IDENTIFICATION AND THERAPEUTIC TARGETING OF NOVEL TRANSFORMING PATHWAYS IN HUMAN GLIOBLASTOMA MULTIFORME

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Tumor Biology

By

David A. Solomon, B.S.

Washington, D.C.
April 14, 2010
Identification and therapeutic targeting of novel transforming pathways in human glioblastoma multiforme

David A. Solomon, B.S.

Thesis advisor: Todd Waldman, M.D., Ph.D.

ABSTRACT

Glioblastoma multiforme (GBM) is the most common primary brain malignancy with approximately 9,000 cases diagnosed per year in the U.S. This devastating cancer is virtually uniformly lethal with an average survival of less than one year after diagnosis. The current standard of care for newly diagnosed GBM is surgical resection, followed by adjuvant radiotherapy plus chemotherapy with temozolomide. This treatment is only marginally successful, rarely extending lifespan after diagnosis past eighteen months. While several oncogenes (e.g. EGFR, PDGFRA) and tumor suppressor genes (e.g. TP53, CDKN2A, PTEN) that contribute to gliomagenesis have been identified, no therapeutics that specifically target these known lesions have yet proved beneficial. As such, a better understanding of the molecular pathogenesis of GBM is required to identify new molecular targets and develop new effective treatments.

To discover novel genes whose alterations drive gliomagenesis, we used Affymetrix SNP microarrays to identify copy number alterations present in a panel of GBM primary tumors, primary xenografts, primary cultures, and cell lines. This analysis demonstrated a significant sample type bias in the frequency of common copy number alterations in GBM and suggested that primary tumors and xenografts are best for the identification of amplifications, whereas xenografts and cell lines are superior for the identification of homozygous deletions.
This analysis also led to identification of homozygous deletions of two genes (PTPRD and CDKN2C), not previously known to be altered in GBM. Using directed sequencing, we subsequently identified frequent somatic mutations of PTPRD, encoding a receptor-type protein tyrosine phosphatase, in both GBM and melanoma tumor samples, demonstrating that phosphatase inactivation (and not just kinase activation) is a major contributor to tumorigenesis. The discovery of frequent deletions of the CDKN2C gene encoding p18\(^{\text{INK4c}}\), a homolog of the well characterized CDKN2A/p16\(^{\text{INK4a}}\) tumor suppressor, identified an additional mechanism leading to aberrant activation of cdk4/6 in GBM and provided further impetus for the testing of cyclin-dependent kinase (cdk)-specific inhibitors against GBM. We found that the cdk4/6-specific inhibitor PD-0332991 potently arrested the growth of all Rb-proficient GBM cells \textit{in vitro} and intracranial xenografts \textit{in vivo}. These findings prompted a clinical trial testing the effectiveness of PD-0332991 in GBM patients.
I would like to thank my mentor Todd Waldman for his support of my research and my growth as an aspiring physician-scientist during the last four years. I am very grateful to have had the opportunity to work so closely with this talented cancer researcher during my time as a PhD student. Jung-Sik Kim has also been an invaluable help both experimentally and emotionally during my four years in the lab. Past and present lab members including Challice Bonifant, Sultan Jenkins, Huifang Li, and Tei Kim have been extremely helpful in assisting with experiments and making the laboratory a pleasurable place to work. Habtom Ressom, Walter Jean, Michael Pishvaian, Hai Yan, Darrel Bigner, Yardena Samuels, Steve Rosenberg, Marcela White and the Brain Tumor Tissue Bank of Canada, Timothy Ryken, Zita Sibenaller, Alisa Goldstein, Karine Michaud, David James, Dana Farber Cancer Institute’s Microarray Core Facility, Lombardi Cancer Center’s Flow Cytometry Core, Tissue Culture Shared Resource, and veterinary technicians (Idalia Cruz, Syid Abdullah, and Aaron Foxworth) all made invaluable contributions to the research presented here. I would also like to thank my thesis committee members Michael Johnson, Careen Tang, Richard Schlegel, and Fred Bunz for their dedication in fostering my progression through this thesis research and allowing me to continue on the course to becoming a physician-scientist.
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Human Glioblastoma Multiforme: Epidemiology, Diagnosis, Current Treatment, Molecular Genetics, and Emerging Therapies

David A. Solomon

Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC
Summary

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults with approximately 9,000 cases diagnosed per year in the U.S. This devastating malignancy is virtually uniformly lethal with an average survival time of less than one year after diagnosis. Around 90% of GBMs occur in adults usually developing supratentorially in the cerebral hemispheres, with an average age at diagnosis in the sixth decade of life. The remaining 10% occur in children, usually developing subtentorially in the brainstem. The current standard of care for newly diagnosed GBM is surgical resection to the extent feasible, followed by adjuvant radiotherapy plus chemotherapy with the alkylating agent temozolomide. This treatment is only marginally successful, rarely extending lifespan after diagnosis past eighteen months. It is this combination of the age of the affected patients, rapid disease progression, high mortality rate, and lack of effective therapy that makes GBM so devastating. While several oncogenes (e.g. EGFR, PDGFRA, PIK3CA) and tumor suppressor genes (e.g. TP53, CDKN2A, PTEN, NF1, RB1) that contribute to gliomagenesis have been identified, no therapeutics that specifically target these known lesions have yet proved successful in the treatment of GBM. As such, a better understanding of the molecular pathogenesis of GBM is required to develop new effective treatment modalities for this neurological disease.
Incidence of malignant brain and other nervous system tumors

The Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute of the National Institutes of Health collects and publishes cancer incidence and survival data from population-based cancer registries for the United States population. The most recent SEER report was released in 2008 and details incidence and survival data in the U.S. from 1975-2006 for malignant/invasive cancers of the brain and other nervous system with age-adjusted incidence rates based on the U.S. Standard Population (1). The reported incidence of cancer of the brain and other nervous system was 7.88 cases per 100,000 population per year for men from 1975-2006, and 5.47 cases per 100,000 population per year for women. Hence, there were approximately 3 brain cancer diagnoses in men for every 2 diagnoses in women during this time. Brain cancer incidence did not change significantly between 1975 and 2006. There exists a significant racial/ethnic disparity in brain cancer incidence in the U.S. as 7.14 cases per 100,000 white population per year were diagnosed between 1975 and 2006, whereas only 4.05 cases per 100,000 black population were diagnosed (see table below and chart on following page).

1. SEER statistics are based on malignant/invasive cancers of the brain and other nervous system. These include all malignant oligodendrogliomas, pilocytic and diffuse astrocytomas, glioblastomas, ependymomas, gangliogliomas, choroid plexus carcinomas, pineal tumors, atypical teratoid/rhabdoid tumors, medulloblastomas, supratentorial primitive neuroectodermal tumors, neuroblastomas, meningiomas, peripheral nerve sheath tumors, perineuriomas, and germ-cell tumors. They exclude all benign/borderline tumors of the brain and other nervous system including choroid gliomas, gliofibromas, benign gangliogliomas, benign ependymomas (e.g. myxopapillary subtype), central neurocytomas, benign meningiomas, neurofibromas, and benign perineuriomas. They also exclude all lymphomas, leukemias, tumors of the pituitary gland, and olfactory tumors of the nasal cavity.
Age-adjusted death rates from cancer of the brain and other nervous system were 5.58 deaths in men and 3.74 deaths in women per 100,000 population per year during 1975-2006. Mortality rate from brain cancers did not improve over this time period – there were 4.11 deaths per 100,000 population in 1975, and 4.17 deaths per 100,000 population in 2006. Similar to the disparity in incidence, there was a significant racial/ethnic disparity in mortality rate from brain cancers with 4.86 deaths per 100,000 white population per year and 2.67 deaths per 100,000 black population per year during 1975-2006 (see table below and charts reproduced from reference 1 on following page).

<table>
<thead>
<tr>
<th>Race/Ethnicity</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Races</td>
<td>5.3</td>
<td>3.5</td>
</tr>
<tr>
<td>White</td>
<td>5.7</td>
<td>3.8</td>
</tr>
<tr>
<td>Black</td>
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<td>2.1</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>American Indian/Alaska Native</td>
<td>2.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Epidemiology, Diagnosis, Treatment, and Molecular Genetics of GBM

U.S. Brain and Other Nervous System Cancers Incidence*

U.S. Brain and Other Nervous System Cancers Mortality*

*Significant data for American Indians/Alaskan Natives not available.
**Data for Hispanics and Asians/Pacific Islanders not available before 1992.
Males have a lifetime risk of 0.67% of being diagnosed with a cancer of the brain and other nervous system and a 0.48% risk of related mortality. Females have a lifetime risk of 0.54% of being diagnosed and a 0.38% risk of death. In other terms, 1 in 149 men will be diagnosed with cancer of the brain and other nervous system during his lifetime, and 1 in 208 men will die from this cancer. 1 in 185 women will be diagnosed during her lifetime, and 1 in 263 women will die from this cancer. From 2002-2006, the median age at diagnosis for cancer of the brain and other nervous system was 56 years of age. 13% were diagnosed in persons under age 20, 52% in persons age 20 to 64, and 35% in persons 65+ years of age (see table below).

| U.S. Incidence Rates per 100,000 Population, 2002-2006 |
|----------------|-------|-------|
| Age at Diagnosis | Males | Females |
| <1               | 3.9   | 3.3    |
| 1-4              | 4.2   | 3.8    |
| 5-9              | 3.2   | 3      |
| 10-14            | 2.8   | 2.3    |
| 15-19            | 2.4   | 1.9    |
| 20-24            | 2.3   | 2.3    |
| 25-29            | 3.1   | 2.5    |
| 30-34            | 3.6   | 2.8    |
| 35-39            | 4     | 3.3    |
| 40-44            | 5.4   | 3.8    |
| 45-49            | 7.1   | 4.6    |
| 50-54            | 9     | 6.5    |
| 55-59            | 12.8  | 8      |
| 60-64            | 16.7  | 10.9   |
| 65-69            | 19.1  | 13.3   |
| 70-74            | 24.8  | 16     |
| 75-79            | 28.5  | 18.4   |
| 80-84            | 26.5  | 17.9   |
| 85+              | 21.8  | 15     |

From 2002-2006, the median age at death for cancer of the brain and other nervous system was 64 years of age (see table below).
### U.S. Death Rates per 100,000 Population, 2002-2006

<table>
<thead>
<tr>
<th>Age at Death</th>
<th>Males</th>
<th>Females</th>
</tr>
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<tbody>
<tr>
<td>&lt;1</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>1-4</td>
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<tr>
<td>5-9</td>
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<td>0.9</td>
</tr>
<tr>
<td>10-14</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>15-19</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>20-24</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>25-29</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>30-34</td>
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</tr>
<tr>
<td>35-39</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>40-44</td>
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<tr>
<td>80-84</td>
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<td>16.6</td>
</tr>
<tr>
<td>85+</td>
<td>20.2</td>
<td>14.5</td>
</tr>
</tbody>
</table>

The 2008 SEER report estimated that there would be approximately 22,000 new cancers of the brain and other nervous system diagnosed (with 12,000 in men and 10,000 in women) and approximately 13,000 related deaths in the U.S. in 2009. Cancers of the brain and other nervous system were predicted to account for 1.5% of the total estimated 1,479,000 new cancers of all tumor types (excluding basal and squamous cell carcinoma of the skin) and 2.3% of the total estimated 562,000 cancer deaths in the U.S. in 2009.
Histologic classification of human brain tumors

Tumors of neuroepithelial origin

Most primary tumors of the brain and central nervous system (CNS) are of neuroepithelial origin and result from malignant transformation of astrocytes, oligodendrocytes, and ependymocytes or their progenitor cells. These three cell types play supportive roles to the neurons of the CNS and are called glial cells (from the Greek word *glia* meaning glue) as a result. Astrocytes have important functions in supporting the blood-brain barrier, the provision of nutrients to neurons, the maintenance of extracellular ion and neurotransmitter balance, and repair processes following traumatic injury. Oligodendrocytes are the myelinating cells of the CNS that produce an insulating myelin sheath around the neuronal axons allowing for efficient propagation of electrical signals. Ependymocytes are the cells that line the ventricles of the brain and spinal cord and produce cerebrospinal fluid (CSF). The tumors that arise from these glial cells or their progenitors are called gliomas.

Astrocytomas are gliomas that arise from the astrocitic cell lineage (*i.e.* either from transformation of mature astrocytes or their progenitor cells). The World Health Organization (WHO) classifies astrocytomas into four grades based on a combination of gross, histologic, and pathologic criteria (see more detailed description of astrocytoma histopathology presented later). Grade I tumors are benign cystic lesions referred to as pilocytic astrocytomas, whereas grade II tumors are well-differentiated but invariably

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invasive lesions referred to as low-grade or diffuse astrocytomas. Grade III tumors called anaplastic astrocytomas are more aggressive tumors than low-grade astrocytomas. Glioblastoma multiforme (GBM), the WHO grade IV astrocytoma, is the most highly malignant and rapidly fatal astrocytoma.

Pilocytic astrocytomas (WHO grade I) typically present in the first two decades of life and are the most common gliomas in children. Few cases have been reported after the age of 40. No gender predilection in incidence has been observed. These tumors account for approximately 5% of all primary brain tumors and compose 10% of all cerebral and 85% of all cerebellar astrocytomas. Approximately 15% of patients with neurofibromatosis type 1 will develop pilocytic astrocytomas, most commonly of the optic nerve and frequently bilaterally.

The diffusely infiltrating astrocytomas (the malignant astrocytomas grades II-IV) account for approximately 60% of all primary brain tumors. Low-grade astrocytoma (WHO grade II) accounts for 10-15% of all astrocytic brain tumors. The peak incidence is in young adults between the ages of 30-40 years, but approximately 10% occur in children and 35% occur in adults over the age of 45 years. Anaplastic astrocytoma (WHO grade III) has a peak incidence in the fourth and fifth decades of life with a mean age older than low-grade astrocytoma patients but younger than GBM patients. GBM (WHO grade IV astrocytoma) is the most common malignant primary brain tumor, accounting for 12-15% of all primary brain tumors and 50-60% of all astrocytic tumors. Two pathways for the development of GBM have been described. In the primary or de novo pathway, the initial lesion presents with sudden onset and is identified as GBM, usually occurring in older patients. In the secondary pathway, a lesser grade lesion transforms over a long period of time into a grade IV lesion and usually occurs in
younger patients. Primary GBM accounts for the vast majority of GBMs (approximately 90%) and has a peak incidence in the sixth and seventh decades of life, while secondary GBM tends to occur in patients below the age of 45 years. Despite their distinct clinical histories, primary and secondary GBMs are histologically indistinguishable.

Oligodendrogliomas, gliomas arising from oligodendrocyte cell lineage, account for approximately 5% of all primary brain tumors and 10-15% of all intracranial gliomas. Histologically, approximately 50% are well-differentiated WHO grade II tumors referred to as low-grade oligodendrogliomas, and 50% are the more aggressive WHO grade III anaplastic oligodendrogliomas. Mixed gliomas with histologic features of both astrocytomas and oligodendrogliomas also occasionally occur (e.g. oligoastrocytomas). These mixed tumors likely result from transformation of the shared progenitor cell that gives rise to both astrocytes and oligodendrocytes.

Ependymomas are gliomas that arise from the transformation of ependymocytes or their progenitor cells and account for approximately 5% of all neuroepithelial tumors. These tumors primarily occur in children and young adults and account for 30% of brain tumors in children younger than 3 years of age. Ependymomas are classified by the WHO as grade II for the more common less aggressive lesion and grade III for the more aggressive anaplastic ependymoma. Two less malignant subtypes, subependymoma and myxopapillary ependymoma (both WHO grade I tumors), account for less than 10% of all ependymomas.

Additional neuroepithelial tumors that do not arise from glial cells can also occur. These include a group of well-differentiated and slow growing tumors composed of neoplastic ganglion cells either alone (gangliocytoma) or in combination with neoplastic glial cells (ganglioglioma). Gangliocytomas correspond histologically to WHO grade I
lesions while gangliogliomas may be grade I or II. A subtype in which the glial component displays anaplastic features has been described, anaplastic ganglioglioma, and corresponds with WHO grade III. Gangliocytomas and gangliogliomas are a rare tumor type and compose approximately 1% of all primary brain tumors.

Central neurocytomas are neoplasms composed of uniform round cell with neuronal differentiation typically located in the lateral ventricles. These tumors are most prevalent in young adults with greater than 70% presenting between the ages of 20 and 40 years. They compose less than 1% of all primary brain tumors and correspond to WHO grade II lesions.

Another group of neuroepithelial tumors are the choroid plexus tumors that arise from the epithelium of the choroid plexus of the cerebral ventricles. Choroid plexus tumors account for less than 1% of all primary brain tumors but compose approximately 5% of those that occur in children and 20% of those that present during the first year of life. These tumors are histologically classified as either the benign slow growing choroid plexus papilloma (WHO grade I) or the less frequent malignant variant choroid plexus carcinoma (WHO grade III).

The pineal gland (also called the epiphysis cerebri) is a small endocrine gland that sits in the midline of the brain at the junction of two thalamic bodies. Its cells called pineocytes produce and secrete melatonin, a hormone involved in regulating circadian (e.g. sleep/wake cycles) and photoperiodic (e.g. seasonal) rhythms. Pineal parenchymal tumors result from transformation of the pineocytes and are quite rare, accounting for less than 1% of all primary brain tumors. Approximately 50% are slow growing tumors called pineocytomas that correspond histologically to WHO grade II lesions. These tumors occur throughout life with highest incidence in young adults age 25-35 years.
The other approximately 50% are highly malignant primitive embryonal tumors called pineoblastomas that correspond histologically to WHO grade IV lesions. They primarily occur in children and young adults, with the highest incidence during the first decade of life.

Another group of neuroepithelial tumors that predominantly occur in children are the embryonal tumors that include medulloblastomas, ependymoblastomas, supratentorial primitive neuroectodermal tumors (PNETs), and atypical teratoid/rhabdoid tumors. Medulloblastomas are malignant invasive tumors of the cerebellum that occur preferentially in children (approximately 70% arise before the age of 16) with a peak incidence of 7 years of age and a marked male bias (approximately 2:1 male to female ratio). These tumors display predominantly neuronal differentiation and correspond to WHO grade IV lesions. The incidence of medulloblastomas has been estimated as 0.5 cases per 100,000 children (age 0-17 years) per year. Ependymoblastoma (WHO grade IV) is a rare highly malignant subtype of ependymoma that is histologically grouped with the embryonal tumors due to its primitive neuroepithelial nature. These tumors occur exclusively in children, most commonly in neonates. Supratentorial PNETs (WHO grade IV) are embryonal tumors occurring in the cerebrum composed of poorly differentiated neuroepithelial cells which may display features of neuronal, astrocytic, ependymal, muscular, or melanocytic lineages. These rare tumors that occur exclusively in the first decade of life are also sometimes referred to as cerebral medulloblastoma and cerebral neuroblastoma depending on their histologic features. The other embryonal tumors are the atypical teratoid/rhabdoid tumors composed of rhabdoid cells, epithelial tissue, and neoplastic mesenchyme. These highly aggressive and malignant tumors occur almost exclusively during the first decade of life and correspond to WHO grade IV lesions.
Meningeal tumors

The brain is encased in three protective layers – the pia, arachnoid, and dura mater – that together make up the meninges. Meningiomas are tumors that arise from the cells that compose this protective encasing of the brain. Meningiomas account for 15-25% of all primary brain tumors and occur throughout life with a peak incidence during the sixth and seventh decades of life. A significant fraction of meningiomas occur in neurofibromatosis type 2 patients (the majority of which will develop meningiomas during their lifetime) and in other non-NF2 families with a hereditary predisposition to meningioma. These familial-linked tumors tend to be multiple, occur in younger patients than sporadic tumors, and affect both sexes equally. On the other hand, sporadic meningioma has a strong female bias with a nearly 2:1 female to male incidence ratio. Several benign subtypes that correspond to WHO grade I lesions include fibrous, transitional, psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, and metaplastic. There also several more aggressive subtypes with a greater likelihood of recurrence after resection including atypical (WHO grade II), clear cell (WHO grade II), chordoid (WHO grade II), rhabdoid (WHO grade III), papillary (WHO grade III), and anaplastic (WHO grade III). Most meningiomas are the benign WHO grade I subtypes, whereas atypical and anaplastic subtypes account for less than 10% and 2% of all meningiomas, respectively.

Hemangiopericytoma is a soft tissue sarcoma that arises from the pericytes that compose the walls of capillaries. Primary hemangiopericytoma of the CNS is a highly cellular, richly vascularized tumor that is almost always attached to the dura and thus classified as a meningeal tumor. This malignant tumor accounts for approximately 0.5% of all primary brain tumors and corresponds histologically to WHO grade II or III.
Primary melanocytic tumors of the CNS are rare tumors that arise from the melanocytes of the leptomeninges. These are a heterogeneous group of tumors that are histologically differentiated into diffuse melanocytosis, melanocytoma, and malignant melanoma. These tumors account for approximately 0.1% of all primary brain tumors and occur at all ages, with the exception of diffuse melanocytosis that usually presents in childhood.

Hemangioblastoma (or capillary hemangioblastoma) is a WHO grade I tumor of uncertain origin composed of stromal cells and abundant capillaries. Approximately 25% of hemangioblastomas occur in patients with von Hippel-Lindau (VHL) disease. Sporadic tumors primarily occur in the cerebellum in adults, while VHL-associated tumors (often multiple per patient) occur in the cerebellum, brain stem, and spinal cord in children and young adults.

**Primary central nervous system lymphomas**

Primary CNS lymphomas are extranodal malignant lymphomas (98% of which are B-cell neoplasms) arising in the CNS in the absence of lymphoma outside the nervous system at the time of diagnosis. While once a rare tumor type (<1% of all primary brain tumors), primary CNS lymphomas have a dramatically increasing incidence (now approximately 5% of all primary brain tumors) as a consequence of the AIDS epidemic. Up to 10% or more of AIDS patients will develop these tumors during their lifetime, mainly during late stages of their disease. Inherited immunodeficiency syndromes and immunosuppressive therapy (i.e. for organ transplantation) also have been shown to increase risk of CNS lymphoma. The peak incidence in immunocompetent individuals is during the sixth and seventh decades of life and occurs approximately equally in both sexes, whereas immunodeficient individuals develop these
tumors at a much younger age and predominantly occurs in males. Up to 90% of primary CNS lymphomas in the setting of AIDS occur in male patients.

**Germ-cell tumors**

Germ-cell tumors of the CNS are a rare group of tumors that mainly affect children and adolescents. Their histopathological profile largely resembles their homologous neoplasms arising in the gonads and other extragonadal sites. The following types of germ-cell tumors can occur in the CNS – germinoma, embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma – although tumors often have overlapping features giving rise to mixed germ-cell tumors. These tumors account for 0.5% of all primary brain tumors and 3% of all brain tumors in children in the U.S., but interestingly have significantly higher incidence in other parts of the world such as Japan.

**Tumors of the sellar region**

Craniopharyngioma (WHO grade I) is a benign, partly cystic tumor of the sellar region derived from the epithelium of the Rathke’s pouch, which forms the anterior pituitary gland during embryogenesis. Craniopharyngiomas account for 2-4% of all primary brain tumors, and are the most common non-neuroepithelial neoplasm in children. Tumors of the pituitary gland (pituitary adenoma and carcinoma) are frequent tumors of the sellar region but are not traditionally grouped together with brain and CNS tumors.

**Metastatic tumors**

While approximately 22,000 malignant primary brain tumors are diagnosed per year in the U.S. at present, metastatic brain tumors are far more common with upwards
of 200,000 cases per year either diagnosed during patient’s lifetimes or more commonly found during autopsy. Intracranial and intraspinal metastases are present in at least 25% and 5% of cancer patients at time of autopsy, respectively. The most frequent primary malignancies that metastasize to the brain are lung cancer (50% of cases), breast cancer (15% of cases), and melanoma (10% of cases). Additionally, greater than 10% of cases have an unknown primary site of malignancy. Other cancers that frequently metastasize to the brain include clear cell renal carcinoma, esophageal cancer, and colorectal cancer, but cases of metastatic disease to the brain from nearly every type of primary malignancy have been reported. It is largely unclear at present why some cancer types frequently metastasize to the brain (e.g. lung) while others do not (e.g. prostate).
## Histologic classification of human brain tumors

### Tumors of neuroepithelial tissue
- Astrocytic tumors
  - Astrocytoma
  - Anaplastic astrocytoma
  - Glioblastoma multiforme
  - Pilocytic astrocytoma
  - Pleomorphic xanthoastrocytoma
  - Subependymal giant-cell astrocytoma
- Oligodendroglial tumors
  - Oligodendroglioma
  - Anaplastic oligodendroglioma
- Mixed gliomas
  - Oligoastrocytoma
  - Anaplastic oligoastrocytoma
- Ependymal tumors
  - Ependymoma
  - Anaplastic ependymoma
  - Myxopapillary ependymoma
  - Subependymoma
- Choroid-plexus tumors
  - Choroid-plexus papilloma
  - Choroid-plexus carcinoma
- Neuronal and mixed neuronal-glia1 tumors
  - Gangliocytoma
  - Dysembryoplastic neuroepithelial tumor
  - Ganglioglioma
  - Anaplastic ganglioglioma
  - Central neurocytoma
- Pineal parenchymal tumors
  - Pineocytoma
  - Pineoblastoma
- Embryonal tumors
  - Medulloblastoma
  - Primitive neuroectodermal tumor

### Meningeal tumors
- Meningioma
- Hemangiopericytoma
- Melanocytic tumor
- Hemangioblastoma

### Primary central nervous system lymphomas

### Germ-cell tumors
- Germinoma
- Embryonal carcinoma
- Yolk-sac tumor
- Choriocarcinoma
- Teratoma
- Mixed-germ-cell tumors

### Tumors of the sellar region
- Pituitary adenoma
- Pituitary carcinoma
- Craniopharyngioma

### Metastatic tumors
In addition to the SEER Program of the National Cancer Institute, another major source of brain cancer incidence data is the Central Brain Tumor Registry of the United States (CBTRUS). CBTRUS collects data from eighteen collaborating state cancer registries (that represent approximately 32% of the U.S. population) on all cases of non-malignant (benign and uncertain) and malignant primary brain and CNS tumors (including lymphomas and leukemias) arising in the brain, meninges, spinal cord, cranial nerves, other parts of the CNS, pituitary and pineal glands, and olfactory tumors of the nasal cavity. The most recent CBTRUS report released in December 2009 contains data on all such tumors newly diagnosed between 2002 and 2006 (n=85,670) with age-adjusted incidence rates based on the Year 2000 U.S. Standard Population (3).

The overall reported incidence rate for 2002-2006 for primary brain and CNS tumors was 18.3 new cases per 100,000 population per year. 7% were in individuals less than 20 years of age at time of diagnosis, and 93% in individuals 20 years of age or older. The overall incidence rate was 4.7 per 100,000 population per year for children 0-19 years of age, and 23.7 per 100,000 population per year for adults age 20+ years.

Of the 51,410 new cases of primary nonmalignant and malignant brain and CNS tumors diagnosed in the U.S. in 2007, 3,750 of those (7%) occurred in children ages 0-19 years. Brain and CNS cancers are the second most common cancers in children (comprising approximately 20% of all childhood cancers) and are the leading cause of solid tumor cancer death. CBTRUS reports that the incidence rate of brain and CNS cancers in children has risen slightly over the past three decades, but the death rate has dropped slightly over this period.
Details on the anatomic location, histologic classification, and gender bias of the primary brain and CNS tumors tracked by CBTRUS between 2002 and 2006 in both the total and pediatric populations are described below. Additionally, details on the anatomic location and histologic subtype for all gliomas reported during this period are presented.

**Distribution of All Primary Brain and CNS Tumors by Site**

**CBTRUS 2002-2006 (n=85,670)**

The distribution by anatomic location of all 85,670 primary brain and CNS tumors reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). The majority of tumors (34%) are located in the meninges. 23% of tumors are located within the frontal, temporal, parietal, and occipital lobes of the brain. Together, the pituitary and pineal glands account for about 12% of tumors. The cranial nerves and the
spinal cord/cauda equina account for 7% and 3% of all tumors, respectively. The cerebellum and brain stem only account for a small fraction of these tumors (3% and 2%, respectively).

The distribution by anatomic location of the 6,320 new cases of primary brain and CNS tumors in children ages 0-19 reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). While the cerebellum and brain stem only account for a small fraction of tumors in the total patient population (3% and 2%, respectively), tumors arising in these subtentorial sites account for a significantly larger fraction of tumors in the pediatric population (16% in the cerebellum and 11% in the brainstem). Tumors of
the pineal gland are also far more common in the pediatric population accounting for 3% of tumors compared to <1% in the total population. On the other hand, meningeal tumors are very rare in children (occurring almost exclusively in children with neurofibromatosis type 2) and account for only 3% of tumors in the pediatric population versus 34% in the total population.

Distribution of All Primary Brain and CNS Tumors by Histology

CBTRUS 2002-2006 (n=85,670)

The distribution by histologic classification of all 85,670 primary brain and CNS tumors reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). Meningiomas, predominantly of non-malignant histological subtypes, account for 34% of these tumors. Overall, gliomas account for 33% of the total 85,670 tumors and 79% of the malignant tumors. Glioblastomas account for 52% of the gliomas and 17% of the total primary brain and CNS tumors.
The distribution by histologic classification of the 4,479 primary brain and CNS tumors arising in children ages 0-14 reported to CBTRUS during 2002-2006 is shown (reproduced from reference 3). Pilocytic astrocytomas are the predominant tumor type occurring in the pediatric population ages 0-14 accounting for 20% of tumors. Together, gliomas account for 57% of the total 4,479 tumors in this children age 0-14, while malignant gliomas of nonspecified histology account for 14% of tumors. Embryonal tumors including medulloblastomas account for 13% of tumors in this pediatric population compared to only 1% in the total patient population. Craniopharyngiomas, ependymomas, and germ-cell tumors also account for a significantly larger fraction of tumors in the pediatric population compared to the total population (4% vs. <1%, 7% vs. 2%, and 4% vs. <1%, respectively). In contrast, pituitary tumors, meningiomas, and glioblastomas account for a significantly smaller fraction of tumors in this pediatric...
population than the total population (2% vs. 11%, 2% vs. 34%, and 3% vs. 17%, respectively).

Incidence Patterns by Gender for Selected Histologies

The incidence ratio in males to females for selected histologies of primary brain and CNS tumors reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). Incidence rates for all primary brain and CNS tumors combined are higher among females than males (19.4 versus 17.1 per 100,000 population per year). Incidence rates for tumors of neuroepithelial tissue are 1.4 times greater in males than females, while tumors of the meninges are 2.3 times greater in females than males. The incidence rate of gliomas is higher in males than in females (7.2 versus 5.0 per 100,000 population per year). Individual histologies with appreciably higher incidence rates in males are anaplastic astrocytomas, glioblastomas, oligodendrogliomas, embryonal
tumors including medulloblastomas, lymphomas, and germ-cell tumors. Meningiomas and pituitary tumors are the two brain and CNS tumor types which are significantly more common in women.

Distribution of All Primary Brain and CNS Gliomas by Site

CBTRUS 2002-2006 (n=28,127)

The distribution by anatomic location of the 28,127 new cases of primary brain and CNS gliomas (33% of the total 85,670 tumors) reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). 61% of these gliomas occurred in the four lobes of the brain, most frequently the frontal and temporal lobes. A minority occur in the cerebellum and brain stem (3% and 4%, respectively), most of which are those arising in children.
The distribution by histologic subtype of the 28,127 new cases of primary brain and CNS gliomas reported to CBTRUS during 2002-2006 is shown above (reproduced from reference 3). Glioblastomas account for the majority of these gliomas (52%), and astrocytic tumors together account for approximately 75% of these gliomas.
Etiology of human brain tumors

Cancer is a disease of genome alterations (e.g. DNA sequence changes, copy number aberrations, chromosomal rearrangements, and modification in DNA methylation) that result from a complex interaction between one’s inherited genetic makeup and a lifetime of environmental exposures. The genetic differences and the environmental factors that cause one individual to develop a brain tumor and another to not are largely unclear at present (reviewed in references 4-6). Herein, the known hereditary syndromes, genetic susceptibility loci, and environmental factors either associated with or being studied for their possible contribution to an increased risk of CNS tumors will be discussed.

Epidemiologic evidence from childhood cancer survivors

The combined influence of heritable and environmental factors in brain tumorigenesis is highlighted by epidemiological examination of childhood cancer survivors. Progress in the treatment of childhood cancer has produced increasing numbers of childhood cancer survivors who are living into adulthood. Unfortunately, children who survive cancer are predisposed to an increased risk of subsequent primary cancers (7). SEER data on childhood cancer survivors (ages 0-17) from 1973-2002 shows an observed-to-expected (O/E) ratio of 5.9 for the development of a second primary malignancy (i.e. childhood cancer survivors are at a nearly 6-fold increased risk of developing a new cancer relative to the general population) (8). The O/E ratio following all childhood brain and CNS cancers was 6.3. The O/E ratios following childhood ependymoma, astrocytoma, and PNET of the brain and CNS were 6.3, 4.3, and 14.6 respectively. The development of a second primary malignancy in these patients may be due to an inherited cancer predisposition (e.g. Li-Fraumeni syndrome)
and/or the genotoxicity of the radiation and chemotherapy treatments these patients received. Additionally, cancers of the brain and CNS are among the most common second cancers that occur in childhood cancer survivors. The O/E ratio for childhood cancer survivors who subsequently developed brain and CNS cancer was 8.4 (e.g. childhood cancer survivors subsequently developed brain and CNS cancer at >8-fold higher incidence than the general population). Brain and CNS cancer occurred as a second cancer in childhood cancer survivors at a statistically significantly increased incidence following a first cancer of acute lymphoblastic leukemia (O/E of 9.0), astrocytoma (O/E of 12.6), and PNET (O/E of 44.6). Brain and CNS cancers also tended to occur at increased frequencies following Hodgkin’s lymphoma (O/E of 4.7), non-Hodgkin lymphoma (O/E of 3.7), neuroblastoma (O/E of 5.3), Wilm’s tumor (O/E of 2.5), and germ-cell cancer (O/E of 2.8). Childhood cancer survivors treated with radiation therapy developed subsequent brain and CNS cancer with an O/E ratio of 16.1, and those children who had no radiation therapy developed subsequent brain and CNS cancer with an O/E of 4.7 (8). Together, these epidemiologic findings clearly link both genetic inheritance and environmental exposures (e.g. radiation therapy) to the development of brain cancer.

**Evidence for familial linkage of brain tumors**

Family members of brain tumor patients have been documented to be at a significantly increased risk for the development of brain tumors (9-12). Several large case-control and retrospective cohort studies have documented that up to 5% of brain tumors, particularly gliomas and meningiomas, arise in patients with a family history of brain tumors (reviewed in reference 13).
For example, one recent study examined the familial clustering of 1,401 primary brain tumors cases defined as astrocytoma or glioblastoma in the Utah Population Data Base with at least three generations of genealogy data. Significantly increased risks to first- and second-degree relatives for astrocytomas were identified for relatives of astrocytoma cases considered separately (n = 744). Significantly increased risks to first-degree relatives, but not second-degree, were observed for astrocytoma and glioblastoma cases considered together, and for glioblastoma cases considered separately (n = 658). This study provides strong evidence for a familial contribution to primary brain cancer risk as well as evidence that this familial aspect includes not only shared environment, but also a heritable component (12).

Another recent cohort study of all primary brain tumor patients in Sweden from 1958-1997 (n = 42,412) investigated whether the familial aggregation of brain tumors is due to environmental or genetic effects by comparing the brain tumor incidence among both the spouses and first-degree relatives of these patients. The brain tumor incidence among first-degree relatives was significantly increased by 2 to 3 times for the same histopathology as the probands, but no increased incidence of any brain tumor type was found in the cohort of spouses (14). This finding strongly indicates a genetic origin of the familial aggregation of brain tumors.

Interestingly, large population based studies of cancer risk among first-degree relatives of cancer probands have revealed that family members of brain tumor patients have a significantly increased risk of cutaneous melanoma in addition to an increased risk of brain tumors (14,15). In one study of 12 Finnish families with two or more glioma patients, relatives of the probands had a 4-fold increased incidence of cutaneous melanoma and a nearly 6-fold increased incidence of meningioma (16). In addition,
brain tumors have been found to aggregate in several melanoma-prone families (17-19). In one study of a series of 904 consecutive melanomas, 17 of the patients had family members with brain tumors (including astrocytomas, glioblastomas, meningiomas, ependymomas, and medulloblastomas) and 9 of the patients developed a primary brain tumor as a second malignancy later in life (18). Kindreds with incidence of both melanoma and nervous system tumors are often termed to be afflicted with “melanoma-astrocytoma syndrome”.

