and thus are likely to rely on p53 function for a therapeutic response[133, 10]. While the effect of p53 mutation on glioma response to radiation is uncertain, p53 status has prognostic significance in other malignant tumors[230, 4]. In cultured glioblastoma cells, p53 mutation promotes radioresistance[266, 74]. Studies by our laboratory and others show that introduction of wild-type p53 into glioma cells using various transduction methods increases radiosensitivity both *in vitro* and *in vivo*[14, 8, 66, 34]. Alterations in other genes, such as *mdm2,* which targets p53 for degradation, represent alternative means of inactivating p53 in gliomas[211]. In Saos-2 osteosarcoma cells (*p53* null), WT1 inhibits p53-mediated apoptosis induced by overexpression of wild-type p53, chemotherapy, and radiation[136]. We recently reported that, in a large sample of glioblastoma tissue specimens, WT1 expression correlates with the presence of wild-type p53[30].

This prompted us to examine if a functional relationship exists between WT1 and p53 in the context of glioma radiosensitivity. In this study, we investigated the effect of both WT1 overexpression and WT1 silencing on p53-mediated cell death and on response to radiotherapy. We found that, while WT1 overexpression does not protect glioma cells against p53 mediated apoptosis or radiation-induced cell death, WT1 silencing sensitizes

glioma cells to radiation-induced arrest and death. These results suggest that WT1 may be a potential target to improve the response of glioblastoma to radiotherapy.

Materials and Methods

Cell culture and tissue preparation

The p53 status of the glioma cell lines used in this study has been previously determined (Table 3-1)[92], and confirmed in our laboratory by RT-PCR and cDNA sequencing[14].

Table 3-1. WT1 and p53 status of the glioblastoma cell lines used in this study

Cell line	*p53* status (codon)[92]	WT1 mRNA / 18S[30]
U87MG	*wild-type*	0 ± 0
T98G	*mutant* (237, Met→Ile)	103.61 ± 7.9
LN-18	*mutant* (238, Cys→Ser)	214.70 ± 20.9
LN-229	*mutant* (98, Pro→Lys)	0 ± 0

LN-229, U87MG, LN-18, and T98G human glioma cells were obtained from American Type Culture Collection and were grown and passaged in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, glutamine, non-essential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. Total RNA was isolated from cell lines using the Trizol extraction protocol (Invitrogen). The concentration of RNA was determined by spectrophotometry. Protein was extracted from cell lines using SDS buffer (50 mM Tris-Cl, 1% SDS, 10% glycerol) supplemented with protease inhibitors. The concentration of protein was determined by DC protein assay (BioRad).

Plasmid construction and transient transfection

WT1 expression plasmids (in pcDNA3, Invitrogen) containing either WT1(+/+) or WT1(-/+) isoforms were kindly provided by Dr. Charles T. Roberts, Jr. and have been described previously[87]. Expression plasmids containing wild-type p53 (in pHCMV-Neo-Bam) were kindly provided by Dr. Sumitra Deb and have also been previously described[39]. Cells were plated in triplicate at a density of 2 X 10^5 in six well plates and allowed to attach overnight in DMEM without penicillin/streptomycin. Cells were transfected with a mixture of 3 µl Lipofectamine 2000 (Invitrogen) and plasmid diluted in 500 µl DMEM without fetal bovine serum or penicillin-streptomycin and incubated for 4 hours at 37° C. After 4 hours, cells were supplemented with 1.5 ml DMEM with 10% fetal bovine serum without penicillin-streptomycin. Twenty four hours after transfection, the conditioned media was removed and replaced with 2.0 ml fresh DMEM with 10% fetal bovine serum without penicillin/streptomycin.

Short interfering RNA transfection

Transfections with WT1 siRNA were carried out using the pre-designed siGENOME SMARTpool containing 4 siRNA sequences directed against WT1 (Dharmacon). A pool of 4 non-targeting siRNA sequences (si*CONTROL* Non-Targeting Pool, Dharmacon) was used as a control and will be referred to as siScramble. Cells were plated at a density of 2.5 X 10^5 cells in six well plates and allowed to attach overnight. Cells were then transfected using Oligofectamine (Invitrogen) diluted in 1 ml 1x Opti-MEM Reduced Serum Medium (Invitrogen) supplemented with 1% penicillin/ streptomycin. After a 4 hour incubation at 37°C, 500 µL of 3x FBS/Opti-MEM solution was added to each well. The final siRNA concentration was 100nM. 24 hours after transfection, cells were trypsinized, resuspended in fresh media, and either lysed for protein/RNA extraction or re-plated for clonogenic cell survival assays and luminescent cell viability assays.

Generation of stable transfectants

LN-229 and U87MG cells were plated at a density of 2 X 10^5 in six well plates and allowed to attach overnight. Cells were transfected with 1.0 µg WT1 isoform expression vectors as described above for transient transfection. Forty eight hours after transfection, cells were selected in DMEM containing 600 µg/ml Geneticin (Invitrogen) for LN229 and 200 µg/ml for U87MG. Selection media was exchanged every 4 days.

Irradiation

Forty eight hours after siRNA transfection or after generation of stable transfectants, cells were treated at room temperature with γ-irradiation using a Cesium-source Mark I Irradiator (Cs-137, 4.149Gy/min) at indicated doses.

Western blot analysis

For protein analysis of cells transfected with WT1, 10 μg of total protein lysate were separated by SDS–PAGE and transferred to nitrocellulose membrane as per manufacturers' protocol (Invitrogen). The membrane was then blocked with 5% nonfat milk solution for 1 hour at room temperature. Rabbit anti-WT1 polyclonal antibody (1:200 dilution, C-19, Santa Cruz Biotechnology), mouse anti-p53 monoclonal antibody (1:1000, Ab-6, Oncogene Research Products), and mouse anti-p21 monoclonal antibody (1:200, DakoCytomation) were diluted in blocking buffer. The membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed six times in Tris buffered saline containing 0.05% Tween-20 before and after a one hour incubation at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000, Rockland, Inc.) for WT1, and anti-mouse secondary antibody (1:2000, Rockland, Inc.) for p53 and p21. Blots were developed using the ECL Detection System (Amersham Biosciences). Anti-Cyclophilin A monoclonal antibody (1:1000, Upstate Biotechnology) was used as a control for protein loading.

Trypan blue exclusion viability assay

After treatment, cells were gently trypsinized from the culture surface, centrifuged, and the cell pellet resuspended in 1 ml of DMEM. A 1:5 dilution of cells was made with a

solution containing 30 µl 0.4% trypan blue in a total volume of 100µl. Viable cells, which had excluded the dye, were counted by light microscopy. All treatments were performed in triplicate.

Luminescent viability assay

Cells were plated in 5 - 10 replicates per treatment group in white 96 well plates at a density of 1000 cells. At the specified time after treatment, CellTiter-Glo Luminescent Cell Viability Assay (Promega) was performed according to the protocol supplied by the manufacturer. Luminescence was detected by Lumi Star Luminometer (BMG). Percent survival was calculated by taking the relative luminescence unit of each irradiated replicate of a treatment group and dividing it by the average of the corresponding non-irradiated treatment group.

Detection of apoptosis

After treatment, cells were trypsinized, washed with PBS, and fixed for 30 minutes in 1% paraformaldehyde at a concentration of 1-2 X 10^6 cells/ml. Prior to analysis, fixed cells were stored at 4° C in 70% ethanol. DNA cleavage was used as an indicator of apoptosis. Treated cells were subjected TUNEL (APO-DIRECT, BD Biosciences) followed by flow cytometry (FACScanto, BD Biosciences) to detect strand breaks. Threshold level for forward scatter was set to exclude cellular debris and TUNEL-positive cells were measured in the FL-2 channel (FITC).

Clonogenic survival assay

Following siRNA transfection, 1 X 10^2 LN-18 cells were plated in triplicate in six well plates. Plates were irradiated 48 hours after transfection as described above. Eight

days after irradiation, cells were fixed for 30 minutes in methanol and stained with 50% Giemsa for one hour. Cells were then washed with distilled water and colonies consisting of ≥ 50 cells were counted.

Statistical analysis

Differences between multiple treatment groups were analyzed by analysis of variance (ANOVA) using the JMPIN 4.0.4 (SAS Institute, Inc.) software. Tukey- Kramer honestly significant difference test (HSD) was performed as a post-hoc analysis. Differences between only two treatment groups were analyzed by Student's t-test.

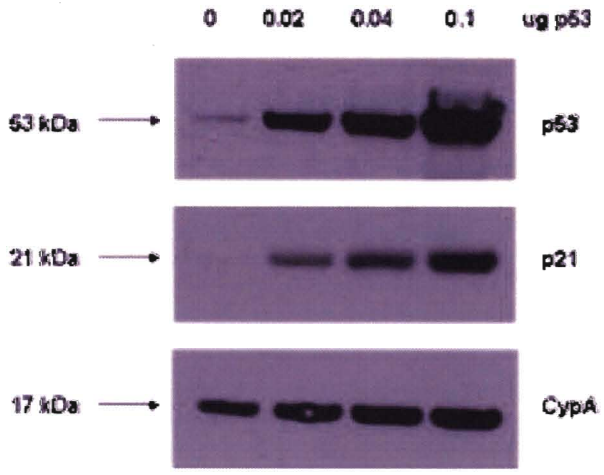
Results

LN-229 glioma cells undergo apoptosis in response to p53 overexpression. To examine the functional relationship between WT1 and p53, we developed a glioma cell model of p53 mediated apoptosis. LN-229 glioma cells do not express endogenous WT1[30] and harbor a p53 mutation. Transfection of LN-229 cells with increasing amounts of wild-type p53 expression vector led to an increase in p53 protein expression and a concomitant up-regulation of the p53 target gene, p21, indicating that the exogenous wild-type p53 is functional (Figure 3-1A). Increasing amounts of wild-type p53 expression vector resulted in a decrease in cell viability relative to cells transfected with the empty vector, which was maximal at a dose of 0.1 μg, and still present at 0.04 μg (Figure 3-1B). The decrease in viability of cells transfected with p53 relative to empty vector was most prominent at 4 days after transfection (Figure 3-1C). Based on the above results, we chose to focus the subsequent experiments on the 0.1 μg p53 treatment dose, and to examine viability at four days after transfection. To determine if the cell death

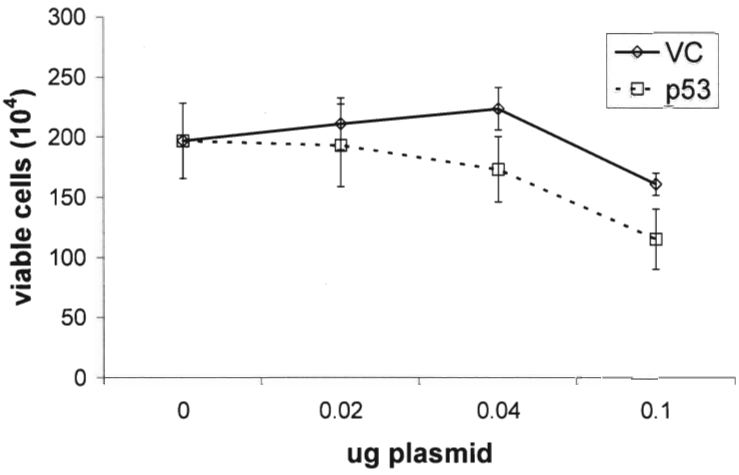
induced by p53 was due to apoptosis, LN-229 cells were transfected with 0.1 μg p53 and subjected to to terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) analysis to detect DNA cleavage which is a hallmark feature of apoptosis. Untransfected control and cells transfected with vector alone exhibited minimal DNA cleavage (1.8 and 1.7 %, respectively, Figure 1D). There was a significant increase in TUNEL positive cells after p53 transfection (10.5 %, $p = 0.007$, Figure 3-1D). This suggests that LN-229 underwent increased apoptosis in response to transient p53 expression.

Figure 3-1. Transient transfection of p53 in LN-229 human glioblastoma cells. A. LN-229 cells were transfected with increasing doses of p53 plasmid. Protein was extracted 24 hours after transfection. Western blot demonstrated p53 protein expression and up-regulation of the p53 target gene, p21. Anti-cyclophilin A was used to estimate equal protein loading. B. LN-229 cells were transfected with increasing amounts of p53 plasmid. Four days after transfection, cells were trypsinized and analyzed for cell viability by trypan blue exclusion assay. All treatments were performed in triplicate. C. LN-229 cells were transfected with 0.1 μg p53 plasmid. Cells were trypsinized at 1, 2, 4, and 6 days after transfection and analyzed by trypan blue exclusion assay. All treatments were performed in triplicate. D. LN-229 cells were transfected with 0.1 μg p53. Four days after transfection cells were subjected to TUNEL. Cells were then analyzed by FACS to detect DNA cleavage indicative of apoptosis (X-axis, FITC). Anotated numbers represent percentage of cells positive for apoptosis. The graphs are respresentative of treatments performed in triplicate. The experiment was repeated with similar results. Left panel, LN229 untransfected control; middle panel, LN229 + empty vector; right panel, LN229 +p53, CypA cyclophilin A; VC vector control.

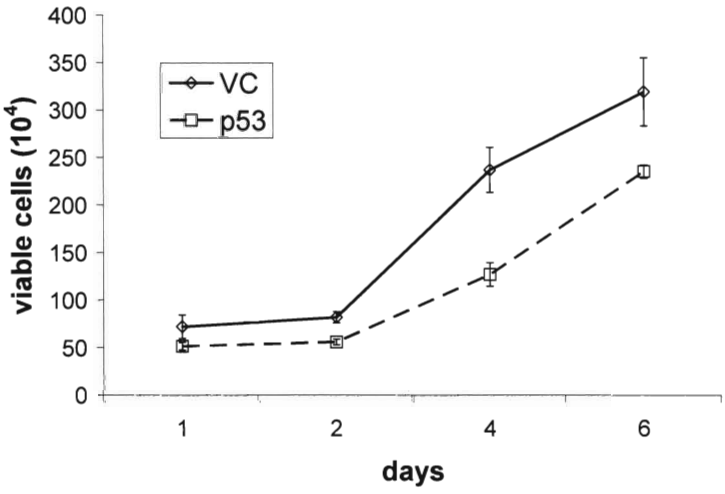
A



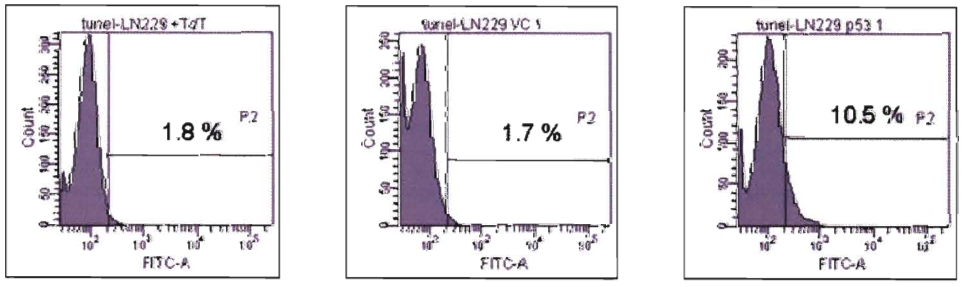
B


300
250
200
150
100
50
0
viable cells (10^4)
VC
p53
0
0.02
0.04
0.1
ug plasmid

C


400
350
300
250
200
150
100
50
0
viable cells (10^4)
VC
p53
1
2
4
6
days

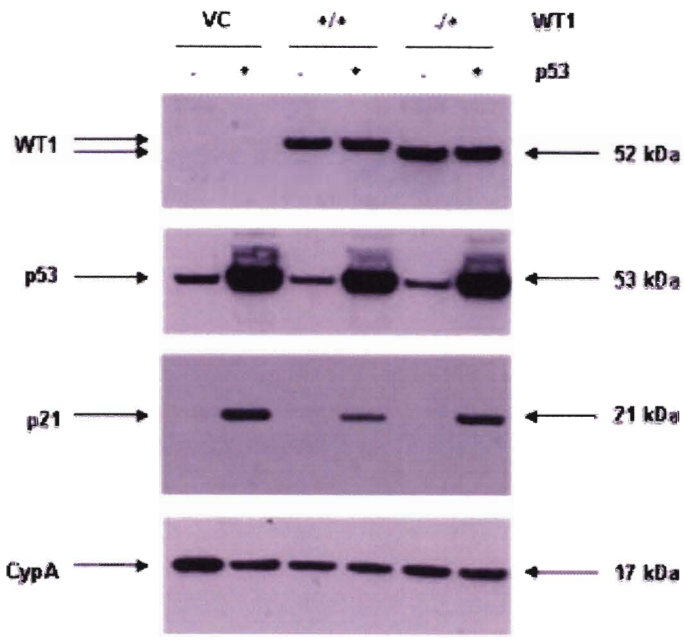
D


tunel-LN229 +TdT
1.8 %
P2
Count
FITC-A
tunel-LN229 VC 1
1.7 %
P2
Count
FITC-A
tunel-LN229 p53 1
10.5 %
P2
Count
FITC-A

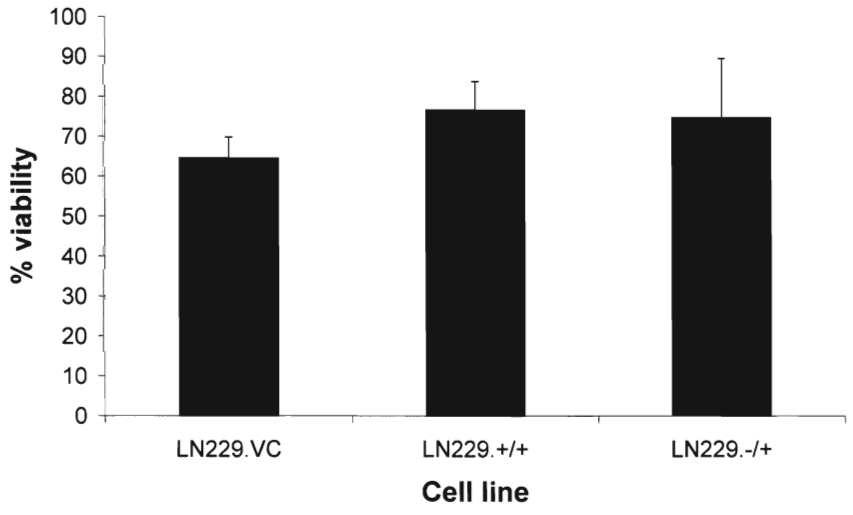
Stable expression of WT1 isoforms does not protect LN-229 cells from p53-induced cell death. Using a liposomal delivery system, we generated pooled LN-229 stable transfectants expressing either empty pcDNA3 vector or pcDNA3 containing WT1(+/+) or WT1(-/+) isoforms, which are the isoforms expressed in human glioblastoma. These cells were named LN229.VC, LN229.+/+, and LN229.-/+, respectively. After maintenance for several months in selection media, the cell lines were analyzed for WT1 isoform expression by western blot. LN229.VC did not express detectable WT1 protein, while LN229.+/+ expressed a 55 kDa protein corresponding to higher molecular weight WT1(+/+) relative to LN229.-/+ which expressed the 52 kDa WT1(-/+) protein (Figure 3-2A and C, lanes 1, 3, and 5). To test the effect of WT1 expression on p53-mediated apoptosis, the cell lines were transfected with either empty vector or wild-type p53 at doses of both 0.1 μg and 0.04 μg. Cells transfected with empty vector expressed baseline levels of p53 protein which is expected as p53 mutations cause stabilization of the protein[5] (Figure 3-2A and C, lanes 1, 3, and 5). Cells transfected with p53 expressed very high levels of p53 and showed up-regulation of p21 (Figure 3-2A and C, lanes 2, 4, and 6). Four days after transfection, there was no difference in percent viability between the stable cell lines after transient p53 expression determined by trypan blue exclusion at either the 0.1 μg or 0.04 μg dose ($p = 0.19$ and 0.67, respectively, Figure 3-2C and D).

Figure 3-2. Effect of transient p53 expression in LN-229 cells stably transfected with WT1 isoforms. LN-229 cells expressed p53 and p21 after transfection with 0.1 μg (A) and 0.04 μg (C) p53 plasmid. Blots were reprobed with anti-cylcophilin A to control for equal protein loading. Trypan blue exclusion assay depicting the change in % viability after transfection with 0.1 μg (B) or 0.04 μg (D) p53 plasmid. Y axis depicts change in viability between cells transfected with p53 and cells transfected with empty vector. LN229.VC, LN229 cells stably expressing empty vector; LN229.+/+, LN229 cells stably expressing WT1 (+/+) isoform; LN229.-/, LN229 cells stably expressing WT1 (-/+) isoform Error bars represent the standard error (B) or standard deviation (D). VC, vector control; CypA, cyclophilin A.

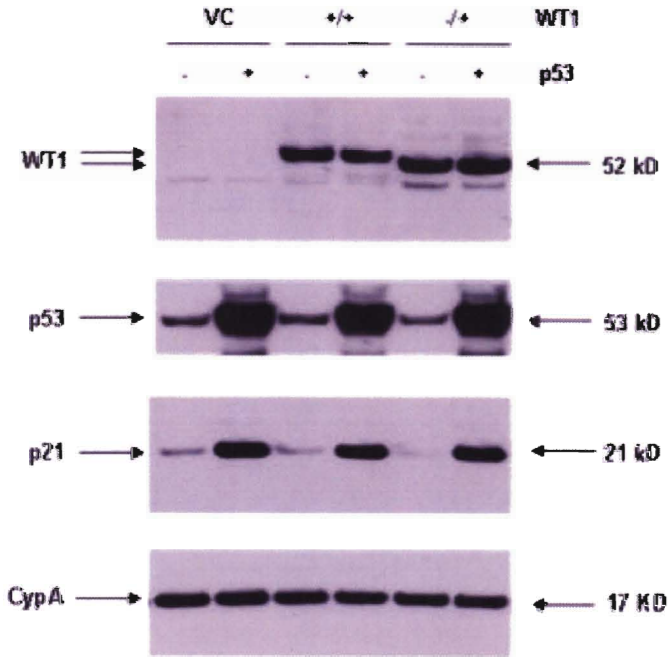
A


VC
+/+
-/+
WT1
-
+
-
+
-
+
p53
WT1
52 kDa
p53
53 kDa
p21
21 kDa
CypA
17 kDa

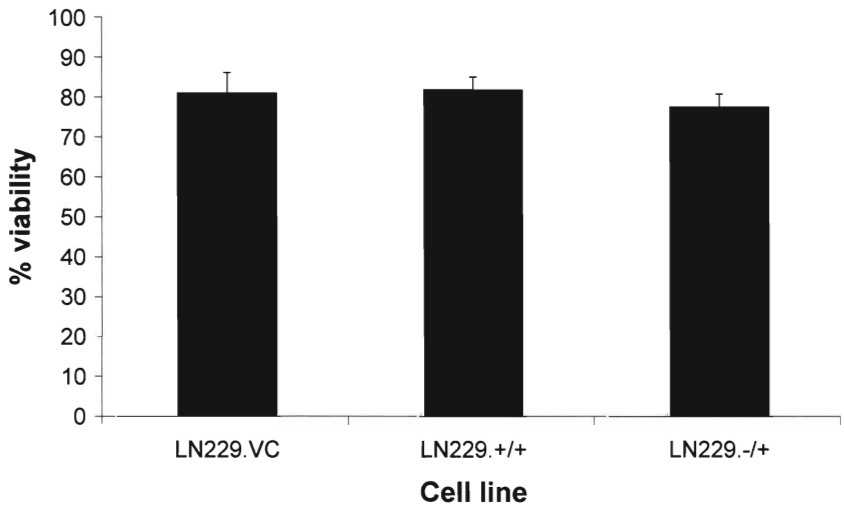
B


100
90
80
70
60
50
40
30
20
10
0
% viability
LN229.VC
LN229.+/+
LN229.-/+
Cell line

C


VC
+/+
-/+
WT1
-
+
-
+
-
+
p53
WT1
52 kD
p53
53 kD
p21
21 kD
CypA
17 KD

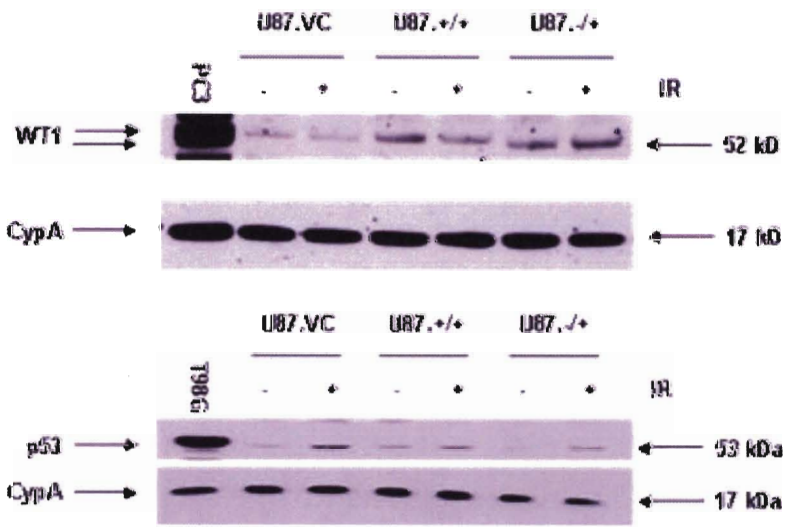
D


100
90
80
70
60
50
40
30
20
10
0
% viability
LN229.VC
LN229.+/+
LN229.-/+
Cell line

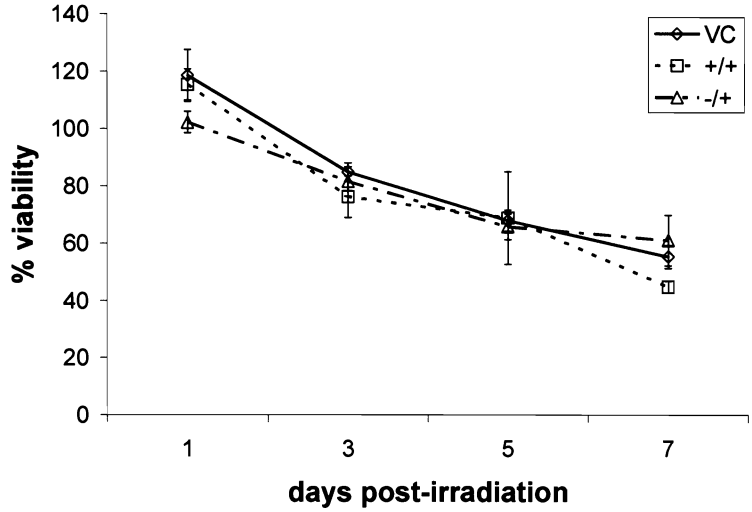
Stable expression of WT1 isoforms does not protect against radiation induced cell death. We then tested the effect of WT1 expression on an endogenous p53 response. U87MG cells have wild-type p53 status and undergo functional p53 signaling pathways following treatment with ionizing radiation[92, 254]. U87MG cells also do not express WT1[30]. We generated pooled U87MG cell lines stably expressing either empty vector, WT1(+/+), or WT1(-/+), referred to as U87.VC, U87.+/+, and U87.-/+, respectively. Western blot showed that U87.VC did not express detectable WT1 protein, while U87.+/+ and U87.-/+ expressed WT1 isoforms of their corresponding molecular weights (Figure 3-3A, top panel, lanes 1, 3, and 5). Twenty four hours after exposure of the cells to 6 Gy of ionizing radiation, there was detectable up-regulation of p53 protein relative to non-irradiated cells (Figure 3-3A, bottom panel). Irradiation caused a time dependent decrease in viability of the U87MG cells reaching 45% relative to non-irradiated cells at 7 days after treatment, however stable expression of WT1 did not cause a change in percent viability (Figure 3-3B). The experiment was repeated 3 times at the 7 day time point with similar results (Figure 3-3C).

