11-14-2011

The Potential Role of Environmental Exposures and Genomic Signaling in Development of Central Nervous System Tumors

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DOI: 10.25148/etd.FI11120809

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THE POTENTIAL ROLE OF ENVIRONMENTAL EXPOSURES AND GENOMIC SIGNALING IN DEVELOPMENT OF CENTRAL NERVOUS SYSTEM TUMORS

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PUBLIC HEALTH

by

Brian William Kunkle

2011

To: Interim Dean Michelle Cicazzo
Robert Stempel College of Public Health and Social Work

This dissertation written by Brian William Kunkle, and entitled The Potential Role of Environmental Exposures and Genomic Signaling in Development of Central Nervous System Tumors, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: November 14, 2011

The dissertation of Brian William Kunkle is approved.

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ACKNOWLEDGMENTS

It is my pleasure to acknowledge some of the colleagues, family and friends who have helped complete this dissertation. I would like to express my deepest gratitude to my advisor, Dr. Deodutta Roy, for his excellent guidance, support, patience, and encouragement during the entire dissertation process. I would also like to thank Dr. Changwon Yoo, Dr. Nasar Ahmed, and Dr. Quentin Felty for guiding my research for the past several years and helping me to develop my background in bioinformatics, gene networking, epidemiology, and biochemistry. Their expertise and assistance proved invaluable at critical points in this dissertation. Financial support from the Stempel College of Public Health & Social Work, as well as the Florida International University Doctoral Evidence Acquisition Fellowship, was greatly appreciated and completion of this dissertation would not have been possible in its absence. Finally, I would like to thank my family and friends. Their support and encouragement was a source of strength for me during this long process, and I greatly value their love and friendship.

ABSTRACT OF THE DISSERTATION

THE POTENTIAL ROLE OF ENVIRONMENTAL EXPOSURES AND GENOMIC SIGNALING IN DEVELOPMENT OF CENTRAL NERVOUS SYSTEM TUMORS

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The etiology of central nervous system tumors (CNSTs) is mainly unknown. Aside from extremely rare genetic conditions, such as neurofibromatosis and tuberous sclerosis, the only unequivocally identified risk factor is exposure to ionizing radiation, and this explains only a very small fraction of cases. Using meta-analysis, gene networking and bioinformatics methods, this dissertation explored the hypothesis that environmental exposures produce genetic and epigenetic alterations that may be involved in the etiology of CNSTs.

A meta-analysis of epidemiological studies of pesticides and pediatric brain tumors revealed a significantly increased risk of brain tumors among children whose mothers had farm-related exposures during pregnancy. A dose response was recognized when this risk estimate was compared to those for risk of brain tumors from maternal exposure to non-agricultural pesticides during pregnancy, and risk of brain tumors among children exposed to agricultural activities. Through meta-analysis of several microarray studies which compared normal tissue to astrocytomas, we were able to identify a list of 554 genes which were differentially expressed in the majority of astrocytomas. Many of

these genes have in fact been implicated in development of astrocytoma, including EGFR, HIF-1α, c-Myc, WNT5A, and IDH3A. Reverse engineering of these 554 genes using Bayesian network analysis produced a gene network for each grade of astrocytoma (Grade I-IV), and 'key genes' within each grade were identified. Genes found to be most influential to development of the highest grade of astrocytoma, Glioblastoma multiforme (GBM) were: COL4A1, EGFR, BTF3, MPP2, RAB31, CDK4, CD99, ANXA2, TOP2A, and SERBP1. Lastly, bioinformatics analysis of environmental databases and curated published results on GBM was able to identify numerous potential pathways and gene-environment interactions that may play key roles in astrocytoma development.

Findings from this research have strong potential to advance our understanding of the etiology and susceptibility to CNSTs. Validation of our 'key genes' and pathways could potentially lead to useful tools for early detection and novel therapeutic options for these tumors.

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INTRODUCTION

Pediatric brain tumors (pBT), which may occur in the brain or the spinal cord, are the second most common malignancy among children less than 20 years of age and the most common solid tumor of childhood. Malignant brain tumors rank second in both incidence and mortality by cancer in children, and they are the leading cause of cancer death in children. Approximately 3,200 pBTs are diagnosed each year in U.S. children under the age of 20, with roughly 800 of these classified as benign. The incidence of these tumors has been steadily increasing since the early 1980s [1;2].

The etiology of pBTs is largely unknown, though several genetic disorders are known risk factors for their development including neurofibromatosis type 1, Li-Fraumeni syndrome, basal cell nevus (Gorlin's) syndrome, Turcot syndrome, and ataxia telangiectasia. Epidemiological evidence supports a genetic component to pBT etiology. Having a sibling or parent with brain cancer has been found to increase risk of pBTs from 3-9 times, while siblings of children with brain tumors have a greater risk of developing other cancers such as leukemia. While the above factors are thought to play a part in less than 5% of all pBTs, evidence for some combination of genetic and epigenetic changes along with environmental factors in their etiology is accumulating. Although only approximately 1% of childhood brain tumors are present at birth or diagnosed within the first few months of life, the majority of pBTs occur before the age of five, suggesting that prenatal as well as postnatal insults may be potential etiologic factors. It is becoming more evident that not only can drugs and environmental stressors interfere with normal fetal development by causing structural malformations, but that exposure to environmental stressor during development can also cause biochemical and functional

abnormalities. In utero exposure to several drugs and environmental toxicants has been proposed to be a primary cause for the increased incidence of childhood brain cancers, which poses a significant clinical problem. Some suspected in utero carcinogens include benzene, pesticides, trichloroethylene, ethanol, arsenic, cigarette smoke, and infectious biological agents. Early childhood exposures may also influence both aBT and pBT development and evidence that pesticides can influence glial development and differentiation well into childhood support this view [3]. Furthermore, many substances, such as 1,3-Butadiene and N-Nitrosomethylurea, have been shown to induce neurogenic tumors in animals, with both the fetus and neonatal animals being at dramatically increased susceptibility to CNST development compared to adults [4].

Aside from the extremely rare genetic conditions mentioned above, the only unequivocally identified risk factor is exposure to ionizing radiation, and this explains only a very small fraction of cases. The most consistent link between exposures and pBT development aside from radiation is that for maternal consumption of cured meats, but whether a dietary component is responsible has not yet been revealed. A majority of epidemiological studies investing pesticides and cancer risk have reported positive associations, with the risks for children generally higher than adults. While the consistency of results may be affected by type of pesticides being evaluated, exposure circumstances, and tumor classification standards, there is a considerable amount of epidemiological evidence that supports a link between pesticide exposure and pCNSTs. Several viruses have been investigated as causative agents in brain tumors. For instance, JC virus, a type of polyoma virus, and T-protein, an oncogenic virus product, have been detected at significant levels in PNETs and other tumors [5], though these results are

challenged by negative results in other studies [6-10]. The ability of MBs to be induced by JC virus in mice models has also been demonstrated however [11;12]. Parental exposure to heat and electromagnetic fields, parental occupational exposure to chemicals, tobacco exposure, maternal hair dye use, and exposure to several medications and vitamins have also been investigated as potential etiologic factors, with equivocal results being found. Many of these exposures could certainly play a role in the etiology of pBT however, as the high rates of cell proliferation and differentiation during development and childhood increases susceptibility of cells to mutagenic and epigenetic alteration. In addition, the blood-brain barrier, which acts as a barrier to potentially harmful substances, is not fully developed in the fetus and can allow harmful substances to reach sensitive organs such as the brain. In fact, in utero DNA damage resulting from environmental pollution has been associated with somatic gene mutation in newborns [13]. Thus, exposure to environmental factors may be involved in the development of pBTs, and they likely interact with a genetic component and differ based on type of tumor.

LITERATURE REVIEWS

Manuscript 1: Gene-environment interaction and susceptibility to pediatric brain tumors*

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*Published as a Book Chapter in 'Environmental Factors, Genes, and the Development of Human Cancers', Deodutta Roy and M. Tevfik Dorak, Elsevier, New York, 2010

Keywords: *Pediatric brain tumors (pBTs), Gene-environment Interactions, Mitochondrial-Nuclear Signaling, Environment, Individual Susceptibility*

OUTLINE

I. Introduction

II. Environmental Epidemiology of Pediatric Brain Tumors

III. Origin and Neurobiology of Pediatric Brain Tumors

IV. Genetic and Epigenetic Alterations in Pediatric Brain Tumors

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I. INTRODUCTION

Pediatric brain tumors (pBT) are a genetically, biochemically, and clinically heterogeneous group of tumors located in both the central and peripheral nervous system. Collectively, they are the most common solid tumor of childhood, and rank second to leukemia in both incidence and mortality for childhood cancers. Approximately 2,200 intracranial pBTs, or 16.6% of all childhood cancers are diagnosed each year in U.S. children under the age of 20, with 78% of these neoplasms being classified as malignant in nature and the remaining classified as benign or of unspecified behavior [1;2]. Astrocytomas comprise 52% of these malignancies, primitive neuroectodermal tumor (PNET) comprise 21%, other gliomas 15%, and ependymomas an additional 9% [1]. Neuroblastomas (NB), the most common solid extracranial nervous system tumor, arise in the adrenal medulla and sympathetic ganglia and account for another 6-10% of all childhood cancers, with approximately 650 new cases occurring in U.S. children each

year [3]. Nearly 50% of these extracranial tumors occur in children under the age of two years old [3]. Given the early age of onset of a majority of pBTs, and the current dogma that most cancers have at least a 10 year period of formation, it appears these tumors may have an etiology that contrasts with some of the traditionally accepted ideas on tumor formation. In fact, recent research is beginning to suggest an etiology focused on early life environmental factors interacting with dysregulated developmental mechanisms (e.g. signaling pathways and stem cell/progenitor cell growth and differentiation) and mitochondrial-nuclear signaling in pBTs.

While links between specific environmental exposures and pBTs are not yet fully established, several aspects of developmental biology increase the likelihood of their involvement in pBT etiology, including 1) the increased vulnerability of the fetus/neonatal nervous system to exposures, 2) the high rates of cellular proliferation and differentiation during development of the nervous system, and 3) the ability of environmental factors to produce and/or influence genetic and epigenetic alterations in pathways involved in both development and tumorigenesis. For instance, high rates of cell proliferation and differentiation during development and childhood increases susceptibility of cells to mutagenic and epigenetic alteration. In fact, in utero DNA damage resulting from environmental pollution has been associated with somatic gene mutation in newborns [4]. Furthermore, it is becoming more evident that not only can drugs and environmental stressors interfere with normal fetal development by causing structural malformations, but that exposure to environmental stressors during development can also cause biochemical and functional abnormalities. Additionally, the blood-brain barrier, which acts as a barrier to potentially harmful substances, is not fully

developed in the fetus and can allow harmful substances to reach sensitive organs such as the brain. Some suspected in utero carcinogens for pBT include pesticides, N-nitroso compounds, tobacco, and infectious biological agents. While evidence linking several of these exposures to pBTs is substantial, the only exposure known to clearly cause pBTs is ionizing radiation. However, chemicals such as 1,3-Butadiene and N-Nitrosomethylurea have been shown to induce neurogenic tumors in animals, with both the fetus and neonatal animals being at dramatically increased susceptibility to BT development compared to adults [5]. Furthermore, evidence showing that pesticides can influence glial development and differentiation well into childhood suggests that early life exposures may influence both pBT and adult BT (aBT) [6]. In fact, evidence from several other cancers suggests that susceptibility to adult tumor development may in fact be influenced by early life environmental exposures and their interaction with epigenetic mechanisms [7]. Given the above factors, it is probable that certain genetic, epigenetic, and environmental factors unique a developing fetus/neonate, are part of a multifactorial cascade of differing events that lead to pBT development. It is likely that these factors exert influences on the growth and differentiation of neural stem cells/progenitor cells, leading to the production of genetic and epigenetic changes that increase an individual's susceptibility to pBTs (Figure 1-1).

The following is a review of the current state of the research on environmental, genetic, and epigenetic factors possibly involved in the development of pBTs. The first section of this review covers epidemiological research on environmental factors that have been investigated in relation to pBTs. Research supporting the involvement of neural stem cells, progenitor cells, and developmental pathways in the etiology of pBTs is then

summarized. This section is followed by a review of the genetic and epigenetic alterations that have been identified in common pBTs, with the final section focusing on how these factors may interact with mitochondrial-nuclear signaling to increase individual susceptibility to pBTs.

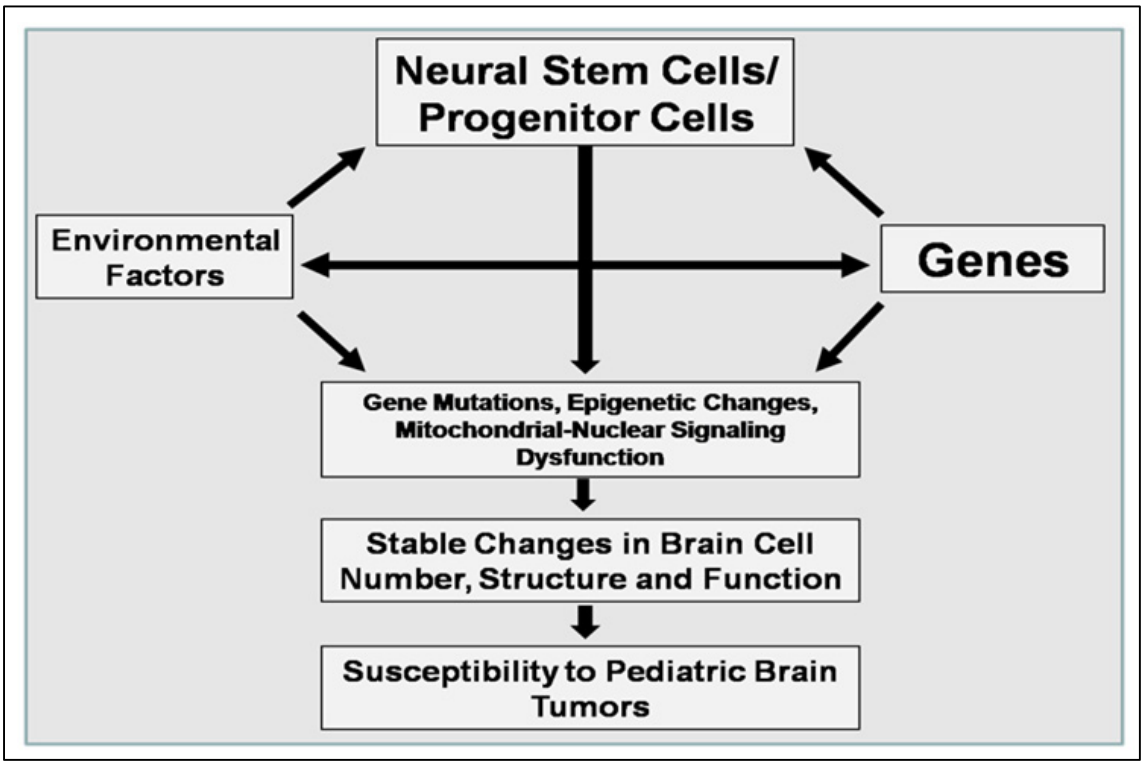

Figure 1-1. A schematic representation of an early life gene-environment interaction model of development of pediatric brain tumors.

II. ENVIRONMENTAL EPIDEMIOLOGY OF PEDIATRIC BRAIN TUMORS

Links between the environment and traditional epidemiology and risk factors

While the incidence of pBTs has been steadily increasing since the early 1980s, debate exists as to the reason for this rise, with some researchers suggesting diagnostic factors as a main cause, while others emphasize the importance of environmental factors [8;9]. In the United States the recorded incidence of pBTs increased by 35% from 1973 to 1994

[10]. Similar increases for these tumors were noted in Europe [11] and in England [12;13]. Considerable debate exists as to how much these large increases are due to an etiologic factor or the result of better diagnostic methods, improved reporting, and changes in classification (i.e. tumors earlier designated as benign being changed to malignant) [14]. Supporting an increase due to better diagnostics and reporting, Smith et al.'s (1998) detailed statistical analysis concluded that a statistical model with a 'jump' in incidence in the mid-1980s fits the incidence data better than a model with a steady increase [10]. A critical analysis of pBTs over this same period by Linet et al. (1999) also supported this view [15]. However, some researchers remain doubtful that the increase is adequately explained with only this type of statistical analysis [14]. Indeed, recent reports from Europe on childhood cancer incidence over this time period argue for a true increase in incidence over this time period [11;16], suggesting that environmental factors have played a role in this rise in incidence. Researchers have also pointed out that while incidence increases in some subgroups of pBTs can be attributed to better diagnostics and changes in classification, these changes do not explain increases in other pBTs [17].

Though the etiology of pBTs is largely unknown, several genetic disorders are known risk factors for their development including neurofibromatosis type 1, Li-Fraumeni syndrome, basal cell nevus (Gorlin's) syndrome, Turcot syndrome, and ataxia telangiectasia. These single gene disorders are thought to play a part in less than 5% of all pBTs however. Still, epidemiological evidence does point to a genetic component in pBTs, as having a sibling or parent with brain cancer has been found to increase risk of pBTs from 3-9 times, while siblings of children with brain tumors have a greater risk of

developing other cancers such as leukemia. These family patterns are likely due to multigenic inheritance patterns ("soft" but interacting mutations), modifiers that alter gene expression or penetrance, or epigenetic gene silencing, rather than the consequence of a highly penetrant gene however [18]. It is also probable that common environmental factors among these family members contribute to this increased risk profile. Gender is another risk factor, as most pBTs have been found to occur at a higher frequency in males than in females, though the reason for this difference is unknown. This difference varies by tumor, as certain tumors including medulloblastoma and ependymoma show incidence rates in males 1.5-2 times that of females, while other tumor types such as astrocytoma show only small differences between these groups [19-21]. While research on the subject is lacking, it is important to consider that these gender-specific differences may in fact be related to gender-specific responses to a given toxicant. Other common risk factors for pBTs include race (they occur more often among Caucasians than other races) and age (they occur more often in children younger than 8 years old than in older children). Geographically, astrocytoma incidence is low in Asia, intermediate in Central and South America, and relatively higher in North America, Europe, Australia, and New Zealand. Additionally, astrocytoma is most common in Sweden, which has a rate 20% higher than any other reported rate. Similar to astrocytoma, PNET, a broad tumor group which includes medulloblastoma (MB), has incidence rates that are relatively low in Asia and higher in Europe and North America. Rates for PNET range from 2 to 12 per million per year with the highest rates occurring in Hawaiians in Hawaii and Maori in New Zealand [22]. Dietary patterns and other differences in environmental factors between these geographies could explain some of these patterns, as has been suggested in other cancers.

Incidence patterns among developed and developing countries may not be comparable however as all brain tumors in developing countries may not come to medical attention and some of the low rates observed might be underestimates.

Studies in North Carolina, Japan, and Norway have found that seasonally, medulloblastoma shows a statistically significant variation in incidence with a peak in October [23-25]. Researchers speculate that exposures during the Spring, when major fetal development would occur for these births, may be involved, including pesticide applications and use of allergic medications. Other tumor types including astrocytoma and ependymoma do not show this seasonal correlation however [26], despite astrocytoma being the tumor most often linked with pesticide exposure. Studies have also assessed clinical parameters and their association with risk of pBTs. Findings on birth weight show only weak or null associations to pBTs, for most studies, although one study which looked at subtypes of tumors found high birth weight to cause an increased OR of 1.71 (95% CI: 1.01-2.90) for astrocytic tumors [27]. Head circumference has also been studied in relation to pBTs and is positively associated with their occurrence [OR = 1.27 (95% CI 1.16-1.38) per 1-cm increase in head circumference] [28].

Epidemiologic research on specific environmental exposures and pBT

Whether specific environmental exposures cause pBTs remains unknown, with only high-intensity external ionizing radiation for therapy of childhood leukemia, tinea capitis and other malignancies the only exposure clearly associated with development of nervous system cancer [29-31]; [19;32]. Diagnostic radiography during pregnancy also appears to result in a small but significant increase in risk of pBT to the fetus, although this

association is disputed [33]. Neither of these exposures is thought to account for a significant number of pBTs today however, as diagnostic radiation has decreased in use and dosage, and therapeutic radiation is now used mostly for malignant conditions that do not expose the brain [34]. Research over the past few decades does point to the relevancy of a few environmental exposures in pBT etiology however. A number of these exposures, including pesticides, N-nitroso compounds, and infection with polyomavirus, have been positively linked to pBT, although findings are inconclusive. While positive results have been found, the rarity of the disease, difficulty in assessing exposure during pregnancy and other critical time periods, assessment of diverse pBTs as a single type, and recall bias, distort and confound results for these investigations. Several work-related exposures have been convincingly linked to aBT however, providing support to the possibility that environmental exposures to a fetus or young child could produce pBTs. These work-related exposures include nuclear workers exposed to radiation, pathologists and embalmers exposed to formaldehyde, plastic workers exposed to vinyl chloride, and textile and plastic workers exposed to acrylonitrile [35]. A summary of the research of several environmental exposures and their relation to pBT is provided below, and Table 1-1 gives an overall 'risk association level' for each exposure.

III. ORIGIN AND NEUROBIOLOGY OF PEDIATRIC BRAIN TUMORS

The majority of pBTs are embryonal in nature, while carcinomas in epithelial tissues, the most frequent type of cancer in adults, are rare in children. A large amount of pediatric gliomas, the most frequent pBTs, arise in the cerebellum and brainstem (infratentorial) from glia, non-neural cells that provide support and nutrition and

Table 1-1. Summary of overall risk evaluation for selected environmental exposures in relation to pediatric brain tumors.

Risk Factors	Risk Evaluation	Risks for Exposures (OR/RR with 95% CI)
Ionizing Radiation	Conclusive	▪ 33.1 (9.4-116.5) for benign nerve sheath tumors of head/neck [31] ▪ 9.5 (3.5-25.7) for meningioma's [31] ▪ 2.6 (0.8-8.6) for malignant gliomas [31] ▪ 21.7-fold increase in CNS neoplasms in a large cohort of 9720 children who had undergone radiation for leukemia [30] ▪ Atomic bomb exposure followed a dose-response curve [(excess relative risk (ERR) = 1.2 (0.6 to 2.1), with the highest ERR found in relation to schwannoma 4.5 (1.9 to 9.2) [36].
Pesticides	Suggestive but not Conclusive	▪ Agricultural pesticide use and pBT risk in children aged 0 to 14 years [RR = 3.37 (1.63-6.94)] [37]. ▪ Increased risk of astrocytoma for exposure to herbicides from residential use [OR = 1.9 (1.2-3.0)] [38]
N-nitroso	Suggestive but not Conclusive	▪ Four of eight studies on cured meat exposure found 2 to 2.5 times increased risk of pBT [39-42] ▪ Mother's frequent ingestion of hot dogs [(RR = 1.33 (1.08-1.66)] and sausage [(RR = 1.44 (1.01–2.06)] during pregnancy [43] ▪ Meta-analysis of cured meat consumption [RR = 1.68 (1.30-2.17)] [43] ▪ Use of metronidazole and neuroblastoma (RR = 2.60 (0.89-7.59)] [44]. ▪ Use of narcotics [OR = 1.3 (1.0–1.6)] and the anasthic penthrane [OR = 1.5 (1.1–2.0)] during delivery [45]. ▪ Anticonvulsant use during pregnancy [(OR = 1.4 (0.6–3.2)] [46]
Infectious Agents	Inconsistent or limited	▪ Viral infection during pregnancy [OR = 10.6 (1.1-503.2) [47] ▪ Influenza during gestation [OR = 3.15 (CI = 1.13-8.77)] [48] ▪ Three siblings, or younger siblings increased risk for astrocytoma (RR=1.34), medulloblastoma (RR=2.30), ependymoma (RR of 2.61), meningioma (RR=3.71), and neuroblastoma (RR=2.31) [49].
Tobacco	Inconsistent or limited	▪ Meta-analysis of 12 observational studies on maternal smoking during pregnancy [RR = 1.05 (0.90-1.21)] [50] ▪ Paternal smoking exposure [RR = 1.22 (1.05-1.40)] [50] ▪ Second meta-analysis on maternal smoking during pregnancy [RR = 1.04 (CI: 0.92-1.18)] [51].
Electromagnetic Frequencies	Inconsistent or limited	▪ Electromagetic Frequencies and childhood exposure [OR = 0.97 (0.46-2.05)] [52].
Trauma	Inconsistent or limited	▪ Medical attention for head injury [OR = 1.4 (1.0 – 1.9)] [53]
Vitamins	Inconsistent or limited	▪ Children of mothers who used multivitamins [OR=0.7 (0.4-1.0)] and had a diet high in iron and folate [OR for iron, 0.5 (0.3-0.9); OR for folate, 0.5 (0.3-0.9)] [54]

participate in signal transmission in the nervous system. While childhood gliomas originating in the cerebellum (supratentorial) are also fairly common, the majority aBTs are supratentorial in nature. Glial tumors are usually broken down into more specific subtypes based on their predicted cell type of origin. The most common glial tumors in patients under 20 years of age are astrocytoma (from astrocytes), oligodendroglioma (from oligodendrocytes), brain stem glioma (from brain stem cells), and ependymoma (from ependymal cells). Some pBTs are composed of primitive or undifferentiated cells, as would be expected given their origin during early life when the nervous system is still developing. These include the primitive neuroectodermal tumors (PNETs) of the brain, notably medulloblastoma (MB), ependymoblastoma, and neuroblastoma (NB). One other common brain tumor in childhood is the germ cell tumor. Germ cells, which are special cells in a developing embryo, can travel to other areas of the body, in this case the brain, and become cancerous. Mixed forms of brain tumors also exist. For instance, mixed gliomas (aka oligoastrocytomas) have both an astrocytic and an oligodendroglial cell component. Additionally, mixed glio-neuronal tumors (tumors displaying a neuronal, as well as a glial component, e.g. gangliogliomas, disembryoplastic neuroepithelial tumors) and tumors originating from neuronal cells (e.g. gangliocytoma, central gangliocytoma) can also develop in the central nervous system (CNS). Other varieties of primary brain tumors include: tumors of the pineal parenchyma (e.g. pineocytoma, pineoblastoma), choroid plexus tumors, and neuroepithelial tumors of uncertain origin (e.g. gliomatosis cerebri, astroblastoma). Additionally, many subtypes of these tumors also exist and are often grouped by histological grade (i.e. Astrocytoma grades I-IV).

Cellular Origins of pBTs

One of the critical issues involving NT biology has been the inability to determine a definitive cell of origin of each individual tumor type, including the three most prevalent tumors of the pediatric CNS: pilocytic astrocytoma, MB, and NB. Identification of these cell types would allow for better comparisons to normal cell counterparts, and a more definitive genomic profile of the tumor cells involved. Currently, tumors are classified by the World Health Organization (WHO) according to the cell type that tumor cells resemble most in the developing embryo or adult. The three main cell types from which tumor cells are thought to arise are neural stem cells, progenitor cells, or differentiated cells, and genetic changes in each cell type have been associated with BT development [55]. It is likely these genetic changes, which may be either inherited or somatic in nature, deregulate normal proliferation and differentiation, and initiate tumor formation. Therefore, most pBTs (which are predominantly immature tumors) likely arise from neural stem cells (which produce both glial and neuronal cells) or progenitor cells in their respective precursor cell type (i.e. granule cell precursors for MBs), while most adult tumors probably form from differentiated cell types (i.e. astrocytes in astrocytoma) that have acquired stem cell-like or progenitor cell-like properties [55]. Recent research on MBs in mice models does in fact suggest that alterations in either cell type can produce MB, with the critical determinant of tumorigenesis being neuronal lineage commitment of the cell [56;57]. Additionally, alterations in genes such as Bmi1, an oncogene involved in proliferation of differentiated cells and self-renewal of stem cells, likely lead to different phenotypic outcomes of BT [58;59]. Also, tumors arising in different regions of the brain retain distinct patterns of gene expression, though cells in different parts of

the brain carry the same genes. This is a result of factors that have modified the use of these genes differently in distinct parts of the brain, suppressing some genes and activating others to allow the cells to take on specialized characteristics as the brain matures. Extensive debate exists within this framework however, as evidence for differing points of view exists in the literature. For example, MB having a neuronal cell of origin as suggested by Wright et al. is currently being opposed by those investigators who support a view by Bailey and Cushing that because MBs generate both glial and neuronal cells, they may originate from a cell they termed as an embryonic neuroepithelial [60;61]. For a complete review of the current information on cell of origin in brain tumors the reader is referred a review by Read et al. [55]. While the cellular origins of these tumors is still debated, recent research is beginning to elucidate how the deregulation of developmental pathways within these cells contributes to pBT etiology, and an overview of this research is provided below.

Disruption of developmental pathways, 'tumor precursor cells', and pBTs

Most pBTs are embryonal tumors that arise from abnormal development of nervous system tissue. Embryonal development is marked by organogenesis from stem cell populations producing 'precursor' cells that give rise to lineage-committed progenitor cells. These progenitor cells then undergo stages of proliferation and differentiation to produce tissue. Several cellular pathways that regulate these developmental processes have been implicated in the development of pBT, including the Sonic Hedgehog (SHh) pathway, the WNT-wingless (WNT) pathway, Mycn signaling, and the NOTCH signaling pathway. During normal nervous system development, these signaling

pathways regulate growth and differentiation of progenitor cells, including granule cell precursors (GCPs) in the cerebellum and neural crest cells in the peripheral neural system (PNS). These cell populations ultimately form the cerebellum, peripheral neural crest, and other nervous system components. In cerebellar development, SHh accomplishes regulation of GCPs in part via expression of N-myc, a commonly expressed tumor suppressor gene thought to be an essential downstream target of SHh signaling in both normal and neoplastic cerebellar growth [62]. Mutations that dysregulate the SHh pathway can cause excessive growth and failure in cell differentiation, producing 'tumor precursor cells' that predispose an individual to MB [63]. Note that we use the term 'tumor precursor cells' as a generic term for all forms of stem, precursor, or progenitor cells that have acquired tumor producing alterations. Similar to dysregulation of SHh pathway in MB, N-myc signaling appears to direct proliferation and differentiation of neural crest cells in PNS development, and its dysregulation appears to make a large contribution to development of neuroblastoma [64]. Finally, several alterations identified in astrocytic tumors are located in genes important for normal differentiation of astroglial cells during development, including changes in several SHh pathway proteins [65-67]. Figure 1-2 provides a schematic showing commonly mutated genes in these and other developmentally regulated pathways thought to be involved in pBT tumorigenesis. There are possible roles for environmental regulation within these pathways. For instance, regulation of N-myc by SHh is thought to occur through activation of phosphoinositide 3-kinase (PI3K), which, through downstream activation of Akt, and inactivation of glycogen synthase kinase-3β (GSK-3β) leads to stabilization of N-myc. High levels of N-myc, which are linked to cellular transformation and aggressive tumors, lead to

proliferation, migration, and cell cycle activation, while reduced levels produce cell cycle exit and differentiation. Thus, when SHh signaling is activated, Akt signaling and amplification of N-myc, promotes cell survival and growth, and possibly tumor initiation and promotion [68]. Interestingly, the PI3K/Akt pathway is implicated in ROS related disorders and has been linked to control of nuclear-receptor factor 1 (NRF-1) signaling in mitochondrial biogenesis [69], linking two biological mechanisms thought to be important in environmental disease.

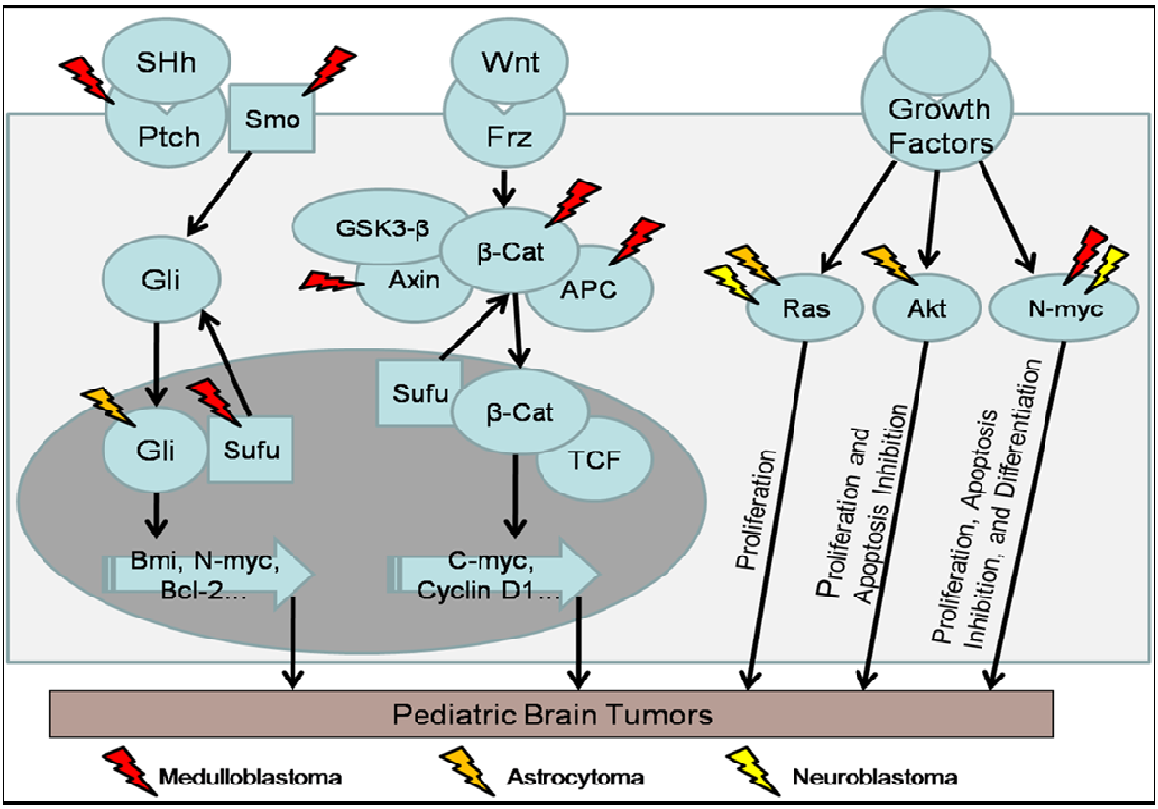

Figure 1-2. Dysregulation of developmental pathways in pediatric brain tumors (pBT). Alterations in pathways important to cellular growth, proliferation and differentiation during development appear to be important to pBT etiology. Cross-talk between these pathways has also been shown. Lightning bolts signify alterations in genes linked to development of their respective pBT.

IV. GENETIC AND EPIGENETIC ALTERATIONS IN PEDIATRIC BRAIN TUMORS

The differing demographic profiles and heterogeneity within and between types of pBT suggests differing etiology for these tumors, with genetic alterations possibly playing a more substantial role in some tumor types than in others, and individual cell types perhaps being more vulnerable to toxins at different stages of development [70]. Additionally, this heterogeneity suggests the probable involvement of epigenetic and mitochondrial mechanisms of disease, both of which have been shown to interact with the environment to produce heterogeneous disease profiles [7]. Despite these probable etiologic differences, the research highlighted above suggests that many pBTs may have a common origin through *in utero* and early life disruption of developmental pathways. Alterations in developmental pathways do not currently account for a majority of pBTs however, and few other significant genetic changes in these tumors have been established. However, when taken as a whole, the collective alterations identified within some of the common pBTs does fit a model where environmental factors interacting with numerous genetic and epigenetic changes influences cellular proliferation and function to produce tumorigenesis. As referenced in section II, several genetic disorders have been linked to development of pBTs. Several mutations in genes causing these familial forms of pBT have been identified (Table 1-2). These genes, most of which are tumor suppressors, also appear altered in sporadic cases of their respective brain tumor types. Moreover, some also function in the developmental processes discussed above.

Table 1-2. Disorders and mutations in genes causing familial forms of pediatric brain tumors.

Disorder	pBT Type	Germline Mutation	Locus	Signaling Pathway
Neurofibromatosis Type-1	Astrocytoma	NF1	17q11.2	Ras
Li-Fraumeni Sydrome	Astrocytoma, PNET	p53	17p13	p53
Basal Cell Nevus (Gorlin's) Syndrome	Medulloblastoma	PTCH	9q22.3-q31	SHh
Turcot Syndrome	Medulloblastoma Malignant Glioma	APC	5q21	Wnt
Ataxia Telangiectasia	Medulloblastoma Pilocytic Astrocytoma	ATM	11q22-23	ATM/p53

V. ENVIRONMENT, MITOCHONDRIAL-NUCLEAR INTERACTIONS, AND DEVELOPMENT OF PEDIATRIC BRAIN TUMORS

Given that mutations in mtDNA have been reported in most cancers (reviewed in [71]), it appears important to consider their effect on tumorigenesis. A key argument in cancer initiation to date however, has been 'what comes first, mitochondrial dysfunction or tumor formation?'. Until recently, most reports have suggested that mitochondrial dysfunction occurs after tumor formation. However, research in 2000 and 2001 showing that inherited and sporadic cases of brain tumors (paraganglioma and pheochromocytoma) are caused by mutation of succinate dehydrogenase, a mitochondrial-specific protein of the Krebs cycle, provided support to the idea that mitochondria (mt) may control tumorigenesis. Subsequently, mutations in another mitochondrial Krebs cycle protein, fumarase, were associated with the development of uterine fibroids, skin leiomyomata and renal cell cancer. Though the specific mechanisms for tumor formation in these instances are yet to be determined, it has been suggested that accumulation of these proteins in mitochondria could lead to a decrease in

the ROS-scavenging activity of the respiratory chain, causing excess superoxide and tumor initiation [72]. Importantly, it appears mutations in either the mitochondrial or nuclear genomes could lead to tumorigenesis, as alterations in both genomes have been shown to lead to mitochondrial diseases [73;74]. Alterations in the epigenome of both the nucleus and mitochondria may also play a role in tumorigenesis, as recent research demonstrating that depletion of mtDNA can regulate epigenetic modification in the nucleus suggests [75]. These tumorigenic scenarios described above fit well with a model where both cell death and proliferation are in large part controlled by the functioning state of the mitochondria. In this model, the redox state of the cell would be of ultimate importance to development of disease. While a low level of ROS would allow for normal cellular function, increasing ROS amounts would signal increased cell proliferation, and an overwhelming excess of ROS would trigger apoptosis. Importantly, research showing that mtDNA-depleted cells increased antioxidant levels (MnSOD) and resisted apoptosis even in an elevated ROS environment, suggest that mitochondrial apoptosis pathways may often be blocked even in dysregulated mitochondria, allowing for cellular transformation to occur [76]. Given that interaction between mitochondrial and nuclear genomes is essential for normal cellular function, variations in the mitochondrial redox state could be of tremendous importance for the fate of the cell, and ROS signaling could have a central role in communication between mitochondria and the nucleus.

Because mitochondria play a large part in brain development [77] and are abundant in brain tissue [78], their health is critical to the wellbeing of the pediatric brain. The pediatric brain is at high risk of oxidative stress and very susceptible to free radical

oxidative damage [79]. The electron transport chain (ETC) found in brain mitochondria can be impaired by both inherited and acquired mutations (Stewart et al. 2000). An impaired ETC leads to decreased ATP production, increased ROS formation, and altered calcium homeostasis. Thus, mutations in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) could affect cellular energy levels, increase oxidative stress, cause ROS-mediated damage to both the mitochondrial and nuclear genome, affect cellular differentiation, and alter the cellular response to apoptosis [80-83]. Figure 1-3 provides a schematic overview of how environmental factors could interact with dysfunction in mitochondrial-nuclear signaling pathways and developmental pathways to produce pBTs.

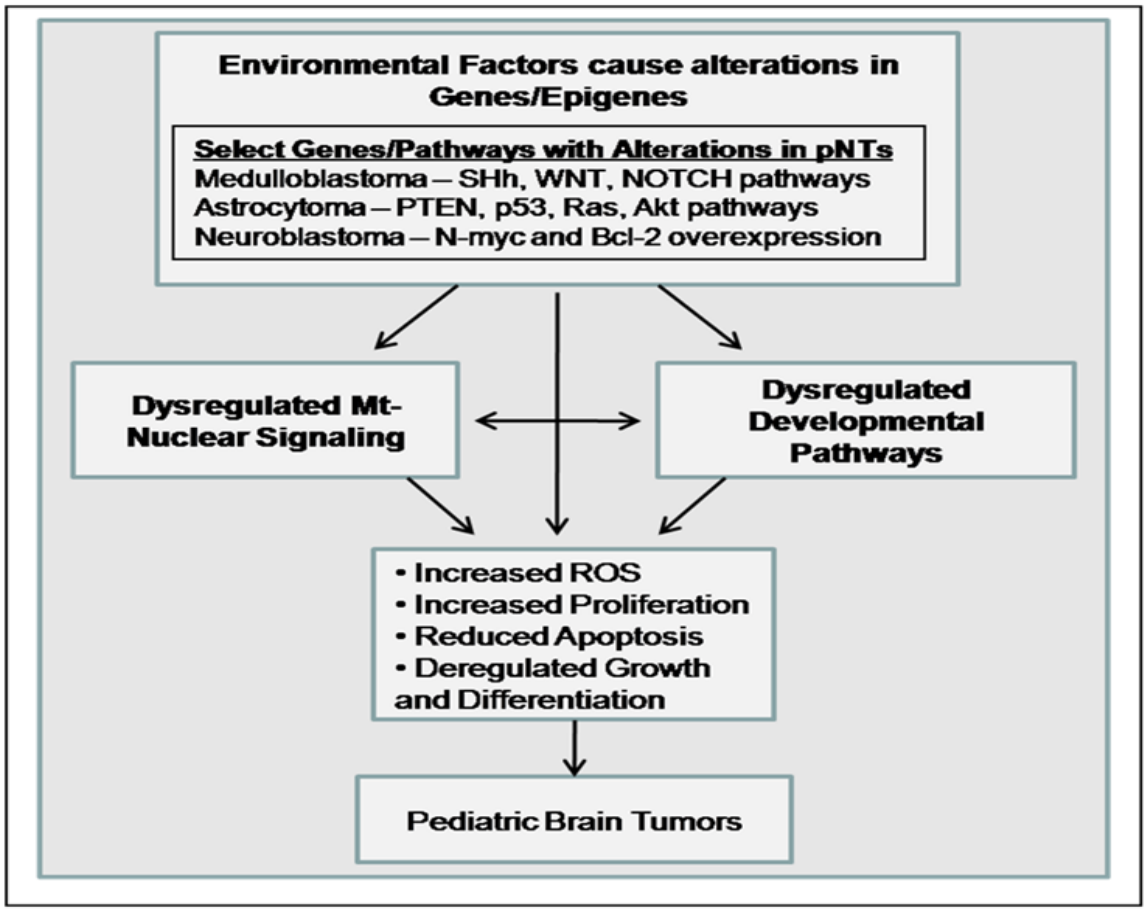

Figure 1-3. Interaction of environmental factors, dysregulation of mt-nuclear signaling and developmental pathways, and development of pediatric brain tumors.