**Hereditary syndromes associated with increased incidence of brain tumors**

Several known hereditary syndromes are associated with an increased risk of CNS tumors including neurofibromatosis type 1 and 2, von Hippel-Lindau, tuberous sclerosis, Li-Fraumeni, Cowden, Turcot, and Gorlin syndromes (reviewed in references 20 and 21). The table below describes the genes involved, the associated nervous system tumors, and other disease pathology present in these hereditary syndromes.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Nervous system tumor(s)</th>
<th>Other disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurofibromatosis 1</td>
<td>NF1</td>
<td>17q11</td>
<td>neurofibromas, malignant peripheral nerve sheath tumors, optic nerve glioma, astrocytoma</td>
<td>iris hamartomas, osseous lesions, pheochromocytoma, leukemia</td>
</tr>
<tr>
<td>Neurofibromatosis 2</td>
<td>NF2</td>
<td>22q12</td>
<td>vestibular and peripheral schwannoma, meningioma, spinal ependymoma, astrocytoma</td>
<td>cerebral calcifications, posterior lens opacities, retinal hamartomas</td>
</tr>
<tr>
<td>von Hippel-Lindau</td>
<td>VHL</td>
<td>3p25</td>
<td>CNS and retinal hemangioblastoma</td>
<td>renal cell carcinoma, pheochromocytoma, visceral cysts</td>
</tr>
<tr>
<td>Tuberous sclerosis</td>
<td>TSC1/TSC2</td>
<td>9q34/16p13</td>
<td>cortical hamartomas (tubers), subependymal giant cell astrocytoma</td>
<td>cutaneous angiomyxomas, subungual fibromas, cardiac rhabdomyomas, adenomatous polyps of the intestine, cysts of the lung and kidney, angiomyolipomas</td>
</tr>
<tr>
<td>Li-Fraumeni</td>
<td>TP53</td>
<td>17p13</td>
<td>astrocytoma, PNET, other tumor types</td>
<td>breast carcinoma, bone and soft tissue sarcomas, adrenocortical carcinoma, leukemia</td>
</tr>
<tr>
<td>Cowden</td>
<td>PTEN</td>
<td>10q23</td>
<td>dysplastic gangliocytoma of the cerebellum</td>
<td>hamartomatous polyps of the colon, thyroid neoplasms, breast carcinoma</td>
</tr>
<tr>
<td>Turcot</td>
<td>APC/MTH1/PMS2</td>
<td>5q21/3p21/7p22</td>
<td>medulloblastoma, glioblastoma multiforme</td>
<td>colorectal polyps</td>
</tr>
<tr>
<td>Gorlin</td>
<td>PTCH</td>
<td>9q31</td>
<td>medulloblastoma</td>
<td>basal cell carcinomas, jaw cysts, ovarian fibromas, skeletal abnormalities</td>
</tr>
</tbody>
</table>

Inherited mutations of the NF1 gene result in neurofibromatosis type 1 (von Recklinghausen’s neurofibromatosis) occurring at an estimated prevalence of 1:4,000 persons in most populations. NF1 patients have numerous characteristic benign
peripheral nerve sheath tumors called neurofibromas and occasionally malignant peripheral nerve sheath tumors as well. Additionally, approximately 15% of NF1 patients develop pilocytic astrocytomas (most commonly of the optic nerve and frequently bilaterally), and a smaller fraction develop diffuse malignant astrocytomas, rhabdomyosarcomas, pheochromocytomas, and chronic myeloid leukemias (22-29).

Inherited mutations and newly acquired germline mutations of the NF2 gene result in neurofibromatosis type 2 occurring at an estimated incidence of 1:40,000 newborns. Affected individuals characteristically develop vestibular schwannomas (typically bilateral). Additionally, the majority of these individuals develop meningiomas during their lifetime, often multiple and in younger patients than sporadic tumors. NF2 patients also have increased incidence of ependymomas (frequently in the medulla of the spine or cauda equine), pilocytic astrocytomas, and diffuse malignant astrocytomas (30,31).

Inherited mutations of the VHL gene result in von Hippel-Lindau disease occurring at an estimated rate of 1:40,000 persons. VHL patients develop characteristic capillary hemangioblastomas, predominantly occurring in the retinas, cerebellum, brain stem, and spinal cord (often multiple and occurring at multiple sites). VHL patients also develop other tumors outside the CNS at high frequency (e.g. clear cell renal carcinoma and pheochromocytoma), and cases of other brain tumors including medulloblastomas, ependymomas, and choroid plexus papillomas have also been reported in these patients (32-34).

Inherited mutations in either the TSC1 or TSC2 genes result in a complex called tuberous sclerosis affecting an estimated 1:10,000 persons. The characteristic lesions of this syndrome are cortical hamartomas, regions of dysmorphic neurons and giant cells
along with calcification of blood vessel walls, that are commonly referred to as “tubers”. Additionally, approximately 10-15% of affected individuals develop supependymal giant cell astrocytomas, benign neoplastic lesions arising in the wall of the lateral ventricles. A number of other benign tumors can also arise in these patients outside the CNS including facial angiofibromas, retinal hamartomas and astrocytomas, renal angiomyolipomas, cardiac rhabdomyomas, hamartomatous rectal polyps, and liver hamartomas (35-38).

Germline mutations in the TP53 gene result in Li-Fraumeni syndrome, an autosomal dominant disorder characterized by multiple primary neoplasms in children and young adults. Approximately 20% of Li-Fraumeni patients will develop breast cancer during their lifetime, ~20% will develop soft tissue sarcomas, ~15% will develop brain tumors, ~5% will develop lung cancers, ~5% will develop stomach cancers, and a smaller fraction will develop a range of other tumor types including cancers of the pancreas, adrenal cortex, bladder, liver, esophagus, and testes. The most common brain tumors arising in Li-Fraumeni patients are malignant astrocytomas, medulloblastomas, and choroid plexus carcinomas. Approximately 50% of kindreds with Li-Fraumeni syndrome have at least one family member with a brain tumor, while some kindreds display remarkable clustering of brain tumors (39-46).

Germline mutations in the PTEN gene result in Cowden disease characterized by multiple trichilemmomas (benign skin appendage tumors) in ~85% of affected individuals, gastrointestinal polyps in ~40% of cases, thyroid tumors in ~70% of cases, and breast cancer in ~30% of cases. A number of these patients will also develop dysplastic gangliocytoma of the cerebellum referred to as Lhermitte-Duclos disease. Dysplastic gangliocytoma of the cerebellum is a benign cerebellar lesion arising
predominantly in young adults and is composed of dysplastic ganglion cells. Additionally, some cases of ependymomas and medulloblastomas have been reported in patients with Cowden disease (47-51).

Turcot syndrome is characterized by the presence of primary brain tumors (95% of which are anaplastic astrocytomas, glioblastomas, and medulloblastomas) in the setting of familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal carcinoma (HNPCC) syndromes. Germline mutations of the PMS2, MSH2, or MLH1 genes that encode DNA mismatch repair proteins result in HNPCC characterized by colorectal cancer that develops from a single to few large adenomas in the proximal colon. The small fraction of HNPCC patients who develop glioblastoma multiforme (or less commonly astrocytomas) are said to be afflicted with Turcot syndrome type 1 (also called mismatch repair-associated Turcot syndrome). These patients develop GBM at a much younger age than sporadic disease (median age at diagnosis in the second decade of life compared to sixth decade), and have a mean survival longer than 2 years compared to less than 12 months for sporadic disease. Germline mutations in the APC gene result in FAP characterized by hundreds of adenomatous polyps in the distal colon that invariably progress to colorectal cancer. The small fraction of FAP patients that develop medulloblastomas are said to be afflicted with Turcot syndrome type 2. These patients typically develop medulloblastoma at a much later age than sporadic disease with a median age at diagnosis of 15 years versus 7 years for sporadic disease (52-56).

Inherited mutations in the PTCH gene result in Gorlin syndrome (also referred to as nevoid basal cell carcinoma syndrome) affecting 1:60,000 persons. Greater than 90% of affected individuals develop multiple basal cell carcinomas and jaw keratocysts by the age of 40 years. In addition, approximately 5% of affected individuals develop
medulloblastomas, primarily in the first three years of life. Multiple cases of other tumor types have been reported in individuals with Gorlin syndrome including meningioma, melanoma, chronic lymphocytic leukemia, non-Hodgkin lymphoma, and breast cancer (57,58).

Despite the relatively high frequency at which individuals with these hereditary cancer syndromes develop brain tumors, these syndromes are all very rare and together account for less than 1% of all primary brain and CNS tumors (20,21). For example, germline mutations in the PTCH gene (i.e. Gorlin syndrome) account for only 1-2% of all medulloblastomas (59). Germline mutations in NF1, NF2, TSC1, and TSC2 genes together account for 1-2% of all gliomas (60). Given that inherited mutations in the genes known to cause these syndromes are seldom responsible for tumorigenesis in the brain, environmental and other genetic factors must clearly be at work in the neoplastic processes that drive human brain cancer.

**Genetic variants associated with an increased risk of brain tumor development**

Several studies have examined the contribution of individual genes to brain tumor susceptibility and have successfully identified a number of genes whose polymorphic variation appear to be associated with an increased risk of brain tumors. For example, one study assessed the PPARG gene, which encodes the gamma isoform of the peroxisome proliferator-activated receptors known to be genetically altered and differentially expressed in human cancer, for the presence of polymorphic variants found in GBM patients but not in population-matched controls. 13/26 GBM patients (50%) were found to carry a C>T polymorphism, a synonymous variant at codon H4449 of the PPARG gene, compared to 10/80 population-matched controls (12%). Carriers of this polymorphic allele were statistically significantly (p<0.001) associated with an increased
risk of GBM with an odds ratio of 4.7 (61). It is unclear how such a synonymous (i.e. silent) variant could itself act as a low-penetrance modifier of GBM risk, but it could exist in linkage disequilibrium with another as yet unidentified polymorphic locus that is the low-penetrance risk-modifying allele. A decade has passed since the publishing of this study without confirmation of the findings in an additional panel of glioma patients.

As all cancers arise from DNA alterations (e.g. point mutations, copy number aberrations, translocations, etc.), one hypothesis has been that variants in genes encoding DNA repair proteins might increase brain tumor susceptibility. Indeed, polymorphic variants in several DNA repair genes have been identified that are associated with an increased incidence of glioma. Nucleotide excision repair (NER) is a repair mechanism that recognizes bulky distortions in the shape of the DNA double helix (such as those resulting from the DNA photoproducts induced by ultraviolet irradiation), removes a short single-stranded DNA segment that includes the lesion, and fills in the single-strand gap using the undamaged complementary strand as template. Both global genomic NER and transcription-coupled NER processes occur in cells, each requiring unique factors that include the excision repair cross-complementing (ERCC) family of proteins, Replication Protein A (RPA), Proliferating Cell Nuclear Antigen (PCNA), a DNA ligase, and a DNA polymerase. In contrast, base excision repair (BER) is a DNA repair mechanism in which small, non-helix distorting single base lesions resulting from deamination, oxidation, and alkylation are removed from the genome. BER is accomplished by DNA glycosylases that remove specific damaged or inappropriate bases (e.g. O-6-methylguanine-DNA methyltransferase [MGMT], the major enzyme responsible for the removal of alkylated bases), the AP endonuclease APE1 which cleaves the damaged base creating a single-strand break, a DNA ligase, XRCC1, a flap
endonuclease (FEN1), and a DNA polymerase. Nonhomologous end-joining (NHEJ) is a repair process in which DNA double-strand breaks are rejoined and requires factors including DNA-PK, XRCC4, DNA Ligase IV, and a DNA polymerase. In one study of the ERCC2 gene (which encodes an NER factor) in 187 adult onset gliomas and 169 controls, glioma cases were significantly more likely than controls to be homozygous AA for the silent polymorphism at codon R156 with an odds ratio of 2.3 (62). In another study of NHEJ factors ERCC4 and LIG4 genes in 772 glioma patients and 752 cancer-free controls, a single polymorphic variant in LIG4 (located in noncoding intronic sequence) was associated with an increased risk of glioma. Moreover, haplotype analysis between single-nucleotide polymorphisms (SNPs) in LIG4 and ERCC4 revealed linkage of three SNP loci that together contributed a more than additive effect in increasing glioma susceptibility (63). Another study examined the association between glioma risk and 18 functional (i.e. nonsilent) SNPs in DNA repair genes in a cohort of 373 Caucasian glioma cases and 365 cancer-free Caucasian controls. Six of these SNPs in ERCC1, XRCC1, APEX1, PARP1, MGMT, and LIG1 genes showed a significant association with glioma risk, with either increased risk or protective effects that varied depending on stratification by patient age, gender, ionizing radiation exposure history, and histologic type of glioma. Furthermore, a significant dosage effect on glioma risk was observed with increasing numbers of adverse genotypes at these six SNPs (64). Mounting evidence from several additional studies are demonstrating that polymorphic variants in these and other DNA repair genes are indeed associated with an increased risk of brain tumors including gliomas and meningiomas (65-70).

It has also been theorized that variants in genes encoding enzymes involved in metabolizing carcinogens may be associated with an increased susceptibility to brain
tumor development. Indeed, a few studies have documented that polymorphic variants in genes encoding cytochrome P450 and glutathione S-transferase enzymes modify brain tumor risk (71-73).

More recently, unbiased genome-wide association studies have been used to identify additional genes whose polymorphic variation are associated with an increased risk of brain tumors. The first genome-wide linkage analysis of familial glioma in 4 glioma-prone pedigrees not linked to known hereditary cancer syndromes found a novel susceptibility locus at 15q23-q26.3 with a nonparametric linkage score of 3.35. Investigation of this locus in 15 familial glioma pedigrees revealed significant association/transmission distortion among affected individuals, providing further evidence that this locus is a novel low-penetration modifier of glioma risk (74). The gene(s) linked with this locus at 15q that potentially modifies glioma susceptibility has yet to be identified. Most recently, high density SNP microarrays have been developed that allow genotyping at hundreds of thousands of SNPs in the genome on a single array. Such SNP microarrays have been used to conduct two genome-wide association studies on glioma patients that have identified novel loci influencing glioma predisposition. One study assessing 275,000 autosomal variants among 692 adult high-grade glioma cases and 3,992 controls discovered SNPs at chromosome 9p21 near the CDKN2B gene ($p = 3.4 \times 10^{-3}$) and at chromosome 20q13.3 intronic to the RTEL1 gene ($p = 1.5 \times 10^{-7}$) associated with increased glioma risk, with odds ratios of 1.4 and 1.6, respectively.(75). The other study assessed 550,000 SNPs in a total of 1,878 glioma cases and 3,670 controls and identified five new susceptibility loci – one at chromosome 5p15.33 intronic to the TERT gene (encoding the enzyme telomerase essential for maintaining telomere length and cellular immortalization), one at 8q24.21 intronic to the CCDC26 gene.
(encoding a retinoic acid modulator of differentiation and death), one at 9p21.3 near the CDKN2A and CDKN2B genes (encoding the known tumor suppressor proteins $p16^{\text{INK4a}}$ and $p15^{\text{INK4b}}$), one at 20q13.33 intronic to the RTEL1 gene (encoding a RAD3-like helicase that regulates homologous recombination), and one at 11q23.3 in the 5’ untranslated region of the PHLDB1 gene (76). With the formation of international collaborations such as GLIOGENE to study familial glioma (13) and the advent of new SNP genotyping microarrays, it is likely that the genetic factors that contribute to brain tumor risk will be largely defined in the coming years.

**Environmental factors associated with increased incidence of brain tumors**

While the human papillomavirus (HPV) and Epstein-Barr virus (EBV) have been shown to definitively play an oncogenic role in the development of some cervical cancers and lymphomas, respectively, no solid evidence exists to demonstrate a viral origin of any CNS tumors. A few small studies have been able to detect DNA sequences from the simian virus 40 (SV40) and JC polyomaviruses in human brain tumor tissue (77-80). While these viruses are known to oncogenic both *in vitro* and in rodents, no causative role has been described for these viruses in human cancer, including brain tumors. SV40 was iatrogenically introduced into human populations in North America and Europe between 1955 and 1962 through SV40-contaminated polio vaccines. One group demonstrated the presence of SV40 in brain tumors from Swiss patients who had received SV40-contaminated vaccines and an absence of SV40 in brain tumors from Finnish patients not exposed to SV40-contaminated vaccines (79,81). No selective increase in the incidence of brain tumors has been reported in populations that received SV40-contaminated polio vaccine, and incidence rates for brain tumors are similar in countries that did (United States and Switzerland) or did not (Finland) use SV40-
contaminated vaccine. Thus, the presence of SV40 probably reflects a bystander infection caused by an intratumoral microenvironment that favors viral replication in humans with latent SV40 infection, rather than a causative role in the development of brain tumors (81).

Numerous other environmental factors once proposed to be linked to brain tumors have now been largely excluded as causative factors. These include the dietary sweeter aspartame and other nutritional factors, exposure to high-tension electrical wires, hair dyes, and head trauma (5,6). Several large epidemiological studies have denied an association of cellular telephone use and increased brain tumor risk (82,83), but other long-term studies are still underway. Tobacco smoking does not appear to affect brain tumor incidence (84-86), except possibly with prenatal exposure where evidence is conflicting at present (87-89). One carcinogen that has been linked to brain tumors is monomeric vinyl chloride, a carcinogen well known to cause angiosarcoma of the liver in workers of polyvinyl chloride (PVC) plants. Several cases of brain tumors, mostly gliomas, have been reported in individuals with high levels of occupational exposure to vinyl chloride and perhaps other industrial compounds (90).

The only environmental exposure unequivocally identified as a causative agent for brain tumors is ionizing radiation. Prior to introduction of griseofulvin in 1960, the world standard for treatment of tinea capitis was scalp irradiation. Several studies have found that children with tinea capitis who were treated with small doses of radiotherapy (on the order of 1 to 2 Gy) prior to 1960 had significantly increased incidence of brain and nervous system tumors (91-94). For example, the cohort in one study compared with population-matched controls exhibited increased incidence of meningiomas,
gliomas, and nerve-sheath tumors (relative risk ratios of 9.5, 2.6, and 18.8, respectively) (94).

While radiotherapy is no longer used to treat tinea capitis, it does continue to play a major therapeutic role in the treatment of childhood cancer. Indeed, children receiving irradiation of the cranium as a treatment for acute lymphocytic leukemia (ALL), pituitary adenoma, craniopharyngioma, pineal tumors, or germ-cell tumors have a dramatically increased risk of developing a secondary brain tumor later in life (95-101). In one study, follow-up of ~1,600 children treated for ALL found secondary brain tumors in 21 of these patients, exclusively occurring in those that had received whole-brain radiotherapy (100). 10 high-grade gliomas were diagnosed after a median latency of 9.1 years, and 11 meningiomas were diagnosed after a median latency of 19 years (cumulative incidence of 1.39%). Risk factors identified for developing a secondary brain tumor included the presence of CNS leukemia at diagnosis and treatment with cranial irradiation, which was dose-dependent. Additionally, age less than 6 years was associated with an increased risk of developing a high-grade glioma. Another study of >9,000 children who had received prophylactic irradiation of the brain for ALL revealed a 22-fold increased risk of developing a secondary CNS tumor, with 2.5% of the cohort being affected (101). Several studies have corroborated that cranial irradiation as well as certain chemotherapeutic agents result in a significantly increased risk of subsequently developing a secondary cancer of the brain (102,103).
Presentation and diagnosis of malignant gliomas

Patients with a CNS tumor will typically present with one or more of the following symptoms – headache, changes in mood or personality, difficulty with concentration, loss of memory, nausea, vomiting, generalized or focal seizures, and focal sensory or motor deficits including urinary incontinence, gait disorder, aphasia, spasticity of the extremities, etc. Different symptoms are more commonly associated with low-grade than high-grade gliomas (e.g. seizures) and vice versa (e.g. hemiparesis). See table below showing the fraction of brain tumor patients that present with four of the most common symptoms (reproduced from reference 5, ©2001 Massachusetts Medical Society).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Low-Grade Glioma</th>
<th>Malignant Glioma</th>
<th>Meningioma</th>
<th>Primary Central Nervous System Lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent with symptom</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>40</td>
<td>50</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>Seizure</td>
<td>65–95</td>
<td>15–25</td>
<td>40</td>
<td>17</td>
</tr>
<tr>
<td>Hemiparesis</td>
<td>5–15</td>
<td>30–50</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Mental-status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>abnormalities</td>
<td>10</td>
<td>40–60</td>
<td>21</td>
<td>61</td>
</tr>
</tbody>
</table>

These symptoms tend to be slowly progressive, rather than acute. Symptoms caused by intracranial tumors result either from the infiltration and destruction of brain parenchyma by tumor cells or the compression of the brain by the tumor and the presence of associated edema (often termed “mass effect”).

After thorough neurologic exam, patients with a suspected CNS tumor should undergo either magnetic resonance imaging (MRI) scan or a contrast-enhanced computed tomography (CT) scan. The identification of a space-occupying lesion by
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despite these imaging techniques is indicative of a brain tumor, infectious abscess, cerebrovascular infarct, or arteriovenous malformation. Characteristic radiologic findings on MRI scan of a malignant glioma (particularly GBM) include a heterogeneous poorly marginated mass, internal cystic areas, necrotic foci, hemorrhagic foci (high signal on T1-weighted images), irregular but intense enhancement of gadolinium-based contrast, internal flow voids (prominent vessels), significant peritumoral edema, and significant mass effect (104). Malignant gliomas can be differentiated from metastatic tumors in the brain that are often multifocal, located at the gray-white junction, and more homogeneous in appearance. New advances in imaging technology are enabling more accurate radiologic differentiation of gliomas versus primary CNS lymphoma, metastatic tumors, and other space-occupying lesions in the brain (104-106). In post-therapy imaging, single-photon emission computed tomography (SPECT) and positron emission tomography (PET) may be useful in differentiating tumor recurrence from radiation necrosis (107,108). A contrast-enhanced CT scan of a malignant glioma in the right cerebral hemisphere with irregularly enhancing margin and central necrosis accompanied by pronounced peritumoral edema and mass effect is shown below (reproduced from reference 109, ©2005 Elsevier Saunders).
Biopsy of a suspected brain tumor is required to make an accurate histologic diagnosis and to rule out non-neoplastic disease such as infectious abscess or hemorrhagic infarct. Biopsy can be performed either by open craniotomy or by MRI- or CT-guided stereotactic technique. Pathologic diagnosis is often made intraoperatively at the time of surgical resection. Tumors are graded pathologically on the basis of the most malignant area identified. All gliomas, and particularly the astrocytic neoplasms, are histologically heterogeneous tumors (e.g., low-grade tumor may be immediately adjacent to highly malignant disease). Thus, small biopsy sections can be misleading, and multiple tumor sections containing abundant tumor tissue are optimal for pathologic examination.

Oligodendrogliomas grossly appear as well-defined soft masses of grayish-pink color. These tumors are typically located in the cortex and white matter (most often of the frontal lobe), and infiltration of the overlying leptomeninges may sometimes be seen. Calcifications are frequently present in the tumor periphery and adjacent cortex. Areas of cystic degeneration and intratumoral hemorrhages may also been seen. Shown below is an oligodendroglioma in the periphery of the cerebral cortex (reproduced from reference 110).
Histology of paraffin-embedded oligodendrogliomas reveals moderate cellularity composed of tumor cells with small round hyperchromatic nuclei surrounded by a swollen clear cytoplasm and a well-defined cell membrane. Pathologists often refer to these cells as having a “fried egg” appearance. These tumors typically contain a dense network of branching capillaries whose appearance is often compared to “chicken wire”. As mentioned previously, the World Health Organization (WHO) classifies oligodendrogliomas into two grades based on a combination of gross, histologic, and pathologic criteria. WHO grade II tumors are referred to as low-grade oligodendrogliomas. These tumors are well-differentiated with moderate nuclear atypia, low to absent mitotic activity, and a MIB-1 index below 5%. Anaplastic oligodendrogliomas (WHO grade III) display increased cellularity, more significant nuclear atypia, abundant mitotic figures, high proliferative activity (MIB-1 index greater than 5%), and prominent microvascular proliferation. Additionally, intratumoral necrosis surrounded by nuclear palisading may be observed (not to be confused with GBM). Shown below is a hematoxylin and eosin stained section of an oligodendroglioma with typical “fried egg” cell appearance as well as a dense capillary network (reproduced from reference 111, ©2005 American Society for Clinical Pathology).
As discussed previously, the WHO classifies astrocytomas into four grades based on a combination of gross, histologic, and pathologic criteria. Pilocytic astrocytomas (WHO grade I astrocytomas) are well-circumscribed, slow growing, and usually cystic tumors. These tumors can appear throughout the neuraxis but are most frequently found at the optic nerve, the hypothalamus, the thalamus and basal ganglia, the cerebellum, and the brainstem. The tumors are typically soft, gray, and discrete. The cystic component can be singular and massive with the tumor existing as a mural nodule, or multiple and smaller with intratumoral lacunae. Infiltration of the leptomeninges may be observed. A minority of chronic tumors will contain calcifications and evidence of previous hemorrhage. A pilocytic astrocytoma in the pons with a nodule of tumor in a large cyst is shown below (reproduced from reference 110).

Histology of pilocytic astrocytomas reveals a low to moderately cellular tumor with a biphasic pattern of (1) densely packed areas of bipolar “piloid” cells with long hair-like cytoplasmic processes with Rosenthal fibers, and (2) loosely packed microcystic areas containing multipolar “protoplasmic” cells with eosinophilic granular bodies. Rosenthal fibers are corkscrew-shaped, brightly eosinophilic hyaline masses commonly observed in regions of compact piloid cells, but are not a requisite feature of pilocytic astrocytoma.
or neoplasia in general, as they are often also observed in chronic reactive gliosis. Absent to rare mitotic figures, moderate nuclear pleomorphism and hyperchromasia, and microvascular proliferation are all common features. Pilocytic astrocytomas always stain positive for GFAP and typically have a MIB-1 index less than 1% (discussed later). Shown below is a hematoxylin and eosin stained section of a pilocytic astrocytoma showing the characteristic biphasic pattern of microcystic areas and dense hypercellular areas (reproduced from reference 110).

Low-grade or diffuse astrocytomas (WHO grade II) are well-differentiated but invariably invasive lesions that occur predominantly in the cerebral hemispheres in both children and adults. They sometimes arise in the brain stem and spinal cord but rarely in the cerebellum. These infiltrative, diffuse tumors cause effacement/blurring of the gross anatomical boundaries with enlargement and distortion of the invaded structures, often producing significant mass effect. Cystic granular areas and zones of firmness and softening may be present. Shown below is a WHO grade II astrocytoma with diffuse
tumor growth extending from the left thalamus to the brainstem apparent (reproduced from reference 110).

Histology of diffuse astrocytomas reveals well-differentiated neoplastic astrocytes on a loosely structured, often microcystic tumor matrix. These tumors are moderately hypercellular, with occasional nuclear atypia and absent to rare mitotic figures. Necrosis and microvascular proliferation (indicative of malignant progression to GBM) are not present in grade II tumors. The neoplastic astrocytes are classified according to three variants – fibrillary, gemistocytic, and protoplasmic. The most common histological variant fibrillary astrocytoma is composed of cells with scant cytoplasm and enlarged, irregular nuclei. GFAP immunoreactivity is usually restricted to a small perinuclear ring. Shown below is a hematoxylin and eosin stained section of a WHO grade II fibrillary astrocytoma showing a monomorphic population of neoplastic fibrillary astrocytes infiltrating the neuropil (reproduced from reference 111, ©2005 American Society for Clinical Pathology).
The gemistocytic variant is composed chiefly of fibrillary astrocytes mixed with a greater than 20% fraction of gemistocytic cells with plump eosinophilic cell bodies and eccentric nuclei. These gemistocytic cells have GFAP immunoreactivity in their perikarya and cell processes. Shown below is a hematoxylin and eosin stained section of a WHO grade II gemistocytic astrocytoma (reproduced without modification from reference 111, ©2005 American Society for Clinical Pathology).
Protoplasmic astrocytoma is a rare histological variant composed of astrocytes with small cell bodies, few flaccid processes, and uniformly round to oval nuclei. Mucoid degeneration, microcyst formation, and scant GFAP immunoreactivity are characteristic features. The MIB-1 index for all diffuse astrocytoma variants is typically below 4%.

WHO Grade III tumors called anaplastic astrocytomas grossly resemble diffuse astrocytomas with a similar preference for arising in the cerebral hemispheres. The high cellularity of anaplastic astrocytoma often produces a more discernible tumor mass allowing a clearer distinction from surrounding brain structures than with diffuse astrocytoma. Their infiltrative growth produces marked enlargement of the invaded structures, usually without macroscopically visible cysts. On histology, these tumors appear as diffusely infiltrating astrocytomas with increased cellularity, marked nuclear atypia, and abundant mitotic activity. Pleomorphic nuclei vary in shape, size, dispersion of chromatin, and increasing nucleolar prominence. However, necrosis and microvascular proliferation (histological features present exclusively in GBM) are absent in these tumors.

Glioblastoma multiforme (GBM), the WHO grade IV astrocytoma, occurs predominantly in the subcortical white matter of the cerebral hemispheres. This rapidly growing and highly malignant tumor often infiltrates into multiple lobes, the adjacent cortex, the basal ganglia, and the contralateral hemisphere. GBM also infrequently arises in the brainstem, almost exclusively occurring in children (e.g. “malignant brainstem glioma”). GBM rarely arises in either the cerebellum or spinal cord. GBMs have a variable appearance macroscopically but typically contain areas of necrosis, abundant vasculature, and both recent and remote hemorrhages visible as red and brown stippling. A hypercellular zone often surrounds areas of necrosis that is visible as
GBMs are poorly demarcated tumors resembling anaplastic astrocytomas histologically with additional characteristic features of prominent endothelial proliferation and intratumoral necrosis. Areas of necrosis can be surrounded by pseudopalisading tumor cells, a feature pathognomonic of GBM. Cellular composition of GBM can be very heterogeneous including pleomorphic poorly differentiated cells, more differentiated cells discernible as neoplastic astrocytes, large granular cells, and multinucleated giant cells. A histologic variant of GBM is giant cell glioblastoma characterized by a predominance of multinucleated giant cells and abundant stromal reticulin network. The stroma of these tumors causes their distinct circumscription and firmness. A hematoxylin and eosin stained section of a GBM tumor is shown below with areas of hemorrhage, necrotic foci, and surrounding pseudopalisading tumor cells all readily apparent (reproduced from reference 109, ©2005 Elsevier Saunders).
A more complete description of the pathology of human gliomas including ependymomas, gliosarcomas, pleomorphic xanthoastrocytomas, and gliomatosis cerebri can be found elsewhere (2).

Various immunohistochemical markers are commonly used to confirm a diagnosis of malignant glioma, to help classify tumors according to WHO grade, to aid in patient prognosis, and to help direct the best course of treatment. For example, immunohistochemistry to the Ki67 protein expressed exclusively in proliferating cells is commonly used to determine a MIB-1 index for glial tumors. The MIB-1 index helps to differentiate between WHO grade II and grade III oligodendrogliomas, which carry different prognoses and often different treatment regimens as well. Glial fibrillary acidic protein (GFAP), an intermediate filament cytoskeletal protein present in differentiated astrocytes and ependymocytes, is widely used as an immunohistochemical marker for astrocytic and ependymal tumors. Shown below is immunohistochemistry to GFAP on a diffuse fibrillary astrocytoma section (reproduced from reference 110).
Oligodendrogliomas rarely stain positive for GFAP, although the gemistocytic and gliofibrillary subtypes are occasionally immunoreactive for GFAP. Myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) are antigens expressed on mature oligodendrocytes and have been used as markers for oligodendrogliomas. These antigens are not present on all oligodendrogliomas (i.e. expression is lost during neoplastic transformation), and newer diagnostic markers such Olig1 and Olig2 are gaining favor with neuropathologists. YKL-40 is a new marker that has shown potential in enhancing differentiation of GBM from anaplastic oligodendroglioma (112). Immunohistochemistry also helps to differentiate glial tumors from other malignancies in the brain. For example, the neuronal markers synaptophysin, neurofilament, and NeuN are used to identify central neurocytomas and medulloblastomas. Chromogranin is a widely used antigen for confirming the diagnosis of a pituitary adenoma. CD20, a differentiation antigen present on B-cell lymphocytes, is the marker most commonly used for B-cell lymphomas, which represent greater than 98% of all primary CNS lymphomas. Metastatic melanoma usually stains positive for the melanosomal antigen HMB-45 (113,114), whereas metastatic carcinomas of epithelial
origin (e.g. breast and lung) usually stain positive for cytokeratin proteins, particularly CK7 and CK20 (115).

While immunohistochemistry has been the standard technique for confirming diagnosis of CNS neoplasms, genetic analysis of tumor tissue is likely to be the future of highly specific and highly sensitive diagnostic methodology. One example in which genetic testing is soon to be incorporated into neuropathology is in the differentiation of pilocytic astrocytoma (WHO grade I) from diffuse astrocytoma (WHO grade II). It was recently discovered that approximately 70% of sporadic pilocytic astrocytomas contain a tandem duplication of ~2 Mb on chromosome 7q34 which results in fusion of the BRAF gene with the KIAA1549 gene (116-118). This in-frame fusion links the kinase domain of BRAF to KIAA1549 and has constitutive kinase activity from lack of the auto-inhibitory N-terminus of BRAF. This oncogenic BRAF-KIAA1549 fusion is found in upwards of 75% of grade I pilocytic astrocytomas but has not been found in >300 diffuse malignant astrocytomas (grades II/III). Additionally, it was recently discovered that approximately 80% of grade II/III astrocytomas carry a point mutation in the IDH1 gene at amino acid residue R132 (119,120). Mutations in IDH1 appear to be exclusive to malignant gliomas and have not been found in >200 pilocytic astrocytomas (see further discussion of IDH1 mutations in gliomagenesis presented later). The combined molecular analysis of BRAF and IDH1 has proven to be a highly specific and sensitive approach for differentiating grade I from grade II astrocytoma and may soon be employed by neuropathologists trying to correctly diagnose these two histologically similar neoplasms with very different prognoses (121).
Current treatment and prognosis for malignant glioma patients

Patients with a suspected brain tumor who develop seizures or are at risk of developing seizures are typically placed on anticonvulsant therapy prior to undergoing biopsy or surgical resection. Additionally, most patients with malignant gliomas have significant brain edema and benefit from glucocorticoid treatment. Dexamethasone is typically preferred due to its long half-life, minimal mineralocorticoid effect, and high oral bioavailability. Mannitol can be administered intravenously to reduce intracranial pressure in patients with life-threatening edema (122).

Surgical resection is attempted in most patients with malignant gliomas. While surgery is not curative for these patients, surgical “debulking” of the tumor may relieve symptoms for several months. While surgical resection has been shown to improve patient survival, a correlation between extent of resection and survival time has not been clearly established (123-126). However, resection is contraindicated/not feasible when the tumor is located in the brainstem, basal ganglia, corpus callosum, or areas of the brain that control language or sensorimotor function because of the risk of death and/or permanent neurologic dysfunction.

Radiation therapy is a typical part of the treatment regimen for malignant gliomas, often performed following surgical resection and either preceding or concurrent with any chemotherapy administered. Conventional external beam radiotherapy for brain tumors is usually delivered via two-dimensional beams (e.g. front and back) to the whole brain. The total dose is often fractionated (i.e. spread out over time) with numerous different dosing strategies commonly used depending on tumor type, the extent of tumor resection, the patient’s overall health, etc. Fractionation allows normal cells time to recover, while tumor cells are generally less efficient at repair between
fractions. Fractionation also allows tumor cells that were in a radioresistant phase of the cell cycle during one treatment to cycle into a sensitive phase of the cycle before the next fraction is given. Similarly, tumor cells that were hypoxic (and therefore more radioresistant) may reoxygenate between fractions, improving the tumor cell kill. The ability to treat a brain tumor with high doses of radiation is limited by the radiation toxicity capacity of healthy brain which receives the same dose of radiation as the tumor with conventional therapy. For this reason, three-dimensional conformal (e.g. stereotactic) radiotherapy is becoming the standard treatment for brain tumors. Stereotactic radiosurgery is a non-surgical procedure that delivers a single high dose of precisely targeted radiation using detailed imaging scans and numerous highly focused gamma-ray beams that converge on the specific area of the brain where the tumor resides, minimizing the amount of radiation to healthy brain tissue. Stereotactic radiotherapy is only appropriate for small well-defined brain tumors though, and the diffuse malignant astrocytomas are still generally treated with conventional whole brain radiation at present.

Pilocytic astrocytomas (WHO grade I) are biologically benign (non-malignant) tumors and can be cured if they can be surgically resected in their entirety. Recurrence, most frequently occurring one to three years after resection, is usually due to reformation of the cystic component rather than regrowth of the tumor component if not completely removed during resection. Unfortunately, a number of pilocytic astrocytomas arise in the brainstem and other regions of the brain essential for basic life functions that are not amenable to surgical intervention. Pilocytic astrocytomas that can not be resected or only partially resected are usually treated with radiation therapy. Chemotherapy is not a typical treatment modality for these tumors. In a series of 55 pilocytic astrocytomas
treated between 1980 and 1994 in Switzerland, survival rates were 100% at 5 years and 96% at 10 years, with deaths only occurring in two patients whose tumors were graded as the rare anaplastic variant of pilocytic astrocytoma (127). A series of 105 cerebellar pilocytic astrocytomas treated at the Mayo Clinic between 1960 and 1984 had survival rates at 5, 10, and 20 years of 85%, 81%, and 79%, respectively (128). 51 supratentorial pilocytic astrocytomas treated at the Mayo Clinic during the same time period had survival rates at 10 and 20 years of 82% each (129). A separate report on 54 cerebellar astrocytomas treated at the Mayo Clinic between 1978 and 1990 showed survival at 5 years of 88% and 10 years of 50% when residual tumor tissue was observed on postoperative imaging, whereas the 10 year survival rate was 100% when total resection was observed (130).