Figure 3-3. Effect of ionizing radiation on U87MG cells, which express wild-type p53, stably transfected with WT1 isoforms. A. U87MG cells were transfected with expression plasmids containing WT1 isoforms, and selected for stable expression. Pooled stable cell lines were treated with 6 Gy ionizing radiation. Protein was extracted 24 hours after irradiation. Western blot demonstrated WT1 expression in U87MG after stable transfection (top panel). Western blot demonstrated p53 expression in U87 stable transfectants after treatment with 6 Gy ionizing radiation (bottom panel). B. U87MG cells were treated with 6 Gy ionizing radiation. Viability assay based on cellular content of ATP demonstrated a decrease in viability of the U87MG stable transfectants at 1, 3, 5, and 7 days relative to non-irradiated cells. All treatments were performed in quintuplicate. C. ATP viability assay demonstrated a decrease in viability of the U87MG stable transfectants at 7 days after irradiation (n = 3).

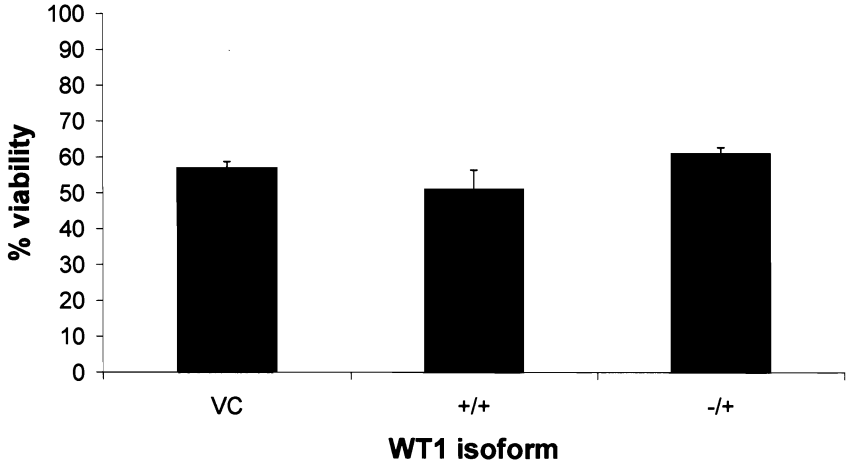
A


U87.VC
U87.+/+
U87./+
Ctl
IR
WT1
52 kD
CypA
17 kD
U87.VC
U87.+/+
U87./+
T98G
IR
p53
53 kDa
CypA
17 kDa

B


140
120
100
80
60
40
20
0
% viability
VC
+/+
-/+
1
3
5
7
days post-irradiation

C


100
90
80
70
60
50
40
30
20
10
0
% viability
VC
+/+
-/+
WT1 isoform

WT1 siRNA transfection increased sensitivity to radiotherapy. We then determined if anti-WT1 strategies would sensitize glioblastoma cells to radiotherapy independently of p53-mediated cell death pathways. We used T98G and LN-18 glioma cells, both of which harbor p53 mutations and express endogenous WT1[30, 92]. To characterize the response of these cells to radiotherapy, cells were treated with increasing doses of radiation and, 5 days after treatment, viability was assessed. Increasing doses of radiation caused a progressive decrease in percent viability relative to untreated controls in both cell lines (Figure 3-4A). Further analysis determined the ED_{50} dose (dose expected to cause a 50% decrease) of ionizing radiation to be 7 Gy for T98G and 5 Gy for LN-18. Cells were then treated with the respective ED_{50} dose of radiation, and percent viability was measured at 1, 3, 5, 7, and 9 days. In both cell lines, viability decreased to a minimum at 5 days after which they began to recover (Figure 3-4B). Subsequent cytotoxicity experiments were performed at the ED_{50} doses and responses measured at 5 days post-irradiation. To confirm that ionizing radiation caused a decrease in the capacity for clonogenic survival, LN-18 cells were subjected to increasing doses of irradiation. Eight days after treatment, Giemsa staining demonstrated a decrease in fractional survival relative to nonirradiated cells with an ED_{50} of 2 Gy (Figure 3-4C).

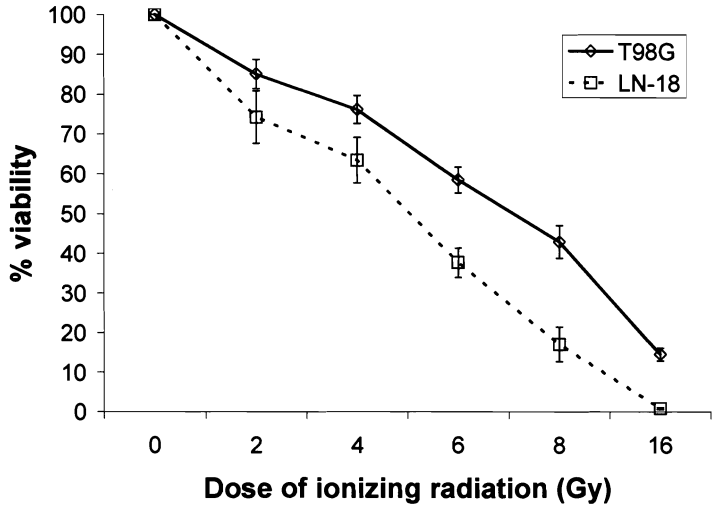
We then tested the efficacy of siRNA targeting WT1 in these cell lines. T98G and LN-18 cells were transfected with siRNA and relative WT1 protein levels were measured 96 hours later. Western blotting demonstrated that WT1 protein expression was inhibited at 96 hours post-transfection in T98G (Figure 3-4D, inset) and LN-18 (Figure 3-4E, inset). Densitometric analysis of levels of WT1 normalized to cyclophilin A showed that WT1

protein expression was decreased by almost 100% at 96 hours in T98G cells and LN-18 cells.

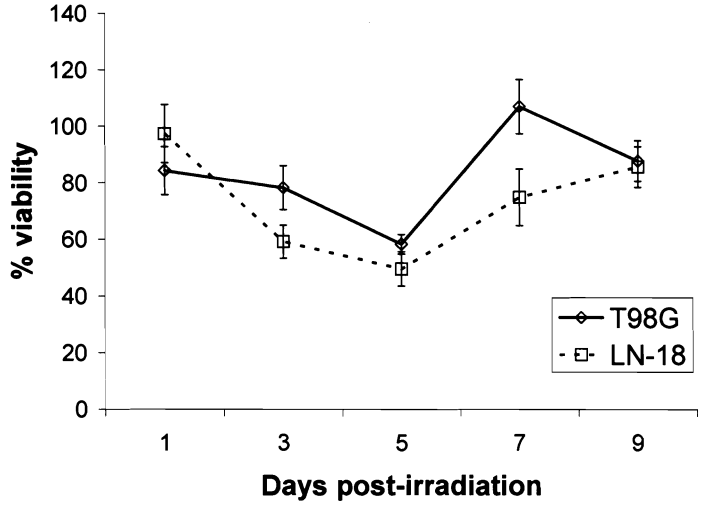
To determine if WT1 targeting could improve the response to radiotherapy, we transfected the cells with WT1 siRNA and 48 hours later subjected them to ED_{50} doses of ionizing radiation. Viability assays performed 5 days after irradiation showed a significantly larger decrease in viable cells pre-treated with WT1 siRNA than either untransfected or vehicle treated T98G (Figure 3-4D) and LN-18 cells (Figure 3-4E). T98G cells transfected with WT1 siRNA followed by irradiation had a 42.5% viability while those untransfected or treated with vehicle alone had 68.8% and 55.8%, respectively ($p < 0.0001$). LN-18 cells had a 43.2% viability after combination treatment, compared to 87.2% and 73.7% for untransfected and vehicle alone, respectively ($p < 0.0001$). In addition, WT1 siRNA caused a smaller, but still significant, decrease in fractional survival after irradiation in LN-18 cells as measured by the clonogenic survival assay ($p < 0.0001$, Figure 3-4F). The fractional survival of cells transfected with WT1 siRNA after irradiation was 0.38, while untransfected cells and those treated with vehicle alone had a fractional survival of 0.50 and 0.47, respectively.

Figure 3-4. Effect of ionizing radiation combined with WT1 siRNA treatment. A. T98G and LN-18 cells were treated with increasing doses of ionizing radiation and at 5 days after treatment percent viability relative to non-irradiated cells was then measured by an ATP based cell viability assay. B. T98G and LN-18 cells were treated with their respective ED_{50} of ionizing radiation and percent viability was measured at 1, 3, 5, 7, and 9 days post-irradiation. C. LN-18 cells plated at very low density were subjected to increasing doses of ionizing radiation. After 8 days colonies were stained with Giemsa and counted to determine the fractional survival relative to untreated cells. T98G (D, inset) and LN-18 (E,inset) cells were treated with siRNA and protein was extracted at 24 and 96 hours after transfection. Western blotting demonstrated knock-down of WT1 protein. Blots were re-probed with anti-cyclophilin A to control for equal protein loading. Anti-WT1 siRNA decreased viability of T98G (D) and LN-18 (E) human glioblastoma cells after treatment with ionizing radiation. Cells either remained un-transfected, or were treated with Oligofectamine vehicle or siRNA. After 48 hours, cells were treated with their respective ED_{50} of ionizing radiation. Luminescent cell viability assay was performed 5 days after irradiation. The data is presented as percent viability of the irradiated cells relative to non-irradiated controls which underwent the same transfection procedure. Error bars represent the standard deviation. Graphs are representative of three experiments with similar results. * $p < 0.01$ by Tukey's HSD. F. LN-18 cells were irradiated 48 hours after treatment with siRNA. Giemsa staining and colony counting were performed 8 days after irradiation ($n = 3$). Error bars represent the s.e.m. * $p < 0.01$ by Tukey-Kramer HSD; UT, untransfected; Of, oligofectamine alone; Si, siRNA

A

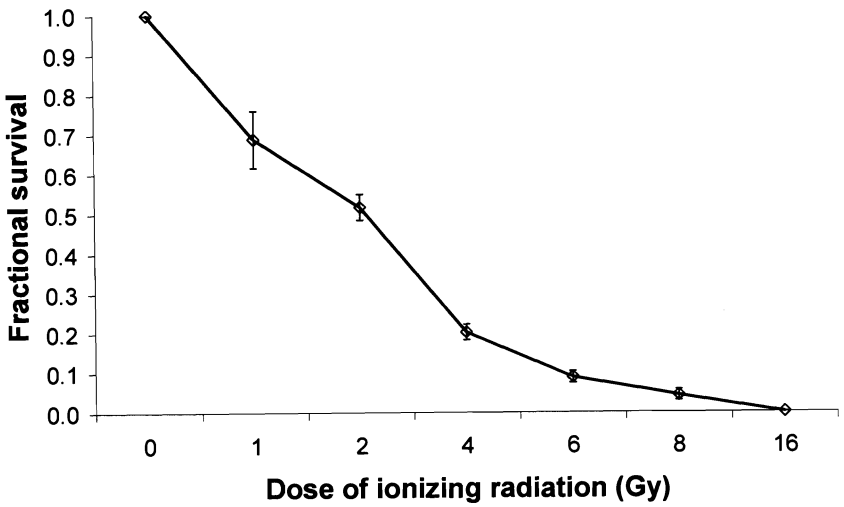


B



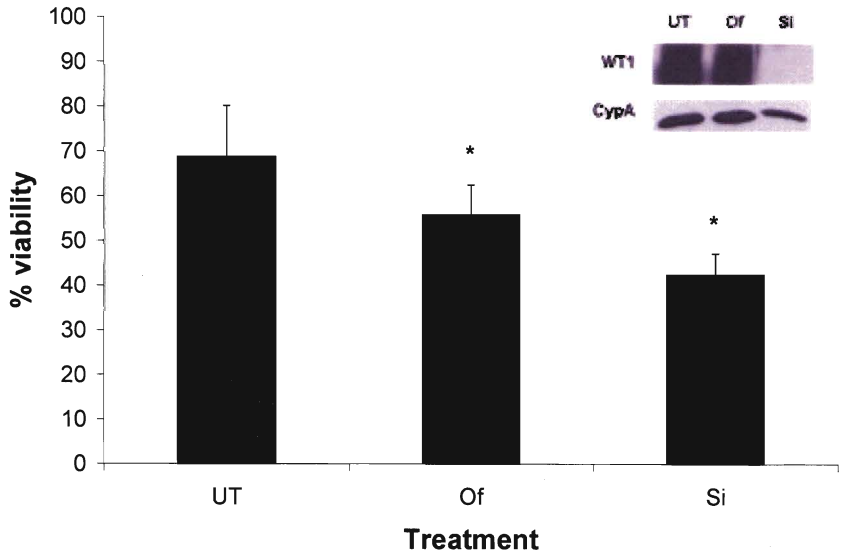
Data courtesy of Dana C Chan, M.S.

C

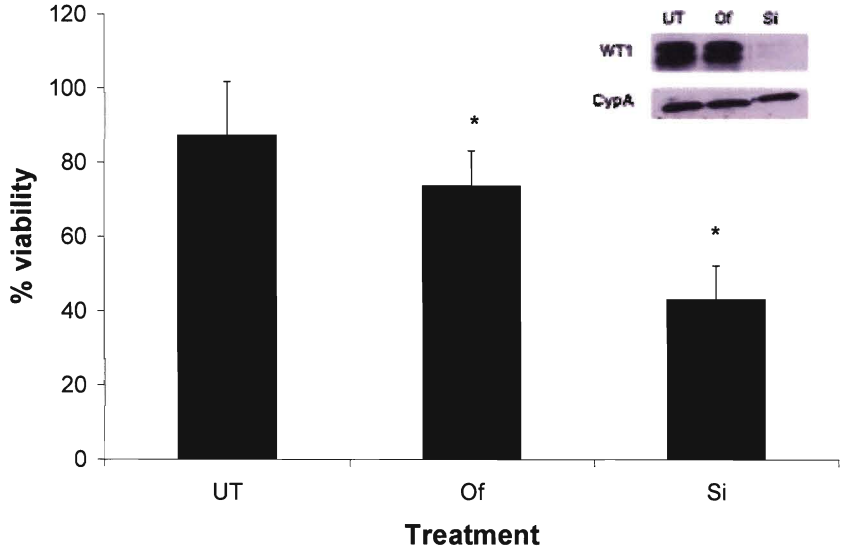


Data courtesy of Dana C Chan, M.S.

D

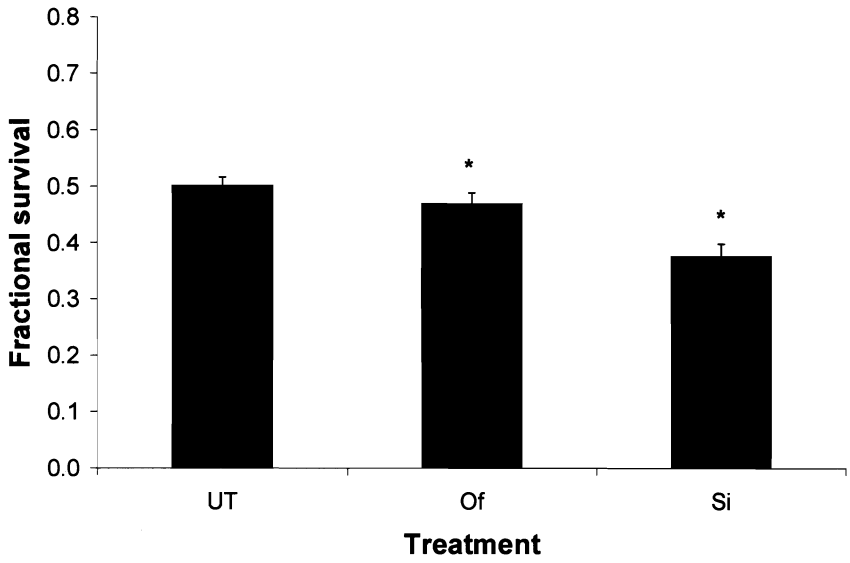


E



Data courtesy of Dana C Chan, M.S.

F



Data courtesy of Dana C Chan, M.S.

Discussion

Based on reports in the literature, we hypothesized that WT1 would be involved in glioma cell response to radiotherapy by inhibiting p53-mediated apoptosis[136]. To examine this possible interaction, we overexpressed WT1(+/+) and (-/+) isoforms in LN-229 glioblastoma cells and found that WT1 expression did not inhibit apoptosis induced by transient p53 transfection. Likewise, we found that WT1 overexpression in U87MG cells did not protect against p53-mediated cell death in response to irradiation. We then used WT1 siRNA to silence WT1 expression in T98G and LN-18 glioblastoma cells, both of which harbor p53 mutations. We found that WT1 silencing increased radiosensitivity as demonstrated by a decrease in viability and clonogenicity after treatment with ionizing radiation.

The effect of WT1 on apoptosis is highly variable. Both pro- and anti-apoptotic effects have been described, depending on the cell type and tissue studied[48, 144]. Low levels of WT1 inhibit apoptosis by interaction with p53[136]. In the same study, the functional interaction was primarily studied using the WT1(-KTS) isoforms, however a more modest anti-apoptotic response was observed with WT1(+KTS) isoforms as well. We chose to study the WT1(+KTS) isoforms in this work because we have previously shown that gliomas express WT1(+KTS) isoforms exclusively[30]. High levels of WT1 induce apoptosis independently of p53 in osteosarcoma cell lines. This apoptosis is associated with down-regulation of the epidermal growth factor receptor (EGFR) and induction of p21[48, 49]. However, these studies only show an effect of WT1(-KTS) isoforms. Nevertheless, apoptosis induced by high levels of WT1 in the stably transfected

cell lines used in this study may have masked the WT1-p53 interaction. Studies examining the effect of lower levels of WT1 on p53 mediated cell death in human glioblastoma cells are ongoing. Here we showed that the cell death associated with wild-type p53 in LN-229 glioblastoma cells correlated with an approximately 10% increase in apoptotic cells. It is therefore possible that some of the decrease in viability was due to p53-dependent non-apoptotic mechanisms, such as cell cycle arrest, autophagy, senescence, or differentiation[33, 248]. No studies to date have examined the ability of WT1 to inhibit these p53 responses.

WT1 also inhibits apoptosis by p53-independent mechanisms. WT1 directly upregulates Bcl-2 in cell lines of different p53 status[144]. This upregulation of the anti-apoptotic protein protects cells from apoptosis induced by DNA damaging chemotherapy agents. Conversely, WT1 siRNA targeting WT1(+exon5) isoforms induces apoptosis in leukemia cell lines which is associated with up-regulation of the pro-apoptotic Bcl-2 family member Bax[93]. In the same study, Ito *et al.* demonstrate that overexpression of WT1 protects leukemia cells from apoptosis induced by chemotherapeutic agents which is associated with downregulation of Bak. In addition, WT1 modulates the expression of EGFR[127] and platelet-derived growth factor (PDGF)[255]. These growth factors and receptors participate in glioma cell resistance to radiotherapy[28, 84]. The effect of WT1 silencing on radiosensitivity observed in this study may be due to p53-independent effects of WT1 on the transcription of these genes.

In addition to p53, other protein-protein interactions modulate the activity of WT1[210]. WT1 physically interacts with the other members of the p53 family of

proteins, p73 and p63[209]. In this study, Scharnhorst *et al.* show that WT1 inhibits p73-mediated transactivation, while p73 reciprocally inhibits DNA binding by WT1. Both p73 and p63 induce apoptosis and are involved in the cellular response to ionizing radiation[268, 207]. WT1(+exon5) isoforms also physically bind to the prostate apoptosis response factor, par4, and inhibit par4-mediated cell death in response to radiation and chemotherapy. Par4 also modulates the function of WT1 as a transcription factor[200]. Therefore, the presence or absence of factors that interact with and modify the function of WT1 in the different cell lines used in this study could be responsible for the differential results obtained from the overexpression and silencing series of experiments.

In contrast to previous studies in other non-glioma cell lines, here we show that in human glioblastoma cell lines, WT1 does not protect against p53-mediated apoptosis induced by either p53 overexpression or ionizing radiation. This study documents for the first time that WT1 in gliomas is unlikely to be functionally related to these cellular responses in gliomas, and underscores the reported cell and tissue type dependence of WT1 function. Nevertheless, we show that WT1 silencing by siRNA can improve the radiosensitivity of glioblastoma cells independently of p53-mediated cell death pathways. This suggests that anti-WT1 therapies may have the potential to improve the response of human brain tumors to radiotherapy. Further study of the molecular mechanisms of WT1 function in gliomas promises to identify strategies to improve glioblastoma radiosensitivity in an effort to improve prognosis of this difficult disease.

ACKNOWLEDGEMENTS

We thank Dr. Jessica McCready for critically reading the manuscript. We also thank Dr. Charles T. Roberts, Jr. and Dr. Sumitra Deb for generously providing WT1 and p53 plasmids. The research presented in this paper was supported in part by a Medical Student Summer Fellowship from the American Brain Tumor Association and by the Hord and Cullather funds of the MCV Foundation.

CHAPTER 4.

WILMS' TUMOR 1 GENE SILENCING DECREASES THE TUMORIGENICITY OF HUMAN GLIOBLASTOMA CELLS

Aaron J Clark, Joy L Ware, Mike Y Chen, Timothy E Van Meter, Martin R Graf, Wagner G Dos Santos, Helen Fillmore, William C Broaddus

Department of Anatomy and Neurobiology, Department of Neurosurgery, Department of Pathology, Department of Human Genetics, Virginia Commonwealth University, School of Medicine, Richmond, Virginia, USA

Abstract

Wilms' tumor 1 (WT1) is overexpressed in many human cancers, including glioblastoma. We have previously shown that transient WT1 silencing increases radiosensitivity in glioma cells. Studies of non-glioma cell lines demonstrate that WT1 promotes cell proliferation and survival. However this has not been rigorously studied in glioblastoma. We tested the efficacy of two sequences of short hairpin RNA (shRNA) directed against WT1 in U251MG human glioblastoma cells and found that one sequence was capable of stably silencing WT1 expression. Stable WT1 shRNA expression

significantly decreased the proliferation of U251MG cells *in vitro* demonstrated by both ATP-based viability assay and tritiated thymidine uptake. Furthermore, stable WT1 silencing caused significantly slower growth after inoculation of tumor cells subcutaneously in the flanks of athymic nude mice and was associated with an increased latency period. These studies provide proof of principle that downregulation of WT1 causes decreased tumorigenicity of a glioblastoma cell line *in vitro* and *in vivo*, and suggest that WT1 may be a promising target for novel glioblastoma molecular therapies perhaps in combination with standard treatment modalities.