Effects of early life exposures on mitochondria and the pediatric brain

There is a growing body of evidence suggesting that there are critical periods of time extending from conception to puberty when the reproductive [84], immune [85], nervous [84], and endocrine [86] systems in children may be more affected by chemical exposures. In fact, a consensus group has concluded that there is sufficient evidence to support the hypothesis that *in utero* and early life exposures can lead to cancer in both children and adults [87]. Evidence supporting this conclusion includes experimental findings that suggest tumors initiated *in utero* can be promoted postnatally, resulting in cancerous growths [88]. Additionally, the ability of environmental exposures to cause micronuclei, chromosomal aberrations, sister chromatic exchanges, DNA adducts, DNA single-strand breaks, and somatic mutations in the DNA of a fetus has been demonstrated (reviewed in [89;90]).

Several characteristics of the developing brain in pediatrics may make agents normally thought not to target the central nervous system harmful to the brain during these critical time periods. These include the blood-brain barrier of the fetus being incompletely developed, the immune system being compromised due to its immaturity, and the increased vulnerability of the developing nervous system to carcinogenesis [91]. Furthermore, several factors including high rates of cell division, lowered detoxification capabilities, and clonal expansion of initiated cells are thought to explain the increased sensitivity of embryos, fetuses, and newborns to chemical carcinogens (reviewed in [92]). Many carcinogens, including ENU, cadmium, and monosodium glutamate, have been shown to freely traverse the blood-brain barrier of a developing fetus in experimental animals, while being blocked from entering the mature brain [93;94]. Furthermore,

numerous agents including rubella virus, lead, methylmercury, retinoids and thalidomide have been shown to be neurotoxic when exposure occurs during gestation. These same agents show no neurotoxicity after adult exposures [94]. Though few quantitative studies on this topic have been completed, one study assessing the blood-brain barrier of sheep during the last two months of gestation and the first month of neonatal compared to adult animals found that the influx of radioactive compounds in all regions of the brain decreased significantly with maturation ($p < 0.0001$). A more in depth review on this topic has been published by Adinolfi 1985. [95]

Experimental evidence linking early life exposures to mitochondrial dysfunction and pBT development

The effects of environmental exposures on BT development have been studied both *in vitro* and in animal models. Several studies have investigated manganese toxicity in brain tumor cell lines. Manganese (Mn) exposure in humans is rare but can occur through occupational pathways. Mn targets brain mitochondria and interferes with energy metabolism in both astrocytoma and neuroblastoma cells through reduction of activity of glycolytic and TCA enzymes such as hexokinase, pyruvate kinase, lactate dehydrogenase, citrate synthase, and malate dehydrogenase [96]. As a result of this metabolic change, Mn exposure may increase oxidative stress via activation of the mitochondrial permeability transition pore (mPTP), a result found in Mn exposed astrocytes [97]. Manganese-induced cell death in both astrocytoma and neuroblastoma cells is primarily necrotic in nature and is enhanced by glutathione depletion [98]. Interestingly, neuroblastoma cells are more susceptible to Mn toxicity than GBM cells,

possibly because of Mn treatments effects on apoptosis in GBM cells. On treatment in GBM cells, Mn increases apoptosis and has differential effects on proliferation pathways as it induces down-regulation of MAPK pathway, but does not significantly affect the AKT pathway. Furthermore, Mn reduces levels of c-Jun, c-Fos, and MMP-2 (an extracellular matrix degrading enzyme), which are all associated with invasiveness of GBM [96].

An *in vitro* study investigating the effect of chlorpyrifos exposure on glioma cells found that the cell signaling interference produced by chlorpyrifos exposure was greater in undifferentiated cells compared to differentiating cells, though effects were still noticed on this cell type as well. However, differentiation enhanced reactive oxygen species (ROS) production from chlorpyrifos exposure and provoked shortage of the nuclear transcription factor Sp1, an essential molecule in differentiation [6]. Other *in vitro* studies include a report which found selenium levels in the cerebrospinal fluid of patients with malignant brain tumors to be lower than those with benign tumors. Furthermore, selenium's anticancer effects are seen in its ability to preferentially target human glioma cells for cell death through induction of superoxide and its subsequent disruption of mitochondria [99]. *In vivo* studies showing environmental exposure effects on BT have been performed on several animal species. Studies in rats and mice showing transplacental induction of BT from chemical exposures have mainly produced glial tumors (most commonly oligodendrogliomas), with some meningeal tumors being induced as well, and peripheral nervous tumors only rare occurrences. Significant incidences of BTs in rats using long-term bioassays have also been produced through exposure to acrylonitrile, acrylamide, 1,3-butadiene, ethylene oxide, glycidol, and

isoprene; the alkylating agents1,3-propane sultone and 2-methylaziridine; and a several hydrazine derivatives including 1,2-diethylhydrazine [5]. Studies in mice show increased susceptibility to brain mutations for prenatal and neonatal (N-ethyl-N-nitrosourea) ENU-exposed mice over adult ENU-exposed mice [100]. It should be noted that while numerous tumor types have been produced from transplacental exposure of rodents, most tumors are not of embryonal origin, but instead are of glial origin and only become clinically evident in adult rats. Other *in vivo* studies have found that phenothiazine chlorpromazine can cause CNS abnormalities in the fetus (Kovacic and Jacintho 2001b), while cadmium has been shown to be toxic to sensory ganglia in many animal species (Habeebu et al. 2001).

Experiments on mitochondrial function in the nervous system point to their involvement in environmentally-induced BTs. Because mitochondria are the main source of superoxide in physiologic conditions, the detoxification of superoxide by mitochondrial MnSOD plays an important role in neuroprotection and ROS homeostasis [101]. Even a modest increase in endogenous ROS could damage both mtDNA and nDNA, leading to cancer development, genetic instability, and disease progression. MnSOD has been shown to have high activity in mice brain when compared to other tissues such as lungs [102]. Because the MnSOD by-product H_2O_2 is itself a ROS, it is interesting to note that overexpression of MnSOD in rat glioma cells increased risk for oxidative damage [103]. However, loss of MnSOD also produces neurodegeneration, DNA oxidative damage and mitochondrial respiratory chain abnormalities, indicating that both overexpression and underexpression of MnSOD can be harmful [104]. A more

focused discussion on mitochondrial ROS production and its effects on the pediatric nervous system is provided below.

Mitochondrial ROS, electron transport chain defects, and pediatric susceptibility

Mitochondrial ETC disorders are believed to occur with an incidence of 1/10,000 live births and research correlates increases in mitochondrial ROS production with both neurodegenerative disease and cancer in animal models [105;106]. In fact, the importance of ROS levels and pBT development can be seen in the fact that numerous genes and signal transduction pathways important to normal fetal brain development (i.e. myc) can be influenced by ROS [107;108]. While, no studies to date have been published on age-dependent differences in brain mitochondrial ROS production in immature animals, research has documented developmental disparities in antioxidant defense systems. Both Mavelli et al. [109] and Khan et al. [110] reported increases in mitochondrial MnSOD in the first weeks of life in mice. Mavelli et al. reported no significant changes with age were noted for Cu, Zn-SOD or GPX activity, while catalase activities were inconclusive. On the contrary, Khan et al. did report an increase in GPX activity near birth. One report also noted a developmental lag in SOD-1 and SOD-2 when compared with GPX and catalase levels present in prenatal telecephalic white matter [111]. On the whole, the above studies describe a profile where a newborn brain must be protected from oxidative stress through increased activity of antioxidant enzymes in utero and around birth.

The large transmembrane protein cytochrome *c* oxidase (aka complex IV or COX) is the last enzyme in the ETC of mitochondria. This complex catalyzes the transfer of

electrons from reduced COX to molecular oxygen to form water, and ultimately, ATP through the coupled process of oxidative phosphorylation. The importance of COX to neurons is seen in this enzyme's reduced expression in neurodegenerative diseases, such as Alzheimer disease [112]. This is because neurons, which are highly dependent upon ATP for their activity and functions [113], receive roughly 90% of their ATP from mitochondria via oxidative phosphorylation [114]. MtDNA diseases exhibit low COX activity and evidence points to clonal expansion of individual mtDNA deletions within single cells as a reason for this decreased action [115]. Moreover, COX deficiency most often manifests in high energy demand organs, such as the brain [78], and is the most prominent ETC defect in infancy and early childhood [116]. Finally, genotype of Tfam, a key regulator of mtDNA copy number and mitochondrial transcription, is associated with a moderate risk for Alzheimer's disease [117]. Evidence suggests that Tfam may protect against mtDNA damage by preventing a decrease in mtDNA copy number and ETC function, an action that ultimately may protect against oxidative stress and its effects on lipid peroxidation, apoptosis, and mtDNA [118]. Importantly, Tfam, appears to accomplish much of these actions through interaction with NRF-1 and other nuclear genes controlling mitochondrial biogenesis and function [119-121]. Furthermore, while nuclear proteins contribute to mitochondrial regulation, mitochondria can also modulate the expression of nuclear cell cycle genes [119], suggesting the importance of cross-talk between the cell nucleus and mitochondria in the apoptosis, proliferation, and differentiation of both normal and malignant cells. We now discuss possible implications of this cross-talk on pBT development.

Evidence of mitochondrial-nuclear interactions and mitochondrial dysfunction in pBTs

Recent evidence is beginning to provide a foundation which does in fact support a model of mitochondrial-nuclear signaling dysfunction in formation of tumors, including pBTs. Studies in yeast models and human cancer cell models showed that depletion of mtDNA increased oxidative stress which then produced extensive damage to the nuclear genome [81;122]. Additionally, it appears that expression of certain nuclear genes may play a key role in mitochondrial mediated tumorigenesis. In fact, APE1, a nuclear encoded gene involved in redox regulation, apoptosis, and DNA repair (both independently and through interaction with p53), may be one such gene, as it was shown to underexpressed in 68% (13 of 19) of CNS tumors [123]. It appears mitochondrial defects can lead to down regulation of APE1 to produce tumors. Restoration of expression of APE1 in mtDNA depleted cells reversed the tumorigenic feature of anchorage independence, suggesting that restoration of mtDNA could reverse tumorigenesis in these cells. Significantly, DNA binding activity of genes regulated by APE1 is sensitive to reduction-oxidation (redox). Proteins for which APE1 functions as a transcriptional cofactor include AP-1 (Fos, Jun) proteins, nuclear factor-κB (NF-κB), polyoma virus enhancer-binding protein, early growth response-1, Myb members of the ATF/CREB family, HIF-1α, HIF-like factor, Oak5 and Pax-8 [123;124]. Interestingly, AP1 and NF-κB have been shown to mediate the expression of proteins involved in neuronal function and survival [124;125], and APE1 itself has been shown to promote survival of neurons after oxidative stress [126] and to be essential for proper embryonic development [125]. Additionally, APE1 has been shown to repair DNA, influence

apoptosis, and interact with p53, a protein found upregulated in many cancers, including many pBTs.

The above evidence highlights a nuclear protein and its links to redox signaling in pBT development. We now present other evidence of mitochondrial dysfunction in pBT, first on mitochondrial genome alterations, and then on mitochondrial apoptotic pathways.

Identified alterations in the mitochondrial genome of BTs: Several features distinguish the mitochondrial and nuclear genomes, some of which allow mtDNA to be far more vulnerable to mutations than nDNA. mtDNA's lack of histone protection, limited repair capacity, and close proximity to the electron transport chain (and hence superoxide radicals), are a few major reasons for this increased susceptibility to damage. While mtDNA is more vulnerable to damage, the fact that only a small percentage of its proteins are coded by mtDNA increases the chances that a mutation that affects mitochondrial function will occur in chromosomal DNA. In fact, of over 3000 mitochondrial proteins, mtDNA only encodes (1) 13 subunits of respiratory chain complexes: seven subunits (ND 1–6 and 4L) of complex I, cytochrome b (Cyt b) of complex III, the COX I–III subunits of cytochrome oxidase or complex IV, ATPase 6 and 8 subunits of F_OF_1 ATP synthase; (2) the 12S and 16S rRNA genes; and (3) 22 tRNA genes. The remainder of the proteins are encoded by nuclear DNA and imported into the mitochondria by chaperones. Still, somatic mtDNA mutations have been linked to several cancers, and while their contribution to tumorigenesis has been debated, recent studies have concluded that mutant mitochondria positively and directly contribute to tumorigenesis by preventing apoptosis [127]. Additionally, novel research showing depletion of mtDNA encoded OXPHOS genes plays a role in tumor cell transformation

supports the involvement of mtDNA alterations and depletion in tumorigenesis [128]. Here we review alterations in mtDNA that have been observed in the mitochondrial genome of both pediatric and adult BTs.

The D-loop region of mitochondria is a region important for replication and mt nucleoid organization found mutated in many cancers. Several BTs, including meningiomas, schwannomas, gliomatosis cerebri, neurofibroma, astrocytoma and GBMs have been shown to have mitochondrial genome instability in the hypervariable regions of the D-loop. Somatic mtDNA mutations of this region have been found in tumors of NF1, a familial disease which predisposes to development of pilocytic astrocytoma [129]. Astrocytic tumors themselves, in fact contain mutations in this region, as a recent study of 42 cases of malignant astrocytomas (39 GBMs, two anaplastic astrocytomas, and one anaplastic oligoastrocytoma) showed alterations in 36% of the cases in the D-loop region, including 16 different somatic alterations [three in the hypervariable 1 region (HV1) and thirteen in the D310 region] [130]. This frequency is comparable to other reports of mtDNA instability in malignant gliomas [131;132]. These alterations do not appear to be associated with increased aggressiveness however [130]. Furthermore, somatic mtDNA mutations have been observed in a series of chemically induced and spontaneous mouse brain tumors in regions that correspond to the hypervariable regions of human mtDNA, though they do not appear to alter the amino acid sequence however, and therefore may not affect disease status [133].

Studies have also found mitochondrial mutations in other regions of the mitochondria as well. One study, which analyzed the entire mitochondrial genome of 15 cases of MB and the cerebrospinal fluid (CSF) of eight of these 15 cases, found that 40% (6 of 15) of

the tumors and 87.5% (7 of 8) of the CSF samples had at least one mtDNA mutation. The somatic mutations identified in this study, which were located primarily in regions of mononucleotide repeats but also in respiratory chain related genes, include: three coding region mutations 1) G7521A mutation in tRNA aspartate that changes a GT base pairing to AT base pairing at the amino acyl stem region, 2) the T15904C mutation in tRNA threonine at the loop region, and 3) the A15937G mutation at the first bp next to the loop, all of which may affect tRNA structure and stability; two missense mutations that can alter mt structure and function: 1) Y496H in cytochrome c oxidase and 2) L96P in NADH subunit 4, which are involved in the substitution of the hydrophobic aromatic tyrosine with positively charged histidine, and the hydrophobic leucine residue with α helix destabilizing secondary amino acid proline; and several noncoding region mutations in areas such as transcription factor binding sites and replication primer sites that can affect mtDNA replication, transcription, and expression. 5 (29.4%) mutations were located in the np 303-315 polyC tract region and 11 (61%) were in the previously discussed D-loop region.

Studies have also identified other alterations in BTs, with the most frequent observation involving changes in the copy number of mtDNA. A study that examined 45 glioma specimens found that mtDNA was highly amplified in 87% of the cases. In comparison, a nuclear-encoded reference gene (erb-b) that is frequently amplified in human cancers, increased in only 18% of the tumor specimens, indicating that mtDNA alterations may be much more frequent in gliomas than nuclear-encoded gene alterations. In addition, a high frequency of mtDNA copy number changes has been found in comparison to normal control tissue in both low and high grade gliomas. Furthermore,

the mt content of gliomas is significantly lower than normal rat brain tissue, as is activity levels of cytochrome c oxidase and citrate synthase. Differential hybridization experiments have also revealed decreased expression of seven mt genes in GBM tumors. Genes downregulated were mt NADH dehydrogenase subunits 1 and 4 (ND1; ND4); mt cytochrome oxidase subunits I, II, and III (COXI; COXII; COXIII); mt ATP synthase subunit 6 (ATP6); and mt 12S rRNA. Regardless of their functional significance, the above studies clearly show that mtDNA alterations are a frequent event in the development and progression of brain disease pathologies and warrant further investigation.

Mitochondrial-directed apoptosis and pBTs: Defects in the regulation of apoptosis (programmed cell death) can contribute to development of cancer by a failure to eliminate harmful cells. The mitochondria's link to control of apoptosis could play an important role in pBT etiology, as most tumors are often relatively resistant to the induction of the mPTP, the rate-limiting step of the intrinsic pathway of apoptosis. Mitochondria play a large role in apoptosis, with their intermembrane space acting as a storage site for numerous pro-apoptotic proteins, including cytochrome c and apoptosis-inducing factor (AIF). Both oxidative stress and mitochondrial calcium (Ca^{2+}) overload can favor activation of the mPTP (Crompton 2004), leading to release of cytochrome c and the induction of caspase-mediated apoptosis [134]. In addition, as mentioned above, a more specific pathway to apoptosis exists in which Bcl-2 family proteins (e.g. Bax, Bid) regulate cytochrome c release through binding to the outer mitochondrial membrane. Other proteins involved in apoptosis, such as AIF, endonuclease G, and Smac/Diablo are also released in this process [135]. It appears environmental factors may be important in

determining which apoptotic pathway is chosen as research on the ERK signaling pathway, shows that H_2O_2-induced apoptosis in glioma cells may be initiated upstream of the mitochondria [136]. In contrast, a study investigating exposure of neuroblastoma cells to thimerosal, an organomercury compound used in vaccines, found that apoptosis was induced through the cytochrome c/caspase mitochondrial cascade described above [137]. Additionally, experiments on the proapoptotic Apoptosis Related Protein in TGF beta Signaling Pathway (ARTS) using astrocytic tumors also point to mitochondrial-mediated apoptosis in brain tumors [138].

Selective programmed apoptosis is an important part of normal brain development. In fact, more than 50% of some neuronal populations actually die during the pre- and postnatal period [139]. Several major apoptotic pathways, working through the mitochondria, play a major part in these early developmental processes, and their dysregulation may affect risk of pBT development. The Bcl-2/Bax, cytochrome c complex governs apoptosis through control of the cytochrome c levels via the mitochondrial permeability transition pore (mPTP) of the mitochondria. The anti-apoptotic Bcl-2 protein strongly protects against free radical-mediated cell death by preventing release of cytochrome c, while other members of the bcl family (i.e. Bax) promote apoptosis [140]. Studies have shown Bcl-2 expression levels in the neocortex and hippocampus of the developing rat to be extremely elevated during development and the first week of life. These levels then show a rapid decrease to low levels in childhood and relatively non-existent levels in adulthood [141]. Caspases, which can be activated by the Bcl-2/cytochrome c complex, also regulate many aspects of apoptosis during development. It appears the interplay between these systems during development may be

important in the etiology of brain-related disorders as deletion of caspase-3, -9 or Apaf-1 in mice results in gross malformations specific to brain because of defective apoptosis and hyperplasia [142]. Furthermore, recent evidence shows that SHh signaling promotes survival of medulloblastoma cells via up-regulation of Bcl-2, linking a developmental pathway and mitochondrial-mediated apoptosis to pBT development [143].

VI. POPULATION STUDIES ON GENE-ENVIRONMENT INTERACTIONS IN PEDIATRIC BRAIN TUMORS

Very few epidemiological studies to date have investigated GEI in relation to BT development, especially in pediatric populations (see Table 1-3 for a summary of current research). To date, only one epidemiologic study has assessed the effect of environmental interactions with gene mutations on development of pBT. In this study, two frequent polymorphisms in Paraoxonase (PON1), a gene that metabolizes two residentially used insecticides (chlorpyrifos and diazinon), were investigated in relation to BT [n = astrocytoma (37), PNET (15), other (14)]. A non-significantly increased risk of pBT in relation to the inefficient PON1 promoter allele ($PON1_{-108T}$ allele, relative to $PON1_{-108CC}$: odds ratio (OR) = 1.4; 95% confidence interval (CI), 1.0–2.2; p-value for trend = 0.07] was found. However, this association was strongest and reached statistical significance among children whose mothers reported chemical treatment of the home for pests during pregnancy or childhood ($PON1_{-108T}$ allele: among exposed, OR = 2.6; 95% CI, 1.2–5.5; among unexposed, OR = 0.9; 95% CI, 0.5–1.6) and when primitive neuroectodermal tumors were assessed alone (per PON1-108T allele: OR = 2.4; 95% CI, 1.1–5.4) [144].

In addition to the above GEI study on pBT, two studies have assessed GEI in adult glioma. A case-control study on lead exposure, the lead toxicity associated gene δ-aminolevulinic acid dehydratase (ALAD), and risk of brain tumors found increased risk of meningioma with occupational lead exposure. Risk of meningioma, a tumor present mainly in later life, was markedly increased in individuals with the ALAD2 variant allele, for whom risks increased in a dose dependent fashion from 1.1 (0.3-4.5) to 5.6 (0.7-45.5) and 12.8 (1.4-120.8) compared to unexposed persons. Risk for glioma was not associated with occupational lead exposure [145]. A second adult GEI study on glutathione transferases (GST) polymorphisms, cigarette smoke exposure, and development of adult glioma failed to find any significant GEI [146]. Several other studies have investigated genes involved in detoxification of carcinogens, and their relation to both pBT and aBT development, though no exposure was considered in these studies. Polymorphisms in the carcinogen metabolizing genes GSTM1, GSTP1, and GSTT1 have been investigated for their effect on risk of pBT. The frequency of the GSTM1 null allele was found to be significantly lower in high-grade pediatric astrocytomas ($p < 0.002$). Additionally, a significant increase in the frequency of the rare GSTP1 variant Val114/Val114 was found in all pediatric astrocytomas combined ($p < 0.002$) and all pediatric brain tumor types that displayed microsatellite instability (MSI) from mismatch repair (MMR) defects (0.003), suggesting this genotype may define a population susceptible to pBT development. This same study assessed these polymorphisms in relation to aBT and found no relation of GST polymorphisms to tumor development [147]. Another study found the relative risk of pBT to be increased 4.9-fold for patients carrying one non-null (GSTM1*A) allele of GSTM1 compared to patients with two null alleles (95% confidence interval 1.5–16, P =

0.009) [148]. Finally, a meta-analysis on GST polymorphisms and adult brain tumor risk also failed to find any association between GST and tumor development, though the GSTT1 null genotype was associated with meningioma development (OR=1.95; 95% CI, 1.02-3.76) [149].

Population studies on folate involvement in BT development have also been performed. Folate is an important micronutrient molecule involved in DNA synthesis and methylation. Studies have linked folate deficiency with both pediatric cancer [150;151] and genomic damage [152]. Disturbances in DNA synthesis, methylation, and repair of this pathway may be involved in BT development [153]. An investigation of single nucleotide polymorphisms (SNPs) in the folate pathway [methylenetetrahydrofolate reductase (MTHFR) C677Tand A1298C, methionine synthase (MTR) A2756G, thymidylate synthase (TS) 28-bp tandem repeat, and reduced folate carrier (RFC) G80A] and their relation to NT susceptibility in pediatrics found that the homozygous CC allele of MTHFR A1298C, which reduces MTHFR activity, conferred an increased risk of BT (medulloblastoma, pineaoblastoma, and PNETs) (OR: 3.9; 95% CI: 1.3–11.4, p = 0.02). SNPs in glial tumors (astrocytoma, oligodendroglioma, and ependymoma) were not related to development of disease [154]. A recent report on folate pathway SNPs in adult meningioma and high grade glioma found that MTHFR C677T and A1298C genotypes associated with increased 5,10 methylenetetrahydrofolate levels elevated disease risk while another report on these tumors failed to associate the MTHFR 677T SNP with tumor development [155]. Dietary influence on brain tumors also find support in animal models as incidence and degree of aggressiveness of gliomas has been shown to be influenced by dietary

Table 1-3. Summary of gene-environment interaction studies conducted on pediatric and adult brain tumors.

Study Reference	Tumor Type (n)	Population	Results (risk estimate; 95% Confidence Interval)
Searles 2005 [144]	Glioma (37); PNET (15); Other (14)	Children 0-20 yrs old from Seattle, WA area	PON1 interaction with insecticide (chlorpyrifos, diazinon) found non-significant risk increase in inefficient promoter allele (1.4; 1.0-2.2); children whose mothers treated home for pests during pregnancy or childhood (2.6; 1.2-5.5) and PNET alone (2.4; 1.1-5.4) reached significance in inefficient allele however
Rajaraman 2006 [145]	Glioma(382); Meningioma (158)	Adults from Phoenix, AZ, Boston, MA, and Pittsburgh, PA	Lead exposure and ALAD gene found significant increased risk for meningioma and ALAD2 variant allele in a dose dependent fashion (1.1; 0.3-4.5 to 5.6; 0.7-45.5 to 12.8; 1.4-120.8)
Schwarzbaum 2007 [146]	Glioblastoma (329); Meningioma (546)	Adults from Sweden, southeast England, Denmark, and Finland.	No associations between the *GSTM3, GSTP1, NQO1, CYP1A1, GSTM1*, or *GSTT1* polymorphisms and adult brain tumor risk with the possible exception of a weak association between the *G-C (Val-Ala) GSTP1 105/114* haplotype and glioma [odds ratio (OR), 0.73; 95% confidence interval (95% CI), 0.54, 0.99], nor was there an interaction between the effects of the *GSTM3* or *GSTP1* polymorphisms and cigarette smoking.
Barnette 2004 [148]	Glioma (32); Medulloblastoma (20); Neuroblastoma (18)	Children 0-18 yrs old from Utah	SNP's in GSTM1, GSTM3, GSTP1, and GSTT1 were analyzed. The relative risk of glial brain tumors was 4.9-fold higher for subjects carrying one non-null (*GSTM1**A) allele of *GSTM1* in comparison to subjects with two null alleles (95% confidence interval 1.5–16, $P = 0.009$).
Ezer 2002 [147]	394 brain tumors (221 adult and 173 pediatric cases consisting of 197 astrocytic and 197 non-astrocytic tumors)	Children and adults	SNP's in GSTM1, GSTT1, and GSTP1 were analyzed. Significant increases in the frequencies of the functional GSTM1 allele in high-grade pediatric astrocytomas ($p < 0.002$), the rare GSTP1 variant Val114/Val114 in pediatric astrocytomas ($p < 0.002$), and the rare GSTP1 Val114/Val114 genotype among pediatric tumors showing microsatellite instability (MSI) due to defects in mismatch repair (MMR) proteins ($p = 0.003$).
Lai 2005 [149]	Glioma (1,630); Meningioma (245)	Meta-analysis of 8 studies on adults, 5 of which were from the United States	No association between any of the GST variants and the risk of glioma (1.08; 0.95-1.22). Subgroups of glioma also showed no association. The T1 null genotype was significantly associated with a risk of meningioma (1.95; 1.02-3.76)
Sirachainan 2008 [154]	Gliomas (31); Embryonal (28); Germ cell (13); Meningioma (1)	Children from Thailand	Increased risk of embryonal CNS tumors for homozygous CC allele of MTHFR A1298C (OR: 3.9; 95% CI: 1.3–11.4, $p = 0.02$)

Study Reference	Tumor Type (n)	Population	Results (risk estimate; 95% Confidence Interval)
Kafadar 2006 [155]	74 tumors total (Glioma; Meningioma)	Adults from Turkey	Though not significant (p =0. 194), the homozygous MTHFR TT genotype was found at a higher frequency in glioma patients compared to controls (15.4% and 7.1%, respectively). The MTHFR genotype was not associated with meningioma patients. Defining patients with the CC genotype as reference, the relative risk of glioma for subjects with the T allele (CT+ TT genotype) was 1.17.

supplementation of rats with phytochemicals. Rats fed a diet high in phytochemicals show both reduced incidence and aggressiveness of tumors and was associated with an increase in bcl-II and catalase and a decrease in ki-67, sod-1 and sod-2 transcripts [156]. This result correlates with an epidemiology study on adult glioma which showed reduced risk of glioma for those consuming a diet high in phytoestrogens [157].

VII. CONCLUSIONS

There is a growing body of scientific evidence suggesting that there are critical periods of time extending from conception to puberty when the central nervous system in children may be more affected by toxic exposures. While only 1% of pediatric brain tumors are diagnosed at birth or in the first few months of life, the majority of pBTs occur early in childhood, strongly suggesting that both prenatal and postnatal exposures may be involved in their etiology. These exposures likely interact with the genome/epigenome of the fetus or young child to produce alterations in their genetic makeup which can predispose to development of disease including pBTs. Importantly, the ability of environmental exposures to cause micronuclei, chromosomal aberrations, sister chromatic exchanges, DNA adducts, DNA single-strand breaks, and somatic

mutations in the DNA of a fetus has been demonstrated. Furthermore, many of these alterations have been shown to predispose to tumor development. These alterations likely play a role in pBT etiology and so we have discussed the frequent alterations. We have also highlighted a role for both developmental pathway alteration and mitochondrial dysfunction in the etiology of these tumors. It is likely, that pBTs result from the interplay of environmental factors with these biological mechanisms at critical developmental periods in a child's life.

Though past research has elucidated several potentially significant environmental, genetic and epigenetic factors in pBTs, evidence linking a majority of pBTs to specific genetic or environmental exposures is limited. Factors such as the relatively low numbers of accessible tumor tissue for pBTs and the heterogeneity of these tumors have contributed to the considerable difficulty involved with determining their etiology. Moreover, most epidemiological research on these tumors has not considered important factors such as timing of exposure, gene-environment interaction, and gene-gene interaction within their design. Additionally, the ability to appropriately measure levels of exposure at time of development or predisposition to disease through molecular biomarkers has been limited. Improved research methods and tools, combined with larger studies involving homogenous tumor types, should help answer questions on the etiology of pBTs in the future.

While several genetic disorders have been linked to development of pBTs, it is likely that the majority of pBTs are a result of low-penetrant gene alterations in common pathways. Importantly, alterations and pathways that may be important to etiology in certain tumor types may not play a role in other pBT types. It is probable, that

heterogeneity in alterations, and possibly even pathways, exists within tumor groups as well. Identification of which pathways are most significant in the etiology of each pBT type will be critical in developing therapies for these tumors. While therapies for single gene mutations have been successful in the past for certain cancers, it appears that therapies based on pathway inhibition will prove to be more successful in the treatment of tumors that have several mutations throughout a pathway such as pBTs.

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Manuscript 2: Environment, genetic and epigenetic alterations and pediatric central nervous system tumors*

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*A version of this manuscript was published in The Encyclopedia of Environmental Health, Five-Volume Set, Jerome Nriagu, Elsevier, New York, 2011.

Keywords: Pediatric central nervous system tumors (pCNSTs), Epigenetics, Gene-environment interactions, Individual Susceptibility

ABSTRACT

Pediatric central nervous system tumors (pCNSTs) are the second most common malignancy among children less than 20 years of age and the most common solid tumor of childhood. The incidence of these tumors has been steadily increasing over the past decades. The etiology as well as the mechanism of the development of a majority of pCNSTs remains elusive. While numerous genetic and epigenetic changes have been identified with both development and outcome of pCNSTs, most of these alterations do not define a majority of tumors. Though ionizing radiation is a risk factor for pCNST development, evidence for the role of other environmental factors such as pesticides, N-nitroso compounds, and viral infections remain debatable. It is probable that the etiology of pediatric brain tumors, as is the case with most cancers, is multifactorial. Interaction of environmental factors with genetic and epigenetic changes may be responsible for the development of the particular type of PCNST in an individual. The assessment of gene–environment interaction in pCNSTs has been more complex because of the lack of sound molecular epidemiological studies with a more complete picture of individual cancer risk associated with environmental exposure and genetic analysis. The increased susceptibility of the developing fetus and child to environmental insults should also be considered as a part in the developmental of pCNSTs. This review describes the chromosomal, genetic and epigenetic changes found in common pCNSTs, and summarizes the alterations found in pCNSTs that may be influence mitochondrial-nuclear signaling and the basis for interaction between environment and genetic factors.

OUTLINE

I. Introduction

II. Environmental Factors and pCNST

III. Molecular Changes in Different Types of pCNST

IV. Mitochondrial-nuclear signaling in CNST development

V. Gene-environment interaction (GEI) and CNST development

VI. Conclusions

I. INTRODUCTION

Pediatric central nervous system tumors (pCNSTs), which may occur in the brain or the spinal cord, are the second most common malignancy among children less than 20 years of age and the most common solid tumor of childhood. The majority of primary pCNSTs arise in the cerebellum and brainstem (infratentorial) from glia and are broadly categorized as gliomas. In contrast, most adult CNSTs (aCNST) originate in the cerebrum (supratentorial), although examples of childhood supratentorial CNSTs exist and vice versa. Glial tumors are usually broken down into more specific subtypes based on their predicted cell type of origin. The most common glial tumors include astrocytoma (from astrocytes), oligodendroglioma (from oligodendrocytes), brain stem glioma (from brain stem cells), and ependymoma (from ependymal cells). Some pCNSTs are composed of primitive or undifferentiated cells, as would be expected given their origin during early life when the nervous system is still developing. These include the primitive neuroectodermal tumors (PNETs) of the brain, notably medulloblastoma (MB), retinoblastoma, ependymoblastoma, and neuroblastoma (NB). Mixed forms of

brain tumors (displaying a neuronal, as well as a glial component) also exist. Other varieties of primary brain tumors include: tumors of the pineal parenchyma (e.g. pineocytoma, pineoblastoma), choroid plexus tumors, and neuroepithelial tumors of uncertain origin (e.g. gliomatosis cerebri, astroblastoma).

The incidence of these tumors has been steadily increasing since the early 1980s [1;2]. The reason for this is unknown. While some of the heritable genetic risk factors thought to play a part in less than 5-10% of all pCNSTs are identified, previous studies have failed to produce strong evidence on the genetic alterations that predispose children to the remaining 95% of sporadic brain tumors. Several cellular pathways important to brain development have been implicated in the development of pCNST, including the Sonic Hedgehog (SHh) pathway, the WNT-wingless (WNT) pathway, and the NOTCH signaling pathway. During normal cerebellar development, these signaling pathways regulate growth and differentiation of granule cell precursors (GCPs) and neural stem cells. Mutations that activate these pathways can cause excessive growth and failure in cell differentiation, and thereby predispose to several types of CNST. However, identified changes in these pathways do not define a majority of pCNSTs. It appears that genetic alterations in pCNST are very subtle. Recent observations implicate a combination of genetic and epigenetic changes induced by environmental factors in the etiology of CNSTs. Therefore, the focus of this paper is on the role of gene mutations, single nucleotide polymorphisms (SNP) of genes, and epigenetic changes in the development of common pCNSTs. Chromosomal, genetic and epigenetic changes found in common pCNSTs, in addition to alterations found in pCNSTs that may be influenced

by mitochondria signaling are described. Finally, environmental exposures and their interaction with genetic influences in relation to CNST development are discussed.

II. ENVIRONMENTAL FACTORS AND pCNST

The etiology of pCNSTs is largely unknown, though several genetic disorders are known risk factors for their development including neurofibromatosis type 1, Li-Fraumeni syndrome, basal cell nevus (Gorlin's) syndrome, Turcot syndrome, and ataxia telangiectasia. The role of environment in the etiology and development of pCNSTs is not very clear. Here the environment is defined as everything that surrounds us, i.e., air, water, diet, home, workplace, etc. It is becoming more evident that not only can drugs and environmental stressors interfere with normal fetal development by causing structural malformations, but that exposure to environmental stressors during development can also cause biochemical and functional abnormalities. In utero exposure to several drugs and environmental hazards has been proposed to be a primary cause for the increased incidence of childhood brain cancers, which poses a significant clinical problem. Some suspected in utero carcinogens include benzene, pesticides, trichloroethylene, ethanol, arsenic, cigarette smoke, and infectious biological agents. Early childhood exposures may also influence both aCNST and pCNST development and evidence that pesticides can influence glial development and differentiation well into childhood support this view [3]. Furthermore, many substances, such as 1,3-Butadiene and N-Nitrosomethylurea, have been shown to induce neurogenic tumors in animals, with both the fetus and neonatal animals being at dramatically increased susceptibility to CNST development compared to adults [4].

Aside from the extremely rare genetic conditions mentioned above, the only unequivocally identified risk factor is exposure to ionizing radiation, and this explains only a very small fraction of cases. The most consistent link between exposures and pCNST development aside from radiation is that for maternal consumption of cured meats, but whether a dietary component is responsible has not yet been revealed. A majority of epidemiological studies investigating pesticides and cancer risk have reported positive associations, with the risks for children generally higher than adults. While the consistency of results may be affected by type of pesticides being evaluated, exposure circumstances, and tumor classification standards, there is a considerable amount of epidemiological evidence that supports a link between pesticide exposure and pCNSTs. Several viruses have been investigated as causative agents in brain tumors. For instance, JC virus, a type of polyoma virus, and T-protein, an oncogenic virus product, have been detected at significant levels in PNETs and other tumors [5], though these results are challenged by negative results in other studies [6-10]. The ability of MBs to be induced by JC virus in mice models has also been demonstrated however [11;12]. Parental exposure to heat and electromagnetic fields, parental occupational exposure to chemicals, tobacco exposure, maternal hair dye use, and exposure to several medications and vitamins have also been investigated as potential etiologic factors, with equivocal results being found. Many of these exposures could certainly play a role in the etiology of pCNST however, as the high rates of cell proliferation and differentiation during development and childhood increases susceptibility of cells to mutagenic and epigenetic alteration. In addition, the blood-brain barrier, which acts as a barrier to potentially harmful substances, is not fully developed in the fetus and can allow harmful substances

to reach sensitive organs such as the brain. In fact, in utero DNA damage resulting from environmental pollution has been associated with somatic gene mutation in newborns [13]. It appears that exposure to environmental factors may influence the development of pCNSTs, and they likely interact with genetic and epigenetic components.

III. MOLECULAR CHANGES IN DIFFERENT TYPES OF pCNST

One of the critical issues involving CNST molecular biology has been the inability to determine the cell of origin of each individual tumor type. The three main cell types from which these tumor precursor cells are thought to arise are neural stem cells, precursor cells, or differentiated cells. Genetic changes in each cell type have been associated with CNST development [14]. It is likely these genetic changes, which may be either inherited or somatic in nature, deregulate normal proliferation and differentiation, and initiate tumor formation. Therefore, most pCNSTs (which are predominantly immature tumors) most likely arise from neural stem cells (which produce both glial and neuronal cells) or progenitor cells in their respective precursor cell type (i.e. neuronal precursor cells for MBs), while most adult tumors probably form from differentiated cell types (i.e. astrocytes in astrocytoma) that have acquired stem cell-like or progenitor cell-like properties [14]. For a complete review of the current information on cell of origin in brain tumors the reader is referred to a 2006 review by Read et al. [14].

Several cellular pathways important to brain development have been implicated in the development of pCNST, including the Sonic Hedgehog (SHh) pathway, the WNT-wingless (WNT) pathway, and the NOTCH signaling pathway. During normal cerebellar development, these signaling pathways regulate growth and differentiation of granule cell

precursors (GCPs) and neural stem cells. Mutations that activate these pathways can cause excessive growth and failure in cell differentiation, and thereby predispose to several types of CNST. Identified changes in these pathways do not define a majority of pCNSTs however, and few other significant genetic changes in these tumors have been established. In fact, tumors arising in different regions of the brain retain distinct patterns of gene expression, though cells in different parts of the brain carry the same genes. This happens because different parts of the brain also contain factors that modify the use of those genes, suppressing some genes and activating others to allow the cells to take on specialized characteristics as the brain matures. Most of molecular and epigenetic changes have been identified in medulloblastoma, astrocytoma and neuroblastoma, and therefore, here, we discuss chromosomal, molecular and epigenetic changes in these three types of CNSTs.

Medulloblastoma (MB)

PNETs are commonly divided by tumor location into infratentorial PNET (iPNET) and supratentorial PNET (sPNET), with iPNET being the more common tumor type. MB, the most common iPNET, is an invasive embryonal tumor that originates in the cerebellum and is most commonly seen in children three to eight years of age. They are the most common non-glial brain tumor, comprising approximately 25% of all pCNSTs [15]. Additionally, they are the most common malignant brain tumor of childhood [15], though sPNETs are generally considered to be a more aggressive tumor group than MB [16]. The two major subtypes of MB are classic MB (75 to 80% of all MBs), the most common tumor type which is found predominantly in children, and desmoplastic MB (15

to 20% of all MBs), which occurs much less frequently and is more common in adults. Classic MB is usually found in the midline, especially in the posterior vermis, while the desmoplastic subtype arises most commonly in the cerebellar hemispheres. A common variant that exists within these two subtypes is a typically more aggressive tumor type described as large cell/anaplastic (LC/A) MB. For an in-depth review of clinical factors related to medulloblastoma development and survival please see Sarkar et al. 2006 [17]. The most common alterations in pediatric MB are summarized in Table 2-1.

Chromosomal Abnormalities: The most frequent genetic alteration, occurring in >70% of MB, is isochromosome 17q [i(17q)], which often occurs in conjunction with partial or complete loss of 17p, found in 30-60% of cases. Monosomy 17, interstitial deletion and unbalanced translocation have been identified within this chromosome and contribute to these alterations [18-33]. Although loss of 17p is not specific for MB, it occurs at a higher frequency in MB than in any other tumor. Specific loss at 17p13.3 has been identified in 38% of MB [34]. Attempts to identify genes on 17p which may be important to tumorigenesis, including p53, have produced negative results [20;35;36]. Whether 17p deletion is associated with poor outcome is debated, with several studies finding these alterations produced poor therapeutic outcome, metastatic disease, and a shorter survival time [18;29;34], while others countered this finding [21;26]. The alterations are more frequent in the more aggressive LC/A MBs compared to classic MBs [37], and are absent in most nodular/desmoplastic MBs [38].

Several other abnormalities occur in most MB, including loss of heterozygosity (LOH) on 7q, 8p, and 16q, which have been reported in 60-70% of cases [33]. 8p contains a homozygous deletion on 8p22-23.1 and therefore could contain a yet to be identified gene

Table 2-1. Common susceptibility genes of pediatric medulloblastoma.