In contrast to benign pilocytic astrocytomas, low-grade/diffuse astrocytomas and oligodendrogliomas (WHO grade II) are well-differentiated but invariably invasive lesions. Although they may follow long clinical courses, early diffuse infiltration of the surrounding brain renders these tumors incurable by surgery. Radiation therapy following resection is standard therapy for all malignant gliomas, but there is some debate whether this therapy should immediately follow resection or should be delayed until disease progression or onset of neurologic deficits in patients with low-grade gliomas (131,132). Chemotherapy is usually withheld until tumor recurrence/progression in patients with low-grade gliomas (see discussion of chemotherapy for gliomas below). It is estimated that approximately 70% of grade II gliomas transform into grade III/IV disease within 5-10 years of diagnosis. In a series of 122 WHO grade II diffuse astrocytomas, oligodendroglialomas, and mixed oligoastrocytomas treated between 1980 and 1994 in Switzerland, survival rates were highest for patients with oligodendroglioma
(78% at 5 years, 51% at 10 years), followed by those with oligoastrocytoma (70% at 5 years, 49% at 10 years) and fibrillary astrocytoma (65% at 5 years, 31% at 10 years). Survival of patients with gemistocytic astrocytoma was poor, with survival rates of 16% at 5 years and 0% at 10 years. Additionally, younger patients (<50 years) were found to survive significantly longer than older patients (>50 years), demonstrating both patient age and tumor histologic type to be significant predictive factors for low-grade glioma patients (133). See Kaplan-Meier survival plot below for these 122 low-grade gliomas (reproduced from reference 133, ©2004 Springer Science, MST = median survival time).

In a separate study on two cohorts of 322 and 288 adults with cerebral low-grade gliomas, median survival was 6.0 and 6.8 year, respectively. In these cohorts age >40 years, astrocytoma histology subtype, largest diameter of the tumor >6 cm, tumor crossing the midline, and presence of neurologic deficit before surgery were all unfavorable prognostic factors significantly impacting survival (134). Several other
studies have demonstrated similar survival rates and prognostic factors for low-grade gliomas (135-139).

While typical ranges of survival are 5 years or more for low-grade glioma patients, patients with more aggressive WHO grade III anaplastic astrocytomas, anaplastic oligodendrogliomas, and anaplastic oligoastrocytomas typically survive 2-5 years after diagnosis. The unfortunate patients with GBM, the most malignant astrocytoma, currently have a life expectancy of less than 1 year after diagnosis. In a series of 1,368 high-grade glioma patients in 10 different treatment protocols at the Mayo Clinic between 1980 and 1999, survival was statistically significantly associated with histologic subtype/grade of the tumor, patient age, patient’s cognitive performance score at time of presentation, extent of surgical resection as observed by postoperative imaging, but not treatment regimen (140). The median survival for the 21 patients with WHO grade III oligoastrocytoma was 61.4 months, 15.6 months for the 47 patients with grade IV oligoastrocytoma, 20.2 months for the 153 patients with grade III anaplastic astrocytoma, and 10.9 months for the 1,147 patients with grade IV glioblastoma multiforme. Similar relationships were observed for progression-free survival. See Kaplan-Meier plots of overall survival for these 1,368 high-grade glioma patients below (reproduced from reference 140, ©2007 Springer Science, OA3 = WHO grade III oligoastrocytoma, AA = WHO grade III anaplastic astrocytoma, OA4 = WHO grade IV oligoastrocytoma, GBM = WHO grade IV glioblastoma multiforme).
In a series of 715 GBMs treated between 1980 and 1994 in Switzerland, the median survival was 4.9 months with observed survival rates of 42% at 6 months, 18% at 1 year, and 3% at 2 years (141). Patient age at diagnosis was a major predictive factor in this cohort with a median survival of 8.8 months for patients <50 years of age compared to 4.1 months for patients >50 years of age and only 1.6 months for patients >80 years of age. Despite their distinct histories, primary GBMs (those arising de novo) and secondary GBMs (those arising from a lesser grade precursor lesion) are clinically indistinguishable as reflected by an equally poor prognosis when adjusted for patient age (141). Several other studies have described a similarly poor prognosis for high-grade glioma patients with similar prognostic factors including patient age, cognitive performance score at time of diagnosis, and extent of surgical resection (123,125,126,137,142-145). Much work has been done and is ongoing to determine the genetic alterations and treatment regimens that modulate survival of patients with malignant gliomas, which will be discussed herein.
Cytogenetic analysis is now becoming part of the diagnostic procedure for glial tumors and has proven useful in predicting patient prognosis and sensitivity to chemotherapy. Allelic loss of chromosomes 1p and 19q is a cytogenetic feature of 60-80% of oligodendrogliomas and oligoastrocytomas and is not commonly present in pure astrocytomas (146-148). The vast majority of oligodendrogliomas with loss of 1p/19q are sensitive to the chemotherapy regimen procarbazine, CCNU, and vincristine (PCV) with approximately half of these patients showing complete neuroradiological responses to PCV therapy. On the other hand, only about 25% of oligodendrogliomas with in-tact 1p/19q respond to PCV therapy, with only rare cases of complete neuroradiological response (149). Moreover, 1p/19q loss is statistically significantly associated with longer recurrence-free survival after chemotherapy (149). Other recurrent cytogenetic abnormalities have been found in both oligodendrogliomas and astrocytomas (e.g. allelic loss of chromosome 10q) but have not yet been conclusively linked to a differential patient prognosis and response to treatment (discussed later in more detail). At present, no cytogenetic tests other than assessment of 1p/19q status have yet become a routine part of the diagnostic procedure for malignant gliomas.

While PCV is the standard first-line chemotherapy regimen for oligodendrogliomas and oligoastrocytomas, the current first-line chemotherapy regimen for malignant astrocytomas including GBM is temozolomide given as a single agent. Early chemotherapy in the 1980’s and 1990’s for malignant gliomas used the highly cytotoxic nitrosourea compounds BCNU (carmustine) and CCNU (lomustine) that caused severe side effects in patients with limited success in slowing tumor progression and extending survival (150-152). Then temozolomide, a new orally available DNA alkylating agent with high CNS penetration, was introduced in the early 1990’s and
proved highly successful in slowing the growth of glioma cells *in vitro* and xenografts *in vivo* (reviewed in reference 153). Early clinical trials in patients with recurrent GBM demonstrated promising efficacy with a milder side effect profile than the traditionally used nitrosoureas (154,155). Then in 2005, a clinical trial on 573 patients with newly diagnosed, histologically confirmed GBM that were randomized to receive either radiotherapy alone or radiotherapy plus concurrent daily temozolomide demonstrated a statistically significant survival benefit of temozolomide addition (156). The median survival was 14.6 months with radiotherapy plus temozolomide compared to 12.1 months with radiotherapy alone. The corresponding two year survival rate was 26.5% with radiotherapy plus temozolomide compared to 10.4% with radiotherapy alone. See Kaplan-Meier survival plot for these 573 patients below (reproduced from reference 156, ©2005 Massachusetts Medical Society).

![Kaplan-Meier survival plot](image)

While temozolomide chemotherapy is now part of the standard of care for newly diagnosed GBM, clinical trials are currently ongoing to determine the benefit of
temozolomide in patients with grade II/III astrocytomas whose current standard of care includes only resection and radiation therapy (157).

Unfortunately, temozolomide is not a cure for GBM patients as some tumors are inherently resistant while the remaining tumors invariably develop resistance leading to disease progression/recurrence. O-6-methylguanine-DNA methyltransferase (MGMT) is the major enzyme responsible for the removal of alkylated nucleotides, the type of DNA damage caused by temozolomide. MGMT expression levels have been shown to dictate sensitivity of tumors to temozolomide, presumably due to the efficiency with which the tumor cells are able to repair the alkylated nucleotides, and these differences in expression have been attributed to differential methylation of the MGMT gene promoter (158-162). A prospective study of 206 patients with newly diagnosed GBM who were randomized to receive either radiation alone or radiation plus temozolomide following resection demonstrated significantly enhanced survival in those patients with a methylated MGMT promoter (present in 45% of the tumors) irrespective of treatment group (161). Patients harboring tumors with a methylated MGMT promoter (and thus no MGMT expression, n = 92) had a median survival of 18.2 months while those patients harboring tumors with an unmethylated MGMT promoter (n = 114) had a median survival of 12.2 months. See Kaplan-Meier survival plot below for these 206 patients grouped by methylation status of the MGMT promoter in the tumor (reproduced from reference 161, ©2005 Massachusetts Medical Society).
Additionally, when both treatment assignment and MGMT promoter methylation status were considered, the longest median survival was in those patients receiving radiation plus temozolomide with methylated MGMT promoters (21.7 months). Patients receiving radiation alone with unmethylated MGMT promoters had the shortest median survival (11.8 months). See Kaplan-Meier survival plot below for these 206 patients grouped by treatment assignment and MGMT promoter methylation status (reproduced from reference 161, ©2005 Massachusetts Medical Society).
It is thus clear that differences in DNA repair efficiency contribute to temozolomide sensitivity, and it stands to reason that alterations in other DNA repair factors active in the repair of alkylated nucleotides might be responsible for the resistance to temozolomide that ultimately develops in virtually all GBMs. Analysis of progressive/recurrent GBM tumors after first-line temozolomide therapy has revealed mutations of the mismatch repair gene MSH6, suggesting that mismatch repair deficiency is another mechanism besides upregulated MGMT expression by which these tumors develop resistance to temozolomide (163-165).

Based on the rapid disease progression, high mortality rate, and lack of effective therapy, advances in the molecular pathogenesis of GBM are desperately needed in order to develop new efficacious treatments for this devastating disease.
Molecular genetics of human glioblastoma multiforme

Genetic alterations such as genomic amplifications, deletions, point mutations, and chromosomal translocations cause the activation of oncogenes and inactivation of tumor suppressor genes that invariably drive the pathogenesis of all cancers. As such, a major focus of cancer research over the past two decades has been to identify the oncogenes and tumor suppressor genes that cause individual tumor types in an effort to better understand cancer pathogenesis and identify targets for therapeutic intervention.

Several oncogenes and tumor suppressor genes that contribute to the pathogenesis of GBM have now been discovered. These include mutational activation or amplification of the EGFR, PDGFRA, PIK3CA, and BRAF oncogenes, and mutational inactivation or deletion of the TP53, CDKN2A, RB1, PTEN, and NF1 tumor suppressor genes (reviewed in references 166 and 167). However, these known hits undoubtedly represent only a fraction of the genes that contribute to the development of these tumors. For example, the IDH1 gene was only recently discovered to be an important oncogene activated by point mutation in ~80% of low-grade gliomas and secondary GBMs. The discovery of the major known GBM oncogenes and tumor suppressor genes and the mechanism by which they drive gliomagenesis, as well as efforts to further genetically characterize GBM, are described in detail below.

The evolution of GBM genetics: early cytogenetic profiling to recent comprehensive genomic analyses

The earliest genetic research on GBM used comparative genomic hybridization (CGH) to document chromosomal gains and losses in tumor samples in the hopes of identifying genes responsible for driving gliomagenesis. Researchers looked for recurrent regions of chromosomal gain/amplification that might harbor GBM oncogenes.
and recurrent regions of chromosomal loss/deletion that might harbor GBM tumor suppressor genes. The earliest studies identified that most GBM tumors and cell lines have gain of chromosomes 7 and 20 and loss of chromosomes 1p, 6q, 9p, 10, 13, 14, 17, and 22. Indeed, several of these recurrent chromosomal gains and losses have been attributed to important GBM oncogenes and tumor suppressor genes (e.g. EGFR at chromosome 7p11.2, CDKN2A at 9p21.3, PTEN at 10q23.31, TP53 at 17p13.1, and NF1 at 17q11.2). However, the gene(s) responsible for driving other recurrent chromosomal gains and losses (e.g. chromosome 1p, 20, and 22) have not yet been identified.

New advances in microarray technology have resulted in DNA microarrays that are capable of interrogating the genomic copy number of a tumor sample at hundreds of thousands of loci on a single chip. For example, the Affymetrix Genome-Wide Human SNP 6.0 Array released in 2008 assays copy number at 1.8 million genetic markers, providing copy number data at a median resolution of 700 bp for the entire human genome in a single experiment. Such DNA microarrays are allowing cancer researchers to document for the first time the genome-wide spectrum of copy number alterations present in tumors at high resolution, including many small focal aberrations not previously appreciated by traditional CGH. Numerous recent studies have used such high-resolution SNP and CGH arrays to document copy number alterations present in GBM tumor samples (168-180). For example, one study found several regions of amplification on chromosome 7p distinct from EGFR and hypothesized that the overexpression of genes in these distinct amplicons may contribute to GBM development (169). Copy number mapping of a series of 58 gliomas of varying histologies and tumor grades revealed aberrations specific to either astrocytic or
oligodendrocytic tumors (e.g. loss of chromosome 1p and 19q in oligodendrogliomas and loss of chromosomes 10 and 22 in astrocytomas) and those specific to either low- or high-grade gliomas (e.g. gains of whole chromosomes 5 and 6 exclusively in low-grade astrocytomas) (170). Also interestingly, another study assessed 39 GBM tumors from rare patients with long-term survival (>3 years) compared to 24 tumors from typical short-term survivors. This study found that chromosome 19q loss was specific to long-term survivors while losses of 6q, 9p, and 10, and gains of 7, 19q, and 20q were more frequent in the short-term survivor group (168). Another study assessed copy number alterations present in a panel of 28 pediatric gliomas and found aberrations both unique to pediatric gliomas and in common with adult gliomas (174). All of these high-resolution genome-wide copy number studies are identifying new candidate cancer genes at an unprecedented rate, but the exact importance of these genes to GBM pathogenesis remains to be seen.

Additionally, new advances in capillary high-throughput sequencing technology have enabled the sequencing of the human genome and are enabling sequencing of entire cancer genomes now as well. In September 2008, Parsons et al. reported the comprehensive sequencing of every coding exon for all 20,661 of the well annotated protein coding genes in the human genome in a panel of 22 GBM primary tumors and xenografts. They identified 685 genes (3.3% of the genes analyzed) with at least one non-silent somatic mutation, and an average of 47 non-silent somatic mutations per tumor. The 21 genes with somatic mutations identified at a frequency >10 mutations per Mb of tumor DNA sequenced were then sequenced in a panel of 83 additional GBM samples. This analysis defined the frequency of somatic mutations in known GBM oncogenes and tumor suppressor genes such as EGFR, PTEN, NF1, RB1, and PIK3CA,
and also led to the discovery of two novel genes altered by point mutation at high frequency in GBM – IDH1 and PIK3R1 (119).

The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/) is a joint effort of the National Cancer Institute and the National Human Genome Research Institute of the National Institutes of Health initiated in 2006 to accelerate understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing. As a culmination of their pilot project using glioblastoma multiforme (the first tumor type TCGA chose to study), TCGA reported in September 2008 the genome-wide copy number analysis of 206 GBM primary tumors and sequencing of the coding exons of 600 selected genes in 91 of these GBM tumors. Their comprehensive genomic characterization of this large panel of GBM primary tumors enabled for the first time an unbiased and accurate measure of the frequency of genes known to be altered either by copy number alteration or point mutation in GBM. This analysis identified three core pathways in GBM (p53 signaling, Rb signaling, and receptor tyrosine kinase/Ras/PI3-kinase signaling) with >80% of tumors having genetic alterations in at least component of each of these three signaling pathways. Furthermore, they discovered frequent somatic mutations of ERBB2 and PIK3R1, genes not previously recognized to be altered in GBM (181).

The advent of “next generation” massively parallel DNA sequencing is now facilitating rapid whole genome sequencing of cancer and is leading to the identification of the complete catalog of mutations, breakpoints, copy number aberrations, and other chromosomal rearrangements in individual tumor samples (182-188). Next generation sequencing technology has most recently allowed the complete genome sequencing of a GBM cell line (U87MG) with >30x coverage that identified 35 interchromosomal
translocation events, 1,315 structural variations (>100 bp), 191,743 small insertions and deletions (<21 bp), and 2,384,470 single nucleotide variations (189). Efforts are underway to comprehensively sequence several GBM primary tumors and paired normals.

**Genetic deregulation of the cdk4/6-cyclin D-INK4-Rb pathway in GBM**

Uncontrolled cellular proliferation is the fundamental process driving cancer. Hence, it is not surprising that genes encoding cell cycle regulatory factors are among the most commonly altered in cancer (reviewed in reference 190). Genes encoding cyclins and cyclin-dependent kinases (cdks) that phosphorylate/inactivate the retinoblastoma susceptibility protein (Rb), the master regulator of cell cycle progression, are frequently amplified and/or overexpressed in most tumor types. Additionally, genes encoding cdk inhibitors and the RB1 gene encoding Rb are commonly deleted and/or mutationally inactivated in most tumor types. The discovery of genetic alterations of cell cycle regulators in GBM and the functional consequences of such alterations will be discussed herein.

The CDKN2A gene at chromosome 9p21.3 encodes the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup>, the prototype member of the INK4 family of cdk4/6 inhibitors that also includes p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup> (191). Shortly after its cloning in 1993, it was discovered that homozygous deletions of the CDKN2A gene were present in the majority of human cancers including GBM, resulting in a lack of p16<sup>INK4a</sup> expression. Furthermore, somatic mutations including nonsense and frameshift mutations accompanied by loss of heterozygosity were present in a fraction of tumors including GBMs without deletions (192-196). Subsequent analysis also found that p16<sup>INK4a</sup> was silenced by promoter methylation in a fraction of gliomas without deletions and mutations.
(197-199). Re-expression of the wild-type $p16^{\text{INK4a}}$ cDNA (but not tumor-derived mutants) in GBM cells with deletions and mutations led to hypophosphorylation of the Rb protein and G1 cell cycle arrest (200,201). Together, these findings clearly defined CDKN2A as a tumor suppressor gene commonly inactivated in GBM, resulting in deregulated cell cycle progression.

The CDKN2B gene, encoding the $p16^{\text{INK4a}}$ homolog $p15^{\text{INK4b}}$, is located at chromosome 9p21.3 just 7 Kb centromeric to the CDKN2A gene. CDKN2B is often, but not always, co-deleted with CDKN2A in cancers including GBM, suggesting that $p15^{\text{INK4b}}$ also has a tumor suppressive function (194-196). However, only a couple human tumors without CDKN2B deletion have ever been found to harbor somatic mutations (195,202). One study has shown that CDKN2B can be inactivated in gliomas without deletions by promoter methylation, independent of CDKN2A inactivation (203). Re-expression of $p15^{\text{INK4b}}$ in GBM cells with deletions induces a G1 cell cycle arrest similar to $p16^{\text{INK4a}}$, demonstrating that $p15^{\text{INK4b}}$ loss does contribute to deregulated cellular proliferation in GBM (202). $p16^{\text{INK4a}}$ and $p15^{\text{INK4b}}$ are thought to have redundant functions in limiting cell cycle progression through inhibition of cdk4/6, therefore resulting in selective pressure for co-deletion of these two genes so close in proximity on chromosome 9p.

In GBMs without loss of $p16^{\text{INK4a}}$, there is often overexpression of cdk4, cdk6, and/or D-type cyclins. Amplification of the CDK4 gene on chromosome 12q14.1 has been found in 10-15% of GBMs leading to its overexpression, occurring exclusively in tumors without deletions of CDKN2A (204-206). Amplification of the CDK6 and CCND1, CCND2, and CCND3 genes encoding the three known D-type cyclins have all been found in human GBM samples at low frequency (207,208).
The RB1 gene was cloned in 1986 as the genetic locus at chromosome 13q14.2 responsible for familial retinoblastoma (209,210). Assessment of this gene demonstrated homozygous deletions, somatic mutations, and inactivation by viral oncoproteins (e.g. T-antigen of SV40 virus, E7 of human papillomavirus) in several other human cancers including osteosarcoma, non-small cell lung cancer, and cervical cancer (reviewed in reference 211). Injection of purified Rb protein or retroviral mediated gene transfer of RB1 into retinoblastoma cells with endogenous gene inactivation produced a G1 cell cycle arrest and reduced cell growth in soft agar and tumorigenicity in nude mice (212,213). Alterations of the RB1 gene in astrocytomas were first reported in 1994 by Henson et al., wherein somatic mutations in 4/54 high-grade tumors but 0/12 low-grade gliomas was reported (214). It was subsequently determined that deletions and mutations of RB1 are present in 10-15% of GBMs, and that virtually all GBM tumors harbor alterations in either CDKN2A, CDK4, or RB1 (119,215-217). Restoration of Rb expression in GBM was found to cause G1 cell cycle arrest and blocked tumorigenicity in nude mice (201,218).

Comprehensive genetic analysis of the cdk4/6-cyclin D-INK4-Rb pathway in 91 GBM primary tumors by The Cancer Genome Atlas revealed genetic alteration in at least one component of this pathway in 80% of samples. Homozygous deletions of CDKN2A were present in 44/91 GBMs (48%), and biallelic/homozygous point mutations of CDKN2A were found in 3/91 GBMs. Their copy number analysis on a panel of 206 GBMs revealed homozygous deletion of CDKN2A in 115 tumors (56%), and CDKN2B was co-deleted with CDKN2A in all but 5/115 of these tumors. No mutations of CDKN2B were found. Genetic alterations of RB1 were present in 10/91 GBMs (11%) including 1 homozygous deletion, 1 heterozygous point mutation, and 8 biallelic point mutations.
6/206 tumors (3%) had homozygous deletions of RB1. Additionally, 29/206 tumors (14%) had CDK4 amplification, 3/206 tumors (1%) had CDK6 amplification, and 5/206 tumors (2%) had CCND2 amplification. No point mutations in CDK4, CDK6, or CCND1/2/3 genes were found in any of the 91 GBM samples. Interestingly, CDK4/6 and CCND1/2/3 genes were often found to be co-amplified in tumors. For example, 4 out of the 5 tumors with CCND2 amplification also harbored amplifications of either CDK4 or CDK6. Additionally, it was observed that amplifications of CDK4/6-CCND2 genes, genetic lesions of CDKN2A, and genetic lesions of RB1 were mutually exclusive in the panel of 91 GBM tumors. There were 16 tumors with CDK4/6-CCND2 amplifications, 47 tumors with genetic lesions of CDKN2A, 10 tumors with genetic lesions of RB1. Together, 73/91 tumors (80%) had an alteration of either CDK4/6-CCND2, CDKN2A, or RB1, demonstrating the fundamental importance of deregulation of the cdk4/6-cyclin D-INK4-Rb pathway in GBM. It was speculated that this frequency could have been confounded by the use of primary tumors which contain admixed non-neoplastic cells (e.g. endothelium, lymphocytes, etc.) and intratumoral heterogeneity that could have masked the identification of genetic lesions in the remaining 20% of samples (181). It is generally appreciated that lesions of this cell cycle regulatory pathway are virtually ubiquitous in GBM tumors and perhaps obligatory to gliomagenesis (219, presented in this thesis). Furthermore, no genetic alterations in other cell cycle regulatory genes were found in GBM by Parsons et al. or The Cancer Genome Atlas panel of GBMs including CDKN1B (encoding p27\textsuperscript{kip1}), CDKN1C (encoding p57\textsuperscript{kip2}), CDK2, CCNA2 (encoding the major cyclin A), CCNB1 (encoding cyclin B), and CCNE1 (encoding cyclin E) (119,181). This highlights that specific deregulation of the cdk4/6-cyclin D-INK4-Rb pathway, and not just generalized cell cycle disruption, drives GBM pathogenesis.
Additionally, it was recently discovered that the CDKN2C gene at chromosome 1p32.3, encoding the \( p16^{INK4a} \) homolog \( p18^{INK4c} \), is genetically altered in a significant fraction of GBM samples (220, presented in this thesis). Solomon et al. reported frequent deletion of CDKN2C in GBM cell lines and xenografts and at a smaller frequency in primary tumors (220). Reconstitution of \( p18^{INK4c} \) expression in GBM cells with CDKN2C deletions resulted in G1 cell cycle arrest and cellular senescence, confirming a growth suppressive role of \( p18^{INK4c} \) in GBM. Shortly thereafter, Wiedemeyer et al. reported the similar identification of CDKN2C deletions in GBM samples and additionally identified two CDKN2C point mutations that blocked the interaction of \( p18^{INK4c} \) with cdk4/6 and inhibited its growth suppression when expressed in GBM cells with CDKN2C deletions (221). The Cancer Genome Atlas found homozygous deletion of CDKN2C in 7/206 GBMs (3%) but no point mutations in 91 GBMs (181). To date, deletions of CDKN2C have been found exclusively in GBM tumors harboring other lesions of the cdk4/6-cyclin D-INK4-Rb pathway. Solomon et al. reported the identification of 4 GBM cell lines and 3 xenografts with homozygous deletions of CDKN2C, all of which also harbored homozygous deletions of CDKN2A/B (220). In the 7 GBM primary tumors TCGA identified with homozygous deletions of CDKN2C, 6 also harbored homozygous deletion of CDKN2A/B and the other tumor harbored amplification of CDK4 (181). This observation has led to the hypothesis that \( p16^{INK4a} \) and \( p18^{INK4c} \) have non-redundant functions in constraining gliomagenesis and that \( p18^{INK4c} \) putatively functions as a tumor suppressor through a unique and as yet unidentified mechanism(s) (219).

Together, these findings have led to a model in which normal, non-transformed astrocytes and other cells in the brain have in-tact Rb and INK4 proteins (e.g. \( p16^{INK4a} \),
p15\(^{\text{INK4b}}\), and p18\(^{\text{INK4c}}\) and a limited quantity of cdk4/6 and D-type cyclins, thereby maintaining Rb in an active hypophosphorylated state that limits cell cycle progression. In contrast, GBM cells have loss of INK4 proteins and amplification/overexpression of cdk4/6 and D-type cyclins that together lead to hyperphosphorylated/inactive Rb and uncontrolled cell cycle progression (see model below).

Based on the genetic data that have demonstrated ubiquitous lesions of this pathway in GBM and functional evidence demonstrating a requirement of this pathway deregulation for tumor growth, therapeutics that specifically target the cdk4/6-cyclin D-INK4-Rb pathway are likely to be highly successful in the treatment of GBM.

**Genetic inactivation of TP53 function in GBM**

The TP53 gene encodes the tumor suppressor protein p53 often described as the “guardian of the genome” and is one of the most frequently somatically altered genes
in human cancer (reviewed in reference 222). p53 is activated in response to a broad range of cellular stress including DNA damage, telomere erosion, osmotic shock, hypoxia, reactive oxygen species, ribonucleotide depletion, and oncogene expression. Macromolecular complexes in the cell responsible for detection of such stresses (e.g. the Mre11/Rad50/Nbs1 complex that recognizes DNA double-strand breaks) activate checkpoint kinases (e.g. Chk2, ATR, ATM) that phosphorylate p53 on its N-terminus. This phosphorylation causes a dramatic increase in p53 stability/accumulation and also a conformational change that induces its transcriptional regulatory activity. p53 modulates the expression of genes that control DNA damage repair, cell cycle arrest, and apoptosis, thereby dictating the appropriate response to the cellular stress. One transcriptional target of p53 is the CDKN1A gene encoding p21WAF1/Cip1, a protein that functions as a potent cyclin-dependent kinase inhibitor and induces cellular senescence.

p53 was originally isolated in 1984 as a cellular target of viral oncoproteins (e.g. T-antigen of SV40 virus, E6 of human papillomaviruses) (223). Researchers investigating colorectal tumors in the 1980’s found frequent allelic loss and chromosomal deletions on the short arm of chromosome 17. Then in 1989 these losses and deletions were eventually narrowed to a region on 17p13.1 that included the TP53 gene, and sequencing of TP53 in two colorectal tumors with allelic loss found homozygous missense mutations in both samples that were not present in constitutional DNA from blood from these patients (224). Subsequent sequencing of more colorectal tumors both with and without 17p allelic loss revealed that somatic TP53 mutations are present in the majority of these tumors as well as >50% of every major human tumor type (225,226). The solitary exception is cervical cancer, in which p53 function is disrupted by the E6 oncoprotein of HPV. Notably, germline mutations of TP53 are known to cause the Li-
Fraumeni cancer predisposition syndrome with a significant fraction (>10%) of affected individuals developing malignant astrocytomas including GBM during their lifetimes as previously discussed. Interestingly, TP53 is most commonly inactivated by missense mutation (>90% of identified lesions) and much less commonly by homozygous deletion or truncating mutation (i.e. nonsense, frameshift, and splice-site mutations). Mutations in TP53 frequently cluster in the central DNA binding domain of the protein, thereby disrupting the ability of p53 to regulate transcription of genes controlling DNA damage repair, cell cycle arrest, and apoptosis. It is currently unclear why TP53 is most commonly inactivated by missense mutation while other tumor suppressor genes are generally inactivated by a spectrum of genetic lesions.

Nigro et al. reported the first identification of TP53 mutations in human brain tumors wherein 5 GBMs were found to harbor somatic missense mutations (225). Mashiyama et al. later reported TP53 mutations in both anaplastic astrocytomas and GBMs (227). One early study found TP53 mutations in 7/25 GBMs (28%), 5/14 anaplastic astrocytomas (36%), and 0/6 low-grade astrocytomas (228), while another study reported mutations in 13/33 GBMs (39%), 1/3 anaplastic astrocytomas (33%), and 2/4 gliosarcomas (50%) (229). It is now clear that TP53 mutations are present in 25-40% of all malignant astrocytomas from low-grade astrocytoma to GBM, indicating that TP53 inactivation is an early event in gliomagenesis (230,231). However, TP53 has been documented to play a more limited role in non-astrocytic brain tumors, as one study reported mutations in only 2/17 oligodendrogliomas, 2/19 medulloblastomas, and 0/15 ependymomas (232), findings that were corroborated by subsequent reports (230). Most recently, Parsons et al. sequencing analysis found somatic TP53 mutations in 37/105 GBMs (35%) (119). The Cancer Genome Atlas reported genetic lesions of TP53
in 32/91 GBMs (35%) that include 1 tumor with homozygous deletion, 13 tumors with heterozygous point mutations, and 18 tumors with biallelic mutations (181). Several studies have documented that TP53 status modulates the properties of GBM cells \textit{in vitro} (e.g. sensitivity to temozolomide, response to radiation, and growth properties/tumorigenicity) (233-236). However, no significant correlation between TP53 status and survival of GBM patients has been found (141).

In addition to alterations of TP53, genetic lesions in factors that regulate p53 function are sometimes present in human cancer and are an alternate means of disrupting this critical tumor suppressor in the presence of wild-type TP53. The MDM2 and MDM4 genes encode proteins that bind to p53 and block its ability to interact with DNA. The Mdm2 and Mdm4 proteins also have E3 ubiquitin ligase activity that targets p53 for proteasomal degradation in the absence of cellular stress. The same checkpoint kinases that phosphorylate and activate p53 after cellular stress also phosphorylate Mdm2 and block its interaction with p53, thereby allowing p53 to bind DNA regulatory sequences and govern the stress response. Somatic point mutations of MDM2 and MDM4 are not commonly present in human cancer, but genomic amplification leading to dramatic overexpression and aberrant p53 sequestration/degradation is observed in certain tumor types including gliomas (237-241). The Cancer Genome Atlas found only 1 point mutation of MDM2 in 91 GBMs but found genomic amplification in 22/206 GBMs (11%). Additionally, they reported 1 point mutation of MDM4 in 91 GBMs, and genomic amplification in 9/206 GBMs (4%). It was observed that genetic alterations in TP53, MDM2, and MDM4 were mutually exclusive in the panel of 91 GBM tumors (\textit{i.e.} tumors with MDM2 amplifications did not harbor TP53 alterations and vice versa). In the panel of 91 GBMs, there were 13 tumors with MDM2 lesions, 6 tumors with MDM4 lesions,
and 32 tumors with TP53 lesions. Together, 50/91 GBMs (55%) had an alteration of either TP53, MDM2, or MDM4, demonstrating that disruption of p53 signaling is critical to the pathogenesis of GBM (181).

Shortly after the discovery of the CDKN2A gene that encodes the cdk inhibitor p16\textsuperscript{ink4a}, it was appreciated that this locus encodes another protein called p14\textsuperscript{arf}, which utilizes a unique exon and shares exon 1 with p16\textsuperscript{ink4a} but is translated in an alternate reading frame (242,243). Given the frequent homozygous deletion and mutation of the CDKN2A locus in cancer, it was speculated that p14\textsuperscript{arf} might have tumor suppressive function as well. Restoration of p14\textsuperscript{arf} expression in cells with CDKN2A deletions was found to cause cell cycle arrest both in G1 and G2 phases dependent on intact p53, but not intact Rb (244,245). Interestingly though, mutations of the CDKN2A locus that alter both p16\textsuperscript{ink4a} and p14\textsuperscript{arf} amino acid sequences were found to disrupt p16\textsuperscript{ink4a} growth suppressive function but did not interfere with growth suppression induced by p14\textsuperscript{arf} (245,246). Thus, it appears that homozygous deletion is the predominant mechanism of p14\textsuperscript{arf} inactivation in cancer, including GBM. It is likely that the presence of p14\textsuperscript{arf} at the same locus as the p16\textsuperscript{ink4a} tumor suppressor enhances selective pressure for homozygous deletions that inactivate both transcripts, rather than point mutations which inactivate only one of the two tumor suppressive transcripts. Further analysis into the mechanism of p14\textsuperscript{arf} action has determined that following cellular stress, it binds and sequesters Mdm2 in the nucleolus thereby promoting p53 release and transcriptional regulatory activity. In tumor cells with deletion of CDKN2A, the lack of p14\textsuperscript{arf} does not allow efficient p53 release by Mdm2, impeding the appropriate checkpoint or apoptotic response to the induced stress (reviewed in reference 247). As described previously, The Cancer Genome Atlas identified homozygous deletions of the CDKN2A locus in
115/206 GBMs (56%). These deletions occurred both in cells with and without TP53/MDM2/MDM4 alterations, suggesting that while p14ARF deletion is an alternate mechanism of p53 inactivation in some tumors, CDKN2A deletions in the other tumors harboring TP53/MDM2/MDM4 lesions were selected for during tumorigenesis as a means of inactivating p16INK4a and not p14ARF/p53 (181).

Genetic activation of EGFR, PDGFRA, and other receptor tyrosine kinases in GBM

Receptor tyrosine kinases (RTKs) are transmembrane receptors with extracellular ligand binding domains and intracellular tyrosine kinase domains that modulate signal transduction pathways in response to growth factor or cytokine stimulation. Binding of ligand normally produces either homo- or hetero-dimerization of receptor monomers causing autophosphorylation of tyrosine residues on the intracellular kinase domain, which creates binding sites for adaptor proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Binding of such adaptor proteins (e.g. Grb2, Shc, Src, IRS-1) to phospho-tyrosine residues on RTKs induces their phosphorylation/activation and leads to activation of intracellular signaling cascades including the Ras-Raf-Mek-Erk (MAP kinase), PI3K-PDK-Akt-mTOR (PI3-kinase), and PLC-DAG-PKC (protein kinase C) pathways. These pathways modulate transcription, translation, and cytoskeletal reorganization through various effectors (e.g. Elk1, NF-KB, c-Myc, c-Jun, c-Fos, Rac1/Rho/Cdc42) that drive cellular survival, proliferation, and motility in response to the initial stimulation of RTKs by ligand. Signaling through RTKs is frequently deregulated in cancer through various mechanisms (e.g. overexpression of ligand, point mutations that constitutively activate RTKs, etc.) leading to the inappropriate cellular survival, proliferation, and invasion that drive human tumorigenesis.
The EGFR gene, which encodes one such RTK (the epidermal growth factor receptor) at chromosome 7p11.2, is the most frequently altered proto-oncogene in GBM identified to date. EGFR was originally identified as the cellular homolog of the v-erbB oncogene found in the acutely transforming avian erythroblastosis virus (248). Genomic amplification of the EGFR gene resulting in overexpression was first reported in GBM primary tumors in 1984 by Libermann et al. (249). EGFR amplifications have since been reported to occur in 40-50% of all GBMs, but are rarely present in anaplastic astrocytomatas and absent in low-grade astrocytomatas (250,251). Interestingly, GBM tumors with EGFR amplifications lose these amplifications when dissociated and cultured in vitro, but do maintain the amplifications when grown as xenografts either subcutaneously or intracranially (252-254). This observation has suggested that EGFR amplification/overexpression is required for in vivo growth possibly through promoting angiogenesis/vascular growth in the tumor or otherwise maintaining an appropriate tumor microenvironment, but is not required for (or is even selected against during) in vitro growth on plastic.

Analysis of GBM tumors with EGFR amplification frequently revealed rearrangement of the amplified alleles, suggesting that rearrangements might be oncogenic (249,251). Sugawa et al. discovered that an in-frame deletion variant lacking exons 2-7 encoding the ligand binding domain (termed EGFRvIII) was recurrent among several of these tumors (255). Expression of this variant EGFRvIII has since been reported in 20-30% of GBMs and >50% of those harboring EGFR amplification (256,257). It has been determined that EGFRvIII is a constitutively active, ligand-independent variant of EGFR that confers enhanced proliferation, survival, and tumorigenicity (258-261). Notably, the fusion junction in EGFRvIII generates a unique
tumor-specific epitope not present in normal cells. An early study immunized a rabbit with a peptide derived from the fusion junction to produce antibodies that specifically recognize EGFRvIII protein (262). An immunotoxin generated from these antibodies, as well as a ribozyme and an siRNA targeting the junction sequence, have all been shown to suppress the growth of GBM cells and tumors expressing EGFRvIII (263-266).