Introduction

Glioblastoma multiforme (GBM) is a WHO grade IV glial tumor that carries a universally poor prognosis with a median survival of one year after diagnosis[27]. Aggressive surgery, combined with radiotherapy and adjuvant chemotherapy, modestly improves survival; however glioma cell resistance to standard treatments remains a major obstacle. This is due, in part, to the high proliferative capacity and profound invasiveness of glioma cells[20, 22]. Decades of intense research have uncovered several molecular mechanisms of glioma cell tumorigenicity[134]. Despite these advances, a significant clinical improvement has not been realized. Therefore, a more complete characterization of the molecular mechanisms of tumorigenicity is necessary to uncover targets for novel therapies. Previous studies by our laboratory and others have examined the expression of the Wilms' tumor 1 (WT1) gene in human brain tumors, including gliomas of all grades, and have found detectable expression in tumor cells but not in normal cells, suggesting an important role for WT1 in glioma biology[30, 178, 152, 43]. We have also shown that

WT1 silencing increases radiosensitivity of glioblastoma cell lines[a]. Oji *et al.*, demonstrate that downregulation of WT1 using antisense oligodeoxynucleotides (ODN) decreases viability of glioma cells 72 hours after transfection[178]. However no studies to date have extensively characterized the relationship between WT1 expression and tumorigenicity in gliomas.

WT1 was originally discovered as mutated in nephroblastoma, a common pediatric kidney cancer also known as Wilms' tumor[23]. It was subsequently found that the *wt1* gene, located at chromosome locus 11p13, encoded a 52-55 kD polypeptide, the structure and function of which designate it as a zinc finger transcription factor[162]. Furthermore, the message is alternatively spliced to generate four main isoforms, designated +/+, -/+, +/-, -/-, depending on the inclusion or exclusion of exon 5 and nine nucleotides in exon 9 known as KTS, respectively[75]. WT1(+KTS) isoforms, which gliomas express[30], may be involved in RNA processing in addition to having both overlapping and unique transcriptional properties relative to WT1(-KTS) isoforms. The functional significance of exon 5 is less well understood, but may affect its interaction with other proteins[200].

Although originally classified as a tumor suppressor, WT1 was later found to be overexpressed in many types of cancer, including breast carcinoma and acute leukemia, suggesting an oncogenic function for the gene[159, 90]. WT1 overexpression causes increased proliferation of cancer cells[239]. Inhibition of WT1 expression in human breast cancer and leukemia cell lines using antisense modalities leads to differentiation, decreased proliferation, and increased sensitivity to treatment[270, 3]. Especially relevant to gliomas

[a] Clark et al., "Down-regulation of Wilms' tumor 1 expression in..." manuscript in prep.

which cause death by direct invasion and destruction of normal brain tissue, WT1 overexpression was recently shown to increase invasion of ovarian cancer cells[95]. Based on the studies described above, we hypothesized that WT1 downregulation would decrease the tumorigenicity of glioma cells. To test the hypothesis, we utilized U251MG human glioblastoma cells, which express moderate levels of endogenous WT1. We generated U251MG cells stably transduced with either empty vector or WT1 shRNA and examined the effect on proliferation, invasion, and *in vivo* tumor growth.

Materials and Methods

Plasmid construction

Short interfering RNA (siRNA, Dharmacon) was tested for efficacy in cell culture per the manufacturer's protocol (data not shown). Forward and reverse oligos were generated using the provided sequence. Plasmid WT1 shRNA expression vectors were constructed according to the manufacturers' protocol (pSUPER.retro.neo+gfp, OligoEngine). Briefly, the pSUPER vector was linearized using HindIII and BclII restriction enzymes. The annealed forward and reverse oligos were ligated into the linearized vector with T4 DNA ligase (New England Biolabs, Inc.) to generate either empty pSUPER vector or pSUPER.shWT1 expression vector. The vectors were then used to transform DH5alpha bacteria (Invitrogen) selected with ampicillin. Plasmid purification was performed using the QIAGEN plasmid mini kit (Qiagen). The presence of positive clones was confirmed by EcoRI and HindIII digestion and sequencing. The packaging cell line 293 T was then transfected with either empty pSUPER vector or pSUPER.shWT1 vector in addition to pCL-ampho packaging vector and pME-VSVg envelope vector using

FuGENE 6 transfection reagent according to the manufacturers' protocol (Roche). After a two day incubation at 37° C, media containing the retrovirus was harvested, filtered, and stored in aliquots at -80° C.

Cell culture, viral transduction, sample preparation

U251MG, LN-18, and U87MG human malignant glioma cells were obtained from American Type Culture Collection and were grown and passaged in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% glutamine, 1% non-essential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. For transduction, cells were plated in six well plates at a density of 0.25 X 10^6 cells per well and allowed to attach overnight. Media was then replaced with 0.5 ml of infection media (without fetal bovine serum) containing virus. Cells were incubated with gentle agitation for 2 hours at which time 1.5 ml of media (with serum) was added to cells. After viral transduction, cells were maintained in DMEM supplemented with 300 μg/ml of G418 (Geneticin, Invitrogen) which was exchanged every three days with fresh selection media. Total RNA was isolated from cell lines using the Trizol extraction protocol (Invitrogen). The concentration of RNA was determined by spectrophotometry. Protein was extracted from cell lines using SDS buffer (50 mM Tris-Cl, 1% SDS, 10% glycerol) supplemented with protease inhibitors. The concentration of protein was determined by DC protein assay (BioRad).

Western blot analysis

20 - 40 μg of protein was separated on a 4-12% Bis-Tris NuPAGE gel and subjected to electrophoresis as per the manufacturer's protocol (Invitrogen). Protein was transferred to a nitrocellulose membrane. The membrane was then blocked with 5% nonfat milk solution for 1 hour at room temperature. Mouse anti-WT1 monoclonal antibody (1:200 dilution, DakoCytomation) was diluted in blocking buffer. The membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed six times in Tris buffered saline containing 0.05% Tween-20 before and after a one hour incubation at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1000, Rockland, Inc.) for WT1. Blots were developed using the ECL Detection System (Amersham Biosciences). Anti-Cyclophilin A monoclonal antibody (1:1000, Upstate Biotechnology) was used as a control for protein loading.

Proliferation assays

Cells were plated in 5 replicate wells of 96 well tissue culture treated plates at a density of 0.5 X 10^3 cells per well. Cell viability was measured using the CellTiter-GLO Luminescent Cell Viability Assay (Promega) at designated time points after plating. Relative luminescence was detected on a Lumistar luminescence plate reader (BMG Technologies). Tritiated thymidine (^{3}H-TdR) uptake was analyzed by plating in quintuplicate 0.5 X 10^3 cells per well in 96 well, flat bottom tissue culture plates and pulsing with 1 μCi of ^{3}H-TdR at 4 days (Amersham Biosciences). Cells were cultured for an additional 18 hours and then stored at -80° C. Incorporation of ^{3}H-TdR was used as a measure of proliferation and was analyzed using a 96-well plate harvester and a beta-plate

reader (Packard). Data are expressed as mean counts per minute (CPM) of quintuplicate experimental cultures.

Invasion assay

A modified 96 well chemotaxis assay (Neuroprobe, Inc.) was used to monitor tumor cell invasiveness. Assay chambers consisted of a fitted manifold with a porous polycarbonate membrane (8 μM pores), pre-coated for 45 minutes at 37 °C with 25 μg/ml growth factor reduced Matrigel (BD Biosciences; 50 μl/well). Cells were plated in 5 replicate wells at a density of 0.5 X 10^4 cells per well. After 48 hours, media and cells which had not invaded through the membrane were removed from the upper surface of the manifold and 0.05 M EDTA/PBS solution added to each well, incubated at 37 °C for 15 minutes, prior to centrifugation of the plate at 300 X g. Cell growth medium was removed from the lower chamber and fresh media added to each well. Collected cells in the lower chamber were lysed for 10 minutes in Cell-titer Glo ATP viability assay reagents, following the manufacturer's protocol (Promega). Cell lysates were transferred to opaque, white-walled 96 well plates and relative luminescence detected on a Lumistar luminescence plate reader (BMG Technologies). Two replicate independent assays were performed.

In vivo tumor growth in nude mice

For inoculation, cells were first washed with sterile PBS and then harvested by trypsin-EDTA treatment. The dispersed cells were resuspended in sterile PBS and adjusted to 3 x 10^7 cells/ml. Then 200 μl (6 x 10^6 cells) of the cell suspension was injected subcutaneously (s.c.) in the middle of backs of male 4 -6 week old athymic BALB/c *nu/nu*

mice (Harlan Sprague Dawley, Inc.). For each of the three cell lines, 5 mice were inoculated. Tumor size after injection was measured starting four days after inoculation every two days for six weeks by dial-caliper and tumor volumes were calculated as (length x (width^2)) / 2. Mice were euthanized when the tumor had reached 800 mm^3. After euthanasia, tumors were dissected from the skin and underlying muscle, snap frozen, and stored at -80° C. Tissue was homogenized and protein extracted by SDS buffer and sonication. Two replicate independent experiments were performed for a total of 30 mice.

Histological analysis

Tumor tissues were fixed in formalin, embedded in paraffin, and 5 μm serial sections were prepared. A representative section was stained with hematoxylin and eosin for histopathology. All slides were read in a blind manner. Microscopy was performed at the VCU - Dept. of Neurobiology & Anatomy Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant (5P30NS047463).

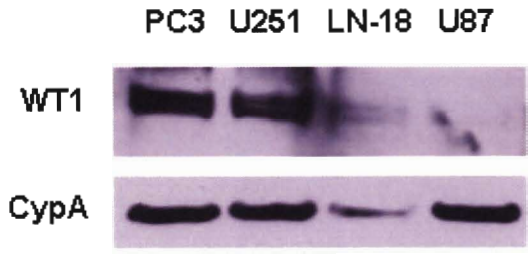
Statistical analysis

Differences between two groups were analyzed by Student's t test. Differences between three groups were analyzed by ANOVA followed by Tukey- Kramer honestly significant difference test (HSD) as a post-hoc analysis. Differences between *in vivo* growth rates were analyzed by repeated measures ANOVA.

Results

WT1 shRNA decreased proliferation. U251MG cells expressed high levels of WT1 (Figure 4-1).

Figure 4-1. U251MG glioblastoma cells expressed WT1 protein. 40 μg protein extracted from U251MG, LN-18, and U87MG cells was separated by SDS-PAGE and examined for WT1 expression by western blot. PC3 prostate carcinoma cell extract was used as a positive control for WT1 expression. U251MG cells expressed high levels of WT1 protein. U87MG cells did not express WT1 protein as we have previously shown and served as a negative control. Anti-cyclophilin A antibody demonstrated low protein loading in LN-18 cells, however a faint WT1 band is observed. CypA, cyclophilin A

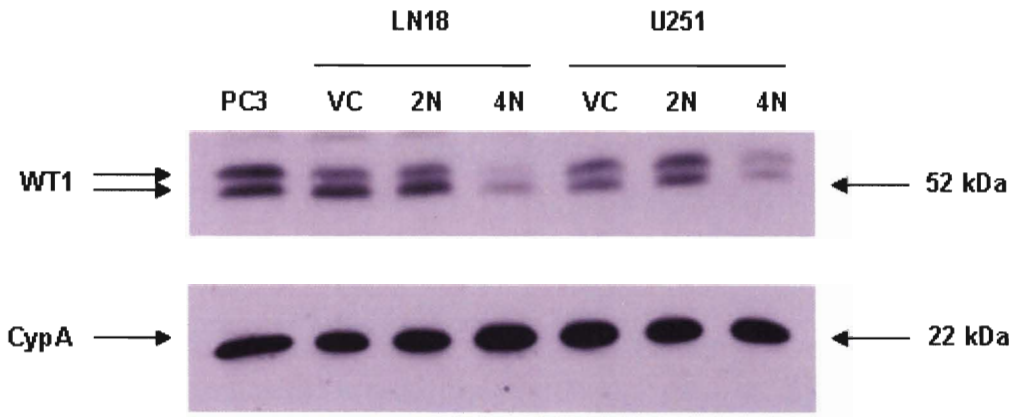

PC3
U251
LN-18
U87
WT1
CypA

Although protein loading was unequal, LN-18 cells expressed detectable WT1 protein while U87MG cells did not. These results confirm our previous characterization of WT1 expression in these cell lines[30]. We tested two sequences of shRNA targeting WT1, referred to as 2N and 4N, for efficacy of WT1 silencing in both U251MG and LN-18. After three weeks of maintenance in selection media, protein was extracted and analyzed by western blot for WT1 expression. Viral transduction with 4N shRNA resulted in a marked decrease in WT1 protein expression in both cell lines relative to cells transduced with empty vector, while 2N did not (Figure 4-2a). All subsequent experiments utilized 4N shRNA transduced cells (now referred to as U251.Sh and LN18.Sh). Each treatment group was maintained as a pooled population of cells to eliminate the possibility of clonal differences in growth characteristics affecting the growth measurements. Viral transduction with either empty vector or WT1 shRNA did not cause a change in morphology of the cells relative to their respective parental cell line (Figure 4-2b). In U251MG cells transduced with either empty vector (U251.VC) or WT1 shRNA containing vector, the majority of cells expressed green fluorescence protein (GFP) demonstrating stable expression of the plasmid vector (Figure 4-2c). The effect of WT1 silencing on *in vitro* proliferation was examined by ATP-based viability assay at 0, 1, 3, 5, and 7 days. U251.Sh cells proliferated at a significantly slower rate than U251.VC cells ($p < 0.0003$, Figure 4-2d). Tritiated thymidine incorporation performed at 5 days confirmed the decrease in proliferation ($p = 0.0003$, Figure 4-2e). WT1 knock-down was maintained throughout these experiments (Figure 4-2e, inset). WT1 silencing in LN-18 cells caused a

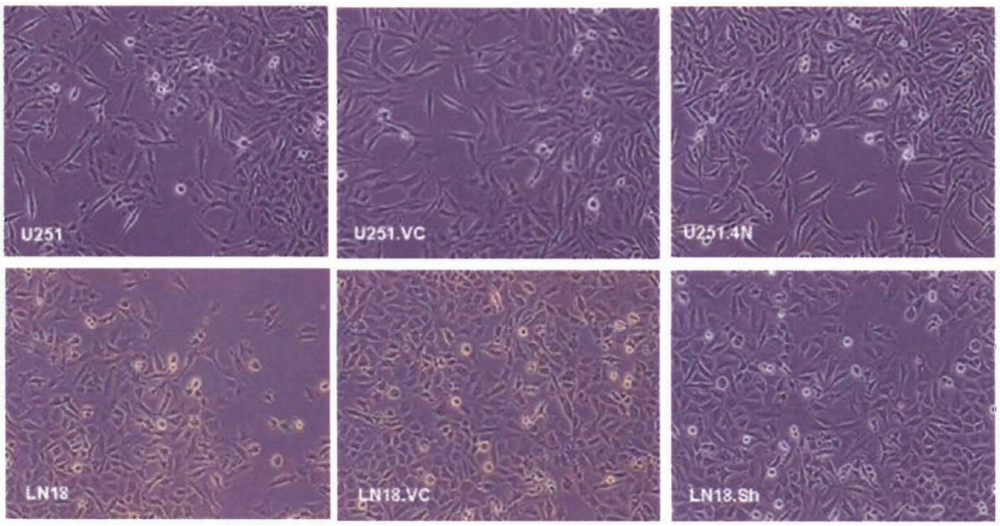
less dramatic, but still statistically significant decrease in proliferation over the course of 5 days (Figure 4-2f).

Figure 4-2. WT1 silencing decreased *in vitro* proliferation. A. U251MG and LN-18 cells were virally transduced with one of two sequences of WT1 shRNA. After several weeks of selection pressure, protein was extracted from the pooled population and analyzed for WT1 silencing by Western blot. B. Phase contrast microscopy demonstrates the morphology of the parental and virally transduced cells. C. Fluorescence microscopy demonstrates that the majority of U251MG cells transduced with the expression vector stably expressed GFP (top panel). Light microscopy images are provided for comparison (bottom panel). Magnification is 40X. D. ATP-based cell viability assay performed on U251MG cells at 0, 1, 3, 5, and 7 days after plating demonstrates the decrease in proliferation of U251.Sh cells relative to cells transduced with empty vector (U251.VC). $*p < 0.0003$ E. ^{3}H-TdR uptake assay performed at 5 days confirms that U251.Sh cells are proliferating more slowly. Inset depicts western blot analysis of WT1 expression confirming that WT1 silencing was maintained at the time of the experiment. $*p = 0.0003$ F. ATP-based cell viability assay demonstrates the proliferation of LN18.Sh cells at 0, 1, 3, and 5 days after plating relative to LN18.VC cells. $*p < 0.05$

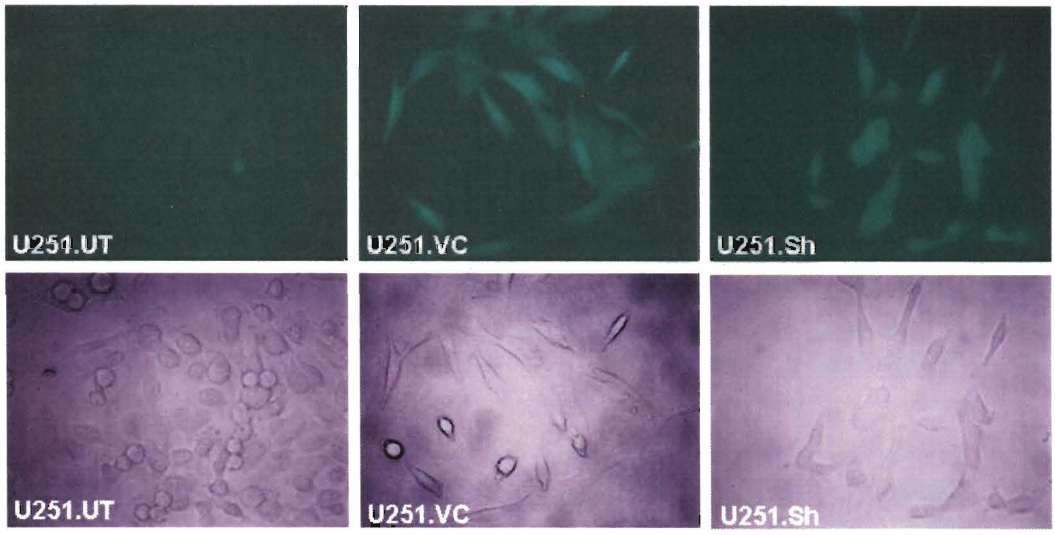
A



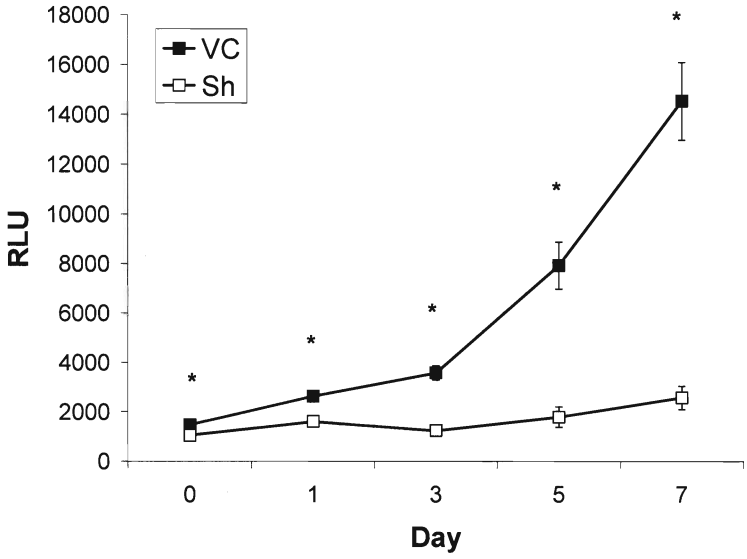
B



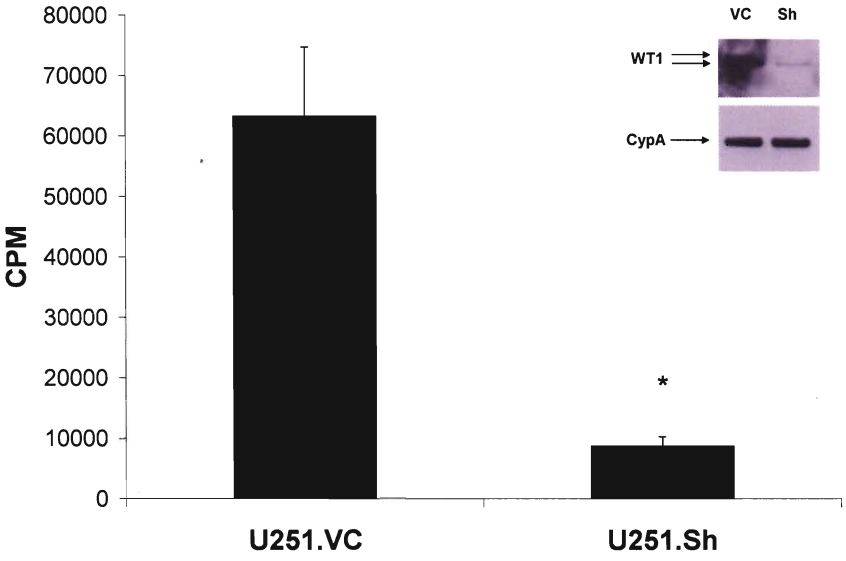
C



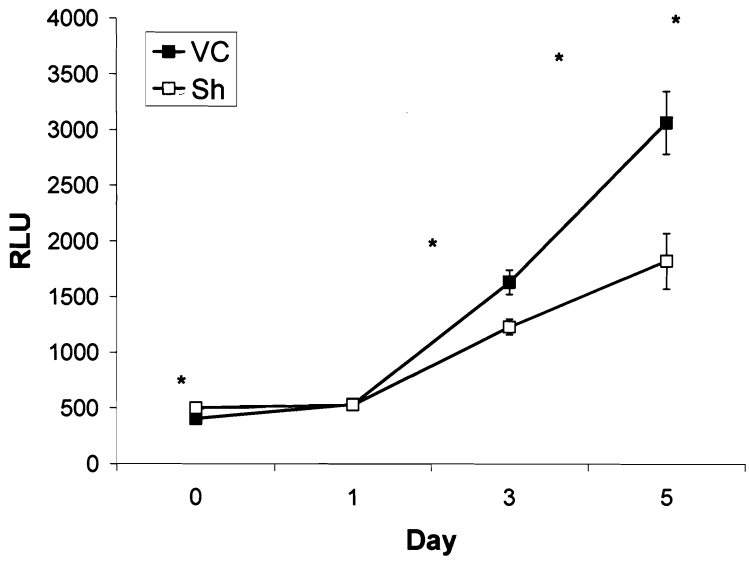
D


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
RLU
VC
Sh
*
0
1
3
5
7
Day

E

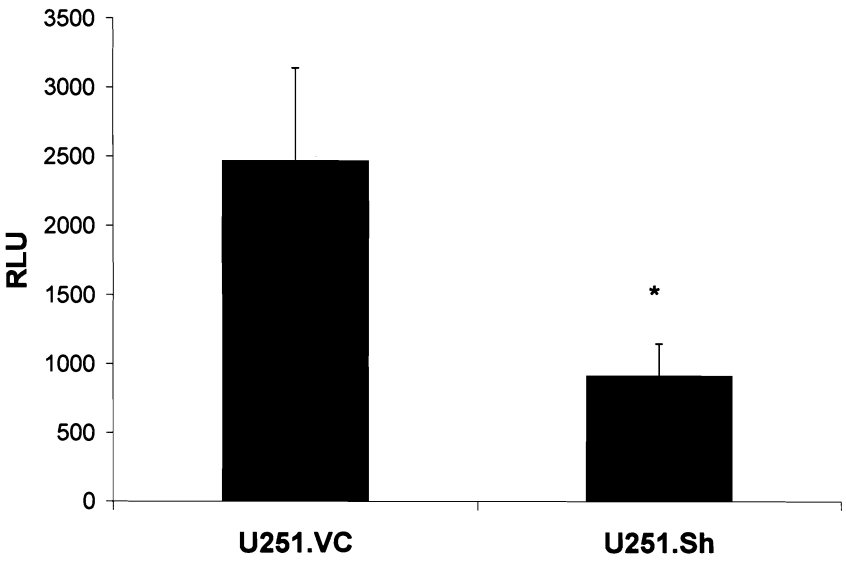

80000
70000
60000
50000
40000
30000
20000
10000
0
CPM
VC
Sh
WT1
CypA
*
U251.VC
U251.Sh

F



WT1 silencing decreased invasion. Glioblastoma cell invasion into normal brain is a major contributor to the tumorigenicity of these cells. We therefore analyzed the affect of WT1 silencing on glioblastoma cell invasion by *in vitro* Matrigel invasion assay. After 48 hours, 63% fewer viable cells were present in the lower chamber of the *in vitro* invasion assay apparatus in U251.Sh cells relative to U251.VC cells ($p = 0.01$, Figure 4-3).