Gene Name	Gene Symbol	Pathway/Function	Type of Alteration	Frequency of Alteration	Reference
Patched	PTCH	Sonic Hedgehog (SHh) Pathway	Somatic mutation	10%	Raffel 1997 [39]; Vorechovsky 1997 [40]; Wolter 1997 [41]
Smoothened	SMO	Sonic Hedgehog (SHh) Pathway	Somatic mutation	10%	Taylor 2002 [42]
Mutated Suppressor of Fused	SUFU	Sonic Hedgehog (SHh) Pathway	Somatic mutation	5%	Reifenberger 1998 [43]
RENKCTD11	RENKCTD 11	Suppressor of Sonic Hedgehog (SHh) Pathway	Allelic Deletion	39%	Di Marcotullio 2004 [25]
Phosphatase and tensin	PTEN	Tumor suppressor-Akt pathway	Allelic Loss	19%	Hartmann 2007 [44]
Adenomatous polyposis coli	APC	WNT-Wingless Pathway	Somatic mutation	3-13%	Koch 2001 [45]; Eberhart 2000 [46]; Zurawel 2000 [47]
β-catenin (cadherin-associated protein)	CTNNB1	WNT-Wingless Pathway	Somatic mutation	6%	Zurawel 1998 [48]; Dahmen 2001 [49]
Axin1/Axin2	Axin1/Axin2	WNT-Wingless Pathway	Point mutation/ deletion	5%	Koch 2007 [50]; Koch 2001 [45]; Dahmen 2001 [49]
v-myc myelocytom-atosis viral oncogene	c-myc or N-myc	Oncogene	Overexpressed	5-15%	Aldorasi 2002 [51]; Avet-Loiseau 1999 [52]
p53	p53	p53-ARF Pathway	Somatic mutation	10%	Frank 2004 [53]
$P14^{ARF}$	$P14^{ARF}$	p53-ARF Pathway	Methylation	10%	Frank 2004 [53]
$INK4A^{ARF}$ (p16)	$INK4A^{ARF}$ (p16)	p53-ARF Pathway	Deletion	10%	Frank 2004 [53]
Neurotrophic tyrosine kinase, receptor, type 3	TrkC	Neurotrophin Pathway	Overexpressed	48-85%	Tajima 1998 [54]; Washiyama 1996 [55]
Platelet-derived growth factor receptor α	PDGFR-α	PDGFR-Ras-MAPK	Synonymous Sequence Variation	7%	Gilbertson 2006 [56]

Gene Name	Gene Symbol	Pathway/Function	Type of Alteration	Frequency of Alteration	Reference
Forkhead box G1	FOXG1	Theorized repressor of TGF-α induced expression of p21cip1 and cellular growth	Copy gain between 2 and 21 fold	93%	Adeesina 2007 [57]
RAS association domain family 1A	RASSF-1A	Candidate tumor suppressor	Transcriptionally silenced	79%	Lusher 2002 [58]
Orthodenticle homeobox 2	OTX2	Oncogene	Overexpressed	93% of Large cell/Anaplas-tic MB	Di Sapio 2005 [59]
Paired box 5	PAX5	Developmental control	Overexpressed	70%	Kozmik 1995 [60]
Caspase 8	Caspase 8	Apoptosis factor	Transcriptionally silenced	62%	Gonzalez-Gomez 2004 [61]
Hypermethyl-ated in cancer	HIC-1	Candidate tumor suppressor	Transcriptionally silenced	72%	Rood 2002 [62]
Secretory granule neuroendocri ne protein 1	SGNE1	Inhibits proliferation	Transcriptionally silenced	50%	Waha 2007 [63]

important to MB development [33]. Loss of chromosome 10q is also frequently seen in MB (40% of cases) [33;64], as is loss of chromosome 1q (20% of cases) [23;32;64-69]. The most frequent abnormalities in chromosome 1 include unbalanced translocations, deletions and duplications and rearrangements of chromosome 1 have been shown to result in trisomy 1q without loss of the p-arm. In addition, deletions in X, 1p, 3q, 6q, 8q, 8p, 9q, 11p, 11q, 16q, and 18 and gains in 1p, 1q, 3q, 4p, 5p, 5q, 7q, 8q, 9p, 11p, 12q, 13q, and 14q have been observed at lower rates [31;32;64;69-72], though some are only common in certain subtypes such as gains of 11p, 13q, and 14q in LC/A MBs [71]. Trisomy of chromosome 7 [19;24;66;73] is also a common chromosomal alteration in MB, though it appears in most tumors with i(17q) and thus is most likely a secondary change unimportant to tumor initiation [74]. Loss of 9q, which contains the tumor

suppressor gene patched (PTCH) at locus 9q22.3, has been linked to poor outcome [75]. Yin et al. 2001 identified the regions 10q25, 11p13-11p15.1 and 16q24.1-24.3 as showing LOH and being localized to the deleted in malignant brain tumors (DMBT1) gene site [69]. Amplification of N-myc and anonymous oncogenes at 2q12-22 and 17p11 have also been detected in a set of LC/A MBs [71]. Finally, deletion of chromosome 22 has also been reported in approximately 10% of these tumors [24;73;76], though some reports argue against the overall importance of this change [19;64], and misdiagnosis has been suggested as a possible explanation for this discrepancy [19].

Several more defined chromosomal regions that may contain oncogenes for MB have been identified by comparative genomic hybridization (CGH) and include chromosomal gains of 1q21.3-q23.1, 1q32.1, 2p21, 2p23.1-p25.3, 7, 5p15.3, 8q24, 9q34.13-q34.3, 11q22.3, 17p11.2-q25.3, and 20q13.31-q13.33, and losses of 1q23.3-q24.2, 2q13.12-q13.2, 3q26.1, 4q31.23-q32.3, 6q23.1-25.3, 8p22-23.3, 10q24.32-26.2, 12q13.12-q13.2, and 16q23.2-q24.3 [77-79]. Oncogene amplifications that have been identified using CGH in MB include D17S1670 (61.5% of tumors), PIK3CA (46.2%), PGY1 (38.5%), MET (38.5%), ERBB2 at 17q21.2 (38.5%), CSE1L (38.5%), PGY1 at 7q21.1, and MDM2 at 12q14.3-q15 [79].

Gene Alterations: Several inherited genetic diseases predispose affected individuals to MB development, including the autosomal dominant disorder Gorlin's syndrome and Turcot's syndrome. Somatic mutations in the Gorlin's syndrome gene PTCH are found in approximately 10% of patients with sporadic MB [39-41;80;81]. This inactivation of PTCH, which leads to upregulation of the SHh pathway, appears to be more prevalent in desmoplastic MBs, along with loss of chromosome 9q, while loss of 17p, HIC-1

inactivation, and myc amplification are more prominent in classic MB [80;82;83]. Mouse models for inactivation of PTCH also show development of MB [84;85].

While PTCH is found mutated in sporadic MB at a relatively low frequency, other members of the SHh pathway are also mutated in MB and aberrant activation of this pathway is found in approximately 25% of cases [40;86;87] Reviewed in [88;89]. The hedgehog signaling pathway is one of the key regulators in brain development as it has been shown to control proliferation and differentiation of granule cell precursors (GCPs). Deregulated proliferation of these progenitor cells through activation of the SHh pathway can result in MB [90;91]. SHh activates patched (PTCH) which then interacts with Smoothened (SMO) and its downstream targets such as Fused (Fu) and Mutated Suppressor of Fused Homolog (SUFU), leading to signaling to Gli proteins to translocate to the nucleus and transcribe target genes [92]. Studies in the brain demonstrate that SHh signaling leads to increased Mycn transcription [93], which as described later, is implicated in CNST development as well. While most SHh mutations are predominantly found in the desmoplastic subtype [40;80;87], they are reported in classic MB as well [39;41]. SUFU is found in approximately 10% of MB [87] while SMO is mutated in approximately 5% of tumors [43]. Furthermore, SUFU activation has been reported to result in overactivity of both the SHh and WNT signaling pathways, leading to excessive proliferation and failure in cell differentiation resulting in MB [94]. Along with mutations in SUFU and SMO, gene mutations in PTCH2, a PTCH homolog have also been identified [95]. In contrast to the above results, Chiappa et al. (1999) found no evidence for mutations in genes of the SHh pathway other than PTCH in 27 medulloblastomas. A negative regulator of the SHh pathway, RENKCTD11 (located at

17p13.2), shows allelic deletion in 39% of sporadic MBs and is significantly underexpressed in MB [25]. RENKCTD11 has been shown to inhibit MB growth by counteracting the SHh induced nuclear transfer of the transcription factor Gli, which regulates expression of several genes including N-myc, cyclin D, PTCH1, and Gli1 [25;96;97]. Many of these target genes have been shown to exist at elevated levels in both major MB subtypes including PTCH1, Gli1, and N-myc [43;82;98;99].

SHh signaling has also been found to interact with other genes that promote cell cycle progression or cell survival in MB. For instance, hyperactive insulin-like growth factor (IGF) signaling enhances SHh-induced formation of MB [93;100], possibly through cooperative control of N-Myc, as SHh stimulates transcription of N-myc while IGF stabilizes N-myc [101]. MBs frequently overexpress the anti-apoptotic factor Bcl-II [102], and dysregulated apoptosis as a result of Bcl-II overexpression cooperating with SHh-stimulated proliferation appears to transform GCPs to form MBs [103]. SHh pathway target Gli1 is also significantly correlated with Bcl-II expression in MB and both proteins have been shown to be present in regions of decreased apoptosis in desmoplastic MB [104]. Alternatively, phosphatidylinositol 3-kinase (PI3K) signaling may also be an anti-apoptotic factor in SHh transformation of GCPs to aggressive MBs as elevated levels of Akt combined with no upregulated expression of Bcl-II was found in SHh induced MBs [103]. In fact, it appears that dysregulation of PTEN leading to activation of the PI3K/Akt pathway may play a significant part in medulloblastoma pathology [100]. Animal models show that SHh induced formation of medulloblastomas is significantly enhanced by activation of the PI3K/Akt signaling pathway [93]. Proliferation of medulloblastoma cell lines is dependent on PI3K/AKT signaling, whereas apoptosis is

not significantly affected [100]. Allelic loss of PTEN has been found in 16% of the medulloblastoma cases and PTEN mRNA and protein levels are significantly lower in medulloblastomas compared with normal cerebellar tissue of different developmental stages, and reduction of PTEN expression was found associated with PTEN promoter hypermethylation in 50% of the MB tissue samples [100].

WNT pathway alterations have also been linked to MB (15% of cases), mostly of the classic MB type [reviewed in [88;89]]. Adenomatous polyposis coli (APC), a gene whose germline mutation is linked to development of Turcot's syndrome, is found mutated in sporadic medulloblastoma (3-13%) [45;47;48;105-107]. In addition, the Wnt-wingless pathway partner of APC, β-catenin (CTNNB1) is also found mutated in a small subset of sporadic MBs (~6%) and converts a GSK-3beta phosphorylation site from serine to cysteine [48;49]. Mutations in GSK-3beta itself have not been identified [108]. Finally, mutations in another WNT pathway gene, Axin1/Axin2, including a single somatic point mutation in exon 1 (Pro255Ser) and seven large deletions, have also been detected in MB (~5%) [45;49;50], and their potential to activate oncogenic activity in the Wnt signaling pathway has been established [50]. All of these alterations influence translocation of β-catenin to the nucleus and activation of its target genes, including myc, in MB. Nuclear accumulation of β-catenin predicts a favorable outcome in pediatric MB [109].

Amplification of the Myc genes (c-myc and N-myc) is present in 5-15% of MBs [32;51;71;110-113], and is more common in the aggressive LC/A type [34;114;115]. Myc is activated by various mitogenic signals including those from pathways important to CNST development such as SHh and WNT. Myc target genes in MB are primarily

involved in cellular growth and metabolism, cell proliferation including rapid expansion of progenitor cells during neurogenesis, cell cycle regulation and differentiation, and stem cell self-renewal [116-119]. While the variations of Myc can perform each other's functions, it appears they function independently as well, modulating the expression of their targets based on cell type and environment [119;120]. New research shows that Myc induces mt gene expression and mt biogenesis, thereby directly linking Myc's regulatory properties to the production of mitochondrial ROS and the promotion of genomic oxidative damage and instability [121]. Several studies of myc amplification or mRNA overexpression have linked the gene to aggressive clinical behavior in MB [51;75;110;112;122-124]. In addition, low N-myc expression has been related to higher survival rates [125]. Furthermore, c-myc alterations in combination with loss of 17p appear to produce a more aggressive MB [75].

Defects in the p53-ARF pathway MB including p53 mutation, methylation of $P14^{ARF}$ or deletion of $INK4A^{ARF}$ exist in ~20% of MB [53]. These pathway alterations are found more frequently in the LC/A MB tumor type, linking them to a more aggressive tumor type [53]. Mutations in the p53 gene itself, located in the 17p region at 17p13.1, are infrequent in MB however [20;36;71;126;127], though they may reduce survival (Woodburn et al. 2001). Overexpression of Bmi1, which represses the ink4a/Arf tumor suppressor and is located on chromosome 10p, is strongly expressed in proliferating cerebellar precursor cells of mice and humans. It is also coexpressed with the SHh pathway gene PTCH in a significant number of MBs and likely plays an important role in the proliferation of GCP cells [128].

Several growth factors and transcription factors involved in neuronal growth, differentiation, survival, and apoptosis have also been investigated in relation to MB development. Several members of the Neurotrophin signaling pathway including the neurothrophins NGF, BDNF, NT3 and the receptors p75, Tyrosine receptor kinase A (TrkA), TrkB, and TrkC are all expressed in MBs. One of these, TrkC, which mediates the functional signals of neurotrophins, is overexpressed in 48-85% of MBs [54;55] and has been reported to be the single most powerful independent predictor of favorable outcome for MBs [17;123;125;129-132]. Only Gajjar et al. have found no evidence of TrkC affecting clinical presentation [133].

The PDGFR-Ras/MAPK pathway, which is potentially involved in N-nitroso compound (NNC)-induced brain tumors in animals [134], has been shown to be activated in MB, and seems to be particularly important for metastatic MB [135;136]. A gene expression profile study found the platelet derived growth factor receptor alpha (PDGFR-α) and the Ras/mitogen-activated protein (MAP) kinase pathway genes to be related to metastasis in MBs [136]. Known mutational 'hotspots' in PDGFR-α, NRas, KRas, HRas, BRAF, and PDGRF-β were not found in one MB study on this pathway, with the exception of a synonymous sequence variation in PDGFR-α (CCG to CCA; PRO 567 PRO) that was found in approximately 7% of tumors (2 of 28 tumors studied), suggesting that this mutation may be associated with MB development in certain cases [56]. Wasson et al. also found no amplification in Hras, Kras, or Nras in 20 analyzed MBs [137]. One study did find N-ras mutations of codon 61 in 10% (3 of 32 cases) of MB however, including a C to A mutation at position 1 (leading to a substitution of a glutamine residue for a lysine) and an A to T mutation at position 3 (glutamine-histidine) [138]. A separate

mutation in pediatric supratentorial PNETs has been found as well. An activating point mutation in KRAS [G to A transition in position 2 of codon 12, substituting aspartate (GAT) for glycine (GGT)] was detected in 1 of 3 sPNETs that developed after prophylactic central nervous system (CNS) treatment for leukemia or lymphoma [139].

ErbB2 (aka HER2/neu) and neuregulin-1b (NRG-1b), part of the ErbB growth factor receptor signaling pathway involved in cerebellar development, appear to play significant roles in MB tumorigenesis also. ErbB2 is expressed in >80% of MBs, while NRG-1b is expressed in 87.5% of MBs [140-144]. Again however, the Gajjar et al. 2004 study disagreed with the consensus as ErbB2 was expressed in only 40% of tumors [133]. Reduced survival and increased ErbB2 expression has also been associated in several studies [133;140;142;143;145]. FOXG1, a theorized repressor of TGF-α induced expression of p21cip1 and cellular growth which interacts with NOTCH signaling, shows copy gain between 2 and 21 fold in 93% of MB [57]. Finally, several members of a family of transcription factors involved in regulation of neuronal differentiation, the NueroD family, were found to be expressed only in PNETs in a study on a series of different brain tumor types, indicating they may be important to MB development as well [146].

Several other genetic changes, some also involved in neuronal differentiation, have been identified in relation to development of MBs. Amplification of the oncogene OTX2 has been described in 93% (14 of 15) anaplastic MBs [59]. Inactivation of RASSF-1A, located at 3p21, was found in 79% of MBs, regardless of pediatric or adult status and/or MB type [58]. The developmental control gene PAX5 is expressed in 70% of MB and was not found expressed in normal neonatal cerebellum tissue, indicating that PAX5 is

deregulated in MB [60]. Pomeroy et al. found the GCP transcription factors Zic1 and SSCL1 to be most closely correlated to MBs [82]. Upregulated genes correlated with favorable outcome in this study were involved in cerebellar differentiation [vesicle coat protein β-NAP, NSC1, Tyrosine receptor kinase C (TrkC), sodium channels], and genes encoding extracellular matrix proteins [procollagen-lysine-2-oxoglutarate 5-dioxygenase, lysyl hydroxylase, collagen type V α-I, elastin]. Poor prognosis was correlated with underexpressed cerebellar differentiation genes and overexpressed cell proliferation and metabolism genes [MYBL2, enolase I, LDH, HMG1 (Y), cytochrome C oxidase] and multidrug resistance [sorcin gene]. Other investigators have also found overexpression of GCP related genes in MB, including $p75^{NTR}$, Math1, and the previously mentioned Zic1 [82;147-149], though GCP marker expression tends to be seen more often in the desmoplastic form of MB found predominantly in adults. In contrast, classic MB are associated with expression of markers of non-granule neurons such as calbindin-28k, a major calcium binding protein in the brain located predominantly in the cytosol [150] . Calbindin appears to play a role in reduction of intracellular calcium levels and calcium induced apoptosis in neurons and astrocytes [151;152]. Calbindin-d(28k)-positive MBs show increased aggressiveness, are associated with a high risk of death and recurrence, and are more frequently seen in a patients younger than 15 years old [150].

Other genetic alterations reported include findings of high levels of a stem-cell marker, the glycoprotein CD133, in both MB tumor subtypes, suggesting that stem cells could provide the origin of both MB types [153;154]. Manganese superoxide dismutase (MnSOD) was overexpressed in MB in one study [155] and decreased in another[156], while catalase (CA) and GSH-Px have not been found to be increased in MB [156].

Epigenetic Alterations: Several recent studies have identified the tumor suppressor genes RASSF1, Caspase 8, and HIC-1 as being transcriptionally silenced in over 60% of MB cases [61;62;157-159]. Hypermethylation of HIC-1, which is located at 17p13.3, has also proven to be a predictor of poor outcome [62]. Recently, Waha et al. 2007 identified a hypermethylated CpG island which led to silencing of the proliferation inhibiting secretory granule neuroendocrine protein 1 (SGNE1) gene in 50% of MBs analyzed (n=50), though no mutations were identified in the SGNE1 (SGNE1 is the human homologue of 7B2 located at 15q11-15). Reports on methylation in MB have also identified $p14^{ARF}$, $p16^{INK4A}$, TP73, TP53, RB1, RASSF1A, HIC1, EDNRB, CASP8, DAPK, CDH1, THBS1, TIMP3, GSTP1, MGMT, INK4C (CDKN2C), and MCJ as showing methylation in MB, albeit at a frequency <20% [158;160].

Astrocytoma

Astrocytoma is the most common CNST, comprising 60% of cases of the disease overall, and 40% of all pediatric cerebral tumors [15;161]. It is divided into four WHO grades: Grade I pilocytic astrocytoma (aka juvenile pilocytic astrocytoma) predominantly occur as a benign cerebellar and midline structures tumor in children; Grade 2 diffuse astrocytomas (includes fibrillary, protoplasmic, and gemistocytic variants) are benign well-differentiated tumors seen primarily in 20-40 year olds, but also occur frequently in pediatrics, especially in the brain stem; Grade III anaplastic tumors are malignant tumors predominantly affecting the cerebrum of 40 year old patients; and the most common and malignant type overall, Grade IV glioblastoma multiforme (GBM), a necrotic tumor which targets patients in their 50's or 60's, though this is the most common tumor of the

brain stem in children. In contrast to adults who more often have an aggressive high-grade tumor (Grades III and IV), the low-grade types (Grade I and II) are the most common type of astrocytomas in childhood, though approximately 15-20% of all pediatric astrocytomas are Grade III and IV astrocytomas [162]. (CBTRUS 2007 Report) A more severe pilocytic astrocytoma subtype, pilomyxoid astrocytoma, also exists and is frequently found in children less than 3 years of age [38]. As with medulloblastoma and all CNST, debate continues as to whether astrocytomas arise from differentiated astrocytes, astroglial progenitor cells, or neural stem cells. In addition, an inherited disease is also associated with development of this brain tumor, as individuals with neurofibromatosis type I (NF1), are at greatly increased risk of developing pilocytic astrocytomas. Table 2-2 summarizes genetic alterations in astrocytoma.

Cytogenetics: Very few studies have evaluated the genetics of pediatric astrocytomas, however most show significant differences in cytogenetic abnormalities between grades of pediatric astrocytomas and between pediatric and adult astrocytomas. While most cytogenetic analysis of pilocytic astrocytoma show normal chromosomal arrangement [163-165], gains of chromosome 7 and 8 are observed in approximately one-third of cases, and loss of 22q is reported in 20-30% of cases [166]. These gains were not found in one study of low-grade astrocytomas [166], however another study identified trisomy 7 in this tumor type [167]. Other abnormalities observed in pilocytic tumors include gains of chromosomes 10p, 11 and 12p, losses on chromosomes XY, 1p, 4q, 9p, 11p, 16p, 18, and 19, and telomeric associations [165;166;168;169]. Some authors have also suggested that chromosomal arrangements define two subtypes of pilocytic astrocytoma, mainly one group showing deletions on chromosome 19 and another group showing gains on 19p

Table 2-2. Common susceptibility genes of pediatric astrocytoma.

Gene Name	Gene Symbol	Pathway/Function	Type of Alteration	Frequency of Alteration	Reference
Neurofibro-matosis 1	NF1	Susceptibility gene for Neurofibromatosis	Overexpress-ion/mutation	Common in pilocytic astrocytoma	Platten 1996 [170]
Epidermal growth factor receptor	EGFR	Member of ErbB family of growth factors	Mutation	Rare in pediatric tumors; common in adult tumors	Bredel 1999 [171]; Cheng 1999 [172]; Sung 2000 [173]
Platelet-derived growth factor receptor- α	PDGFR-α	PDGFR-Ras-MAPK	Mutation	Rare in pediatric tumors; common in adult tumors	Bredel 1999 [171]; Di Sapio 2002 [174]; Nakamura 2007 [175]
PTEN	PTEN	Tumor Suppressor	Homozygous deletion	8% in high-grade pediatric astrocytoma	Cheng 1999 [176]; Raffel 1999 [177]; Watanabe 1996 [178]
p53	p53	p53-MDM2-$p14^{ARF}$	Mutation	28-38% of high-grade pediatric astrocytoma	Cheng 1999 [176]; Felix 1995 [179]; Nakamura 2007 [175]; Pollack 1997 [180]; Pollack 2001 [181]; Pollack 2002 [182]; Sure 1997 [183]
MDM2	MDM2	p53-MDM2-$p14^{ARF}$	Overexpressi on	67% of pediatric malignant astrocytoma	Sung 2000 [173]
$p14^{ARF}$	$p14^{ARF}$	p53-MDM2-$p14^{ARF}$	Homozygous Deletion	10% of pediatric glioblastoma	Newcomb 2000 [184]
Ras-Akt pathway	Ras-Akt pathway	Ras-Akt pathway	Pathway overexpressio n	100% of sporadic pilocytic astrocytomas	Sharma 2005 [185]
Ras	Ras	Ras-Akt pathway	Mutation	Rare	Sharma 2005 [185]; Janzarik 2007 [186]; Maltzman 1997 [187]; Sharma 2005 [185]
RB-cyclinD1-cyclin dependent kinase-p16	RB cyclinD1 CDK4 p16	RB-cyclinD1-cyclin dependent kinase 4 (CDK4)-p16 pathway	Inactivation of pathway	25% of pediatric high-grade astrocytoma	Sung 2000 [173]

and 22q [188]. Cytogenetic abnormalities appear unrelated to pediatric low-grade astrocytoma prognosis [189].

In contrast to the relatively low cytogenetic changes in grade I astrocytoma, pediatric malignant astrocytomas demonstrate a number of chromosomal changes and differ in several respects their adult tumor counterparts, though a few of these tumors have shown normal karyotype at well [190]. In a study on malignant childhood astrocytomas (Grade II-IV) (NOTE: included young adults less than 25 years old), the most common aberrations were loss of 16p (54% of cases), 17p (38%), 19p (38%), and 22 (38%) and gain on 2q (38%), 12q (38%), 13 (38%), 4q (31%), 5q (31%), and 8q (31%). Other studies on these tumors have found loss of heterozygosity (LOH) on 1p/19q and 10p/10q, and gains of 7p and 9p at a lower frequency than adults, however LOH on 22q is comparable in frequency (44% of diffuse, 40% of anaplastic, and 61% of glioblastoma) [73;175]. LOH on 19q and 22q was more frequent in children older than six years of age compared to younger children. Rickert et al. 2001 found that compared with adult cases, gain of 1p, 2q, and 21q as well as loss of 6q, 11q, and 16q were more frequent in pediatric malignant astrocytomas [191]. Furthermore, microsatellite instability, which is absent in adult astrocytomas, is seen in 27% (12/45) of pediatric malignant astrocytomas [192]. It also appears that survival is not predicted by cytogenetic changes in pediatric malignant gliomas, in contrast to adult malignant gliomas [193], though this is disputed by a study that showed survival was significantly shorter for pediatric high-grade astrocytomas with amplification of chromosome 1q [191]. Differences in chromosomal aberrations also exist between malignant grades of pediatric astrocytoma. Grade III pediatric tumors show differing cytogenetic changes (gain of 5q; loss of 6q, 9q, 12q, and

22q) compared to Grade IV pediatric tumors (gain of 1q, 3q, 16q, and loss of 8q and 17p) [191]. Specific regions showing high copy number amplification in pediatric malignant astrocytomas include 8q21-22, 7q22-23, 1p21-22, 2q22, 12q13-pter, 12q15-21, and 13q11-14 [190].

Gene Alterations and Characteristics: NF-1 is caused by a mutation of a gene on chromosome 17q which encodes a protein known as neurofibromin (*Nf1*). This mutation also predisposes individuals to development of pilocytic astrocytoma. In contrast to individuals with NF1 who show loss of *NF1* expression [194], sporadic pilocytic astrocytomas often have overexpression of *NF1* [170;195]. The reason for this discrepancy is currently unknown [38]. Beyond the *NF1* gene, many of the other identified mutations found in astrocytomas are important for normal astrocyte development. For instance, alterations in growth factor genes important for glial differentiation from neural stem cells such as mutations in the epidermal growth factor receptor (EGFR) (approximately 30% to 50% frequency in grade IV astrocytoma and 15% of grade III astrocytoma) and overexpression of platelet-derived growth factor alpha (PDGFR-α) (found in many primary grades and secondary glioblastomas), are common in adult astrocytoma [196-201]. These common adult astrocytic alterations are detected rarely in pediatric low-grade astrocytomas however, and in fact, PDGFR-α amplification has only been observed in one case of one study on these tumors [137;171;173-175;177;180;183]. Overexpression of EGFR is also rarely observed in pediatric anaplastic astrocytoma and glioblastoma [171-173;176;177], despite them being histologically indistinguishable from adult glioblastoma. Interestingly, a malignant form of pediatric low-grade astrocytoma, disseminated astrocytoma (5-10% of all pediatric

low-grade astrocytoma), show a high rate of EGFR amplification and protein expression [202], as do pediatric non-brainstem high-grade tumors (80% show high expression levels though only 2 of 27 show amplification and EGFR changes did not influence outcome) [171]. Thus, it appears EGFR may drive progression of astrocytomas from low to high grade [198].

A common signature of astrocytic tumor progression is angiogenesis and an increasingly hypoxic environment. A report on angiogenic factors in childhood astrocytoma identified the EGFR/FK506-binding protein (FKBP)12/HIF pathway as being significantly overexpressed in childhood glioblastomas compared to low-grade childhood astrocytomas [203]. Peroxisome-proliferator-activated receptor-c coactivator-1a (PGC-1a), a key mt-nuclear signaling gene involved in oxidative phosphorylation, mt biogenesis and respiration, is induced by a lack of nutrients and oxygen and is a powerfully regulator of the PDGFR family member vascular endothelial growth factor (VEGF) [204]. VEGF is upregulated in adult astrocytic tumors compared to normal brain [205]. Interleukin-6 (IL-6) upregulates VEGF expression in human glioblastoma cells and the activation of IL-6 is dependent on its interaction with STAT3. This process may play an important role in angiogenesis and its contribution to glioma progression from lower-grade to high-grade GBMs [206]. VEGF, which can be induced by IL-6 and hypoxia [207], can also be upregulated by TNF-α and this process appears to be mediated by Sp-1 in human glioblastoma cells [208]. A role for Hypoxia-inducable factor-1 (HIF-1) and its function in a hypoxic environment in the progression of astrocytic tumors to a more aggressive state has also been suggested and is supported by evidence that HIF-1 mediated gene expression is modulated by alterations in genes important to astrocytoma

development such PTEN, TP53, p16(CDKN2A), p14ARF, EGFR, and PDGFR [209]. Furthermore, recent work shows that loss-of-function mutations in fumarate hydratase (FH) and succinate hydrogenase (SDH) in the mt TCA cycle result in activation of HIF-1. HIF-1 activation can then change the expression of numerous genes important to cellular metabolism, and increase angiogenesis, glucose uptake and glycolosis [210-213]. Additionally, HIF-1 modulated gene expression can be influenced by reactive oxygen species (ROS), a signaling molecule primarily produced in mt [214]. This work has strengthened the notion that HIF-1 activation could play a role beyond promotion of angiogenesis and actually be involved in initiation of cancer.

PTEN mutations, which occur in ~30% of adult high-grade astrocytoma, are rare (8% homozygous deletion) in childhood high-grade astrocytoma [176-178;201], and are non-existent in pediatric pilocytic astrocytoma [215;216]. PTEN protein expression is present in 38% of pilocytic tumors however [215], and mutations in PTEN predict poor prognosis in pediatric grade III and IV malignant astrocytomas [177]. PTEN interacting with wild-type p53 has been shown to inhibit angiogenesis in malignant astrocytoma cells [217;218], suggesting that mutations in p53 could be important in development of higher grade tumors. Mutations of p53 in astrocytoma are thought to result in decreased apoptosis [219] and cellular transformation [220;221]. In fact, mutations in the p53 pathway (mutation of p53/overexpression of MDM2/deletion of $p14^{ARF}$) have been found to be >95% in high grade pediatric astrocytomas [173], with mutations in p53 itself occurring in 28-38% of both childhood and adult high-grade astrocytoma, though these mutations are much lower in children younger than 4 with high-grade astrocytoma [175;176;179-183]. Still, they are the most commonly observed alteration in pediatric

malignant astrocytomas (Grades II-IV), although some early studies did not find p53 to be a frequent mutation [222-224]. There is also controversy concerning p53 mutation frequency in pilocytic tumors as combination of reports show the frequency to be as low as 1.3% [215;224-226], while one report showed p35 mutation frequency to be 35% [227]. Overexpression of MDM2 occurs in 67% of pediatric malignant astrocytomas [173], while homozygous deletions of CDKN2A/p14^{ARF} at 9p21 are found in 10% of pediatric malignant glioblastomas [184]. No MDM2 gene amplification was found in pediatric high-grade and pilocytic astrocytomas in one study however, although this study found p53 mutations to be rare in these tumors as well [224]. As mentioned above, Ink4a is a member of the p53 signaling pathway, being a gene product of p16. Although Ink4a is frequently lost in a large fraction of human GBM samples, mice lacking Ink4a seldom develop spontaneous brain tumors [228]. Aberrant copy numbers of the Ink4a antagonist Bmi1, have been identified in 59% of gliomas (low and high-grade combined), and increased copy number of Bmi1 at 10p13 (3-5 copies), while found in all glioma types, are especially elevated in GBMs. In addition, deletions of the Bmi1 locus are found in most types of tumors and are associated with poor prognosis, while increased copy number of the gene locus is not associated with poor outcome [229]. Some authors have suggested two alternative pathways to GBM involving the p53 pathway, one that transforms astrocytes and is Ink4a/Arf dependent and p53 independent, and another that transforms neural stem cells and is p53 dependent [230]. For a more detailed discussion of these mechanisms the reader is referred to Perotin et al. 2006 [230].

Studies in glioma cells have demonstrated the ability of oxidative stress to induce p53 dependent mt mediated apoptosis through MDM2 degradation promoting p53

signaling to the mt followed by cytochrome c release and nucleosomal fragmentation [231]. This apoptotic pathway involves both pro-apoptotic factors such as bax and Fas, and anti-apoptotic genes such as bcl-II [232]. Glial tumors (46 tumors including astrocytomas, oligodendrogliomas, oligo-astrocytomas, and glioblastomas) show upregulation of the apoptosis-promoting factors bax, Fas, Fas-L, and the apoptotic inhibitor bcl-II but none of these genes correlated with malignancy [233;234]. Bcl-II expression levels have been shown to increase with astrocytic tumor grade in glioma cell studies (included WHO grades I-IV), and an increase in proliferation and decrease in apoptosis accompanies this increased expression in each tumor grade [234]. Furthermore, overexpression of Bax, an antagonist of Bcl-II, has been shown to accelerate apoptosis in rat glioma and human glioblastoma (GBM) cells, while Bcl-II overexpression inhibits apoptosis by repressing neutral sphingomyelinase and the subsequent cascade of ceramide formation, mt cytochrome c release and caspase activation [235]. Importantly, environmental stimuli such as heat shock, irradiation, and chemotherapeutic drugs [236], have been shown to activate sphingomyleinase mediated apoptosis. Thus, Bcl-II gene over-expression inhibiting apoptosis coupled with gene alterations in other genes promoting cell proliferation may interact with environmental factors to play a significant role in tumor development and progression.

NF1 is a Ras-guanosine triphosphatase-activating protein that, as previously described, contains germline mutations that predispose individuals to development of pilocytic astrocytoma. Ras/Akt pathway overexpression has been reported in 100% of sporadic pilocytic astrocytomas in one study of 21 tumors [185], in ~70% of aGBM, and in most pGBM and aGBM cell lines [237]. A report by Faury et al. 2007 found that pediatric

glioblastomas can be divided by subtype based on Ras/Akt pathway activation. In their analysis, the subset defined by Ras and Akt pathway activation had very poor prognosis and exhibited increased expression of genes related to proliferation and to a neural stem-cell phenotype (expression of markers of neural stem cells such as nestin, dlx2, CD133, vimentin and ELK). These findings were similar to those of the aggressive adult glioblastoma phenotype they observed. A second subset not showing Ras/Akt pathway activation, showed better prognosis and expressed no markers of a proliferative/stem-cell phenotype, suggesting they may instead originate from astroglial progenitors. [238] Overexpression of H- or N-ras has been reported in 0, 43, and 71% of grade I, II, and high-grade astrocytomas respectively [239], though age was not accounted for in this study. Several studies have found Ras mutation, including one which found a somatic G12A Kras mutation in pilocytic astrocytoma [186], one which found a Kras codon 13 mutation in pilocytic astrocytoma [185], and another which found 3 of 25 (12%) of astroglial tumors to have mutation of K-ras-61. As previously mentioned, mutations in Ras are possibly important given that they may be related to development of cancer from environmental exposure [240]. Overall however, mutations in Ras appear to be rare in pediatric astrocytoma, with some studies reporting no mutations of Ras variants in all tumors analyzed [185-187].

Mutations of SHH pathway, which regulates astroglial and oligodendroglial differentiation from their neural progenitor cells, are also found in astrocytomas, though they have been primarily investigated in adult tumors. Three members of the Gli family (Gli1, Gli2, Gli3), which mediate SHh-induced transcriptional activation [241], are reported to be amplified and highly expressed in GBM [242] and Gli1 was found over-

expressed in several low grade astrocytomas [243]. Levels of GLi1 were not found to correlate with prognosis however, while both PTCH and SMO mRNA expression correlated significantly with malignancy, suggesting their involvement in suppression of astrocytic tumors [244].

Inactivation of the retinoblastoma (RB) tumor suppressor pathway (pRB/cyclinD1/cyclin dependent kinase 4 (CDK4)/p16) is found in approximately 25% of pediatric high grade astrocytoma, compared to over 80% of high grade adult astrocytomas showing these alterations [173]. Progression from astrocytoma to anaplastic astrocytoma has been shown to involve mutations in other tumor suppressor genes including the retinoblastoma (RB) gene on chromosome 13q. Pilocytic astrocytomas show protein expression of CDK4 in 61% of cases and protein expression of p16 in 73% of cases [215], while loss of expression of p16 was found in 11/18 (61%) of pediatric glioblastomas [183].

A majority of pediatric glioblastomas show overexpression of Y-box-protein-1 (YB1) (38 of 53 = 72%) [238], an RNA-binding protein/transcription factor involved in brain development. This gene may help drive oncogenesis in this tumor as its nuclear localization has been associated with poorer outcome and tumor progression in other cancers [245]. Other findings which point to the importance of precursor cells in tumor development include immunohistochemical studies that have astrocytoma cells express markers of glial progenitor cells such as GFAP, nestin, brain lipid-binding protein, and OLIG-2 [246-248]. PEN5, which is expressed in oligodendrocyte precursor cells (ODPCs), is also observed at a high frequency in pilocytic astrocytomas, suggesting ODPCs may give rise to pilocytic astrocytoma [249;250]. Lastly, as with MB, all grades

of astrocytoma have been found to harbor CD133 stem cells, providing for more debate on their cell of origin [154;251].

Other genetic findings in pediatric astrocytomas include studies showing MnSOD is strongly expressed in all glioma types (46 tumors including astrocytomas, oligodendrogliomas, oligo-astrocytomas, and glioblastomas) but is not correlated with tumor grade [155;233]. Another study found that MnSOD expression was decreased in gliomas however, while catalase (CAT) levels were significantly increased in ascending order in high-grade astrocytoma and low-grade astrocytomas, and GSH-Px was not increased in any astrocytomas [156]. Finally, pilocytic astrocytomas show upregulation of several immune defense-related genes including HLA-DRalpha, HLA-DPA1, HLA-DPB1, HLA-DQB1, IgG3, IgGK, FCER1G, A2M, FCRN, IFI-56K, DAP12, TIMP1, TIMP2, CDKN1A, and SOCS3 [252].

Epigenetic Alterations: Very few studies have specifically assessed epigenetic changes in pediatric astrocytomas. In one study however, both GSTP1 and $p14^{ARF}$ were both found to be unmethylated in pilocytic astrocytomas. This was in comparison to MBs which showed an 18% methylation rate for GSTP1 and a 45% rate for $p14^{ARF}$ [253].

Other epigenetic changes have been identified primarily in adult astrocytomas. Grade II gliomas (astrocytomas, oligodendrogliomas, and oligoastrocytomas), grade III astrocytomas, and secondary GBMs commonly display a methylated PTEN promoter. This event is absent in non-tumor brain specimens and rare in primary GBMs [254]. That grade III astrocytomas and secondary GBMs contain methylation of PTEN is consistent with the hypothesis that they arise from progression of grade II gliomas. PTEN promoter methylation also appears to phosphorylate protein kinase B (PKB/Akt)

and activate the PI3K pathway, which can also be activated by PTEN mutations [254]. Significant global hypomethylation is frequent in adult GBMs and is associated with specific copy number alterations, a low-functioning methylenetetrahydrofolate reductase (MTHFR) allele status, and increased proliferation, suggesting that either low MTHFR levels and increased cell proliferation lead to hypomethylation or decreased MTHFR causes hypomethylation which promotes cell proliferation [255]. Finally, secondary glioblastomas show promoter methylation of RB1, TIMP-3, and HRK [256-258].

Neuroblastoma (NB)

Neuroblastoma, a tumor located in the peripheral neural crest, is the most common extracranial solid tumor in childhood and the most common cancer in infancy, causing 8-10% of all infant malignancies [259]. Approximately 75% of cases of neuroblastoma (NB) occur in children under the age of 2 [260]. Table 2-3 summarizes genetic alterations in neuroblastoma.

Table 2-3. Common susceptibility genes of neuroblastoma.

Gene Name	Gene Symbol	Pathway/Function	Type of Alteration	Percent of Cases	Reference
v-myc myelocytomatosis viral oncogene	N-myc	Oncogene	Overexpression	25-35%	Park 2008 [261]
Ras	Ras	Ras-Akt pathway	Mutation	Rare	Ballas 1988 [262]
bcl-II	bcl-II	Governs mitochondrial outer membrane permeabilization	Expressed	Majority of tumors	Ikegaki 1995 [263]

N-myc protoncogene is amplified in 25 to 35% of neuroblastomas [261]. It is the key genetic feature used to stratify patients into risk groups [264-266]. As with other CNSTs,

myc target genes in neuroblastoma are primarily involved in cellular growth and metabolism, cell proliferation, and cell cycle regulation [119]. Phenotype of NB cell lines have been transformed, supporting the oncogenic potential of N-myc [267;268]. Antisense experiments using N-myc have also shown that downregulation of N-Myc result in decreased proliferation and cell growth in NB [269;270]. Finally, mice experiments using N-myc as a target gene have shown its ability to produce NB in vivo [271-273]. The adverse prognostic significance of *N-myc* amplification in NB has been well established and correlates with increased metastases and chemotherapy resistance [264;266;274;275]. Its importance in aggressive NB is also supported by studies showing amplification of N-Myc in NB cell lines exhibit increased proliferation, underexpression of inhibitors of angiogenesis, and increased invasive potential [266;268;276-282].

Ikegaki et al. found that bcl-II is expressed in a majority of NB tumors and cell lines, however it was not correlated with clinical outcome or myc amplification or expression [263]. Phosphatidylinositol 3-kinase (PI3K) inhibition in mice neuroblastoma produces decreased tumor size and decreased N-myc protein levels. In addition knock-down of PI3K in N-myc amplified human neuroblastoma cells causes decreased proliferation and increased apoptosis [283]. Finally, Ras mutations were identified in one study on neuroblastoma [262].

IV. MITOCHONDRIAL-NUCLEAR SIGNALING IN CNST DEVELOPMENT

Because mitochondria play a large part in brain development and are abundant in brain tissue, their health is critical to the wellbeing of the pediatric brain. Mitochondria in the

developing brain are very different from those of mature animals and these developmental differences in mitochondria can make the immature brain more vulnerable to environmental factors.

Mt have been shown to play a crucial role in energy metabolism through oxidative phosphorylation, programmed cell death, and the generation of DNA-damaging ROS as side products of their normal function. Mitochondrial DNA (mtDNA) is an easy target for oxidative DNA damage due to its close proximity to ROS production, its lack of protective histone proteins, and its limited repair capabilities. Environmental exposures causing dysregulation of mtDNA and ROS production may contribute to increased nuclear gene mutagenesis. The ability of mt dysfunction and mt mutations, to cause cancer has been the subject of intense debate, however. Recent evidence that mtDNA mutations seem to promote tumorigenesis by preventing apoptosis [284] supports a role for mt dysfunction in cancer. In addition, evidence that mutated nuclear genes that encode subunits of complex II may cause loss of this complex's tumor suppressor role and promote development of hereditary paraganglioma [285;286], suggests that mt-nuclear signaling may be important to tumorigenesis as well.

Evidence of mitochondrial changes in CNST: Mitochondrial abnormalities in tumor tissues generally have been considered to be a consequence, rather than the cause of tumorigenesis. However, recent reports argue against this concept and provide support to the idea that mitochondria (mt) may control the growth of cancer tissues [287]. For example, genetic and sporadic cases of brain tumors (paraganglioma and pheochromocytoma) are caused by mutation of a mitochondrial-specific protein,

succinate dehydrogenase, a Krebs cycle enzyme [285;288;289]. Recently, mutations in another mitochondrial Krebs cycle protein, fumarase, have been associated with the development of uterine fibroids, skin leiomyomata and renal cell cancer [290]. Mutations in these proteins appear to be involved in familial predisposition to benign and malignant tumors, such as malignant phaeochromocytomas and renal cell carcinomas.