In addition to the EGFRvIII variant, missense mutations of EGFR have also been identified in GBM tumors that cause increased receptor activation and signaling downstream. While mutations of the tyrosine kinase domain are present in 10-20% of lung cancers as well as at lower frequency in prostate, thyroid, pharyngeal, and other cancers, tyrosine kinase domain mutations have not been found in GBM (267-269). Rather, sequencing of EGFR has revealed the presence of missense mutations in the extracellular portion of the protein, first reported in 3/66 GBM tumors which all harbored somatic changes in the second extracellular cysteine-rich domain (257). A subsequent study detected missense mutations in 19/132 GBMs (14%), 18/19 of which occurred in the extracellular ligand binding or cysteine-rich domains of EGFR (the other single mutation was present in the intracellular kinase domain). These extracellular mutations were associated with increased EGFR gene dosage and conferred tumorigenicity to NIH3T3 cells (270). Interestingly, extracellular mutations appear to be unique to GBM as such extracellular mutations are not present in >30,000 non-CNS tumors reported in the COSMIC database of the Cancer Genome Project of the Wellcome Trust Sanger Institute (271). Most recently, Parsons et al. found somatic mutations of EGFR in 15/105 GBMs (14%) (119). In the recent analysis by The Cancer Genome Atlas, genetic alterations of EGFR were present in 41/91 GBMs (45%) including 26 tumors with amplification, 3 tumors with point mutations, and 12 tumors with both amplification and
point mutations. Copy number analysis in the larger panel of 206 GBMs revealed amplification in 88 tumors (43%). Assessment of EGFRvIII was not included in this analysis (181). Together, these studies have defined EGFR as one of the most commonly altered genes in GBM and, as such, one of the most promising molecular targets for therapeutic intervention (discussed later in more detail).

In addition to EGFR, the PDGFRA gene encodes another RTK (the platelet-derived growth factor receptor α) at chromosome 4q12 that is also frequently altered in GBM. Early analysis determined that PDGFRα protein and its ligands PDGF-A and PDGF-B are expressed in gliomas, with highest expression in high-grade tumors, while PDGFRβ is strongly expressed in proliferating endothelial cells in GBMs (272,273). These findings led to speculation that a PDGF autocrine loop is important for tumor growth and angiogenesis in gliomas (274,275). The first somatic alteration of the PDGFRA proto-oncogene described in human cancer was in a GBM in which a 2,100 bp genomic deletion encompassing exons 8-9 was identified causing an 81 amino acid in-frame deletion in the extracellular ligand binding domain of PDGFRα (276). This deletion mutant, similar to EGFRvIII, was subsequently found to be constitutively phosphorylated/active even in the absence of ligand and induced transformation when expressed in immortalized cells (277). The PDGFRA was later found to amplified and overexpressed in GBM. One study found PDGFRA amplification in 4/50 GBMs (8%) and high levels of expression in 4/17 tumors analyzed, including those with amplifications (278). Another study found amplifications in 12/43 primary GBMs (29%) (279). One recent study sequenced 20 receptor tyrosine kinase genes in a panel of 19 GBMs with matched normal and found one sample that harbored a somatic 2 bp deletion at codon 1048 of the PDGFRA gene causing a frameshift and loss of the C-terminal tail
(amino acids 1049-1089) but not affecting the kinase domains of the encoded protein (280). The Cancer Genome Atlas copy number analysis found amplification of PDGFRA in 22/206 tumors (11%), one of which also harbored a somatic point mutation (heterozygous G>C change causing W349C change in the extracellular ligand binding domain). Interestingly, PDGRFA amplification was not mutually exclusive with EGFR alterations as 6 of these 22 tumors had amplification of both EGFR and PDGFRA, demonstrating selective pressure for activation of multiple RTK signaling cascades in a single tumor (181). Targeting GBM cells and xenografts with high levels of PDGFRα activity via dominant-negative mutants or a monoclonal antibody has been demonstrated to suppress growth in vitro and in vivo (281,282). The small molecule imatinib that selectively inhibits PDGFRα in addition to a couple of other kinases (discussed in more detail later) has been shown to inhibit GBM growth both in primary cultures and intracranial xenografts (283,284). Together, these findings reveal that PDGFRα is an important RTK for GBM development or progression and is a potential therapeutic target in the 10-15% of GBM patients with PDGFRA alterations.

Besides EGFR and PDGFRA, genetic alterations in other RTKs have been found in GBM, albeit at much lower frequencies. The MET gene at chromosome 7q31.2 encodes the mesenchymal-epithelial transitional factor (c-Met) also called the hepatocyte growth factor (HGF) or scatter factor (SF) receptor. Germline mutations in the MET gene were identified in 1997 as the cause of hereditary papillary renal carcinoma, and somatic mutations were found to be present in the vast majority of sporadic cases as well (285). MET has since been appreciated as a proto-oncogene activated in various human cancers, particularly childhood hepatocellular carcinoma, through genomic amplification, overexpression, and somatic point mutations (286,
reviewed in reference 287). Analysis of small panels of human gliomas and GBMs has revealed individual cases of MET gene amplification and one GBM with a homozygous missense mutation in the tyrosine kinase domain (288-290). The recent TCGA analysis identified 6/206 GBM tumors (3%) with amplification and 2/91 tumors (2%) with point mutations (181). Despite infrequent amplification and mutation, c-Met is frequently overexpressed in GBM tumors and has been found to promote both proliferation and invasion of GBM cells (291-293). In one study, immunohistochemical analysis of c-Met expression in a panel of 62 GBMs revealed overexpression in 18/62 cases (29%). GBMs overexpressing c-Met in this panel were statistically significantly associated with a more invasive, multifocal phenotype and a shorter median survival (11.7 months) compared to the 44 tumors with little or no detectable c-Met protein (14.3 months; reference 294). RNA interference, monoclonal antibodies, and specific small molecule inhibitors of c-Met have all been found to slow tumor growth and induce apoptosis of GBM cells and xenografts (295-299). Together, these findings indicate that MET activation occurs in a small fraction of GBMs, and the c-Met signaling pathway is a potential therapeutic target in GBM patients.

The FGFR1 gene at chromosome 8p11 encodes another RTK, the fibroblast growth factor receptor-1, that is genetically altered in human cancer. Translocations resulting in fusion of the FGFR1 gene with multiple other gene loci produce fusion proteins with constitutively active tyrosine kinase activity that are present in various hematologic malignancies (300-303). Additionally, amplifications of the FGFR1 gene are found in approximately 10% of breast cancers and at lower frequency in a number of other tumor types, and somatic mutations in the kinase domain have been found in a breast and a colorectal tumor (304-307). High levels of FGFR1 expression in human
GBM tumor samples have been documented by immunohistochemistry with low or no expression observed in endothelial cells of the tumor capillaries, adjacent normal brain tissue, and white matter from non-neoplastic brains (308-310). Sequencing of 20 RTK genes in 19 GBM samples revealed two tumors with heterozygous missense mutations in the tyrosine kinase domain of FGFR1 (280). The recent TCGA analysis of 91 GBMs found one tumor with a heterozygous missense mutation in the kinase domain but did not find any amplifications in 206 tumors (181). RNA interference of FGFR1, a monoclonal antibody to FGFR1, and a small molecule inhibitor of FGF receptors have all been found to slow tumor growth and induce apoptosis of GBM cells and xenografts, suggesting that FGFR1 could be another useful therapeutic target in GBM (311-314).

The ERBB2 gene at chromosome 17q12 encodes another RTK which is amplified/overexpressed in up to 20% of breast cancers and at lower frequency in gastric, esophageal, ovarian, and lung cancers (315). Additionally, somatic point mutations or small in-frame insertions have been found in the kinase domain of ERBB2 in up to 5% of these tumors as well (316). No such amplifications or mutations had ever been described in GBM – an analysis of 47 gliomas including 37 GBMs found no focal amplifications of ERBB2 (317). The Cancer Genome Atlas copy number analysis of 206 GBMs found no tumors with ERBB2 amplification. However, they did find 11 somatic mutations in 7/91 GBMs (8%) including 3 point mutations in the kinase domain and 8 mutations in the extracellular domain of the protein, uncovering a potentially important role of this proto-oncogene in gliomagenesis (181).

**Genetic activation of the PI3K-Akt signaling pathway in GBM**

The phosphatidylinositol 3'-kinase (PI3K)-protein kinase B (Akt) signaling pathway is now appreciated as one of the most commonly altered pathways in human
cancer and is essential for promoting cancer cell proliferation, survival, and invasion. Genetic alterations in at least one component of this signaling pathway are present in the vast majority of GBM tumors as described in detail below, and this pathway thus represents a promising target for the development of targeted therapeutics with great potential to inhibit GBM progression.

One of the most commonly altered genes in GBM and the most frequently altered component of the PI3K-Akt signaling pathway in human cancer is the PTEN tumor suppressor gene. Early cytogenetic investigation of GBM tumors and cell lines identified frequent chromosomal loss and LOH of chromosome 10, and further analysis eventually mapped a minimal region of recurrent LOH and homozygous deletions to the long arm at chromosome 10q23.3 where the PTEN gene resides (318-320). Then in 1997, two independent groups simultaneously reported the identification of mutations of the PTEN gene in multiple human cancer cell lines and primary tumors of several tumor types including GBM (321,322). The mutations identified in these reports included nonsense and splice-site mutations as well as small insertions/deletions causing frameshifts, all inactivating mutations that confirm the theory that PTEN activity is required to suppress tumorigenesis. Several subsequent studies confirmed the frequent inactivation of PTEN in GBM tumor samples by homozygous deletion and somatic point mutations (323-326). For example, Wang et al. sequenced the PTEN gene in 34 tumor-normal pairs and identified 4 homozygous deletions and 15 missense, nonsense, or frameshift mutations, which together accounted for PTEN inactivation in 56% of their GBM primary tumors (323). Another study reported that PTEN mutations were found only in high-grade but not low-grade gliomas, suggesting that PTEN inactivation occurs during the progression (and not the initiation) of gliomagenesis (324). Most recently, Parsons et al. genomic
analysis of 105 GBM tumor samples found somatic mutations in 27 samples (26% frequency), and The Cancer Genome Atlas identified 33 samples with homozygous deletions or somatic mutations in their panel of 91 GBM primary tumors (36% frequency) (119,181).

After the discovery of frequent PTEN genetic alterations in human cancer, numerous groups worked to determine how PTEN inactivation contributes to tumorigenesis. The PTEN protein was found to have significant homology to the actin binding protein Tensin and to possess dual-specificity protein phosphatase activity (i.e. the ability to dephosphorylate both tyrosine and serine/threonine residues) (327,328). PTEN was later appreciated to also possess 3’ phosphoinositol phosphatase activity (329). Restoration of PTEN expression in GBM cells with endogenous PTEN mutations caused a G1 cell cycle arrest and reduced their tumorigenic potential (330,331). PTEN re-expression was also found to inhibit cancer cell migration, spreading, and focal adhesions (332). Furthermore, the catalytic phosphatase domain was required for these growth suppressive effects, but the downstream target(s) of dephosphorylation was still unknown (333,334). It was subsequently determined that GBM cells with inactivated PTEN have high levels of Akt activity, and it was postulated that PTEN might function as a tumor suppressor through negative regulation of PI3K-dependent Akt signaling (335). Indeed, restoration of PTEN expression in cells with endogenous PTEN lesions led to decreased Akt activation, an effect dependent on its lipid phosphatase activity (336-339). It is now clear that PTEN is a critical regulator of the levels of membrane-associated phosphatidylinositol which signal through 3-phosphoinositide-dependent protein kinase-1 (PDK1) to activate Akt and promote cellular proliferation, survival, adhesion, and
survival through various effectors including mTOR and the FOXO transcription factors (reviewed in references 340 and 341).

As PTEN inactivation leads to increased phosphatidylinositol levels that promote tumorigenesis, it was hypothesized that PI3-kinase activation might occur in tumors without PTEN lesions as an alternate means of increasing phosphatidylinositols levels. Indeed, PI3-kinases are activated by genetic alterations in human cancer as first described by Samuels et al. in 2004 (342). Therein, the identification of frequent somatic mutations of the PIK3CA gene in multiple human tumor types was reported, including 4/15 GBM samples studied (27% frequency). The mutations identified clustered in the C2 helical and kinase domains of PIK3CA, which encodes the p110α catalytic subunit of the PI3Kα complex (342). The clustering of mutations discovered in PIK3CA was similar to that observed for activating mutations in oncogenes including KRAS, BRAF, and CTNNB1. Biochemical and genetic studies demonstrated that these PIK3CA mutations are oncogenic causing increased lipid kinase activity (343,344). Further genetic studies demonstrated that PIK3CA mutations are indeed very prevalent in diverse human cancer types including both pediatric and adult GBM as well as other gliomas and medulloblastomas (345-347). Most recently, Parsons et al. genomic analysis of 105 GBM tumor samples found somatic point mutations in 10 samples (10% frequency), and The Cancer Genome Atlas identified 6 samples with somatic mutations in their panel of 91 GBM primary tumors (119,181). Copy number analysis on a larger panel of 206 GBM primary tumors by The Cancer Genome Atlas found 4 samples with focal amplifications of the PIK3CA gene (181). Together, these studies have established PIK3CA as an important oncogene activated by genomic amplification and somatic point mutation in 5-10% of GBMs.
In addition to PIK3CA, other PI3-kinase subunits have recently been identified to harbor genetic alterations in human cancers including GBM. Mizoguchi et al. discovered a somatic small intragenic deletion in the PIK3R1 gene in a GBM tumor sample, resulting in a truncation of the encoded p85α protein (348). p85α is the regulatory subunit that together with catalytic p110α subunit encoded by PIK3CA form the class 1 PI3Kα complex. This GBM tumor harboring mutated PI3KR1 had both wild-type PIK3CA and PTEN and was found to have activated Akt, suggesting that genetic inactivation of the regulatory subunit is an additional mechanism of PI3K-Akt pathway activation in human GBM. Genomic analysis by Parsons et al. identified somatic mutations of the PIK3R1 gene in 8/105 GBM tumor samples (8% frequency) that included inactivating frameshift and splice-site alterations (119). Similarly, The Cancer Genome Atlas identified 9 somatic mutations of the PI3KR1 gene in their 91 GBM primary tumors (10% frequency) (181). These 9 mutations, which included 5 small in-frame deletions, occurred exclusively in tumors not harboring mutations of PIK3CA or PTEN. Interestingly, 8/9 of these mutations clustered in the SH2 domain of PI3KR1 and based on crystal structure analysis were predicted to disrupt the interaction of the encoded p85α regulatory subunit with the C2 helical domain of the catalytically active p110α subunit (181).

In addition to the class 1 PI3Kα complex, genetic alterations in class 2 PI3-kinase genes have recently been reported in GBM. Amplification of the PIK3C2B gene was reported in 6/103 GBM samples (6% frequency) in one study (349). Copy number and sequencing analysis by The Cancer Genome Atlas uncovered genomic amplification and somatic mutations of PIK3C2A in 1/91 GBMs, PIK3C2B in 6/91 GBMs, and PIK3C2G in 4/91 GBMs. Interestingly, these class 2 PI3K lesions were mutually exclusive with
lesions of class 1 PI3K genes (i.e. tumors had alteration in one or the other but not both) but were found to be present both in tumors with and without PTEN alterations (181). It remains to be determined if these lesions in class 2 PI3-kinase genes do indeed contribute to gliomagenesis and how alterations in class 1 versus class 2 PI3K complexes might differ in oncogenic mechanism.

In addition to regulators of phosphatidylinositol levels (e.g. PTEN and PI3-kinases), alterations in downstream signaling molecules are also present in GBM. For example, The Cancer Genome Atlas found amplification of AKT1 in 1/206 GBMs, and amplification of AKT3 in 4/206 GBMs. Additionally, deletions and mutations of multiple FOXO genes were present in individual tumors (181).

Together, genetic and biochemical studies have demonstrated that the PI3K-Akt signaling pathway is commonly altered in human GBM, promotes the tumorigenic and invasive properties of GBM cells, and represents a promising target for the development of novel therapeutics with great potential to inhibit GBM progression.

**Activation of Ras/Raf signaling in GBM by NF1 inactivation or BRAF mutation**

Ras/Raf are critical effectors of mitogenic signaling downstream of several growth factor and cytokine receptors (e.g. EGFR, PDGFRA, and MET receptor tyrosine kinases previously discussed). Upon ligand stimulation, receptors recruit and activate specific adaptor proteins (e.g. Grb-2). These adaptor proteins can then recruit and bind guanine nucleotide exchange factors (GEFs; e.g. Sos, cdc25) via their SH3 domains. The RTK/SH3-adaptor/GEF complex causes GEF activation that promotes the removal of GDP from inactive GDP-bound Ras. The small G protein Ras can then bind GTP causing its activation. GTP-bound Ras has high affinity for numerous effectors whose binding causing their activation, including PI3-kinases discussed previously and the
serine-threonine kinase Raf. Activated Raf then phosphorylates and activates downstream signaling pathways such as the Raf-Mek-Erk (MAP kinase) cascade that promote cellular proliferation.

Ras/Raf signaling is frequently activated in cancer through various mechanisms. First, overexpression of growth factor ligands can cause increased RTK and downstream pathway activation. Similarly, amplification/overexpression or point mutations in RTKs can also result in increased downstream signaling activation. Apart from alterations in RTKs, Ras/Raf and their regulators are frequently somatically altered in cancer. Mutations in RAS genes (e.g. KRAS G12V) can cause their constitutive activation by locking the protein in its active GTP-bound state. Mutations also occur in RAF genes (e.g. BRAF V599E) causing their constitutive kinase activation. RAS and RAF genes can also be amplified causing their significant overexpression and pathway activation. Additionally, the GTPase activating proteins (GAPs) that facilitate Ras GTP hydrolysis (e.g. NF1) can be inactivated by point mutations or homozygous deletions. As discussed previously, Ras/Raf signaling in GBM is frequently activated through alterations in RTKs including the EGFR, PDFGRA, and MET. Ras/Raf signaling is also frequently activated in GBM through inactivation of the Ras-GAP NF1 and through V600E point mutation of the BRAF proto-oncogene as described below.

NF1 was originally identified as the gene whose inherited alterations are responsible for neurofibromatosis type 1 (von Recklinghausen's neurofibromatosis), characterized by numerous benign peripheral nerve sheath tumors called neurofibromas as well as increased incidence of malignant peripheral nerve sheath tumors, pilocytic astrocytomas, and diffuse malignant astrocytomas. Germline heterozygous alterations of the NF1 gene were found to be accompanied by LOH or independent somatic
mutation of the second allele in the tumors that arise in affected individuals (22-29). Subsequent analysis of the NF1 gene in sporadic cancers from non-NF1 patients has revealed the presence of somatic mutations and homozygous deletions in colon adenocarcinomas, lung adenocarcinomas, primitive neuroectodermal tumors, malignant peripheral nerve sheath tumors, and malignant astrocytomas (350-354). Interestingly, NF1 mutations are not present in sporadic pilocytic astrocytomas, although inherited mutations of NF1 commonly give rise to pilocytic astrocytomas in neurofibromatosis type 1 patients (355,356). The first study of NF1 alterations in sporadic gliomas identified 1/22 anaplastic astrocytomas with a somatic missense mutation in codon 1423 changing this lysine residue to glutamine, a residue commonly altered in NF1 patients (350). An additional study looked for somatic alterations in a panel of 31 gliomas and found mutation events in 1 astrocytoma, 2 GBMs, and 1 ependymoma (352). Other studies of malignant astrocytomas failed to find alterations of NF1 (357), suggesting that NF1 alterations only rarely contribute to glioma development. However, recent genetic analyses of GBM have found frequent somatic alterations of NF1. Parsons et al. sequencing analysis identified somatic mutations in 16/105 GBMs (119). The Cancer Genome Atlas analysis found genetic lesions in 16/91 GBM tumors (18%) including 3 tumors with homozygous deletions, 7 tumors with multiple or biallelic/homozygous point mutations, and 6 tumors with heterozygous point mutations. These alterations occurred both in GBMs with and without alterations of the RTKs EGFR, ERBB2, and PDGFRA (181). Western blot analysis of GBM cell lines, primary cultures, and primary tumor tissue has revealed that the neurofibromin protein encoded by NF1 is frequently destabilized and proteasomally degraded in a large fraction of those samples without NF1 mutations, demonstrating an additional mechanism of Ras activation in GBM (358).
Together, these recent studies have demonstrated that NF1 is indeed inactivated in a significant fraction of spontaneous GBMs and suggest that Ras is a promising therapeutic target in these tumors.

BRAF somatic mutations were first described in human cancer by Davies et al. in 2002. Mutations were found in greater than 60% of malignant melanomas and at lower frequency in a broad spectrum of tumor types. All mutations observed were present in the kinase domain with a single substitution (V599E) accounting for >80%, and were found to cause increased kinase activity of B-Raf protein and transformation when expressed in NIH3T3 cells. In this report, 4/38 malignant glioma cell lines (11%) were found to harbor BRAF V599E mutations (359). Sequencing of BRAF and the three known Ras genes (KRAS, NRAS, and HRAS) in a panel of 94 GBM tumors found 3 samples with BRAF V599E mutations and 2 samples with NRAS G12D mutations. All of these 5 GBMs with activating NRAS/BRAF mutations were not found to harbor alterations in the upstream RTKs EGFR and PDGFRA, suggesting that RAS/RAF mutations are an alternate mechanism of activating this mitogenic signaling cascade in tumors without RTK alterations (360). Another study examined 49 astrocytomas and 33 oligodendrogliomas for the presence of BRAF mutations and found V599E mutations in 2/34 GBMs (6% frequency) and no mutations in lower grade astrocytomas or oligodendrogliomas (361). The recent analysis of 91 GBMs by The Cancer Genome Atlas found no tumors with BRAF mutations (181). Most recently however, an analysis of 31 pediatric malignant astrocytomas WHO grades II-IV found 7 tumors (23%) with BRAF mutations, 5/7 of which also harbored CDKN2A deletion, demonstrating that combined BRAF/CDKN2A alterations define a subset of pediatric malignant astrocytomas (362).
Together, these findings of frequent receptor tyrosine kinase, NF1, and BRAF alterations in GBM define the critical importance of this mitogenic signaling pathway to gliomagenesis and suggest that combined therapeutic targeting of activated RTKs upstream and Ras/Raf further downstream is of significant potential benefit to GBM patients.

**Cancer genes altered at high frequency in other tumor types but at low or zero frequency in GBM**

Just as informative as the genes discussed above that are frequently altered in GBM are the genes that are rarely or never altered and appear to have little relevance to GBM pathogenesis. For example, it is now clear that KRAS is mutationally activated in >90% of pancreatic adenocarcinomas and at high frequencies in other tumor types including carcinoma of the stomach and large intestine (363-366). However, sequencing and copy number analysis of KRAS in 50 malignant glioma cell lines by the Cancer Genome Project of the Wellcome Trust Sanger Institute failed to detect any mutations or gene amplifications (http://www.sanger.ac.uk/genetics/CGP/). Accordingly, copy number analysis of 206 GBM primary tumors and sequencing of KRAS (as well as the other known Ras genes HRAS and NRAS) in 91 of these tumors by The Cancer Genome Atlas failed to detect any amplifications and found only one sample each with a KRAS mutation and NRAS mutation (181). It is clear that KRAS, HRAS, and NRAS are not drivers of tumorigenesis in the vast majority of GBMs (360). As described above however, Ras/Raf signaling is frequently deregulated in GBM by alternative mechanisms, primarily through NF1 inactivation or BRAF mutation. It is not clear at this time why KRAS mutation is of fundamental importance to cancer of the pancreas and
not the brain, and conversely why NF1 is frequently inactivated in GBM but not pancreatic adenocarcinoma.

Besides KRAS, several additional genes altered at high frequency in other tumor types appear to be rarely or never altered in GBM. Two such genes are MYC and MYCN that encode basic helix-loop-helix transcription factors which promote expression of genes driving cellular proliferation. MYC is amplified and overexpressed in 10-30% of breast, esophagus, stomach, lung, and other cancers, while MYCN is amplified and overexpressed in >50% of neuroblastomas but only rarely in other tumor types (367-369). Only a couple reports have documented rare MYC and MYCN amplifications in GBM (170,176,370,371). Notably, The Cancer Genome Atlas reported found focal amplifications of MYCN in 5/206 GBM tumors (3%) and no MYC amplifications (181). Germline mutations of the VHL tumor suppressor gene at chromosome 3p25 cause von Hippel-Lindau disease characterized by a unique cancer predisposition discussed previously, and somatic mutations and deletions are commonly present in sporadic renal cell carcinomas, hemangioblastomas of the CNS, and pheochromocytomas (372-374). Sequencing of the VHL gene in 38 gliomas of various histological grades found a grade II astrocytoma and a grade II oligodendroglioma with biallelic/homozygous missense mutations (375). However, no other studies have identified alterations of VHL in gliomas, and The Cancer Genome Atlas found no mutations in their panel of 91 GBMs and no deletions in their panel of 206 GBMs (181). The SMAD4 tumor suppressor gene, encoding a transcriptional regulatory factor that mediates TGFβ signaling, is somatically mutated in >50% of pancreatic and colorectal cancers and at a smaller frequency in several other tumor types (376,377). Analysis of SMAD4 in 27 GBMs by Bleeker et al. found no alterations, and analysis in 91 GBMs by The Cancer Genome Atlas found only
1 tumor with a heterozygous nonsense mutation (378,181). CTNNB1 is a proto-oncogene encoding β-catenin, a subunit of the cadherin complex at adherens junctions and an integral component of the Wnt signaling pathway. Mutations of CTNNB1 are common (10-50% frequency) in cancers of the adrenal cortex, biliary tract, cerebellum (medulloblastoma), endometrium, kidney, colon, liver, ovary, pancreas, pituitary, skin, and thyroid (379-382). However, no mutations of CTNNB1 have ever been reported in GBM, and The Cancer Genome Atlas failed to detect any mutations in their 91 GBM tumor samples. Germline mutations of the BRCA1 and BRCA2 genes are found in a significant fraction of familial breast and ovarian cancers, and are also somatically mutated at low frequency in sporadic breast, colorectal, esophageal, and ovarian cancers (383-386). Sequencing of these two genes by TCGA in 91 GBM tumors found no BRCA1 mutations and 3 tumors with heterozygous missense mutations of BRCA2 (181). Together, these findings are consistent with a limited or negligible role of MYC/MYCN, VHL, SMAD4, CTNNB1, and BRCA1/2 genes in pathogenesis of the vast majority of GBM tumors.

**Genetic and expression profiling distinguishes primary and secondary GBM**

As discussed previously, two pathways for the development of GBM have been described. A distinct sequence of genetic alterations had been predicted to underlie the difference in the primary versus secondary pathway to GBM, but these genetic events were largely unknown prior to the discovery of IDH1 mutations in gliomas (reviewed in reference 387). One study used high-resolution CGH arrays to compare copy number profiles of 20 primary GBMs and 17 secondary GBMs all of pure astrocytic histology and arising from histologically verified low-grade astrocytomas. This study found copy number alterations shared between both primary and secondary GBM that include
known genes including PDGFRA, CDK4, MDM2, CDKN2A, and PTEN, as well as novel recurrent amplifications/deletions present only in primary or secondary GBM (388).

A population based study of 715 GBMs treated in Zurich, Switzerland between 1980 and 1994 included only 38 tumors (5%) with clinical and pathologic evidence of progression from a less malignant precursor lesion (i.e. secondary GBM; reference 141), confirming other reports that primary tumors comprise the vast majority of GBMs diagnosed (389). The mean age of primary GBM patients was 62 years of age with a 1.33 male:female predominance, whereas the mean age of secondary GBM patients was 45 years with a slight female predominance (0.65 male:female ratio). Of the GBMs with tumor tissue available for genetic analysis, TP53 mutations were found in 28% of the primary GBMs compared to 65% of the secondary GBMs. This finding is consistent with data demonstrating frequent TP53 mutations in grade II/III astrocytomas and shows that genetic lesion of TP53 is not a requirement for the development of de novo GBM. In contrast, EGFR amplification and PTEN mutation were found at statistically significantly higher frequency in primary versus secondary GBM (36% vs. 8% and 25% vs. 4%, respectively). LOH of chromosome 10q and CDKN2A deletion were found at comparable frequency between primary and secondary GBM (~70% and ~30%, respectively). Based on this limited genetic analysis, it was nonetheless appreciated that primary and secondary GBM represent distinct disease entities likely driven by as then undiscovered distinct genetic alterations and sensitive to distinct therapeutic regimens (141).

Discovery of IDH1 as a frequently mutated gene in gliomas and secondary GBMs

Comprehensive exomic sequencing of 22 GBM samples by Parsons et al. discovered somatic point mutations at codon 132 of the IDH1 gene at chromosome 2q33
in 5 tumor samples (119). Sequencing of the IDH1 gene in an additional panel of 127 GBMs revealed 13 additional somatic mutations for a total frequency of 12% (18/149 samples). The IDH1 gene encodes isoform 1 of isocitrate dehydrogenase, an enzyme that catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate in the citric acid cycle resulting in the production of NADPH. All mutations identified were heterozygous substitutions affecting codon 132, changing the arginine-132 residue to either a histidine or serine, and were present only in the tumor but not in normal DNA from blood of these patients. R132 is an evolutionary conserved residue located within the isocitrate binding site. The recurrent nature of the mutations within a single amino acid residue of the active site without evidence of loss of heterozygosity or other genetic alterations (e.g., deletions, truncating mutations) suggests that these mutations are activating/oncogenic, very reminiscent of mutations activating the KRAS and BRAF proto-oncogenes. While the other four IDH genes encode isoforms that are localized to the mitochondria, the IDH1 gene encodes the only isoform localized within the cytoplasm and lysosomes. None of the other IDH genes were found to be genetically altered in this study. Interestingly, the IDH1 mutations occurred in a significantly younger patient population (mean age = 33 years) than those GBM patients with wild-type IDH1 (mean age = 53 years). In patients under 35 years of age, nearly 50% (9 out of 19) had mutations in IDH1. Mutations in IDH1 were found in nearly all patients with secondary GBMs (5 out of 6) while mutations were relatively uncommon in patients with primary GBM (7 out of 99). Additionally, patients with IDH1 mutations had a significantly improved prognosis with a median overall survival of 3.8 years, compared to 1.1 years for patients with wild-type IDH1. Mutation of TP53 was present in 83% of tumors harboring IDH1 mutations while mutation of PTEN, RB1, EGFR, or NF1 was not present in these tumors. In
contrast, only 27% of tumors harboring wild-type IDH1 had TP53 mutations while 60% had mutations in either PTEN, RB1, EGFR, or NF1 (119). Together, these findings indicate that IDH1 alterations could identify a biologically-specific subgroup of GBM patients with distinct clinical characteristics and genetic profile, and could potentially represent a new therapeutic target in this subgroup of GBM patients.

In a follow up to this study, Yan et al. sequenced IDH1 and the related IDH2 gene in a panel of 445 CNS tumors and 494 non-CNS tumors (120). Mutations of IDH1 were identified in more than 70% of WHO grade II and III astrocytomas, oligodendrogliomas, and secondary glioblastomas that developed from these lower-grade lesions. Interestingly, tumors without mutations in IDH1 often had mutations affecting the analogous R172 amino acid residue in the active site of the IDH2 gene. Together, IDH1 or IDH2 mutations were found in 27/30 grade II astrocytomas (90%), 43/51 grade II oligodendrogliomas (84%), 3/3 grade II oligoastrocytomas (100%), 38/52 anaplastic astrocytomas (73%), 34/36 anaplastic oligodendrogliomas (94%), 7/7 anaplastic oligoastrocytomas (100%), 11/13 secondary glioblastomas (85%), and 6/123 primary adult glioblastomas (5%). No IDH1 or IDH2 mutations were observed in 21 pilocytic astrocytomas, 30 ependymomas, 15 pediatric glioblastomas, 55 medulloblastomas, and the 494 non-CNS tumors including 35 lung cancers, 57 gastric cancers, 27 ovarian cancers, 96 breast cancers, 114 colorectal cancers, 95 pancreatic cancers, 7 prostate cancers, and 63 hematologic cancers. The IDH gene mutations and the frequency of mutation by tumor histologic type observed are shown below (reproduced from reference 120, ©2009 Massachusetts Medical Society).
Furthermore, this study demonstrated that exogenous expression of wild-type but not tumor-derived mutant forms of IDH1 and IDH2 in an oligodendroglioma cell line increased NADPH production in a biochemical assay using total cell lysate, demonstrating that these tumor-derived mutations in the active sites of IDH1 and IDH2 reduce their enzymatic activity. Survival analysis of malignant glioma patients with or without IDH gene mutations revealed significantly improved prognosis for patients with IDH mutant tumors versus those with wild-type tumors. For patients with anaplastic astrocytoma, the median survival was 65 months for the 38 patients with mutated IDH1 or IDH2, as compared with 20 months for the 14 patients with wild-type IDH1 and IDH2. For patients with glioblastomas, the median survival was 31 months for the 14 patients with mutated IDH1 or IDH2, as compared with 15 months for the 115 patients with wild-type IDH1 and IDH2 (see Kaplan-Meier survival plot for these 129 GBM patients below reproduced from reference 120, ©2009 Massachusetts Medical Society).
Together these findings defined IDH1/2 gene mutation as a signature event in the pathogenesis of malignant gliomas and secondary glioblastomas, not occurring at appreciable frequency in any other major tumor type. Furthermore, this genetic alteration distinguished gliomas into two subgroups (e.g. those with and those without IDH gene mutation) that are characterized by distinct genetic profiles and clinical courses. While these mutations were found to diminish the enzymatic activity associated with IDH1 and IDH2, these data did not provide any conclusions as to how IDH mutations drive gliomagenesis.

Following this study by Yan et al., several other reports confirmed the presence of frequent IDH1 mutations associated with decreased enzymatic function in malignant gliomas and secondary GBMs and only the rare presence of such mutations in other human tumor types, notably acute myelogenous leukemia (390-398). However, it remained unclear as to why these mutations were arising exclusively in malignant gliomas and acute myeloid leukemias and how they were involved in driving the pathogenesis of these two specific tumor types.
One study demonstrated that heterozygous mutations of IDH1 dominantly inhibit wild-type IDH1 through the formation of catalytically inactive heterodimers, reducing normal enzymatic production of α-ketoglutarate (399). In cells expressing mutant IDH1, decreased levels of α-ketoglutarate were found to stabilize HIF-1α, a transcription factor that promotes tumor growth under hypoxic conditions, leading to its accumulation at high levels. They also found that HIF-1α levels were higher in gliomas harboring IDH1 mutations than gliomas without mutations. They thus postulated that IDH1 functions as a tumor suppressor in glial cells by constraining HIF-1α levels that drive tumorigenesis (399). However, this model fails to explain the remarkable tissue specificity of IDH1 mutations in gliomas and is inconsistent with the hypothesis that IDH1 mutations are activating/oncogenic based on their clustering at a mutational hot spot within the active site (similar to KRAS and BRAF). If IDH1 does truly function as a tumor suppressor, then one would expect to find other mutations throughout the protein including truncating (i.e. nonsense, splice site, frameshift) mutations, LOH of the remaining wild-type allele, and/or homozygous deletions of the gene as is classically observed with tumor suppressor genes such as PTEN, TP53, and RB1. These other genetic lesions are definitively not present in human gliomas, strongly suggesting that the observed heterozygous mutations cause an activating gain-of-function for the IDH1 protein that drives tumorigenesis through an as yet unknown mechanism.

This hypothesis was later realized by a metabolomics study of glioma cells expressing wild-type or mutant IDH1 alleles (400). Metabolic profiling of the parental U87MG GBM cell line and after transfection with wild-type and R132H mutant IDH1 by liquid chromatography-mass spectrometry found no significant differences except for a >100 fold increase of a single species in mutant IDH1 transfected cells, R(−)-2-
hydroxyglutarate (2HG). Biochemical assays of purified IDH1 protein revealed that while wild-type IDH1 enzymatically converts isocitrate to $\alpha$-ketoglutarate, mutant IDH1 has decreased binding affinity for isocitrate and a 1,000-fold decrease in catalytic production of $\alpha$-ketoglutarate. Purified mutant IDH1 protein was instead found to catalyze the reduction of $\alpha$-ketoglutarate to 2HG, an acquired gain-of-function not possessed by the wild-type protein. X-ray analysis of crystallized R132H mutant IDH1 protein revealed changes in the structure of the active site producing a closed conformation that excludes isocitrate from entering as well as shifts in the position of highly conserved amino acid residues in the active site pocket consistent with a change in substrate specificity. Analysis of citric acid cycle metabolites in a panel of 22 malignant gliomas (12 with R132 mutant IDH1 alleles and 10 with wild-type alleles) revealed >100-fold increased levels of 2HG in IDH1 mutant tumors. They thus concluded that IDH1 mutation results in an oncogenic gain-of-function that causes production of the “oncometabolite” 2HG (400). It was also noted that the rare genetic disorder with 2-hydroxyglutarate dehydrogenase deficiency that results in accumulation of 2HG in the brain is associated with an increased risk of developing brain tumors (401). These discoveries have identified 2HG as an important new glioma biomarker and raised the intriguing possibility that small molecule inhibitors of mutant IDH1 that block production of 2HG might be beneficial to the large fraction of glioma and secondary GBM patients with IDH1 mutations.