Figure 4-3. WT1 silencing decreased *in vitro* invasion. U251 parental, U251.VC, and U251.Sh cells were plated in the top chamber of a modified chemotaxis manifold on a Matrigel-coated porous membrane. Two days after plating, the amount of cells that had invaded through the membrane was quantified by viability assay and demonstrated that U251.Sh cells exhibited lower invasion through the membrane. *$p = 0.01$, RLU, relative luciferase units

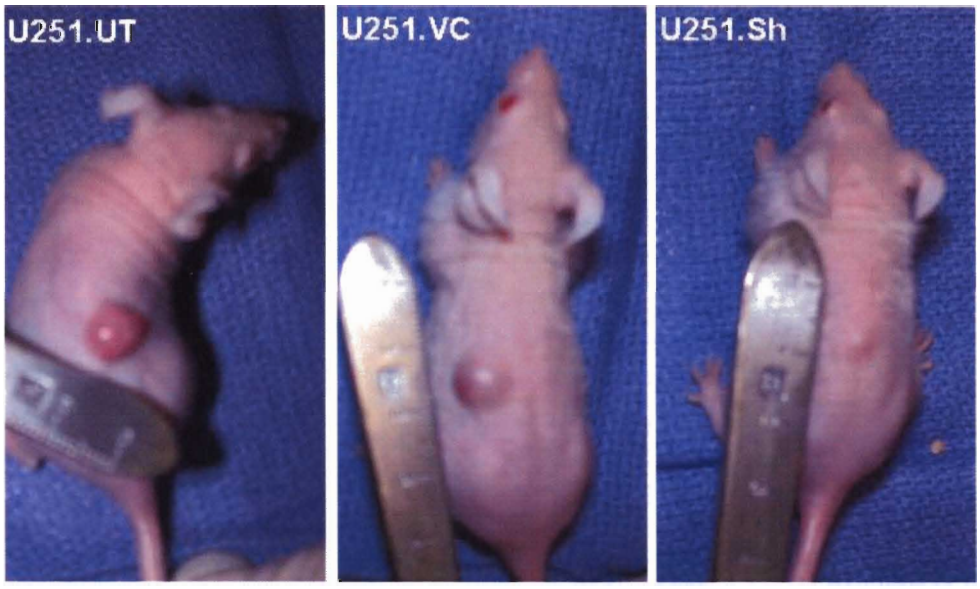

3500
3000
2500
2000
1500
1000
500
0
RLU
*
U251.VC
U251.Sh

WT1 silencing decreased growth of tumor xenografts in nude mice. The ultimate test of tumorigenicity is *in vivo* tumor formation. To test this, U251.Sh cells were used to inoculate five nude mice subcutaneously in the flank and compared to five mice inoculated with U251 parental and five mive inoculated with U251.VC cells. U251 parental and U251.VC cells formed tumors with very little latency period. U251.Sh tumors remained undetectable for a significantly longer period of time relative to either parental U251 cells or U251.VC cells (9 vs. 4 days, $p = 0.01$). Eventually tumors formed in 100% of animals inoculated with either of the three cell lines. At all days, mice bearing U251.Sh tumors had visibly smaller tumors than either U251 parental or U251.VC bearing animals (Figure 4-4a). At 22 days post-inoculation, when the first animal was euthanized for a tumor > 800 mm^3, U251.Sh tumors were significantly smaller than U251 parental or U251.VC tumors (U251.Sh, 156.0 mm^3; U251, 570.6 mm^3; U251.VC, 720.5 mm^3, $p = 0.0004$). U251 parental and U251.VC tumors were not significantly different ($p > 0.05$). Analysis of the growth curves revealed a significant decrease in growth rate of U251.Sh tumors relative to U251 parental and U251.VC tumors (Figure 4-4b top panel, $p < 0.0001$). Despite differences in growth rate, 100% of tumors reached 800 mm^3 by 34 days post-inoculation and were euthanized. However, U251.Sh tumors reached 800 mm^3 in 31.3 days while U251 parental and U251.VC tumors took 26.4 and 23.6 days, respectively ($p = 0.0028$). A second independent experiment was performed with similar results (Figure 4-4b, lower panel). Histologic analysis of a subset of tumors demonstrated that all tumors examined were densely cellular. Although the sample size is too small to derive any conclusions, the U251.Sh tumors appeared to contain less cells undergoing mitosis,

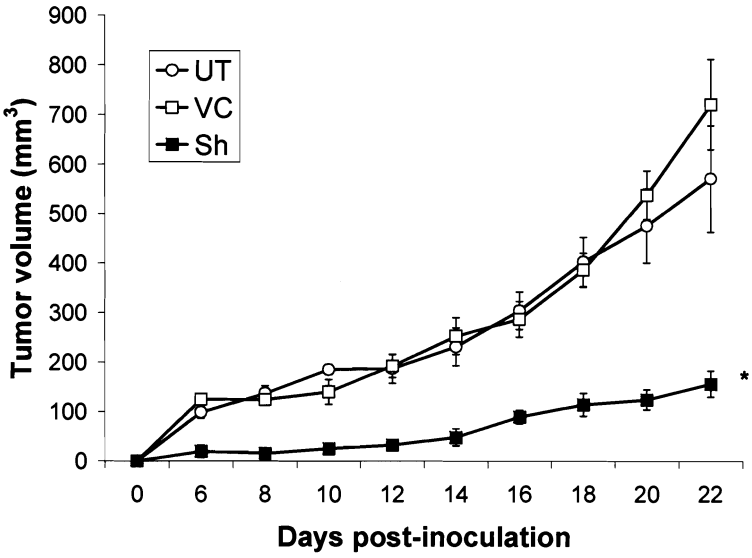
consistent with a lower proliferation rate(Figure 4-4c). Since the U251.Sh tumors eventually formed large tumors, western blotting was performed on the excised tumors and probed for WT1 expression to determine if WT1 shRNA expression had been lost. While WT1 silencing was maintained at the time of inoculation in cell lines growing in culture, all U251.Sh tumors regained WT1 expression by the time they had reached 800 mm^3 (Figure 4-4d).

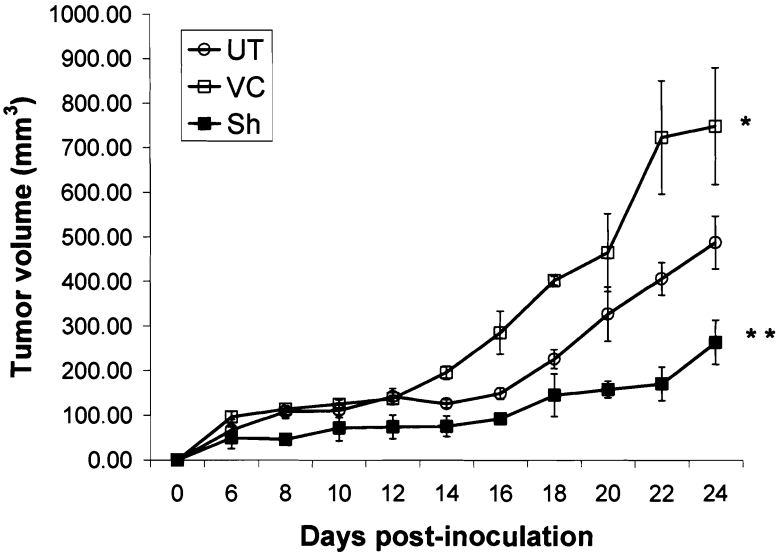
Figure 4-4. WT1 silencing decreased *in vivo* growth rate. Five athymic nude mice each were inoculated with 6 X 10^6 U251 parental, U251.VC, and U251.Sh cells subcutaneously in the middle of the back. Tumor growth was measured every two days until tumor had reached 800 mm^3. A. Photograph taken of representative animals from each group at day 16 post-inoculation demonstrated the difference in tumor size of U251.Sh tumors relative to U251 parental and U251.VC tumors. The scalpel handle is shown for scale. B. Growth curve demonstrated the slower growth rate of U251.Sh tumors. The *in vivo* growth experiment was repeated with similar results (lower panel). Error bars represent the standard error. C. When tumors reached 800 mm^3, tumors were excised and formalin fixed for tissue sectioning and hematoxylin and eosin staining. Histopathology demonstrated densely cellular tumors. Arrows indicate mitotic bodies. Magnification is indicated at the left margin. D. After excision, tumor tissue was snap frozen and protein extracted for western analysis. Representative western blot of excised tumor protein lysates demonstrated that WT1 was expressed by all tumors. WT1 expression in protein extracts from cells growing in culture is demonstrated for comparison.

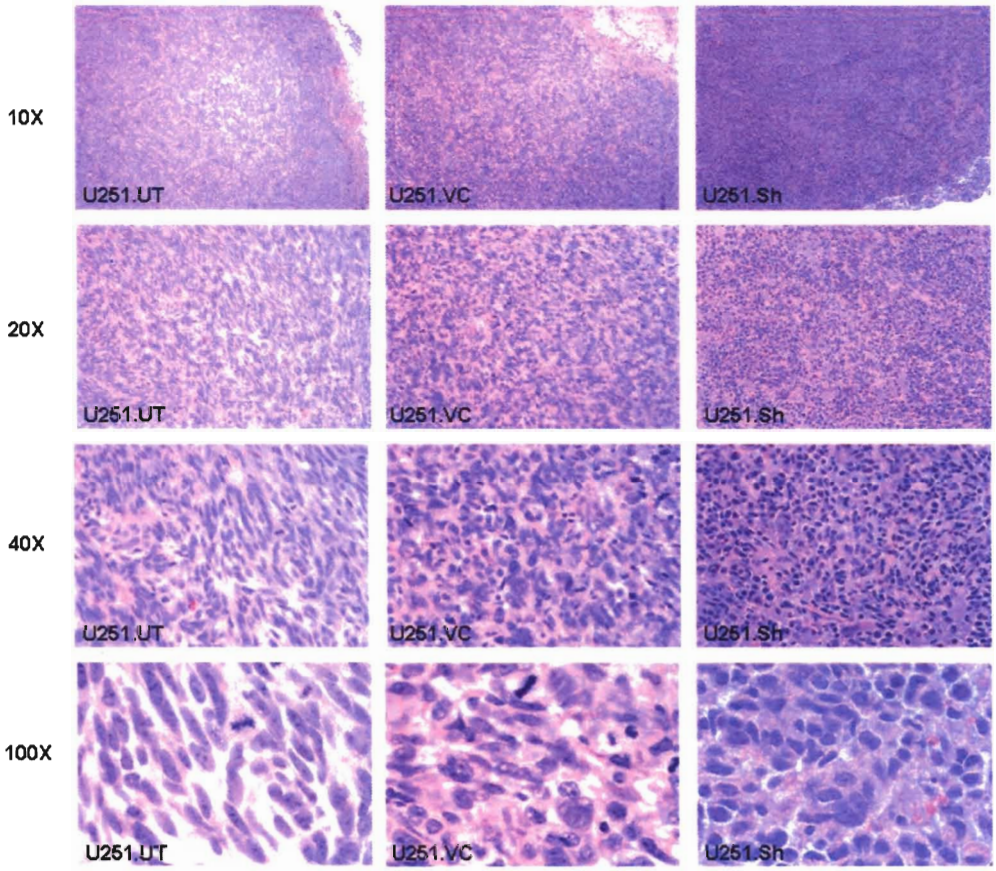
A



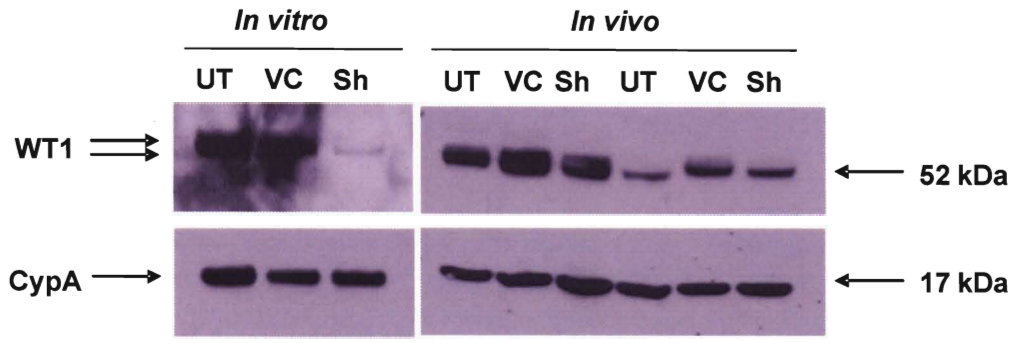
C


900
800
700
600
500
400
300
200
100
0
Tumor volume (mm3)
UT
VC
Sh
*
0
6
8
10
12
14
16
18
20
22
Days post-inoculation


1000.00
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Tumor volume (mm3)
UT
VC
Sh
*
* *
0
6
8
10
12
14
16
18
20
22
24
Days post-inoculation


10X
20X
40X
100X
U251.UT
U251.VC
U251.Sh

D



Discussion

To examine the affect of WT1 expression on glioblastoma cell tumorigenicity, we generated U251MG and LN-18 human glioblastoma cells stably expressing shRNA targeting WT1. Viral transduction with WT1 shRNA was capable of stably silencing of the WT1 gene over the course of multiple passages. WT1 silencing significantly decreased the proliferation of U251MG and LN-18 cells in culture. Interestingly, we observed a decrease in viable cells that had migrated through a Matrigel-coated membrane suggesting that WT1 silencing decreased the invasive potential of U251MG cells. Most importantly, WT1 silencing was associated with increased tumor latency and decreased growth rate *in vivo* after inoculation in athymic nude mice.

The role of WT1 in glioblastoma has not been extensively characterized. The results of this study suggest that one function of WT1 in glioblastoma is to maintain the high proliferative rate characteristic of the tumor. One previous study describes a decrease in viability 72 hours after treatment of U87MG, A172, and T98G glioblastoma cells with WT1 antisense ODN[178]. Although their results correlate well with the cell proliferation results presented here, our laboratory has shown in this study and previously that U87MG cells do not express endogenous WT1[30]. Strangely, the manuscript does not include a depiction of U87MG protein expression or of the efficiency of WT1 knock-down in any of the cell lines examined[178]. Studies of other cancer cell types also support a role for WT1 in increasing proliferation. Inhibition of WT1 expression by antisense ODN in K562 and MM6 leukemia cell lines causes decreased proliferation and viability. Zapata-Benavides *et al*[270] show that WT1 antisense ODN treatment of breast cancer cell lines

causes decreased proliferation which is associated with decreased cyclin D1, however direct transactivation is not demonstrated. Furthermore, in MCF-7 and MDA486 breast cancer cells, WT1 directly up-regulates the expression of the proto-oncogene c-myc, a known stimulator of proliferation[76]. These *in vitro* functional studies are potentially clinically relevant as high WT1 expression in both leukemia and breast cancer correlates with worse prognosis[90, 159].

Our results suggest a role for WT1 in glioblastoma cell invasion. However, due to the strong anti-proliferative effect of WT1 silencing observed in U251MG cells, we cannot discount the possibility that the difference in viable cells that had migrated through the Matrigel-coated membrane is due to a difference in proliferation and not invasion. Although the relationship between WT1 and glioblastoma cell invasion has not been previously reported, recent evidence suggests a role for WT1 in cancer cell migration and invasion. Jomgeow *et al* demonstrates that WT1 increases the invasion of TYK ovarian cancer cells[95]. WT1 overexpression causes an increase in cell invasion as measured by in vitro invasion assay. This is associated with a change in morphology possibly due to WT1-mediated changes in cytoskeletal components; a decrease in α-actinin and cofilin expression and an increase in gelsolin expression. A separate study demonstrates that WT1 regulates the E-cadherin promoter, a cell adhesion molecule involved in cancer cell invasion[85]. Studies to confirm the role of WT1 in glioblastoma cell invasion and to determine the underlying molecular mechanism are ongoing in the laboratory.

Several studies have examined the effect of WT1 over-expression on *in vivo* tumor growth in nude mice. The results, however, have been contradictory. WT1(-KTS) isoform

expression in transformed baby rat kidney cells increases tumor growth rate, while WT1(+KTS) isoforms suppress tumor growth[150]. Transfection of all isoforms of WT1 in Wilms' tumor cells increases tumor latency and decreases growth rate. Stable expression of WT1(-KTS) in prostate cancer cells decreases *in vivo* tumorigenicity which is associated with increased apoptosis and decreased expression of the WT1 target Bcl-2[51]. In contrast to *in vitro* data described above in breast cancer cells, transfection of MDA-MB-231 breast cancer cells with WT1 completely prevents tumor formation in nude mice which is associated with decreases β-catenin expression and activity. Likewise subcutaneous or intraperitoneal injection of M1 murine leukemia cells overexpressing WT1(+KTS) results in decreased tumor formation in severe combined immunodeficient mice[226]. To our knowledge, ours is the first study to examine the affect of WT1 silencing on *in vivo* tumor growth. The results of our study relative to others may be indicative of the well described cell-type specific responses of WT1 or may reflect differences inherent in comparing the response of overexpression of an exogenously applied gene versus down-regulation of an endogenously expressed gene.

Although stable expression of WT1 shRNA caused increased tumor latency and decreased tumor growth in vivo, within 34 days the U251.Sh tumors reached 800mm^3. The outgrowth of a tumor cell subpopulation could explain the accelerated growth rate after a latency period. In fact, at the time of harvest, all tumors had regained WT1 expression as demonstrated by western blot. Protein contamination by non-tumor cells in the tissue sample was not likely responsible for this observation as histologic examination demonstrated that anaplastic tumor cells comprised the vast majority of the specimen.

This suggests that the *in vivo* environment provided a selective pressure for the outgrowth of clones which had lost WT1 shRNA expression allowing increased proliferation. This finding clearly demonstrates the importance of WT1 in supporting glioblastoma cell proliferation and provides further evidence in support of an oncogenic function of WT1 in glioblastoma. Future studies focused on modalities capable of maintaining full WT1 silencing potentially may be able to halt *in vivo* experimental glioblastoma growth.

ACKNOWLEDGEMENTS

The authors would like to thank Amanda Richardson for invaluable technical assistance. The research presented in this paper was supported in part by a Medical Student Summer Fellowship from the American Brain Tumor Association and by the Hord and Cullather funds of the MCV Foundation.

CHAPTER 5.

GENERAL DISCUSSION

In this series of studies we aimed to characterize the expression of WT1 in human glioblastoma and determine its function therein. This manuscript presents several novel findings pertaining to the role of WT1 in glioma biology. We show for the first time that 80% of glioblastoma tissue specimens express WT1(+KTS) isoforms, the expression of which correlates with wild-type p53. Interestingly, lower grade gliomas, including those of oligodendroglial origin, also express WT1. Unexpectedly, we found that WT1 does not protect against p53-mediated apoptosis induced by either transient transfection with wild-type p53 or ionizing radiation. We also show that WT1 silencing using transient siRNA transfection increases radiosensitivity of glioma cell lines. This is a finding that transcends the field of neuro-oncology as it has not, to our knowledge, been reported in any other type

of cancer. We utilized retroviral transduction with shRNA to stably inhibit WT1 expression which caused a decrease in proliferation and invasion *in vitro*. Furthermore, glioblastoma cells stably expressing WT1 shRNA demonstrated decreased *in vivo* growth rate in nude mice. To date, no other studies have shown that WT1 silencing causes decreased *in vivo* growth of cancer cells. We, therefore, have conclusively demonstrated for the first time that WT1 plays a major role in the tumorigenicity and resistance to treatment of glioblastoma.

While several other studies have conclusively shown that WT1 physically and functionally interacts with p53[136, 137, 87], we were unable to show such an interaction. It could be argued that utilization of a different model of p53 induction could have yielded superior results. However, in our studies we used both artificial (transient transfection) and endogenous (ionizing radiation) methods to induce a p53 response. In both cases, cells underwent cell death in response to p53 activation. Both of these sets of experiments failed to show a modulation of p53-mediated cell death by WT1 expression. In addition, we attempted to use an LN-Z308 glioblastoma derived cell line stably expressing wild-type p53 under the control of a doxycycline-inducible promoter[243]. Induction of p53 with doxycycline resulted in upregulation of p53 protein, with no resultant decrease in viability or detectable cell cycle arrest, thus negating its utility in studying a WT1/p53 interaction (Appendix C). A potential alternative method of p53 overexpression is adenoviral transduction which effectively induces apoptosis in glioblastoma cells[68], however this was not used in this study. Nevertheless, based on the variety of techniques and cell

backgrounds tested in our studies, it is not probable that WT1 interacts functionally with p53 in relation to cell viability in glioma cells.

We also showed that, while WT1 overexpression in WT1 non-expressing p53 wild-type glioblastoma cells (U87MG) did not affect radiosensitivity, WT1 silencing in WT1-expressing glioblastoma cells increased radiosensitivity. We also generated glioblastoma cells of different p53 status (LN-229, p53 mutant; LN-Z308, p53 null) stably expressing WT1 and also found no difference in radiosensitivity, suggesting that the lack of WT1 induced radioresistance is applicable in general to glioma cells (Appendix D). An explanation is that, when dealing with an aggressive transformed cell that is maximally resistant to therapies, it is unlikely that the addition of one factor, such as WT1 expression, will cause an increase in resistance. This is particularly true in a complex disease process such as glioblastoma in which several overlapping pathways contribute to radioresistance. In this situation, it is more feasible to decrease resistance by silencing a gene that is partially responsible for the resistance as we showed in our studies. The question as to how WT1 mechanistically contributes to radioresistance remains unanswered. Our studies suggest that it is not due to interaction with p53 and subsequent inhibition of p53-mediated apoptosis.

Thus, although these studies clearly support an oncogenic role of WT1 in glioblastoma, the mechanism by which it functions remains unknown. As mentioned above, in breast cancer cells WT1 transactivates c-myc which is known to promote proliferation[76]. Because c-myc is also overexpressed in glioblastoma, we examined the effect of WT1 silencing on c-myc protein expression by western blot and detected no

difference in U251.Sh cells relative to U251.VC (Appendix E). There exist several other candidates for mediators of the WT1 effect on proliferation. EGF family members and EGFR are known WT1 targets[127, 124]. They play a critical role in glioblastoma biology, in part by stimulating proliferation. In glioblastoma tissue, amplification and mutation are the most common causes of EGFR hyperactivity. However, in established glioma cell lines, EGFR amplification and EGFRvIII mutation are generally lost. Only one described cell line exhibits EGFR amplification[233]. Therefore, other mechanisms of up-regulation and activation of EGFR may be relevant to glioma cell behavior in culture. WT1 transactivation of EGFR could be a potential mechanism. Likewise, WT1 mediated up-regulation of EGFR stimulating ligands, such as Amphiregulin, may be responsible. Likewise, in certain settings WT1 activates PDGF, a known regulator of glioblastoma proliferation[255]. Unlike EGFR, amplification and/or mutation of PDGFR and its ligands are rare events. The mechanism of PDGF overexpression in glioblastoma is currently unknown. Future examination of the relationship between WT1 and PDGF in gliomas may uncover an unrecognized mechanism of PDGF upregulation.

The decrease in viability observed in cells transduced with WT1 shRNA could also be due to increased apoptosis due to WT1 silencing. As previously described, WT1 up-regulates the anti-apoptotic protein Bcl-2 and may also regulate apoptosis via the pro-apoptotic protein Bax[144, 93]. Again, we performed western blot analyses of U251.VC and U251.Sh cell lysates and did not detect any difference in these proteins (Appendix E). Other potential mediators of apoptosis potentially modulated by WT1 are pro-apoptotic Bak and the anti-apoptotic Bcl-2 family member A1/BFL1[93, 225]. Most of these studies

were performed in leukemia cells which undergo apoptosis much more readily than glioblastoma cells and, therefore, may display distinct patterns of gene expression in response to pro-apoptotic stimuli. We have not yet performed apoptosis analysis of these cells to determine the effect of WT1 silencing.

We detected a significant decrease in glioblastoma cell invasion following WT1 silencing. Glioblastoma cell invasion and destruction of normal brain continues to be one of the most difficult components in the clinical management of the disease. To date, no studies have examined the role of WT1 in glioma invasion and few have examined its role in invasion in other types of cancer. The few studies in other cell types have yielded mixed results. A recent study demonstrates that WT1 overexpression in TYK ovarian cancer cells leads to increased motility and invasion[95]. This increase in invasion is associated with changes in cytoskeletal proteins, but a direct mechanism of WT1 function is not described. In NIH-3T3 fibroblasts, WT1 overexpression leads to up-regulation of E-cadherin[85]. This observation contrasts with our results and those of the previous study as E-cadherin loss is associated with increased invasion, therefore WT1 mediated transactivation of E-cadherin would be expected to decrease invasion. When interpreting the applicability of these studies, it is important to consider the cell-type dependence of WT1 transcriptional activity. Because the MT2-MMP promoter contains several WT1 binding sites and is relevant to glioma invasion, we examined the expression of MT2-MMP after WT1 silencing (Appendix E). We found no change in the levels of MT2-MMP protein. Screening of other MMPs relevant to gliomas is currently underway in the laboratory.