Somatic mtDNA mutations have been found in tumors of NF1, an autosomal dominantly inherited disease which predisposes to development of pilocytic astrocytoma. These mutations were all found in the D-loop region [7 of 19 patients with diffuse cutaneous neurofibromas (avg. of 1 mutation per tumor) and 9 of 18 patients with benign plexiform neurofibromas (average of 3 mutations per tumor) had at least 1 somatic mtDNA mutation] and the proportion of mutant mtDNA in the neurofibroma tumors was increased in relation to non-tumor tissues Additionally, the D-loop np 303 to 309 C8 mutation was always dominant in tumors, while the C7 variant was either reduced or absent in tumors and was dominant in non-tumor tissue [291]. Kiebish and Seyfried 2005 sequenced the entire mitochondrial genome in a series of chemically induced and spontaneous mouse brain tumors with differing metastasis, malignancy, and vascularity. They reported that the tumors showed somatic mtDNA mutations in regions of mononucleotide repeats, which are similar to those found previously in hypervariable regions of the D-loop in human brain tumors. However, none of these mutations were considered pathogenic since they did not change amino acid sequence and therefore could not alter gene function [292].

Mt Changes in Medulloblastoma: Studies have found mitochondrial mutations in MB. One study analyzed the entire mitochondrial genome of 15 cases of MB and the

cerebrospinal fluid (CSF) of eight of these 15 cases. 40% (6 of 15) of the tumors studied had at least one mitochondrial mutation and seven of eight of the CSF samples were found to have mtDNA mutations as well. Three tumors had one somatic mtDNA mutation, 2 had two mutations, and 1 had 11 mutations. The somatic mutations identified in this study, which were located primarily in regions of mononucleotide repeats rather than in respiratory chain related genes, include: three coding region mutations 1) G7521A mutation in tRNA aspartate that changes a GT base pairing to AT base pairing at the amino acyl stem region, 2) the T15904C mutation in tRNA threonine at the loop region, and 3) the A15937G mutation at the first bp next to the loop, all of which may affect tRNA structure and stability; two missense mutations that can alter mt structure and function: 1) Y496H in cytochrome c oxidase and 2) L96P in NADH subunit 4, which are involved in the substitution of the hydrophobic aromatic tyrosine with positively charged histidine, and the hydrophobic leucine residue with α helix destabilizing secondary amino acid proline; and several noncoding region mutations in areas such as transcription factor binding sites and replication primer sites that can affect mtDNA replication, transcription, and expression. 5 (29.4%) mutations were located in the np 303-315 polyC tract region and 11 (61%) were in the D-loop region. [293]

Mt Changes in Astrocytomas and Other Gliomas: Malignant glioma is the best-characterized type of brain tumor with respect to mtDNA alterations. The most frequent observation involves changes in the copy number of mtDNA. A study that examined 45 glioma specimens found that mtDNA was highly amplified in 87% of the cases. In comparison, a nuclear-encoded reference gene (erb-b) that is frequently amplified in human cancers, increased in only 18% of the tumor specimens, indicating that mtDNA

alterations may be much more frequent in gliomas than nuclear-encoded gene alterations [294]. Analysis of 15 low grade gliomas (5 pilocytic astrocytomas, 3 gangliogliomas, 5 low-grade astrocytomas, and 2 neurocytomas) confirmed this finding as all samples analyzed showed evidence of mt sequence localization within the nucleus [295]. Interestingly, the authors also noted a transfer of mtDNA to the nucleus with nuclear localization of the mtDNA segment significantly correlated to an increase of mtDNA copy numbers. This is significant given that incorporation of mtDNA into the nuclear genome may serve as a mechanism of oncogene activation. In addition, a high frequency of mtDNA copy number changes has been found in comparison to normal control tissue in both low and high grade gliomas [294;295]. Furthermore, the mt content of gliomas is significantly lower than normal rat brain tissue, as is activity levels of cytochrome c oxidase and citrate synthase [296]. This low amount of normally functioning mt has been linked to a hexokinase dependent shift of energy metabolism to glycolysis in gliomas [296].

Astrocytic tumors show high sequence variability, as well as a loss of the heteroplasmy present in a polymorphic D-loop region site of normal cells [297]. Differences in percentage of mtDNA mutations between astrocytic tumor grades has not been found [298]. A recent study of 42 cases of malignant astrocytomas (39 GBMs, two anaplastic astrocytomas, and one anaplastic oligoastrocytoma) showed alterations in 36% of the cases in the D-loop region, including 16 different somatic alterations (three in the HV1 region and thirteen in the D310 region) [299]. This frequency is comparable to other reports of mtDNA instability in malignant gliomas [297;300]. These alterations do not appear to be associated with increased aggressiveness however [299].

Recent work in differential hybridization of GBM tumors revealed decreased expression of seven mt genes. Genes downregulated were mt NADH dehydrogenase subunits 1 and 4 (ND1; ND4); mt cytochrome oxidase subunits I, II, and III (COXI; COXII; COXIII); mt ATP synthase subunit 6 (ATP6); and mt 12S rRNA. The authors noted however, that the reason for this decreased expression could be due to a decrease in mt genome transcription activity, increased mutations in mtDNA, and/or a decrease in number of mt in GBMs [301]. Others have found hypoxia-tolerant GBM cells to reduce their rate of oxygen consumption in response to oxygen shortage, a defense mechanism that contributes to their survival in this environment. In contrast, hypoxia-sensitive GBM cells maintain their rate of oxygen consumption and show reduced survival [302;303]. This inability of hypoxia-sensitive cells to respond to reduced oxygen may result from an amino acid change in the gene encoding the ND6 subunit of Complex I which may alter Complex I function in hypoxia-sensitive cells [304].

Other reports have noted mt genome instability in the hypervariable regions in the D-loop of meningiomas, schwannomas, gliomatosis cerebri, low-grade astrocytomas, GBMs, and neurofibromas [291;297;298;300;305] and the presence of 23 separate mtDNA alterations in 4 separate GBM cell lines [304].

Some authors have reported that the mtDNA instabilities (e.g. mutations, polymorphisms, etc.) found in CNST tumors (primarily adult astrocytomas, meningioma, and shwannoma) are simply common human polymorphisms and ‘mutational hotspots’ [300]. The same authors found no correlation between nuclear mitochondrial instability (nMI) and D-loop changes or mtDNA instability and patient characteristics such as sex, age, tumor size, and clinical outcome [300]. Regardless of their functional significance,

the above studies clearly show that mtDNA alterations are a frequent event in the development and progression of brain disease pathologies and warrant further investigation. Information on specific mitochondrial-nuclear signaling genes that may play a role in brain disorders can be found in the succeeding section.

V. GENE-ENVIRONMENT INTERACTION (GEI) AND CNST DEVELOPMENT

Very few studies to date have investigated GEI in relation to CNST development, especially in pediatric populations. To date, only one epidemiologic study has assessed the effect of environmental interactions with gene mutations on development of pCNST. In this study, two frequent polymorphisms in Paraoxonase (PON1), a gene that metabolizes two residentially used insecticides (chlorpyrifos and diazinon), were investigated in relation to CNST [n = astrocytoma (37), PNET (15), other (14)]. A nonsignificantly increased risk of CNST in relation to the inefficient PON1 promoter allele ($PON1_{-108T}$ allele, relative to $PON1_{-108CC}$: odds ratio (OR) = 1.4; 95% confidence interval (CI), 1.0–2.2; p-value for trend = 0.07] was found. However, this association was strongest and reached statistical significance among children whose mothers reported chemical treatment of the home for pests during pregnancy or childhood ($PON1_{-108T}$ allele: among exposed, OR = 2.6; 95% CI, 1.2–5.5; among unexposed, OR = 0.9; 95% CI, 0.5–1.6) and when primitive neuroectodermal tumors were assessed alone (per PON1-108T allele: OR = 2.4; 95% CI, 1.1–5.4) [306]. An in vitro study investigating the effect of chlorpyrifos exposure on glioma cells found that the cell signaling interference produced by chlorpyrifos exposure was greater in undifferentiated cells compared to differentiating cells, though effects were still noticed on this cell type as well. However,

differentiation enhanced ROS production from chlorpyrifos exposure and provoked shortage of the nuclear transcription factor Sp1, an essential molecule in differentiation [3].

In addition to the above GEI study on pCNST, two studies have assessed GEI in adult glioma. A case-control study on lead exposure, the lead toxicity associated gene δ-aminolevulinic acid dehydratase (ALAD), and risk of brain tumors found increased risk of meningioma with occupational lead exposure. Risk of meningioma, a tumor present mainly in later life, was markedly increased in individuals with the ALAD2 variant allele, for whom risks increased in a dose dependent fashion from 1.1 (0.3-4.5) to 5.6 (0.7-45.5) and 12.8 (1.4-120.8) compared to unexposed persons. Risk for glioma was not associated with occupational lead exposure [307]. A second adult GEI study on glutathione transferases (GST) polymorphisms, cigarette smoke exposure, and development of adult glioma failed to find any significant GEI [308]. Several other studies have investigated genes involved in detoxification of carcinogens, and their relation to both pCNST and aCNST development, though no exposure was considered in these studies. Polymorphisms in the carcinogen metabolizing genes GSTM1, GSTP1, and GSTT1 have been investigated for their effect on risk of pCNST. The frequency of the GSTM1 null allele was found to be significantly lower in high-grade pediatric astrocytomas ($p < 0.002$). Additionally, a significant increase in the frequency of the rare GSTP1 variant Val114/Val114 was found in all pediatric astrocytomas combined ($p < 0.002$) and all pediatric brain tumor types that displayed microsatellite instability (MSI) from mismatch repair (MMR) defects (0.003), suggesting this genotype may define a population susceptible to pCNST development. This same study assessed these polymorphisms in

relation to aCNST and found no relation of GST polymorphisms to tumor development [309]. Another study found the relative risk of pCNST to be increased 4.9-fold for patients carrying one non-null (GSTM1*A) allele of GSTM1 compared to patients with two null alleles (95% confidence interval 1.5–16, P = 0.009) [310]. Finally, a meta-analysis on GST polymorphisms and adult brain tumor risk also failed to find any association between GST and tumor development, though the GSTT1 null genotype was associated with meningioma development (OR=1.95; 95% CI, 1.02-3.76) [311].

Population studies on folate involvement in CNST development have also been performed. Folate is an important micronutrient molecule involved in DNA synthesis and methylation. Studies have linked folate deficiency with both pediatric cancer [312;313] and genomic damage [314]. Disturbances in DNA synthesis, methylation, and repair of this pathway may be involved in CNST development [255]. An investigation of single nucleotide polymorphisms (SNPs) in the folate pathway [methylenetetrahydrofolate reductase (MTHFR) C677Tand A1298C, methionine synthase (MTR) A2756G, thymidylate synthase (TS) 28-bp tandem repeat, and reduced folate carrier (RFC) G80A] and their relation to CNST susceptibility in pediatrics found that the homozygous CC allele of MTHFR A1298C, which reduces MTHFR activity, conferred an increased risk of embryonal CNS tumors (medulloblastoma, pinealoblastoma, and PNETs) (OR: 3.9; 95% CI: 1.3–11.4, p = 0.02). SNPs in glial tumors (astrocytoma, oligodendroglioma, and ependymoma) were not related to development of disease [315]. A recent report on folate pathway SNPs in adult meningioma and high grade glioma found that MTHFR C677T and A1298C genotypes associated with increased 5,10 methylenetetrahydrofolate levels elevated disease risk

while another report on these tumors failed to associate the MTHFR 677T SNP with tumor development [316].

While very few population studies have investigated GEI in CNST, some effects of environmental exposures on CNST development can be seen from cell line and tissue studies such as the aforementioned study on glioma cells exposed to chlorpyrifos. For instance, several studies have investigated manganese toxicity in brain tumor cell lines. Manganese (Mn) exposure in humans is rare but can occur through occupational pathways. Mn targets brain mt and interferes with energy metabolism in both astrocytoma and neuroblastoma cells through reduction of activity of glycolytic and TCA enzymes such as hexokinase, pyruvate kinase, lactate dehydrogenase, citrate synthase, and malate dehydrogenase [317]. As a result of this metabolic change, Mn exposure may increase oxidative stress via activation of the mitochondrial permeability transition pore, a result found in Mn exposed astrocytes [93]. Manganese-induced cell death in both astrocytoma and neuroblastoma cells is primarily necrotic in nature and is enhanced by glutathione depletion [318]. Interestingly, neuroblastoma cells are more susceptible to Mn toxicity than GBM cells, possibly because of Mn treatments effects on apoptosis in GBM cells. On treatment in GBM cells increases apoptosis and has differential effects on proliferation pathways as it induces down-regulation of MAPK pathway, but does not significantly affect the AKT pathway. Furthermore, Mn reduces levels of c-Jun, c-Fos, and MMP-2 (an extracellular matrix degrading enzyme), which are all associated with invasiveness of GBM [317].

Other *in vitro* studies include a report which found selenium levels in the cerebrospinal fluid of patients with malignant brain tumors to be lower than those with

benign tumors. Furthermore, selenium's anticancer effects are seen in its ability to preferentially target human glioma cells for cell death through induction of superoxide and its subsequent disruption of mitochondria [319]. *In vivo* studies showing environmental exposure effects on CNST include studies in mice that show increased susceptibility to brain mutations for prenatal and neonatal (N-ethyl-N-nitrosourea) ENU-exposed mice over adult ENU-exposed mice [320]. Additionally, incidence and degree of aggressiveness of gliomas has been shown to be influenced by dietary supplementation of rats with phytochemicals. Rats fed a diet high in phytochemicals show both reduced incidence and aggressiveness of tumors and was associated with an increase in bcl-II and catalase and a decrease in ki-67, sod-1 and sod-2 transcripts [321]. This result correlates with an epidemiological study on adult glioma which showed reduced risk of glioma for those consuming a diet high in phytoestrogens [322].

VI. CONCLUSIONS

A growing body of evidence suggesting that there are critical periods of time extending from conception to puberty when the central nervous system in children may be more affected by toxic exposures. While only 1% of pediatric brain tumors are diagnosed at birth or in the first few months of life, the majority of pCNSTs occurs before age five, strongly suggesting that both prenatal and postnatal exposures may be involved in their etiology. These exposures likely interact with the genome/epigenome of the fetus or young child to produce alterations in their genetic makeup which can predispose to development of disease including pCNSTs (Figure 2-1). Importantly, the ability of environmental exposures to cause micronuclei, chromosomal aberrations, sister

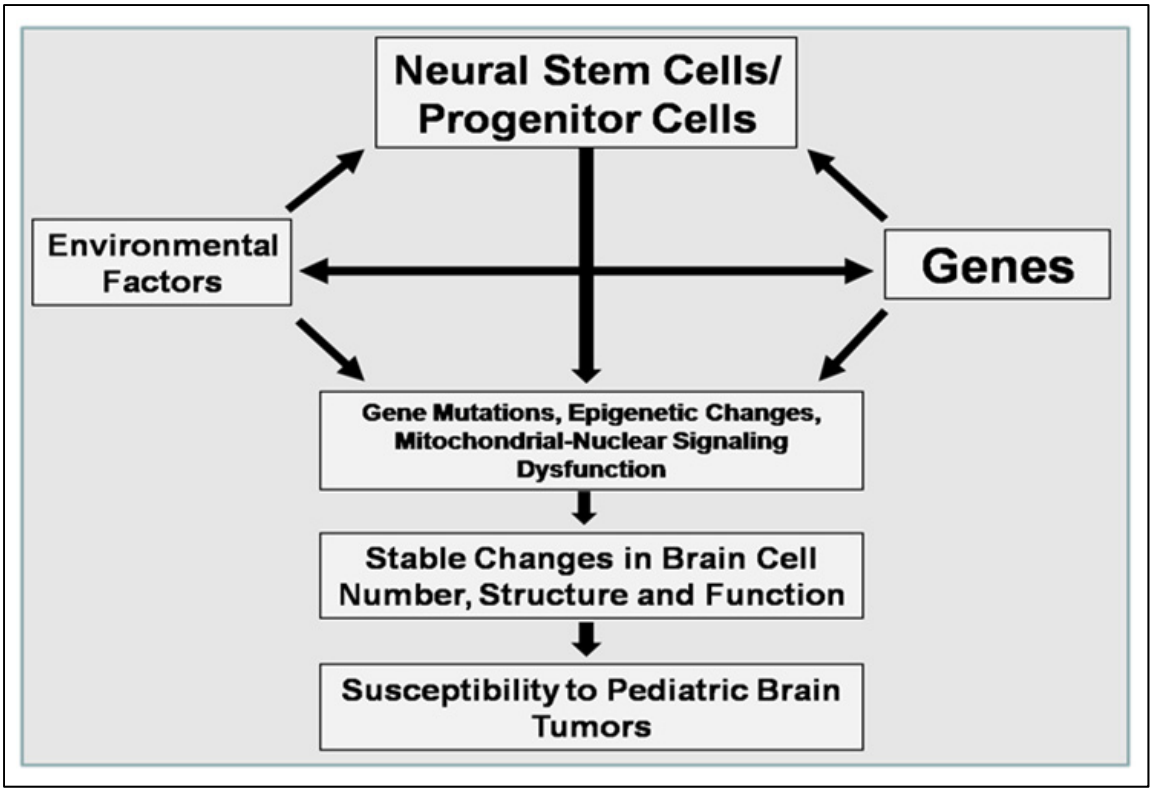

Figure 2-1. A schematic representation of an early life gene-environment interaction model for development of pediatric brain tumors.

chromatic exchanges, DNA adducts, DNA single-strand breaks, and somatic mutations in the DNA of a fetus has been demonstrated. Furthermore, many of these alterations have been shown to predispose to tumor development. These alterations likely play a role in pCNST etiology and so we have discussed the frequent alterations in several common pCNSTs and have reviewed current research on GEI in these tumors. While past research has elucidated several potentially significant environmental, genetic and epigenetic factors in pCNSTs, evidence linking a majority of pCNSTs to specific genetic or environmental exposures is limited. Factors such as the relatively low numbers of accessible tumor tissue for pCNSTs and the heterogeneity of these tumors have contributed to the considerable difficulty involved with determining their etiology.

Moreover, most past research on these tumors has not considered important factors such as timing of exposure, GEI, and gene-gene interaction within their design. Additionally, the ability to appropriately measure levels of exposure at time of development or predisposition to disease through molecular biomarkers has been limited. Improved research methods and tools, combined with larger studies involving homogenous tumor types, should help answer questions on the etiology of pCNSTs in the future.

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HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: In utero environmental exposures are hypothesized to produce genetic and epigenetic changes in mitochondrial-nuclear signaling genes, such as, PGC1, mtTFA, NRF-1. These changes, in turn, may be involved in the development of pediatric central nervous system tumors (pCNTSs).

Specific Aim 1: To evaluate activation and/or expression of genetic and epigenetic components of the mt-nuclear signaling pathway in brain tumors.

Specific Aim 2: To estimate pre- and postnatal environmental risk factors (organic solvents, pesticides, illicit drugs, infections, etc.) for childhood brain cancer. Correlations of these risk factors to our genetic and epigenetic changes of interest will be determined to establish whether there is any evidence of gene-environment interactions (GEI) between our genes and/or epigenes of interest and *in utero* and early life exposures.

Specific Aim 3: To identify how many genetic and/or epigenetic changes are required for the development of pCNST.

RESEARCH MANUSCRIPTS

Manuscript 3: Early life exposure of the brain to pesticides and development of childhood brain cancer: A Meta-analysis*

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ABSTRACT

Objective: Relatively little is known about the etiology of childhood brain tumors (CBT). While there are several studies which link pesticide exposure to increased risk of CBT, findings have been inconsistent. A meta-analysis of 17 published epidemiological studies was performed to test the concept that imprinting as a result of *in utero* exposure to pesticides is involved in the development of brain cancer in children.

Material and methods: Meta-analysis was performed using the general variance-based method and homogeneity was tested by means of the Q statistic. Summary relative risk (RR) estimates were calculated for risk of childhood brain cancer and 1) paternal exposure to pesticides prior to conception, 2) both maternal and paternal exposure to pesticides during pregnancy, 3) maternal exposure during pregnancy to a. agricultural and b. non-agricultural activities, and 4) childhood exposure to a. agricultural and b. non-agricultural activities up to date of diagnosis with CBT.

Results: Results reveal a significantly increased risk of CBT among children whose mothers had farm-related exposures during pregnancy (RR=1.48, 95% CI=1.18–1.84). A dose response is recognized when this risk estimate is compared to those for risk of CBT from maternal exposure to non-agricultural pesticides (e.g. home extermination, pest strips) during pregnancy (RR=1.36, 1.10–1.68), and risk of CBT among children exposed to agricultural activities (RR=1.32, 1.04–1.67).

Conclusions: Our findings suggest that *in utero* imprinting of brain development by pesticides may be involved in the etiology of CBT.

INTRODUCTION

Links between pesticide exposure and childhood tumors were first noted in case reports in the 1970s [1-3]. Since then, numerous studies have been published that support a role for pesticides in childhood brain tumor (CBT) etiology. To date, three separate reviews of pesticide exposure and CBTs have been published [4-6]. In 1997, Daniels et al. found that the studies to date showed that 1) farm residence was associated with CBT and 2) pesticide use in the home was fairly consistently associated with CBT despite the small sample sizes of studies. However, they concluded that "it remains unclear whether a specific time window of exposure may be of greater importance in studying the effects of home pesticide use." Zahm and Ward's 1998 review noted that nine of the 17 studies through 1998 had reported increased risk of CBT from pesticide exposure, five additional studies found a positive relationship that was not statistically significant, and only three studies reported no excess risk associated with pesticide exposure. Finally, Jurewics and Hanke 2006 reported that most studies since 1998 found associations between CBT and both agricultural employment and home use of pesticides. In addition, they commented on new positive associations being found with prenatal and preconception time periods of exposure.

Most studies on the relation between pesticide exposure and CBT have used small sample sizes and to date no meta-analysis of these studies has been published. While not a pediatric study, a meta-analysis of adult BT and farming did find an increased RR of 1.30 (95% CI: 1.09 – 1.56) [7]. By conducting a meta-analysis of the studies on CBT and pesticides we aim to synthesize past information on this topic and produce more robust risk estimates of any relations that may exist. Questions that we attempted to answer

with this study include: 1) Does exposure to pesticides increase risk of CBT?; 2) Are maternal and paternal exposures both important in CBT etiology and if so, does one increase risk more than the other?; 3) Are certain exposure time periods important for etiology of CBT?; and, 4) Is there a difference in risk for agricultural and non-agricultural pesticides exposures?

BACKGROUND

Several mechanisms for CBT development from pesticide exposure have been suggested, occurring at three separate time periods:

(1) exposure during preconception resulting in genetic alterations;

(2) in utero exposure causing genetic or teratogenic effects; and

(3) postnatal exposure via the mother's breast milk or direct exposure to the child (e.g. diet, childhood play habits, etc.).

While occupational exposure is an established pathway for significant pesticide exposure, contact through home use of pesticides has been of less concern. However, substantial pesticide exposure to children can occur in and around the home, with those being used outdoors being tracked into the home on shoes and by pets. 82% of United States households use pesticides with an average of 3 to 4 different pesticide products being used per home [8]. These exposures can become long-lasting as the pesticide residues can remain in carpets, furniture, and toys without being degraded by processes that exist outdoors (e.g., rain and sun) [6]. Furthermore, Whyatt et al. 2003 examined pesticide levels in plasma of 230 NYC mothers and infant pairs, finding that 7 pesticides were detected in up to 83% of the plasma sample. Maternal plasma levels were

correlated with cord blood levels, indicating placental transfer from pregnant mother to fetus [9].

METHODS

A PubMed search was conducted to identify studies of the association between CBT and pesticides. This search included articles published from 1966 until December 17, 2010. Search terms used in various combinations were brain, cancer, tumor, childhood, adolescent, *in utero*, pregnancy, central nervous system, pesticides, farming, and agriculture. References cited in the studies identified by this search were also reviewed for inclusion in the meta-analyses.

Inclusion criteria for initial search were: 1) Published in English language peer-reviewed journals between 1966 and 2006; 2) Provided sufficient data to determine an estimator of risk for CBT and its confidence interval (CI); and, 3) Study evaluated pesticide and/or farm exposure and CBT. Exclusion criteria for initial search were: 1) Did not report original results (reviews, comments, letters, etc.); 2) Results already reported in another study or in a more comprehensive study; 3) Geographic studies using GIS, etc.; 4) Study had less than 4 cases in subgroup of interest; and, 5) Study did not report timing of exposure. For timing of exposure a study was excluded if the exposure period was not specific to one of our identified time periods. For example, Kristensen et al. 1997 used a 5 year period of exposure and was thus excluded from the final analyses [10].

CBT was defined as any child diagnosed with CBT until age 19. This conformed to the standard SEER (Surveillance, Epidemiology, and End Results) childhood cancer

classification. CBT included all central nervous system tumors including brain tumors such as astrocytoma and primitive neuroectodermal tumor (PNET) and other central nervous system tumors such as neuroblastoma. Pesticide exposures were defined as either agricultural or non-agricultural in nature. Agricultural pesticide exposures were defined as exposures to pesticides from farming, farm related activities, living on a farm, mixing and preparing agricultural pesticides, horticulture, nursery work, and professional gardening. Non-agricultural pesticide exposures were those that occurred during *home use* such as flea/tick pesticide use, garden pesticide use, yard treatment, pest strip use, and home extermination.

The data were stratified three separate ways and several separate meta-analyses were performed among these groups. Stratification methods included 1) exposure categories separated into maternal exposures, paternal exposures, and child exposures; 2) exposure time period separated into preconception, pregnancy, and childhood; and 3) type of exposure split into agricultural pesticide exposures and non-agricultural pesticides exposures.

A general variance-based method using confidence intervals developed independently by Prentice and Thomas [11] and Greenland [12] was used to calculate odds ratio risk estimates. This method requires only information on each study's estimate of risk and its 95% confidence interval, reducing the amount of studies excluded from the analysis because of missing data. The weight given to each study is chosen to be the inverse of the variance of the effect estimate, meaning that larger studies with smaller standard errors are given more weight than smaller studies that have larger standard errors. In addition, because adjusted rate ratios and confidence intervals are used in the analysis,

the method takes confounding into consideration, unlike other meta-analyses methods which may not include confounding in their estimates. Analysis was completed using Comprehensive Meta-Analysis Version 2.2.046 from Biostat, Inc. which can be downloaded at www.Meta-Analysis.com [13].

Risk estimates were combined following several pre-determined rules. For studies that reported risk estimates for different types of CBT separately without reporting a summary estimate for all CBT, we calculated a summary risk estimate for use in our meta-analyses. For example, one summary risk estimate was calculated by combing the odds ratio for risk of astrocytoma and PNET reported by Bunin et al. 1994. In addition, when a study reported more than one risk estimate for different pesticide exposure types or situations, the general exposure category was used for the meta-analyses calculations. Finally, the studies that reported home exposure/use of pesticides during pregnancy without specifying it as either maternal or paternal specifically were included in the maternal exposure during pregnancy category. This was based on the concept that the mother is the ultimate exposure during this period.

RESULTS

The initial literature search produced 33 studies that fit the inclusion criteria. 17 studies were rejected by the exclusion criteria, leaving 16 studies for the final meta-analyses calculations. Table 3-1 lists the characteristics of these 16 studies, including their design, case and control totals, and tumor types analyzed. Of the 16 studies, 15 were case-control studies and one was a cohort study. Results of the meta-analyses performed can be found in Table 3-2, organized by time period and exposure category.

Table 3-1. Characteristics of studies included in the meta-analyses of parental pesticide exposure and risk of childhood brain tumor development.

Study First Author and Year	Study Design	Study Subjects (n)	Tumor Type(s)
Feychting 2001 [14]	Cohort	152 cases; 11 exposed cases; 235,635 cohort size	Nervous System Tumor
Kuijten 1992 [15]	Case-control	163 cases and control mothers; 158 cases and control fathers	Astrocytoma
Wilkins 1990 [16]	Case-control	110 cases; 193 controls	Brain Cancer
Efird 2003 [17]	Case-control	1218 cases; 2223 controls	Astroglial, PNET, Other Glial, Other
Kerr 2000 [18]	Case-control	183 cases; 372 controls	Neuroblastoma
Holly 1998 [19]*	Case-control	540 cases; 801 controls	Astroglial, PNET, Other Glial
Bunin 1994 [20]	Case control	322 cases; 321 controls	Astrocytoma, PNET
McCredie 1994 [21]	Case-control	82 cases; 164 controls	Brain and cranial nerve tumors
Daniels 2001 [22]	Case-control	538 cases and 504 control mothers; 405 cases and 304 control fathers	Neuroblastoma
Pogoda 2001 [23]	Case-control	224 cases; 218 controls	Brain, cranial nerve, and cranial meninges tumors
Leiss 1995 [24]	Case-control	17 cases; 118 controls	Brain Cancer
Davis 1993 [25]	Case-control	45 cases; 85 no disease controls; 108 cancer controls	Astrocytoma, Medulloblastoma, Other
Preston-Martin 1982 [26]	Case-control	209 cases; 209 controls	Astrocytoma, medulloblastoma, ependymoma, other glioma, meningioma, neuroma, other
McKean-Cowdin 1998 [27]*	Case-control	540 cases; 801 controls	Astroglial, PNET, Other Glial, Other
Wilkins 1988 [28]	Case-control	30 cases; 19 controls	Brain Cancer
Gold 1982 [29]	Case-control	70 cases	Brain Cancer

*These manuscripts used the same study population. However, different exposure analyses results were reported in each study and therefore, both manuscripts were included in our meta-analyses.

Table 3-2. Summary of results of meta-analyses of the association between pesticides and childhood brain tumors.

Time Period	Exposure Category	Studies in Estimate	Summary Risk Estimate Odds Ratio (95% CI)
Pre-conception	Maternal Exposures	1	0.87 (0.29 – 2.60)
	Paternal Exposures	3	2.29 (1.39 – 3.78)
Pregnancy	Maternal Exposures (Agricultural)	5	1.48 (1.18 – 1.84)
	Maternal Exposures (Non-agricultural)	7	1.36 (1.10 – 1.68)
	Paternal Exposures	5	1.63 (1.16 – 2.31)
Childhood	Agricultural Exposures	4	1.35 (1.08 – 1.70)
	Non-agricultural Exposures	5	1.32 (1.04 – 1.67)

Exposure during Pre-conception

No meta-analysis was performed on maternal exposure to pesticides during preconception as only one study fit the criteria for this grouping. This study, McKean-Cowdin et al. 1998, reported no increased risk for CBT from maternal exposure to pesticides during preconception (OR: 0.87, 95% Confidence Interval (CI): 0.29 – 2.6) [27]. Three studies were combined for the paternal exposure to pesticides during preconception, producing a calculated summary risk estimate of odds ratio (OR) = 2.29 (95% CI: 1.39 – 3.78) (Figure 3-1).

Exposures during Pregnancy

All three summary risk estimates for exposure during pregnancy showed a significant association between pesticide exposure and CBT. A combination of 5 studies for maternal exposure to agricultural pesticides during pregnancy generated an increased risk of OR = 1.48 (95% CI: 1.18 – 1.84) (Figure 3-2).

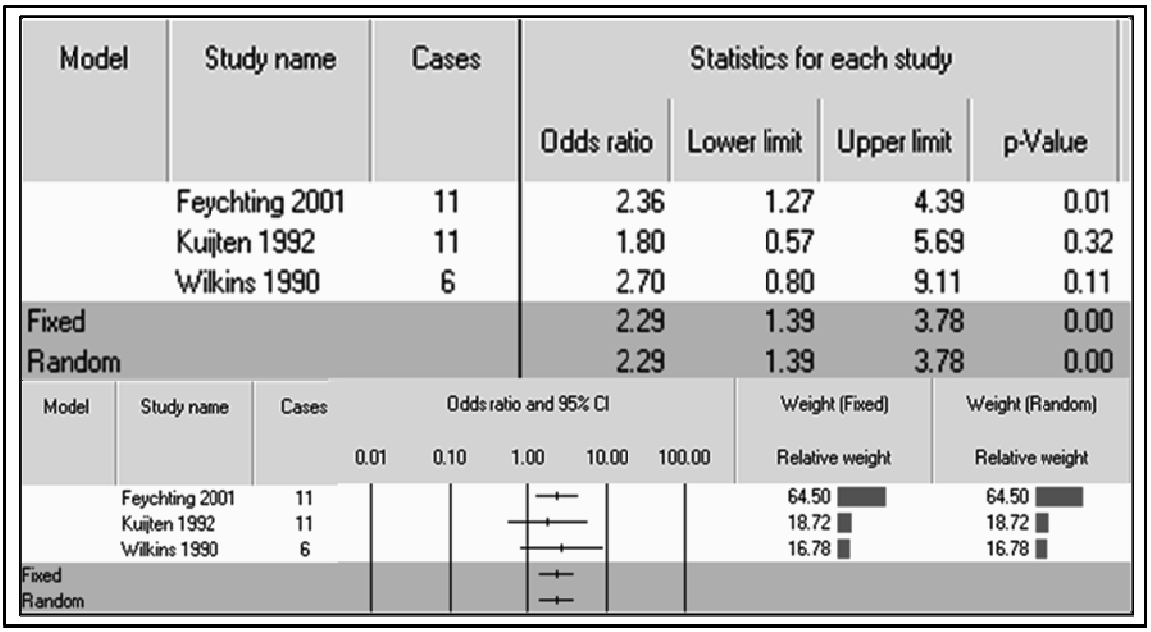

Figure 3-1. Meta-analysis results for paternal exposure to pesticides during pre-conception.

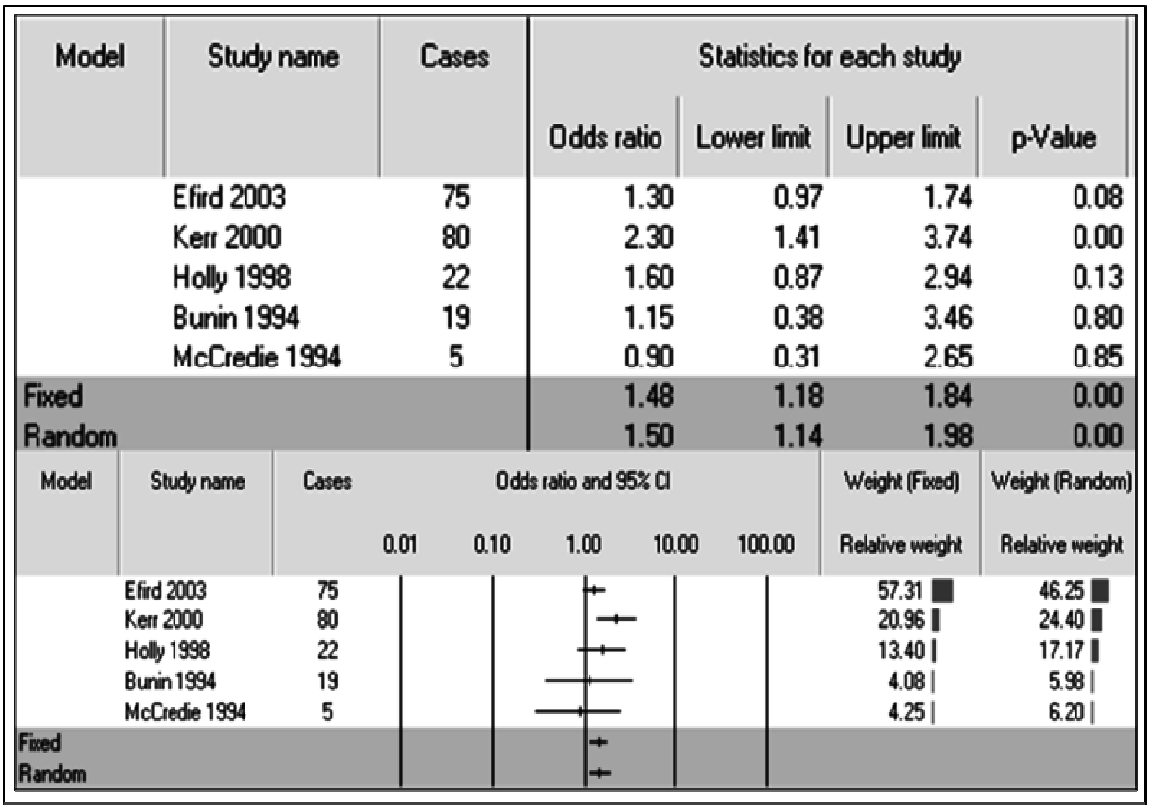

Figure 3-2. Meta-analysis results for maternal exposure to pesticides during pregnancy (agricultural/work exposures only).

Seven studies of maternal exposure to non-agricultural pesticides during pregnancy produced a summary risk estimate of OR = 1.36 (95% CI: 1.10 – 1.68) (Figure 3-3). Finally, the paternal exposure to pesticides during pregnancy meta-analysis grouped five studies for a final calculated summary risk estimate of OR = 1.63 (95% CI: 1.16 – 2.31) (Figure 3-4).

Exposures during Childhood

Combining four studies of childhood exposure to agricultural pesticides produced a summary risk estimate of OR = 1.35 (95% CI: 1.08 – 1.70) (Figure 3-5). Non-agricultural pesticide exposures during childhood were also found to be significantly associated with development of CBT as a meta-analysis of five studies found an increased risk of OR = 1.32 (95% CI: 1.04 – 1.67) (Figure 3-6).

DISCUSSION

Our meta-analyses produced several estimates of increased risk of CBT from exposure to pesticides. Comparing results from our categories of exposure, pre-conception and pregnancy exposure estimates were slightly higher than childhood exposure estimates, paternal exposures produced slightly higher risk estimates compared to maternal exposures, and agricultural exposures produced slightly higher risk estimates compared to non-agricultural exposures.

Evidence supporting a possible role for paternal exposure to pesticides during preconception and increased risk of CBT is limited but does exist. Tomatis et al. 1981 reported male rats given a single dose of ethylnitrosourea prior to mating with untreated

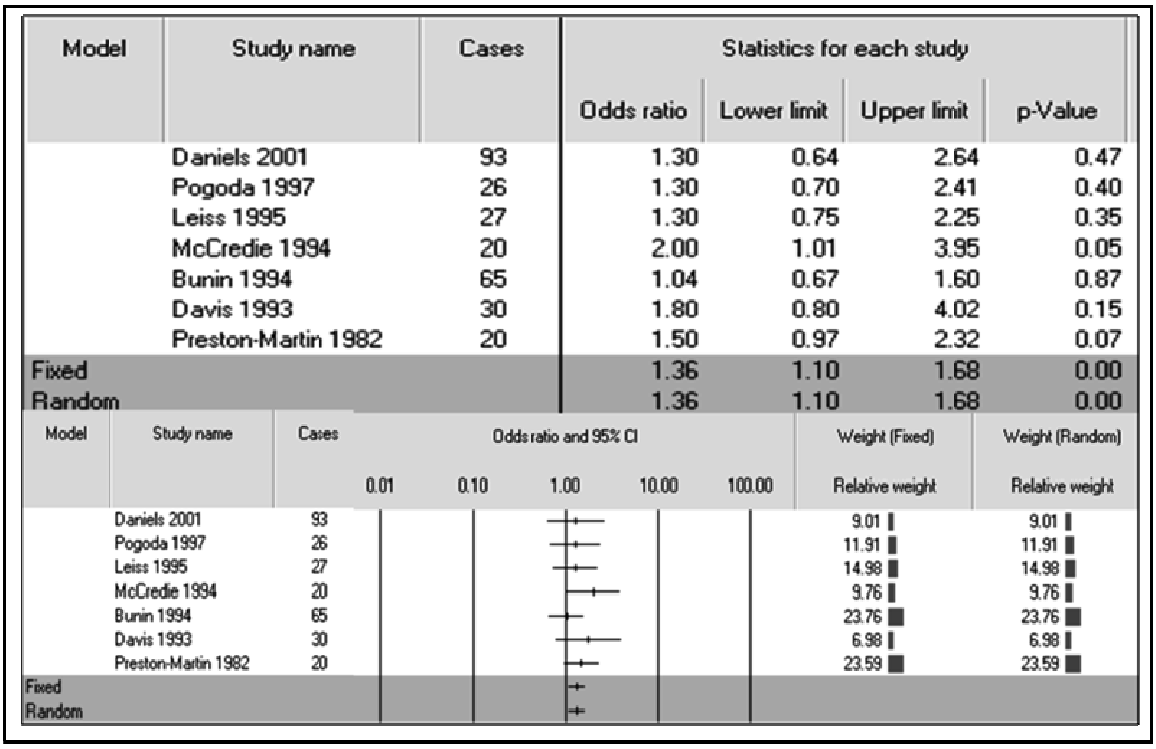

Figure 3-3. Meta-analysis results for maternal exposure to pesticides during pregnancy (non-agricultural exposures only).

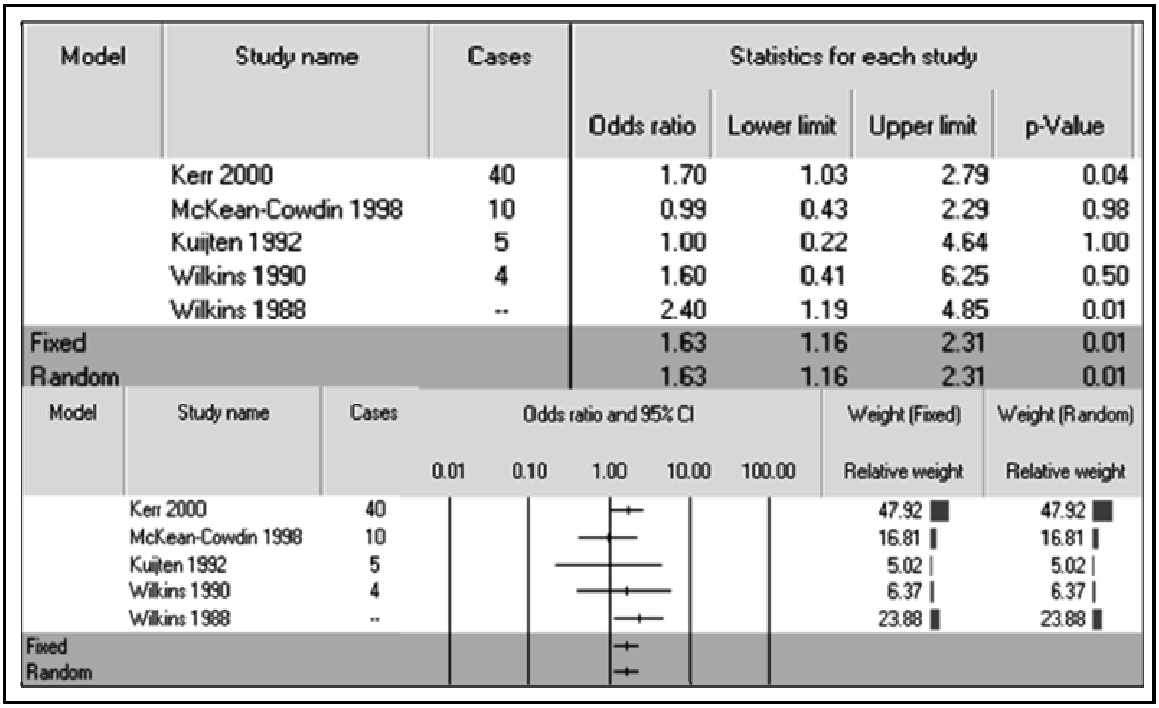

Figure 3-4. Meta-analysis results for paternal exposure to pesticides during pregnancy (agricultural/work exposures only).