Genetic and expression profiling distinguishes clinically relevant GBM subtypes

Numerous studies have attempted to correlate GBM patient survival data with individual gene expression levels or genetic alterations present in patient’s tumors. For examples, studies have assessed whether high levels of VEGF expression correlate with poor patient prognosis, and whether CDKN2A deletion correlates with
increased/decreased patient survival (402-405). None of these studies have successfully identified such genetic factors that reproducibly correlate with GBM patient survival, apart from MGMT promoter methylation and IDH1 mutations as previously discussed. The largest such study to date analyzed a series of >300 newly diagnosed GBMs treated in Switzerland between 1980 and 1994 (141). The 244 GBMs with EGFR amplification had a median survival of 7.6 months versus 6.8 months for the 127 GBMs without EGFR amplification (p = 0.40). The 102 GBMs with CDKN2A deletion had a median survival of 8.5 months versus 8.0 months for the 226 GBMs without CDKN2A deletion (p = 0.54). The 127 GBMs with TP53 mutation had a median survival of 8.2 months versus 7.2 months for the 259 GBMs with wild-type TP53 (p = 0.02), however age-adjusted analysis showed this difference to be non-significant. The 77 GBMs with PTEN mutations had a median survival of 8.8 months versus 8.0 months for the 247 GBMs with wild-type PTEN. The 185 GBMs with LOH of chromosome 10q had a median survival of 7.7 months versus 9.3 months for the 84 GBMs without LOH of 10q. The only predictive prognostic factor found to be significant in this large study was patient age at time of diagnosis (141), previously discussed as a prognostic factor in GBM.

As expression differences and genetic alterations of individual genes have proved largely uninformative in predicting patient survival and sensitivity to therapeutics, studies have subsequently assessed how global gene expression profiles and combinations of genetic alterations might stratify GBM into subtypes with distinct therapeutic sensitivity and patient survival characteristics (406-420). Mohapatra et al. first described evidence for subgroups in primary GBM – for example, one group contained tumors with both chromosome 7p gain and chromosome 10p loss, and the
other group contained tumors with neither copy number alteration. However, none of the identified subgroups significantly correlated with altered patient survival in this study (406). The first study to document the molecular classification of histologically indistinguishable GBM into subgroups with different overall survival was reported in 2005 (412). Therein, a set of ~70 genes was found to be more highly expressed in one subgroup of GBMs with median survival of 4 months than another subgroup of GBMs with median survival of 25 months. This set of differentially expressed genes included those with known function in regulating cellular differentiation (e.g. OLIG2) and invasion (e.g. BCAN and FABP7). Other recent studies have also documented that molecular subclassification of GBM based on combinations of genetic, transcriptional, and proteomic profiling can predict patient survival. For example, one recent study stratified 256 grade III/IV astrocytomas into proneural, proliferative, and mesenchymal subtypes that had median survival of 175 weeks, 61 weeks, and 65 weeks, respectively. The proneural tumors had Notch pathway activation and high levels of expression of genes involved in neurogenesis including OLIG2, DLL3, and BCAN. The proliferative tumors were characterized by high MIB-1 index and high expression of PCNA and topoisomerase IIα, whereas the mesenchymal tumors were distinguished by Akt activation and high expression YKL-40, CD44, and VEGF ligand and its receptors (414). Continued characterization, validation, and application of this molecular stratification technology will ultimately lead to improved prognostication and selection of the most effective therapies for GBM patients.
Emerging therapies for the treatment of GBM

Standard DNA damaging chemotherapeutics are highly cytotoxic compounds capable of slowing tumor growth and extending lifespan, often at the expense of devastating and even life-threatening side effects. These agents are not directed specifically to tumor cells and cause cytotoxicity throughout the body, hence their adverse effects. One method of increasing drug dosage to the tumor while avoiding such unwanted side effects is through local delivery methods. For example, biodegradable wafers impregnated with the nitrosourea BCNU (Gliadel wafers) have been developed that can be placed into the tumor bed at time of surgical resection which release BCNU (also called carmustine) directly onto residual tumor cells in the brain (421). One study randomized 240 patients with primary malignant gliomas to receive either placebo wafers or BCNU-impregnated wafers at resection plus postoperative external beam radiation and found a median survival of 13.9 months in the intent-to-treat group versus 11.6 months for the placebo group (422). The use of Gliadel wafers in combination with radiation therapy plus temozolomide has also been shown to improve survival in a subsequent clinical trial of 33 GBM patients (423). Such enhanced drug delivery methods will likely prove beneficial to GBM patients.

Another strategy likely to improve the success of current therapeutics while reducing undesirable side effects is the addition of agents that increase sensitivity to radiation and/or temozolomide. Numerous compounds have been found to increase radiation sensitivity of cancer cells including carboplatin, taxols, mTOR inhibitors, and many others, and various clinical trials are underway aimed at improving the effectiveness of radiation therapy for gliomas (reviewed in references 424 and 425). Similarly, several strategies are evolving for increasing sensitivity of cancer cells to
temozolomide (reviewed in reference 426). As discussed previously, increased expression of the DNA repair enzyme MGMT has been correlated with resistance to temozolomide and shorter survival following temozolomide therapy (158-162). In vitro studies have shown that ectopic overexpression of MGMT in cancer cells causes temozolomide resistance while depletion of MGMT expression causes increased sensitivity. Additionally, two inhibitors of MGMT, O-6-methylguanine and O-6-benzylguanine, while not cytotoxic on their own, have been shown to potentiate the cytotoxic effects of temozolomide both in vitro and in xenografts models (427-430). Clinical trials evaluating the combination of temozolomide and O-6-benzylguanine are currently underway (431-434). In addition, poly-(ADP-ribose)- polymerases (PARP) are a group of enzymes involved in the repair of DNA damage, including base excision repair of alkylated nucleotides (as is caused by the DNA alkylating agent temozolomide). PARP inhibitors have been developed that also increase sensitivity of GBM cells to temozolomide both in vitro and in vivo in animal models (435-437).

In spite of the promise such sensitizing agents present, they are unlikely to ever render standard DNA damaging therapies “curative”. The future of successful treatment for cancer undoubtedly lies with immune therapy and other targeted therapeutics such as monoclonal antibodies and small molecule inhibitors directed at oncoproteins either uniquely present or specifically activated in tumor cells.

One such targeted therapy that has already proved successful in the treatment of recurrent GBM is a humanized monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A) called bevacizumab (marketed as Avastin). Bevacizumab functions as an angiogenesis inhibitor by blocking the formation of new blood vessels driven by VEGF-A (reviewed in reference 438). Bevacizumab was first approved by the
U.S. Food and Drug Administration (FDA) in 2004 for use in metastatic colorectal cancer and has subsequently been approved for use in breast and non-small cell lung cancer. Several open-label phase II clinical trials have tested the efficacy of bevacizumab either alone as a single agent or in combination with the DNA damaging agents irinotecan or etoposide in patients with recurrent GBM and found that it prolonged survival (439-446). Bevacizumab was approved for treatment of progressive/recurrent GBM by the FDA in May 2009 and is currently under investigation for potential benefit as a first-line therapy in newly diagnosed GBM.

Given the frequent activation of EGFR in GBM by amplification/overexpression and point mutations in the extracellular ligand binding domain, therapeutics targeting EGFR were presumed to be highly effective in GBM patients. A monoclonal antibody to the EGFR, cetuximab (marketed as Erbitux), and small molecule inhibitors of EGFR, erlotinib and gefitinib (marketed as Tarceva and Iressa, respectively), have demonstrated efficacy in the treatment of certain tumor types. Cetuximab has proven efficacious and is FDA approved for the treatment of EGFR expressing, KRAS wild-type colorectal cancer and in combination with radiation or as a single agent after failed platinum therapy for the treatment of squamous cell carcinoma of the head and neck. Several GBM xenograft studies have documented efficacy of cetuximab (447-450), and clinical trials in GBM patients are underway with conflicting results about the potential benefit (451,452).

Erlotinib and gefitinib reversibly block the ATP-binding site in the kinase domain of EGFR thereby preventing kinase signaling even in the presence of ligand. Erlotinib has proven effectiveness and is FDA approved for the treatment of non-small cell lung cancer (NSCLC) that has failed at least one prior chemotherapy regimen and in
combination with gemcitabine for the treatment of pancreatic cancer that has failed prior chemotherapy. Erlotinib is currently favored over gefitinib, except in those patients with a documented response to gefitinib. Approximately 10% of NSCLC patients have a dramatic response to erlotinib or gefitinib, while the remainder show very little or no response. Response in these 10% of patients was found to be correlated with kinase domain mutations of EGFR while non-responders had NSCLC tumors with wild-type EGFR (453). Numerous clinical trials have now examined the efficacy of erlotinib and gefitinib in the treatment of both newly diagnosed and recurrent GBM either alone as a single agent or in combination with radiation and/or temozolomide (454-466). Erlotinib and gefitinib were largely unsuccessful at slowing tumor growth and extending survival in these trials, with only 10-20% of patients showing response to these inhibitors. One study showed that amplification of EGFR or low levels of phosphorylated Akt were significantly correlated with a clinical response to erlotinib (455). In another study, coexpression of EGFRvIII and PTEN were significantly correlated with response to EGFR kinase inhibitors (456), but such predictive factors have not been corroborated in subsequent studies in separate patient cohorts (462,464). Other studies have examined the genetic and expression profiles of GBM cell lines and xenografts that correspond with sensitivity to erlotinib/gefitinib. While kinase domain mutations in EGFR are known to correlate with sensitivity of NSCLC to gefitinib (453), such kinase domain mutations are not commonly present in GBM (267-270). Rather, missense mutations in the extracellular ligand binding domain of EGFR were recently identified to be present in 10-15% of GBM tumors, and such extracellular domain mutations were found to increase sensitivity to EGFR kinase inhibitors in cell culture models (270). The presence of EGFR extracellular domain mutations in GBM patients treated with erlotinib/gefitinib and
associated responsiveness to therapy have not yet been determined. Recently, an *in vitro* study found that STATI, PTGER4, and MYC expression were correlated with sensitivity and FKBP14 and RAC1 expression were correlated with resistance of GBM cells to erlotinib (467). Another study examined the sensitivity of GBM xenografts to erlotinib and found that sensitivity was correlated with expression of wild-type PTEN and amplification of aberrant EGFR, either through extracellular domain mutation or EGFRvIII rearrangement (468). Determination of the genetic factors that dictate sensitivity to EGFR kinase inhibitors will direct their use in those GBM patients who will benefit from EGFR targeted therapy.

Imatinib (marketed as Gleevec) is a small molecule initially developed as an inhibitor of the translocation-fusion oncoprotein BCR-ABL uniquely present in chronic myelogenous leukemia (CML). It has been implemented in the treatment of CML with enormous success and now serves as a model for the promise of targeted therapeutics in the treatment of cancer. Imatinib was subsequently determined to also potently inhibit the PDGFα and c-Kit receptors, which led to its successful testing and FDA approval as a treatment for gastrointestinal stromal tumors (90% of which harbor c-Kit mutations, reference 469). Given the moderate frequency of PDGFRA amplification and occasional point mutations in GBM, it was hypothesized that imatinib might also be effective in the treatment of GBM patients. Several clinical trials have tested the efficacy of imatinib, either alone or in combination with temozolomide or hydroxyurea (an antimetabolite agent), in patients with progressive/recurrent GBM with very limited success (470-476). However, none of these clinical trials stratified their patient population so as to include only those patients with PDGFRA amplification or mutation. It is thus unclear at present
if imatinib is or is not effective in this subpopulation of GBM patients for which it holds the greatest potential benefit.

PI3K-Akt signaling is frequently deregulated in GBM through PIK3CA mutation, PIK3R1 mutation, PTEN deletion/mutation, AKT3 amplification, and lesions activating upstream receptor tyrosine kinases as discussed previously. Thus, therapies targeting this important signaling axis hold great potential for improving outcome in GBM patients. Recently developed PI3-kinase inhibitors such as PX-866 are being tested in vitro and in xenograft models at present, with demonstrated growth suppression in all KRAS wild-type tumors (477-479). A few highly specific Akt inhibitors have recently been synthesized including KP-372-1 and GSK690693 that should have therapeutic efficacy in the vast majority of GBMs with lesions in this pathway (480-483). Downstream in the PI3K-Akt signaling pathway is the mammalian target of rapamycin (mTOR) complex that is one effector driving increased survival, proliferation, and invasion in cells with upstream PI3K-Akt lesions. mTOR inhibitors including sirolimus and everolimus have well documented efficacy in suppressing the growth of GBM cells in vitro and in animal models, and are currently being tested in several clinical trials of patients with recurrent malignant gliomas both as a single agent and in combination with EGFR inhibitors erlotinib and gefitinib (484-491).

Given the frequent and perhaps obligatory genetic alterations affecting the cdk4/6-cyclin D-INK4-Rb growth regulatory axis in GBM, inhibitors of cdk4/6 are likely to be effective in suppressing the growth of Rb-proficient tumors (reviewed in reference 492). The successful preclinical evaluation of one such cdk4/6 specific inhibitor (PD-0332991) both in vitro and in vivo in intracranial xenografts was recently documented
Clinical trials are currently beginning to determine the efficacy of such cdk inhibitors in the treatment of GBM.

The Cancer Genome Atlas discovery of ERBB2 mutations in the extracellular ligand binding domain and intracellular kinase domain in 7/91 GBM tumors (181) suggests that small molecule inhibitors of ERBB2 may have efficacy in a fraction of GBM patients. Preclinical evaluation of the newly developed, highly specific ERBB family inhibitors such as lapatinib (approved by the FDA in February 2010 for first-line therapy in the treatment of ERBB2+ breast cancer) against GBM tumors harboring ERBB2 mutations is clearly warranted.

Numerous other small molecule inhibitors have been developed that are currently being tested both in preclinical studies and in clinical trials against GBM. Besides imatinib and erlotinib/gefitinib, other small molecule inhibitors of tyrosine kinases being evaluated include sorafenib (a broad spectrum inhibitor of RAF and VEGF, PDGF, and c-Kit receptors), sunitinib (a broad spectrum inhibitor of PDGF, VEGF, RET, c-Kit, and Flt3 receptors), and SGX523 (a specific MET inhibitor) (494-497). A highly specific inhibitor of V600E mutant BRAF, PLX4032, was recently developed (498,499) that might be efficacious in the small fraction (<5%) of GBM patients with this activating BRAF mutation. A review of small molecule kinase inhibitors being tested in clinical trials of GBM patients was recently written (500). Beyond kinase inhibitors, several other small molecule inhibitors are also being evaluated (reviewed in reference 501). For example, an inhibitor of telomerase, essential for preventing erosion of telomeres and activated in most cancer cells, called Imetelstat has been developed and is being tested against GBM (502).
As previously discussed, the EGFR variant found at high frequency in GBM tumors, EGFRvIII, is the result of an in-frame deletion of nucleotides 275-1075 in the EGFR cDNA sequence creating a novel epitope at the fusion junction. Studies have demonstrated that a peptide derived from the fusion junction when conjugated to a hapten can serve as an antitumor vaccine in GBM patients capable of eliciting a cytotoxic immune attack specifically of tumor cells expressing EGFRvIII on their surface. Clinical trials are underway to determine the efficacy of such EGFRvIII peptide vaccines (e.g. CDX-110) and have shown promising results in some patients to date (503-508).

As multiple genetic lesions in multiple growth factor signaling pathways are present in every human cancer, no single agent targeted therapeutic is likely to be successful on its own. One recent study demonstrated co-activation of multiple receptor tyrosine kinases (RTKs) in GBM cells – 19/20 GBM cell lines had 3 or more activated RTKs including EGFR, PDGFRα and β, MET, ERBB3, insulin receptor, colony stimulating factor 1 receptor, and Ephrin A receptors, as determined through an unbiased RTK antibody array followed by anti-phosphotyrosine analysis. This finding was recapitulated in GBM xenotransplants and primary tumors. As such, they found that combinations of RTK inhibitors and/or RNA interference were required to decrease downstream signaling, cell survival, and anchorage-independent growth of GBM cells (509). Several clinical trials evaluating combinations of targeted therapeutics in GBM patients are currently underway (452,486-488,491).

Given the vast number of novel therapeutics that are being tested against GBM, it is likely that new successful therapies will emerge over the next several years. Undoubtedly though, it will be a combination of several such targeted therapies tailored
to the specific genetic lesions present in each patient’s tumor that are ultimately successful in the treatment of GBM in the coming era of “personalized medicine”.
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Identification of p18^{INK4c} as a Tumor Suppressor Gene in Glioblastoma Multiforme

David Solomon¹, Jung-Sik Kim¹, Sultan Jenkins¹, Habtom Ressom¹, Michael Huang², Nicholas Coppa², Lauren Mabanta¹, Darell Bigner³, Hai Yan³, Walter Jean², and Todd Waldman¹

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC
²Department of Neurosurgery, Georgetown University School of Medicine, Washington, DC
³Department of Pathology, Duke University School of Medicine, Durham, NC


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Abstract

Genomic alterations leading to aberrant activation of cyclin/cdk complexes drive the pathogenesis of many common human tumor types. In the case of glioblastoma multiforme (GBM), these alterations are most commonly due to homozygous deletion of p16\(^{INK4a}\), and less commonly due to genomic amplifications of individual genes encoding cyclins or cyclin-dependent kinases (cdks). Here we describe deletion of the p18\(^{INK4c}\) cdk inhibitor as a novel genetic alteration driving the pathogenesis of GBM. Deletions of p18\(^{INK4c}\) often occurred in tumors also harboring homozygous deletions of p16\(^{INK4a}\). Expression of p18\(^{INK4c}\) was completely absent in 43% of GBM primary tumors studied by immunohistochemistry. Lentiviral reconstitution of p18\(^{INK4c}\) expression at physiological levels in p18\(^{INK4c}\)-deficient but not p18\(^{INK4c}\)-proficient GBM cells led to senescence-like G1 cell cycle arrest. These studies identify p18\(^{INK4c}\) as a GBM tumor suppressor gene, revealing an additional mechanism leading to aberrant activation of cyclin/cdk complexes in this terrible malignancy.
Introduction

Glioblastoma multiforme (GBM) is the most lethal primary brain tumor. Approximately 10,000 GBMs are diagnosed each year in the United States, with an average survival of approximately one year. Activation of the EGF signaling pathway by amplification and/or mutation of the EGFR is found in most GBM tumors (1). Additionally, activation of the PI3K signaling pathway via mutational inactivation of the PTEN tumor suppressor or mutational activation of the PIK3CA oncogene is also very common in GBM (2,3). Finally, virtually all GBMs harbor genomic alterations that lead to the constitutive activation of cdks.

Several different genomic alterations are present in GBM tumors that lead to activation of cdks. A remarkable 60%-80% of GBMs harbor homozygous deletions of the p16\textsuperscript{INK4a} tumor suppressor, which binds to and inhibits cdk4 and cdk6 (4,5). Another 5-10% of GBMs have amplifications of individual cyclins and cdks, including cdk4, cdk6, and cyclin D1 (5-7). As such, it is by now clear that aberrant activation of cdks is a particularly important genetic event contributing to the pathogenesis of GBM.

We have employed high resolution SNP arrays to interrogate the genomes of GBM samples in an effort to identify recurrent copy number alterations that may drive the pathogenesis of GBM. Using this approach, we have identified inactivation of the p18\textsuperscript{INK4c} cdk inhibitor as an additional genomic alteration that drives the pathogenesis of GBM.
Materials and Methods

Cell Lines, Xenografts, and Primary Tumors. Cell lines were obtained from the American Type Culture Collection (U87MG, U138MG, M059J, Hs683, H4, A172, LN18, LN229, CCF-STTG1, T98G, DBTRG-05MG), DSMZ (8MGBA, 42MGBA, DKMG, GAMG, GMS10, LN405, SNB19), and the Japan Health Sciences Foundation Health Science Research Resources Bank (AM38, NMC-G1, KG-1-C). Normal human astrocytes (NHAs) were obtained from Clonetics (Walkersville, MD) and AllCells (Emeryville, CA). All cell lines were grown in DMEM + 10% FBS at 37° in 5% CO₂.

Subcutaneous xenografts in immunodeficient mice were obtained from the Duke University Brain Tumor Center or created in the Lombardi Comprehensive Cancer Center Animal Shared Resource from tissue taken from patients undergoing craniotomy at Georgetown University Hospital (IRB #2006-344).

Snap frozen primary GBM tumors and paired blood samples were obtained from the Brain Tumour Tissue Bank (London Health Sciences Centre, Ontario, Canada) funded by the Brain Tumour Foundation of Canada. All tumors were graded by a neuropathologist as good or moderate on a scale of good to poor depending on the amount of tumor cells present (as opposed to hemorrhagic, necrotic, or fibrous tissue). All tumor samples were further categorized as “tumor center”.

Microarrays and bioinformatics. Genomic DNA derived from GBM cell lines and xenografts was interrogated with Affymetrix 250K Nsp I Human Gene Chip microarrays using protocols described by the manufacturer. Data processing was performed using dCHIP (8,9). Additional details regarding the protocols used for data acquisition and processing, as well as the complete raw and processed datasets will be presented elsewhere.
PCR. Conventional PCR was performed using Taq Platinum (Invitrogen, Carlsbad, CA) as described by the manufacturer. qPCR was performed in an iCycler (Bio-Rad, Hercules, CA) using the Platinum Taq SYBR Green PCR Supermix (Invitrogen) according to the manufacturer’s instructions. DNA copy number was calculated using the $2^{-\Delta\Delta C_T}$ method, normalizing to the copy number of an arbitrarily chosen region on chromosome 9 that was neither amplified nor deleted in the samples studied. All assays were performed at least in triplicate.

Western blot. Primary antibodies used were: p18$^{INK4c}$ clone DCS118 (Cell Signaling, Danvers, MA), p16$^{INK4a}$ #554079 (BD-Pharmingen, Franklin lakes, NJ), α-tubulin Ab-2 clone DM1A (Neomarkers, Fremont, CA), and cdk6 clone C-21 (Santa Cruz Biotechnology, Santa Cruz, CA).

DNA sequencing. Individual exons of p18$^{INK4c}$ were PCR amplified using conditions and primer pairs recently described by Sjoblom et al. (10). PCR products were purified using the Exo/SAP method followed by a Sephadex spin column. Sequencing reactions were performed using Big Dye v3.1 (Applied Biosystems, Foster City, CA) using an M13F primer, and analyzed on an Applied Biosystems 3730XL capillary sequencer. Sequences were analyzed using Mutation Surveyor (Softgenetics, State College, PA). Traces with putative mutations were re-amplified and sequenced from both tumor and matched normal DNA.

Immunohistochemistry. GBM tissue microarrays were obtained from US Biomax (Rockville, MD) and contained 35 cases of GBM spotted in duplicate. Immunohistochemistry was performed using standard techniques using the same antibodies as for Western blot except using p16$^{INK4a}$ antibody #554070 (BD-Pharmingen). Antigen-antibody complexes were detected with the avidin-biotin
peroxidase method using 3,3'-diaminobenzididine as the chromogenic substrate (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), and sections were counterstained with hematoxylin.

**p18<sup>INK4c</sup> Lentivirus.** A full-length, wild-type p18<sup>INK4c</sup> cDNA (MGC 3907917) was obtained from Open Biosystems (Huntsville, AL) and cloned into the pCDF1-MCS2-EF1-Puro lentiviral expression vector backbone (System Biosciences, Mountain View, CA). To make virus, this construct was co-transfected into 293T cells with pVSV-G (Addgene, Cambridge, MA) and pFIV-34N (System Biosciences) helper plasmids using Fugene 6 (Roche, Indianapolis, IA) as described by the manufacturer. Virus-containing conditioned media was harvested 48 h. after transfection, filtered, and used to infect recipient cells in the presence of 8 µg/mL polybrene.

**Flow cytometry.** Cells were pulsed with 10 µmol/L BrdU for 1 hr, trypsinized, and centrifuged. Cells were fixed and stained using the BrdU Flow Kit (PharMingen, San Diego, CA) and analyzed by flow cytometry in a BD FACSort instrument using FCS Express v.3 software (DeNovo Software, Los Angeles, CA).

**β-Galactosidase Staining.** Cells grown on coverslips were stained with the Senescence β-Galactosidase Staining Kit (Cell Signaling) as described by the manufacturer.

**Microscopy.** All imaging was performed on an Olympus BX61 light microscope with a 40X Plan-Apochromat objective.
Results

Homozygous Deletion of p18\textsuperscript{INK4c} in GBM. In an effort to identify novel copy number alterations that drive the pathogenesis of GBM, we initially interrogated genomic DNA derived from 35 GBM cell lines and xenografts with Affymetrix SNP microarrays, as described in Materials and Methods. This analysis revealed focal deletions of chromosome 1p in 7 out of 35 (20\%) of samples (Fig. 1A).

This approximately 200-300 kb region of chromosome 1 contains two annotated genes – p18\textsuperscript{INK4c} and FAF1. p18\textsuperscript{INK4c} is a cyclin-dependent kinase inhibitor that binds to and inhibits cdk4 and cdk6, and is a known tumor suppressor in mice (11,12). FAF1 binds to the intracellular domain of Fas and is a pro-apoptotic signal transduction molecule (13). To determine whether either of these genes was the likely target of the deletion, we examined the copy number information at individual probesets to identify the consensus region of deletion (Supplemental Fig. 1). This analysis revealed that three probesets were deleted in all samples studied, narrowing the consensus region of deletion to a 56-133 kb interval containing only p18\textsuperscript{INK4c}. These deletions of p18\textsuperscript{INK4c} were confirmed by PCR and qPCR analysis using primer pairs specific to p18\textsuperscript{INK4c} exon 1 (Fig. 1B and Supplemental Fig. 2). Finally, Western blot analysis demonstrated the absence of p18\textsuperscript{INK4c} expression in GBM cells with confirmed homozygous deletions (Supplemental Fig. 3).

p18\textsuperscript{INK4c} Deletions in Primary Tumor Samples from The Cancer Genome Atlas. The NIH has recently sponsored a large scale project to perform genomic analysis on GBM and other tumor types. As part of this project, they have recently released raw Affymetrix SNP microarray data on 106 primary GBM tumors. We analyzed this data with dCHIP to determine the copy number status of p18\textsuperscript{INK4c} in primary GBM tumors. As
depicted in Fig. 1C, deletions of p18<sup>INK4c</sup> were present in five tumors (5%). These deletions were not present in constitutional DNA from these patients studied with the same Affymetrix SNP microarrays (data not shown), demonstrating that the deletions are somatic. The available clinical and pathological details of these samples are presented in Supplemental Fig. 4. These data demonstrate that deletions of p18<sup>INK4c</sup> are identifiable in uncultured primary human GBM samples, albeit at a lower apparent frequency than in our cultured samples.

**Admixed non-neoplastic cells complicate the identification of p18<sup>INK4c</sup> deletions in uncultured GBMs.** We hypothesized that the lower apparent frequency of p18<sup>INK4c</sup> deletions in uncultured GBM samples was due, at least in part, to the presence of admixed non-neoplastic human cells that are eliminated during *ex vivo* growth. To directly test this, we performed SNP microarray analysis on a primary GBM tumor and a matched first-passage xenograft that we derived from the tumor. Deletion of p18<sup>INK4c</sup> was easily detectable in the first passage xenograft, but was much less apparent (and would have been missed) in the primary tumor from which the xenograft was derived (Fig. 2A). This experiment clearly demonstrates that the presence of admixed normal human cells is one factor that confounds the identification of p18<sup>INK4c</sup> deletions in primary tumors. Xenograft growth eliminates admixed non-neoplastic human cells, enabling more efficient detection of p18<sup>INK4c</sup> deletions with available technologies.

**Heterogeneity of p18<sup>INK4c</sup> deletion in GBM.** Despite eliminating the influence of admixed non-neoplastic human cells by *ex vivo* growth, we did not observe *complete* copy number reduction at the p18<sup>INK4c</sup> locus in either GBM xenografts or uncultured primary tumors (Figs. 1A and C). To determine whether this was an issue with sample quality or instead reflected a more fundamental underlying biology, we examined copy...
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number at the p16\textsuperscript{INK4a} locus in the same GBM samples that harbored deletions of p18\textsuperscript{INK4c}. Deletions of p16\textsuperscript{INK4a} were present in 12 of these 14 samples (86%). As expected, in cell lines the copy number for both p16\textsuperscript{INK4a} and p18\textsuperscript{INK4c} was zero, reflecting the homogeneity of cultured cell lines (compare Fig. 2B to identical samples in Fig. 1A). In contrast, in more genetically heterogeneous xenografts and uncultured primary tumors, the copy number for p16\textsuperscript{INK4a} was near zero whereas the copy number for p18\textsuperscript{INK4c} was 0.5-1.0 (compare Fig. 2B to identical samples in Fig. 1A and C). This analysis clearly demonstrates that GBMs are homogenous with regard to p16\textsuperscript{INK4a} deletions and more heterogeneous with regard to p18\textsuperscript{INK4c} deletions.

**Loss of p18\textsuperscript{INK4c} Expression in GBM Primary Tumors.** We next employed immunohistochemistry to measure p18\textsuperscript{INK4c} expression in individual cells in primary GBM tumors. In particular, we measured the expression of p18\textsuperscript{INK4c} protein in 35 primary GBM specimens as part of a GBM tissue microarray (Fig. 3A). Remarkably, expression of p18\textsuperscript{INK4c} was completely lost in 15 of the 35 (43%) samples studied by IHC (examples in subpanels i,ii). In samples expressing p18\textsuperscript{INK4c}, staining was primarily nuclear, with some cytoplasmic staining observable as has been reported by Bartkova et al. (14). By comparison, expression of p16\textsuperscript{INK4a} (known to be commonly deleted in GBM) was lost in 21 of the 35 (60%) samples studied (examples in subpanels iii, iv). 9 of the 15 samples (60%) lacking expression of p18\textsuperscript{INK4c} also lacked expression of p16\textsuperscript{INK4a}. Staining for α-tubulin was positive in all but one of the 70 tumor cores, demonstrating that all p18\textsuperscript{INK4c} - negative tumors were accessible for staining with other antibodies (example in subpanel v). This experiment demonstrated that p18\textsuperscript{INK4c} expression is lost in approximately 40-50% of GBMs (Fig. 3B), and therefore its inactivation is likely to play a major role in the pathogenesis of GBM.
Absence of p18\textsuperscript{INK4c} Missense Mutations in 82 GBM Samples. We next sequenced p18\textsuperscript{INK4c} in 82 GBM samples lacking homozygous deletions, including 51 primary tumors, 14 xenografts, and 17 cell lines as described in Materials and Methods. The p18\textsuperscript{INK4c} coding region was wild-type in each of the 82 samples sequenced. Heterozygosity at a previously-annotated synonymous SNP is depicted in Supplemental Fig. 5. This experiment demonstrated that as for p16\textsuperscript{INK4a}, homozygous deletion of p18\textsuperscript{INK4c} is the predominant genetic mechanism leading to its inactivation during GBM pathogenesis.

Amplification of cdk6 in GBMs Harboring Intact p18\textsuperscript{INK4c} Genes. cdk6 is thought to be a particularly important target of inhibition by p18\textsuperscript{INK4c}, and Costello et al. have previously described cdk6 amplifications in GBM samples (6). Therefore, we determined whether amplification of cdk6 might be present in our GBM samples with intact p18\textsuperscript{INK4c} genes. To test this, we first analyzed SNP microarray data and found amplifications of cdk6 in both a GBM cell line and xenograft with wild-type p18\textsuperscript{INK4c} (Supplemental Fig. 6A). Next, we expanded the analysis by employing qPCR to measure cdk6 copy number in a panel of 51 GBM primary tumors, and in this way identified an additional sample with cdk6 amplification (Supplemental Fig. 6B).

Lentiviral reconstitution of p18\textsuperscript{INK4c} expression in GBM cells leads to G1 cell cycle arrest. To determine the phenotypic consequences of p18\textsuperscript{INK4c} deletion in GBM cells, we created a p18\textsuperscript{INK4c}-expressing lentivirus as described in Materials and Methods. Next, we infected five GBM cell lines - LN229, U87MG, T98G, SNB19 (each deleted for p18\textsuperscript{INK4c}), and M059J (wild-type p18\textsuperscript{INK4c}) - with either empty vector or p18\textsuperscript{INK4c} virus. Infected cells were studied by Western blot (Fig. 4A), flow cytometry/BrdU incorporation
Identification of p18\textsuperscript{INK4c} as a GBM Tumor Suppressor Gene

(Supplemental Fig. 7 and Fig. 4B), phase-contrast microscopy (Fig. 4C), and staining for senescence-associated β-galactosidase activity (Fig. 4D).

Infection with the p18\textsuperscript{INK4c} lentivirus led to a physiological level of expression, comparable to the endogenous levels of expression found in M059J GBM cells harboring an intact p18\textsuperscript{INK4c} gene (Fig. 4A). Importantly, ectopic expression of p18\textsuperscript{INK4c} led to a rapid and complete senescence-like G1 cell cycle arrest in cells with homozygous deletions of p18\textsuperscript{INK4C}, but not in cells with an intact p18\textsuperscript{INK4C} gene (Fig. 4B-D, Supplemental Fig. 7, and data not shown). Interestingly, expression of p18\textsuperscript{INK4c} in p18\textsuperscript{INK4c}-deficient SNB19 cells led to rapid and complete cell death (Fig. 4C).
Discussion

Here we identify p18\textsuperscript{INK4c} as a new GBM tumor suppressor gene by describing homozygous deletions in GBM cell lines, xenografts, and primary tumors, and complete loss of expression in 43% of GBMs studied by immunohistochemistry. Furthermore, we show that re-expression of p18\textsuperscript{INK4c} at physiological levels in GBM cells that lack it leads to immediate senescent-like arrest in the G1 phase of the cell cycle.

p18\textsuperscript{INK4c} is a member of the INK4 family of cyclin-dependent kinase inhibitors, which includes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. Members of this family bind to cdk4 and cdk6 and inhibit their ability to bind to D-type cyclins, thereby inhibiting the formation of an active cdk/cyclin complex and leading to cell cycle arrest. Deletions at the p18\textsuperscript{INK4c} locus have previously been implicated in the pathogenesis of other tumor types, including those of the brain (12,15-18). Intriguingly, loss of both p18\textsuperscript{INK4c} and PTEN (both GBM tumor suppressors) has been shown to have synergistic effects on tumor formation in mice (19).

INK4 family members differ from each other in their patterns of expression and in the potency with which they bind individual cyclin/cdk complexes. p18\textsuperscript{INK4c} is thought to bind most potently to cdk6-specific complexes, though there is conflicting data on this point (11). The phenotypic consequences of binding to and inhibiting cdk4 and cdk6 with differing affinities are not well understood.

We have demonstrated that the presence of admixed non-neoplastic cells and intratumoral heterogeneity complicates the efficient identification of p18\textsuperscript{INK4c} deletions in uncultured primary tumors using conventional technologies. However, it is also a formal possibility that the greater frequency of p18\textsuperscript{INK4c} deletions in cell lines and xenografts are artifacts of \textit{ex vivo} culture.
It is also notable that the same GBM samples harboring heterogeneous deletions of \( p18^{\text{INK4c}} \) also often harbor remarkably homogeneous deletions of \( p16^{\text{INK4a}} \). This finding was important as it enabled us to rule out issues of sample quality as a trivial explanation for our finding of heterogeneity. Furthermore, it suggests that homozygous deletion of \( p16^{\text{INK4a}} \) is an early event in the pathogenesis of GBM, whereas inactivation of \( p18^{\text{INK4c}} \) appears to occur later in the neoplastic process.

\( p18^{\text{INK4c}} \) appears to be inactivated in GBM predominantly by homozygous deletion. This is similar to the situation for \( p16^{\text{INK4a}} \), in which homozygous deletion is the major mechanism of inactivation in GBM (though point mutations in \( p16^{\text{INK4a}} \) also occur, albeit at a lower frequency) (20). In the case of \( p16^{\text{INK4a}} \), this has been rationalized by suggesting that there is selection pressure for loss of \( p14^{ARF} \) as well. It is possible that there is similar selection pressure for simultaneous co-deletion of \( p18^{\text{INK4c}} \) and FAF1 (or an adjacent as yet uncharacterized gene or non-coding RNA) during the pathogenesis of GBM.

In summary, here we have identified \( p18^{\text{INK4c}} \) as a tumor suppressor gene that is genetically inactivated by homozygous deletion during the pathogenesis of GBM. Additional detailed studies are warranted to identify the phenotypic consequences of \( p18^{\text{INK4c}} \) deletion during the pathogenesis of GBM.
Acknowledgements

We thank Michelle Lombard and Karen Cresswell for assistance with flow cytometry, and Aaron Foxworth and Syid Abdullah for assistance with animal husbandry. We also thank Marcela White of the Brain Tumor Tissue Bank at the London Health Sciences Centre in Ontario, Canada for her assistance with the procurement of high quality GBM specimens, and Yardena Samuels for her comments on the manuscript.

The results published here are in part based upon data generated by The Cancer Genome Atlas pilot project established by the NCI and NHGRI. Information about TCGA and the investigators and institutions who constitute the TCGA research network can be found at http://cancergenome.nih.gov.
References


Figure Legends

Figure 1. Homozygous deletions of p18\textsuperscript{INK4c} in GBM. (A) Copy number analysis of Affymetrix 250K SNP microarray data demonstrates focal homozygous deletions of chromosome 1p in four GBM cell lines (left panel) and three GBM xenografts (right panel) but not in normal human astrocytes (NHAs). (B) PCR on genomic DNA derived from GBM cell lines confirms the presence of homozygous deletions in all four GBM cell lines with putative deletions (SNB19 not shown). (C) Copy number analysis of Affymetrix SNP microarray data demonstrates p18\textsuperscript{INK4c} deletions in five uncultured primary GBM tumors. The raw SNP microarray data on the primary tumors was generated as part of The Cancer Genome Atlas.