The role of WT1 in glioblastoma may be more far-reaching than examined in this set of studies. As we detected WT1 expression in astrocytomas of all grades, it is possible that WT1 is also functioning at earlier stages of gliomagenesis. In the study of the earliest stages of gliomagenesis, an important question is the cell of origin of glioblastoma. The determination of the cell of origin has been difficult, particularly since the discovery of adult neural progenitor cells capable of proliferating and differentiating into the mature cells of the brain and, therefore, hypothetically able to undergo malignant transformation to form a glioblastoma[194]. A theory which incorporates these ideas posits that astrocytomas arise from mature astrocytes that dedifferentiate into highly proliferative immature cells in response to genetic mutations. Recent evidence in mouse models supports this theory. Uhrbom *et al* report increased frequency of glial fibrillary acidic protein (GFAP) positive brain tumors with histologic features of glioblastoma in transgenic $p16^{Ink4a\text{-}Arf}$ null mice in which mature astrocytes were specifically infected with a virus containing *Akt* and *Ras*[242]. These data suggest that a combination of genetic changes can induce dedifferentiation of astrocytes which are then capable of forming glioblastoma. Given that WT1 is preferentially expressed by undifferentiated leukemia and breast cancer specimens[185, 223], WT1 overexpression decreases differentiation in leukemia cell lines[91, 229, 3, 44], and WT1 inhibition causes differentiation in the breast cancer cell lines[239], it seems probable that WT1 functions in some cancer types to maintain the undifferentiated phenotype. In fact, several WT1 target genes have been shown to induce dedifferentiation in astrocytes and subsequent tumor formation. Viral transduction of the proto-oncogene c-myc in combination with Akt and Ras dedifferentiates astrocytes in

culture and increases glioblastoma formation when used to infect mature astrocytes *in vivo*[122]. WT1 transactivates c-myc as described above, however we showed that WT1 silencing in U251MG cells did not effect levels of c-myc protein. This represents an observation in one glioblastoma cell line and needs to be replicated in others before a generalization can be made. In contrast to the previous examples which required combinations of gene transduction for dedifferentiation, infection of mature astrocytes with PDGF caused recapitulation of the undifferentiated phenotype *in vitro* and increased tumor formation *in vivo*[35]. As discussed previously, PDGF is a WT1 target gene and, similarly to WT1, is expressed at an early stage in gliomagenesis. One can envision a mechanism by which WT1 is aberrantly overexpressed by unknown factors which leads to transactivation of c-myc and PDGF causing dedifferentiation of mature astrocytes and maintenance of the immature phenotype. The affect of WT1 on astrocyte differentiation has not been studied, but it would be interesting to determine the outcome of WT1 transfection into mature astrocytes alone or in combination with other genes.

Future directions

Athymic nude mice have been used successfully for decades to model human tumor growth when inoculated subcutaneously with tumor cells[189]. This model has provided invaluable data regarding tumor cell proliferation and efficacy of novel treatment modalities. There are, however, several major limitations to this type of animal model, particularly when applied to brain tumors such as glioblastoma. First is the absence of the unique environment of the brain. Second is the lack of invasion of tumor cells into the

normal tissue. Third is the lack of immune response. Future studies should include models which are compatible with the examination of these important components.

Numerous studies have used U251MG heterotopic subcutaneous xenografts to model human glioblastoma growth *in vivo*[155, 103]. Although the U251MG glioblastoma cell line was used to inoculate nude mice subcutaneously in the flank, this cell line possesses tumorigenic capacity in nude mouse brains also[204]. Future studies should focus on replicating the subcutaneous flank tumor growth data in mice inoculated orthotopically with U251MG cells stably expressing WT1 shRNA in the caudate nucleus. In this model, the animal is followed until onset of neurological decline, at which time the animal is euthanized and the intracranial tumor is examined. The data is reported as survival after inoculation. After replication of the data using stable cell lines, more therapeutically relevant methods of knock-down should be tested. Our data demonstrating a decrease in tumor growth by stable expression of WT1 shRNA provides proof of principle that down-regulation of WT1 decreases *in vivo* tumor growth. A more stringent test of therapeutic potential is decreased growth rate or tumor regression after infusion of WT1 shRNA, which more closely mimics the clinical scenario. Our laboratory has extensive experience in the study of therapeutic delivery modalities in the brain which could be combined with our use of WT1 shRNA[15, 190]. Recently, direct intratumoral injection of shRNA plasmid expression vectors into established intracranial gliomas has induced down-regulation of target genes and subsequently affected tumor regression[69]. Replication of these results using WT1 shRNA would be a major step toward a potential clinical trial.

Studies examining the behavior of glioma cell lines and the tumor from which they were derived show that *in vitro* invasion assays correlate well with *in vivo* invasiveness[29]. This suggests that *in vitro* assays are valid models for studying glioma invasion. Nevertheless, it is critical to test anti-invasive therapies *in vivo* to better extrapolate efficacies in animal models to the clinical setting. Unfortunately, orthotopic xenografts of established glioblastoma cell lines grow as discrete masses that compress, but do not invade the mouse brain. Recently developed nude mouse models generate tumors which more closely recapitulate the invasive characteristics of human glioblastoma. Researchers obtain tissue biopsies during glioblastoma resections, process the fresh specimens, and immediately implant the suspension in the flanks of nude mice. After several heterotopic passages in nude mice, the tumors are excised and cultured briefly. Inoculation of the resulting cultures orthotopically in nude mice brains produces densely cellular, mitotically active, and rapidly lethal tumors characteristic of malignant astrocytomas[61]. The study also shows that the tumors retained several genetic characteristics of the patient tumors, including EGFR amplification. The tumors also readily invaded the host brain along white matter tracts in some cases leading to leptomeningeal spread and in one case gliomatosis cerebri mimicking the human disease. A follow-up study demonstrates the utility of the model. Using this model, EGFR inhibition combined with radiation therapy caused increased survival[206]. After characterization of the xenografts for WT1 expression, an experiment could be designed to examine the efficacy of WT1 silencing strategies in decreasing *in vivo* glioma invasion.

The models described above utilize immunodeficient mice to study the behavior of human glioblastoma cells *in vivo*, but preclude the examination of the dynamic immune response in brain tumor biology. Recent studies suggest, however, that the immune system plays an important role in gliomagenesis. Schwartzbaum *et al* report an inverse correlation between asthma and other allergic conditions and glioblastoma in a large case-control study[213]. In addition, they screened patients for polymorphisms in 5 genes known to be involved in both allergic conditions and glioma or brain biology. They found that polymorphisms in the pro-inflammatory genes interleukin receptor-4α (IL-4A) and interleukin-13 (IL-13) were inversely correlated with glioblastoma and positively correlated with asthma, suggesting that modulation of pro-inflammatory responses confers risk of glioma development. Animal studies in immunologically intact rats suggest that these molecules may increase the immune system response to experimental gliomas. Therefore, study of gliomas must include analysis of immune function. *In vitro* and *in vivo* models exist to study and modulate the immune response to glioblastoma. Since WT1 is expressed by the majority of glioma specimens and not by normal cells, it is an attractive target for immunotherapy. Several mouse and rat brain tumor cell lines exist that could be utilized to study the immune response to WT1 expressing tumors. These cell lines could be characterized for WT1 expression. An animal could then be immunized with WT1 peptide plus adjuvant or WT1-pulsed antigen presenting cells after which the animal is challenged either subcutaneously or intracranially with the WT1 expressing tumor cell to determine if the tumor is rejected.

In spite of a lack of supportive animal data in glioblastoma, a clinical trial is underway to examine the safety and preliminary efficacy of WT1 peptide vaccination for the treatment of refractory WT1-positive glioblastoma. The group reports that in a sample of 5 glioblastoma patients, 1 demonstrated a partial response and 4 maintained stable disease during the 12 week treatment period with minimal toxicity[161]. Despite a paucity of studies in experimental glioma, several studies have examined the efficacy of immunotherapy targeting WT1 in other malignancies. Cytotoxic T lymphocytes (CTL) generated against WT1 peptide specifically lyse WT1 positive leukemia cell lines and patient samples but not those that do not express WT1[172]. This response is specific to undifferentiated leukemia cells which express a higher level of WT1 than either more differentiated leukemia cells or WT1 positive normal hematopoetic progenitor cells which are spared[55]. Mice vaccinated with either WT1 peptide or WT1 encoding plasmide DNA generated WT1 specific CTL capable of killing WT1 expressing leukemia cells and induced immunity against tumor challenge without evidence of autoimmunity[182, 238]. A small scale clinical trial involving 2 breast cancer, 10 lung cancer, and 14 leukemia patients consisted of vaccination with WT1 peptide and evaluation for change in tumor size and adverse effects[181]. Of the 20 patients available for evaluation, 12 demonstrated the following clinical responses; decrease in tumor size (breast cancer), decrease in tumor markers (lung cancer), decrease in blast cells (leukemia), or decrease in WT1 expression (leukemia). An additional 2 patients maintained stable disease. A recent report demonstrates that, although WT1-specific CTL are present in draining lymph nodes of a small sample of patients with stage 1 or 2 breast cancer, they were not capable of killing

breast cancer cells without adjuvant stimulation[64]. Taken together, these results indicate that vaccination strategies to activate endogenous impaired WT1-specific immune cells can induce clinically detectable responses in patients with leukemia and solid tumors, including glioblastoma.

Conclusion

Here we have shown that the majority of gliomas express WT1 while remaining undetectable in normal human astrocytes. Functional studies demonstrated that WT1 is critical for glioblastoma cell proliferation, invasion, radioresistance, and *in vivo* tumor formation and growth. Future studies will undoubtedly discern the molecular mechanisms by which WT1 exerts these effects and most likely will uncover an expanded list of functions in glioma biology. As molecular diagnostic modalities become more refined and prevalent, WT1 may possess clinical utility in glioblastoma diagnosis and treatment.

Literature Cited

Literature Cited

1. Kleihues, P. and Cavenee, W. K., eds. Pathology and Genetics of Tumours of the Nervous System, Lyon: International Agency for Research on Cancer, 1997. 255p

2. Acs G, Pasha T, Zhang PJ. 2004. WT1 is differentially expressed in serous, endometrioid, clear cell, and mucinous carcinomas of the peritoneum, fallopian tube, ovary, and endometrium. ***International Journal of Gynecological Pathology*** **23**:110-118

3. Algar EM, Khromykh T, Smith SI, Blackburn DM, Bryson GJ, Smith PJ. 1996. A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. ***Oncogene*** **12**:1005-1014

4. Alsner J, Sorensen SB, Overgaard J. 2001. TP53 mutation is related to poor prognosis after radiotherapy, but not surgery, in squamous cell carcinoma of the head and neck. ***Radiother Oncol*** **59**:179-185

5. Anker L, Ohgaki H, Ludeke BI, Herrmann HD, Kleihues P, Westphal M. 1993. p53 protein accumulation and gene mutations in human glioma cell lines. ***Int J Cancer*** **55**:982-987

6. Arafat WO, Buchsbaum DJ, Gomez-Navarro J, Tawil SA, Olsen C, Xiang J, El Akad H, Salama AM, Badib AO, Stackhouse MA, Curiel DT. 2003. An adenovirus encoding proapoptotic Bax synergistically radiosensitizes malignant glioma. ***Int J Radiat Oncol Biol Phys*** **55**:1037-1050

7. Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JB. 1993. The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. ***Mech Dev*** **40**:85-97

8. Badie B, Kramar MH, Lau R, Boothman DA, Economou JS, Black KL. 1998. Adenovirus-mediated p53 gene delivery potentiates the radiation-induced growth inhibition of experimental brain tumors. ***J Neurooncol*** **37**:217-222

9. Bahuau M, Vidaud D, Jenkins RB, Bieche I, Kimmel DW, Assouline B, Smith JS, Alderete B, Cayuela JM, Harpey JP, Caille B, Vidaud M. 1998. Germ-line deletion involving the INK4 locus in familial proneness to melanoma and nervous system tumors. ***Cancer Res*** **58**:2298-2303

10. Barker FG, Simmons ML, Chang SM, Prados MD, Larson DA, Sneed PK, Wara WM, Berger MS, Chen PC, Israel MA, Aldape KD. 2001. EGFR overexpression and radiation response in glioblastoma multiforme. ***International Journal of Radiation Oncology Biology Physics*** **51**:410-418

11. Bleehen NM and Stenning SP. 1991. A Medical Research Council trial of two radiotherapy doses in the treatment of grades 3 and 4 astrocytoma. The Medical Research Council Brain Tumour Working Party. ***Br J Cancer*** **64**:769-774

12. Bogler O, Huang HJS, Kleihues P, Cavenee WK. 1995. The P53 Gene and Its Role in Human Brain-Tumors. ***Glia*** **15**:308-327

13. Breslow N, Beckwith JB, Ciol M, Sharples K. 1988. Age distribution of Wilms' tumor: report from the National Wilms' Tumor Study. ***Cancer Res*** **48**:1653-1657

14. Broaddus WC, Liu Y, Steele LL, Gillies GT, Lin PS, Loudon WG, Valerie K, Schmidt-Ullrich RK, Fillmore HL. 1999. Enhanced radiosensitivity of malignant glioma cells after adenoviral p53 transduction. ***J Neurosurg*** **91**:997-1004

15. Broaddus WC, Prabhu SS, Gillies GT, Neal J, Conrad WS, Chen ZJ, Fillmore H, Young HF. 1998. Distribution and stability of antisense phosphorothioate oligonucleotides in rodent brain following direct intraparenchymal controlled-rate infusion. ***J Neurosurg*** **88**:734-742

16. Bruening W, Gros P, Sato T, Stanimir J, Nakamura Y, Housman D, Pelletier J. 1993. Analysis of the 11p13 Wilms' tumor suppressor gene (WT1) in ovarian tumors. ***Cancer Invest*** **11**:393-399

17. Bruening W, Moffett P, Chia S, Heinrich G, Pelletier J. 1996. Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product. ***FEBS Lett*** **393**:41-47

18. Bruening W and Pelletier J. 1996. A non-AUG translational initiation event generates novel WT1 isoforms. ***J Biol Chem*** **271**:8646-8654

19. Buckler AJ, Pelletier J, Haber DA, Glaser T, Housman DE. 1991. Isolation, Characterization, and Expression of the Murine Wilms-Tumor Gene (Wt1) During Kidney Development. ***Molecular and Cellular Biology*** **11**:1707-1712

20. Burger PC, Heinz ER, Shibata T, Kleihues P. 1988. Topographic anatomy and CT correlations in the untreated glioblastoma multiforme. ***J Neurosurg*** **68**:698-704

21. Burton EC, Lamborn KR, Feuerstein BG, Prados M, Scott J, Forsyth P, Passe S, Jenkins RB, Aldape KD. 2002. Genetic aberrations defined by comparative genomic hybridization distinguish long-term from typical survivors of glioblastoma. ***Cancer Res*** **62**:6205-6210

22. Burton EC, Lamborn KR, Forsyth P, Scott J, O'Campo J, Uyehara-Lock J, Prados M, Berger M, Passe S, Uhm J, O'Neill BP, Jenkins RB, Aldape KD. 2002. Aberrant p53, mdm2, and proliferation differ in glioblastomas from long-term compared with typical survivors. ***Clin Cancer Res*** **8**:180-187

23. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE. 1990. Isolation and Characterization of A Zinc Finger Polypeptide Gene at the Human Chromosome-11 Wilms Tumor Locus. ***Cell*** **60**:509-520

24. Caricasole A, Duarte A, Larsson SH, Hastie ND, Little M, Holmes G, Todorov I, Ward A. 1996. RNA binding by the Wilms tumor suppressor zinc finger proteins. ***Proc Natl Acad Sci U S A*** **93**:7562-7566

25. Carrington D and Algar E. 2000. Overexpression of murine WT1 + / + and - / - isoforms has no effect on chemoresistance but delays differentiation in the K562 leukemia cell line. ***Leuk Res*** **24**:927-936

26. Castillo J, Erroba E, Perugorria MJ, Santamaria M, Lee DC, Prieto J, Avila MA, Berasain C. 2006. Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells. ***Cancer Res*** **66**:6129-6138

27. CBTRUS. 2006. Statistical report: Primary brain tumors in the United States, 1998-2002

28. Chakravarti A, Dicker A, Mehta M. 2004. The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data. ***Int J Radiat Oncol Biol Phys*** **58**:927-931

29. Chicoine MR and Silbergeld DL. 1995. The in vitro motility of human gliomas increases with increasing grade of malignancy. ***Cancer*** **75**:2904-2909

30. Clark AJ, Dos Santos WG, McCready J, Chen MY, Van Meter TE, Ware JL, Wolber SB, Fillmore H, Broaddus WC. 2007. Wilms' tumor 1 expression in malignant gliomas and correlation of +KTS isoforms with p53 status. ***J Neurosurg***

31. Cohen HT, Bossone SA, Zhu GM, McDonald GA, Sukhatme VP. 1997. Sp1 is a critical regulator of the Wilms' tumor-1 gene. ***Journal of Biological Chemistry*** **272**:2901-2913

32. Coppes-Zantinga AR and Coppes MJ. 1999. The eponym "Wilms": a reminder of a surgeon's lifelong contributions to medicine. ***Med Pediatr Oncol*** **32**:438-439

33. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. 2006. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. ***Cell*** **126**:121-134

34. D'Avenia P, Porrello A, Berardo M, Angelo MD, Soddu S, Arcangeli G, Sacchi A, D'Orazi G. 2006. Tp53-gene transfer induces hypersensitivity to low doses of X-rays in glioblastoma cells: a strategy to convert a radio-resistant phenotype into a radiosensitive one. ***Cancer Lett*** **231**:102-112

35. Dai C, Celestino JC, Okada Y, Louis DN, Fuller GN, Holland EC. 2001. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. ***Genes Dev*** **15**:1913-1925

36. Dai C, Lyustikman Y, Shih A, Hu X, Fuller GN, Rosenblum M, Holland EC. 2005. The characteristics of astrocytomas and oligodendrogliomas are caused by two distinct and interchangeable signaling formats. ***Neoplasia*** 7:397-406

37. Damon SE, Plymate SR, Carroll JM, Sprenger CC, Dechsukhum C, Ware JL, Roberts CT, Jr. 2001. Transcriptional regulation of insulin-like growth factor-I receptor gene expression in prostate cancer cells. ***Endocrinology*** **142**:21-27

38. Davies RC, Calvio C, Bratt E, Larsson SH, Lamond AI, Hastie ND. 1998. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. ***Genes Dev*** **12**:3217-3225

39. Deb S, Jackson CT, Subler MA, Martin DW. 1992. Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. ***J Virol*** **66**:6164-6170

40. Dechsukhum C, Ware JL, Ferreira-Gonzalez A, Wilkinson DS, Garrett CT. 2000. Detection of a novel truncated WT1 transcript in human neoplasia. ***Molecular Diagnosis*** **5**:117-128

41. DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR. 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. ***Cell*** **69**:265-273

42. Dejong V, Degeorges A, Filleur S, Ait-Si-Ali S, Mettouchi A, Bornstein P, Binetruy B, Cabon F. 1999. The Wilms' tumor gene product represses the transcription of thrombospondin 1 in response to overexpression of c-Jun. ***Oncogene*** **18**:3143-3151

43. Dennis SL, Manji SSM, Carrington DP, Scarcella DL, Ashley DM, Smith PJ, Algar EM. 2002. Expression and mutation analysis of the Wilms' tumor 1 gene in human neural tumors. ***International Journal of Cancer*** **97**:713-715

44. Deuel TF, Guan LS, Wang ZY. 1999. Wilms' tumor gene product WT1 arrests macrophage differentiation of HL-60 cells through its zinc-finger domain. ***Biochem Biophys Res Commun*** **254**:192-196

45. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. 1994. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. ***Genes Dev*** **8**:2540-2551

46. Discenza MT, Vaz D, Hassell JA, Pelletier J. 2004. Activation of the WT1 tumor suppressor gene promoter by Pea3. ***Febs Letters*** **560**:183-191

47. Dupont J, Wang X, Marshall DS, Leitao M, Hedvat CV, Hummer A, Thaler H, O'Reilly RJ, Soslow RA. 2004. Wilms tumor gene (WT1) and p53 expression in endometrial carcinomas: a study of 130 cases using a tissue microarray. ***Gynecologic Oncology*** **94**:449-455

48. Englert C, Hou X, Maheswaran S, Bennett P, Ngwu C, Re GG, Garvin AJ, Rosner MR, Haber DA. 1995. WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. ***EMBO J*** **14**:4662-4675

49. Englert C, Maheswaran S, Garvin AJ, Kreidberg J, Haber DA. 1997. Induction of p21 by the Wilms' tumor suppressor gene WT1. ***Cancer Res*** **57**:1429-1434

50. Forsyth PA, Wong H, Laing TD, Rewcastle NB, Morris DG, Muzik H, Leco KJ, Johnston RN, Brasher PM, Sutherland G, Edwards DR. 1999. Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. ***Br J Cancer*** **79**:1828-1835

51. Fraizer G, Leahy R, Priyadarshini S, Graham K, Delacerda J, Diaz M. 2004. Suppression of prostate tumor cell growth in vivo by WT1, the Wilms' tumor suppressor gene. ***Int J Oncol*** **24**:461-471

52. Fraizer GC, Shimamura R, Zhang XH, Saunders GF. 1997. PAX 8 regulates human WT1 transcription through a novel DNA binding site. ***Journal of Biological Chemistry*** **272**:30678-30687

53. Francke U, Holmes LB, Atkins L, Riccardi VM. 1979. Aniridia-Wilms' tumor association: evidence for specific deletion of 11p13. ***Cytogenet Cell Genet*** **24**:185-192

54. Furstenberger G and Senn HJ. 2002. Insulin-like growth factors and cancer. ***Lancet Oncol*** **3**:298-302

55. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, Stauss HJ. 2000. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. ***Blood*** **95**:2198-2203

56. Gaspar LE, Fisher BJ, Macdonald DR, LeBer DV, Halperin EC, Schold SC, Jr., Cairncross JG. 1992. Supratentorial malignant glioma: patterns of recurrence and implications for external beam local treatment. ***Int J Radiat Oncol Biol Phys*** **24**:55-57

57. Gerald WL, Rosai J, Ladanyi M. 1995. Characterization of the genomic breakpoint and chimeric transcripts in the EWS-WT1 gene fusion of desmoplastic small round cell tumor. ***Proc Natl Acad Sci U S A*** **92**:1028-1032

58. Gessler M, Konig A, Bruns GA. 1992. The genomic organization and expression of the WT1 gene. ***Genomics*** **12**:807-813

59. Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GA. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. ***Nature*** **343**:774-778

60. Giangaspero F and Burger PC. 1983. Correlations between cytologic composition and biologic behavior in the glioblastoma multiforme. A postmortem study of 50 cases. ***Cancer*** **52**:2320-2333

61. Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, Schroeder MA, James CD. 2005. Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. ***Neuro -oncol*** **7**:164-176

62. Giese A, Loo MA, Norman SA, Treasurywala S, Berens ME. 1996. Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins. ***J Cell Sci*** **109 (Pt 8)**:2161-2168

63. Giese A, Rief MD, Loo MA, Berens ME. 1994. Determinants of human astrocytoma migration. ***Cancer Res*** **54**:3897-3904

64. Gillmore R, Xue SA, Holler A, Kaeda J, Hadjiminas D, Healy V, Dina R, Parry SC, Bellantuono I, Ghani Y, Coombes RC, Waxman J, Stauss HJ. 2006. Detection of Wilms' tumor antigen--specific CTL in tumor-draining lymph nodes of patients with early breast cancer. ***Clin Cancer Res*** **12**:34-42

65. Gire V and Wynford-Thomas D. 1998. Reinitiation of DNA synthesis and cell division in senescent human fibroblasts by microinjection of anti-p53 antibodies. ***Mol Cell Biol*** **18**:1611-1621

66. Gjerset RA, Turla ST, Sobol RE, Scalise JJ, Mercola D, Collins H, Hopkins PJ. 1995. Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. ***Mol Carcinog*** **14**:275-285

67. Glaser T, Driscoll DJ, Antonarakis S, Valle D, Housman D. 1989. A highly polymorphic locus cloned from the breakpoint of a chromosome 11p13 deletion associated with the WAGR syndrome. ***Genomics*** **5**:880-893

68. Gomez-Manzano C, Fueyo J, Kyritsis AP, Steck PA, Roth JA, McDonnell TJ, Steck KD, Levin VA, Yung WK. 1996. Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. ***Cancer Res*** **56**:694-699

69. Gondi CS, Lakka SS, Dinh DH, Olivero WC, Gujrati M, Rao JS. 2004. Downregulation of uPA, uPAR and MMP-9 using small, interfering, hairpin RNA (siRNA) inhibits glioma cell invasion, angiogenesis and tumor growth. ***Neuron Glia Biol*** **1**:165-176