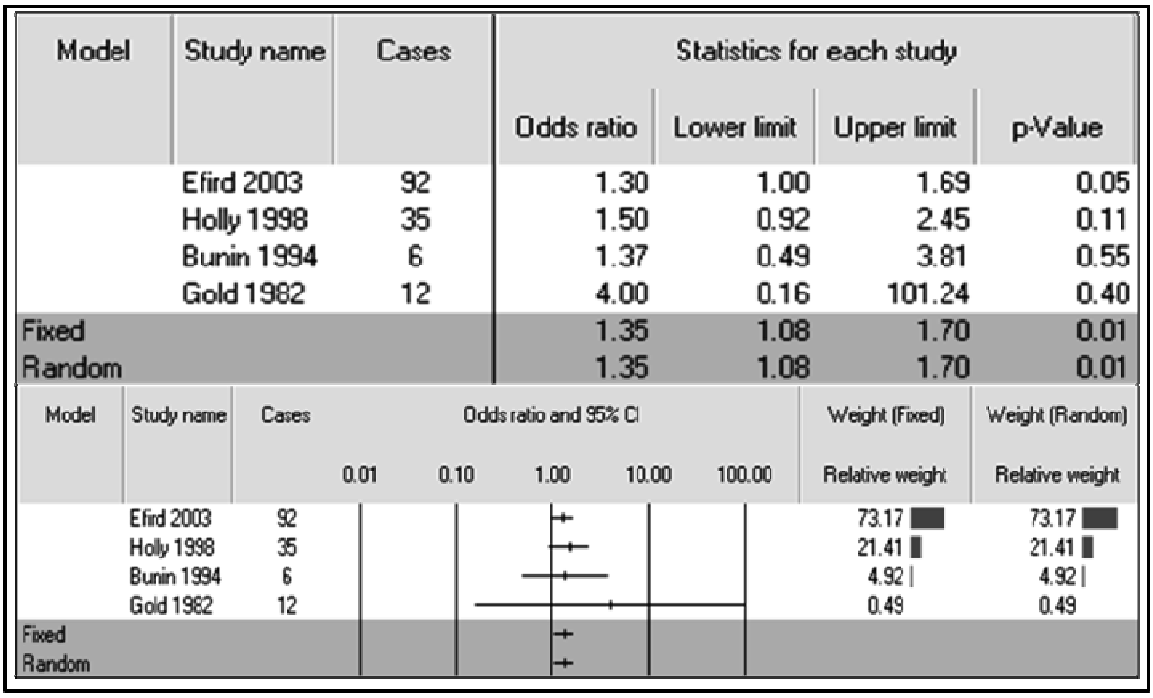

Figure 3-5. Meta-analysis results for childhood exposure to agricultural pesticides.

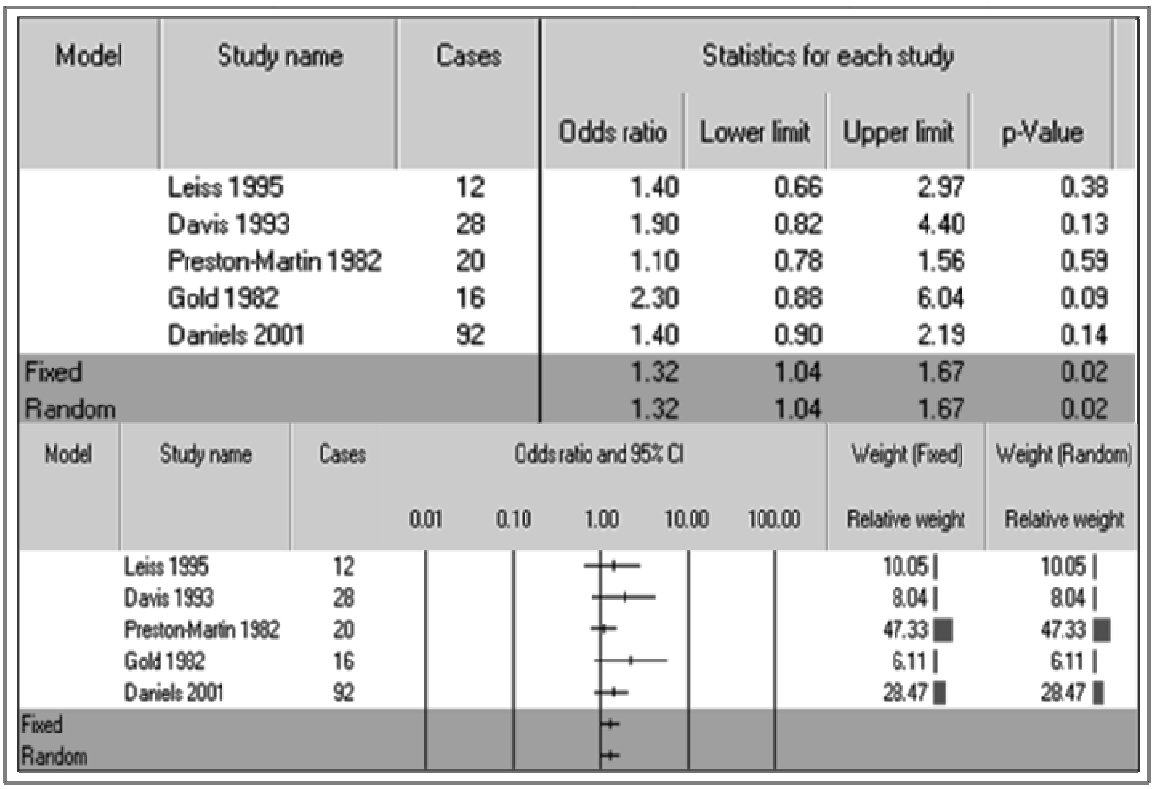

Figure 3-6. Meta-analysis results for childhood exposure to non-agricultural pesticides.

females produced offspring at increased risk for CNS tumors (5.6% compared to 1.9% controls; $p = 0.08$) [30]. Yu et al. 1999 found that offspring of male mice treated with chromium two weeks before conception were at increased risk of tumors. This study also reported that methylation changes were noted in the sperm and could have altered parental imprinting [31]. Lastly, a population study by Infante-Rivard et al. 1999 found increased risk of leukemia in children whose fathers were exposed to pesticides during preconception [32].

There are several strengths of this meta-analysis. As mentioned earlier, the use of the general variance based method gave more weight to larger studies, considered confounding, and limited the number of studies excluded because of missing data. Most studies used interview data to assess exposure, providing a more direct accounting of exposure. Finally, the combining of similar exposure time periods and splitting of maternal/paternal and agricultural/non-agricultural exposures allowed for assessment of the range of possible external etiological factors involved in CBT development.

Limitations of the study include those typical of the epidemiological studies combined in meta-analyses such as publication bias, recall bias and exposure misclassification. Also, pesticide and CBT type, along with individual practices of participants, were not distinguished in most studies. Lastly, a few studies had correlations for exposures across three time periods, thus limiting our ability to evaluate fully the independent effects of pesticide exposure during specific time periods.

Based on the collective results of these meta-analyses it appears pesticide exposure does increase risk of CBT, with preconception and prenatal exposures being especially important factors in increasing risk of its development. Interestingly, paternal exposure

may be as important, if not more important than maternal exposures, particularly during the preconception period. Whether this is a result of paternal exposures being more prevalent than maternal exposures or the consequence of a biological process, is a question that deserves further attention in future investigations of CBT etiology. The need for CBT exposure studies with better exposure time period characterization, including the division of pregnancy exposures into trimester, is also apparent.

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Manuscript 4: Identifying gene alterations required for the development of astrocytoma

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ABSTRACT

Astrocytomas are neoplasms of the brain that originate in a type of glial cell called an astrocyte. Recent advances in high-throughput microarrays have produced a wealth of information concerning molecular biology of glioma. In particular, microarrays have been used to obtain genetic and epigenetic changes between normal non-tumor tissue and glioma tissue. Due to the relative rarity of gliomas, microarray data for these tumors is often the product of small studies, and thus pooling this data becomes desirable. Additionally, analysis of microarray data has been an evolving field as techniques such as cluster analysis, networking analysis and principal components analysis have been used in order to tease biologically relevant information from the large amount of data produced from microarrays. We chose to combine these analytic approaches through first combining available microarray data on gliomas using a meta-analysis approach, and then conducting gene pathway networking analysis on results of this meta-analysis. Our goal in this study was to identify key genes and/or pathways that are critical in the development of astrocytic tumors. We also aim to identify genes and/or pathways that may further the understanding of the differences between low and high grade astrocytic tumors. Several steps were involved in our analysis, including: 1) identification of a significant set of over- and under-expressed genes through meta-analysis of several astrocytoma microarray studies; 2) enrichment analysis of the set of significant genes; 3) network analysis of the set of significant genes; and 4) investigation and validation of the network analysis. Through meta-analysis of 12 sub-studies which compared normal tissue to astrocytomas, we were able to identify a list of 554 genes which were differentially expressed in the majority of these studies. Many of the genes have in fact

been implicated in development of astrocytoma, including EGFR, HIF-1α, c-Myc, WNT5A, and IDH3A. We then performed reverse engineering of our gene list using Bayesian network analysis. Four networks of genes were produced, one for each grade of Astrocytoma (Grade I-IV). Genes most influential to the highest grade of astrocytoma, Glioblastoma (Grade IV) were: COL4A1, EGFR, BTF3, MPP2, RAB31, CDK4, CD99, ANXA2, TOP2A, and SERBP1. This study was able to identify a set of key genes significantly dysregulated during the development of astrocytoma. Our results suggest that alterations in the expression of eight to ten key genes may be required for the development of astrocytomas.

BACKGROUND

Astrocytomas are neoplasms of the brain that originate in a type of glial cell called an astrocyte. They are the most common glioma and their most aggressive form, glioblastoma multiforme, has a median survival of less than one year. While recent studies have characterized much of their basic biology, the major mechanisms behind the development of these tumors still remain unknown. Importantly, while some glioblastomas are thought to evolve from lower grade astrocytomas (secondary glioblastomas), most are thought to arise *de novo* (primary glioblastomas). Identifying genetic differences between the typically benign lower grade astrocytomas (Grade I-II) malignant higher grade astrocytomas (Grade III-IV) could be an important step in better characterization of these highly malignant tumors (Figure 4-1). In addition, determination of the main pathways and genes involved in their development could provide for better therapies in the future.

Recent advances in high-throughput microarrays have produced a wealth of information concerning glioma biology. In particular, microarrays have been used to obtain differences in gene expression between normal non-tumor tissue and glioma tissue. Due to the relative rarity of gliomas, microarray data for these tumors is often the product of small studies, and thus pooling this data becomes desirable. Additionally, analysis of microarray data has been an evolving field as techniques such as cluster analysis, networking analysis and principal components analysis have been used in order to tease biologically relevant information from the large amount of data produced from microarrays. We chose to combine these analytic approaches through first combining available microarray data on gliomas using a meta-analysis approach, and then

conducting Bayesian analysis on results of this meta-analysis. Our goal in this approach was to identify key genes and/or pathways that are critical in the development of astrocytic tumors. Our results point to the involvement of several key genes in the development and progression of astrocytoma (8 – 18 genes depending on the grade of tumor).

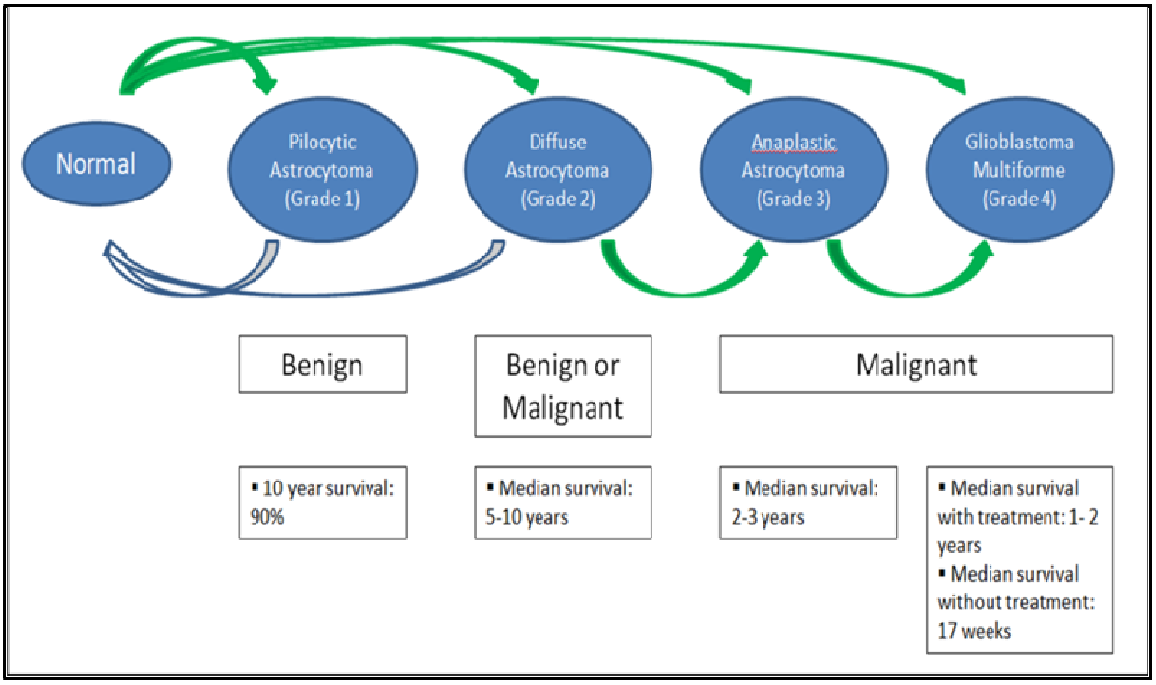

Figure 4-1. Characteristics of progression of Astrocytoma from Grade 1 to Grade 4.

METHODS

Several steps were involved in our analysis, including: 1) identification of a significant set of over- and under-expressed genes through meta-analysis of several astrocytoma microarray studies; 2) enrichment analysis of the set of significant genes; 3) network analysis of the set of significant genes; and 4) investigation and validation of the network analysis. A more detailed description of these steps follows.

Meta-analysis of Microarray Datasets: Oncomine (Compendia Bioscience, Ann Arbor, MI), a web-based cancer microarray database, was used to perform meta-analysis of cancer vs. normal studies in Astrocytoma [1]. The goal of this analysis was to identify a set of significantly over-and under-expressed genes in Astrocytoma for further investigation. An Oncomine query for 'Differential Analysis - Cancer vs. Normal Analysis' and 'Cancer Type - Brain and CNS Cancer' was performed to identify studies that compared Astrocytoma to normal tissue. Pilocytic Astrocytoma (WHO Grade I), Diffuse Astrocytoma (WHO Grade II), Anaplastic Astrocytoma (WHO Grade III), and Primary and Secondary Glioblastoma Multiforme (WHO Grade IV) 'sub-studies' were chosen. Only studies analyzing microarray mRNA expression were used for the analysis. For purposes of this paper, 'sub-studies' are defined as studies on brain tumor sub-types within a larger overall study on brain tumors. Studies from our query that compared Astrocytic tumors to normal tissue were then selected for the meta-analysis. Oncomine ranks genes within each individual study based on a gene's p-value compared to all other genes within the study. In meta-analysis, two heat-maps are returned: one for top over-expressed genes and one for top under-expressed genes. Genes in these heat-maps ordered based on their median rank across the selected individual analyses. For our study, the top 600 significantly under-expressed and the top 600 significantly over-expressed genes from meta-analysis were narrowed to our 'significant gene list' by discarding all genes from these 1200 over- and under-expressed genes that were identified in 6 or less of the sub-studies. Thus, a gene was included in our final list of significant genes if it was identified as over- or under-expressed in at least 7 of the 10

sub-studies. This final set of genes was then subjected to enrichment and pathway analysis with several different tools.

Gene Set Enrichment Analysis: FuncAssociate (Roth Laboratory, Harvard) and Ingenuity Pathway Analysis (IPA) (Redwood City, California) were used to identify pathways and other systems biology characteristics of our top set of genes. FuncAssociate is a web-based tool which performs a Fisher's Exact Test to determine a list of Gene Ontology (GO) attributes that are over- (or under-) represented among a set of genes entered by the user [2]. GO Terms, curated by the Gene Ontology Consortium, identify significant cellular components (e.g. rough endoplasmic reticulum, ribosome), biological processes (e.g. signal transduction, pyrimidine metabolic process), and molecular functions (e.g. catalytic activity, binding, adenylate cyclase activity) of a set of genes [3]. Our significant gene list from Oncomine was entered into FuncAssociate for analysis. Settings were species: Homo sapiens; namespace: HGNC_Symbol; mode: ordered; simulations: 1000; over/under: both; and p-value cutoff: 0.05. The HGNC Symbol namespace setting resulted in our choosing the entire known human genome as our universe of comparison genes for the enrichment analyses.

IPA was also used to analyze our Oncomine gene list. This web-based program uses a manually curated database of findings from the scientific literature, along with data obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG), to analyze connections between genes, proteins, and other molecules. It also uses its own terminology for functional classifications of these molecules that is similar but not exact to the terminology used by GO. Enrichment analysis was performed using IPA's "Core Analysis" function. Whereas GO Terms do not relate significant pathways of a set of

genes, IPA Core Analysis does have this ability and therefore was used both to identify significant biological processes/molecular functions and to identify any pathways that were more commonly activated or inactivated in our set of genes. Significance of the identified processes and pathways is given by the right-tailed Fisher exact test p-value, meaning only overrepresented attributes are returned by IPA. The IPA default reference set of molecules, which includes all functionally-characterized molecules in IPA, was used as the universe of comparison genes. Several groups of processes are identified, including: biological functions ('Bio Functions'), toxicological functions ('Tox Functions'), and established pathways ('Canonical Pathways'). The number of molecules from a set of data found to be in a pathway, divided by the total number of molecules in the identified canonical pathway is given.

Reverse Engineering Bayesian Network Analysis: Bayesian networks have been widely used and accepted in modeling molecular networks from microarray data [4;5]. These networks are probabilistic graphical models that produce directed acyclic graphs (DAG) that represent a set of random variables and their conditional dependencies. Nodes of the DAG represent genes or other variables such as disease and are assumed to be conditionally independent of each other. The structures produced by Bayesian network analysis naturally represent causal hypotheses.

We used the software application Banjo (Duke University, NC) for probabilistic structure learning of static Bayesian networks from our steady state expression data from Oncomine [6]. Banjo performs structure inference using a local search strategy termed Bayesian Dirichlet equivalence (BDe) scoring metric for discrete variables. This strategy

makes incremental changes in the structure aimed at improving the score of the structure. A score for the 'best network', influence scores for the edges of the best network, and a dot graphical layout file are returned as results of the search. The dot file is a DAG indicating regulation among genes and their possible influence on disease outcome.

The goal of this Bayesian analysis was to identify what may be the most critical genes for development of astrocytoma from our significant set of meta-analysis genes. This was accomplished by identifying a Markov blanket of each network output chosen as the 'best network' for each grade of astrocytoma. In a Bayesian network, the Markov blanket of any node *A* is its set of neighboring nodes composed of a nodes parents, children, and the parents of its children. This defined set of neighboring nodes shields node *A* from the rest of the network, and thus the Markov blanket of node *A* is the only knowledge needed to predict the behavior of node *A*.

Though its sensitivity is low, Banjo has been shown to have a very high positive predictive value for 100 plus case sets (regardless of the number of genes) composed of the type of 'global', steady-state gene data we analyzed [7]. For an overview of Bayesian network probability structures the reader is referred to Charniak 1991 [8]. Several other papers provide more detailed information on their construction and examples of their use with molecular modeling [5;9-12].

To perform the analysis on our data, expression values for our significant set of genes were downloaded from Oncomine and loaded into Microsoft Excel. The top 100 over-expressed genes and top 100 under-expressed genes were then considered for analysis in Banjo. In order to increase our sample size, missing cases imputation was performed on cases with missing expression data for a particular gene using average of all expression

values across the gene as the imputation. Cases without Grade identification and/or identified as non-tissue cases (i.e. cell lines) were excluded from the analysis. Studies from our meta-analysis with missing data for a large amount of genes were also excluded. The expression data for the remaining genes was then separated by Grade, discretized per study (due to Oncomine normalizing expression values per study), and combined for analysis in Banjo. Discretization of the data into three tiers expression (under-, median-, and over-expressed) was performed using the programming software tool Perl. Assuming normally distributed data, the three tiers were selected based on a one standard deviation confidence interval (i.e. ~68% of the values will have 'median-expression', with ~16% of the values under-expressed and ~16% of the values over-expressed). Discretized files were then run in Banjo for four separate analyses: 1) Normal Tissue vs. Grade I Pilocytic Astrocytoma cases, 2) Normal Tissue vs. Grade II Diffuse Astrocytoma cases, 3) Normal Tissue vs. Grade III Anaplastic Astrocytoma, and 4) Normal Tissue vs. Grade IV Glioblastoma Multiforme cases. Analyses was performed on the four Grades three separate times (three hours in length for each network search), with the 'best network' from these three runs being chosen as our 'final best network' for each Grade. Best network score significance was calculated using a log calculation of all three network scores, with a percent of the total score returned for each network.

Investigation of Bayesian Network Results: Several methods were used to investigate and validate both the prediction capabilities and the biological plausibility of our Markov network genes. They included literature and biological database searches, prediction analysis and clustering analysis, and curated gene and pathway analysis. The literature

and database search of our Markov genes gathered information on gene cellular localization and function, and published research supporting the genes involvement in tumor formation by searching biological databases such The Human Gene Compendium's GeneCards (www.genecards.org), PubMed (www.pubmed.com), the Information Hyperlinked over Proteins (iHOP) Database (www.ihop-net.org), and the Glioblastoma Multiforme Database (GBMBase) (www.gbmbase.org).

To assess the ability of our Markov genes to distinguish between normal and tumor samples in our analysis, we performed several prediction analyses including receiver operating characteristic (ROC) curve analysis representing the Bayesian network discretized results; and linear regression, logistic regression, cross validation and support vector machine (SVM) analysis to assess the predictability of both the discretized and raw expression values of our Markov genes. Hierarchical Clustering was also performed on each set of Markov genes in order to further explore how these genes separated our set of non-tumor and tumor patients. These analyses were performed using both IBM SPSS Statistics 19.0 and Multi-Experiment Viewer (MeV) version 4.7.1.

Genie, a software tool for analyzing Bayesian networks developed by the University of Pittsburgh [13], was used to predict the probability of developing Astrocytoma given certain expression states for its gene network. In Bayesian network analysis this is done by learning the parameters of a given DAG structure. To accomplish this task, the discretized results files for each Grade of astrocytoma were loaded into the Genie software. Additionally, the Banjo network structure results were recreated in Genie. Genie's 'learn parameters' function was then used to predict probabilities of outcomes for certain network structures. Given our small sample sizes, we did not allow a probability

of 0 to be assigned to any result, choosing instead to use 0.01 for any probability calculated as 0. This allowed us to perform parameter assessment under the assumption that a low probability case may still have a very small chance of occurring in our data. Once our network parameters were established in Genie, we analyzed the probability of developing each grade of astrocytoma given differentially expressed states of the Markov genes of each grade. This was done assuming the 2005-2007 Surveillance, Epidemiology and End Results (SEER) calculated lifetime probability of diagnosis of cancer of the brain and other nervous system of 0.61% [14]. (NOTE: This analysis was performed for Grade IV Astrocytoma only).

In order to investigate existing literature and ontology based connections between our Markov gene lists we used programs in both IPA and PathJam [15]. The goal of these analyses was to investigate a) the quality of our network analysis findings in Banjo and Genie, and b) the biological relationships of our Markov genes from these analyses. The initial investigation was done using the Path Explorer feature of IPA. Path Explorer uses curated literature and experimental evidence of biochemical interactions to produce networks of existing connections between a set of user imputed genes. This function was used to search for any existing connections among the Grade IV Astrocytoma Markov Genes.

IPA's Core Analysis was then performed on these same Grade IV Astrocytoma Markov Genes in order to produce connections for a set of genes independent of their established pathways. This analysis generated gene networks produced by including genes in pathways of the inputted gene list. Networks are ordered in importance by an IPA-defined significance score. Settings for this analysis were Direct and Indirect

Relationships, All Data Sources, All Species, and All Tissues & Cell Lines. The Human Genome U133 Plus 2.0 Array (19,079 genes) was selected as our reference universe of genes as it contained the largest gene set from our meta-analysis and was used in 2 of the 5 meta-analysis studies used for our Banjo analysis. The top identified network from the Core Analysis was compared to our Banjo/Genie generated results.

Complementary to this Core Analysis's production of top biological and disease related functions was our investigation of our Markov genes using PathJam [15]. This public server-based tool allows for interpretation of gene lists by integrating pathway-related annotations from several public sources including Reactome, KEGG, NCBI Pathway Interaction Database, and Biocarta. Using this tool we were able to produce interactive graphs linking all four Astroctyoma Markov gene lists with pathway annotations, allowing for graphical pathway investigation into our gene lists.

RESULTS

Meta-analysis of Microarray Datasets: A total of 12 studies (with 27 sub-studies) conducting cancer vs. normal analysis on 'Brain and CNS Cancer' were identified in Oncomine. Non-astrocytic tumor studies and studies analyzing DNA (i.e. acCGH arrays) were then discarded, leaving seven studies (10 sub-studies) on astrocytoma for the meta-analysis. These 10 sub-studies are listed in Table 4-1.

The top 600 significantly over-expressed and top 600 significantly under-expressed genes were identified from a total of 10 'sub-studies'. The narrowing of the initial list of 1200 genes produced a total of 646 genes for further analysis (372 significantly over-expressed genes and 274 significantly under-expressed genes). A list of these genes can

be found in Table 1 and Table 2 of the Appendix. It should be noted that Primary and Secondary Glioblastomas were separated within only one of the nine studies identified as Astrocytoma in Oncomine (Bredel: 27 Primary vs. 2 Secondary Glioblastomas). Therefore, separation of these subtypes of Glioblastomas was not considered in our study.

Table 4-1. List of Oncomine studies in meta-analysis of Astrocytoma vs. Normal Studies.

Oncomine Study ID, *Publication Journal*, Date	Study Astrocytoma Type*	n (tumor/normal)
Bredel Brain 2, *Cancer Res*, 2005 [16]	Glioblastoma	27 / 4
Gutmann Brain, *Cancer Res*, 2002 [17]	Pilocytic Astrocytoma	8 / 3
Lee Brain, *Cancer Cell*, 2006 [18]	Glioblastoma	22 / 3
Liang Brain, *Proc Natl Acad Sci USA*, 2005 [19]	Glioblastoma	30 / 3
Rickman Brain, *Cancer Res*, 2001 [20]	Astrocytoma	45 / 6
Shai Brain, *Oncogene*, 2003 [21]	Astrocytoma	5 / 7
Shai Brain, *Oncogene*, 2003 [21]	Glioblastoma	27 / 7
Sun Brain, *Cancer Cell*, 2006 [22]	Anaplastic Astrocytoma	19 / 23
Sun Brain, *Cancer Cell*, 2006 [22]	Diffuse Astrocytoma	7 / 23
Sun Brain, *Cancer Cell*, 2006 [22]	Glioblastoma	81 / 23

*** All studies are Astrocytoma tissue type vs. normal tissue.**

Gene Set Enrichment Analysis: In order to identify significant biological processes, molecular functions, and pathways of the final set of 646 genes, we conducted enrichment analysis on this set of genes. As described in the methods, two separate programs were used for this analysis: FuncAssociate and IPA.

FuncAssociate Results: FuncAssociate identified 60 GO Terms as being over-represented and 1 GO Term as being under-represented among our set of 314 over-expressed genes (Table 4-2 and Table 4-3). Several significant processes were related to nervous system processes (axon part, postsynaptic density, synapse part, synaptic transmission, neuron projection), developmental processes (cell part morphogenesis, cellular component morphogenesis, regulation of anatomical structure morphogenesis,

anatomical structure morphogenesis, regulation of developmental process, anatomical structure development, development process), and several cellular processes associated with cancer (cell adhesion, biological adhesion, regulation of cell proliferation, regulation of apoptosis). Several genes involved in developmental processes have been linked to brain tumor development. 147 genes of our list of 646, in fact, were categorized in the GO developmental process terms listed above. Several of these genes, including MYC, EGFR, HIF1A, HGF, APOE, TIMP3, and WNT5A have been identified as being important to development of astrocytoma.

Table 4-2. Significant over-represented GO Terms for differentially expressed genes in Astrocytoma.

Rank	p-adjusted	GO ID	GO Name
1	0.006	GO:0019829	Cation-transporting ATPase activity
2	0.036	GO:0033267	Axon part
3	0.04	GO:0005938	Cell cortex
4	0.04	GO:0014069	Postsynaptic density
5	0.013	GO:0044420	Extracellular matrix part
6	0.019	GO:0032990	Cell part morphogenesis
7	0	GO:0032989	Cellular component morphogenesis
8	0	GO:0019900	Kinase binding
9	0	GO:0044456	Synapse part
10	0.011	GO:0019901	Protein kinase binding
11	0.001	GO:0010035	Response to inorganic substance
12	0.002	GO:0007268	Synaptic transmission
13	0	GO:0043005	Neuron projection
14	0	GO:0044419	Interspecies interaction between organisms
15	0.002	GO:0044057	Regulation of system process
16	0.013	GO:0022603	Regulation of anatomical structure morphogenesis
17	0	GO:0019899	Enzyme binding
18	0	GO:0008092	Cytoskeletal protein binding
19	0	GO:0007155	Cell adhesion
20	0	GO:0022610	Biological adhesion
21	0	GO:0042127	Regulation of cell proliferation
22	0	GO:0042995	Cell projection
23	0	GO:0005515	Protein binding
24	0.007	GO:0005509	Calcium binding
25	0	GO:0008150	Biological process
26	0.001	GO:0010033	Response to organic substance
27	0	GO:0023034	Intracellular signaling pathway
28	0	GO:0044459	Plasmamembrane part
29	0.013	GO:0045907	Intracellular transport
30	0.001	GO:0009653	Anatomical structure morphogenesis
31	0.011	GO:0050793	Regulation of developmental process

Rank	p-adjusted	GO ID	GO Name
32	0.011	GO:0051239	Regulation of multicellular organismal process
33	0.016	GO:0010646	Regulation of cell communication
34	0.013	GO:0035466	Regulation of signaling pathway
35	0.035	GO:0045184	Establishment of protein localization
36	0.035	GO:0042981	Regulation of apoptosis
37	0	GO:0005488	Binding
38	0.033	GO:0050790	Regulation of catalytic activity
39	0.015	GO:0065009	Regulation of molecular function
40	0.03	GO:0031226	Intrinsic to plasma membrane
41	0	GO:0048518	Positive regulation of biological process
42	0.006	GO:0042221	Response to chemical stimulus
43	0	GO:0048522	Positive regulation of cellular process
44	0.015	GO:0065008	Regulation of biological quality
45	0	GO:0043234	Protein complex
46	0.001	GO:0048519	Negative regulation of biological process
47	0	GO:0009987	Cellular process
48	0.016	GO:0048856	Anatomical stucture development
49	0.011	GO:0048523	Negative regulation of cellular process
50	0	GO:0005737	Cytoplasm
51	0	GO:0032502	Developmental process
52	0.002	GO:0005886	Plasma membrane
53	0.007	GO:0016043	Cellular component organization
54	0.005	GO:0023052	Signaling
55	0	GO:0044444	Cytoplasmic part
56	0.006	GO:0032991	Mactromolecular complex
57	0	GO:0065007	Biological regulation
58	0.002	GO:0050789	Regulation of biological process
59	0.005	GO:0050794	Regulation of cellular process
60	0.013	GO:0044424	Intracellular part

Table 4-3. Significant under-represented GO Terms for differentially expressed genes in Astrocytoma.

Rank	p-adjusted	GO ID	GO Name
1	0.006	GO: 0004984	Olfactory receptor activity

Ingenuity Pathway Analysis Results: IPA produced similar and contrasting results to the above analysis using FuncAssociate. Top Canonical Pathways identified for the over-expressed gene list include: 'Synaptic Long Term Potentiation' (p-value: 6.25E-07; Ratio of molecules in pathway from user list/total molecules in pathway: 16/113), 'IL-8 Signaling' (p-value: 7.41E-07; Ratio: 20/186), 'G Beta Gamma Signaling' (p-value: 9.48E-07; Ratio: 15/119), 'CXCR4 Signaling' (p-value: 1.1E-06; Ratio: 19/167), and

'Cholecystokinin/Gastrin-mediated Signaling' (p-value: 1.39E-06; Ratio: 15/104) (Figure 4-2). Several pathways known to be important to glioma development were also at the top of the significant canonical pathways list, including 'WNT/beta-Catenin Signaling' (CD44, CDH2, DVL3, LRP1, MYC, SOX4, SOX9, SOX13, TCF3, TCF4, TLE3, WNT5A) and 'mTOR Signaling' (EIF3B, EIF3E, EIF3F, EIF4A1, HIF1A, PRKD1, RHOC, RND2, RND3). Confirming our gene list as involved with brain tumor development, 'Glioma Invasiveness Signaling' (CD44, F2R, ITGAV, MMP9, RHOC, RND2, RND3, TIMP3, TIMP4) and 'Glioblastoma Multiforme Signaling' (CDK6, CDKN1A, EGFR, ITPR2, MYC, RHOC, RND2, RND3, TCF3, WNT5A) were returned as significant pathways as well.

IPA Core Analysis also returns what are termed 'Top Bio Functions', grouped into three categories: Diseases and Disorders, Molecular and Cellular Functions, and Physiological System Development and Function. Significant functions are returned with their associated p-value and # of input molecules in the function. The top 5 Disease and Disorders for our list of 554 astrocytoma differentially expressed genes were: 'Neurological Disease' (p-value: 1.17E-25 – 4.98E-04; 270 molecules from our list), 'Cancer' (3.83E-24 – 5.61E-04; 240 molecules), 'Skeletal and Muscular Disorders' (2.32E-19 – 4.42E-04; 206 molecules), 'Genetic Disorder' (3.04E-17 – 5.27E-04; 354 molecules), and 'Inflammatory Disease' (2.20E-16 – 4.92E-04; 195 molecules). The top 5 Molecular and Cellular Functions were 'Cell Death' (1.36E-21 – 5.81E-04; 205 molecules), 'Cellular Growth and Proliferation' (1.23E-14 – 4.48E-04; 203 molecules), 'Cell Morphology' (2.51E-14 – 4.82E-04; 99 molecules), 'Cellular Movement' (1.08E-11 – 4.58E-04; 116 molecules), and 'Cell Cycle' (5.83E-11 – 5.61E-04; 91 molecules).

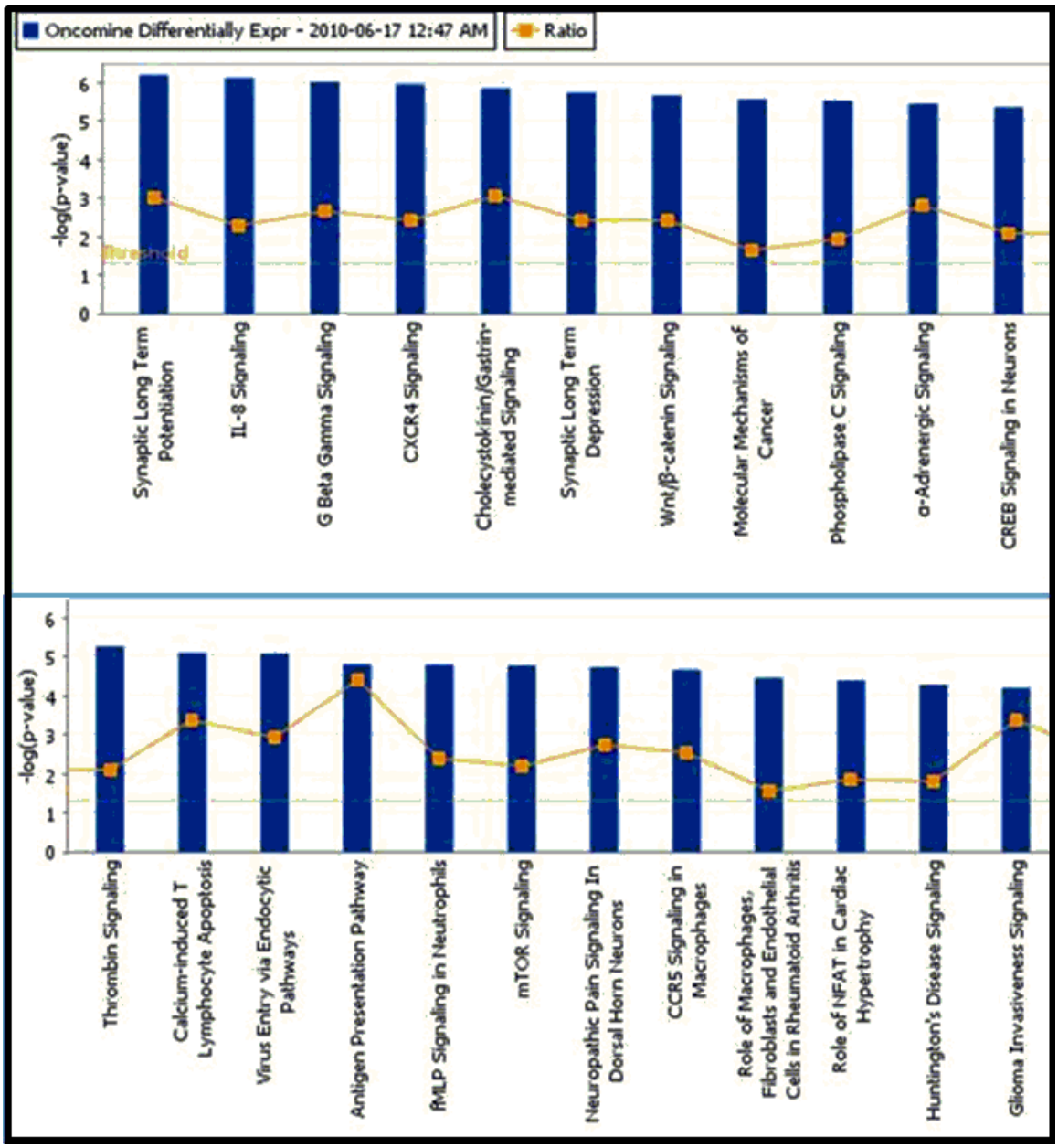

Figure 4-2. Top Canonical Pathways for Astrocytoma differentially expressed genes. The threshold line denotes the cutoff for significance (p-value = 0.05). Ratio is the number of molecules in the input list vs. the total number of molecules in the function.

The top 5 Physiological System Development and Functions were 'Tissue Development' (1.65E-09 – 5.79E-04; 105 molecules), 'Skeletal and Muscular System Development and Function' (1.67E-09 – 2.96E-04; 54 molecules), 'Tissue Morphology'

(7.04E-08 – 1.35E-04; 78 molecules), 'Nervous System Development and Function' (1.48E-07E – 3.65E-04; 96 molecules), and 'Behavior' (1.67E-07 – 3.09E-04; 47 molecules). Figure 4-3 shows these top Bio Functions in order of significance. When interpreting these results, it is important to keep in mind that the p-values refer to the High Level Functions rather than to individual Lower-Level Functions, and therefore, if a High Level Function contains two or more specific Lower-Level Functions, a range of significances is displayed.

Core Analysis also produces Top Toxicity Profiles. The Top 5 profiles for our 554 differentially expressed genes were 'Hepatic Fibrosis' (p-value: 3.59E-06; Ratio of molecules: 13/85), 'Hepatic Cholestasis' (p-value: 4.77E-03; Ratio: 11/135), 'G1/S Transition of the Cell Cycle' (p-value: 5.02E-03; Ratio: 6/49), 'Oxidative Stress' (p-value: 1.05E-02; Ratio: 6/57), and 'VDR/RXR Activation' (p-value: 1.28E-02; Ratio: 7/77) (Figure 4-4).

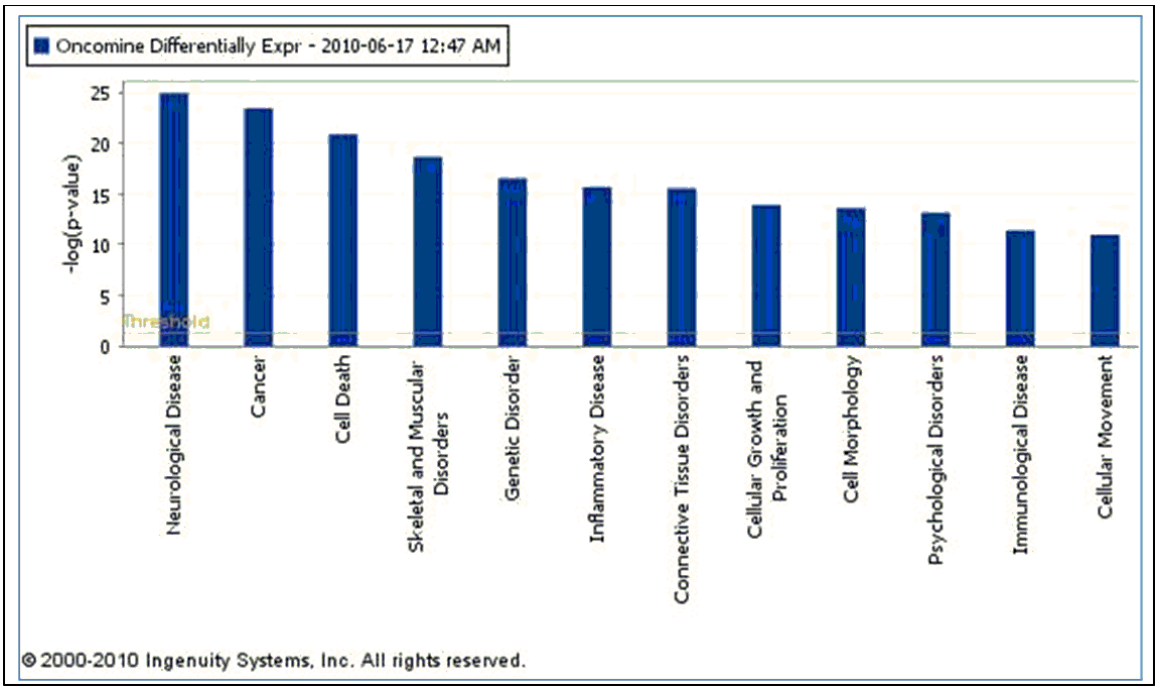

Figure 4-3. Top Biological Functions for Astrocytoma differentially expressed genes. The threshold line denotes the cutoff for significance (p-value = 0.05).