Figure 2. Heterogeneity of p18\textsuperscript{INK4c} deletions in GBM. (A) Copy number analysis of Affymetrix SNP microarray data demonstrates deletion of p18\textsuperscript{INK4c} in a first-passage xenograft that is largely obscured by admixed normal cells in the primary tumor from which it was derived. (B) Copy number analysis of the p16\textsuperscript{INK4a} gene in cell lines (left panel), xenografts (middle panel), and primary tumors (right panel) harboring deletions of p18\textsuperscript{INK4c} reveals striking homogeneity with regard to p16\textsuperscript{INK4a} deletion in all sample types.

Figure 3. Loss of p18\textsuperscript{INK4c} Expression in GBM Primary Tumors. IHC to GBM tissue microarrays was performed as described in Materials and Methods. (A) Representative GBMs that (i) express p18\textsuperscript{INK4c}, (ii) fail to express p18\textsuperscript{INK4c}, (iii) express p16\textsuperscript{INK4a}, (iv) fail to express p16\textsuperscript{INK4a}. (v) Representative staining for α-tubulin. (B) Bar graph depicting the percentage of samples lacking expression of p18\textsuperscript{INK4c}, p16\textsuperscript{INK4a}, and α-tubulin.
Fig. 4. Reconstitution of p18\textsuperscript{INK4c} Leads to Senescence in GBM Cells. (A) Western blot for p18\textsuperscript{INK4c} 24 h. post-infection demonstrates lentiviral reconstitution of p18\textsuperscript{INK4c} expression in p18\textsuperscript{INK4c}-null LN229, U87MG, and T98G GBM cells (SNB19 not shown). The level of reconstituted expression is only slightly higher than the endogenous levels present in p18\textsuperscript{INK4c}-proficient M059J cells. (B) Cell cycle distributions 60 h. post-infection with control or p18\textsuperscript{INK4c}-expressing lentiviruses. Infection with p18\textsuperscript{INK4c} lentivirus causes G1 cell cycle arrest in p18\textsuperscript{INK4c}-deleted LN229 and T98G cells, but not in p18\textsuperscript{INK4c}-proficient M059J cells. (C) Phase contrast microscopy of cells seven days post-infection demonstrates that reconstitution of p18\textsuperscript{INK4c} expression in p18\textsuperscript{INK4c}-deficient LN229 and T98G cells leads to morphological changes resembling senescence, whereas expression of p18\textsuperscript{INK4c} in SNB19 cells leads to frank cell death. (D) Reconstitution of p18\textsuperscript{INK4c} expression in LN229 cells leads to induction of senescence-associated β-galactosidase activity. Similar results were observed in T98G cells but not in p18\textsuperscript{INK4c}-proficient M059J cells (not shown).
Identification of p18^{INK4c} as a GBM Tumor Suppressor Gene

Figure 1

A

B

C

NHAS, LN229, T98G, U87MG, SNB19, xenograft 245, xenograft 263, xenograft 368

Position along ch 1 in Mb

0 2 4 : copy number

FAF1, p18^{INK4c}

exon 1 of p18^{INK4c}

distal region on ch 1

T172, LN118, CCF-STTG1, T98G, DBTRG-05MG

normal, TCGA-02-0028, TCGA-02-0074, TCGA-06-0137, TCGA-06-0168, TCGA-06-0241
Identification of p18\textsuperscript{INK4c} as a GBM Tumor Suppressor Gene

Figure 3

A

p18\textsuperscript{INK4c}

\begin{enumerate}
\item[i] \hspace{1cm} ii
\end{enumerate}

p16\textsuperscript{INK4a}

\begin{enumerate}
\item[iii] \hspace{1cm} iv
\end{enumerate}

\textit{\alpha}-tubulin

\begin{enumerate}
\item[v]
\end{enumerate}

B

\begin{enumerate}
\item \textbf{P18 INK4c}
\item \textbf{P16 INK4a}
\item \textbf{\textit{\alpha}-tubulin}
\end{enumerate}

% LOSS

\begin{enumerate}
\item 0 \hspace{1cm} 20 \hspace{1cm} 40 \hspace{1cm} 60 \hspace{1cm} 80
\end{enumerate}
Identification of p18\textsuperscript{INK4c} as a GBM Tumor Suppressor Gene

Figure 4

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Panel A: Western blot analysis for p18\textsuperscript{INK4c}

Panel B: Cell cycle analysis

Panel C: Morphological changes in glioblastoma cell lines

Panel D: Confocal microscopy of LN229 cells with/without p18\textsuperscript{INK4c}
Identification of p18\(^{\text{INK4c}}\) as a GBM Tumor Suppressor Gene

Supplemental Figure Legends

Supplemental Figure 1. Identification of the Minimal Region of Deletion. Detailed analysis of SNP microarray probeset data reveals the common area of deletion on chromosome 1 to be 51.166 Mb to 51.222, which includes a single gene – p18\(^{\text{INK4c}}\).

Supplemental Figure 2. qPCR confirms copy number reduction of p18\(^{\text{INK4c}}\) in GBM samples. (A) Examples of qPCR traces from normal human astrocytes (NHAs) and two GBM samples for p18\(^{\text{INK4c}}\) exon 1 (top panel) and a region on chromosome 9 without copy number alteration (bottom panel). (B) Copy number estimate at the p18\(^{\text{INK4c}}\) locus by SNP microarray and qPCR analysis for NHAs and those GBM cell lines and xenografts with identified deletions of p18\(^{\text{INK4c}}\).

Supplemental Figure 3. Expression of p18\(^{\text{INK4c}}\) in GBM Cells. Western blots using antibodies to p18\(^{\text{INK4c}}\) confirms that each of the four cell lines with homozygous deletions of p18\(^{\text{INK4c}}\) fails to express p18\(^{\text{INK4c}}\) protein. All of the cell lines with deletions of p18\(^{\text{INK4c}}\) also fail to express p16\(^{\text{INK4a}}\), demonstrating that inactivation of these related INK4 family members is not mutually exclusive during the pathogenesis of GBM.

Supplemental Figure 4. Clinical and Pathological Details of The Cancer Genome Atlas Samples. Information available for the primary tumor samples harboring deletions of p18\(^{\text{INK4c}}\) is shown.

Supplemental Figure 5. Sequencing of p18\(^{\text{INK4c}}\) in GBM Samples. Analysis of sequence traces from 51 primary tumors, 14 xenografts, and 17 cell lines revealed the presence of a common synonymous SNP present in both tumor and normal DNAs (shown) but no somatic or non-synonymous alterations.
Supplemental Figure 6. Amplification of cdk6 in p18\textsuperscript{INK4c}-Proficient GBM Cells and Tumors. (A) Copy number analysis of Affymetrix SNP microarray data demonstrates genomic amplifications of cdk6 in GBM xenograft 561 and cell line CCF-STTG1 but not in normal human astrocytes (NHAs). (B) qPCR confirms the amplifications in xenograft 561 and cell line CCF-STTG1, identifies an additional amplification in primary tumor 1118, and identifies several additional samples with copy number gains.

Supplemental Figure 7. Reconstitution of p18\textsuperscript{INK4c} Leads to G1 Cell Cycle Arrest in GBM Cells. Flow cytometry 60 h. post-infection reveals that the p18\textsuperscript{INK4c} lentivirus causes G1 cell cycle arrest in p18\textsuperscript{INK4c}-deleted LN229 and T98G cells, but not in p18\textsuperscript{INK4c}-proficient M059J cells.
### Supplemental Figure 1

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Identification of p18\textsuperscript{NK4c} as a GBM Tumor Suppressor Gene

Supplemental Figure 2

A

p18\textsuperscript{NK4c} exon 1

Relative fluorescent units

0 5 10 15 20 25 30 35 40 45 50
Cycle number

NHAs

xenograft 245

LN229

chromosome 9 region without copy number alteration

Relative fluorescent units

0 5 10 15 20 25 30 35 40 45 50
Cycle number

NHAs

xenograft 245

LN229

B

Copy number estimate at the p18\textsuperscript{NK4c} locus

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Supplemental Figure 3

Identification of $p18^{INK4c}$ as a GBM Tumor Suppressor Gene
Identification of p18<sup>INK4c</sup> as a GBM Tumor Suppressor Gene

Supplemental Figure 4

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sequence traces of p18^INK4c exon 2

base 5912, Gly114
primary tumor 1209 homozygous C/C
primary tumor 785 heterozygous C/T
Identification of p18\textsuperscript{NK4c} as a GBM Tumor Suppressor Gene

Supplemental Figure 6

A

B

CCF-STTG1, 43.6 +/- 6.5
xenograft 561, 8.7 +/- 1.0
primary tumor 1118, 42.6 +/- 4.8
Identification of $p18^{\text{NK4c}}$ as a GBM Tumor Suppressor Gene

Supplemental Figure 7

[Diagram showing flow cytometry data for LN229, T98G, and M059J cells with and without $p18^{\text{NK4c}}$.]
Conspirators in a Capital Crime: 

Co-Deletion of $p18^{\text{INK}4c}$ and $p16^{\text{INK}4a/p14\text{ARF}/p15^{\text{INK}4b}}$ in Glioblastoma Multiforme

David A. Solomon$^1$, Jung-Sik Kim$^1$, Walter Jean$^2$, and Todd Waldman$^1$

$^1$Department of Oncology, Lombardi Comprehensive Cancer Center, and
$^2$Department of Neurosurgery, Georgetown University School of Medicine, Washington, DC


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Abstract

Glioblastoma multiforme (GBM) is one of the most dreaded cancer diagnoses due to the limited treatment options and poor prognosis. Homozygous deletion of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus is among the most common genetic alterations in GBM. Two recent studies have demonstrated that deletion and mutation of another INK4 family member, p18\textsuperscript{INK4c}, also drives the pathogenesis of GBM. This minireview will discuss the known roles for p18\textsuperscript{INK4c} in the initiation and progression of cancer and suggest opportunities for future studies.
Glioblastoma multiforme (GBM) is one of the most devastating human cancers, notorious for its fast growing nature, infiltrative growth, resistance to radiotherapy, and the rapid progression from diagnosis to death (1). Homozygous deletion of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus on chromosome 9p21.3 is a signature genetic event that drives the pathogenesis of GBM (2,3). Deletion of this locus is thought to be strongly selected for during the pathogenesis of GBM and other tumor types because it simultaneously inactivates three tumor suppressor genes - the p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} cyclin-dependent kinase inhibitors (CDKIs) and p14\textsuperscript{ARF}, which modulates the activity of the p53 tumor suppressor (4,5).

p16\textsuperscript{INK4a} is the prototype member of the INK4 family of CDKIs, which includes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. These four proteins have approximately 30% amino acid sequence similarity and share potent cyclin-dependent kinase (cdk) inhibitory activity, but are thought to differ from each other in their patterns of expression and their relative affinities for different cdk5s. Of the four genes, p16\textsuperscript{INK4a} plays the most prominent role as a tumor suppressor gene in human cancer, as it is deleted or mutated in the majority of common human tumor types and the cause of an inherited cancer predisposition syndrome. In contrast, p15\textsuperscript{INK4b} and p18\textsuperscript{INK4c} are thought to play more limited roles as human tumor suppressors, and p19\textsuperscript{INK4d} is thought not to be involved in cancer pathogenesis.

Two recent studies have demonstrated that deletions and mutations of p18\textsuperscript{INK4c}, together with deletions and mutations of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} genes, drive the pathogenesis of human GBM (6,7). When taken together with other prior studies identifying deletions and mutations of p18\textsuperscript{INK4c} in several other tumor types and demonstrating that p18\textsuperscript{INK4c}-deficient mice are tumor prone, these recent studies have
suggested that inactivation of p18\textsuperscript{INK4c} may play a perhaps underappreciated role in human cancer pathogenesis. As such, this review will provide a brief history of the discovery of p18\textsuperscript{INK4c}, summarize the studies that link it to cancers in mice and humans, and provide a forward-looking assessment of the demonstrated role of p18\textsuperscript{INK4c} inactivation in the pathogenesis of GBM.

**Discovery of p18\textsuperscript{INK4c}**. The human p18\textsuperscript{INK4c} gene was initially discovered in 1994 by Guan et al. in a yeast two-hybrid screen to identify proteins that interacted with human cdk6 (8). The mouse homolog was reported the following year by Hirai et al. (9). Human p18\textsuperscript{INK4c} is a 168 amino acid cdk-interacting protein with multiple ankyrin repeats and substantial similarity to both human Notch and the yeast CDKI PHO8. p18\textsuperscript{INK4c} bound with high affinity to cdk6, with intermediate affinity to cdk4, and did not bind to cdk2. This discovery of a new p16\textsuperscript{INK4a} homolog immediately raised the exciting possibility that p18\textsuperscript{INK4c} might be a new tumor suppressor gene commonly inactivated in wide range of human cancers. However, initial genetic analysis of p18\textsuperscript{INK4c} status in >100 human tumor samples derived from >20 different tissues failed to identify homozygous deletions and found only a single sample with a putative somatic missense mutation (10).

Detailed biochemical studies provided further information on the role of p18\textsuperscript{INK4c} in inhibition of cdks. Initial studies suggested that p18\textsuperscript{INK4c} preferentially inhibited cdk6-containing complexes whereas p16\textsuperscript{INK4a} preferentially inhibited cdk4-containing complexes (8)(8). This suggested that despite their status as homologs, these proteins have evolved for the purpose of providing subtly distinct negative regulation of cdks in the human cell cycle. However, this notion that INK4 proteins have distinct binding specificities for cdk4 and cdk6 remains somewhat controversial (11).
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**Mouse Models of p18\textsuperscript{INK4c} Deficiency Demonstrate Its Role as a Potent Tumor Suppressor.** In contrast to the early data suggesting that p18\textsuperscript{INK4c} might play a limited role in human cancer pathogenesis, data from knockout mouse studies have demonstrated a clear role for p18\textsuperscript{INK4c} in cellular proliferation, organ size, and cancer. p18\textsuperscript{INK4c}-deficient mice are viable but demonstrate a variety of intriguing phenotypes. Franklin et al. reported that p18\textsuperscript{INK4c}-deficient mice displayed pronounced gigantism, organomegaly, lymphoma, and pituitary hyperplasia; phenotypes that were strikingly reminiscent of those seen in Rb\textsuperscript{-/-} mice (12). Shortly thereafter, Latres et al. reported that p18\textsuperscript{INK4c}-deficient mice also displayed deregulated epithelial cell growth in the kidneys and breast (13). Interestingly, Bai et al. reported that p18\textsuperscript{INK4c} heterozygous (+/-) knockout mice developed tumors at an accelerated rate when treated with a chemical carcinogen. These tumors retained the remaining allele of p18\textsuperscript{INK4c}, demonstrating that p18\textsuperscript{INK4c} is a haploinsufficient tumor suppressor in mice (14).

Subsequent studies in which p18\textsuperscript{INK4c}-deficient mice were crossed with mice harboring targeted deletions of other tumor suppressor genes uncovered additional roles for p18\textsuperscript{INK4c} in cancer pathogenesis. A subset of these studies are described here. Mice lacking both p18\textsuperscript{INK4c} and p53 were predisposed to medulloblastomas, hemangiosarcomas, and other tumors not present in either of the parental strains (15). Deletion of p18\textsuperscript{INK4c} in Ptc1\textsuperscript{1-/-} mice led to the rapid development of medulloblastoma with high penetrance, and further examination of these and related mice indicated that p18\textsuperscript{INK4c} plays an important role in normal cerebellar development (16-18). Deletion of p18\textsuperscript{INK4c} in PTEN\textsuperscript{1-/-} mice led to an enhanced predisposition to a variety of tumors when compared to mice with deletion of either gene alone, suggesting that these genes could synergize during the development of human malignancies harboring inactivation of both...
genes (such as GBM) (19). Finally, mice lacking both p18\textsubscript{INK4c} and p16\textsubscript{INK4a} developed unusually aggressive pituitary tumors, suggesting that these GBM-related genes also synergize in tumor formation and suggesting that these INK4 family members could at least partially compensate for each other (20).

Evidence that p18\textsubscript{INK4c} is a Tumor Suppressor Gene in Human Cancer.

Despite the early negative results in human cancer samples, the potential promise of p18\textsubscript{INK4c} as a human tumor suppressor gene and the mouse studies implicating p18\textsubscript{INK4c} as a bona fide tumor suppressor gene prompted several groups to continue to search for homozygous deletions and inactivating point mutations in a variety of common human cancer types. These efforts continued to prove largely frustrating, in that deletions/point mutations of p18\textsubscript{INK4c} were identified rarely, albeit in a range of human tumor types including adenocarcinoma of the breast, multiple myeloma, acute lymphoblastic leukemia, oligodendroglioma, and meningioma (10,21-30). Epigenetic inactivation of p18\textsubscript{INK4c} in human medulloblastoma has also been reported (16).

Of these tumor sample types, cell lines derived from multiple myeloma (MM) harbored by far the highest frequency of p18\textsubscript{INK4c} deletions – approximately 30%. Two studies in particular highlighted the frequency and impact of p18\textsubscript{INK4c} deletion in MM. In 2002, Kulkarni \textit{et al.} identified homozygous deletions of p18\textsubscript{INK4c} in 6 of 16 MM cell lines and demonstrated that these deletions often occurred in tumor cells also harboring deletions of the p16\textsubscript{INK4a}/p14\textsubscript{ARF}/p15\textsubscript{INK4b} locus (23). In 2006, Dib \textit{et al.} confirmed and extended this work, identifying homozygous deletions of p18\textsubscript{INK4c} in 13 of 40 MM cell lines, but in only 2-10% of primary tumor samples (24). There were two opposing possible explanations for these data - the worrisome possibility that the majority of these p18\textsubscript{INK4c} deletions in MM cell lines could be artifacts of \textit{ex vivo} culture, and the exciting
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possibility that deletions of $p^{18\text{INK}4c}$ could be present in a similarly high fraction of primary tumor samples as well but that their presence was masked by intratumoral heterogeneity. These same issues would re-emerge in the more recent studies implicating $p^{18\text{INK}4c}$ in the pathogenesis of GBM.

**Identification of Co-Deletion of $p^{18\text{INK}4c}$ and $p^{16\text{INK}4a/p14\text{ARF}/p15\text{INK}4b}$ in Human Glioblastoma Multiforme.** In April 2008, Solomon *et al.* and Wiedemeyer *et al.* reported homozygous deletions of $p^{18\text{INK}4c}$ in glioblastoma multiforme (6,7). These deletions were identified using copy number analysis in GBM cell lines, xenografts, and primary tumors. Notably, Wiedemeyer *et al.* also identified three missense mutations of $p^{18\text{INK}4c}$ that interfered with binding to cdk6. There were two notable similarities between the data presented in these studies and those previously reported for MM. First, while $p^{18\text{INK}4c}$ deletions were identifiable in GBM primary tumor samples, they were most commonly found in GBM cell lines and xenografts. Second, as in MM, GBM samples with deletions of $p^{18\text{INK}4c}$ often also harbored deletions of the $p^{16\text{INK}4a/p14\text{ARF}/p15\text{INK}4b}$ locus. Follow up functional studies clearly demonstrated that $p^{18\text{INK}4c}$ could play a tumor suppressor role in human and murine GBM, and Wiedemeyer *et al.* suggested that $p^{18\text{INK}4c}$ could functionally compensate for $p^{16\text{INK}4a}$ homozygous deletions during the pathogenesis of GBM.

**Why the Disparity in $p^{18\text{INK}4c}$ Deletions Between Cultured and Uncultured GBMs?** One clear result of the GBM and MM studies is that deletions of $p^{18\text{INK}4c}$ are much easier to identify in cultured samples than in uncultured samples. There are at least two possible explanations for this disparity.

One possibility is that deletions of $p^{18\text{INK}4c}$ are more easily identifiable in cultured samples because *ex vivo* growth enriches for $p^{18\text{INK}4c}$-deficient cells already present in
Co-deletion of \( p18^{INK4c} \) and \( p16^{INK4a/p14ARF/p15INK4b} \) in GBM

the tumor. In fact, Solomon et al. demonstrated just this – whereas one primary GBM tumor they studied clearly harbored a deletion of \( p18^{INK4c} \), identification of the deletion was obscured by intratumoral heterogeneity. However, that same deletion was easily identifiable in a first-passage xenograft derived from that tumor (6). That same primary tumor was homogeneous for deletion of the \( p16^{INK4a/p14ARF/p15INK4b} \) locus, providing support for the notion that genetic heterogeneity and not the presence of admixed non-neoplastic cells obscured detection of the \( p18^{INK4c} \) deletion in the primary tumor. Of note, it appears unlikely that the genetic heterogeneity displayed by these and other heterogeneous deletions was due to true haploinsufficiency, since all \( p18^{INK4c} \) deletions identified by Solomon et al. and Wiedemeyer et al. in genetically homogenous cell lines appeared to be biallelic/homozygous. Based on these types of data and immunohistochemistry studies identifying loss of \( p18^{INK4c} \) expression in approximately 40% of GBMs, Solomon et al. suggested that deletions of \( p18^{INK4c} \) could be common in GBM primary tumors, but that intratumoral heterogeneity might obscure their easy identification.

However, it is also a formal possibility that the deletions of \( p18^{INK4c} \) identified by Solomon et al. and Wiedemeyer et al. in cultured GBM samples could have occurred de novo during ex vivo culture, and as such represent true culture artifacts. Importantly, however, both groups clearly identified deletions of \( p18^{INK4c} \) in both cultured and uncultured samples, providing strong evidence that deletions of \( p18^{INK4c} \) are not artifacts of culture. Furthermore, it is arguable that any putative cancer-related gene has been shown to be deleted/mutated only in samples grown in an ex vivo setting, and some believe that the enthusiasm for the idea that ex vivo culture leads to artifactual genetic lesions exceeds the data to support it.
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**Homogeneity of p16\textsuperscript{INK4a/p14ARF/p15INK4b} Deletions in GBM.** A related point brought up by Solomon *et al.* is that unlike the heterogeneous deletions of p18\textsuperscript{INK4c}, GBMs are remarkably homogeneous with regard to deletions of the p16\textsuperscript{INK4a/p14ARF/p15INK4b} locus. Emerging copy number analyses of GBM samples makes it obvious that deletion of this locus is by far the most common homozygous deletion present in GBM (in >75% of samples), and that genetically heterogeneous uncultured primary GBMs tend to be homogeneous for deletion of this locus. This degree of homogeneity suggests that deletion of the p16\textsuperscript{INK4a/p14ARF/p15INK4b} locus is unusually fundamental to the pathogenesis of GBM. And, when taken together with the heterogeneity of deletions in p18\textsuperscript{INK4c}, suggests that deletion of the p16\textsuperscript{INK4a/p14ARF/p15INK4b} locus is an early event in the pathogenesis of GBM, whereas deletion of p18\textsuperscript{INK4c} is likely to be a later event associated with progression of GBM (see Fig. 1). These data further suggest that deletion of the p16\textsuperscript{INK4a/p14ARF/p15INK4b} locus could even be the initiating event in GBM, as this would simultaneously explain the remarkable homogeneity of these deletions and the shockingly explosive growth of primary GBM.

**Possible Explanations for Co-Deletion of the p18\textsuperscript{INK4c} Gene and the p16\textsuperscript{INK4a/p14ARF/p15INK4b} Locus in GBM.** One intriguing observation is that deletions of p18\textsuperscript{INK4c} generally co-exist in the same GBM cells also harboring deletions of the p16\textsuperscript{INK4a/p14ARF/p15INK4b} locus. There are several possible explanations for this surprising finding. Wiedemeyer *et al.* demonstrated that depletion of p16\textsuperscript{INK4a} led to transcriptional upregulation of p18\textsuperscript{INK4c} by the E2F1 transcription factor, suggesting that p18\textsuperscript{INK4c} is induced to compensate for deletion of p16\textsuperscript{INK4a}, and that this provides selective pressure for the loss of p18\textsuperscript{INK4c} (7,31). Such a model might be considered a “serial model,” in that
there is a linear mechanistic relationship between the tumor suppression activities of these two genes. However, it is also worth noting that: (a) deletions of p16\textsuperscript{INK4a} are at least twice as common in GBM than deletions of p18\textsuperscript{INK4c}, (b) GBMs are quite heterogeneous for deletions of p18\textsuperscript{INK4c} yet remarkably homogeneous for deletions of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus, and (c) the CDKIs encoded by these loci probably have distinct specificities for binding to different cdks. These observations suggest that INK4 genes could play biologically distinct roles in the initiation (p16\textsuperscript{INK4a}) and progression (p18\textsuperscript{INK4c}) of GBM. Such a model might be termed a “parallel model,” in that inactivation of these genes is mechanistically unrelated and loss of each gene has distinct biochemical and phenotypic sequelae during the pathogenesis of GBM.

**Conclusions and Future Directions.** Numerous studies have now implicated p18\textsuperscript{INK4c} as a tumor suppressor whose genetic inactivation plays a role in the pathogenesis of glioblastoma multiforme, multiple myeloma, and a variety of other common cancers. Deletions of p18\textsuperscript{INK4c} are clearly less common than deletions of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus and appear to demonstrate substantial intratumoral heterogeneity. They often occur in tumors also harboring deletions of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus but probably occur later in tumorigenesis, raising fundamental questions about the relative contributions of these two loci to the initiation and progression of human neoplasia. Further studies are clearly warranted to better define the relative biochemical and phenotypic roles of these INK4 family members in the pathogenesis of glioblastoma multiforme and other human tumor types.
References


Figure Legend

Figure 1. Depiction of sequential INK4 inactivation during GBM progression resulting in a tumor with homogeneous loss of p16\textsuperscript{INK4a} and heterogeneous loss of p18\textsuperscript{INK4c}.
Co-deletion of $p18^{\text{INK4c}}$ and $p16^{\text{INK4a}}/p14^{\text{ARF}}/p15^{\text{INK4b}}$ in GBM

**Figure 1**

- Astrocyte or precursor
- $p16^{\text{INK4a}}$ loss
- Initial INK4 inactivation

- $p18^{\text{INK4c}}$ loss
- Other genetic lesions

- Subsequent INK4 inactivation

- Heterogeneous tumor
Mutational Inactivation of PTPRD in Glioblastoma Multiforme and Malignant Melanoma

David A. Solomon¹, Jung-Sik Kim¹, Julia C. Cronin², Zita Sibenaller³, Timothy Ryken³, Steven A. Rosenberg⁴, Habtom Ressom¹, Walter Jean⁵, Darell Bigner⁶, Hai Yan⁶ Yardena Samuels², Todd Waldman¹

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC
²Cancer Genetics Branch, National Human Genome Research Institute, Bethesda, MD
³Department of Neurosurgery, University of Iowa College of Medicine, Iowa City, IA
⁴Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD
⁵Department of Neurosurgery, Georgetown University School of Medicine, Washington, DC
⁶Department of Pathology, Preston Robert Tisch Brain Tumor Center, Duke University School of Medicine, Durham, NC


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Abstract

An additional tumor suppressor gene on chromosome 9p telomeric to the CDKN2A/B locus has long been postulated to exist. Using Affymetrix 250K SNP arrays to screen for copy number changes in glioblastoma multiforme (GBM), we detected a high frequency of deletions of the PTPRD gene, which encodes a receptor protein tyrosine phosphatase at chromosome 9p23-24.1. Missense and nonsense mutations of PTPRD were identified in a subset of the samples lacking deletions, including an inherited mutation with somatic loss of the wild-type allele. We then sequenced the gene in melanoma and identified 10 somatic mutations in 7 out of 47 tumors (12%). Reconstitution of PTPRD expression in GBM and melanoma cells harboring deletions or mutations led to growth suppression and apoptosis that was alleviated by both the somatic and constitutional mutations. These data implicate PTPRD in the pathogenesis of tumors of neuroectodermal origin and, when taken together with other recent reports of PTPRD mutations in adenocarcinoma of the colon and lung, suggest that PTPRD may be one of a select group of tumor suppressor genes that are inactivated in a wide range of common human tumor types.
**Introduction**

Cancer is a genetic disease that results from the disruption of signaling pathways that regulate cellular proliferation, differentiation, and programmed cell death. Though it was originally hoped that there would be a small, finite number of genes controlling these signaling pathways whose dysregulation was common to many tumor types, most current research supports the notion that the majority of cancer-causing genes contribute to neoplasia at low frequency and in a limited tumor spectrum (1). Nonetheless, the discovery of new oncogenes and tumor suppressor genes commonly altered during tumorigenesis remains a major goal of modern cancer research, since such genes and the pathways they control are the most exciting potential targets for anticancer drug development.

Constitutive activation of tyrosine phosphorylation signaling pathways is one biochemical hallmark of cancer. This is most well known to occur via activation of tyrosine kinase receptors, such as amplification HER2/Neu in breast cancer and mutation of EGFR in lung cancer. However, given the obvious importance of constitutive activation of tyrosine kinase signaling to human neoplasia, one might expect to find inactivation of protein tyrosine phosphatases (PTPs) in human tumors as well. Though inactivating mutations of individual PTPs have recently been reported in human colon cancer (2), at present there is no single tyrosine phosphatase thought to play a generally important role as a tumor suppressor gene in multiple tumor types.

PTPRD is one of 21 known human receptor-type PTPs, a group of genes which are increasingly thought to be important in cancer development and progression (for reviews see 3,4). Deletions of PTPRD in human cancer cell lines were first identified by Cox *et al.* in 2005 (5). Subsequent studies have reported homozygous deletions of
PTPRD in multiple human tumor types (6-11), and missense mutations of unknown functional significance have recently been reported in adenocarcinoma of the colon and lung (11,12).

Here we identify frequent deletion and mutation of PTPRD in glioblastoma multiforme and malignant melanoma and demonstrate that these mutations are inactivating. These data provide the first functional evidence that PTPRD is a tumor suppressor gene and, when taken together with other recent studies identifying mutations in adenocarcinoma of the colon and lung, suggest that inactivation of PTPRD contributes to the pathogenesis of a wide range of common human cancers.
Materials and Methods

**Tumor tissues.** A panel of 21 GBM cell lines were obtained from the American Type Culture Collection (U87MG, U138MG, M059J, Hs683, H4, A172, LN18, LN229, CCF-STTG1, T98G, DBTRG-05MG), DSMZ (8MGBA, 42MGBA, DKMG, GAMG, GMS10, LN405, SNB19), and the Japan Health Sciences Foundation Health Science Research Resources Bank (AM38, NMC-G1, KG-1-C). Normal human astrocytes (NHAs) were obtained from Clonetics and AllCells. All cell lines were growth in DMEM + 10% FBS at 37° in 5% CO₂.

Subcutaneous xenografts in immunodeficient mice were obtained from the Duke University Brain Tumor Center or created in the Lombardi Comprehensive Cancer Center Animal Shared Resource from tissue taken from patients undergoing craniotomy at Georgetown University Hospital (IRB #2006-344).

Snap frozen primary GBM tumors and paired blood samples were obtained from the Brain Tumour Tissue Bank (London Health Sciences Centre, Ontario, Canada) funded by the Brain Tumour Foundation of Canada. All tumors were graded by a neuropathologist as good or moderate on a scale of good to poor depending on the amount of tumor cells present (as opposed to hemorrhagic, necrotic, or fibrous tissue). All tumor samples were further categorized as “tumor center”.

A panel of 10 primary GBM cell cultures were derived from primary tumor samples at time of surgical resection at the University of Iowa Medical Center by dissociation with collagenase and then cultured in DMEM/F12 containing 15% FBS, 10 ug/mL insulin, and 5 ng/mL bFGF at 37° in 5% CO₂.

A panel of 47 malignant melanoma tumor and paired blood samples were collected during surgical resection at the National Cancer Institute. The primary cell cultures 16T
and 86T used for functional analysis were derived from melanoma tumor samples by
dissociation with collagenase and then cultured in RPMI + 10% FBS at 37° in 5% CO₂.

**Microarrays and bioinformatics.** Genomic DNA derived from GBM tumor
samples was interrogated with Affymetrix 250K Nsp I Human Gene Chip Arrays using
protocols described by the manufacturer. Data processing was performed using dCHIP
(13). The scanned array images and processed data sets have been deposited in the

**Western blot.** Primary antibodies used were PTPRD clone C-18 (Santa Cruz
Biotechnology) and α-tubulin Ab-2 clone DM1A (Neomarkers).

**DNA sequencing.** Individual exons of PTPRD were PCR amplified from genomic
DNA using conditions and primer pairs described by Sjoblom *et al.* (12). PCR products
were purified using the Exo/SAP method followed by a Sephadex spin column.
Sequencing reactions were performed using Big Dye v3.1 (Applied Biosystems) using an
M13F primer, and analyzed on an Applied Biosystems 3730XL capillary sequencer.
Sequences were analyzed using Mutation Surveyor (Softgenetics). Traces with putative
mutations were re-amplified and sequenced from both tumor and matched normal DNA.

**PTPRD Lentivirus.** A wild-type PTPRD cDNA (MGC 119751) was obtained from
Open Biosystems and cloned into the pCDF1-MCS2-EF1-Puro lentiviral expression
vector backbone (System Biosciences). To make virus, this construct was co-transfected
into 293T cells with pVSV-G (Addgene) and pFIV-34N (System Biosciences) helper
plasmids using Fugene 6 (Roche) as described by the manufacturer. Virus-containing
conditioned media was harvested 48 hrs. after transfection, filtered, and used to infect
recipient cells in the presence of 8 ug/mL polybrene.
Site-directed mutagenesis. Mutations identified in GBM and melanoma tumors were engineered into the pCDF1-PTPRD construct by site-directed mutagenesis using the QuikChange II XL kit (Stratagene) as directed by the manufacturer. The coding sequence of all expression vectors was verified by DNA sequencing.

Flow cytometry. Cells were pulsed with 10 µmol/L BrdU for 1 hr, trypsinized, and centrifuged. Cells were fixed and stained using the BrdU Flow Kit (PharMingen) and analyzed by flow cytometry in a BD FACSsort instrument using FCS Express v.3 software (DeNovo Software).

Apoptosis quantification assay. Cells were collected by trypsinization, centrifuged, and simultaneously fixed and stained in a solution containing 3.7% formaldehyde, 0.5% Igepal, and 10 µg/mL Hoechst 33258 in PBS. Fluorescence microscopy was used to visualize and score apoptotic nuclei. At least 200 cells were counted for each determination in triplicate.

Microscopy. All imaging was performed on an Olympus BX61 light microscope with a 40X Plan-Apochromat objective.

Statistical Analysis. Two-tailed unpaired t-test analysis of BrdU incorporation data was performed using GraphPad Prism software.
Results

In an effort to discover genes that contribute to the pathogenesis of GBM, we used Affymetrix 250K Gene Chip Arrays to identify recurrent copy number alterations in a panel of 58 GBM tumor samples (Fig. 1A). Focal deletions of the PTPRD gene on chromosome 9p23-24.1 were among the most prevalent deletions detected, present in 14% of the GBM samples studied (Fig. 1B and Supplementary Table 1). This frequency of focal deletion is higher than that of PTEN (9%) and similar to that of CDKN2C (also named p18INK4c, 16%), a recently identified GBM tumor suppressor gene (14,15). Larger scale loss of the PTPRD gene was present in an additional 33% of the samples (Fig. 1C and Supplementary Table 2). Intriguingly, several studies have suggested the presence of another important tumor suppressor gene on chromosome 9p telomeric to the CDKN2A/B locus in tumor types including astrocytoma, melanoma, and lung adenocarcinoma (16-20). We therefore considered PTPRD to be an attractive candidate as a GBM tumor suppressor gene, and possibly relevant to a range of other tumor types as well.

To determine if PTPRD is genetically altered by mutation during GBM tumorigenesis, we sequenced the 35 coding exons of the PTPRD gene in tumor samples lacking focal deletions of PTPRD and in corresponding normal tissue (when available). This sequence analysis identified somatic mutations of the PTPRD gene in three samples, including two missense mutations and one nonsense mutation (Fig. 2A and Supplementary Fig. 1). Additionally, we identified a heterozygous germline mutation that was accompanied by somatic loss of the wild-type allele in the tumor of a GBM patient with a history of multiple primary malignancies (Fig. 2A,B). This mutation is not a reported SNP, and was not present in any of >100 alleles of PTPRD sequenced during
the course of this study. Together, these data demonstrate that PTPRD is altered by somatic mutation during GBM pathogenesis, and raise the intriguing possibility that germline mutation of PTPRD might lead to a predisposition to the development of GBM and other tumor types.

To determine if mutations of PTPRD were present in a second tumor type proposed to harbor an additional 9p tumor suppressor gene, we sequenced PTPRD in 47 melanoma tumor samples. Two somatic nonsense mutations and eight somatic missense mutations were identified (Table 1, Supplementary Fig. 2, and Supplementary Fig. 3) in a total of seven samples. All of these mutations were C/G>T/A transversions, consistent with UV-induced DNA damage. Additionally, three of the mutations were dinucleotide CC>TT mutations caused by the formation of UV-induced cyclobutane pyrimidine dimers (Supplementary Fig. 3). Three of the seven samples harboring somatic mutations of PTPRD displayed LOH of the wild-type allele. Furthermore, tumor 76T harbored four independent mutations of the gene, increasing the likelihood that both alleles of the gene had been targeted by mutation in this sample. Interestingly, 5 of these 7 samples with PTPRD mutation also harbor activating mutations of either B-Raf or N-Ras (data not shown). This 12% mutation frequency makes PTPRD among the most commonly mutated genes in sporadic melanoma reported to date, which include B-Raf (~60%), p53 (0-25%), N-Ras (10-15%), PTEN (~10%), p16\(^{\text{INK4a}}\) (0-5%), and PIK3CA (<1%) (21,22).