70. Green DM, Breslow NE, Beckwith JB, Finklestein JZ, Grundy P, Thomas PR, Kim T, Shochat S, Haase G, Ritchey M, Kelalis P, D'Angio GJ. 1998. Effect of duration of treatment on treatment outcome and cost of treatment for Wilms' tumor: a report from the National Wilms' Tumor Study Group. ***J Clin Oncol*** **16**:3744-3751

71. Guha A, Dashner K, Black PM, Wagner JA, Stiles CD. 1995. Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop. ***Int J Cancer*** **60**:168-173

72. Guo P, Hu B, Gu W, Xu L, Wang D, Huang HJ, Cavenee WK, Cheng SY. 2003. Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. ***Am J Pathol*** **162**:1083-1093

73. Gurney JG, Severson RK, Davis S, Robison LL. 1995. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. ***Cancer*** **75**:2186-2195

74. Haas-Kogan DA, Kogan SS, Yount G, Hsu J, Haas M, Deen DF, Israel MA. 1999. p53 function influences the effect of fractionated radiotherapy on glioblastoma tumors. ***Int J Radiat Oncol Biol Phys*** **43**:399-403

75. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. 1991. Alternative Splicing and Genomic Structure of the Wilms-Tumor Gene-Wt1. ***Proceedings of the National Academy of Sciences of the United States of America*** **88**:9618-9622

76. Han Y, San Marina S, Liu J, Minden MD. 2004. Transcriptional activation of c-myc proto-oncogene by WT1 protein. ***Oncogene*** **23**:6933-6941

77. Hastie ND. 2001. Life, sex, and WT1 isoforms - Three amino acids can make all the difference. ***Cell*** **106**:391-394

78. Haupt Y, Maya R, Kazaz A, Oren M. 1997. Mdm2 promotes the rapid degradation of p53. ***Nature*** **387**:296-299

79. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R. 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. ***N Engl J Med*** **352**:997-1003

80. Henson JW, Schnitker BL, Correa KM, von Deimling A, Fassbender F, Xu HJ, Benedict WF, Yandell DW, Louis DN. 1994. The retinoblastoma gene is involved in malignant progression of astrocytomas. ***Ann Neurol*** **36**:714-721

81. Herzer U, Crocoll A, Barton D, Howells N, Englert C. 1999. The Wilms tumor suppressor gene wt1 is required for development of the spleen. ***Curr Biol*** **9**:837-840

82. Hess KR, Broglio KR, Bondy ML. 2004. Adult glioma incidence trends in the United States, 1977-2000. ***Cancer*** **101**:2293-2299

83. Hewitt SM, Fraizer GC, Wu YJ, Rauscher FJ, III, Saunders GF. 1996. Differential function of Wilms' tumor gene WT1 splice isoforms in transcriptional regulation. ***J Biol Chem*** **271**:8588-8592

84. Holdhoff M, Kreuzer KA, Appelt C, Scholz R, Na IK, Hildebrandt B, Riess H, Jordan A, Schmidt CA, Van Etten RA, Dorken B, le Coutre P. 2005. Imatinib mesylate radiosensitizes human glioblastoma cells through inhibition of platelet-derived growth factor receptor. ***Blood Cells Mol Dis*** **34**:181-185

85. Hosono S, Gross I, English MA, Hajra KM, Fearon ER, Licht JD. 2000. E-cadherin is a WT1 target gene. ***J Biol Chem*** **275**:10943-10953

86. Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, Huang CM, Gill GN, Wiley HS, Cavenee WK. 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. ***J Biol Chem*** **272**:2927-2935

87. Idelman G, Glaser T, Roberts CT, Werner H. 2003. WT1-p53 interactions in insulin-like growth factor-I receptor gene regulation. ***Journal of Biological Chemistry*** **278**:3474-3482

88. Inoue K, Ogawa H, Sonoda Y, Kimura T, Sakabe H, Oka Y, Miyake S, Tamaki H, Oji Y, Yamagami T, Tatekawa T, Soma T, Kishimoto T, Sugiyama H. 1997. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. ***Blood*** **89**:1405-1412

89. Inoue K, Ogawa H, Yamagami T, Soma T, Tani Y, Tatekawa T, Oji Y, Tamaki H, Kyo T, Dohy H, Hiraoka A, Masaoka T, Kishimoto T, Sugiyama H. 1996. Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. ***Blood*** **88**:2267-2278

90. Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K, Kyo T, Dohy H, Nakauchi H, Ishidate T, Akiyama T, Kishimoto T. 1994. Wt1 As A New Prognostic Factor and A New Marker for the Detection of Minimal Residual Disease in Acute-Leukemia. ***Blood*** **84**:3071-3079

91. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Akiyama T, Kishimoto T, Sugiyama H. 1998. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. ***Blood*** **91**:2969-2976

92. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, Van Meir EG. 1999. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. ***Brain Pathol*** **9**:469-479

93. Ito K, Oji Y, Tatsumi N, Shimizu S, Kanai Y, Nakazawa T, Asada M, Jomgeow T, Aoyagi S, Nakano Y, Tamaki H, Sakaguchi N, Shirakata T, Nishida S, Kawakami M, Tsuboi A, Oka Y, Tsujimoto Y, Sugiyama H. 2006. Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. ***Oncogene*** **25**:4217-4229

94. Jensen RL, Ragel BT, Whang K, Gillespie D. 2006. Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. ***J Neurooncol*** **78**:233-247

95. Jomgeow T, Oji Y, Tsuji N, Ikeda Y, Ito K, Tsuda A, Nakazawa T, Tatsumi N, Sakaguchi N, Takashima S, Shirakata T, Nishida S, Hosen N, Kawakami M, Tsuboi A, Oka Y, Itoh K, Sugiyama H. 2006. Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion in vitro. ***Cancer Sci*** **97**:259-270

96. Jones TR, Bigner SH, Schold SC, Jr., Eng LF, Bigner DD. 1981. Anaplastic human gliomas grown in athymic mice. Morphology and glial fibrillary acidic protein expression. ***Am J Pathol*** **105**:316-327

97. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. ***Exp Cell Res*** **284**:31-53

98. Kaneuchi M, Sasaki M, Tanaka Y, Shiina H, Yamada H, Yamamoto R, Sakuragi N, Enokida H, Verma M, Dahiya R. 2005. WT1 and WT1-AS genes are inactivated by promoter methylation in ovarian clear cell adenocarcinoma. ***Cancer*** **104**:1924-1930

99. Karnofsky DA and Burchenal JH. 1949. The Clinical Evaluation of Chemotherapeutic Agents In Cancer191-205

100. Kennedy D, Ramsdale T, Mattick J, Little M. 1996. An RNA recognition motif in Wilms' tumour protein (WT1) revealed by structural modelling. ***Nat Genet*** **12**:329-331

101. Kilic T, Alberta JA, Zdunek PR, Acar M, Iannarelli P, O'Reilly T, Buchdunger E, Black PM, Stiles CD. 2000. Intracranial inhibition of platelet-derived growth factor-mediated glioblastoma cell growth by an orally active kinase inhibitor of the 2-phenylaminopyrimidine class. ***Cancer Res*** **60**:5143-5150

102. King-Underwood L and Pritchard-Jones K. 1998. Wilms' tumor (WT1) gene mutations occur mainly in acute myeloid leukemia and may confer drug resistance. ***Blood*** **91**:2961-2968

103. Kioi M, Kawakami K, Puri RK. 2004. Analysis of antitumor activity of an interleukin-13 (IL-13) receptor-targeted cytotoxin composed of IL-13 antagonist and Pseudomonas exotoxin. ***Clin Cancer Res*** **10**:6231-6238

104. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK. 2002. The WHO classification of tumors of the nervous system. ***J Neuropathol Exp Neurol*** **61**:215-225

105. Kleymenova EV, Yuan X, LaBate ME, Walker CL. 1998. Identification of a tumor-specific methylation site in the Wilms tumor suppressor gene. ***Oncogene*** **16**:713-720

106. Knudson AG, Jr. and Strong LC. 1972. Mutation and cancer: a model for Wilms' tumor of the kidney. ***J Natl Cancer Inst*** **48**:313-324

107. Kokkinakis DM, Bocangel DB, Schold SC, Moschel RC, Pegg AE. 2001. Thresholds of O6-alkylguanine-DNA alkyltransferase which confer significant resistance of human glial tumor xenografts to treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolomide. ***Clin Cancer Res*** 7:421-428

108. Koufos A, Hansen MF, Lampkin BC, Workman ML, Copeland NG, Jenkins NA, Cavenee WK. 1984. Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumour. ***Nature*** **309**:170-172

109. Kragh M, Quistorff B, Tenan M, Van Meir EG, Kristjansen PE. 2002. Overexpression of thrombospondin-1 reduces growth and vascular index but not perfusion in glioblastoma. ***Cancer Res*** **62**:1191-1195

110. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. 1993. WT-1 is required for early kidney development. ***Cell*** **74**:679-691

111. Kumar-Singh S, Segers K, Rodeck U, Backhovens H, Bogers J, Weyler J, Van Broeckhoven C, Van Marck E. 1997. WT1 mutation in malignant mesothelioma and WT1 immunoreactivity in relation to p53 and growth factor receptor expression, cell-type transition, and prognosis. ***J Pathol*** **181**:67-74

112. Kunkel P, Ulbricht U, Bohlen P, Brockmann MA, Fillbrandt R, Stavrou D, Westphal M, Lamszus K. 2001. Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. ***Cancer Res*** **61**:6624-6628

113. Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F, Lang FF, McCutcheon IE, Hassenbusch SJ, Holland E, Hess K, Michael C, Miller D, Sawaya

R. 2001. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. ***J Neurosurg*** **95**:190-198

114. Ladomery MR, Slight J, Mc GS, Hastie ND. 1999. Presence of WT1, the Wilm's tumor suppressor gene product, in nuclear poly(A)(+) ribonucleoprotein. ***J Biol Chem*** **274**:36520-36526

115. Laity JH, Chung J, Dyson HJ, Wright PE. 2000. Alternative splicing of Wilms' tumor suppressor protein modulates DNA binding activity through isoform-specific DNA-induced conformational changes. ***Biochemistry*** **39**:5341-5348

116. Laity JH, Dyson HJ, Wright PE. 2000. Molecular basis for modulation of biological function by alternate splicing of the Wilms' tumor suppressor protein. ***Proc Natl Acad Sci U S A*** **97**:11932-11935

117. Lal A, Glazer CA, Martinson HM, Friedman HS, Archer GE, Sampson JH, Riggins GJ. 2002. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. ***Cancer Res*** **62**:3335-3339

118. Lamborn KR, Chang SM, Prados MD. 2004. Prognostic factors for survival of patients with glioblastoma: Recursive partitioning analysis. ***Neuro-Oncology*** **6**:227-235

119. Lang FF, Miller DC, Koslow M, Newcomb EW. 1994. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. ***J Neurosurg*** **81**:427-436

120. Laperriere N, Zuraw L, Cairncross G. 2002. Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review. ***Radiother Oncol*** **64**:259-273

121. Larsson SH, Charlieu JP, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, Van H, V, Hastie ND. 1995. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. ***Cell*** **81**:391-401

122. Lassman AB, Dai C, Fuller GN, Vickers AJ, Holland EC. 2004. Overexpression of c-MYC promotes an undifferentiated phenotype in cultured astrocytes and allows elevated Ras and Akt signaling to induce gliomas from GFAP-expressing cells in mice. ***Neuron Glia Biol*** **1**:157-163

123. Laux DE, Curran EM, Welshons WV, Lubahn DB, Huang TH. 1999. Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas. ***Breast Cancer Res Treat*** **56**:35-43

124. Lee SB, Huang K, Palmer R, Truong VB, Herzlinger D, Kolquist KA, Wong J, Paulding C, Yoon SK, Gerald W, Oliner JD, Haber DA. 1999. The Wilms tumor suppressor WT1 encodes a transcriptional activator of amphiregulin. ***Cell*** **98**:663-673

125. Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. ***Nature*** **313**:144-147

126. Little MH, Prosser J, Condie A, Smith PJ, Van H, V, Hastie ND. 1992. Zinc finger point mutations within the WT1 gene in Wilms tumor patients. ***Proc Natl Acad Sci U S A*** **89**:4791-4795

127. Liu XW, Gong LJ, Guo LY, Katagiri Y, Jiang H, Wang ZY, Johnson AC, Guroff G. 2001. The Wilms' tumor gene product WT1 mediates the down-regulation of the rat epidermal growth factor receptor by nerve growth factor in PC12 cells. ***J Biol Chem*** **276**:5068-5073

128. Loeb DM, Evron E, Patel CB, Sharma PM, Niranjan B, Buluwela L, Weitzman SA, Korz D, Sukumar S. 2001. Wilms' tumor suppressor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation. ***Cancer Res*** **61**:921-925

129. Loeb DM, Korz D, Katsnelson M, Burwell EA, Friedman AD, Sukumar S. 2002. Cyclin E is a target of WT1 transcriptional repression. ***J Biol Chem*** **277**:19627-19632

130. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. 2002. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. ***Cancer Res*** **62**:3729-3735

131. Louis DN and Gusella JF. 1995. A Tiger Behind Many Doors - Multiple Genetic Pathways to Malignant Glioma. ***Trends in Genetics*** **11**:412-415

132. Lowe SW and Lin AW. 2000. Apoptosis in cancer. ***Carcinogenesis*** **21**:485-495

133. Lowe SW, Ruley HE, Jacks T, Housman DE. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. ***Cell*** **74**:957-967

134. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DM, Cavenee WK, DePinho RA. 2001. Malignant glioma: genetics and biology of a grave matter. ***Genes & Development*** **15**:1311-1333

135. Mahesparan R, Tysnes BB, Read TA, Enger PO, Bjerkvig R, Lund-Johansen M. 1999. Extracellular matrix-induced cell migration from glioblastoma biopsy specimens in vitro. ***Acta Neuropathol (Berl)*** **97**:231-239

136. Maheswaran S, Englert C, Bennett P, Heinrich G, Haber DA. 1995. The Wt1 Gene-Product Stabilizes P53 and Inhibits P53-Mediated Apoptosis. ***Genes & Development*** **9**:2143-2156

137. Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ, Hill DE, Haber DA. 1993. Physical and Functional Interaction Between Wt1 and P53 Proteins. ***Proceedings of the National Academy of Sciences of the United States of America*** **90**:5100-5104

138. Malik KT, Poirier V, Ivins SM, Brown KW. 1994. Autoregulation of the human WT1 gene promoter. ***FEBS Lett*** **349**:75-78

139. Malkin D, Li FP, Strong LC, Fraumeni JF, Jr., Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, . 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. ***Science*** **250**:1233-1238

140. Mares J, Kriz V, Weinhausel A, Vodickova S, Kodet R, Haas OA, Sedlacek Z, Goetz P. 2001. Methylation changes in promoter and enhancer regions of the WT1 gene in Wilms' tumours. ***Cancer Lett*** **166**:165-171

141. Mariani L, Beaudry C, McDonough WS, Hoelzinger DB, Demuth T, Ross KR, Berens T, Coons SW, Watts G, Trent JM, Wei JS, Giese A, Berens ME. 2001.

Glioma cell motility is associated with reduced transcription of proapoptotic and proliferation genes: a cDNA microarray analysis. ***J Neurooncol*** **53**:161-176

142. MATSUKADO Y, MacCarty CS, KERNOHAN JW. 1961. The growth of glioblastoma multiforme (astrocytomas, grades 3 and 4) in neurosurgical practice. ***J Neurosurg*** **18**:636-644

143. Matsuzawa K, Fukuyama K, Hubbard SL, Dirks PB, Rutka JT. 1996. Transfection of an invasive human astrocytoma cell line with a TIMP-1 cDNA: modulation of astrocytoma invasive potential. ***J Neuropathol Exp Neurol*** **55**:88-96

144. Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, Weissman BE, Baldwin AS. 1999. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. ***EMBO J*** **18**:3990-4003

145. McConnell MJ, Cunliffe HE, Chua LJ, Ward TA, Eccles MR. 1997. Differential regulation of the human Wilms tumour suppressor gene (WTI) promoter by two isoforms of PAX2. ***Oncogene*** **14**:2689-2700

146. McCready J, Broaddus WC, Sykes V, Fillmore HL. 2005. Association of a single nucleotide polymorphism in the matrix metalloproteinase-1 promoter with glioblastoma. ***Int J Cancer*** **117**:781-785

147. McDonough W, Tran N, Giese A, Norman SA, Berens ME. 1998. Altered gene expression in human astrocytoma cells selected for migration: I. Thromboxane synthase. ***J Neuropathol Exp Neurol*** **57**:449-455

148. McLendon RE and Halperin EC. 2003. Is the long-term survival of patients with intracranial glioblastoma multiforme overstated? ***Cancer*** **98**:1745-1748

149. Melean G, Sestini R, Ammannati F, Papi L. 2004. Genetic insights into familial tumors of the nervous system. ***Am J Med Genet C Semin Med Genet*** **129**:74-84

150. Menke AL, Riteco N, van Ham RC, de Bruyne C, Rauscher FJ, III, van der Eb AJ, Jochemsen AG. 1996. Wilms' tumor 1 splice variants have opposite effects on the tumorigenicity of adenovirus-transformed baby-rat kidney cells. ***Oncogene*** **12**:537-546

151. Menke AL, van der Eb AJ, Jochemsen AG. 1998. The Wilms' tumor 1 gene: Oncogene or tumor suppressor gene? ***International Review of Cytology - A Survey of Cell Biology, Vol 181*** **181**:151-212

152. Menssen HD, Bertelmann E, Bartelt S, Schmidt RA, Pecher G, Schramm K, Thiel E. 2000. Wilms' tumor gene (WT1) expression in lung cancer, colon cancer and glioblastoma cell lines compared to freshly isolated tumor specimens. ***Journal of Cancer Research and Clinical Oncology*** **126**:226-232

153. Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R, Thiel E. 1995. Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. ***Leukemia*** **9**:1060-1067

154. Mierau GW, Beckwith JB, Weeks DA. 1987. Ultrastructure and histogenesis of the renal tumors of childhood: an overview. ***Ultrastruct Pathol*** **11**:313-333

155. Mineo JF, Bordron A, Isabelle QR, Maurage CA, Virginie B, Severine L, Dubois F, Blond S, Berthou C. 2006. Increasing of HER2 membranar density in human glioblastoma U251MG cell line established in a new nude mice model. ***J Neurooncol*** **76**:249-255

156. Misra A, Chattopadhyay P, Dinda AK, Sarkar C, Mahapatra AK, Hasnain SE, Sinha S. 2000. Extensive intra-tumor heterogeneity in primary human glial tumors as a result of locus non-specific genomic alterations. ***Journal of Neuro-Oncology*** **48**:1-12

157. Miwa H, Beran M, Saunders GF. 1992. Expression of the Wilms' tumor gene (WT1) in human leukemias. ***Leukemia*** **6**:405-409

158. Miyashita T and Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. ***Cell*** **80**:293-299

159. Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Sugiyama H, Noguchi S. 2002. High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. ***Clinical Cancer Research*** **8**:1167-1171

160. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. 1999. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. ***Development*** **126**:1845-1857

161. Morita S, Oka Y, Tsuboi A, Kawakami M, Maruno M, Izumoto S, Osaki T, Taguchi T, Ueda T, Myoui A, Nishida S, Shirakata T, Ohno S, Oji Y, Aozasa K, Hatazawa J, Udaka K, Yoshikawa H, Yoshimine T, Noguchi S, Kawase I, Nakatsuka S, Sugiyama H, Sakamoto J. 2006. A phase I/II trial of a WT1 (Wilms' tumor gene) peptide vaccine in patients with solid malignancy: safety assessment based on the phase I data. ***Jpn J Clin Oncol*** **36**:231-236

162. Morris JF, Madden SL, Tournay OE, Cook DM, Sukhatme VP, Rauscher FJ, III. 1991. Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. ***Oncogene*** **6**:2339-2348

163. Morrison AA and Ladomery MR. 2006. Presence of WT1 in nuclear messenger RNP particles in the human acute myeloid leukemia cell lines HL60 and K562. ***Cancer Lett***

164. Nagane M, Coufal F, Lin H, Bogler O, Cavenee WK, Huang HJ. 1996. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. ***Cancer Res*** **56**:5079-5086

165. Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. 1998. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. ***Proc Natl Acad Sci U S A*** **95**:5724-5729

166. Nakagama H, Heinrich G, Pelletier J, Housman DE. 1995. Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. ***Mol Cell Biol*** **15**:1489-1498

167. Nakagawa T, Kubota T, Kabuto M, Sato K, Kawano H, Hayakawa T, Okada Y. 1994. Production of matrix metalloproteinases and tissue inhibitor of metalloproteinases-1 by human brain tumors. ***J Neurosurg*** **81**:69-77

168. Nakahara Y, Okamoto H, Mineta T, Tabuchi K. 2004. Expression of the Wilms' tumor gene product WT1 in glioblastomas and medulloblastomas. ***Brain Tumor Pathol*** **21**:113-116

169. Neglia JP, Meadows AT, Robison LL, Kim TH, Newton WA, Ruymann FB, Sather HN, Hammond GD. 1991. Second neoplasms after acute lymphoblastic leukemia in childhood. ***N Engl J Med*** **325**:1330-1336

170. Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, Pohl U, Hartmann C, McLaughlin ME, Batchelor TT, Black PM, von Deimling A, Pomeroy SL, Golub TR, Louis DN. 2003. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. ***Cancer Res*** **63**:1602-1607

171. Ohgaki H and Kleihues P. 2005. Epidemiology and etiology of gliomas. ***Acta Neuropathol (Berl)*** **109**:93-108

172. Ohminami H, Yasukawa M, Fujita S. 2000. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. ***Blood*** **95**:286-293

173. Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Nakatsuka S, Koga S, Ikeba A, Abeno S, Honjo Y, Yamamoto Y, Iwai S, Yoshida K, Oka Y, Ogawa H, Yoshida J, Aozasa K, Kubo T, Sugiyama H. 2003. Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. ***Cancer Sci*** **94**:523-529

174. Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka SI, Yao M, Takahashi E, Nakano Y, Hirabayashi H, Shintani Y, Oka Y, Tsuboi A, Hosen N, Asada M, Fujioka T, Murakami M, Kanato K, Motomura M, Kim EH, Kawakami M, Ikegame K, Ogawa H, Aozasa K, Kawase I, Sugiyama H. 2002. Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. ***International Journal of Cancer*** **100**:297-303

175. Oji Y, Miyoshi S, Takahashi E, Koga S, Nakano Y, Shintani Y, Hirabayashi H, Matsumura A, Iuchi K, Ito K, Kishimoto Y, Tsuboi A, Ikegame K, Hosen N, Oka Y, Ogawa H, Maeda H, Hayashi S, Kawase I, Sugiyama H. 2004. Absence of mutations in the Wilms' tumor gene wt1 in de novo non-small cell lung cancers. ***Neoplasma*** **51**:17-20

176. Oji Y, Miyoshi Y, Koga S, Nakano Y, Ando A, Nakatsuka S, Ikeba A, Takahashi E, Sakaguchi N, Yokota A, Hosen N, Ikegame K, Kawakami M, Tsuboi A, Oka Y, Ogawa H, Aozasa K, Noguchi S, Sugiyama H. 2003. Overexpression of the Wilms' tumor gene WT1 in primary thyroid cancer. ***Cancer Science*** **94**:606-611

177. Oji Y, Nakamori S, Fujikawa M, Nakatsuka S, Yokota A, Tatsumi N, Abeno S, Ikeba A, Takashima S, Tsujie M, Yamamoto H, Sakon M, Nezu R, Kawano K, Nishida S, Ikegame K, Kawakami M, Tsuboi A, Oka Y, Yoshikawa K, Aozasa K, Monden M,

Sugiyama H. 2004. Overexpression of the Wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma. ***Cancer Sci*** **95**:583-587

178. Oji Y, Suzuki T, Nakano Y, Maruno M, Nakatsuka S, Jomgeow T, Abeno S, Tatsumi N, Yokota A, Aoyagi S, Nakazawa T, Ito K, Kanato K, Shirakata T, Nishida S, Hosen N, Kawakami M, Tsuboi A, Oka Y, Aozasa K, Yoshimine T, Sugiyama H. 2004. Overexpression of the Wilms' tumor gene W T1 in primary astrocytic tumors. ***Cancer Sci*** **95**:822-827

179. Oji Y, Yamamoto H, Nomura M, Nakano Y, Ikeba A, Nakatsuka S, Abeno S, Kiyotoh E, Jomgeow T, Sekimoto M, Nezu R, Yoshikawa Y, Inoue Y, Hosen N, Kawakami M, Tsuboi A, Oka Y, Ogawa H, Souda S, Aozasa K, Monden M, Sugiyama H. 2003. Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. ***Cancer Sci*** **94**:712-717