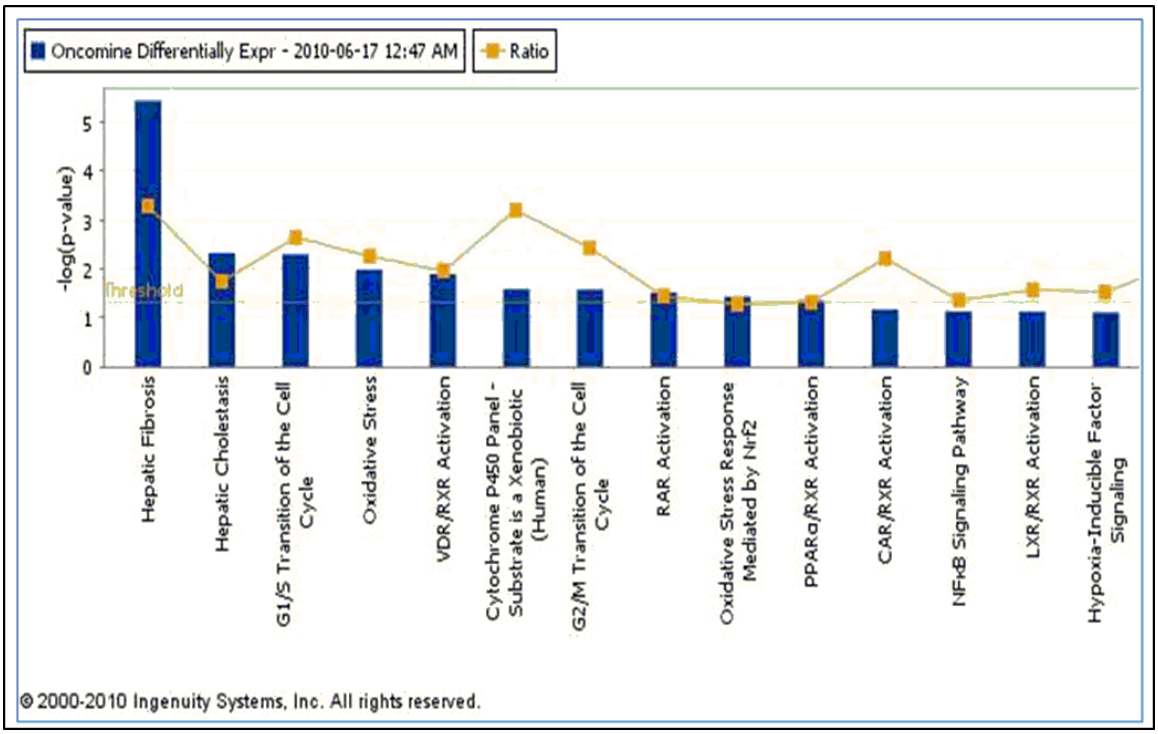

Figure 4-4. Top Toxicological Functions for Astrocytoma differentially expressed genes. The threshold line denotes the cutoff for significance (p-value = 0.05). Ratio is the number of molecules in the input list vs. the total number of molecules in the function.

Reverse Engineering Bayesian Network Analysis: Four separate analyses were run in Banjo in order to search for genes critical for Grade I, II, III and IV Astrocytoma development. As discussed in the methods, studies and/or genes with missing expression data were excluded from the network analysis. Studies removed for both analyses were Bredel 2005, Liang 2005, and Rickman 2001. Additionally, Gutmann 2002 was removed from the Grade IV analyses as it did not contain Grade IV tumors. Genes were removed from our top 200 genes list (100 over- and 100 under-expressed genes) for each analyses based on availability per Grade. 77 genes were removed for Grade 1, 68 for Grade 2, and 23 each for Grades 3 and 4. Please see Table 4-4 for sample size and search score results for each grade of tumor. Markov blanket genes identified for each grade were as follows:

- Grade I Pilocytic Astrocytoma: IGFB5, TIMP4, SSR2, LPL, DUSP7, GABRA5, SH3GL3, C1S, WNT10B, SRPX, ANK3, HLAA, EIF4A1, PTGER3, CCND2
- Grade II Diffuse Astrocytoma: FN1, MARCKS, PRDX4, NONO, SPARC, WNT5A, CD44, EIF4A1, CD99, CALCRL, EMP1, VCAN, CDH11, VAMP1, RAB3B, DUSP7, PPP2R2B, SERINC3
- Grade III Anaplastic Astrocytoma: LPL, MARCKS, SERBP1, DPYSL3, SNRPE, EIF4A1, ANXA1, MCM3, BTN3A3, MTHFD2, DAB2, RCAN2, RUSC2, TPPP, MAST3, CNTN2
- Grade IV Glioblastoma Multiforme: COL4A1, EGFR, BTF3, MPP2, RAB31, CDK4, CD99, ANXA2, TOP2A, SERBP1

DAG structures for these Markov genes can be seen in Figures 4-5, 4-6, 4-7, and 4-8. A comparison of these Markov genes across each grade of Astrocytoma can be found in Appendix Table 3.

Table 4-4. Sample statistics and significance of search score results of Bayesian network analysis.

	Studies in Analysis	Normal	Tumor	Genes Analyzed for Network	Bayesian Analysis Network Score Significance*		
					Search 1	Search 2	Search 3
Normal vs. Grade 1	Bredel, Gutmann, Rickman	13	30	122	1.37%	98.16%	0.46%
Normal vs. Grade II	Rickman, Shai, Sun	36	14	131	9.11%	6.62%	84.62%
Normal vs. Grade III	Bredel, Shai, Sun	34	23	176	1.98%	0.04%	99.95%
Normal vs. Grade IV	Bredel, Shai, Sun	34	137	176	0.00%	0.00%	99.99%

***Significance score for each network equals percent of total score for all three networks combined.**

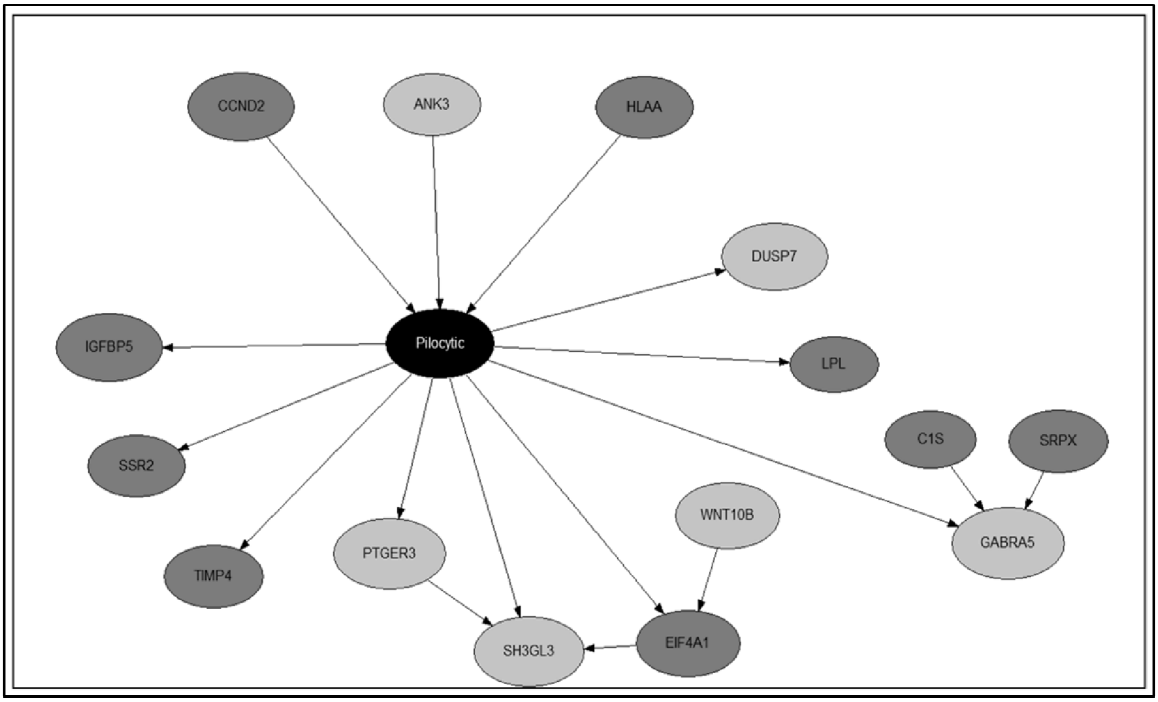

Figure 4-5. Markov blanket genes for Bayesian network of Pilocytic Astrocytoma. Darker shade genes are overexpressed genes and lighter shade genes are underexpressed genes from the Oncomine meta-analysis.

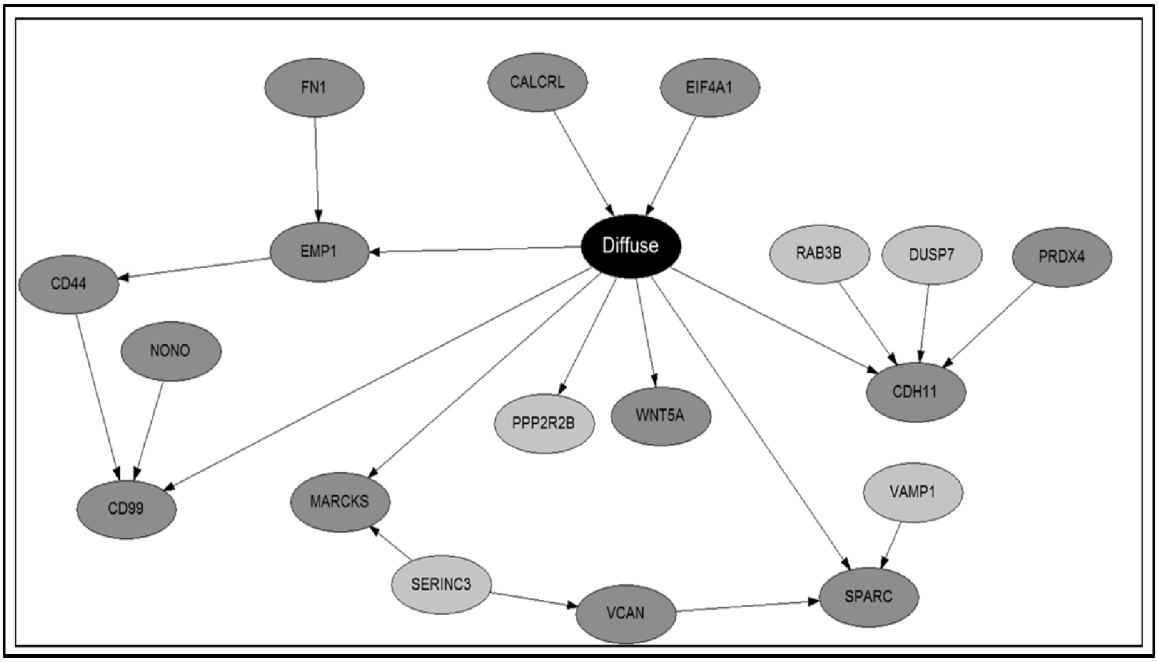

Figure 4-6. Markov blanket genes for Bayesian network of Diffuse Astrocytoma. Darker shade genes are overexpressed genes and lighter shade genes are underexpressed genes from the Oncomine meta-analysis.

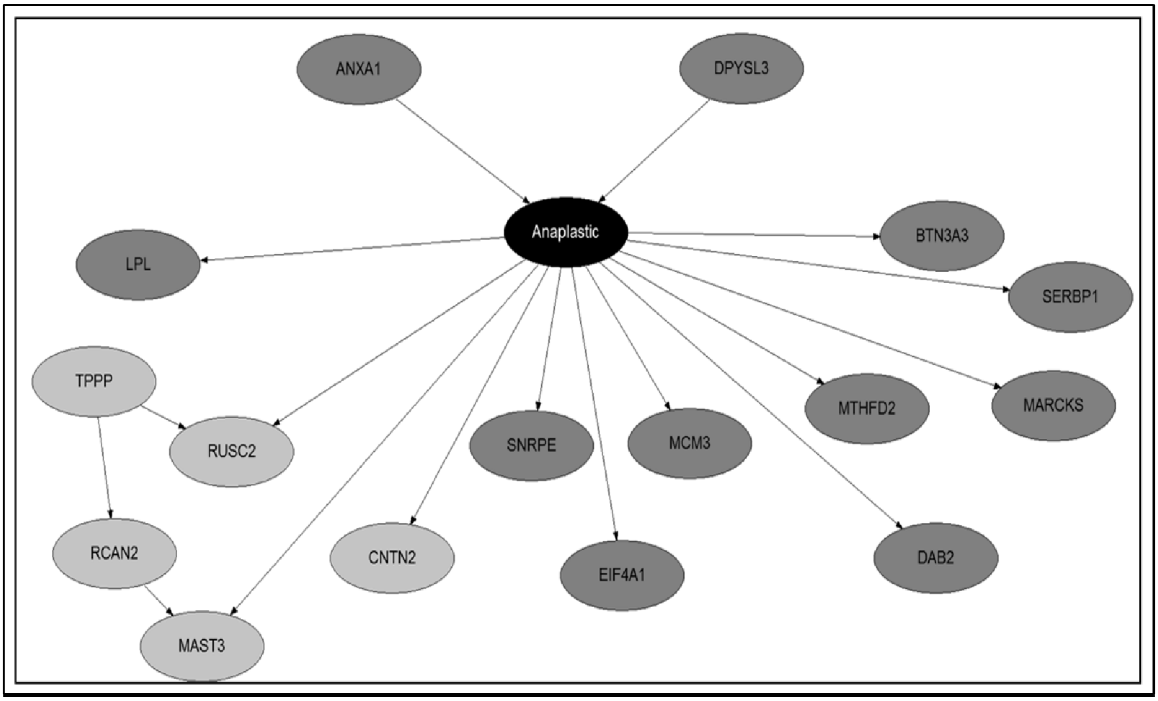

Figure 4-7. Markov blanket genes for Bayesian network of Anaplastic Astrocytoma. Darker shade genes are overexpressed genes and lighter shade genes are underexpressed genes from the Oncomine meta-analysis.

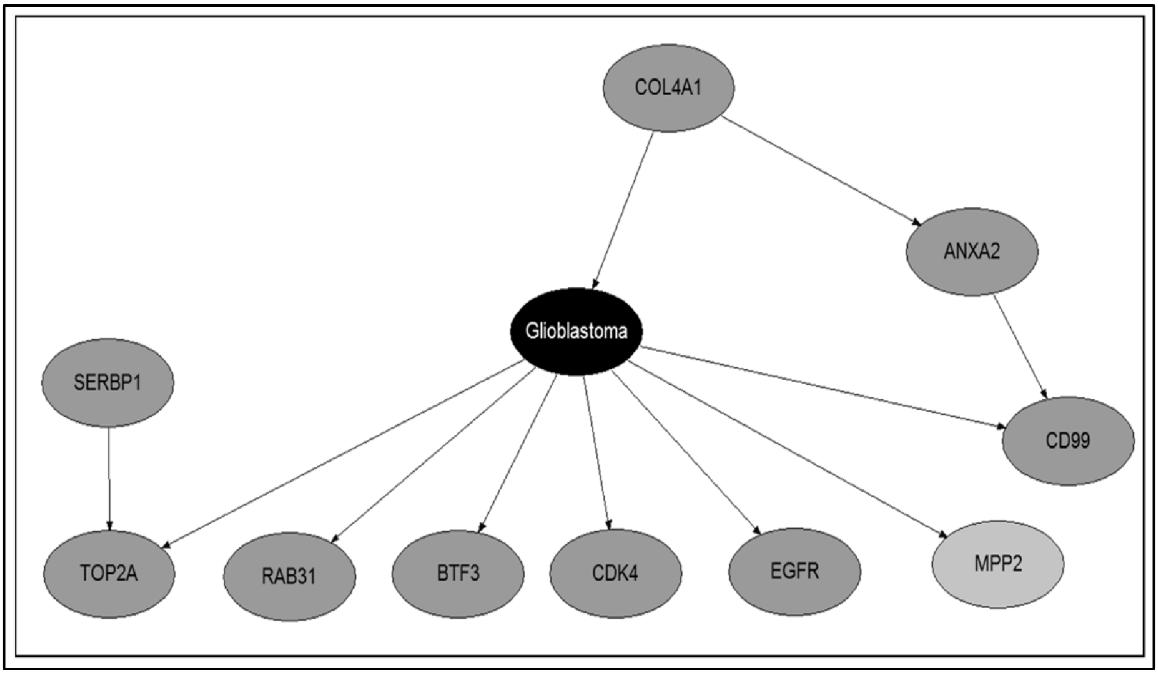

Figure 4-8. Markov blanket genes for Bayesian network of Glioblastoma Multiforme. Darker shade genes are overexpressed genes and lighter shade genes are underexpressed genes from the Oncomine meta-analysis.

Analysis of Bayesian Network Results

Normal Tissue vs. Grade I Astrocytoma: Prediction analysis of the 15 Grade I Markov genes showed that the 15 gene set we identified as important for Pilocytic Astrocytoma development was able to predict tumor status consistently in each analysis type (Table 4-5). Results of hierarchical clustering using expression data from our Markov genes can be found in Figure 4-9. Results of the PathJam analysis can be found in Appendix Figure 1.

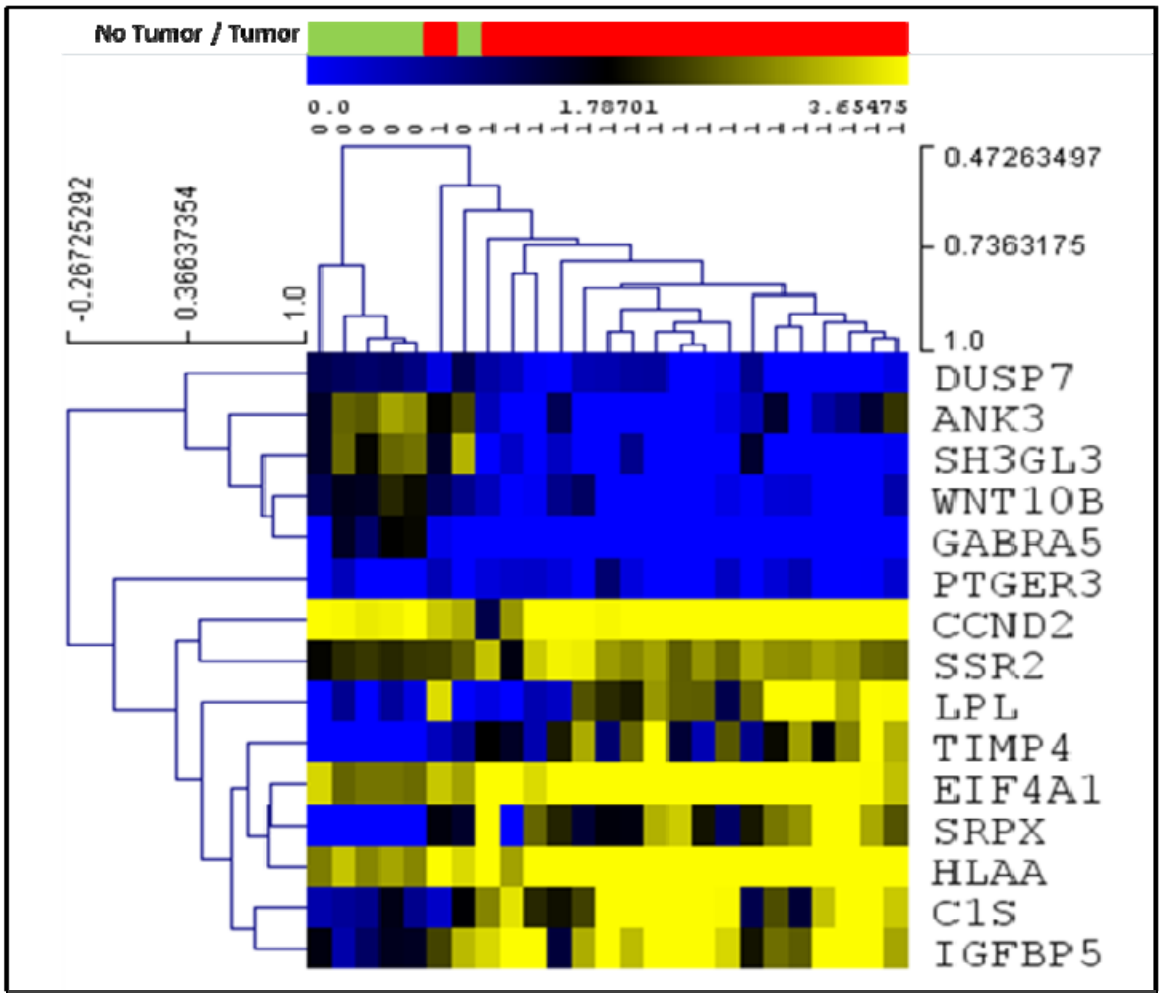

Figure 4-9. Hierarchical clustering of Pilocytic Astrocytoma Markov genes using raw expression values of Rickman study only. No Tumor /Tumor Bar: Red bar areas represent samples without tumors and green bar areas represent tumor cases. Expression: Blue squares represent underexpression; yellow squares represent overexpression.

Table 4-5. Pilocytic Astrocytoma prediction analysis summary. 15 total Markov genes were used in the analysis, except in linear stepwise regression, which used only 10 significant predictor genes in the analysis.

Type of Prediction Analysis	Case Counts (No Tumor/Tumor)	Predictability
Bayesian Network Results using ROC Curve	13/30	1.000 AUC (.000 sig.)
Linear Regression (15 genes together)	13/30	.873 (aR square) (.000 sig.)
Linear Regression (stepwise) (10 gene model)	13/30	.880 (aR square) (.000 sig.)
Logistic Regression (discretized expression)	13/30	Error: Perfect fit detected
Logistic Regression (raw expression)	6/19	Error: Perfect fit detected
Cross Validation (discretized expression)	13/30	79.1%
Cross Validation (raw expression)	6/19	88%
Support Vector Machine (SVM)	6/19	100%

Normal Tissue vs. Grade II Astrocytoma: Prediction analysis of the 18 Grade II Markov genes showed that our set of 18 genes was able to predict tumor status consistently when using logistic regression, cross validation and SVM analysis. Linear regression seemed to predict that a signature of 10 genes would predict tumor status just as well as our 18 total genes, though each gene set only predicted approximately 21% of the tumor status's variability in either case (Table 4-6). Results of hierarchical clustering using expression data from our Markov genes can be found in Figure 4-10. Results of the PathJam analysis can be found in Appendix Figure 2.

Normal Tissue vs. Grade III Astrocytoma: Prediction analysis of the 18 Grade III Markov genes showed that our set of 18 genes was able to predict tumor status, especially when using logistic regression, raw expression in cross validation, and the SVM analysis. Linear regression seemed to again predict that a signature of much less than the total genes (10 of 18) would predict tumor status (Table 4-7). Results of hierarchical clustering using expression data from our Markov genes can be found in Figure 4-11. Results of the PathJam analysis can be found in Appendix Figure 3.

Table 4-6. Diffuse Astrocytoma prediction analysis summary. 18 total Markov genes were used in the analysis, except in linear stepwise regression, which used only 10 significant predictor genes in the analysis.

Type of Prediction Analysis	Case Counts (No Tumor/Tumor)	Predictability
Bayesian Network Results using ROC Curve	36/14	1.000 AUC (.000 sig.)
Linear Regression (18 genes together)	36/14	.207 (aR square) (.092 sig.)
Linear Regression (stepwise) (10 gene model)	36/14	.880 (aR square) (.028 sig.)
Logistic Regression (discretized expression)	36/14	88% (1.000 sig.)
Logistic Regression (raw expression)	23/8	Error: Perfect fit detected
Cross Validation (discretized expression)	36/14	70%
Cross Validation (raw expression)	23/7	80%
Support Vector Machine (SVM)	23/7	100%

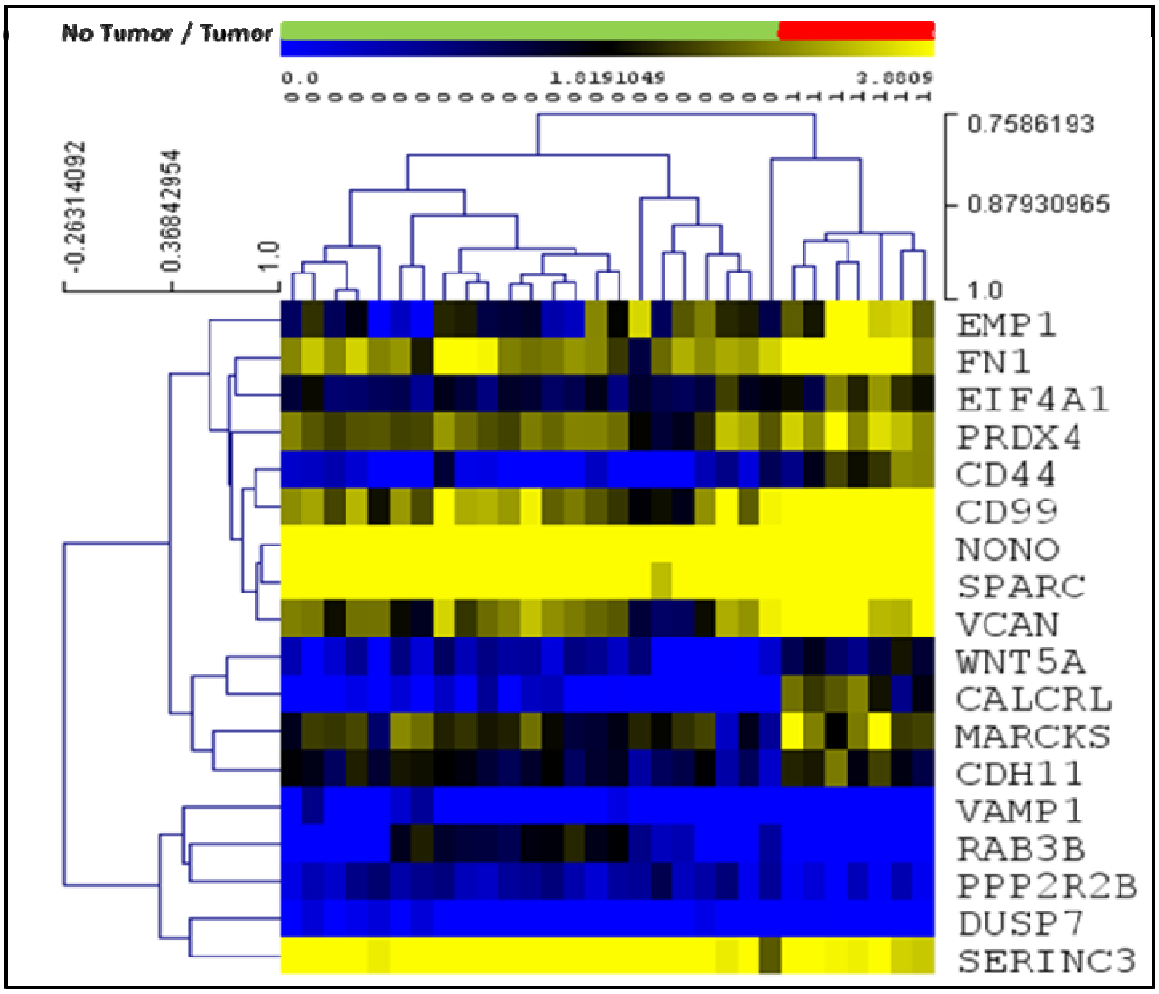

Figure 4-10. Hierarchical clustering of Diffuse Astrocytoma Markov genes using raw expression values of Sun Study Only. No Tumor /Tumor Bar: Green bar areas represent samples without tumors and red bar areas represent tumor cases. Expression: Blue squares represent underexpression; yellow squares represent overexpression.

Table 4-7. Anaplastic Astrocytoma prediction analysis summary. 18 total Markov genes were used in the analysis, except in linear stepwise regression, which used only 11 significant predictor genes in the analysis.

Type of Prediction Analysis	Case Counts (No Tumor/Tumor)	Predictability
Bayesian Network Results using ROC Curve	34/23	1.000 AUC (.000 sig.)
Linear Regression (18 genes together)	34/23	.631 (aR square) (.000 sig.)
Linear Regression (stepwise) (11 gene model)	34/23	.645 (aR square) (.000 sig.)
Logistic Regression (discretized expression)	34/23	96.5% (1.000 sig.)
Logistic Regression (raw expression)	23/19	Error: Perfect fit detected
Cross Validation (discretized expression)	34/23	84.2%
Cross Validation (raw expression)	23/19	100%
Support Vector Machine (SVM)	23/19	100%

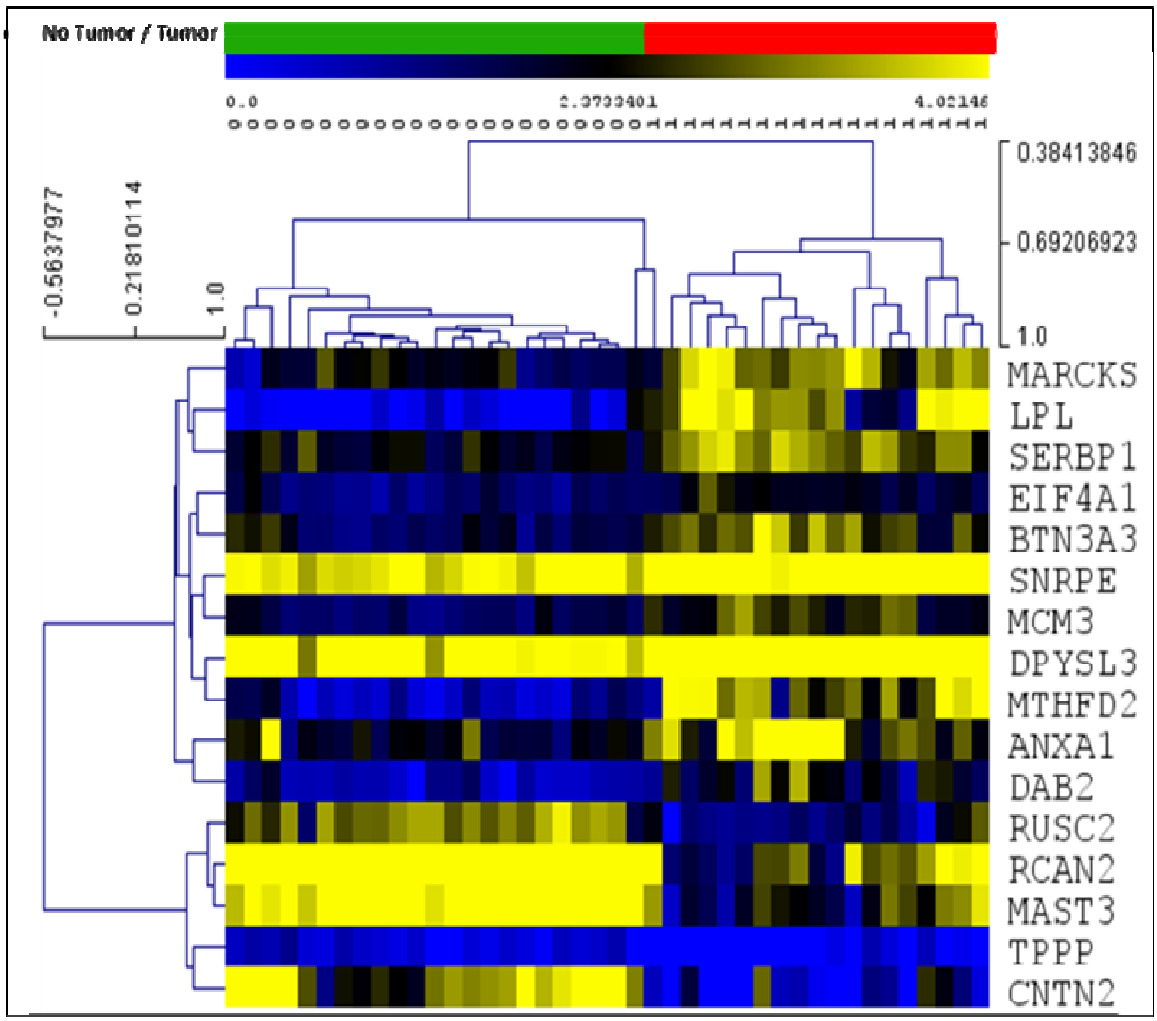

Figure 4-11. Hierarchical clustering of Anaplastic Astrocytoma Markov genes using raw expression values of Sun study only. No Tumor /Tumor Bar: Green bar areas represent samples without tumors and red bar areas represent tumor cases. Expression: Blue squares represent underexpression; Yellow squares represent overexpression.

Normal Tissue vs. Grade IV Astrocytoma: Prediction analysis of the 10 Grade IV Markov genes showed that our set of 10 genes was again able to consistently predict between non-tumor and tumor cases with all analysis (Table 4-8). Please see Appendix Table 4 for results of the biological database and literature search. Research supporting the potential involvement and importance of all 10 genes in development of glioblastoma was found in the literature. Results of hierarchical clustering using expression data from

our Markov genes can be found in Figure 4-12. Assessment of increased lifetime risk of development of development of astrocytoma due to deregulation of our Markov genes showed that differential expression of all 10 of our genes at once increased your lifetime risk of brain tumor development to 85.90%. In contrast, differential expression of two separate sets of 10 genes found outside the Markov blanket in our Bayesian network increased lifetime risk of brain tumor development to 2.61% and 0.98% respectively (Table 4-9).

Results of the IPA and PathJam analysis can be found in Appendix Figures 4 through 6. The investigation using IPA's Path Explorer produced a network of genes showing empirical evidence of interaction among our 10 Glioblastoma Markov blanket genes. IPA Core Analysis of these same Markov genes added genes and molecules such as NFkB, ERK, MAPK, Vegf, growth hormone and collagen to produce a network whose top biological functions are cancer, neurological disease, and cellular movement (Appendix Figure 6). Our analysis of these same Markov genes using PathJam found that three of the 10 genes in particular seemed to be potential 'hubs of activity' and had functions that they shared. These genes, EGFR, COL4A1, and CDK4 all shared the 'pathways to cancer' annotation; and EGFR and COL4A1 were shown to be involved specific cancers such as glioma, melanoma, lung cancer, bladder cancer, and pancreatic cancer. Additionally, COL4A1 and EGFR shared involvement in axon guidance and focal adhesion. For a full list of the biological pathways and gene ontology terms associated with each gene please see Appendix Figure 4.

Table 4-8. Glioblastoma Multiforme prediction analysis summary. 10 total Markov genes were used in the analysis, except in linear stepwise regression, which used only 6 significant predictor genes in the analysis.

Type of Prediction Analysis	Case Counts (No Tumor/Tumor)	Predictability
Bayesian Network Results using ROC Curve	34/23	1.000 AUC (.000 sig.)
Linear Regression (18 genes together)	34/23	.631 (aR square) (.000 sig.)
Linear Regression (stepwise) (11 gene model)	34/23	.645 (aR square) (.000 sig.)
Logistic Regression (discretized expression)	34/23	96.5% (1.000 sig.)
Logistic Regression (raw expression)	23/19	Error: Perfect fit detected
Cross Validation (discretized expression)	34/23	84.2%
Cross Validation (raw expression)	23/19	100%
Support Vector Machine (SVM)	23/19	100%

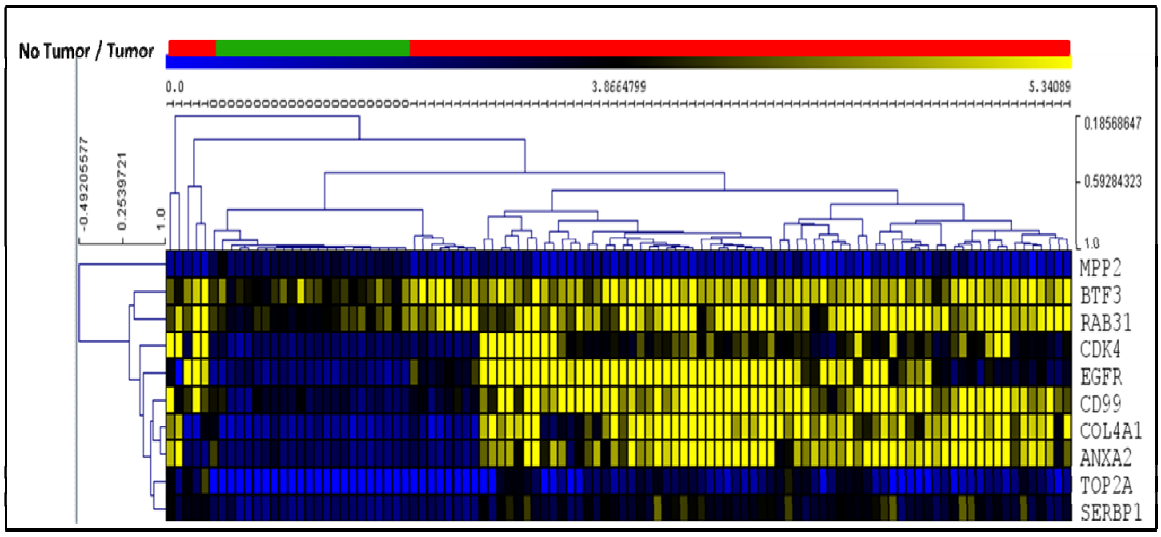

Figure 4-12. Hierarchical clustering of Glioblastoma Multiforme genes using raw expression values of Sun study only. No Tumor /Tumor Bar: Green bar areas represent samples without tumors and red bar areas represent tumor cases. Expression: Darker squares represent underexpression; Lighter squares represent overexpression.

Table 4-9. Risk associated with Markov genes vs. non-Markov genes in Glioblastoma Multiforme. Normal Gene Set Risk represents the SEER calculated 0.61% chance of a person developing a brain tumor in their lifetime.

Gene Set	Risk
Normal Gene Set	0.61
Markov Differentially Expressed (COL4A1, CD99, ANXA2, MPP2, EGFR, CDK4, BTF3, RAB31, TOP2A, SERBP1)	85.90
D-Connected Differentially Expressed (KIF5C, PALLD, S100A10, TPPP, PCNA, FN1, IGFBP2, CDH11, LYPLA1)	2.61
D-Separated Differentially Expressed (PKP4, SYNCRIP, CALCRL, GABRA5, MOBP, PLOD2, WNT5A, C1S, RPS2, FCHO1)	0.98

DISCUSSION

This study produced several major findings including identification of a list of top over- and under-expressed genes among 10 sub-studies on astrocytoma, identification of several genes important to development of both low and high grade astrocytomas, identification of important signaling pathways in astrocytic tumors, and identification of possible mechanisms which explain the genes and pathways identified as important to development of astrocytoma.

Through meta-analysis of 10 sub-studies which compared normal tissue to astrocytomas, a list of 646 genes which were differentially expressed in the majority of these studies was identified. Many of the genes identified through this meta-analysis have in fact been implicated in development of astrocytoma including EGFR (amplification occurs in ~40% of primary glioblastomas [23;24]), HIF-1α, c-Myc, WNT5A, and IDH3A. Enrichment analysis of the 646 genes using FuncAssociate identified several processes associated with these genes, many of which are related to nervous system, developmental, and tumor promoting processes. Ingenuity Pathway Analysis also produced a list of processes that are significantly associated with these genes, including two pathways which have previously been linked to development of astrocytomas [1. 'WNT/beta-Catenin Signaling' (Genes from our set in pathway: CD44, CDH2, DVL3, LRP1, MYC, SOX4, SOX9, SOX13, TCF3, TCF4, TLE3, WNT5A) and 2. 'mTOR Signaling' (Genes: EIF3B, EIF3E, EIF3F, EIF4A1, HIF1A, PRKD1, RHOC, RND2, RND3)] and two pathways associated with brain tumor development [1. 'Glioma Invasiveness Signaling' (Genes: CD44, F2R, ITGAV, MMP9, RHOC, RND2, RND3,

TIMP3, TIMP4) and ‘Glioblastoma Multiforme Signaling' (Genes: CDK6, CDKN1A, EGFR, ITPR2, MYC, RHOC, RND2, RND3, TCF3, WNT5A)].

In order narrow our large set of genes down into a few genes which could be most influential to development of astrocytomas, we performed reverse engineering of our gene list using Bayesian network analysis. Four networks of genes were produced, one for each grade of Astrocytoma.

To investigate the biological mechanisms of our set of significant Grade IV network genes we used biological databases such as The Human Gene Conpendium's GeneCards, PubMed, the Information Hyperlinked over Proteins (iHOP) Database, and the Glioblastoma Multiforme Database (GBMBase). Results of this investigation can be found in Table 4. Much of the biology described in this table was considered in the context of current knowledge on signaling pathways of astrocytoma. Major patterns in these tumors have been described [25], and include components of the Ras-MAPK and PI3K-AKT-mTOR signaling pathways being affected in the plurality (88%; 80 of 91) of malignant gliomas and disruption of the p53 and RB tumor suppressor networks also occurring in a high proportion of glioblastomas: 87% (79 of 91) and 78% (71 of 91), respectively [25].

There are several strengths and limitations involved with our analyses. Several characteristics of microarray expression studies must be considered. First, expression levels of many genes differ among individuals and thus gene expression can be analyzed like other quantitative phenotypes such as height and blood glucose levels. This allowed us to separate each gene into expression categories of over-, median, and under-expression. Expression changes can also reflect many types of alterations significant to

tumor development, including chromosomal translocations and epigenetic alterations. Additionally, several studies have established causal links between differential gene expression and complex disease risk and thus identification of over- and under-expressed genes in tumor tissue compared to normal tissue could provide important clues to the development of tumors. Furthermore, it has also been shown that genes with similar expression patterns form complexes and/or pathways that are part of regulatory circuits that may lead to tumors and other diseases, lending support to the validity of our pathway analyses. Our meta-analysis, which took the top 600 over- and top 600 under-expressed genes from a set of studies, should also have produced the most important differentially expressed genes across all astrocytic tumors. Analysis that shows 143 genes in GBM are expressed on average at 10-fold higher levels than normal tissue confirms that the most highly expressed genes in GBM were considered in our analysis [26].

There are also several limitations of expression values. Foremost are the discrepancies between protein and mRNA levels in studies correlating their expression, a clear sign that interactions outside the classical DNA to mRNA to protein pathway are taking place inside the cell. Additionally, it has been shown that known genes may not necessarily be differentially expressed in diseases due to the ability of mutations in the coding regions of genes and post-translational modifications affecting gene function without affecting its expression level. However, our approach of meta-analysis and focusing on networks of genes rather than single genes may lessen the effect of missing important genes (i.e. while one gene in a pathway may not be expressed, another may). Finally, only looking at mean expression changes of genes could lead to incorrect conclusions about the

involvement of a pathway in a disease condition, and so as suggested by de la Fuente 2010, co-expression of genes should also be considered [27].

Limitations of enrichment analysis in general apply [28] to our analysis, including: a) incomplete annotation databases as a result of only a subset of known genes being functionally annotated; b) annotation databases may not be completely updated with all literature results; c) some annotation assignments may be erroneous, especially those which are electronically inferred; d) singling out the most important processes for genes involved in several biological processes is limited. This can be overcome by looking at the gene in context of other over- and/or under-expressed genes however; and e) annotation bias due to some biological processes being studied in more detail than others (e.g. proliferation).

Another limitation of our approach is that because our set of significant genes was chosen through meta-analysis of micro-array studies that used differing platforms and differing gene totals per study, we were unable to input a set of genes as for our 'total gene universe' in our gene enrichment analyses. This limited us to choosing the entire genome as our universe of comparison genes for the enrichment analyses. However, 4 of the 9 studies contained 18,800+ genes and one other study contained 14,584 genes, making it likely that most of our selected significant genes represent most of the appropriate over- and/or under-expressed genes in astrocytoma.