The 14 mutations of PTPRD reported here are distributed roughly evenly throughout the various extracellular and intracellular domains of the encoded PTPRD protein (Fig. 3), though there appears to be a mini-hotspot in the first and second fibronectin type-III repeat.
Mutational Inactivation of PTPRD in GBM and Melanoma

Despite its potential importance, functional data implicating PTPRD deletion or mutation in tumorigenesis are lacking. To determine if PTPRD has the growth suppressing properties expected of a broad spectrum tumor suppressor gene, we examined the functional consequences of reconstituting PTPRD expression in GBM and melanoma cells. A 5.1 kb human PTPRD cDNA was obtained, cloned into a lentiviral expression vector, and packaged into infectious lentivirus as described in Materials and Methods. Infection of H4 cells that harbor biallelic deletion of PTPRD (Fig. 1B and Supplementary Table 1) with lenti-PTPRD led to expression of both the PTPRD proprotein and its mature cleavage products (which then reassemble at the cell membrane to form a heterodimer (23)) (Fig. 4A). Infection of H4 cells with lenti-PTPRD but not vector alone led to a transient growth arrest evidenced by a reduction in BrdU incorporation and an increase in both G1 and sub-G1 cell populations (Fig. 4B). Infection with lenti-PTPRD had a similar effect on 8MGBA cells, which also harbor a focal deletion of PTPRD (Fig. 1B and data not shown).

We next infected two primary melanoma cell cultures harboring homozygous missense mutations of PTPRD (16T and 86T with G446E in the second FN-III domain and V1565I in the first PTPc domain, respectively). Infection of both primary cell cultures with wild-type PTPRD but not vector alone led to significant growth inhibition and decrease in cell viability (Fig. 4C), as well as a substantial, time-dependent increase in apoptotic cells (Fig. 4D). These are the first reported data indicating that PTPRD has growth suppressive properties when expressed in human cancer cells, supporting the hypothesis that PTPRD is a bona fide human tumor suppressor gene.

We next sought to examine the consequences of tumor-derived mutations on PTPRD function in these assays. To do this, five tumor-derived mutations were
introduced into lenti-PTPRD as described in Materials and Methods including two mutations in the second FN_III domain mini-hotspot (one each from GBM and melanoma), one mutation in the first PTPc domain (melanoma), and two mutations flanking the proprotein cleavage site (one each from GBM and melanoma). Initially, H4 cells were infected with wild-type and mutant lenti-PTPRD, protein lysates prepared, and PTPRD expression documented by Western blot. As shown in Fig. 5A, infection of H4 cells with lentivirus expressing either wild-type or mutant PTPRD resulted in similar levels of protein expression. However, there was a marked decrease in growth inhibition as measured by BrdU incorporation, indicating that each of the five tumor-derived mutants alleviated the growth suppression activity of PTPRD, albeit to differing extents (Fig. 5B). Next, 16T melanoma cells were similarly infected with wild-type and mutant PTPRD lentiviruses. As depicted in Fig. 5C, wild-type PTPRD led to apoptosis of approximately 75% of the cells at 10 days post-infection, whereas the mutant PTPRD lentiviruses led to a substantially reduced fraction of cells that had undergone programmed cell death. When taken together, these experiments demonstrate that tumor-derived mutations of PTPRD attenuate its function, confirming that the mutations of PTPRD are likely to be pathogenic.
Discussion

Aberrant regulation of signaling pathways governed by tyrosine phosphorylation is common to virtually all types of human cancer. Whereas activation of tyrosine kinase growth factor receptors by amplification and/or mutation is well established as a major mechanism leading to constitutive tyrosine phosphorylation, the role of inactivation of PTPs in tumorigenesis is comparatively poorly understood. Here we implicate one such PTP as a tumor suppressor gene in two major tumor types and demonstrate that its reconstitution in human cancer cells harboring deletions and mutations leads to cell cycle arrest and apoptosis.

Receptor-type PTPs are transmembrane proteins whose extracellular immunoglobulin-like and fibronectin domains are known to promote cell-cell adhesions (3,4,24,25). It is postulated that these PTPs transduce information regarding cell contacts across the membrane to the intracellular phosphatase domains which control cytoplasmic tyrosine phosphorylation levels appropriate for the current state of cell adhesion. PTPRD is highly expressed in the neuroepithelium during early development of the nervous system (26) – an environment where cell-to-cell contacts are known to be essential for patterning and directing appropriate synaptic connections (27). Accordingly, homozygous knockout of PTPRD in the mouse results in impaired learning, enhanced hippocampal long-term potentiation, and early postnatal lethality due to insufficient food intake (28). Whether heterozygous knockout mice are tumor prone has not yet been established.

Interestingly, PTPRD is also known to be expressed in the adult, with highest levels present in the brain and kidney (23). The function of PTPRD in these tissues after completion of development has not yet been described. Current evidence suggests that
PTPRD is a homophilic cell adhesion molecule, but it is also possible that its extracellular domain binds to other as yet unidentified ligands present in the extracellular space. Furthermore, the substrate(s) of its phosphatase domains remain unidentified (29).

Other than the fact that inactivation of PTPRD is predicted to increase tyrosine phosphorylation of signaling molecules, it is not clear at this time how PTPRD suppresses cancer development and/or progression. It is possible that in the hypercellular microenvironment of a neoplastic lesion, PTPRD senses an increasing abundance of receptor-type PTP molecules present on neighboring neoplastic cells causing activation of its phosphatase domains resulting in decreased tyrosine phosphorylation of signaling molecules that promote cellular proliferation. In this hypothesized model, it is easy to speculate how inactivation of PTPRD tumor suppressive function through deletion or mutation might promote tumorigenesis. It is also possible that PTPRD more simply functions to maintain a normal homeostasis of intracellular tyrosine phosphorylation levels in tissues of the adult organism. In this model, inactivation of PTPRD would lead to the increase in tyrosine phosphorylation of as yet unidentified signaling molecule(s) in a way that promotes proliferation and neoplasia.

In summary, we believe that the identification of deletions and mutations of PTPRD in the wide spectrum of human tumors reported to date, the presence of an inherited mutation in a patient with LOH of the wild-type allele in the tumor, and the functional data presented herein provide strong evidence that PTPRD is a bona fide human tumor suppressor gene. Future studies appear warranted to identify the complete range of tumors in which PTPRD is inactivated, to determine if PTPRD plays a more
general role in inherited cancer predisposition syndromes, and to identify and pharmacologically inhibit the signaling pathway(s) that are activated in cells harboring deletions and mutations of this receptor-type PTP.
Acknowledgements

We thank Marcela White of the Brain Tumor Tissue Bank at the London Health Sciences Centre in Ontario, Canada for her assistance with the procurement of high quality GBM specimens. We also thank Karen Creswell for assistance with flow cytometry.

T.W. is funded by the National Cancer Institute and the American Cancer Society. Y.S. is funded by the National Human Genome Research Institute and The Harry J. Lloyd Charitable Trust.
Mutational Inactivation of PTPRD in GBM and Melanoma

References


Mutational Inactivation of PTPRD in GBM and Melanoma

Figure and Table Legends

Figure 1. PTPRD is deleted at high frequency in GBM. (A) Most frequently deleted genes in 58 GBM tumor samples as determined by Affymetrix 250K SNP microarray analysis. (B) Copy number analysis of SNP microarray data demonstrates focal (<10 Mb) deletions of chromosome 9p23-24.1 in 8/58 GBM samples (four shown) but not in normal human astrocytes (NHAs). x, primary xenograft. (C) Copy number analysis of SNP microarray data demonstrates large-scale (>10 Mb) chromosomal loss of the PTPRD locus in 19/58 GBM samples (five shown). p, primary tumor; c, primary culture.

Figure 2. Identification of somatic and inherited mutations of PTPRD in GBM. (A) One nonsense and three missense mutations were identified in GBM samples. ^Genomic position is based on the hg18 genome assembly. #Transcript ENST00000381196 was used for annotation of the nucleotide and amino acid changes. *Assignment of functional domains was based on UniProtKB/Swiss-Prot P23468-1. FN_III, fibronectin type-III domain. (B) Sequence traces depicting an inherited heterozygous mutation of PTPRD in constitutional DNA (blood) and somatic loss of the wild-type allele in GBM primary tumor p1118.

Table 1. Identification of somatic mutations of PTPRD in malignant melanoma.
Ten somatic mutations of PTPRD were identified in 7 out of 47 malignant melanoma tumor samples. ^Genomic position is based on the hg18 genome assembly. #Transcript ENST00000381196 was used for annotation of the nucleotide and amino acid changes. *Assignment of functional domains was based on UniProtKB/Swiss-Prot P23468-1. Ig_C2, immunoglobulin-like C2-type domain; FN_III, fibronectin type-III domain; PTPc, protein tyrosine phosphatase catalytic domain.
Figure 3. Schematic of the PTPRD protein and the location of all mutations reported to date in human cancer. The mutations in colon cancer and lung cancer were previously reported in references 11 and 12. S, signal peptide; Ig, immunoglobulin-like C2-type domain; FN, fibronectin type-III domain; broken line, cleavage site; PTPc, protein tyrosine phosphatase catalytic domain.

Figure 4. Expression of PTPRD in GBM and melanoma cells harboring deletions and mutations causes growth suppression and apoptosis. (A) Western blot for PTPRD demonstrates reconstitution of PTPRD expression in 8MGBA and H4 GBM cells infected with lenti-PTPRD. Both cell lines harbor focal deletions of PTPRD (Fig. 1B), and no endogenous PTPRD protein was detected in either cell line. The C-18 antibody recognizes a C-terminal epitope present on both the full-length PTPRD proprotein (~175 kDa) and one of the two mature cleavage products (~75 kDa). (B) Flow cytometry of H4 GBM cells at 48 hrs. post-infection reveals that lenti-PTPRD causes a 32% reduction in BrdU incorporation and a 2.5-fold increase in sub-G1 cells. Cell cycle distributions are shown. (C) Phase contrast microscopy of cells ten days post-infection demonstrates that reconstitution of PTPRD expression leads to frank cell death in 16T and 86T melanoma cells harboring homozygous missense mutations of PTPRD (Table 1). (D) Lenti-PTPRD causes a time dependent increase in apoptosis in 16T and 86T cells. Hoechst-stained nuclei of cells undergoing apoptosis after infection with lenti-PTPRD are depicted in the upper panel, and quantification of apoptotic cells is depicted in the lower panel.

Figure 5. Tumor-derived mutations compromise the growth suppressive function of PTPRD in GBM and melanoma cells. Five tumor-derived mutations were introduced into lenti-PTPRD. (A) Western blot analysis for PTPRD protein demonstrates equivalent expression of wild-type and mutant proteins in infected H4 GBM cells. (B)
Infection of H4 cells with wild-type PTPRD led to growth suppression, as measured by BrdU incorporation using a 1 hr. pulse 48 hrs. after infection. In contrast, ectopic expression of the PTPRD cDNA harboring tumor-derived mutations led to less potent growth suppression, albeit to varying extents (25-60% of the activity relative to wild-type). This experiment was performed in triplicate, and the inactivation of growth suppressive activity was shown to be statistically significant ($p<0.05$ for each of the mutants, except D1248N with $p=0.06$). (C) Infection of 16T melanoma cells with wild-type PTPRD led to apoptosis of approximately 75% of the infected cells at 10 days post-infection, whereas the mutant PTPRD lentiviruses led to a substantially reduced fraction of cells that had undergone programmed cell death.
Mutational Inactivation of PTPRD in GBM and Melanoma

Figure 1

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<th>Consensus gene(s)</th>
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<th>Large-scale loss (&gt;10 Mb)</th>
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*CDKN2B (p15^NBN*) is co-deleted with CDKN2A (p16^NBN*) in 44 of these 45 samples.
Figure 2

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<th>Nucleotide change</th>
<th>Amino acid change</th>
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</table>

B

reference

血

继承性突变

肿瘤

体细胞丢失


 homozygous T3486C, I1115T mutation in p1118
## Table 1

<table>
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<th>Amino acid change</th>
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Figure 3

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<td>Lung adenocarcinoma</td>
<td>11/188 (6%)</td>
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<tr>
<td>Glioblastoma multiforme</td>
<td>4/65 (6%)</td>
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<td>Malignant melanoma</td>
<td>7/57 (12%)</td>
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Mutational Inactivation of PTPRD in GBM and Melanoma

Figure 5

A

PTPRD alleles

- empty vector
- wild-type
- G446E
- P459L
- I1115T
- D1248N
- V1565I

anti-PTPRD

anti-\(\alpha\)-tubulin

B

\% relative growth inhibition

- wild-type
- G446E
- P459L
- I1115T
- D1248N
- V1565I

C

\% apoptotic cells

- empty vector
- wild-type
- G446E
- P459L
- I1115T
- D1248N
- V1565I

PTPRD alleles
Supplementary Table and Figure Legends

Table S1. Focal deletions (<10 Mb) of the PTPRD gene identified in 8 out of 58 GBM tumor samples by Affymetrix 250K SNP array.

Table S2. Large-scale chromosomal loss (>10 Mb) encompassing the PTPRD gene identified in 21 out of 58 GBM tumor samples by Affymetrix 250K SNP array.

Table S3. Complete list of nonsense and missense mutations identified in PTPRD to date in human cancer.

Figure S1. Examples of somatic nonsense and missense mutations of PTPRD in GBM primary tumors. Sequence traces of PCR products amplified from paired tumor and normal (blood) genomic DNA are shown.

Figure S2. Examples of somatic single-nucleotide point mutations of PTPRD in malignant melanoma tumor samples. Sequence traces of PCR products amplified from paired tumor and normal (blood) genomic DNA are shown.

Figure S3. Examples of somatic dinucleotide mutations of PTPRD in malignant melanoma tumor samples. Sequence traces of PCR products amplified from paired tumor and normal (blood) genomic DNA are shown. Three of the ten mutation events identified in melanoma samples were CC→TT dinucleotide mutations likely resulting from the presence of UV-induced cyclobutane pyrimidine dimers. The first dinucleotide mutation we identified (G4474A, G4475A) affects the third nucleotide of codon 1444 and the first nucleotide of codon 1445. The G4474A change results in mutation of W1444 to a Stop codon that terminates translation. The G4475A change results in mutation of E1445 to K, but this change has no effect on the translated protein as it now follows a
Stop codon. The second dinucleotide mutation we identified (C5210T, C5211T) affects the second and third nucleotides of codon 1690. These mutations together result in the P1690F amino acid substitution. The third dinucleotide mutation we identified (C5533T, C5534T) affects the third nucleotide of codon 1797 and the first nucleotide of codon 1798. The C5533T change is a synonymous mutation that does not change S1797, and the C5534T change is a nonsense mutation that results in change of R1798 to a Stop codon.
Supplementary Table 1

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*Based on hg18 genome assembly.
## Supplementary Table 2

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\(^a\)Based on hg18 genome assembly.
## Supplementary Table 3

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*Based on hg17 human genome assembly for CRC and LA mutations, hg18 assembly for GBM and MM mutations.

ENST00000381196 is the transcript used for annotating the mutations.

* Domain structure based on UniProtKB/Swiss-Prot P23468-1.

Ig_C2, immunoglobulin-like C2-type; FN_III, fibronectin type-III; PTPc, protein tyrosine phosphatase catalytic.

CRC, colorectal carcinoma; LA, lung adenocarcinoma; GBM, glioblastoma multiforme; MM, malignant melanoma.

Nucleotide/codon is not present in the annotated transcript ENST00000381196.
Figure S1

Mutational Inactivation of PTPRD in GBM and Melanoma

blood

C1421C/T, R427R/X in p1151

tumor

blood

G3957G/T, G1272G/V in p898

tumor
Mutational Inactivation of PTPRD in GBM and Melanoma

Figure S2

- **blood**
  - G342G/A, G61G/E in 34T

- **tumor**
  - G3884A, D1248N in 21T

- **blood**
  - G4835A, V1565I in 86T

- **tumor**
Figure S3

Mutational Inactivation of PTPRD in GBM and Melanoma

- G4474G/A, G4475G/A
- W1444W/Stop in 76T

- C5210C/T, C5211C/T
- P1690P/F in 6T

- C5533C/T, C55534C/T
- R1798R/Stop in 76T
Lack of Inherited Mutations of PTPRD in Familial Melanoma and Melanoma-Astrocytoma Syndrome

David A. Solomon¹, Jung-Sik Kim¹, Xiaohong R. Yang², Margaret A. Tucker², Alisa M. Goldstein², Yardena Samuels³, and Todd Waldman¹

¹Department of Oncology, Lombardi Cancer Center, Georgetown University School of Medicine, Washington, DC

²Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD

³Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD


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Abstract

Inherited susceptibility to malignant melanoma has long been linked to genetic anomalies on chromosome 9p. Approximately 20-40% of melanoma-prone families have germline mutations in CDKN2A, which encodes the cyclin-dependent kinase inhibitor p16\(^{\text{INK4a}}\) at chromosome 9p21.3. However, the susceptibility gene(s) in the 9p-linked families without CDKN2A alteration remains unidentified. Recently we and others have identified frequent deletions and somatic mutations of the PTPRD tumor suppressor gene on chromosome 9p in sporadic melanoma. We thus considered the possibility that inherited mutations of PTPRD could be responsible for familial melanoma or melanoma-astrocytoma syndrome in kindreds lacking mutations in CDKN2A. To test this hypothesis, we sequenced the PTPRD gene in probands from 12 familial melanoma and melanoma-astrocytoma kindreds that lack mutations in CDKN2A. In each case the PTPRD gene was wild-type. Our findings indicate that despite frequent lesions of PTPRD in sporadic melanomas, we find no evidence that PTPRD is the long sought “other” 9p susceptibility gene in familial melanoma.
Lack of Inherited Mutations of PTPRD in Familial Melanoma

The CDKN2A gene on chromosome 9p21.3 is the major known high-risk melanoma susceptibility gene. However, despite the fact that more than half of familial melanoma has been linked to chromosome 9p, only about 20-40% of melanoma-prone families have mutations in CDKN2A (de Snoo and Hayward, 2005; Goldstein et al., 2006). Several groups have found recurrent regions of LOH and deletion on chromosome 9p in both sporadic and familial melanoma that do not encompass the CDKN2A locus (Fig. 1A; Puig et al., 1995; Parris et al., 1999; Pollock, Welch, and Hayward, 2001). Therefore, an additional unidentified tumor suppressor gene(s) on chromosome 9p may also be responsible for inherited susceptibility to melanoma.

The PTPRD gene at chromosome 9p23-24.1 encodes one of 21 known human receptor-type protein tyrosine phosphatases, a family of proteins which are increasingly thought to be important in cancer development and progression (for reviews see Tonks, 2006; Ostman, Hellberg, and Bohmer, 2006). PTPRD was first theorized to be a tumor suppressor by Urushibara et al. (1998) who described a selective reduction in PTPRD expression in hepatomas. This theory was supported by the discovery of genomic deletions of PTPRD in several human cancer cell lines by Cox et al. (2005). Subsequent studies have reported homozygous deletions of PTPRD in a broad spectrum of human tumor types including lung adenocarcinoma (Zhao et al., 2005; Sato et al., 2005; Nagayama et al., 2007; Weir et al., 2007), pancreatic carcinoma (Calhoun et al., 2006), melanoma (Stark and Hayward, 2007), neuroblastoma (Stallings et al., 2006), glioblastoma multiforme (Solomon et al., 2008), and cutaneous squamous cell carcinoma (Purdie et al., 2007).

Somatic mutations of PTPRD in human cancer were first discovered by Sjoblom et al. (2006) who identified 3 missense substitutions in a panel of 35 colorectal cancers.
Two additional studies have since described mutation of PTPRD in lung adenocarcinoma (Weir et al., 2007; Ding et al., 2008). Most recently, somatic mutations of PTPRD were discovered in glioblastoma multiforme (GBM) and melanoma (Solomon et al., 2008). While PTPRD was mutated in only a modest fraction of lung cancers (6%) and GBMs (6%), the 12% mutation frequency in melanoma makes PTPRD among the most commonly mutated genes in sporadic melanoma reported to date, which include B-Raf (~60%), p53 (0-25%), N-Ras (10-15%), PTEN (~10%), CDKN2A (0-5%), and PIK3CA (<1%) (Curtin et al., 2005; Fecher et al., 2007). Functional experiments have now definitively established that PTPRD has a growth suppressive role in human cancer cells and that tumor-derived mutations compromise the tumor suppressive function of PTPRD (Solomon et al., 2008).

Intriguingly, this study by Solomon et al. (2008) also reported an inherited mutation of PTPRD in a GBM patient with a history of multiple primary tumors. This inherited mutation was accompanied by somatic loss of the wild-type allele in the tumor, suggesting that inherited mutations of this emerging tumor suppressor gene might result in a predisposition syndrome for GBM and other tumor types. Interestingly, melanoma-prone families often have a high rate of gliomas and other neural system tumors, a syndrome that has been termed “melanoma-astrocytoma syndrome” (Kaufman et al., 1993; Azizi et al., 1995). Based on 1) frequent somatic alterations in sporadic melanoma, 2) its location on chromosome 9p, and 3) the presence of an inherited mutation in a GBM patient with a history of multiple primary malignancies, we thus considered PTPRD an attractive candidate as a cancer susceptibility gene in familial melanoma and melanoma-astrocytoma syndrome.
Lack of Inherited Mutations of PTPRD in Familial Melanoma

To test this hypothesis, we sequenced the PTPRD gene in constitutional DNA from probands of twelve melanoma-prone families lacking mutations of CDKN2A. Five of these families had multiple melanoma patients plus at least one brain cancer patient, six families showed putative linkage to chromosome 9p or a haplotype consistent with linkage in the CDKN2A region, and the twelfth family had seven melanoma patients (see Fig. 1B for details on these 12 probands). The 35 coding exons and flanking intronic sequence of PTPRD were PCR amplified from genomic DNA isolated from whole blood using conditions and primer pairs described by Sjoblom et al. (2006). PCR products were purified using the Exo/SAP method followed by a Sephadex spin column. Sequencing reactions were performed using Big Dye v3.1 (Applied Biosystems) and M13-Fwd primer, and were run on an Applied Biosystems 3730XL capillary sequencer. Sequence traces were analyzed using Mutation Surveyor (Softgenetics). Traces with putative mutations were re-amplified and re-sequenced.

Though several common SNPs were identified, no mutations of PTPRD were found (Fig. 1C). Although the presence of common somatic mutations and homozygous deletions has clearly implicated inactivation of PTPRD in the pathogenesis of sporadic melanomas, these data provide no evidence for PTPRD as the long sought “other” chromosome 9p familial melanoma susceptibility gene.
Lack of Inherited Mutations of PTPRD in Familial Melanoma

Acknowledgements

T.W. is supported by the National Cancer Institute and the American Cancer Society. This work was supported in part by the National Institutes of Health Intramural Research Program of the National Cancer Institute, Division of Cancer Epidemiology and Genetics (X.Y., M.T., and A.G.) and the National Human Genome Research Institute (Y.S.).
References


Lack of Inherited Mutations of PTPRD in Familial Melanoma


Figure legend

Figure 1. (A) Schematic of chromosome 9p showing the location of the CDKN2A gene at 9p21.3 (21.958-21.984 Mb) and the PTPRD gene at 9p23-24.1 (8.304-10.603 Mb). Recurrent regions of LOH in a panel of 37 melanomas (Pollock, Welch, and Hayward, 2001) are depicted with black boxes. (B) Details of the 12 familial melanoma probands assessed in this study. *, 3 of the 4 patients in family A2 have 9p21-haplotype sharing. ND, not determined. (C) Frequency of PTPRD and CDKN2A alterations in sporadic melanoma tumors (top) and kindreds with familial melanoma (bottom).
Lack of Inherited Mutations of PTPRD in Familial Melanoma

Figure 1

A

B

C

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Sample Type Bias in the Analysis of Cancer Genomes

David A. Solomon¹, Jung-Sik Kim¹, Habtom W. Ressom¹, Zita Sibenaller², Timothy Ryken², Walter Jean³, Darell Bigner⁴, Hai Yan⁴, and Todd Waldman¹

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC

²Department of Neurosurgery, University of Iowa College of Medicine, Iowa City, IA

³Department of Neurosurgery, Georgetown University School of Medicine, Washington, DC

⁴Department of Pathology, Preston Robert Tisch Brain Tumor Center, Duke University School of Medicine, Durham, NC

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Abstract

There is widespread agreement that cancer gene discovery requires high quality tumor samples. However, whether primary tumors or cultured samples are superior for cancer genomics has been a longstanding subject of debate. This debate has recently become more important since federally-funded cancer genomics has been centralized under The Cancer Genome Atlas (TCGA), which has chosen to focus exclusively on primary tumors. Here we provide a data-driven “Perspective” on the effect of sample type selection on cancer genomics research. We demonstrate that, in the case of glioblastoma multiforme (GBM), primary tumors and xenografts are best for the identification of amplifications, whereas xenografts and cell lines are superior for the identification of homozygous deletions. We also note that many of the most important oncogenes and tumor suppressor genes have been discovered through the use of cell lines and xenografts, and highlight the lack of published evidence supporting the dogma that ex vivo culture generates artifactual genetic lesions. Based on this analysis, we suggest that cancer genomics projects such as TCGA should include a variety of sample types such as xenografts and cell lines in their integrated genomic analysis of cancer.
**Introduction**

After several decades in which cancer genomics research was performed in individual laboratories and funded by single-investigator grants, the field has recently been centralized and expanded under the auspices of The Cancer Genome Atlas (TCGA), which is performing integrated genomic analysis on a large number of samples from a wide range of common human tumor types. TCGA was initiated in December 2005, recently completed a three year pilot project (focused on glioblastoma multiforme, ovarian cancer, and lung cancer), and is currently organizing itself to begin the production phase of genomic analysis on a wider range of tumor types.

The procurement of high quality cancer samples is the critical first step for cancer genomics projects such as TCGA. There are four principle types of human cancer samples available for such studies – primary tumors, primary cultures, primary xenografts, and established cell lines. The availability of each sample type is somewhat tumor-type specific (e.g. breast cancers do not efficiently form xenografts). Each of these sample types has unique advantages and disadvantages that are thought to affect the success of genomic analyses (see Supplemental Table 1).

Unlike other ongoing cancer genomics projects (1,2,3), TCGA has chosen to focus exclusively on the collection and analysis of primary tumor samples. This decision was based on considerations such as the fact that primary tumors can most easily be collected in large numbers in a prospective fashion, and the concern that ex vivo culture could induce artifactual genetic lesions. However, this decision was not based strictly on scientific data, as few (if any) published studies have directly evaluated the advantages and disadvantages of various sample types for genetic analysis.
We initially became interested in this issue of sample type selection for cancer genomics because, as TCGA was performing copy number analysis on GBM primary tumor samples (4), we were performing similar analysis on a panel of all four GBM sample types (5,6). The results of these studies, described comprehensively for the first time in detail below, suggested that while primary tumors are an ideal sample type for the identification of genomic amplifications, they are inferior to xenografts and cell lines for the identification of genomic deletions. As such, this “Perspective” will describe the effects of sample type on copy number analysis in GBM, examine the evidence supporting the widely accepted idea that cultured sample types contain artifactual genetic lesions, and review the role of different sample types in the history of cancer gene discovery.

Comparative Copy Number Analysis of Diverse GBM Sample Types

In an effort to experimentally address issues in sample type selection for cancer genomics projects, copy number analysis was performed on 58 GBM samples derived from all four GBM sample types – primary tumors, primary cultures, primary xenografts, and established cell lines. Copy number data from an additional panel of 50 cell lines were also analyzed.

Initially we identified amplifications and deletions of the major GBM oncogenes and tumor suppressor genes (Table 1A and Supplemental Table 2). There was a substantial discrepancy in the frequency of oncogene amplification between sample types. For example, amplification of EGFR was commonly found in primary tumors and xenografts, but rarely found in primary cultures and cell lines. This phenomenon of loss of amplifications in GBM cell lines has been previously described but was thought to be specific to EGFR (7,8). However, our data indicate that amplification of other GBM
oncogenes such as PDGFRA, CDK4, and MDM4 is similarly lost during in vitro culture, and suggest that primary tumors and xenografts are the best sample type for the identification of novel amplicons containing candidate oncogenes.

Of note, this loss of oncogene amplification during tissue culture appears to be tumor-type specific, as there are examples of tumor types in which oncogenes are amplified at a similar frequency in both cultured and uncultured samples. For example, MYC or MYCN are amplified in 28 out of 37 neuroblastoma cell lines (76%)\(^3\), comparable to that observed in neuroblastoma primary tumor samples (9).

There was also a discrepancy in the frequency of identifiable tumor suppressor gene deletion between sample types. For example, deletions of the CDKN2A/B locus were identifiable in a much higher fraction of xenografts and cell lines than in primary tumors and primary cultures (Table 1A and Supplemental Table 2). Importantly, this disparity was not limited to CDK inhibitors, but was also present for PTEN, NF1, and PTPRD. In the case of PTPRD, deletions in primary tumors were very rarely identified, and therefore TCGA did not sequence the gene in their GBM pilot project (4). It was only the use of additional sample types that enabled identification of frequent deletions and somatic mutations of this emerging tumor suppressor gene in GBM (6).

To determine whether the presence of admixed non-neoplastic cells and intratumoral genetic heterogeneity was responsible for impeding the identification of deletions in primary tumor samples, we analyzed CDKN2A/B and CDKN2C in both a first passage xenograft and the primary tumor from which it was derived. Deletions of both loci were present in the xenograft, but were largely masked in the primary tumor by the presence of admixed non-neoplastic cells and intratumoral genetic heterogeneity (5,10). This same observation is evident when comparing copy number at each of the major
Sample Type Bias in the Analysis of Cancer Genomes

tumor suppressor genes – deletions in primary tumors are more difficult to identify because their average copy number is significantly higher and their boundaries are less discrete (Table 1B, Fig. 1, and Supplemental Table 3).

Taken together, these data indicate that xenografts and cell lines are superior to primary tumors for the identification of genomic deletions. The presence of non-neoplastic cells and heterogeneity in even the most homogeneous tumor types such as GBM results in substantial “noise” in the analysis, which hinders the identification of deletions and leads to a high rate of false negatives. Such noise would be expected to pose similar problems in other cancer genomics assays as well, including DNA sequencing.

No Evidence of Artifactual Genetic Lesions Caused by Ex Vivo Culture

Many cancer researchers favor using primary tumors rather than cultured samples because of the widespread belief that ex vivo culture can lead to the accumulation of spurious genetic alterations. Concerns of this type reached a pinnacle fifteen years ago, when there was substantial controversy about whether the recently identified deletions and mutations of the p16\textsuperscript{INK4a} tumor suppressor gene could be artifacts of ex vivo culture (11,12). After substantial high profile debate, this concern was eventually refuted and it is now universally accepted that p16\textsuperscript{INK4a} is one of the most commonly inactivated tumor suppressor genes in human cancer. However, such concerns remain firmly entrenched in the minds of most cancer researchers.

To test whether these concerns are valid, we catalogued all the copy number alterations present in each of our 58 samples. Strikingly, there were no examples of recurrent deletions or amplifications present exclusively in cultured samples. Additionally, if ex vivo culture specifically enriches for cells with deleted tumor
Sample Type Bias in the Analysis of Cancer Genomes

Suppressor genes, one would similarly expect culture to enrich for cells with amplified oncogenes. Yet as we show in Table 1A, *ex vivo* culture leads to a decrease in oncogene amplification in GBM cells, not the predicted increase.

Next, a comprehensive search of the literature was performed in an effort to identify studies that document copy number alterations and/or mutations present exclusively in cultured samples but not in primary tumors. While we were able to identify several studies that demonstrate expression differences between primary tumors and cultured samples (13,14), we were unable to identify any studies documenting genetic lesions unique to cultured samples.

In contrast, Jones et al. recently provided remarkably strong evidence in support of the idea that cultured samples faithfully recapitulate the genetic profile present in the tumor from which they were derived. In their study, 287/289 mutations (99.3%) initially discovered in human colon cancer xenografts and cell cultures were similarly present in the primary tumors from which the cultured samples were derived (15). These data indicate that *ex vivo* culture of colon tumors does not lead to the formation or accumulation of spurious genetic aberrations.

Based on these findings, we believe that there is little convincing evidence to support the dogma that *ex vivo* culture leads to artifactual deletions, amplifications, and somatic mutations. As such, the risk of failing to identify deletions in human cancer samples due to an exclusive focus on primary tumors is likely to be substantially greater than the risk of identifying spurious genetic events by including other sample types in the analysis. This is especially true since it is relatively trivial to determine whether an event initially discovered in cultured samples is similarly present in primary tumors, as was the
case, for example, with the recent identification of CDKN2C as a GBM tumor suppressor gene (5,16).

Cultured Samples Have Been Used in the Discovery of Most Oncogenes and Tumor Suppressors

Finally, we looked back through the modern history of cancer genetics to identify the sample types used to discover the most commonly altered oncogenes and tumor suppressor genes (Table 2). Notably, most somatically altered cancer genes that were not discovered via linkage analysis were initially identified using xenografts and cell lines. This includes p53, PTEN, p16\(^{\text{INK4a}}\), K-Ras, PIK3CA, B-Raf, and others (11,17-30). Based on this history, it seems prudent to include cultured samples in any cancer genomics initiative whose major goal is the identification of novel somatically altered cancer genes.

Conclusions

Here we provide three rationales for the inclusion of cultured samples in TCGA and other cancer genomics efforts. First, we show that in the case of one major human tumor type, there are significant differences in the utility of different sample types for the identification of copy number alterations. Second, we document that there is little evidence supporting the popular notion that \textit{ex vivo} culture of human tumors leads to spurious genetic alterations. And third, we show that most major somatically altered cancer genes discovered to date were identified using xenografts and cell lines. Based on these arguments, we believe it would be prudent for TCGA to include a range of sample types in their burgeoning analysis of cancer genomics. We also note that the use of cultured samples is supported by the Cancer Genome Project of the Wellcome Trust.
Sanger Institute and is within the agreed guidelines of the International Cancer Genome Consortium (ICGC).
Footnotes

1. These data were generated using Affymetrix 250K Nsp I SNP arrays and analyzed using dChip, a publicly available software program (http://biosun1.harvard.edu/complab/dchip/). These data have been reported on previously (see references 5 and 6), and the raw and processed data sets have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE13021.

2. Copy number data for a panel of 50 malignant glioma cell lines using Affymetrix SNP 6.0 arrays was generated by the Cancer Genome Project of the Wellcome Trust Sanger Institute, and is publicly available at http://www.sanger.ac.uk/genetics/CGP.

3. Copy number data for a panel of 37 neuroblastoma cell lines using Affymetrix SNP 6.0 arrays was generated by the Cancer Genome Project of the Wellcome Trust Sanger Institute, and is publicly available at http://www.sanger.ac.uk/genetics/CGP.
Acknowledgments

T.W. is supported by grants from the National Cancer Institute, American Cancer Society, and Georgetown University School of Medicine.
References


Table and Figure Legends

Table 1. Significant sample type effects on copy number alterations in GBM. A, The percentage of tumor samples with focal (<10 Mb) genomic deletion and high-level (copy number >7) focal amplification of the indicated gene loci is shown. B, The mean copy number and standard deviation at the indicated gene loci in those tumor samples with focal genomic deletion is shown. Two-tailed unpaired t-test analysis was used to compare the statistical significance of any difference in frequency of copy number alteration (A) and mean copy number (B) between the TCGA primary tumors and other GBM tumor samples. Statistically significant differences ($p$<0.05) in frequency (A) and copy number means (B) are highlighted in bold. -, no samples with focal genomic deletion at the indicated gene loci. *, less than 3 samples with genomic deletion, no standard deviation calculation or t-test analysis possible.

Figure 1. Copy number plots along chromosome 9p for four TCGA primary tumors (reported to have homozygous deletion of CDKN2A/B), two xenografts, two cell lines, and normal human astrocytes (NHAs). Each of the depicted xenografts and cell lines have homozygous deletion of the CDKN2A/B locus with copy number < 0.2, whereas the four TCGA primary tumors have hemizygous/heterogeneous deletion with copy numbers of 1.07, 1.32, 1.22, and 1.29 for TCGA-06-0122, TCGA-06-0133, TCGA-06-0143, and TCGA-06-0169, respectively.

Table 2. Sample types used in the initial discovery of major somatically altered cancer genes.
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<th>15 xenografts</th>
<th>21 cell lines</th>
<th>50 Sanger CGP cell lines</th>
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**B**

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Sample Type Bias in the Analysis of Cancer Genomes

**Figure 1**

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<th>TCGA primary tumors</th>
<th>xenografts</th>
<th>cell lines</th>
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CDK2A1B

0124 : copy number
Sample Type Bias in the Analysis of Cancer Genomes

### Table 2

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<table>
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<th>Gene</th>
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<th>Sample type(s)</th>
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Supplemental Table Legends

Supplemental Table 1. Description and features of the four major human cancer sample types.

Supplemental Table 2. Copy number alterations of the major known GBM tumor suppressor genes (a) and oncogenes (b) present in a panel of 50 malignant glioma cell lines. These copy number data were generated on Affymetrix 6.0 SNP arrays by the Cancer Genome Project of the Wellcome Trust Sanger Institute.