180. Oji Y, Yano M, Nakano Y, Abeno S, Nakatsuka S, Ikeba A, Yasuda T, Fujiwara Y, Takiguchi S, Yamamoto H, Fujita S, Kanato K, Ito K, Jomgeow T, Kawakami M, Tsuboi A, Shirakata T, Nishida S, Hosen N, Oka Y, Aozasa K, Monden M, Sugiyama H. 2004. Overexpression of the Wilms' tumor gene WT1 in esophageal cancer. ***Anticancer Res*** **24**:3103-3108

181. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu F, Fujiki F, Murakami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase L, Sugiyama H. 2004. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. ***Proceedings of the National Academy of Sciences of the United States of America*** **101**:13885-13890

182. Oka Y, Udaka K, Tsuboi A, Elisseeva OA, Ogawa H, Aozasa K, Kishimoto T, Sugiyama H. 2000. Cancer immunotherapy targeting Wilms' tumor gene WT1 product. ***J Immunol*** **164**:1873-1880

183. Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, . 1993. The Wilms tumour gene WT1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. ***Nat Genet*** **4**:415-420

184. Pasquier B, Pasquier D, N'Golet A, Panh MH, Couderc P. 1980. Extraneural metastases of astrocytomas and glioblastomas: clinicopathological study of two cases and review of literature. ***Cancer*** **45**:112-125

185. Patmasiriwat P, Fraizer GC, Claxton D, Kantarjian H, Saunders GF. 1996. Expression pattern of WT1 and GATA-1 in AML with chromosome 16q22 abnormalities. ***Leukemia*** **10**:1127-1133

186. Paulus W, Baur I, Beutler AS, Reeves SA. 1996. Diffuse brain invasion of glioma cells requires beta 1 integrins. ***Lab Invest*** **75**:819-826

187. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, . 1991. Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. ***Cell*** **67**:437-447

188. Pollack IF, Finkelstein SD, Woods J, Burnham J, Holmes EJ, Hamilton RL, Yates AJ, Boyett JM, Finlay JL, Sposto R. 2002. Expression of p53 and prognosis in children with malignant gliomas. ***New England Journal of Medicine*** **346**:420-427

189. Povlsen CO and Rygaard J. 1971. Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant Nude. A study of nine consecutive transplantations. ***Acta Pathol Microbiol Scand [A]*** **79**:159-169

190. Prabhu SS, Broaddus WC, Gillies GT, Loudon WG, Chen ZJ, Smith B. 1998. Distribution of macromolecular dyes in brain using positive pressure infusion: a model for direct controlled delivery of therapeutic agents. ***Surg Neurol*** **50**:367-375

191. Quezado MM, Dechsukhum C, Garrett CT, Ware JL, Burks RT, Fillmore HL, Broaddus WC, Elmore LW. 2000. WT1 expression in ovarian and brain cancer cell lines. ***United States and Canadian Academy of Pathology, Annual Meeting (abstract)***

192. Rauscher FJ, III, Morris JF, Tournay OE, Cook DM, Curran T. 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. ***Science*** **250**:1259-1262

193. Reardon DA, Rich JN, Friedman HS, Bigner DD. 2006. Recent advances in the treatment of malignant astrocytoma. ***J Clin Oncol*** **24**:1253-1265

194. Recht L, Jang T, Savarese T, Litofsky NS. 2003. Neural stem cells and neuro-oncology: quo vadis? ***J Cell Biochem*** **88**:11-19

195. Reddy JC, Morris JC, Wang J, English MA, Haber DA, Shi Y, Licht JD. 1995. WT1-mediated transcriptional activation is inhibited by dominant negative mutant proteins. ***J Biol Chem*** **270**:10878-10884

196. Reifenberger G, Liu L, Ichimura K, Schmidt EE, Collins VP. 1993. Amplification and Overexpression of the Mdm2 Gene in A Subset of Human-Malignant Gliomas Without P53 Mutations. ***Cancer Research*** **53**:2736-2739

197. Reizner N, Maor S, Sarfstein R, Abramovitch S, Welshons WV, Curran EM, Lee AV, Werner H. 2005. The WT1 Wilms' tumor suppressor gene product interacts with estrogen receptor-alpha and regulates IGF-I receptor gene transcription in breast cancer cells. ***J Mol Endocrinol*** **35**:135-144

198. Renshaw J, Orr RM, Walton MI, Te PR, Williams RD, Wancewicz EV, Monia BP, Workman P, Pritchard-Jones K. 2004. Disruption of WT1 gene expression and exon 5 splicing following cytotoxic drug treatment: antisense down-regulation of exon 5 alters target gene expression and inhibits cell survival. ***Mol Cancer Ther*** **3**:1467-1484

199. Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BK, Dobra A, Dressman HK, Bigner DD, Nevins JR, West M. 2005. Gene expression profiling and genetic markers in glioblastoma survival. ***Cancer Res*** **65**:4051-4058

200. Richard DJ, Schumacher V, Royer-Pokora B, Roberts SG. 2001. Par4 is a coactivator for a splice isoform-specific transcriptional activation domain in WT1. ***Genes Dev*** **15**:328-339

201. Rooprai HK, Rucklidge GJ, Panou C, Pilkington GJ. 2000. The effects of exogenous growth factors on matrix metalloproteinase secretion by human brain tumour cells. ***Br J Cancer*** **82**:52-55

202. Rubio MP, von Deimling A, Yandell DW, Wiestler OD, Gusella JF, Louis DN. 1993. Accumulation of wild type p53 protein in human astrocytomas. ***Cancer Res*** **53**:3465-3467

203. Rupprecht HD, Drummond IA, Madden SL, Rauscher FJ, III, Sukhatme VP. 1994. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. ***J Biol Chem*** **269**:6198-6206

204. Saito R, Krauze MT, Noble CO, Drummond DC, Kirpotin DB, Berger MS, Park JW, Bankiewicz KS. 2006. Convection-enhanced delivery of Ls-TPT enables an effective, continuous, low-dose chemotherapy against malignant glioma xenograft model. ***Neuro -oncol*** **8**:205-214

205. Sakamoto Y, Yoshida M, Semba K, Hunter T. 1997. Inhibition of the DNA-binding and transcriptional repression activity of the Wilms' tumor gene product, WT1, by cAMP-dependent protein kinase-mediated phosphorylation of Ser-365 and Ser-393 in the zinc finger domain. ***Oncogene*** **15**:2001-2012

206. Sarkaria JN, Carlson BL, Schroeder MA, Grogan P, Brown PD, Giannini C, Ballman KV, Kitange GJ, Guha A, Pandita A, James CD. 2006. Use of an orthotopic xenograft model for assessing the effect of epidermal growth factor receptor amplification on glioblastoma radiation response. ***Clin Cancer Res*** **12**:2264-2271

207. Sasaki Y, Morimoto I, Ishida S, Yamashita T, Imai K, Tokino T. 2001. Adenovirus-mediated transfer of the p53 family genes, p73 and p51/p63 induces cell cycle arrest and apoptosis in colorectal cancer cell lines: potential application to gene therapy of colorectal cancer. ***Gene Ther*** **8**:1401-1408

208. Scharnhorst V, Dekker P, van der Eb AJ, Jochemsen AG. 1999. Internal translation initiation generates novel WT1 protein isoforms with distinct biological properties. ***J Biol Chem*** **274**:23456-23462

209. Scharnhorst V, Dekker P, van der Eb AJ, Jochemsen AG. 2000. Physical interaction between Wilms tumor 1 and p73 proteins modulates their functions. ***J Biol Chem*** **275**:10202-10211

210. Scharnhorst V, van der Eb AJ, Jochemsen AG. 2001. WT1 proteins: functions in growth and differentiation. ***Gene*** **273**:141-161

211. Schiebe M, Ohneseit P, Hoffmann W, Meyermann R, Rodemann HP, Bamberg M. 2000. Analysis of mdm2 and p53 gene alterations in glioblastomas and its correlation with clinical factors. ***J Neurooncol*** **49**:197-203

212. Schumacher V, Schuhen S, Sonner S, Weirich A, Leuschner I, Harms D, Licht J, Roberts S, Royer-Pokora B. 2003. Two molecular subgroups of Wilms' tumors with or without WT1 mutations. ***Clinical Cancer Research*** **9**:2005-2014

213. Schwartzbaum J, Ahlbom A, Malmer B, Lonn S, Brookes AJ, Doss H, Debinski W, Henriksson R, Feychting M. 2005. Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. ***Cancer Res*** **65**:6459-6465

214. Scott CB, Scarantino C, Urtasun R, Movsas B, Jones CU, Simpson JR, Fischbach AJ, Curran WJ, Jr. 1998. Validation and predictive power of Radiation Therapy Oncology Group (RTOG) recursive partitioning analysis classes for malignant glioma patients: a report using RTOG 90-06. ***Int J Radiat Oncol Biol Phys*** **40**:51-55

215. Sekiya M, Adachi M, Hinoda Y, Imai K, Yachi A. 1994. Downregulation of Wilms' tumor gene (wt1) during myelomonocytic differentiation in HL60 cells. ***Blood*** **83**:1876-1882

216. Sharma PM, Bowman M, Madden SL, Rauscher FJ, III, Sukumar S. 1994. RNA editing in the Wilms' tumor susceptibility gene, WT1. ***Genes Dev*** **8**:720-731

217. Sharma PM, Yang X, Bowman M, Roberts V, Sukumar S. 1992. Molecular cloning of rat Wilms' tumor complementary DNA and a study of messenger RNA expression in the urogenital system and the brain. ***Cancer Res*** **52**:6407-6412

218. Shih AH and Holland EC. 2006. Platelet-derived growth factor (PDGF) and glial tumorigenesis. ***Cancer Lett*** **232**:139-147

219. Shu HK, Kim MM, Chen P, Furman F, Julin CM, Israel MA. 1998. The intrinsic radioresistance of glioblastoma-derived cell lines is associated with a failure of p53 to induce p21(BAX) expression. ***Proc Natl Acad Sci U S A*** **95**:14453-14458

220. Siehl JM, Thiel E, Heufelder K, Snarski E, Schwartz S, Mailander V, Keilholz U. 2003. Possible regulation of Wilms' tumour gene 1 (WT1) expression by the paired box genes PAX2 and PAX8 and by the haematopoietic transcription factor GATA-1 in human acute myeloid leukaemias. ***Br J Haematol*** **123**:235-242

221. Silbergeld DL, Rostomily RC, Alvord EC, Jr. 1991. The cause of death in patients with glioblastoma is multifactorial: clinical factors and autopsy findings in 117 cases of supratentorial glioblastoma in adults. ***J Neurooncol*** **10**:179-185

222. Silberstein GB, Dressler GR, Van Horn K. 2002. Expression of the PAX2 oncogene in human breast cancer and its role in progesterone-dependent mammary growth. ***Oncogene*** **21**:1009-1016

223. Silberstein GB, VanHorn K, Strickland P, Roberts CT, Daniel CW. 1997. Altered expression of the WT1 Wilms tumor suppressor gene in human breast cancer. ***Proceedings of the National Academy of Sciences of the United States of America*** **94**:8132-8137

224. Simpson JR, Horton J, Scott C, Curran WJ, Rubin P, Fischbach J, Isaacson S, Rotman M, Asbell SO, Nelson JS, . 1993. Influence of location and extent of surgical resection on survival of patients with glioblastoma multiforme: results of three consecutive Radiation Therapy Oncology Group (RTOG) clinical trials. ***Int J Radiat Oncol Biol Phys*** **26**:239-244

225. Simpson LA, Burwell EA, Thompson KA, Shahnaz S, Chen AR, Loeb DM. 2006. The antiapoptotic gene A1/BFL1 is a WT1 target gene that mediates granulocytic differentiation and resistance to chemotherapy. ***Blood*** **107**:4695-4702

226. Smith SI, Down M, Boyd AW, Li CL. 2000. Expression of the Wilms' tumor suppressor gene, WT1, reduces the tumorigenicity of the leukemic cell line M1 in C.B-17 scid/scid mice. ***Cancer Res*** **60**:808-814

227. Stewart LA. 2002. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. ***Lancet*** **359**:1011-1018

228. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO.

2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. ***N Engl J Med*** **352**:987-996

229. Svedberg H, Chylicki K, Baldetorp B, Rauscher FJ, Gullberg U. 1998. Constitutive expression of the Wilms' tumor gene (WT1) in the leukemic cell line U937 blocks parts of the differentiation program. ***Oncogene*** **16**:925-932

230. Tada M, Matsumoto R, Iggo RD, Onimaru R, Shirato H, Sawamura Y, Shinohe Y. 1998. Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutations. ***Cancer Res*** **58**:1793-1797

231. Tamaki H, Ogawa H, Ohyashiki K, Ohyashiki JH, Iwama H, Inoue K, Soma T, Oka Y, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Fuchigami K, Tomonaga M, Toyama K, Aozasa K, Kishimoto T, Sugiyama H. 1999. The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. ***Leukemia*** **13**:393-399

232. Thangnipon W, Mizoguchi M, Kukita Y, Inazuka M, Iwaki T, Fukui M, Hayashi K. 1999. Distinct pattern of PCR-SSCP analysis of p53 mutations in human astrocytomas. ***Cancer Letters*** **143**:S43-S43

233. Thomas C, Ely G, James CD, Jenkins R, Kastan M, Jedlicka A, Burger P, Wharen R. 2001. Glioblastoma-related gene mutations and over-expression of functional

epidermal growth factor receptors in SKMG-3 glioma cells. ***Acta Neuropathol (Berl)*** **101**:605-615

234. Tohma Y, Gratas C, Biernat W, Peraud A, Fukuda M, Yonekawa Y, Kleihues P, Ohgaki H. 1998. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. ***J Neuropathol Exp Neurol*** **57**:684-689

235. Toms SA, Ferson DZ, Sawaya R. 1999. Basic surgical techniques in the resection of malignant gliomas. ***J Neurooncol*** **42**:215-226

236. Trent J, Meltzer P, Rosenblum M, Harsh G, Kinzler K, Mashal R, Feinberg A, Vogelstein B. 1986. Evidence for rearrangement, amplification, and expression of c-myc in a human glioblastoma. ***Proc Natl Acad Sci U S A*** **83**:470-473

237. Tribius S, Pidel A, Casper D. 2001. ATM protein expression correlates with radioresistance in primary glioblastoma cells in culture. ***Int J Radiat Oncol Biol Phys*** **50**:511-523

238. Tsuboi A, Oka Y, Ogawa H, Elisseeva OA, Li H, Kawasaki K, Aozasa K, Kishimoto T, Udaka K, Sugiyama H. 2000. Cytotoxic T-lymphocyte responses elicited to Wilms' tumor gene WT1 product by DNA vaccination. ***J Clin Immunol*** **20**:195-202

239. Tuna M, Chavez-Reyes A, Tari AM. 2005. HER2/neu increases the expression of Wilms' Tumor 1 (WT1) protein to stimulate S-phase proliferation and inhibit apoptosis in breast cancer cells. ***Oncogene*** **24**:1648-1652

240. Ueda T, Oji Y, Naka N, Nakano Y, Takahashi E, Koga S, Asada M, Ikeba A, Nakatsuka S, Abeno S, Hosen N, Tomita Y, Aozasa K, Tamai N, Myoui A, Yoshikawa H, Sugiyama H. 2003. Overexpression of the Wilms' tumor gene WT1 in human bone and soft-tissue sarcomas. ***Cancer Sci*** **94**:271-276

241. Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A, Louis DN. 1996. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. ***Cancer Res*** **56**:150-153

242. Uhrbom L, Dai C, Celestino JC, Rosenblum MK, Fuller GN, Holland EC. 2002. Ink4a-Arf loss cooperates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt. ***Cancer Res*** **62**:5551-5558

243. Van Meir EG, Polverini PJ, Chazin VR, Su Huang HJ, de Tribolet N, Cavenee WK. 1994. Release of an inhibitor of angiogenesis upon induction of wild type p53 expression in glioblastoma cells. ***Nat Genet*** **8**:171-176

244. Van Meter TE, Broaddus WC, Rooprai HK, Pilkington GJ, Fillmore HL. 2004. Induction of membrane-type-1 matrix metalloproteinase by epidermal growth factor-mediated signaling in gliomas. ***Neuro -oncol*** **6**:188-199

245. VanMeter TE, Rooprai HK, Kibble MM, Fillmore HL, Broaddus WC, Pilkington GJ. 2001. The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis. ***J Neurooncol*** **53**:213-235

246. Varanasi R, Bardeesy N, Ghahremani M, Petruzzi MJ, Nowak N, Adam MA, Grundy P, Shows TB, Pelletier J. 1994. Fine structure analysis of the WT1 gene in sporadic Wilms tumors. ***Proc Natl Acad Sci U S A*** **91**:3554-3558

247. Varela M, Ranuncolo SM, Morand A, Lastiri J, Kier Joffe EB, Puricelli LI, Pallotta MG. 2004. EGF-R and PDGF-R, but not bcl-2, overexpression predict overall survival in patients with low-grade astrocytomas. ***J Surg Oncol*** **86**:34-40

248. Vousden KH and Lu X. 2002. Live or let die: The cell's response to p53. ***Nature Reviews Cancer*** **2**:594-604

249. Wagner KD, Wagner N, Vidal VP, Schley G, Wilhelm D, Schedl A, Englert C, Scholz H. 2002. The Wilms' tumor gene Wt1 is required for normal development of the retina. ***EMBO J*** **21**:1398-1405

250. Wagner KD, Wagner N, Wellmann S, Schley G, Bondke A, Theres H, Scholz H. 2003. Oxygen-regulated expression of the Wilms' tumor suppressor Wt1 involves hypoxia-inducible factor-1 (HIF-1). ***Faseb Journal*** **17**:

251. Wagner N, Wagner KD, Schley G, Coupland SE, Heimann H, Grantyn R, Scholz H. 2002. The Wilms' tumor suppressor Wt1 is associated with the differentiation of retinoblastoma cells. ***Cell Growth Differ*** **13**:297-305

252. Walker MD, Alexander E Jr, Hunt WE, MacCarty CS, Mahaley MS, Jr., Mealey J, Jr., Norrell HA, Owens G, Ransohoff J, Wilson CB, Gehan EA, Strike TA. 1978. Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. ***J Neurosurg*** **49**:333-343

253. Walker MD, Strike TA, Sheline GE. 1979. An analysis of dose-effect relationship in the radiotherapy of malignant gliomas. ***Int J Radiat Oncol Biol Phys*** **5**:1725-1731

254. Wang X, Ohnishi K, Takahashi A, Ohnishi T. 1998. Poly(ADP-ribosyl)ation is required for p53-dependent signal transduction induced by radiation. ***Oncogene*** **17**:2819-2825

255. Wang ZY, Qiu QQ, Deuel TF. 1993. The Wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains. ***J Biol Chem*** **268**:9172-9175

256. Wang ZY, Qiu QQ, Enger KT, Deuel TF. 1993. A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. ***Proc Natl Acad Sci U S A*** **90**:8896-8900

257. Wang ZY, Qiu QQ, Gurrieri M, Huang J, Deuel TF. 1995. WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. ***Oncogene*** **10**:1243-1247

258. Watanabe K, Tachibana O, Sata K, Yonekawa Y, Kleihues P, Ohgaki H. 1996. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. ***Brain Pathol*** **6**:217-223

259. Wehrle-Haller B and Imhof BA. 2003. Actin, microtubules and focal adhesion dynamics during cell migration. ***Int J Biochem Cell Biol*** **35**:39-50

260. Werner H, Karnieli E, Rauscher FJ, LeRoith D. 1996. Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. ***Proc Natl Acad Sci U S A*** **93**:8318-8323

261. Werner H, Shen-Orr Z, Rauscher FJ, III, Morris JF, Roberts CT, Jr., LeRoith D. 1995. Inhibition of cellular proliferation by the Wilms' tumor suppressor WT1 is associated with suppression of insulin-like growth factor I receptor gene expression. ***Mol Cell Biol*** **15**:3516-3522

262. Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, Whittle IR, Jaaskelainen J, Ram Z. 2003. A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. ***Neuro -oncol*** **5**:79-88

263. Wu Y, Fraizer GC, Saunders GF. 1995. GATA-1 transactivates the WT1 hematopoietic specific enhancer. ***J Biol Chem*** **270**:5944-5949

264. Yamagami T, Sugiyama H, Inoue K, Ogawa H, Tatekawa T, Hirata M, Kudoh T, Akiyama T, Murakami A, Maekawa T. 1996. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. ***Blood*** **87**:2878-2884

265. Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR. 1996. Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. ***EMBO J*** **15**:5606-5615

266. Yount GL, Haas-Kogan DA, Vidair CA, Haas M, Dewey WC, Israel MA. 1996. Cell cycle synchrony unmasks the influence of p53 function on radiosensitivity of human glioblastoma cells. ***Cancer Res*** **56**:500-506

267. Yu J, Zhang H, Gu J, Lin S, Li J, Lu W, Wang Y, Zhu J. 2004. Methylation profiles of thirty four promoter-CpG islands and concordant methylation behaviours of

sixteen genes that may contribute to carcinogenesis of astrocytoma. ***BMC Cancer*** **4**:65-

268. Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R, Kufe D. 1999. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. ***Nature*** **399**:814-817

269. Yung WK, Albright RE, Olson J, Fredericks R, Fink K, Prados MD, Brada M, Spence A, Hohl RJ, Shapiro W, Glantz M, Greenberg H, Selker RG, Vick NA, Rampling R, Friedman H, Phillips P, Bruner J, Yue N, Osoba D, Zaknoen S, Levin VA. 2000. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. ***Br J Cancer*** **83**:588-593

270. Zapata-Benavides P, Tuna M, Lopez-Berestein G, Tari AM. 2002. Downregulation of Wilms' tumor 1 protein inhibits breast cancer proliferation. ***Biochemical and Biophysical Research Communications*** **295**:784-790

271. Zhou J, Ahn J, Wilson SH, Prives C. 2001. A role for p53 in base excision repair. ***EMBO J*** **20**:914-923

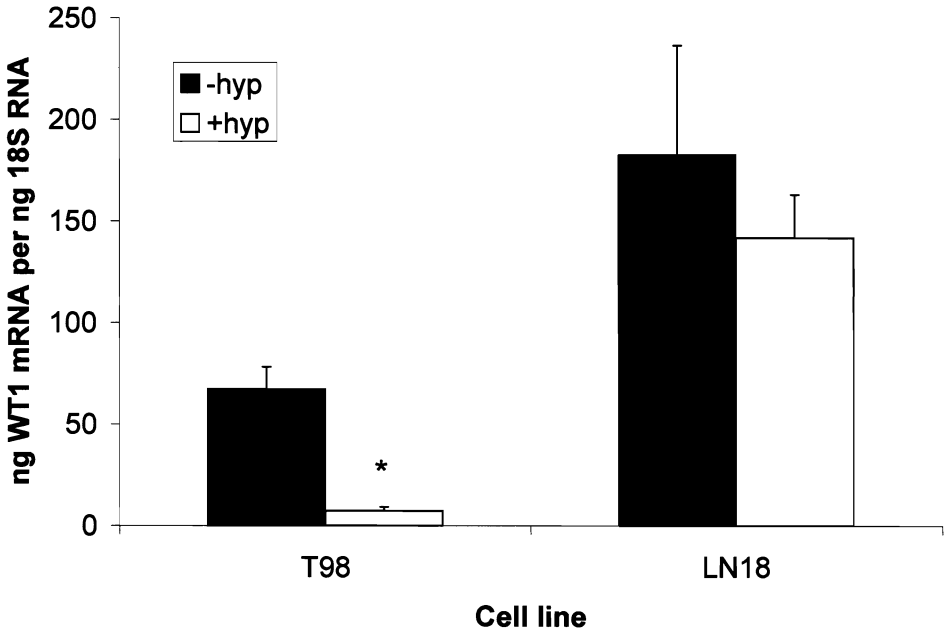
APPENDIX A

Decrease in WT1 mRNA expression in T98G glioma cells after 24 hours of hypoxia.

Rationale. HIF-1α transactivates the WT1 promoter in osteosarcoma cells exposed to hypoxia[250]. Since HIF-1α may play a critical role in the response of glioma cells to intra-tumoral hypoxia, we examined the response of the WT1 gene to hypoxia[94].

Methods. Cells were plated at a density of 1 X 10^6 cells in 100 mm^2 sterile tissue culture dishes and incubated overnight for attachment. Cultures were then placed in a humidified portable isolation Chamber (Billups-Rothenberg), flushed with a gas mixture of 1% O_2 plus 5% CO_2 plus 94%N_2. A rate of 15 liters per minute was used to purge the chamber for 3 minutes which yielded a concentration of 3% O2. The entire chamber was incubated 37° C for 24 hours, after which RNA was extracted from the cells followed by real-time PCR analysis to detect WT1 mRNA expression as described previously. Differences between (+)hypoxia and (–)hypoxia within each cell line was analyzed by Student's t-test. *denotes $p = 0.014$

Results and discussion. T98G cells demonstrated a significant decrease in WT1 mRNA expression after exposure to hypoxic conditions while LN-18 cells demonstrated no change. This possibly indicates a cell-type specific difference in WT1 response to hypoxia, the mechanism of which is currently not known.