Reverse network engineering methods have evolved greatly over the past decade, with recent reports lending credibility to their ability to correctly predict biological interactions [29;30]. However, limitations associated with their use must be considered. In particular, static Bayesian networks cannot contain feedback loops, due to the steady

state nature of the data. Thus, a characteristic common to biological systems was not considered in our network. Also, because Bayesian networks model probabilistic dependencies among variables and not causality, we cannot conclusively say that the parents of a node are direct causes of its behavior [4]. A causal link can be inferred however, if the Causal Markov Condition holds true. Simply, this condition states that any node in a Bayesian network is conditionally independent of its non-descendants, given its parents; and, a node is conditionally independent of the entire network, given its Markov blanket. A strength of our approach is the exploration of gene networks in tumors without a priori genetic interaction networks being assumed. This has been mentioned as a limitation of previous work on gene networks in gliomas [31]. Incorporation of biological evidence that directs our Bayesian network search could serve to strengthen our approach in the future however.

Finally, limitations concerning the data used in our study must be considered. For example, our inability to separate pediatric astrocytomas from adult astrocytomas, secondary glioblastomas from secondary glioblastomas, and male vs. female cases does limit the extent to which we can draw conclusions from our data. The possibility that 'a fraction of GBMs designated as primary tumors may follow a sequence of genetic events similar to that of secondary lesions but not come to clinical attention until malignant progression to a GBM has occurred', lessens the concern of dividing types of glioblastomas however. Additionally, our method could be considered non-biased in this respect, as it does not pre-condition results based on priors, thus allowing for a search which may provide key genes across all hypothesized glioblastoma subtypes.

This research was able to identify several genes and pathways that are associated in the development of astrocytoma. Though these molecules could be causally linked to astrocytoma, further detailed analysis is necessary. Experiments involving system perturbations of these genes (e.g. gene knockout experiments) are needed to establish directionality in our network and to provide validity of our findings.

Reference List

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Manuscript 5: Modeling gene-environment interaction in Glioblastoma Multiforme via an integrated bioinformatics approach

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ABSTRACT

Recent developments in bioinformatics, including integration of large amounts of diverse biological and environmental data, allow for investigation of gene-gene and gene-environment interactions. Bioinformatics resources were used to develop hypotheses for investigation of GEI in the development of glioblastoma multiforme (GBM), the most common and aggressive type of human brain tumor. Genes responsive to environmental exposures were identified using the Environmental Genome Project, Comparative Toxicology, and Seattle SNPs databases. These genes were then compared to a curated list of genes altered in GBM. The list of genes responsive to the environment and important to GBM was then further investigated using gene networking tools such as RSpider and Cytoscape. A total of 173 environmentally responsive genes were found to be altered in GBM. The main biological functions of these genes included Signaling by Nerve Growth Factor (NGF), DNA Repair, Integrin Cell Surface Interactions, Biological Oxidations, Apoptosis, Synaptic Transmission, Cell Cycle Checkpoints, and Arachidonic Acid Metabolism. Importantly, NGF, a protein essential to axonal growth and differentiation, and maintenance and survival of its targeted nerve cells, has been implicated in development of several cancers, including GBM. Generation of GEI data relevant to GBM etiology through this bioinformatics method, can provide highly useful information for hypothesis generation.

INTRODUCTION

The etiology of brain tumors is mainly unknown. Aside from extremely rare genetic conditions, such as neurofibromatosis and tuberous sclerosis, the only unequivocally identified risk factor is exposure to ionizing radiation, and this explains only a very small fraction of cases. Research over the past few decades does point to the relevancy of a few environmental exposures in brain tumor etiology however. A number of these exposures, including pesticides, N-nitroso compounds, non-ionizing radiation from long-term cell phone use, and infection with polyomavirus, have been positively linked to brain tumors, although findings are inconclusive. Several work-related exposures have been convincingly linked to brain tumors however, including nuclear workers exposed to radiation, pathologists and embalmers exposed to formaldehyde, plastic workers exposed to vinyl chloride, and textile and plastic workers exposed to acrylonitrile [1].

Very few epidemiological studies to date have investigated gene-environment interactions (GEI) in relation to brain tumor development. To date, two studies have assessed GEI in adult glioma. A case-control study on lead exposure, the lead toxicity associated gene δ-aminolevulinic acid dehydratase (ALAD), and risk of brain tumors found increased risk of meningioma with occupational lead exposure. Risk of meningioma, a tumor present mainly in later life, was markedly increased in individuals with the ALAD2 variant allele, for whom risks increased in a dose dependent fashion from 1.1 (0.3-4.5) to 5.6 (0.7-45.5) and 12.8 (1.4-120.8) compared to unexposed persons. Risk for glioma was not associated with occupational lead exposure [2]. A second adult GEI study on glutathione transferases (GST) polymorphisms, cigarette smoke exposure, and development of adult glioma failed to find any significant GEI [3]. While few GEI

studies in glioblastoma exist, computational modeling of 600 adults with glioma has shown that a polygenic-environment-interactive model best explained the pattern of occurrence of brain tumors [4].

Recent developments in bioinformatics such as tools that allow for the assessment of pathway and gene relationships, text mining of published literature, and integration of large amounts of diverse biological and environmental data allow for hypothesis driven investigations of gene-gene and gene-environment interactions. Methods that exploit these tools have been applied to modeling of gene-environment interactions in depression and alcohol use [5], and bipolar disorder and its interaction with both tobacco use [6] and lithium treatment [7]. Integration of toxicological and pharmacological databases such as the Comparative Toxicological Database (CTD) [8] and Environmental Genome Projects (EGP) [9] with data on genetic alterations has also proven useful in developing hypothesis for research into GEI related diseases [10;11]. We chose to use these environmental bioinformatics resources to develop hypotheses for investigation of gene-environment interactions in the development of glioblastoma multiforme (GBM), the most common and aggressive type of human brain tumor.

BACKGROUND ON GENE ALTERATIONS AND ENVIRONMENTAL EXPOSURES

Alterations in genes that interact with environmental influences are thought to play a large part in this variable response and its subsequent risk for disease. The genetic alterations that result from environmental exposures can occur at several levels, including in single nucleotides, small stretches of DNA (microsatellites), whole genes, structural

components of chromosomes, or complete chromosomes. In principle, complex diseases such as cancer might be more susceptible to the lower levels, or 'softer' forms, of variation such as variation in noncoding sequences and copy number, which alter gene dose without abolishing gene function. Thus, variability in responsiveness of these altered genes to environmental influences may be directly related to disease susceptibility.

Among rearrangements frequently involved in tumor progression is DNA amplification, which drastically modifies gene dosage in cancer cells [12;13] and has been linked to glioma development through growth factors such as EGFR [14]. Amplification of a gene refers to existence of at least five copies of a DNA segment (or deletion of a segment for loss of copies) that is less than 20 megabases in length, whereas a low (<5) copy number increase or decrease (also referred to as gain or loss), is directed towards intact chromosomes, entire chromosome arms, or larger chromosomal regions with only one intra-chromosomal breakpoint. Mechanisms of gene amplification have been reviewed in detail by Myllykangas and Knuutila [15]. In this review, the authors cite a 0.440 amplification frequency per case of GBM (268 amplifications in 609 reviewed cases) vs. 0.047 amplification per case of astrocytoma (43 amplifications in 920 reviewed cases). This difference may be due to the more progressed solid tumor nature of GBM compared to astrocytoma. For the purposes of this paper, amplification or deletion, and gain or loss of gene copy number will be referred to as copy number variation (CNV).

A recent study of gene expression variation sought to determine the fraction of gene expression 'traits' associated with either SNPs or CNVs in complex phenotypes. SNP

genotypes and CNV measurements were associated with 83% and 18% of those gene expression traits for which statistically significant associations were found [16]. While CNVs accounted for a small role, this study may still underestimate the role of CNVs, given the greater completeness and accuracy with which SNPs can be queried at present. Regardless, the import of both SNPs and CNVs to variation in phenotype and disease susceptibility is becoming clearer. Recent research is also finding that, surprisingly, most of the SNP variations associated with disease are not in the region of DNA that codes for a protein. Instead, they are usually in the large non-coding regions on the chromosome between genes, or in the intron sequences that are edited out of the DNA sequence when proteins are processed. These are presumably sequences of DNA that control other genes, but usually, their protein function is not known [17].

Evidence of environmental exposures causing CNV is still developing, while the environments ability to cause SNP mutations is quite established [18;19]. Although evidence suggests that most common copy number variants are inherited and therefore caused by ancestral structural mutations [20], there is growing evidence that many or most normal and sporadic, nonrecurring CNVs, which account for the majority of disease-associated CNVs in humans and those in cancers, arise via mechanisms coupled to aberrant DNA replication and/or non-homologous repair of DNA damage [21]. This suggests an unexpected mitotic, rather than meiotic, cell origin for many CNVs and has a number of important implications for the role of environmental exposures in their formation. This evidence has led some to hypothesize that the two types of environmental agents most likely to be associated with CNV formation are: 1) agents that lead to replication stress, which might lead to CNVs through secondary breakage or

replicative template switching, and 2) agents that directly induce DNA double-strand breaks (DNA DSBs), which might lead to CNVs through inappropriate joining of broken ends. Moreover, the ability of environmental agents to cause CNVs and induce epigenetic transgenerational effects in the sperm epigenome separate from methylation effects has recently been established [22].

In an attempt to identify genes potentially important in environmentally related alterations in GBM, we apply a method similar to the bioinformatics methods of Herbert et al. in their research on identifying potential environmentally-related autism genes [23]. We have modified and extended this method to include a search for specific gene-chemical observations important for our 'environmentally responsive genes', and develop a gene network using these genes and alterations in GBM.

METHODS

A summary of the methodology and workflow outline for this research can be seen in Figure 5-1. Briefly, significant copy number alterations and SNP mutations in GBM were curated from published literature and the COSMIC database [24]. Gene lists from six studies on GBM were used for this curation (Table 5-1) [24-30]. Design of study, analysis platform, sample size and region of genome analyzed were criteria used to select studies to include in our alteration results.

Gene lists from three environmental databases were used for the compilation of possible environmentally important genes in GBM. These databases were:

a. The Environmental Genome Project (EGP) located at: http://egp.gs.washington.edu/finished_genes.html. The National Institute of Environmental Health Sciences (NIEHS) EGP is a database which identifies and genotypes genes with functions related to cell cycle, cell division, cell signaling, cell structure, DNA repair, gene expression, homeostasis, metabolism, immune and inflammatory response, hormone metabolism, nutrition, oxidative metabolism and stress, membrane pumps and/or drug resistance, and signal transduction [9]. All genes from this database were included in our environmentally responsive genes list.

b. Seattle SNPs located at: http://pga.gs.washington.edu/finished_genes.html. The Seattle SNP database is a National Heart Lung and Blood Institute (NHLBI) funded project focused on identifying, genotyping, and modeling associations between SNPs in candidate genes and pathways that underlie inflammatory responses in humans. All genes from this database were included in our inflammatory genes list.

c. The Comparative Toxicogenomics Database (CTD) located at: http://www.mdibl.org/research/ctd.shtml. The CTD, operated by the Mount Desert Island Biological Laboratory (MDIBL) with support from the National Institutes of Health (NIH), collects gene-environment-disease interactions information from published literature. It is searchable by several methods including disease, gene, chemicals and gene-chemical interactions. We searched GBM to obtain a list of genes found to be modulated by chemical exposures.

Table 5-1. List of Glioblastoma studies used for compilation of alterations list.

Alteration Type	Study Author and Year							Total
	TCGA 2008 [25]	Korshunov 2006 [28]	Freire 2008 [27]	Margareto 2009 [29]	Parson 2008 [30]	Carter 2009 [26]	COSMIC [24]	
Amplifications	15	35	98	x	69	x	x	217
Deletions	12	x	44	x	77	x	x	350
Copy Gains	158	34	x	22	x	x	x	214
Copy Losses	126	34	x	1	x	x	x	161
SNP Mutated Genes	222	x	x	x	42	17	2129	2410
Driver Mutations	x	x	x	x	42	17	x	59

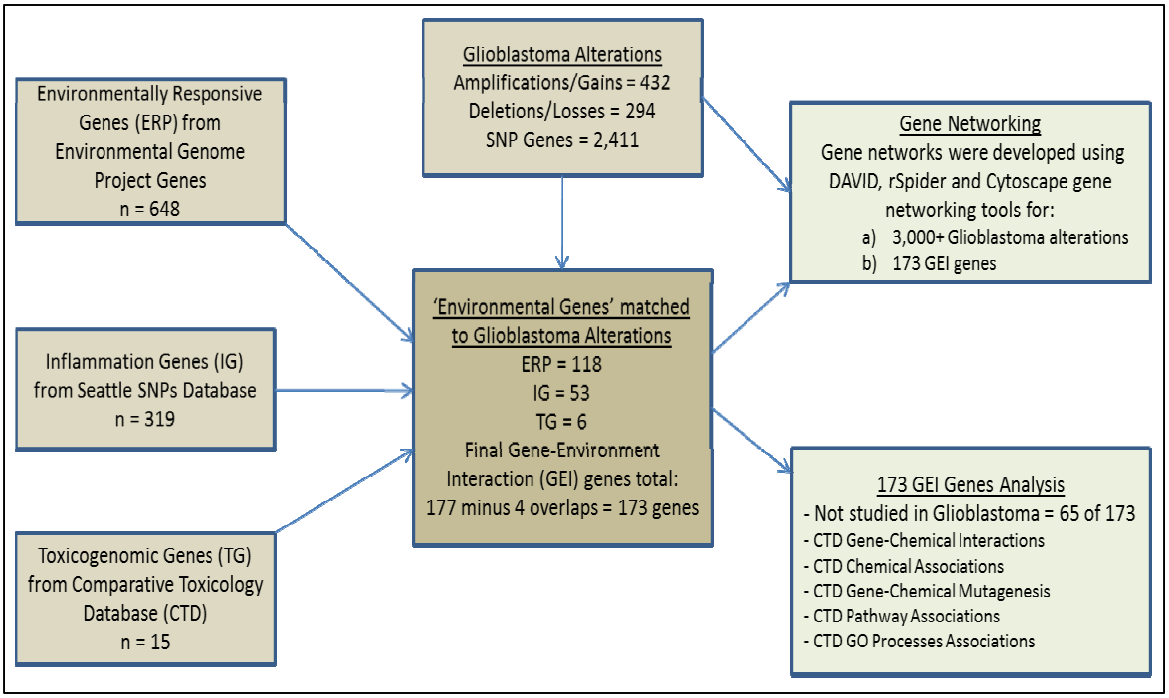

Figure 5-1. Bioinformatics methodology. This flowchart illustrates steps outlined in the methods section by which we identified interactions between three environmentally relevant genomics databases and Glioblastoma alterations.

The gene lists from the glioblastoma alterations search and our environmental genes database search were then inputted into the GeneVenn program [31] to assess their overlap. Gene overlaps between the three environmental gene databases and our

glioblastoma alterations list were determined. Overlapping genes, referred to as the 'GEI gene list' from this point forward, were used for further analysis including:

1. Pubmatrix (http://pubmatrix.grc.nia.nih.gov), an NIH tool which allows cross referencing of gene lists with search terms, was used to assess whether overlapping genes had been previously studied in relation to glioblastoma.
2. The GEI gene list was searched in the Comparative Toxicology Database for relevant gene-chemical interactions and chemical associations.
3. Both the GEI gene list and the entire Glioblastoma alterations list were subjected to gene networking analysis using the bioinformatics tools RSpider [32] and DAVID [33]. RSpider results were visualized using Cytoscape [34].

RESULTS

A total of 217 amplified genes, 214 copy number gain genes, 350 deleted genes, 161 copy number loss genes, and 2410 SNP mutated genes were found in the 6 total studies and 1 database we searched (Table 5-1). According to the COSMIC database, 2,129 genes have been found to be mutated in glioblastoma, while 15,733 genes have been sequenced in glioblastoma where no mutation has been found. The top 20 mutated GBM genes in our COSMIC search, with the percentage of mutated genes per tumors analyzed, can be seen in Figure 5-2. In a GeneVenn comparison of our alterations lists, 67 amplified/gain genes and 45 deleted/loss genes were shown to also be mutated in our gene sets, while 6 amplified/gain genes also have copy number deletion or loss in GBM (Figure 5-3).

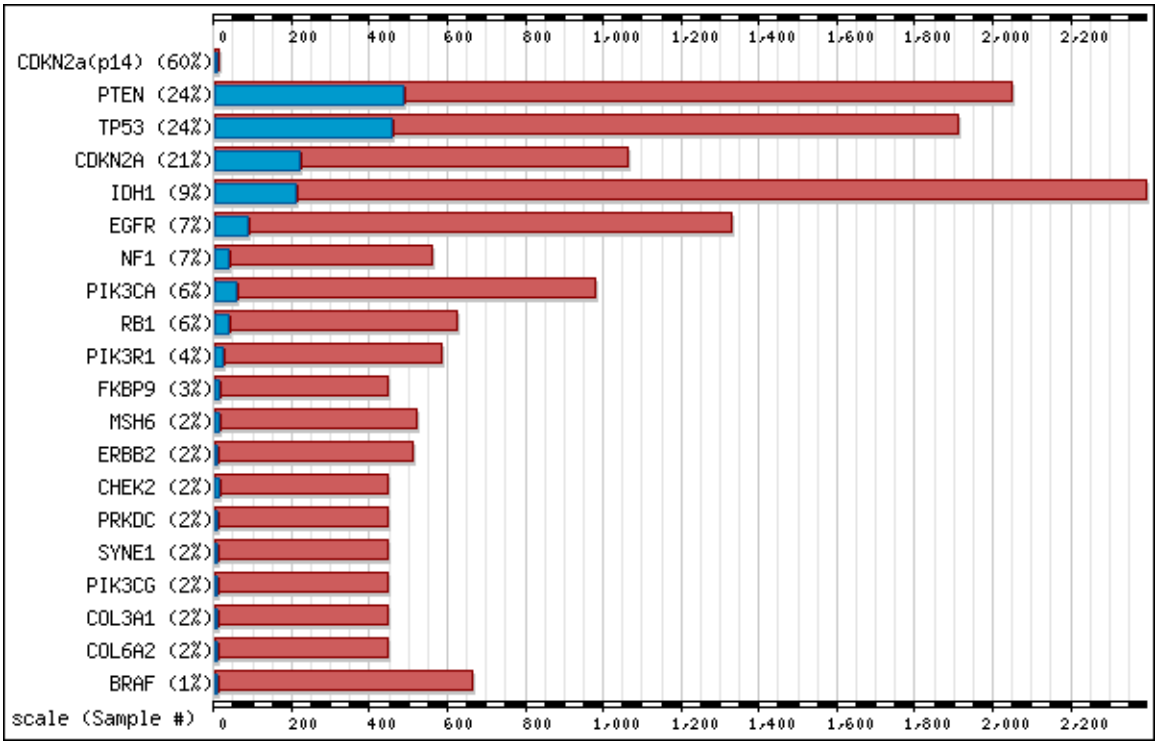

Figure 5-2. Top 20 genes mutated in Glioblastoma according to COSMIC database search. Blue bars represent tissue samples with mutations; Red bars represent all tissue samples.

The search of the environmental databases returned 648 Environmental Genome Project (EGP) genes (environmentally responsive genes), 319 Seattle SNP (SSNP) genes (inflammatory genes), and 15 Comparative Toxicology Database (CTD) genes (toxicogenomic genes). Very little overlap existed between these gene sets (4 between EGP and CTD, 3 between SSNP and CTD, 8 between EGP and SSNP, and 1 between all three databases). Overlapping of our final list of GBM alterations with the environmental genes found 173 genes that have an environmental exposure link and are altered in glioblastoma. These genes are listed in Appendix Table 5 with their gene symbol, gene name, and whether they have ever been previously studied in glioblastoma. Of these 173 genes, a Pubmatrix search found that 65 of our overlapping genes had not been previously assessed in glioblastoma research. The list of 173 genes potentially important

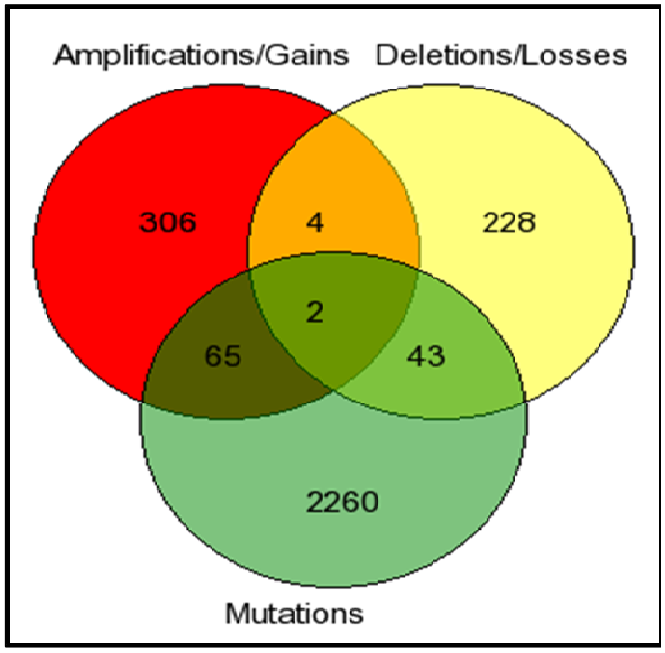

Figure 5-3. Overlap between our Glioblastoma amplified/gain genes, deleted/loss genes, and SNP mutated genes.

in GEI in glioblastoma formation was then subjected to analysis in the Comparative Toxicology Database. Results indicate substantial potential for chemical interaction with these set of genes including interactions with chemicals such as arsenic and pesticides such as chlorpyrifos for gene ABCB1, benzene and bisphenol A for EGFR, and estradiol for MDM2 and NCOA1. In total, our list of 173 genes produced 30,983 gene-chemical interactions and showed 13,779 chemical associations. A specific search for chemical-gene interactions producing mutagenesis in our genes found 68 results. A list of these genes accompanied their interacting chemical and a brief description of their interaction effect can be found in Appendix Table 6.

The final section of our analysis involved the development of a gene network for our 173 potentially environmentally important GBM genes and a second network for our entire list of over 3,000 GBM alterations. These networks were produced using R spider, a web-based tool for that develops biological networks from user-inputted gene lists by querying the core pathways and reactions contained in the Reactome and KEGG databases. These databases, which use data extracted from biomedical experiments and literature, are currently two of the most complete and best-curated pathway databases.

RSpider and Cytoscape analysis of our list of 173 GEI GBM genes produced a network with main biological functions of Signaling by Nerve Growth Factor (NGF), DNA Repair, Integrin Cell Surface Interactions, Biological Oxidations, Apoptosis, HIV Infection, Synaptic Transmission, Metabolism of Amino Acids and Derivatives, Signaling by Insulin Receptor, Cell Cycle Checkpoints, and Arachidonic Acid Metabolism. The RSpider/Cytoscape network for the list of GBM alterations again found Signaling by NGF to be the top biological function. Other functions for this gene list were Integrin Cell Surface Interactions, Cell Cycle Checkpoints, Signaling by PDGF, Synaptic Transmission, Signaling by Rho GTPases, Signaling by Insulin Receptor, DNA Repair, Apoptosis, Inositol Phosphate Metabolism, and Metabolism of Carbohydrates. Genes from our list of 173 GEI genes involved in NGF signaling, according to our search of the CTD database include AKT2, BRAF, CASP9, MDM2, NRAS, PTEN, and STAT3.

Analysis by DAVID of the 173 GEI GBM genes found 35 KEGG ontologies significantly represented in the gene list (Table 5-2). The top ontology was 'Pathways in cancer' which contained 42 of the 173 GEI genes. The ontology 'Glioma' was also significant as 13 of the 173 GEI are known glioma genes according to the KEGG database. The top KEGG ontology for the Glioblastoma alterations list according to DAVID analyses was also 'Pathways in Cancer', as 123 genes from the list were in this ontology. A total of 62 KEGG ontologies were significantly represented in this list of over 3,000 mutated genes. Gene ontology results for this list of alterations provide a broad overall picture of pathways potentially affected by or effecting Glioblastoma development. Representations of the 'Pathways in Cancer' and 'Glioma' KEGG

pathways/ontologies with genes contained in the GEI gene list and GBM gene list highlighted are presented in the Appendix (Appendix Figures 7-10).

DISCUSSION

Our review of current research on GBM found very few studies that assessed the potential role of gene-environment interaction in their development. Several plausible environmental exposures have been linked to their development however, and therefore the possible biological mechanisms involved need more investigation. Most research on GBM biology to date has focused on a few mechanisms and pathways in their development, including those promoting cell cycle dysregulation and enhanced glioma cell proliferation. These mechanisms include the Rb pathway, the p53 pathway, mitogenic signaling pathways (MAPK, PI3K, PTEN, AKT) and receptor tyrosine kinase signaling molecules (EGFR and PDGFR) [35]. Our bioinformatics investigation found 173 genes with the potential to be involved in GEI in GBM. 65 of these environmentally responsive genes have not been investigated with regards to Glioblastoma, and several of them were shown to have significant potential for response to chemicals and subsequent disease related actions. For example, Chlorpyrifos, an organophosphate insecticide associated with development of neurological disorders [36;37], was shown to bind to ABCB1 gene in our CTD search. While this gene has been studied in Glioblastoma treatment [38], to our knowledge, its potential for involvement in development of GBM through GEI has not been assessed. In fact, 174 of our 176 Glioblastoma altered genes with GEI potential had evidence of gene-chemical relationships in our CTD search, including many with mutagenic relationships (Appendix Table 6). We have shown that

Table 5-2. KEGG Gene Ontology Results produced by DAVID analysis of 173 GEI Glioblastoma gene list.

KEGG Gene Ontology	Gene Count	% of Genes in Ontology	P-Value	Genes in KEGG Gene Ontology Category
hsa05200:Pathways in cancer	42	24.4186	1.38E-20	FGFR2, FGFR1, FGFR3, PTGS2, ERBB2, MLH1, CDH1, PTEN, MMP2, SHH, CTNNB1, ARNT, IGF1R, CDKN2A, CDKN2B, CASP9, BCL2, CSF3R, EGF, MYC, FGF2, AKT2, EGFR, MSH6, IL6, BRAF, MSH2, TGFBR2, TP53, BRCA2, CDK6, RB1, HGF, STAT1, CDK4, STAT3, NRAS, BAX, MDM2, MAPK9, PTCH2, ABL1
hsa05218:Melanoma	17	9.883721	2.78E-12	EGFR, FGFR1, BRAF, TP53, CDK6, CDH1, RB1, HGF, CDK4, PTEN, NRAS, IGF1R, CDKN2A, MDM2, EGF, FGF2, AKT2
hsa05219:Bladder cancer	14	8.139535	4.20E-12	EGFR, FGFR3, BRAF, ERBB2, TP53, CDH1, RB1, CDK4, MMP2, NRAS, CDKN2A, MDM2, EGF, MYC
hsa05212:Pancreatic cancer	16	9.302326	4.73E-11	EGFR, BRAF, ERBB2, TGFBR2, TP53, BRCA2, CDK6, RB1, STAT1, CDK4, STAT3, CDKN2A, CASP9, MAPK9, EGF, AKT2
hsa05215:Prostate cancer	17	9.883721	1.08E-10	EGFR, FGFR2, FGFR1, BRAF, ERBB2, TP53, IGF2, RB1, PTEN, CTNNB1, NRAS, IGF1R, CASP9, BCL2, MDM2, EGF, AKT2
hsa04115:p53 signaling pathway	15	8.72093	2.59E-10	TP53, CDK6, CHEK1, CHEK2, CDK4, PTEN, ATM, CDKN2A, CASP9, CCND2, BAX, SERPINE1, GADD45G, MDM2, MDM4
hsa05213:Endometrial cancer	13	7.55814	1.31E-09	EGFR, BRAF, ERBB2, TP53, MLH1, CDH1, PTEN, CTNNB1, NRAS, CASP9, EGF, MYC, AKT2
hsa04110:Cell cycle	18	10.46512	2.54E-09	CDC7, E2F4, TP53, PRKDC, CDK6, CHEK1, RB1, CHEK2, CDK4, ATM, CDKN2A, CDKN2B, CCND2, CDKN2C, GADD45G, MDM2, ABL1, MYC
hsa05210:Colorectal cancer	15	8.72093	4.89E-09	EGFR, MSH6, BRAF, MSH2, TGFBR2, TP53, MLH1, CTNNB1, IGF1R, CASP9, BAX, BCL2, MAPK9, MYC, AKT2
hsa05214:Glioma	13	7.55814	1.37E-08	EGFR, BRAF, TP53, CDK6, RB1, CDK4, PTEN, NRAS, IGF1R, CDKN2A, MDM2, EGF, AKT2

KEGG Gene Ontology	Gene Count	% of Genes in Ontology	P-Value	Genes in KEGG Gene Ontology Category
hsa05223:Non-small cell lung cancer	12	6.976744	2.71E-08	EGFR, NRAS, CDKN2A, CASP9, BRAF, ERBB2, TP53, CDK6, RB1, EGF, CDK4, AKT2
hsa05220:Chronic myeloid leukemia	12	6.976744	9.21E-07	NRAS, CDKN2A, BRAF, TGFBR2, TP53, MDM2, CDK6, RB1, ABL1, CDK4, MYC, AKT2
hsa05222:Small cell lung cancer	11	6.395349	2.01E-05	PTGS2, CASP9, CDKN2B, BCL2, TP53, CDK6, RB1, CDK4, MYC, PTEN, AKT2
hsa04010:MAPK signaling pathway	19	11.04651	3.50E-05	EGFR, FGFR2, FGFR1, FGFR3, BRAF, NF1, TGFBR2, TP53, DDIT3, NRAS, TNFRSF1A, MAP3K8, GADD45G, MAPK9, RAP1B, EGF, FGF2, MYC, AKT2
hsa03430:Mismatch repair	6	3.488372	1.49E-04	MSH6, RFC2, MSH2, POLD1, MLH1, PMS2
hsa04012:ErbB signaling pathway	10	5.813953	1.61E-04	EGFR, NRAS, BRAF, ERBB2, MAPK9, EGF, ABL1, ABL2, MYC, AKT2
hsa04060:Cytokine-cytokine receptor interaction	17	9.883721	3.14E-04	EGFR, IL3, IL6, TNFRSF25, TGFBR2, TNFRSF8, HGF, IFNAR1, TNFRSF1A, TNFRSF9, IFNA1, TNFRSF1B, TNFRSF11B, IL17B, IL4R, CSF3R, EGF
hsa05216:Thyroid cancer	6	3.488372	4.70E-04	NRAS, BRAF, TP53, CDH1, MYC, CTNNB1
hsa04510:Focal adhesion	14	8.139535	6.96E-04	EGFR, BRAF, ERBB2, HGF, PTEN, CTNNB1, VWF, IGF1R, CCND2, BCL2, MAPK9, RAP1B, EGF, AKT2
hsa04722:Neurotrophin signaling pathway	10	5.813953	0.002176	NRAS, BRAF, BCL2, BAX, TP53, MAPK9, RAP1B, ABL1, CSK, AKT2
hsa04630:Jak-STAT signaling pathway	11	6.395349	0.002988	IL3, IFNA1, IL6, CCND2, IL4R, CSF3R, STAT1, MYC, STAT3, IFNAR1, AKT2
hsa04210:Apoptosis	8	4.651163	0.003823	TNFRSF1A, IL3, CASP9, BCL2, BAX, TP53, ATM, AKT2
hsa05014:Amyotrophic lateral sclerosis (ALS)	6	3.488372	0.007297	TNFRSF1A, TNFRSF1B, CASP9, BCL2, BAX, TP53
hsa04520:Adherens junction	7	4.069767	0.00853	EGFR, IGF1R, FGFR1, ERBB2, TGFBR2, CDH1, CTNNB1

KEGG Gene Ontology	Gene Count	% of Genes in Ontology	P-Value	Genes in KEGG Gene Ontology Category
hsa04620:Toll-like receptor signaling pathway	8	4.651163	0.008628	IKBKE, IFNA1, IL6, MAP3K8, MAPK9, STAT1, IFNAR1, AKT2
hsa03420:Nucleotide excision repair	5	2.906977	0.018309	ERCC5, RFC2, DDB1, POLD1, POLE
hsa04920:Adipocytokine signaling pathway	6	3.488372	0.019018	TNFRSF1A, PPARA, TNFRSF1B, MAPK9, STAT3, AKT2
hsa04610:Complement and coagulation cascades	6	3.488372	0.021341	F13B, VWF, F3, F13A1, SERPINE1, SERPING1
hsa05211:Renal cell carcinoma	6	3.488372	0.022569	NRAS, BRAF, RAP1B, HGF, ARNT, AKT2
hsa04810:Regulation of actin cytoskeleton	11	6.395349	0.026801	EGFR, FGFR2, FGFR1, NRAS, ITGAL, FGFR3, BRAF, IGF2, EGF, CSK, FGF2
hsa05221:Acute myeloid leukemia	5	2.906977	0.044768	NRAS, BRAF, MYC, STAT3, AKT2
hsa04914:Progesterone-mediated oocyte maturation	6	3.488372	0.048636	PGR, IGF1R, BRAF, MAPK9, IGF2, AKT2
hsa00980:Metabolism of xenobiotics by cytochrome P450	5	2.906977	0.049693	CYP3A4, CYP1B1, CYP2C19, CYP2E1, UGT1A1
hsa04370:VEGF signaling pathway	5	2.906977	0.095568	NRAS, PTGS2, CASP9, NOS3, AKT2

many of these genes have not been studied in Glioblastoma, and therefore their potential for involvement in GBM should be assessed in future investigations into the etiology of GBM.

Through gene network and gene ontology analysis, we were able to obtain significant biological functions involved in our set of GEI and GBM alteration genes lists. The top function in both gene sets involved NGF, a small secreted protein important in axonal growth and integral in the differentiation, maintenance and survival of its targeted nerve cells. NGF has been implicated in several cancers including liver cancer [39], oral cancer [40], breast cancer [41], and several brain cancers [42-44], including glioblastoma [45;46]. The survival and proliferation of breast cancer cells are in fact, strongly stimulated by NGF [47], and NGF, through direction of downstream signaling of PI3K, ERK, and VEGF, may be an important stimulator of breast cancer angiogenesis [48]. Furthermore, the potential importance of NGF on the development of tumors through shifting proapoptotic signals in cancer cells has been shown in GBM cells [45]. Importantly, environmental factors such as PCBs are believed to have the ability to influence the NGF neurotrophic system [49]. One mechanism for the influence of NGF to environmental factors may be through Nrf2, a redox-sensitive transcription factor that has been shown to direct expression of cytoprotective phase 2 genes such as thioredoxin reductase 1 (TXNRD1) in GBM cells [50]. There are obvious limitations to this type of bioinformatics analyses of course. While this analysis provides numerous hypotheses for potential GEI interactions, it can only suggest possibilities, and therefore further research in a lab setting is necessary to validate their involvement in GBM. Another limitation is the nonrandom choice of our set of GBM alterations. While we did choose studies and

databases we felt would provide a comprehensive set of alterations, we did not assess the entire set of literature on GBM alterations, and therefore may have missed some potential alterations in our analysis. Furthermore, we have not included epigenetic genes in our analysis and therefore may have missed other potential GEI pathways to GBM through these mechanisms. While these are shortcomings of this type of research, the clear benefit of this study in particular, is the production of a list of genes with potential to contribute to GBM that have not previously been studied. Furthermore, generation of GEI data relevant to GBM etiology through this bioinformatics method provides highly use information for hypothesis generation.

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APPENDIX

Appendix Table 1. Significantly over-expressed gene list from Oncomine meta-analysis of normal vs. astrocytoma studies. *Genes are ranked by significance in Oncomine. **Genes were considered significant in the meta-analysis, and chosen for further analysis, if they were found in at least in at least 7 of 10 meta-analyzed studies.

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
1	SYNCRIP	9	**187**	PLAU	10
2	RAB31	10	**188**	HLA-DRB1	10
3	HLA-G	8	**189**	SERPING1	10
4	FN1	10	**190**	RB1	8
5	HLA-J	7	**191**	NFKB1A	10
6	SOX4	10	**192**	PTTG1IP	10
7	C1R	10	**193**	RPL12	9
8	SNRPG	9	**194**	CXCR4	9
9	NAMPT	9	**195**	FYB	10
10	TIMP3	10	**196**	ITGAV	10
11	GNB2L1	10	**197**	ISG15	10
12	IGFBP7	9	**198**	GALNT1	10
13	HIF1A	10	**199**	SEC11A	8
14	RPLP0	9	**200**	EMP3	10
15	ANXA2	10	**201**	IFI44	8
16	LPL	10	**202**	CAV1	10
17	ID3	7	**203**	RIT1	10
18	PRPF40A	9	**204**	ATP6V0E1	9
19	MARCKS	10	**205**	RBMS1	9
20	P4HB	10	**206**	SMAD1	10
21	PALLD	8	**207**	LPCAT1	8
22	COL4A1	10	**208**	FOXD1	9
23	PRDX4	10	**209**	EIF4A3	10
24	SERBP1	9	**210**	AEBP1	10
25	IFI16	10	**211**	MYCBP	10
26	PROS1	10	**212**	DDX39	8
27	DPYSL3	10	**213**	CTSO	10
28	IDH1	8	**214**	IL13RA1	10
29	DYNLT1	9	**215**	CFI	9
30	NONO	10	**216**	TFPI	10
31	SOD2	10	**217**	G3BP1	10
32	SPARC	10	**218**	DAG1	10
33	TIMP4	10	**219**	CKS2	10
34	DBI	9	**220**	PABPC1	10
35	CD151	10	**221**	AK2	9

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
36	SNRPE	9	**222**	PTPN12	10
37	LYPLA1	9	**223**	ILF2	10
38	ZFP36L2	10	**224**	SP3	10
39	HLA-F	10	**225**	SSR4	10
40	HLA-B	8	**226**	SOX6	7
41	PTBP1	9	**227**	LAPTM4B	9
42	PCNA	10	**228**	CDKN2C	9
43	COL1A2	10	**229**	F13A1	10
44	BTF3	9	**230**	SP110	7
45	WNT5A	10	**231**	TOP1	10
46	CCND2	10	**232**	ANXA5	10
47	CD44	10	**233**	MUC1	10
48	EIF4A1	10	**234**	VAT1	10
49	CSDA	10	**235**	UBE2J1	9
50	PDLIM5	9	**236**	KCTD12	7
51	HLA-E	9	**237**	INPPL1	10
52	NNMT	10	**238**	TAP1	10
53	CD99	10	**239**	STAT1	10
54	IGFBP5	10	**240**	GBP1	10
55	ANXA1	10	**241**	PHLDA1	10
56	CALCRL	10	**242**	COPB2	10
57	IGFBP2	9	**243**	DECR1	10
58	EMP1	8	**244**	COL4A2	10
59	BTG1	10	**245**	SNRNP200	8
60	SRPX	10	**246**	SOX9	10
61	SSR2	9	**247**	KIF14	10
62	PRRX1	8	**248**	ACBD3	9
63	TRAF3IP2	9	**249**	DARS	10
64	CDK4	10	**250**	TAPBP	9
65	EGFR	10	**251**	LYN	10
66	TOP2A	10	**252**	CHI3L2	10
67	PTPRZ1	10	**253**	BCL6	10
68	SAT1	10	**254**	ILF3	10
69	ABCC3	9	**255**	LMNB1	9
70	WEE1	10	**256**	TGIF1	10
71	TAGN2	9	**257**	NID1	10
72	S100A10	10	**258**	PPP4C	10
73	MCM3	10	**259**	CANX	10
74	STAT3	10	**260**	NMI	10
75	RPS2	9	**261**	COL1A1	7

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
76	BTN3A3	9	**262**	SEC61G	9
77	MTHFD2	10	**263**	TRIO	10
78	RPS19	10	**264**	DNALI1	9
79	VCAN	10	**265**	F2R	10
80	IRAK1	10	**266**	PSMB9	9
81	CDH11	10	**267**	C1orf61	10
82	RPL18A	9	**268**	CEBPD	9
83	DAB2	10	**269**	SLC2A5	10
84	TMX1	9	**270**	TCF4	10
85	PSMB8	7	**271**	LSM7	8
86	HLA-A	10	**272**	EDNRA	10
87	CD63	8	**273**	PLXNB2	8
88	HSD17B10	10	**274**	SSR1	10
89	DAP	10	**275**	SCP2	10
90	UBE2L6	9	**276**	SUPR1	9
91	DFNA5	7	**277**	PXDN	10
92	TGFB1	10	**278**	OSMR	9
93	YBX1	8	**279**	RNASE6	10
94	C1S	10	**280**	IL1RAP	9
95	FCGBP	10	**281**	GLB1	10
96	PYGL	10	**282**	SON	10
97	SHMT2	10	**283**	LIMS1	10
98	PLOD2	10	**284**	NEDD4	10
99	ODC1	10	**285**	ZNF22	10
100	VIM	10	**286**	WTAP	10
101	PSMA2	10	**287**	RPL29	10
102	LTF	7	**288**	EIF3B	10
103	IGFBP3	10	**289**	TM9SF1	10
104	ABCA1	8	**290**	EIF3E	10
105	WDR1	8	**291**	CD14	10
106	NCK1	10	**292**	TNX8	7
107	B2M	10	**293**	ENTPD1	10
108	KDELR2	10	**294**	RBBP4	10
109	CTSC	10	**295**	GPR56	8
110	GNAS	10	**296**	STAB1	8
111	CHI3L1	10	**297**	BUD31	10
112	SFRS3	10	**298**	DTYMK	10
113	RAB13	8	**299**	LRP10	8
114	ADAM9	10	**300**	TRIB2	10
115	TNC	10	**301**	GNA12	9

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
116	HNMT	9	**302**	RBMX	10
117	CALD1	10	**303**	HEBP2	9
118	APOC1	10	**304**	SFRS1	9
119	PSME2	10	**305**	ARHGDIB	10
120	MCL1	10	**306**	FLNA	10
121	PHB2	10	**307**	VCAM1	10
122	MYC	10	**308**	CSNK1A1	10
123	RELA	9	**309**	PSMF1	10
124	PLTP	8	**310**	PDIA6	9
125	SOAT1	7	**311**	GNS	10
126	NME4	10	**312**	RUNX1	10
127	RHOC	10	**313**	RPL17	8
128	HLA-C	7	**314**	SMC5	9
129	ZC3HAV1	9	**315**	ETV1	10
130	JAG1	10	**316**	LAMC1	10
131	CSRP2	10	**317**	PCOLCE	10
132	BARD1	10	**318**	RECQL	10
133	LAMA4	9	**319**	VAMP8	8
134	PRCP	9	**320**	ARHGEF6	10
135	TIMP1	10	**321**	BNIP2	10
136	CBX3	10	**322**	FADD	10
137	NME2	7	**323**	HLA-DRA	10
138	CPNE3	8	**324**	MDK	9
139	CD163	10	**325**	GALNT10	9
140	POLR2J	10	**326**	TNPO1	10
141	CKAP4	10	**327**	HMGN1	9
142	PTN	9	**328**	EIF4EBP1	8
143	LYPD1	9	**329**	BTN3A2	9
144	DLG5	9	**330**	TMCO1	9
145	LAPTM4A	9	**331**	KARS	10
146	ID4	10	**332**	POSTN	10
147	WWTR1	9	**333**	ELF1	10
148	CALU	9	**334**	TPM2	10
149	GBE1	10	**335**	GTF3C2	10
150	FYN	10	**336**	GBP2	9
151	ERGIC3	9	**337**	TP53	9
152	BACH1	8	**338**	GAS1	10
153	SERPINE1	10	**339**	CYFIP1	10
154	POLD2	10	**340**	JUN	10
155	AIF1	10	**341**	RCN1	10

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
156	FCGRT	10	**342**	HNRNPUL1	9
157	H19	8	**343**	HAT1	9
158	H2AFV	9	**344**	MAPK7	10
159	MAPRE1	10	**345**	TRIM14	10
160	KIAA0040	7	**346**	UBE2C	10
161	TPR	10	**347**	MAPKAPK2	10
162	RPL23	10	**348**	SP100	10
163	COL3A1	10	**349**	SFPQ	10
164	DPY19L1	9	**350**	FKBP5	10
165	APOE	10	**351**	HLA-DMA	10
166	PDPN	9	**352**	H3F3A	10
167	GNG5	9	**353**	NPM1	7
168	NUP205	10	**354**	NAGA	7
169	SSRP1	10	**355**	CTGF	10
170	HLA-DQB1	10	**356**	GUSB	10
171	LAMB2	10	**357**	RPA1	10
172	LHFPL2	10	**358**	MPZL1	9
173	BAT1	8	**359**	LY96	8
174	OBSL1	9	**360**	HLA-DPB1	10
175	CNN3	10	**361**	AIMP2	9
176	PPIB	10	**362**	ARPC1B	10
177	STK17A	9	**363**	EEF1G	10
178	ACLY	10	**364**	SH3BP2	10
179	FCGR1A	9	**365**	UBA7	10
180	COL6A1	10	**366**	PTK7	10
181	RPL7A	7	**367**	FHL1	10
182	ZNF207	9	**368**	CLEC2B	10
183	TLE3	10	**369**	CCBL2	9
184	NMB	9	**370**	VEGFA	10
185	SRI	10	**371**	CLIC1	7
186	PAICS	10	**372**	SEPT9	8

Appendix Table 2. Significantly under-expressed gene list from Oncomine meta-analysis of normal vs. astrocytoma studies. *Genes are ranked by significance in Oncomine. **Genes were considered significant in the meta-analysis, and chosen for further analysis, if they were found in at least in at least 7 of 10 meta-analyzed studies.