Supplemental Table 3. Mean copy number values reported by the TCGA of the genomic deletions in primary tumor samples containing homozygous deletions of CDKN2A (a), PTEN (b), CDKN2C (c), and NF1 (d).
Supplemental Table 1

Primary Tumors
- Tumor tissue obtained directly from the operating room
- Faithfully represent the genetic, epigenetic, and transcriptional profile of human tumors
- Contain admixed non-neoplastic cells
- Contain intratumoral genetic heterogeneity
- Often contain substantial necrosis
- Limited in quantity (i.e. individual tumors are non-renewable)
- Susceptible to ischemia and degradation due to delays between resection and freezing/fixation

Primary Xenografts
- Primary tumors that have been implanted into immunodeficient mice and have grown into tumors
- Largely free of admixed non-neoplastic human cells
- Unlimited in quantity (i.e. xenografts can be repeatedly passaged)
- Some tumor types do not efficiently form xenografts
- Xenograft growth can require several months

Primary Cultures
- Early passage cultures of dissociated primary tumors
- Often contain admixed non-neoplastic cells
- Limited in quantity

Established Cell Lines
- Late passage cultures of dissociated primary tumors
- Do not contain admixed non-neoplastic human cells
- Unlimited in quantity (i.e. cell lines can be repeatedly passaged)
- Some tumor types do not efficiently form cell lines
- Establishment can require several months
- Matched normal tissue is often not available
### Supplemental Table 2a

<table>
<thead>
<tr>
<th>cell line</th>
<th>CDKN2A</th>
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*U251 cell line is synonymous with B2-17 cell line that was also analyzed.
## Supplemental Table 2b

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*U251 cell line is synonymous with B2-17 cell line that was also analyzed.
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Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts

Karine Michaud†*, David A. Solomon2*, Eric Oermann2, Jung-Sik Kim2, Wei-Zhu Zhong3, Michael D. Prados1, Tomoko Ozawa1, C. David James††, and Todd Waldman2††

1Department of Neurological Surgery, Helen Diller Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA
2Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC
3Pfizer Oncology, La Jolla, CA

*Contributed equally to this report
†To whom correspondence should be addressed


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**Abstract**

Activation of cyclin-dependent kinases 4 and 6 (cdk4/6) occurs in the majority of glioblastoma multiforme (GBM) tumors, and represents a promising molecular target for the development of small molecule inhibitors. In the current study we investigated the molecular determinants and *in vivo* response of diverse GBM cell lines and xenografts to PD-0332991, a cdk4/6 specific inhibitor. *In vitro* testing of PD-0332991 against a panel of GBM cell lines revealed a potent G1 cell cycle arrest and induction of senescence in each of 16 Rb-proficient cell lines regardless of other genetic lesions, whereas each of 5 cell lines with homozygous inactivation of Rb were completely resistant to treatment. shRNA depletion of Rb expression conferred resistance of GBM cells to PD-0332991, further demonstrating a requirement of Rb for sensitivity to cdk4/6 inhibition. PD-0332991 was found to efficiently cross the blood-brain barrier and proved highly effective in suppressing the growth of intracranial GBM xenograft tumors, including those that had recurred after initial therapy with temozolomide. Remarkably, no mice receiving PD-0332991 died as a result of disease progression while on therapy. Additionally, the combination of PD-0332991 and radiation therapy resulted in significantly increased survival benefit compared with either therapy alone. In total, our results support clinical trial evaluation of PD-0332991 against newly diagnosed as well as recurrent GBM, and indicate that Rb status is the primary determinant of potential benefit from this therapy.
Introduction

Deregulation of the cdk4/6-cyclin D-INK4-Rb signaling pathway is among the most common aberrations found in human cancer (1). In the case of glioblastoma multiforme (GBM), this pathway is most commonly altered by homozygous deletion of CDKN2A/B, and less commonly by deletion/mutation of CDKN2C and RB1, or genomic amplification of CDK4, CDK6, and individual D-type cyclins (2-8). Recent molecular profiling of GBM has further highlighted the critical role of cdk4/6 activation in the pathogenesis of this devastating tumor (9,10).

Despite the ubiquitous nature of cdk4/6 activation in human cancer, surprisingly little work has been reported describing efforts to target activated cdk4/6 with pharmacological inhibitors. This is due to the difficulty of identifying inhibitors specific to cdk4/6, and also because cdk inhibition is predicted to be cytostatic (not cytotoxic), and therefore of uncertain therapeutic utility (11).

PD-0332991 is a recently developed cdk4/6 specific inhibitor. Initial reports detailed the synthesis of this pyridopyrimidine derivative, demonstrated its in vitro specificity against cdk4/6, and documented its potent anti-proliferative activity against Rb-proficient subcutaneous human tumor xenografts (12,13). A large phase I study of this compound is ongoing and has already demonstrated clinical efficacy against otherwise untreatable teratomas (14). Additional xenograft studies modeling disseminated multiple myeloma (15) and breast carcinoma (16) helped to motivate recently initiated phase I/II clinical trials testing PD-0332991 against these cancers (http://www.clinicaltrials.gov).

Given its effectiveness in a variety of tumor types and its specificity for activated cdk4/6, we hypothesized that PD-0332991 could be useful in the treatment of GBM. To
evaluate this, PD-0332991 was initially tested *in vitro* against a panel of 21 human GBM cell lines of defined genetic backgrounds. Next, its activity was assayed *in vivo* against three different GBM intracranial xenografts using a luciferase reporter to allow longitudinal monitoring of tumor growth and response to therapy. Finally, PD-0332991 was tested in combination with ionizing radiation against intracranial xenografts, as well as in tumors that had recurred following initial therapy with temozolomide, an alkylating agent routinely used in upfront treatment of GBM (17). Our findings demonstrate that PD-0332991 crosses the blood-brain barrier and works effectively both as a single agent and in combination with radiation to suppress the growth of all Rb-proficient GBM cells and tumors tested.
Materials and Methods

**GBM cell lines and xenografts.** A panel of 21 GBM cell lines was obtained directly from the American Type Culture Collection (U87MG, U138MG, M059J, Hs683, H4, A172, LN18, LN229, CCF-STTG1, T98G, and DBTRG-05MG), DSMZ (8MGBA, 42MGBA, DKMG, GAMG, GMS10, LN405, and SNB19), and the Japan Health Sciences Foundation Health Science Research Resources Bank (AM38, NMC-G1, and KG-1-C). These repositories authenticate all human cell lines prior to accession by DNA fingerprinting, and independent evidence of authenticity is also provided by cytogenetic and immunophenotypic tests. All experiments were performed on cell lines that had been passaged for less than six months after receipt. Cell line SF767 was established from a GBM at the UCSF Brain Tumor Research Center, and has been previously genotyped for CDKN2A, TP53, and PTEN status (18). All cell lines were grown in DMEM + 10% fetal bovine serum at 37°C in 5% CO2. GBM 39 is a human GBM primary xenograft maintained as a subcutaneous heterotransplant in athymic mice, as previously described (19).

**Western blots.** Total cell lysate was collected from asynchronously proliferating cells in RIPA buffer, resolved by SDS-PAGE, and then immunoblotted using standard techniques. Primary antibodies used were obtained from Santa Cruz Biotechnology (Mdm2, clone SMP14), Cell Signaling (cyclin D1, clone DCS6; cyclin D3, clone DCS22; cdk4, clone DCS156; cdk6, clone DCS83; p18INK4c, clone DCS118; p21WAF1/CIP1, clone DCS60; p14ARF, clone 4C6/4; Rb, clone 4H1), BD PharMingen (p16INK4a, #554079), NeoMarkers (α-tubulin, Ab-1 clone DM1A; EGFR, Ab-12), CalBiochem (p53, Ab-6 clone DO-1), and Cascade Bioscience (PTEN, clone 6H2.1).
**Flow cytometry.** Cells were pulsed with 10 μmol/L bromodeoxyuridine (BrdU) for 1 h, trypsinized, and centrifuged. Cells were fixed and stained using the BrdU Flow kit (Pharmingen), counterstained with propidium iodide, and analyzed by flow cytometry in a BD FACSort instrument using FCS Express v.3 software (DeNovo Software).

**β-Galactosidase staining.** Cells grown on coverslips were stained with the Senescence β-Galactosidase Staining kit (Cell Signaling) as described by the manufacturer.

**Microscopy.** All imaging was performed on an Olympus BX61 light microscope with Plan-Apochromat objectives.

**Rb knockdown.** Five unique shRNAs to the RB1 mRNA in the pLKO.1-Puro lentiviral expression vector were obtained from Open Biosystems. To make virus, empty pLKO.1-Puro or each of these 5 shRNA clones were cotransfected into 293T cells with pVSV-G (Addgene) and pH'-CMV-8.2ΔR (Addgene) helper plasmids using Fugene 6 (Roche). Virus-containing conditioned medium was harvested 48 h after transfection, filtered, and used to infect recipient cells in the presence of 8 µg/mL polybrene. Infected cells were selected with 2 µg/mL puromycin until all mock-infected cells were dead and then maintained in 0.2 µg/mL puromycin.

**Generation and therapy of mice with intracranial GBM xenograft tumors.** All intracranial therapy response experiments involved the use of 5-6 week-old female athymic mice (nu/nu genotype, BALB/c background, Simonsen Laboratories). Animals were housed and fed under aseptic conditions, and all animal research was approved by The University of California, San Francisco Institutional Animal Care and Use Committee. Procedures used for intracranial tumor establishment, including monitoring of tumor growth and response to therapy by bioluminescence imaging, have previously
been described (20). Treatments for the experiments reported here were as follows: oral administration of vehicle only for control groups (50 mM sodium lactate, pH 4, for PD-0332991, and/or OraPlus for temozolomide), oral administration of PD-0332991 (Pfizer) at 150 mg/kg/day, clinical-grade temozolomide (Schering-Plough) at 10 mg/kg/day, and radiation at 2 Gy/day from a Cesium-137 source (J. L. Shepherd & Associates).

**Brain tumor analysis.** PD-0332991 concentrations in mouse brain or brain tumor were determined using an LC-MS/MS method following liquid-liquid extraction (LLE). Brain or brain tumor samples were homogenized with purified water at 1:4 dilution (w/v). A 100 µL aliquot of the homogenate was used for LLE with the addition of 100 µL of potassium bicarbonate buffer, 10 µL of the stable labeled internal standard (IS), and 0.4 mL methyl tert-butyl ether. Following centrifugation, the organic layer was evaporated to dryness and reconstituted in 100 µL of methanol for LC-MS/MS analysis. The LC system (Shimadzu) was coupled to an MDS Sciex API 4000 triple quadrupole mass spectrometer, and operated in the positive ionization mode using multiple reaction source. PD-0332991 and internal standard were monitored using specific precursor ion → product ion transitions of \( m/z \ 448 \rightarrow 380 \) and \( m/z \ 451 \rightarrow 383 \), respectively. The final concentration of PD-0332991 was normalized based on the weight of mouse brain or brain tumor collected.
Results

Ubiquitous genetic lesions of the cdk4/6-cyclin D-INK4-Rb signaling pathway in GBM cell lines. Copy number and sequencing data from our previous analyses (7,21,22) and from the Cancer Genome Project of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP) were analyzed to identify the genetic status of the major known GBM oncogenes and tumor suppressor genes in a panel of 21 GBM cell lines. This analysis demonstrated genetic lesions in at least one component of the cdk4/6-cyclin D-INK4-Rb signaling pathway in each cell line, without exception (Table 1). Fifteen of the cell lines have homozygous deletion of CDKN2A and CDKN2B, five have homozygous deletion or mutation of RB1 (resulting in a lack of detectable Rb protein), four have homozygous deletion of CDKN2C, and one (CCF-STTG1) harbors amplifications of both CDK6 and CCND3, with high levels of expression of corresponding encoded proteins (Table 1 and Supplemental Fig. S1). Lesions of CDKN2A/B, RB1, and CDK6/CCND3 were mutually exclusive in these 21 cell lines, except for 42MGBA cells which harbor homozygous deletion of CDKN2A/B and express no detectable Rb protein. In contrast, homozygous deletion of CDKN2C occurred exclusively in cell lines also harboring homozygous deletions of CDKN2A/B. A number of additional genetic lesions in other signaling pathways were identified among the cell lines, including PTEN deletion and mutation, TP53 mutation, BRAF mutation, EGFR rearrangement, and MDM2 amplification (Supplemental Table S1). However, no concordance was detected between any of these other genetic lesions and lesions of cdk4/6-cyclin D-INK4-Rb signaling pathway genes.

PD-0332991 induces cell cycle arrest and senescence of Rb-proficient GBM cells. Each of the 21 GBM cell lines was cultured in vitro in the presence of either 1 µM
PD-0332991 Arrests the Growth of GBM Intracranial Xenografts

PD-0332991 or vehicle alone. At 48 hours post-treatment, cell cycle distribution was assessed by BrdU incorporation and flow cytometry analysis (Fig. 1A-B and Supplemental Fig. S2). In 16/21 cell lines PD-0332991 treatment significantly inhibited BrdU incorporation and resulted in an increased fraction of cells in G1 phase of the cell cycle compared to vehicle alone (Table 1). While some cell lines displayed a greater than 95% reduction in BrdU incorporation after treatment with 1 µM PD-0332991 for 48 hrs (e.g. A172, CCF-STTG1, DBTRG-05MG, DKMG, Hs683, KG-1-C, NMC-G1, SNB19, U87MG), others displayed a more intermediate response (e.g. AM38, GAMG, H4, LN18). Of note, 5/21 of the cell lines displayed no difference in cell cycle distribution between treatment with vehicle or 1 µM PD-0332991 (8MGBA, 42MGBA, GMS10, LN405, and M059J). Remarkably, each of the five resistant cell lines harbors homozygous deletion or mutation of the RB1 gene that result in the absence of detectable Rb protein (see Supplemental Fig. S1).

To further explore the differential sensitivity of GBM cell lines to PD-0332991, the 21 cell lines were next cultured in the presence of 0, 1, 2, or 10 µM PD-0332991, and cell cycle distribution was again assessed at 48 hrs post-treatment (Supplemental Fig. S3). Cells that initially displayed >95% reduction in BrdU incorporation at 1 µM demonstrated similar inhibition at doses of 2 and 10 µM PD-0332991, and without evidence of apoptosis or cytotoxicity (i.e. floating cell debris or sub-G1 DNA content) at any dose (data not shown). Importantly, Rb-proficient cells that displayed intermediate reductions in BrdU incorporation at 1 µM PD-0332991 displayed increased reductions when incubated with 2 and 10 µM PD-0332991. For example, GAMG cells displayed a 53% reduction in BrdU incorporation at 1 µM PD-0332991, but had 78% and 96% reductions at 2 and 10 µM, respectively. In contrast, the five Rb-deficient cell lines
displayed no reduction in BrdU incorporation or alterations in cell cycle distribution at any of the doses tested.

We next examined the effects of long-term culture in the presence of PD-0332991. Microscopic analysis of Rb-proficient GBM cells treated with 1 µM PD-0332991 for 7 days revealed not only sustained growth arrest, but also morphological changes characteristic of cells that have exited the cell cycle and entered a senescent-like state (i.e. large and flat: Fig. 1C). Senescence-associated β-galactosidase activity was evident in Rb-proficient GBM cells after 14 days of treatment with PD-0332991 (Fig. 1D). However, Rb-deficient cells did not display these morphologic and senescent-like changes (Fig. 1C-D). Together, these findings indicate that PD-0332991 potently induces G1 cell cycle arrest of GBM cells at doses of <1 to 10 µM, and that sustained treatment induces cellular senescence in Rb-proficient cells.

**Stable depletion of Rb expression alleviates the growth arrest induced by PD-0332991.** To unequivocally determine if Rb status dictates the sensitivity of GBM cells to PD-0332991, U87MG cells were infected with lentiviruses expressing five unique shRNAs to RB1, and stably expressing pooled clones were generated by puromycin selection. Two of the clones (19 and 63) demonstrated greater than 99% knockdown of Rb expression relative to cells infected with the pLKO.1 vector alone (Fig. 2A). BrdU incorporation and flow cytometry analysis at 48 hrs post-treatment revealed that Rb knockdown had substantially mitigated the cell cycle inhibitory effects of PD-0332991 in clones 19 and 63, relative to the empty vector pLKO.1 clone (Fig. 2B). Long-term culture of clones 19 and 63 with 1 µM PD-0332991 demonstrated their failure to arrest in the presence of drug, resulting in eventual growth to confluence (Fig. 2C), albeit at a slower rate than cells treated with vehicle alone.
**PD-0332991 suppresses the growth of GBM intracranial xenografts.** Next we tested whether orally-administered PD-0332991 could efficiently cross the blood-brain barrier and effectively suppress the growth of intracranial GBM tumors comprised of cells with known sensitivity *in vitro*. To do this, we established luciferase-modified U87MG xenografts in the brains of a series of athymic mice, with half receiving daily PD-0332991 by oral gavage, and the other half receiving vehicle only. Bioluminescence monitoring showed sustained anti-proliferative activity of PD-0332991 throughout the four-week course of treatment (Fig. 3A), with tumor growth only evident upon completion of therapy. Consistent with these results, the survival of PD-0332991 treated mice was significantly extended relative to mice receiving vehicle only (Fig. 3B: p < 0.001). Importantly, none of the mice receiving PD-0332991 succumbed to tumor-related death while on therapy. In contrast, intracranial xenografts derived from the Rb-deficient cell line SF767 were completely resistant to PD-0332991 treatment (Supplemental Fig. S4).

To provide formal proof that PD-0332991 crosses the blood-brain barrier, we dissected the brain of a mouse and measured the levels of PD-0332991 in the U87MG xenograft tumor, in surrounding normal brain, and in brain from contralateral hemisphere. Notably, LC-MS/MS analysis revealed the presence of PD-0332991 in all three intracranial tissues. Furthermore, the drug was present at a 25-35x higher concentration in tumor tissue than in normal tissue (Fig. 3C). Next, the brains from treated and untreated mice with intracranial U87MG tumors (matched with respect to their time of sacrifice) were formalin fixed, embedded in paraffin, and sectioned for MIB-1 immunohistochemistry. Treatment with PD-0332991 led to a greater than 8-fold reduction in MIB-1 index (Fig. 3D).
It is widely appreciated that established GBM cell lines do not recapitulate several aspects of GBM biology (*e.g.* in vivo invasiveness, the presence of genomic amplifications, gene expression profiles [19,22-24]). Therefore, we next tested the efficacy of PD-0332991 against a model system that has been shown to preserve patient tumor characteristics that are lost and/or suppressed with extended *in vitro* propagation – primary intracranial xenografts generated from surgically resected human tissue that have been directly implanted and serially passaged in nude mice (19). To do this, mice harboring primary intracranial xenografts of luciferase-modified GBM 39 (which harbors a homozygous deletion of the CDKN2A/B locus and is Rb-proficient [25]) were randomized to control and treatment groups at 25 days post injection of tumor cells. Daily treatment with PD-0332991 resulted in sustained growth suppression (Fig. 3E), with significant survival benefit to treatment group mice (Fig. 3F).

**PD-0332991 combined with radiation therapy enhances survival of mice with GBM intracranial xenografts.** Radiation has long been used as standard of care for treating newly diagnosed GBM following surgical debulking of tumor (26), and investigational drugs for treating newly-diagnosed GBM are likely to see use with radiotherapy. To assess combined effects of radiation and PD-0332991 therapy, we established U87MG intracranial tumors in the brains of athymic mice and randomized them to four treatment groups at day 13 post-injection of tumor cells: control (mock treated), radiotherapy only (2 Gy/day, days 13-17), PD-0332991 only (150 mg/kg/day, days 13-26), or combination therapy with radiation administered either concurrently (RTc) or sequentially (RTs), with PD-0332991 and radiation combination regimens dosed identically to their corresponding monotherapy regimens. Both bioluminescence
and survival analysis showed that anti-tumor activity was most pronounced for mice receiving combination therapy, either concurrent or sequential (Fig. 4A-B).

**PD-0332991 effectively suppresses the growth of recurrent GBM.** An additional clinical scenario that often provides a setting for initial testing of an investigational agent is the treatment of recurrent cancer. For modeling this situation, mice with intracranial U87MG tumors were treated with 10 mg/kg/day temozolomide for 5 consecutive days, and subsequently monitored for initial anti-tumor activity of therapy, as well as for tumor regrowth from therapy (Fig. 5A). At time of tumor regrowth, mice were randomized to four treatment groups: (i) no additional treatment, (ii) repeat treatment with the same temozolomide regimen, (iii) treatment with PD-0332991 at 150 mg/kg/day for 2 weeks, or (iv) combined treatment with temozolomide and PD-0332991 administered concurrently at identical doses as the monotherapies. Both bioluminescence monitoring (Fig. 5B) and survival analysis (Fig. 5C) indicated that each therapy resulted in improved survival compared to no additional treatment. Furthermore, there was a trend favoring combination therapy with temozolomide and PD-0332991 as the most efficacious.
**Discussion**

Frequent and perhaps obligatory genetic alteration affecting the cdk4/6-cyclin D-INK4-Rb growth regulatory axis in GBM is well documented (2-8; Table 1 in the current report), and has been recently corroborated in two large-scale, multi-institutional genomic analyses of GBM (9,10). The most common alteration of this pathway in GBM is homozygous deletion of CDKN2A/B, encoding p16^{INK4a} and p15^{INK4b}, present in greater than 50% of tumors. Other alterations include amplification and overexpression of CDK4 (15-20%) and homozygous deletion/mutation of RB1 (~10%). Amplification of CDK6 and individual D-type cyclins, and homozygous deletion of CDKN2C encoding p18^{INK4c} are less common. Of these alterations, only genetic inactivation of RB1 itself is thought to render a tumor resistant to inhibition of cdk4/6. Since genetic inactivation of RB1 occurs infrequently in GBM, a substantial majority of GBM patients are predicted to be candidates for therapies targeting cdk4/6.

Several previous studies have tested the effects of kinase inhibitors against GBM (e.g. imatinib, erlotinib, flavopiridol), both in a preclinical setting and in clinical trials without significant efficacy (27-29). While some of these compounds promiscuously inhibit cyclin-dependent kinases (e.g. flavopiridol), none of the previously tested compounds display selectivity for cyclin-dependent kinases relative to other kinases that may or may not be activated in GBM. Our study for the first time tests a cdk-specific inhibitor in the treatment of GBM, demonstrating significant efficacy both in vitro and in vivo as a single agent and in combination with radiation therapy.

PD-0332991 is an orally available pyridopyrimidine derivative that selectively inhibits cyclin-dependent kinases 4 and 6 (12), leading to a reduction in Rb phosphorylation and subsequent cell cycle arrest. The in vitro and in vivo results
presented here further emphasize that Rb is the primary determinant of sensitivity to cdk4/6 inhibition. This aspect of PD-0332991 efficacy should be especially attractive with respect to clinical trial evaluation, since there are excellent reagents and protocols established for immunohistochemical detection of Rb in paraffin-embedded tissues (14,30).

Though Rb expression is clearly the primary determinant of tumor cell response to PD-0332991, variable growth inhibition among Rb-proficient cell lines (Table 1) suggests the existence of secondary factors that influence tumor cell sensitivity to PD-0332991, such as tumor CDK4/6 amplification vs. CDKN2A homozygous deletion. For the cell lines examined here, the single case with CDK6 amplification (CCF-STTG1) showed substantial sensitivity to PD-0332991 (Table 1).

Analysis of GBM response to PD-0332991 in vivo, using three different GBM tumor cell sources for establishing intracranial tumors (Fig. 3), yielded results entirely consistent with the in vitro data. PD-0332991 arrested the growth of xenografts generated from U87MG cells and GBM 39 (both Rb-proficient) and led to significantly improved survival (Fig. 3). The growth inhibitory effect of PD-0332991 was remarkably durable during the period of treatment.

Recent studies point to interest in investigating anti-tumor effects of PD-0332991 in combination with therapeutics that affect tumor properties other than cell cycling. For instance, it has been shown that PD-0332991, which by itself does not promote apoptotic response of cancer cells, markedly enhances myeloma cell killing by dexamethasone (31) as well as by bortezomib (32). Similarly, PD-0332991 has been shown to enhance breast cancer cell sensitivity to tamoxifen in vitro (33). Here, our bioluminescence and survival analysis of mice with intracranial U87MG tumors indicate
that the anti-tumor activity of PD-0332991 when used with radiation, either concurrently or sequentially, is superior to using either agent as a monotherapy (Fig. 4). These results, therefore, could help motivate clinical trial testing of PD-0332991 against newly-diagnosed GBM, for which the use of radiotherapy is the standard of care.

Finally, treatment of intracranial GBM tumors that had re-grown following initial therapy with temozolomide demonstrated that PD-0332991 has activity against recurrent GBM (Fig. 5). These results additionally suggest a general approach for pre-clinical animal model testing of therapies for patients with recurrent brain tumors, which is a mostly neglected area of neuro-oncology research.

In total, these findings provide strong support for evaluating the efficacy of PD-0332991 in treating GBM patients. For future investigation, it will be interesting to examine combination therapies involving PD-0332991 with small molecule inhibitors targeting activated gene products in other GBM core pathways, such as erlotinib for inhibiting EGFR. Determination of the full range of applications of this cdk4/6 inhibitor will undoubtedly prove informative, and will hopefully lead to improved treatment for this devastating cancer.
Acknowledgements

We thank Karen Creswell and Annie Park for assistance with flow cytometry, Raquel Santos for assistance in conducting the intracranial xenograft therapy response experiments, Minerva Batugo for assistance with the LC-MS/MS analysis of brain tumor samples, and Michael Pishvaian and Erik Knudsen for helpful discussions.
References


Table and Figure Legends

Table 1. Genetic lesions of the cdk4/6-cyclin D-INK4-Rb signaling pathway and in vitro sensitivity to PD-0332991 for a panel of 21 GBM cell lines. †A panel of 21 GBM cell lines was grown in vitro in the presence of vehicle or 1 μM PD-0332991 for 48 hrs, pulsed with BrdU for 1 hr, then fixed and stained with propidium iodide and FITC-conjugated BrdU antibody for flow cytometry analysis (see Supplemental Fig. 2). A sensitivity score of +++ was assigned to cells with greater than 95% reduction in BrdU incorporation after treatment with 1 μM PD-0332991 compared to vehicle, ++ for 75-95% reduction, + for 10-75% reduction, and – for less than 10% reduction. ‡Genetic data are derived from exonic sequencing and Affymetrix 250K SNP arrays performed by Solomon et al. (7,21,22) and from Affymetrix 6.0 SNP arrays and the COSMIC database of the Cancer Genome Project of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/). HD, homozygous deletion; MUT, homozygous mutation; AMP, genomic amplification; *, mutation status of gene not determined.

Figure 1. PD-0332991 induces cell cycle arrest and senescence of GBM cells dependent on Rb status. (A) Flow cytometry analysis of three Rb-proficient GBM cell lines (DBTRG-05MG, LN229, and U87MG) and one Rb-deficient cell line (42MGBA) following culture in the presence of vehicle or 1 μM PD-0332991 for 48 hrs and then pulsed with BrdU for 1 hr. Cell cycle distribution plots of BrdU vs. propidium iodide (PI) intensity are shown. (B) Quantification of reduction in S phase cells depicted in (A). (C) Photomicrographs of the Rb-proficient cell lines after culture in the presence of 1 μM PD-0332991 for 7 days reveal growth inhibition and morphological changes resembling cellular senescence, whereas the Rb-deficient cell line (42MGBA) shows no growth
inhibition or morphological changes. (D) Staining for senescence-associated β-galactosidase activity in two Rb-proficient cell lines and one Rb-deficient cell line after culture in the presence of vehicle or 1 µM PD-0332991 for 2 weeks.

**Figure 2. Depletion of Rb expression alleviates growth arrest induced by PD-0332991.** (A) Five unique shRNAs to RB1 were transduced into U87MG cells, and total protein from stably expressing pooled clones was resolved by SDS-PAGE and immunoblotted with an Rb antibody. Clones 19 and 63 show greater than 99% knockdown of Rb expression relative to the empty pLKO.1-infected clone. (B) Flow cytometry analysis following a 1 hr BrdU pulse of U87MG Rb shRNA clones 19 and 63 after 48 hrs of treatment with vehicle or 1 µM PD-0332991. (C) Photomicrographs of clones 19 and 63 following incubation with 1 µM PD-0332991 for 3 weeks demonstrates their failure to arrest and eventual growth to confluence in spite of sustained incubation with the cdk4/6 inhibitor.

**Figure 3. PD-0332991 crosses the blood-brain barrier and potently suppresses the growth of intracranial human GBM xenografts.** U87MG cells (A-D) and primary xenograft GBM 39 (E-F) were modified to express luciferase and injected into the brains of nude mice to establish intracranial tumors. Mice were randomized to control (vehicle treated) and PD-0332991 treatment groups, with PD-0332991 administered daily by oral gavage at 150 mg/kg (4 weeks for U87MG; 12 weeks for GBM 39: see gray horizontal arrows in A,B,E,F). Mice were imaged 1-2x weekly for bioluminescence intensity (see examples in A,E), with luminescence values of individual mice normalized to their corresponding luminescence at the start of therapy, and mean normalized values plotted (A,E). (C) One mouse from the U87MG treatment group was euthanized and its brain was dissected for the isolation of pure tumor, brain adjacent to tumor (BAT), and normal
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tissue from the opposite hemisphere (OH). These specimens were analyzed by LC-MS/MS for determination of PD-0332991 content which demonstrated dissemination of PD-0332991 into the brain and accumulation in tumor tissue. (D) One mouse from the U87MG treatment group and one mouse from the control group were euthanized on the same day post tumor cell injection, and their brains dissected, fixed, and embedded in paraffin. Immunohistochemistry with MIB-1 antibody revealed >8-fold reduction in MIB-1 index in the tumor from the PD-0332991 treated mouse relative to the control. (B,F) Kaplan-Meier survival plots for the U87MG and GBM 39 experiments demonstrating significant survival benefit from PD-0332991 treatment (p < 0.001 in each case).

**Figure 4. PD-0332991 combined with radiation therapy shows enhanced anti-tumor activity and extends survival.** Luciferase-modified U87MG cells were injected into the brains of nude mice to establish intracranial tumors. Mice were randomized to four treatment groups: (i) untreated (Control), radiation only (RT, 2 Gy/day x 5 days, horizontal gray arrow), PD-0332991 only (150 mg/kg/day x 14 days, horizontal black arrow), PD-0332991 and concurrent radiation (RTc), and PD-0332991 with subsequent radiation (RTs). (A) Bioluminescence monitoring was conducted 1-2x weekly throughout the course of the experiment, with mean normalized values plotted for each treatment group. (B) Kaplan-Meier survival plots demonstrating survival benefit from PD-0332991 and radiation combination therapy (p value comparisons < 0.05 are shown in bold).

**Figure 5. Evaluation of PD-0332991 activity against intracranial GBM xenografts that have recurred following initial treatment with temozolomide.** (A) Luciferase-modified U87MG cells were injected into the brains of athymic mice. Mice with proliferating tumors were randomized into untreated (Control) and temozolomide treated groups (TMZ, 10 mg/kg/day for 5 days). (B) When successive mean normalized
luminescence values indicated tumor recurrence of TMZ treated mice (day 35), these mice were randomized to 4 treatment groups: no additional treatment, repeat treatment with the same TMZ regimen, treatment with PD-0332991 (150 mg/kg/day for 14 days), or combined TMZ + PD-0332991 treatment, and then monitored for bioluminescent intensity 1-2x weekly during therapy. (C) Kaplan-Meier survival plots demonstrating significant survival benefit from PD-0332991 therapy of recurrent tumors (p value comparisons < 0.05 are shown in bold).
### Table 1

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PD-0332991 Arrests the Growth of GBM Intracranial Xenografts
PD-0332991 Arrests the Growth of GBM Intracranial Xenografts

Figure 2

A

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 & \text{empty pLKO.1} & \text{RB shRNAs} & 19 & 63 \\
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\text{lane 4}
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B

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C

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PD-0332991 Arrests the Growth of GBM Intracranial Xenografts

Figure 3

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Figure 4

A

Control
RT
PD0332991 + RTs
PD0332991 + RTc

Normalized luminescence

Days

B

Control
RT
PD0332991 + RTs
PD0332991 + RTc

Percent survival

Days

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PD-0332991 Arrests the Growth of GBM Intracranial Xenografts

**Figure 5**

A.  
![Graph A](image1.png)

B.  
![Graph B](image2.png)

C.  
![Graph C](image3.png)

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Supplemental Table and Figure Legends

Supplemental Table 1. Genetic lesions of the major known GBM oncogenes and tumor suppressor genes in a panel of 21 GBM cell lines. These data are derived from exonic sequencing and Affymetrix 250K SNP arrays performed by Solomon et al. (Cancer Res 68:2564-9, 2008; Cancer Res 68:10300-6, 2008; Cancer Res 69:5630-3, 2009), TP53 sequencing from Van Meir et al. (Cancer Res 54:649-52, 1994), Affymetrix 6.0 SNP arrays performed by the Cancer Genome Project of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/), and from the COSMIC database also of the Cancer Genome Project. *, mutation status of gene not determined.

Supplemental Figure 1. Western blots for the major known GBM oncogenes and tumor suppressor genes on a panel of 21 GBM cell lines. Genetic lesions of the cdk4/6-cyclin D-INK4-Rb signaling pathway present in these 21 cell lines detailed in Supplemental Table 1 that result in altered gene expression can be seen. 15/21 of these cell lines have homozygous deletion of CDKN2A/B and make no detectable p16INK4a protein, 5/21 have homozygous deletion or mutation of RB1 that result in no detectable Rb protein, 4/21 have homozygous deletion of CDKN2C that results in no detectable p18INK4c protein, and 1/21 has amplifications of both CDK6 and CCND3 that result in significant overexpression of cdk6 and cyclin D3 proteins. Lesions of CDKN2A/B, RB1, and CDK6/CCND3 are mutually exclusive in these 21 cell lines, except in 42MGBA cells which harbor homozygous deletion of CDKN2A/B and are also Rb-deficient. In contrast, homozygous deletions of CDKN2C are not mutually exclusive with CDKN2A/B, RB1, and CDK6/CCND3 lesions, and in fact are present exclusively in cells with homozygous deletions of CDKN2A/B. A number of additional genetic lesions
in other signaling pathways detailed in Supplemental Table 1 that result in altered gene expression in these cells can also be seen. 11/21 of these cell lines have homozygous deletion or mutation of PTEN resulting in scant or absent protein levels, 2/21 have EGFR rearrangement resulting in EGFR protein at aberrant molecular weights, and 2/21 have MDM2 amplification resulting in significant overexpression of Mdm2 protein. No significant concordance was detected between any of these other genetic lesions and lesions of cdk4/6-cyclin D-INK4-Rb signaling pathway genes.

**Supplemental Figure 2. PD-0332991 induces cell cycle arrest of GBM cells dependent on Rb status.** A panel of 21 GBM cell lines was cultured *in vitro* in the presence of vehicle or 1 µM PD-0332991 for 48 hrs, pulsed with BrdU for 1 hr, then fixed and stained with propidium iodide and FITC-conjugated BrdU antibody for flow cytometry analysis. Cell cycle distribution plots of BrdU vs. propidium iodide (PI) intensity are shown.

**Supplemental Figure 3. Dose response of GBM cell lines with partial or no response to 1 µM PD-0332991.** The panel of 21 GBM cell lines was cultured *in vitro* in the presence of 0, 1, 2, or 10 µM PD-0332991, and cell cycle distribution was assessed at 48 hrs post-treatment by staining with propidium iodide and FITC-conjugated BrdU antibody following a 1 hr BrdU pulse before fixation. Cell lines with homozygous lesions of RB1 (*e.g.* 8MGBA, GMS10, and LN405) displayed no significant reduction in BrdU incorporation or other alteration in cell cycle distribution at any of the doses tested. Of the Rb-proficient cells, those that displayed a 10-95% reduction in BrdU incorporation at a dose of 1 µM PD-0332991 (*e.g.* AM38, Hs683, T98G, GAMG, and LN18) demonstrated increased reductions at doses of 2 and 10 µM. Cells that displayed >95% reduction in BrdU incorporation at 1 µM PD-0332991 demonstrated similar responses at
doses of 2 and 10 µM, and no evidence of cytotoxicity/apoptosis was apparent (e.g. sub-G1 DNA content) at any dose tested (data not shown).

**Supplemental Figure 4. PD-0332991 fails to suppress the growth of intracranial xenografts generated from an Rb-deficient cell line SF767.** Bioluminescence monitoring of mice bearing intracranial xenografts generated from Rb-proficient U87MG cells and Rb-deficient SF767 cells during treatment with vehicle (Control) or PD-0332991 (150 mg/kg/day from day 13-27).
### Supplemental Table 1

#### Genetic lesions present in cells

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Figure S1

PD-0332991 Arrests the Growth of GBM Intracranial Xenografts

EGFR
Myc
PTEN
MDM2
p53
p14ARF
p21WAF1/CIP1
Cyclin D1
Cyclin D3
CDK4
CDK6
p16INK4a
p16INK4c
RB
α-tubulin
Figure S2

+ vehicle
+ 1 μM PD-0332991

U87MG

T98G

CCF-STTG1

H4

LN229

8MGBA

M059J
Figure S2 continued

+ vehicle  + 1 \mu M PD-0332991

SNB19

GAMG

AM38

GMS10

A172

LN18

42MGBA
Figure S2 continued

+ vehicle  
+ 1 μM PD-0332991

Hs683

LN405

DBTRG-05MG

DKMG

NMC-G1

KG-1-C

U138MG
Figure S3

8MGBA

GMS10

LN405

AM38

Hs683

T98G

GAMG

LN18

PD-0332991 Arrests the Growth of GBM Intracranial Xenografts
Figure S4

![Graph showing the growth of GBM Intracranial Xenografts](image-url)
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In total – Michaud et al. Cancer Res 70:3228-38, 2010

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Akron Children's Hospital
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