250
200
150
100
50
0
ng WT1 mRNA per ng 18S RNA
-hyp
+hyp
*
T98
LN18
Cell line

APPENDIX B

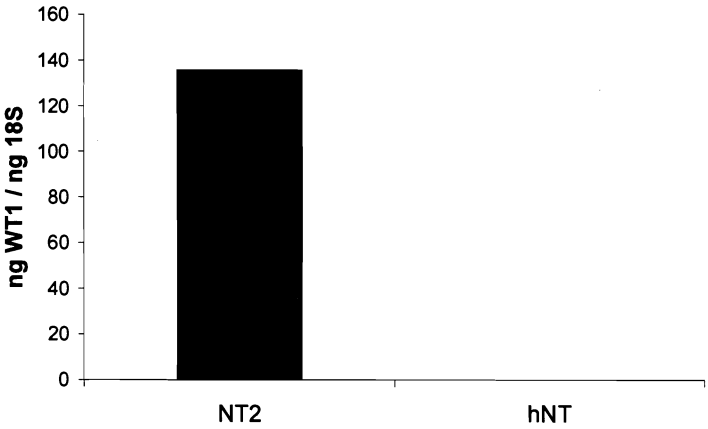
Decrease in WT1 mRNA expression after neuronal differentiation of human NTera-2 cells.

Rationale. WT1 is down-regulated in PC12 cells induced to differentiate into neurons by NGF which is functionally important because it may be directly responsible for the down-regulation of EGFR during the differentiation procells[127]. In an effort to generalize this observation, we examined the expression of WT1 during the neuronal differentiation of a different model system; retinoic acid induced differentiation of NTera-2 (NT-2) cells.

Methods. NT2 cells were maintained in ATCC modified DMEM (ATCC cat #30-2002) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For differentiation, 2 X 10^6 cells were seeded in a 75 mm^2 flask and treated with 10 μM retinoic acid (RA) dissolved in DMSO. Cells were incubated at 37° C 5% CO_2 for four weeks with RA-containing media replaced three times a week. After four weeks of RA treatment, cells were replated at a dilution factor of 1:6 in fresh 75 mm^2 flasks and then incubated for 24 hours at 37° C. Cells were then mechanically dislodged and floating cells were replated on Matrigel (BD Biosciences) diluted 1:60 in 100 mm^2 plates. After a 24 hour incubation period, cells were again mechanically dislodged and collected. 7.5 X 10^6 cells were replated on Matrigel coated plates and incubated in media containing the mitotic inhibitors cytosine arabinoside (1 μM), 5-fluoro-2'-deoxyuridine (10 μM), and uridine (10 μM). After 1 week in culture, media was changed to media containing mitotic inhibitor

minus cytosine arabinoside, and maintained for 3 weeks. Cells were then trypsinized and replated in six well plates coated with poly-L-lysine.

Results and discussion. WT1 mRNA was detectable in undifferentiated NT-2 cells but fell to undetectable levels after retinoic acid induced neuronal differentiation. This suggests that WT1 may play a role in the differentiation process of NT-2 cells and PC12 cells, and may therefore by important for neuronal differentiation.


160
140
120
100
80
60
40
20
0
ng WT1 / ng 18S
NT2
hNT

APPENDIX C

Lack of p53 response in LN-Z308 cells stably expressing doxycycline inducible p53.

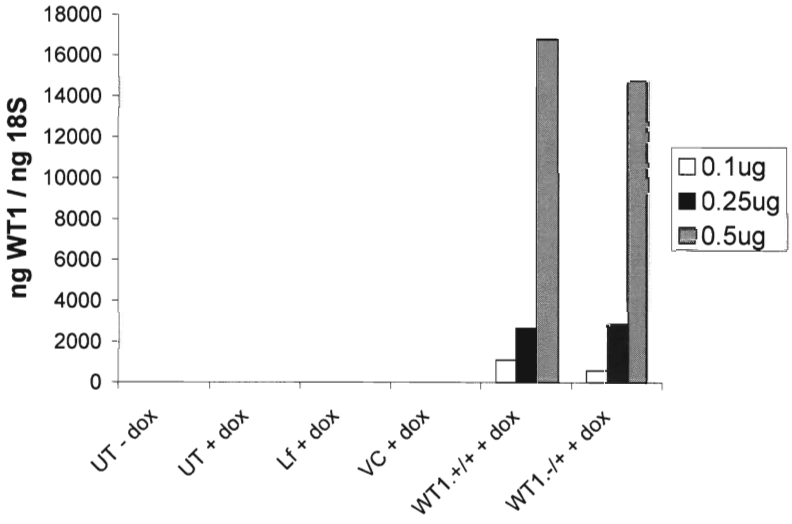
Rationale. To examine the effect of WT1 on p53 mediated apoptosis and cell cycle arrest we utilized a glioma cell line expressing wild-type p53 under the control of an inducible promoter.

Methods. LN-Z308 cells expressing p53 under the control of a doxycycline inducible promoter (LNZ.C2024, kindly provided by Dr. Erwin Van Meir) were plated at a density of 2 X 10^5 cells per well of a six well plate and allowed to attach overnight. Cells were then transfected as described previously with a mixture 0.5 μg plasmid expression vector containing WT1(+/+) or (-/+) isoforms and 3 μl Lipofectamine2000 (Invitrogen). For viability analysis, the next day, cells were gently harvested by trypsin-EDTA treatment, counted, and re-plated in 96 well tissue culture plates at a density of 1 X 10^3 cells per well. After a 24 hour incubation, cells were treated with doxycycline to induce p53 expression. Viability was analyzed at 1 and 3 days after induction by ATP-based viability assay (C). At 24 hours after doxycycline treatment, RNA and protein were extracted. WT1 mRNA was expressed after transient transfection and was unaffected by doxycycline (A). WT1 and p53 were expressed after transient transfection and doxycycline treatment (B). For cell cycle analysis, cells were treated with doxycycline in six well plates for 24 hours and harvested. Cells were stained with propidium iodide and analyzed by flow cytometry (D).

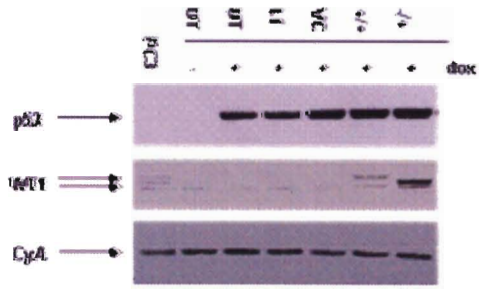
Surprisingly, p53 induction did not cause a change in either cell viability or cell cycle distribution.

Results and discussion. Transient transfection of LNZ.C2024 cells with WT1 caused a dose dependent increase in WT1 mRNA expression (Figure A). Addition of doxycycline caused an increase in p53 expression which was not affected by WT1 transfection (Figure B). However, p53 expression did not induce and change in viability or cell cycle distribution (Figures C and D). We can conclude that LNZ.C2024 cells possess a functional inactivation of p53. The mechanism is not known.

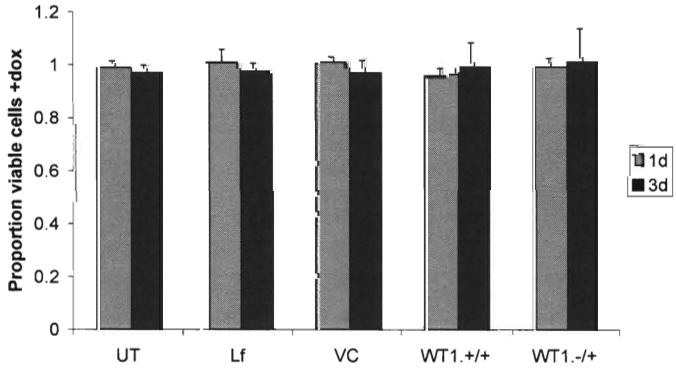
A.


ng WT1 / ng 18S
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.1ug
0.25ug
0.5ug
UT - dox
UT + dox
Lf + dox
VC + dox
WT1.+/+ + dox
WT1.-/+ + dox

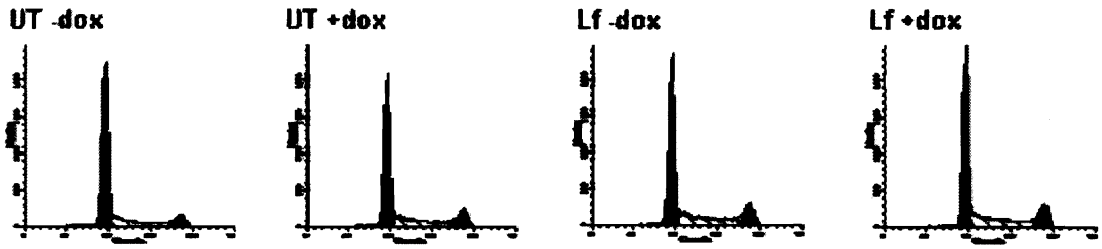
B.


dox
p53
WT1
CyA

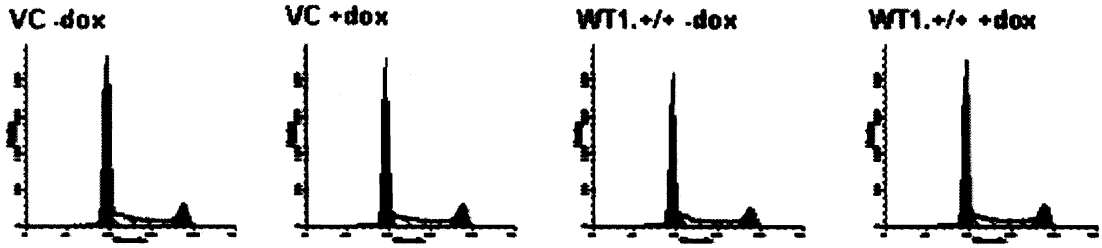
C.


Proportion viable cells +dox
1.2
1
0.8
0.6
0.4
0.2
0
1d
3d
UT
Lf
VC
WT1.+/+
WT1.-/+

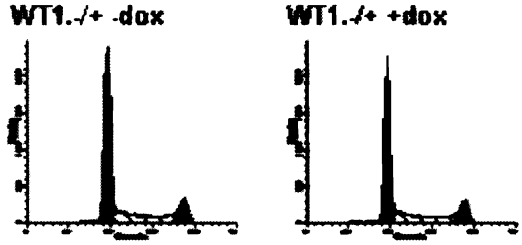
D.



	UT -dox	UT +dox	Lf -dox	Lf +dox
Go/G1	62.97	57.7	57.57	52.15
S	27.59	29.13	28.98	34.29
G2/M	9.44	13.17	13.44	13.56



	VC -dox	VC +dox	WT1.+/+ -dox	WT1.+/+ +dox
Go/G1	52.67	53.22	56.41	51.26
S	33.32	32.18	30.44	33.99
G2/M	14.01	14.60	13.15	14.75



	WT1.-/+ -dox	WT1.-/+ +dox
Go/G1	54.44	53.79
S	31.61	32.2
G2/M	13.94	14.01

APPENDIX D

Appendix D. WT1 expression does not protect LN-229 or LN-Z308 cells from ionizing radiation induced cell death.

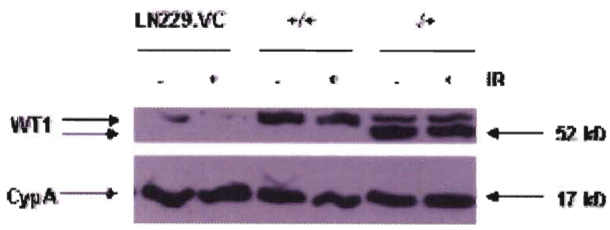
Rationale. We examined the effect of WT1 overexpression on radiosensitivity in LN-229 and LN-Z308 cells.

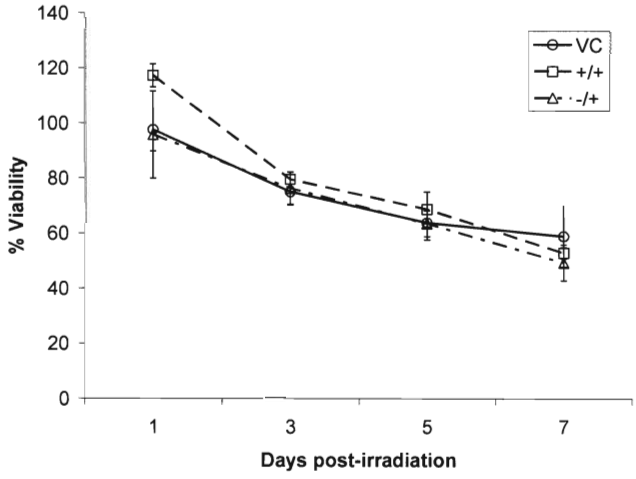
Methods. For protein extraction, LN-229 and LN-Z308 glioblastoma cells stably expressing WT1(+/+) or (-/+) isoforms were plated at a density of 1 X 10^6 cells/100 mm^2 plate and exposed to 6 Gy of ionizing radiation. 24 hours after irradiation, protein was extracted and Western blotting performed as described above. For ATP-based viability assay, 1 X 10^3 cells were plated per well of 96 well flat bottom tissue culture plates and exposed to 6 Gy of ionizing radiation. Cells were lysed and viability extrapolated based on ATP content at 1, 3, 5, and 7 days after irradiation. Results are reported as % viability of irradiated cells relative to matched non-irradiated cells. All treatment groups were performed in quintuplicate.

Results and discussion. A. Western blotting demonstrates that LN-229 cells transfected with WT1 isoforms were stably expressing WT1 protein (top panel). WT1 expression did not change after irradiation. ATP-based viability assay demonstrated a time-dependent decrease in viability in LN-229 cells after irradiation (bottom panel). Stable WT1 expression did not protect LN-229 cells from cell death. B. Similar results were obtained from LN-Z308 glioblastoma cells. C. ATP-based viability assays were repeated three times at the 7 day time point and demonstrated the same lack of protective effect of WT1

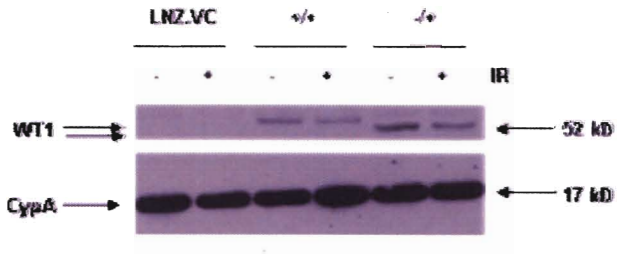
expression. We can conclude that in these two glioma cell lines, WT1 ovexpression does not protect agains ionizing radiation-induced cell death.

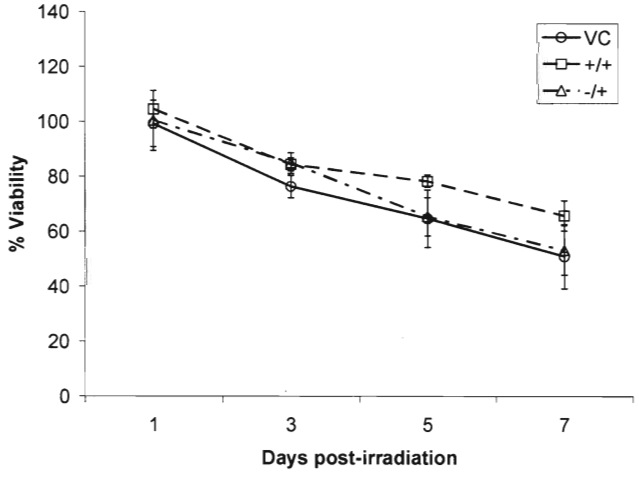
A.


LN229.VC
+/+
-/+
IR
WT1
52 kD
CypA
17 kD

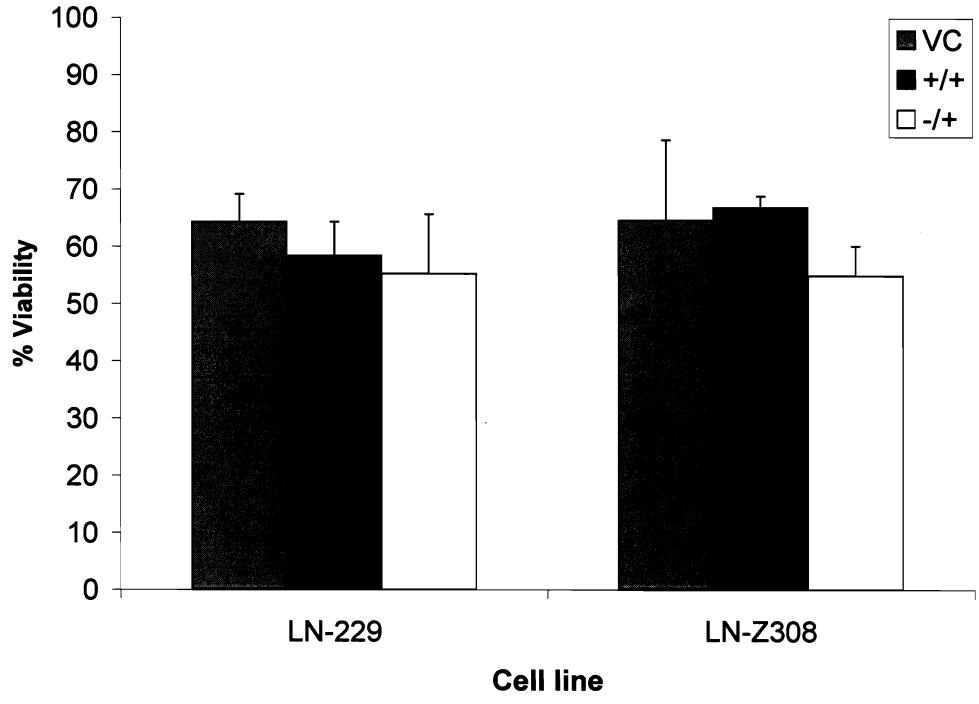

140
120
100
80
60
40
20
0
% Viability
1
3
5
7
Days post-irradiation
VC
+/+
-/+

B.


LNZ.VC
+/+
-/+
IR
WT1
52 kD
CypA
17 kD


140
120
100
80
60
40
20
0
% Viability
1
3
5
7
Days post-irradiation
VC
+/+
-/+

C.


100
90
80
70
60
50
40
30
20
10
0
% Viability
VC
+/+
-/+
LN-229
LN-Z308
Cell line

APPENDIX E

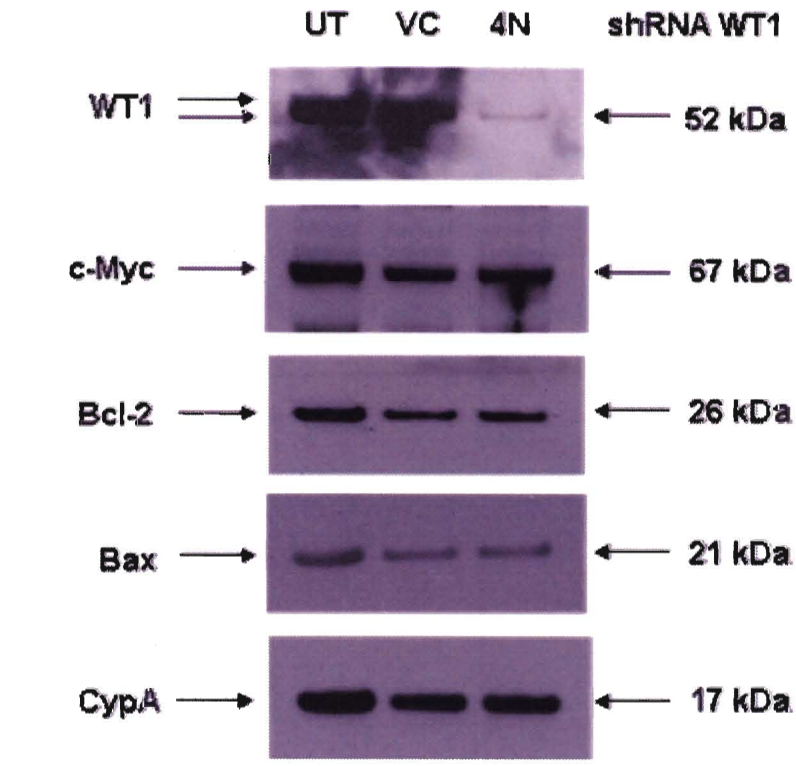
Changes in previously described WT1 targets are not responsible for decrease in proliferation and invasion after WT1 silencing

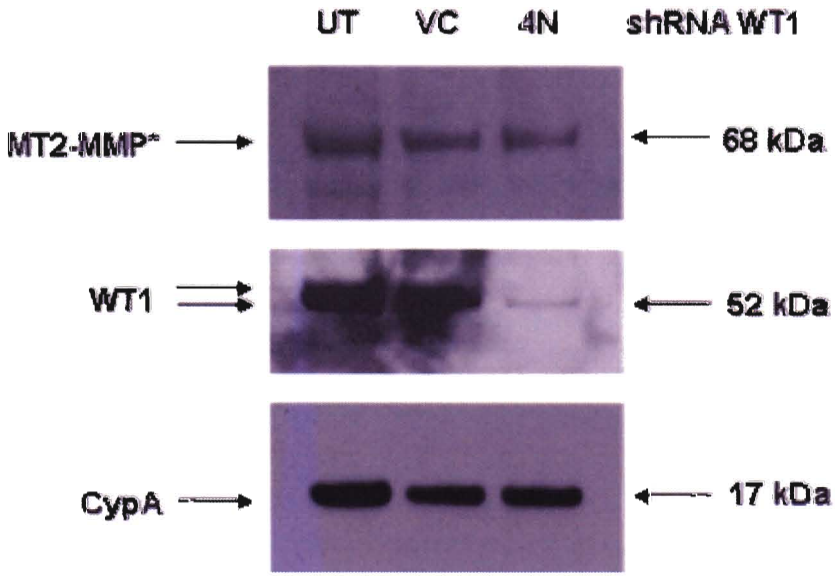
Rationale. WT1 decreases the proliferation of U251MG glioma cells *in vitro* and *in vivo* and may possibly affect *in vitro* invasion. In U251MG cells stably expressing WT1 shRNA we examined the expression of known WT1 target genes that were potentially involved in these processes.

Methods. U251, U251.VC, and U251.Sh cells were plated at a density of 1 X 106 cells per 100 mm2 plate and allowed to attach overnight. The following day, protein was extracted by SDS lysis buffer as described previously. 20 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking for 1 hour in 5% milk, membranes were incubated overnight at 4° C with anti-WT1 (1:200, Dako Cytomation), anti-c-myc (1:100 dilution, Santa Cruz Biotechnology), anti-Bcl-2 (1:100, Calbiochem), anti-Bax (1:300, Dako Cytomation), or anti-MT2-MMP (1:2000, Chemicon). Membranes were washed six times with TBS-T and incubated with their corresponding secondary antibody for 1 hour at room temperature; anti-rabbit (1:2000) for Bax and c-myc, anti-rabbit (1:5000) for MT2-MMP, anti-mouse (1:2000) for Bcl-2, and anti-mouse (1:1000) for WT1. Membranes were again washed and protein was detected using the ECL detection system.

Results and discussion. There were no significant differences in WT1 target gene expression between U251.VC and U251.Sh protein lysates, suggesting that the decreases

in proliferation and invasion are due to WT1 mediated changes in other targets that remain to be discerned.


UT
VC
4N
shRNA WT1
WT1
52 kDa
c-Myc
67 kDa
Bcl-2
26 kDa
Bax
21 kDa
CypA
17 kDa


UT
VC
4N
shRNA WT1
MT2-MMP*
68 kDa
WT1
52 kDa
CypA
17 kDa

VITA

Aaron John Clark was born in San Francisco, California on May 10, 1977 and is currently a citizen of the United States of America. He graduated from Lowell High School in San Francisco, California in 1995. He then attended the University of California, San Diego in La Jolla, California where he received his Bachelor of Science in 1999. He graduated *cum laude* with a major in Microbiology and a minor in Humanities. Aaron was also inducted into Phi Beta Kappa in 1999. After working as a research assistant in a Cardiology laboratory at the San Diego Veterans' Affairs Medical Center, he began the combined M.D./Ph.D program at the Virginia Commonwealth University in Richmond, Virginia. While a student Aaron was awarded numerous honors including first place in the VCU School of Medicine Student Honors Day abstract presentations, the C.C. Clayton Award from the Department of Anatomy and Neurobiology, and the Lucien J. Rubinstein Memorial Award from the American Brain Tumor Association. He also served as a teaching assistant for the Dental gross anatomy course, a tutor for Occupational Therapy neuro-anatomy, and as lecturer in the graduate neuro-anatomy course. He completed his doctoral work in the Broaddus Neuro-oncology laboratory in November, 2006 and completes the dual degrees of Doctor of Medicine and Doctor of Philosophy in May, 2008. He has authored several peer-reviewed papers and abstracts and presented his work at several local and national conferences. In his free time, Aaron enjoys surfing and snowboarding. In pursuit of the perfect ride, he has traveled extensively in northern and southern California, Colorado, Montana, West Virginia, North and South Carolina, Puerto Rico, Mexico, Costa Rica, Nicaragua, Fiji, and Western Samoa.