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
1	ANK3	10	**139**	RAB11FIP2	9
2	DCNTN1	7	**140**	DLGAP2	9
3	BCL2L2	10	**141**	MADD	10
4	WDR7	9	**142**	TNK1	9
5	PLEKHB2	9	**143**	CD200	10
6	PRKCZ	10	**144**	SORBS2	9
7	GRM3	10	**145**	RAP1GAP	10
8	OPA1	9	**146**	NR1D1	10
9	USP12	9	**147**	IDI1	10
10	ATP6V0A1	10	**148**	MAL	10
11	VAMP1	8	**149**	ELMO1	10
12	EPB41L3	9	**150**	PURA	10
13	IDH3A	10	**151**	KRT17	10
14	MAPRE2	10	**152**	CD47	10
15	SH3GL3	10	**153**	RUFY3	9
16	PTGER3	10	**154**	COX7A1	9
17	ATP6V1E1	9	**155**	MPHOSPH8	9
18	RCAN2	10	**156**	BIN1	10
19	SBF1	7	**157**	C5orf30	9
20	TSPYL1	9	**158**	CAMTA1	9
21	RANGAP1	9	**159**	PRDM2	10
22	ZNF365	7	**160**	GSTM5	10
23	SNCG	9	**161**	EDIL3	9
24	STAU2	9	**162**	NEFM	10
25	ASPHD1	7	**163**	SYT5	10
26	PTAFR	10	**164**	PRSS3	9
27	RAB3B	10	**165**	LARGE	9
28	PRKCB	10	**166**	STK39	8
29	STMN1	10	**167**	SV2B	9
30	CNNM2	9	**168**	XK	10
31	CACNA1B	8	**169**	CLCN4	10
32	CHIC1	7	**170**	MEG3	9
33	VAMP2	10	**171**	NECAB3	7
34	WNT10B	9	**172**	YWHAZ	10
35	DUSP7	10	**173**	C22orf9	8

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
36	GRIN2C	10	**174**	INPP5F	9
37	RUSC2	9	**175**	RAB3A	10
38	MAPK8IP2	7	**176**	DBP	10
39	SSX2IP	9	**177**	KCNAB2	10
40	TPM3	9	**178**	GABRB2	8
41	TPPP	9	**179**	SLC17A7	9
42	PICK1	8	**180**	AMPH	10
43	APLP1	10	**181**	PDE1A	10
44	GABRA5	9	**182**	QDPR	10
45	SOCS7	9	**183**	FOXO4	10
46	PPP1R16B	9	**184**	KCNMA1	10
47	TSPAN5	9	**185**	MYH10	10
48	MAPT	10	**186**	CYP26A1	9
49	MAGI1	10	**187**	RAB40B	10
50	RASGRF1	10	**188**	SEMA4D	10
51	MAST3	9	**189**	NCKIPSD	9
52	CNTN2	10	**190**	PRR4	10
53	SERPINI1	10	**191**	DCLK1	9
54	PKP4	10	**192**	UROS	9
55	CACNB1	10	**193**	PDE2A	9
56	NUAK1	9	**194**	KIAA0232	10
57	SNCA	10	**195**	TRIM3	8
58	KIF17	7	**196**	ARHGEF4	8
59	GABRA2	10	**197**	SYNPO	9
60	TTBK2	7	**198**	MBOAT7	10
61	BTRC	8	**199**	RIMS2	9
62	DYNC1I1	9	**200**	TERF2IP	9
63	PRKCG	10	**201**	PSD3	9
64	ZBTB7A	9	**202**	STX1A	10
65	LMTK2	9	**203**	ATP6V1C1	10
66	RYBP	9	**204**	FXR2	10
67	KIF5C	9	**205**	RAB40C	9
68	AGTPBP1	9	**206**	KIT	10
69	RYR2	10	**207**	UBE3A	10
70	LDOC1	8	**208**	ZC3H13	9
71	TPD52	10	**209**	CLASP2	9
72	MOBP	10	**210**	MAN1A2	9
73	TUBB4	10	**211**	HLF	10
74	LDB3	8	**212**	SYN2	10
75	CACNA1A	9	**213**	NEBL	9

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
76	BSCL2	9	**214**	NR2F6	9
77	MPP2	9	**215**	CPLX2	10
78	PPP2R2B	10	**216**	JAKMIP1	8
79	RAP1GDS1	9	**217**	MYCBP2	9
80	GOT1	10	**218**	DGKZ	10
81	WASF1	10	**219**	DOCK3	9
82	FAIM2	9	**220**	KCNC3	7
83	FCH01	9	**221**	HABP4	9
84	CDKN2D	10	**222**	PRKCQ	9
85	EFR3B	9	**223**	SLC13A3	9
86	MBP	10	**224**	RAPGEF5	8
87	SNRPN	10	**225**	PET112L	8
88	CA11	9	**226**	KCNC4	10
89	SERINC3	10	**227**	GRM7	8
90	MICAL3	9	**228**	NPY1R	10
91	EFNA5	9	**229**	CCKBR	10
92	FGF13	9	**230**	DLG1	10
93	IQSEC1	9	**231**	GRM1	8
94	PAK1	10	**232**	PEBP1	10
95	ULK2	9	**233**	RNMT	9
96	ATP2B1	10	**234**	CCDC64	8
97	MAP2K4	10	**235**	PEG3	10
98	GABARAPL1	9	**236**	GRLF1	10
99	APBB1	10	**237**	KLK3	10
100	ATP8A1	9	**238**	NAV3	8
101	AUH	10	**239**	CLDN9	9
102	NIPAL3	10	**240**	PRKAR1A	10
103	DNM1L	10	**241**	CRHR2	8
104	EMX1	9	**242**	PPP1R7	10
105	SCAMP1	10	**243**	ICAM5	10
106	FAAH	10	**244**	CRELD1	8
107	LPGAT1	10	**245**	PRKCE	10
108	PTPRD	10	**246**	ARFGEF2	8
109	GHITM	8	**247**	PPFIA2	9
110	AAK1	9	**248**	FABP6	9
111	DGK1	10	**249**	PDIA2	9
112	CACNA1C	10	**250**	OSBPL1A	9
113	FUT9	9	**251**	RAPGEF3	10
114	GRIN2A	10	**252**	FAM190B	10
115	PDS5B	8	**253**	CALM1	9

Gene Significance Rank*	Gene Symbol	Studies (of 10)**	Gene Significance Rank*	Gene Symbol	Studies (of 10)**
116	KCNJ9	8	**254**	LOC157627	8
117	APBA1	10	**255**	PCDH9	9
118	DIP2C	9	**256**	DOCK9	9
119	IQSEC3	9	**257**	FRMPD4	8
120	TYRO3	9	**258**	HSPH1	10
121	KIAA0284	7	**259**	C17orf108	9
122	HK1	7	**260**	SEPT11	9
123	PAFAH1B1	10	**261**	DNAJC6	9
124	FXYD1	9	**262**	GDE1	9
125	SLC25A4	10	**263**	EXOC6B	7
126	KHDRBS2	7	**264**	CAMK2G	10
127	HRAS	10	**265**	OPTN	9
128	ACTR1A	10	**266**	ANXA3	9
129	AP3M2	10	**267**	PPFIA3	9
130	MEF2C	10	**268**	NDEL1	9
131	CLTB	9	**269**	CDC42	9
132	ATP2B3	10	**270**	CDH8	10
133	TLN2	10	**271**	ARPP19	9
134	SEC14L5	9	**272**	DTNB	9
135	SLC6A12	10	**273**	PPM1A	10
136	RB1CC1	10	**274**	ATP58	10
137	ATP5L	7	**275**	MTMR9	9
138	SPOCK3	7			

Appendix Table 3. Comparison of Markov blanket genes across Grades I to IV Astrocytoma. Over-expressed genes are red; Underexpressed genes are blue. Genes highlighted in grey are Markov blanket genes for the corresponding tumor grade.

PILOCYTIC	DIFFUSE	ANAPLASTIC	GLIOBLASTOMA
ANK3	ANK3	ANK3	ANK3
ANXA1	ANXA1	ANXA1	ANXA1
ANXA2	ANXA2	ANXA2	ANXA2
BTF3	BTF3	BTF3	BTF3
BTN3A3	BTN3A3	BTN3A3	BTN3A3
C1S	C1S	C1S	C1S
CALCRL	CALCRL	CALCRL	CALCRL
CCND2	CCND2	CCND2	CCND2
CD44	CD44	CD44	CD44
CD99	CD99	CD99	CD99
CDH11	CDH11	CDH11	CDH11
CDK4	CDK4	CDK4	CDK4
CNTN2	CNTN2	CNTN2	CNTN2
COL4A1	COL4A1	COL4A1	COL4A1
DAB2	DAB2	DAB2	DAB2
DPYSL3	DPYSL3	DPYSL3	DPYSL3
DUSP7	DUSP7	DUSP7	DUSP7
EGFR	EGFR	EGFR	EGFR
EIF4A1	EIF4A1	EIF4A1	EIF4A1
EMP1	EMP1	EMP1	EMP1
FN1	FN1	FN1	FN1
GABRA5	GABRA5	GABRA5	GABRA5
HLAA	HLAA	HLAA	HLAA
IGFBP5	IGFBP5	IGFBP5	IGFBP5
LPL	LPL	LPL	LPL
MARCKS	MARCKS	MARCKS	MARCKS
MAST3	MAST3	MAST3	MAST3
MCM3	MCM3	MCM3	MCM3
MPP2	MPP2	MPP2	MPP2
MTHFD2	MTHFD2	MTHFD2	MTHFD2
NONO	NONO	NONO	NONO
PPP2R2B	PPP2R2B	PPP2R2B	PPP2R2B
PRDX4	PRDX4	PRDX4	PRDX4
PTGER3	PTGER3	PTGER3	PTGER3
RAB31	RAB31	RAB31	RAB31

PILOCYTIC	DIFFUSE	ANAPLASTIC	GLIOBLASTOMA
RAB3B	RAB3B	RAB3B	RAB3B
RCAN2	RCAN2	RCAN2	RCAN2
RUSC2	RUSC2	RUSC2	RUSC2
SERBP1	SERBP1	SERBP1	SERBP1
SERINC3	SERINC3	SERINC3	SERINC3
SH3GL3	SH3GL3	SH3GL3	SH3GL3
SNRPE	SNRPE	SNRPE	SNRPE
SPARC	SPARC	SPARC	SPARC
SRPX	SRPX	SRPX	SRPX
SSR2	SSR2	SSR2	SSR2
TIMP4	TIMP4	TIMP4	TIMP4
TOP2A	TOP2A	TOP2A	TOP2A
TPPP	TPPP	TPPP	TPPP
VAMP1	VAMP1	VAMP1	VAMP1
VCAN	VCAN	VCAN	VCAN
WNT5A	WNT5A	WNT5A	WNT5A
WNT10B	WNT10B	WNT10B	WNT10B

Appendix Table 4. Characteristics of Grade IV Glioblastoma Multiforme Markov blanket genes. References are listed below text of manuscript.

Gene Symbol / Name	Genomic Location / Cellular Localization	Function	Cancer / Disease Link
COL4A1 Collagen, type IV, alpha 1	13q34 extracellular matrix	Inhibits angiogenesis and tumor formation	Are upregulated in malignant and metastatic brain tumors [32]
EGFR Epidermal growth factor receptor	7p12 membrane	Growth factor	Consistently linked to development of glioblastoma [33]; Has been linked to glioma tumor invasiveness, proliferation, and angiogenesis [34;35]; Mutations have been found in EGFR in primary glioblastomas [36] and have been linked to poor prognosis in GBM [37]. Its signaling has been shown to cooperate with loss of tumor suppressor gene functions in promotion of gliomagenesis
BTF3 Basic transcription factor 3	5q13.2 nucleus	Transcription factor	Found to be highly expressed in glioblastoma multiforme [38]; regulates tumor-associated genes in pancreatic cancer cells [39]
MPP2 Membrane protein, palmitoylated 2	17q12-q21 membrane	Tumor suppressor; Coupling of cytoskeleton to cell membrane	Contributes to cell proliferation and resistance in cisplatin treatment in medulloblastoma cells [40]
RAB31 Member RAS oncogene family	18q11.3 membrane	Vesicle and granule targeting	May have role in regulating EGFR in astrocyte development and oncogenesis (Ng 2009) [41]; associated with survival in glioblastoma [42]
CDK4 Cyclin-dependent kinase 4	12q14 cytoplasm	Cell cycle regulation; inhibits RB protein family members	Known target of glioblastoma anticancer therapy [43]; thought to be a driver mutation gene in glioblastoma [44]
CD99 CD99 molecule	Xp22.32 membrane	Leukocyte migration, T-cell adhesion, protein transport, and T-cell death	May act as an oncosuppressor in osteosarcoma; Is a useful marker for diagnosis of brain tumor types [45]
ANXA2 Annexin A2	15q22.2 Extracellular space,	Regulation of cell growth	Involved in migration of neural stem cells to glioma sites [46]; potentially involved in glioma invasion [47]

Gene Symbol / Name	Genomic Location / Cellular Localization	Function	Cancer / Disease Link
	extracellular matrix, membrane		
TOP2A Topoisomerase (DNA) II alpha 170kDa	17q21-q22 Cytosplasm, nucleus, nucleoplasm	Resolves topological problems in genomic DNA resulting from replication, transcription and repair	Is target of several anticancer agents; mutations in this gene have been associated with development of drug resistance; common significantly altered gene in cancer [48]; May be involved in network of genes controlling cell cycle regulation in glioblastoma [49]; very high copy number gain in glioblastoma [50]
SERBP1 SERPINE1 mRNA binding protein 1	1p31 Cytoplasm, nucleus	Regulation of mRNA stability	Significantly overexpressed in ovarian cancer, especially in advanced disease [51]

Appendix Table 5. List of 173 genes present in environmental gene databases and having mutations in Glioblastoma, with number of studies conducted on the gene in relation to Glioblastoma.

Gene Symbol	Gene Name	Number of GBM Studies
ABCA3	ATP-binding cassette, sub-family A (ABC1), member 3	1
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	80
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	16
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	1
ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	0
ADAMTS13	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 13	2
ADM	adrenomedullin	13
AKT2	thymoma viral proto-oncogene 2	6
ALDH1A2	aldehyde dehydrogenase family 1, subfamily A2	0
ALOX12	arachidonate 12-lipoxygenase	0
AOC3	amine oxidase, copper containing 3	0
API5	apoptosis inhibitor 5	0
APOBEC3G	Apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G	0
ARNT	aryl hydrocarbon receptor nuclear translocator	2
ATM	ataxia telangiectasia mutated homolog (human)	23
ATRX	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	0
BAX	BCL2-associated X protein	155
BCL2	B-cell leukemia/lymphoma 2	25
BMI1	Bmi1 polycomb ring finger oncogene	12
BRAF	Braf transforming gene	8
BRCA1	breast cancer 1	7
BRCA2	breast cancer 2	5
CALCA	calcitonin/calcitonin-related polypeptide, alpha	1
CAPN3	calpain 3	0
CASP9	caspase 9	60
CCK	cholecystokinin	5
CCND2	cyclin D2	4
CDC7	cell division cycle 7 (S. cerevisiae)	0
CDH1	cadherin 1	4
CDK4	cyclin-dependent kinase 4	92
CDK6	cyclin-dependent kinase 6	12
CDKN2A	cyclin-dependent kinase inhibitor 2A	177
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	18
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	7
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	0
CES3	carboxylesterase 3A	0
CHEK1	checkpoint kinase 1 homolog (S. pombe)	0
CHEK2	CHK2 checkpoint homolog (S. pombe)	2
CHRNA4	cholinergic receptor, nicotinic, alpha polypeptide 4	0
CIZ1	CDKN1A interacting zinc finger protein 1	0
CKS2	CDC28 protein kinase regulatory subunit 2	1

Gene Symbol	Gene Name	Number of GBM Studies
CLSPN	claspin homolog (Xenopus laevis)	0
COCH	coagulation factor C homolog (Limulus polyphemus)	0
CPSF4	cleavage and polyadenylation specific factor 4	0
CSF3R	colony stimulating factor 3 receptor (granulocyte)	7
CSK	c-src tyrosine kinase	4
CTNNB1	catenin (cadherin associated protein), beta 1	44
CTNND2	catenin (cadherin associated protein), delta 2	0
CYP1B1	cytochrome P450, family 1, subfamily b, polypeptide 1	1
CYP27B1	cytochrome P450, family 27, subfamily b, polypeptide 1	7
CYP2C19	cytochrome P450, family 2, subfamily c, polypeptide 50	0
CYP2E1	cytochrome P450, family 2, subfamily e, polypeptide 1	1
CYP3A4	cytochrome P450, family 3, subfamily a, polypeptide 41A	15
DDB1	damage specific DNA binding protein 1	0
DDC	dopa decarboxylase	2
DDIT3	DNA-damage inducible transcript 3	10
E2F4	E2F transcription factor 4	4
EDNRA	endothelin receptor type A	0
EGF	epidermal growth factor	252
EGFR	epidermal growth factor receptor	747
ELN	elastin	1
ENO1	enolase 1, alpha non-neuron	1
EPHB6	Eph receptor B6	1
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	73
ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5	0
ESR1	estrogen receptor 1 (alpha)	3
F13A1	coagulation factor XIII, A1 subunit	0
F13B	coagulation factor XIII, beta subunit	0
F2RL1	coagulation factor II (thrombin) receptor-like 1	0
F3	contactin 1	5
FANCA	Fanconi anemia, complementation group A	2
FGF2	fibroblast growth factor 2	68
FGFR1	fibroblast growth factor receptor 1	20
FGFR2	fibroblast growth factor receptor 2	11
FGFR3	fibroblast growth factor receptor 3	3
FOXA2	forkhead box A2	0
FOXM1	forkhead box M1	9
FUT2	fucosyltransferase 2	0
GABPA	GA repeat binding protein, alpha	0
GAD2	glutamic acid decarboxylase 2	0
GADD45G	growth arrest and DNA-damage-inducible 45 gamma	0
GAS6	growth arrest specific 6	3
GATA3	GATA binding protein 3	1
GCLC	glutamate-cysteine ligase, catalytic subunit	0
GYPC	glycophorin C	0

Gene Symbol	Gene Name	Number of GBM Studies
HGF	hepatocyte growth factor	58
IFNA1	interferon alpha 1	0
IFNAR1	interferon (alpha and beta) receptor 1	0
IGF1R	insulin-like growth factor I receptor	45
IGF2	insulin-like growth factor 2	7
IGFBP7	insulin-like growth factor binding protein 7	5
IKBKE	inhibitor of kappaB kinase epsilon	3
IL17B	interleukin 17B	0
IL3	interleukin 3	1
IL4R	interleukin 4 receptor, alpha	6
IL6	interleukin 6	12
ITGAL	integrin alpha L	1
ITM2B	integral membrane protein 2B	0
JUNB	Jun-B oncogene	3
KEL	Kell blood group	0
KLF4	Kruppel-like factor 4 (gut)	0
MAP3K8	mitogen-activated protein kinase kinase kinase 8	0
MAPK9	mitogen-activated protein kinase 9	0
MDM2	transformed mouse 3T3 cell double minute 2	114
MDM4	transformed mouse 3T3 cell double minute 4	4
MLH1	mutL homolog 1 (E. coli)	22
MLL	myeloid/lymphoid or mixed-lineage leukemia 1	3
MMP10	matrix metallopeptidase 10	0
MMP15	matrix metallopeptidase 15	2
MMP2	matrix metallopeptidase 2	23
MN1	meningioma 1	0
MSH2	mutS homolog 2 (E. coli)	17
MSH6	mutS homolog 6 (E. coli)	12
MUC5AC	mucin 5, subtypes A and C, tracheobronchial/gastric	1
MYBPC3	myosin binding protein C, cardiac	0
MYC	myelocytomatosis oncogene	132
NCOA1	nuclear receptor coactivator 1	1
NF1	neurofibromatosis 1	44
NOS3	nitric oxide synthase 3, endothelial cell	1
NRAS	neuroblastoma ras oncogene	3
PARK2	Parkinson disease (autosomal recessive, juvenile) 2, parkin	3
PDIA2	protein disulfide isomerase associated 2	0
PGR	progesterone receptor	3
PMS1	postmeiotic segregation increased 1 (S. cerevisiae)	0
PMS2	postmeiotic segregation increased 2 (S. cerevisiae)	11
POLD1	polymerase (DNA directed), delta 1, catalytic subunit	0
POLE	polymerase (DNA directed), epsilon	4
POLG2	polymerase (DNA directed), gamma 2, accessory subunit	0
POR	P450 (cytochrome) oxidoreductase	16
PPARA	peroxisome proliferator activated receptor alpha	0
PRKCB1	protein kinase C, beta	1

Gene Symbol	Gene Name	Number of GBM Studies
PRKDC	protein kinase, DNA activated, catalytic polypeptide	36
PROM1	prominin 1	2
PTCH2	patched homolog 2	0
PTEN	phosphatase and tensin homolog	391
PTGDR	prostaglandin D receptor	0
PTGS2	prostaglandin-endoperoxide synthase 2	57
RAD52	RAD52 homolog (S. cerevisiae)	1
RAP1B	RAS related protein 1b	2
RB1	retinoblastoma 1	25
RFC2	replication factor C (activator 1) 2	2
SEMA7A	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	0
SERPINE1	serine (or cysteine) peptidase inhibitor, clade E, member 1	23
SERPING1	serine (or cysteine) peptidase inhibitor, clade G, member 1	0
SFTPB	surfactant associated protein B	0
SHH	sonic hedgehog	11
SLC14A1	solute carrier family 14 (urea transporter), member 1	0
SLC4A1	solute carrier family 4 (anion exchanger), member 1	0
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	6
STAT1	signal transducer and activator of transcription 1	20
STAT3	signal transducer and activator of transcription 3	89
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0
TAF6	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0
TERT	telomerase reverse transcriptase	25
TGFBR2	transforming growth factor, beta receptor II	1
TGM1	transglutaminase 1, K polypeptide	0
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	0
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1a	1
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1b	0
TNFRSF25	tumor necrosis factor receptor superfamily, member 25	0
TNFRSF8	tumor necrosis factor receptor superfamily, member 8	0
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	1
TP53	transformation related protein 53	211
TRPM2	transient receptor potential cation channel, subfamily M, member 2	4
TRPV5	transient receptor potential cation channel, subfamily V, member 5	0
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1	1
UNG	uracil DNA glycosylase	4
VCAM1	vascular cell adhesion molecule 1	0
VDR	vitamin D receptor	2
VLDLR	very low density lipoprotein receptor	0
VWF	Von Willebrand factor homolog	17
XDH	xanthine dehydrogenase	1

Appendix Table 6. List of genes mutated in Glioblastoma and showing mutagenic actions in response to chemicals in studies curated in the Comparative Toxicology Database.

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
ATRX	Ethylnitrosourea	Rn	Ethylnitrosourea results in increased mutagenesis of ATRX gene	increases mutagenesis	16495775
CYP2E1	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride affects the mutagenesis of KRAS gene]	affects mutagenesis |increases reaction	12705718
CYP2E1	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]	affects mutagenesis increases reaction	12705718
CYP2E1	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride results in increased mutagenesis of KRAS gene]	increases mutagenesis increases reaction	17384900
CYP2E1	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride results in increased mutagenesis of TP53 gene]	increases mutagenesis increases reaction	12010862|17384900
EGFR	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of EGFR gene	increases mutagenesis	15366372|16724327
ERBB2	Ethylnitrosourea	Rn	Ethylnitrosourea results in increased mutagenesis of ERBB2 gene	increases mutagenesis	16651423
IGF1R	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of IGF1R gene	increases mutagenesis	16461637
MLL	Genistein	Hs	Genistein results in increased mutagenesis of MLL gene	increases mutagenesis	17468513
MLL	kaempferol	Hs	kaempferol results in increased mutagenesis of MLL gene	increases mutagenesis	17468513
MLL	Permethrin	Hs	Permethrin results in increased mutagenesis of MLL gene	increases mutagenesis	12937054
MLL	Quercetin	Hs	Quercetin results in increased mutagenesis of MLL gene	increases mutagenesis	17468513
MSH2	Ethylnitrosourea	Rn	Ethylnitrosourea results in increased mutagenesis of MSH2 gene	increases mutagenesis	16495775

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
MSH6	Ethylnitrosourea	Rn	Ethylnitrosourea results in increased mutagenesis of MSH6 gene	increases mutagenesis	16495775|18417481
MYC	Estradiol	Mm	AICDA protein affects the reaction [Estradiol results in increased mutagenesis of MYC gene]	affects reaction increases mutagenesis	19139166
MYC	Estradiol	Mm	Estradiol results in increased mutagenesis of MYC gene	increases mutagenesis	19139166
PARK2	Ethylnitrosourea	Ol	Ethylnitrosourea results in increased mutagenesis of PARK2 exon	increases mutagenesis	17156454
PTEN	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of PTEN gene	increases mutagenesis	15755804|16724327
SHH	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of SHH enhancer	increases mutagenesis	17049204
SLC4A1	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of SLC4A1 gene	increases mutagenesis	16724327
STAT1	Ethylnitrosourea	Mm	Ethylnitrosourea results in increased mutagenesis of STAT1 gene	increases mutagenesis	16688530|16724327
TP53	2,4,6-trichlorophenol	Dr	2,4,6-trichlorophenol results in increased mutagenesis of TP53 gene	increases mutagenesis	18939895
TP53	2-phenylphenol	Hs	[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	10334203
TP53	2-phenylphenol	Hs	bathocuproine inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	2-phenylphenol	Hs	CAT protein inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Acrolein	Hs	Acrolein results in increased mutagenesis of TP53 gene	increases	17030796

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
				mutagenesis	
TP53	Acrylamide	Hs	Acrylamide results in increased mutagenesis of TP53 gene	increases mutagenesis	15240786
TP53	Aflatoxin B1	Hs	Aflatoxin B1 results in increased mutagenesis of TP53 gene	increases mutagenesis	12619106|15946497
TP53	Aflatoxins	Hs	Aflatoxins results in increased mutagenesis of TP53 gene	increases mutagenesis	16007211
TP53	Arsenic	Hs	Arsenic results in increased mutagenesis of TP53 gene	increases mutagenesis	15967209
TP53	Arsenic	Hl	Arsenic results in increased mutagenesis of TP53 gene	increases mutagenesis	19203779
TP53	Arsenic	Of	Arsenic results in increased mutagenesis of TP53 gene	increases mutagenesis	19203779
TP53	bathocuproine	Hs	bathocuproine inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	bathocuproine	Hs	bathocuproine inhibits the reaction [[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	chlorophyllin	Rn	chlorophyllin inhibits the reaction [Cyclophosphamide results in increased mutagenesis of TP53 exon]	decreases reaction increases mutagenesis	19227835
TP53	NAD	Hs	[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	10334203
TP53	NAD	Hs	bathocuproine inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
TP53	NAD	Hs	CAT protein inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Copper	Hs	[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	10334203
TP53	Copper	Hs	bathocuproine inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Copper	Hs	bathocuproine inhibits the reaction [[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Copper	Hs	CAT protein inhibits the reaction [[2-phenylphenol metabolite co-treated with NAD co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Copper	Hs	CAT protein inhibits the reaction [[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	Copper	Hs	[N-hydroxy-4-aminobiphenyl co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	11275476
TP53	Copper	Hs	[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	10334203

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
TP53	Curcumin	Rn	Curcumin inhibits the reaction [Cyclophosphamide results in increased mutagenesis of TP53 exon]	decreases reaction increases mutagenesis	19227835
TP53	Cyclophosphamide	Rn	chlorophyllin inhibits the reaction [Cyclophosphamide results in increased mutagenesis of TP53 exon]	decreases reaction increases mutagenesis	19227835
TP53	Cyclophosphamide	Rn	Curcumin inhibits the reaction [Cyclophosphamide results in increased mutagenesis of TP53 exon]	decreases reaction increases mutagenesis	19227835
TP53	Cyclophosphamide	Rn	Cyclophosphamide results in increased mutagenesis of TP53 exon	increases mutagenesis	19227835
TP53	Ethylnitrosourea	Ol	Ethylnitrosourea results in increased mutagenesis of TP53 exon	increases mutagenesis	17156454
TP53	Ethylnitrosourea	Rn	Ethylnitrosourea results in increased mutagenesis of TP53 gene	increases mutagenesis	16495775
TP53	glycidamide	Hs	glycidamide results in increased mutagenesis of TP53 gene	increases mutagenesis	15240786
TP53	N-hydroxy-4-aminobiphenyl	Hs	[N-hydroxy-4-aminobiphenyl co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	11275476
TP53	Nitrofurazone	Hs	Nitrofurazone results in increased mutagenesis of TP53 gene	increases mutagenesis	15488632
TP53	phenylhydroquinone	Hs	bathocuproine inhibits the reaction [[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203
TP53	phenylhydroquinone	Hs	CAT protein inhibits the reaction [[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene]	affects cotreatment decreases reaction increases mutagenesis	10334203

Gene Symbol	Chemical Name	Organism	Interaction	Interaction Actions	PubMed IDs
TP53	phenylhydroquinone	Hs	[phenylhydroquinone co-treated with Copper] results in increased mutagenesis of TP53 gene	affects cotreatment increases mutagenesis	10334203
TP53	potassium diazoacetate	Hs	potassium diazoacetate results in increased mutagenesis of TP53 gene	increases mutagenesis	16926174
TP53	Tobacco Smoke Pollution	Hs	Tobacco Smoke Pollution results in increased mutagenesis of TP53 gene	increases mutagenesis	10824542
TP53	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]	affects mutagenesis increases reaction	12705718
TP53	Vinyl Chloride	Hs	CYP2E1 gene polymorphism promotes the reaction [Vinyl Chloride results in increased mutagenesis of TP53 gene]	increases mutagenesis increases reaction	12010862|17384900
TP53	Vinyl Chloride	Hs	GSTM1 gene polymorphism affects the reaction [XRCC1 gene polymorphism affects the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]]	affects mutagenesis affects reaction	16097394
TP53	Vinyl Chloride	Hs	GSTT1 gene polymorphism affects the reaction [XRCC1 gene polymorphism affects the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]]	affects mutagenesis affects reaction	16097394
TP53	Vinyl Chloride	Hs	Vinyl Chloride results in increased mutagenesis of TP53 gene	increases mutagenesis	12010862|12670519|16881598|17384900
TP53	Vinyl Chloride	Hs	XRCC1 gene polymorphism affects the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]	affects mutagenesis affects reaction	16097394
TP53	Vinyl Chloride	Hs	XRCC1 gene polymorphism promotes the reaction [Vinyl Chloride affects the mutagenesis of TP53 gene]	affects mutagenesis increases reaction	14602524
TP53	Vinyl Chloride	Hs	XRCC1 gene polymorphism promotes the reaction [Vinyl Chloride results in increased mutagenesis of TP53 gene]	increases mutagenesis increases reaction	12010862|16881598

Appendix Figure 1. Results of PathJam analysis of Grade 1 Astrocytoma Markov genes. Arcs represent interactions between genes and pathways. Databases for curated pathways and gene ontology terms are identified by their respective emblems. See Additional File 1.

Appendix Figure 2. Results of PathJam analysis of Grade 2 Astrocytoma Markov genes. Arcs represent interactions between genes and pathways. Databases for curated pathways and gene ontology terms are identified by their respective emblems. See Additional File 2.

Appendix Figure 3. Results of PathJam analysis of Grade 3 Astrocytoma Markov genes. Arcs represent interactions between genes and pathways. Databases for curated pathways and gene ontology terms are identified by their respective emblems. See Additional File 3.

Appendix Figure 4. Results of PathJam analysis of Grade 4 Astrocytoma Markov genes. Arcs represent interactions between genes and pathways. Databases for curated pathways and gene ontology terms are identified by their respective emblems. See Additional File 4.

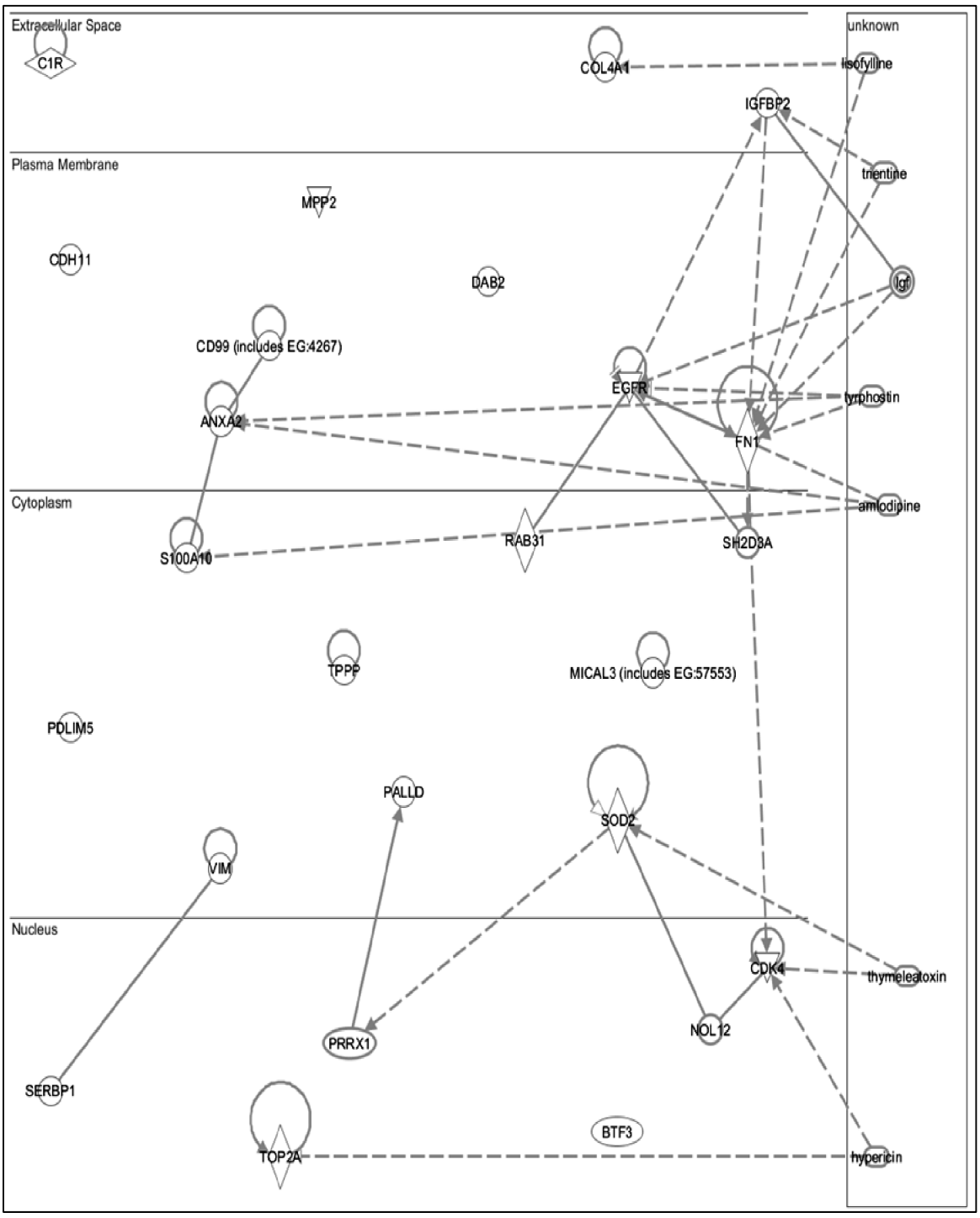

Figure 5. IPA connections of Grade IV Glioblastoma Markov genes. Direct interactions between genes (genes/gene products make direct physical contact with each other) are represented by solid lines. Indirect interactions (genes/gene products do not make direct physical contact with each other but instead may influence each other through some intermediate factor) are represented as dotted lines.

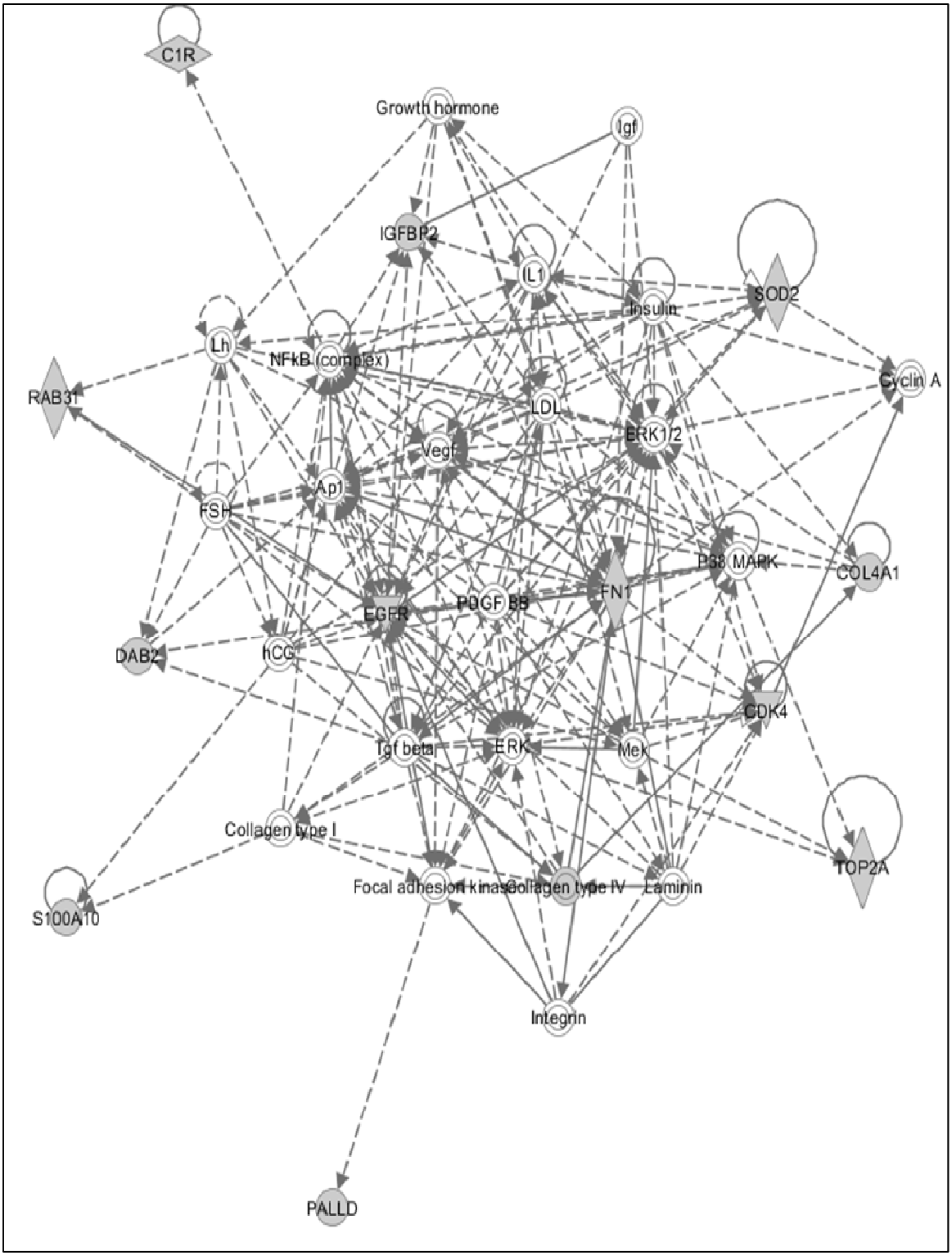

Figure 6. Top IPA Grade IV Glioblastoma Markov genes network. IPA defined the network as related to 'cancer, neurological disease, and cellular movement'. Shaded genes represent our Markov genes. Non-shaded molecules were added by IPA during the analyses.

Appendix Figure 7. KEGG Pathway 'Pathways in Cancer' found to contain 42 genes from our GEI Glioblastoma alterations list. Analyses run using DAVID. Stars represent genes in the GEI gene list and pathways significantly represented in the GEI gene list.
See Additional File 5.

Appendix Figure 8. KEGG Pathway 'Glioma' found to contain 13 genes from our GEI Glioblastoma alterations list. Analyses run using DAVID. Stars represent genes in the GEI gene list and pathways significantly represented in the GEI gene list.
See Additional File 6.

Appendix Figure 9. KEGG Pathway 'Pathways in Cancer' found to contain 123 genes from our Glioblastoma alterations list. Analyses run using DAVID. Stars represent genes in the GBM gene list and pathways significantly represented in the GBM gene list.
See Additional File 7.

Appendix Figure 10. KEGG Pathway 'Glioma' found to contain 30 genes from our Glioblastoma alterations list. Analyses run using DAVID. Stars represent genes in the GBM gene list and pathways significantly represented in the GBM gene list.
See Additional File 8.

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