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IGFBP2 is Overexpressed by Pediatric Malignant Astrocytomas and Induces the Repair Enzyme DNA-PK

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Abstract

To identify receptor tyrosine kinases (RTKs) critical to malignant childhood astrocytoma, we compared the expression of RTK-associated genes between low- and high-grade pediatric astrocytomas. The highest differentially overexpressed RTK-associated gene in high-grade astrocytoma is *insulin-like growth factor-binding protein-2* (*IGFBP2*; *P* = .0006). Interestingly, other members of the IGF family are similarly expressed, suggesting that *IGFBP2* regulation in childhood astrocytomas is independent of the IGF system. Immunohistochemistry confirmed overexpression of IGFBP2 protein (*P* = .027). IGFBP2 stimulation had no effect on astrocytoma cell growth and migration, and minimally inhibited IGF-I-, but not IGF-II-, mediated migration. However, IGFBP2 stimulation significantly upregulated the major DNA repair enzyme gene, *DNA-PKcs*, and induced DNA-PKcs protein expression in a time- and dose-dependent manner, whereas IGF-I had no effect. *DNA-PKcs* is also highly overexpressed by high-grade astrocytomas. These findings suggest IGFBP2 plays an IGF-independent role in astrocytoma progression by promoting DNA-damage repair and therapeutic resistance.

Introduction

Astrocytomas are the most common central nervous system neoplasm of childhood.[1] While most pediatric low-grade astrocytomas (World Health Organization grades I and II) respond to surgery, radiation, and chemotherapy (overall survival, 70% to 90%), high-grade astrocytomas (World Health Organization grades III and IV) remain resistant to current treatment regimens (overall survival, 25% to 30%).[2–4] Thus, there is significant interest in identifying biologic markers that promote malignant progression and confer treatment resistance, and that in turn could be targeted for novel therapeutic intervention. There is substantial evidence that the malignant progression of astrocytomas in adults results in part from perturbation of growth factor receptor tyrosine kinase signaling pathways that promote cell proliferation, survival, migration, invasion, and other crucial functions characteristic of malignant cell behavior.[5–9] For example, progression from low-grade to high-grade astrocytoma in adults is characterized by amplification and overexpression of epidermal growth factor receptor (*EGFR*), and to a lesser degree, platelet-derived growth factor receptor (*PDGFR*).[10–12] Whether there are similar patterns of differential receptor tyrosine kinase expression in childhood astrocytomas is not known.

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This distinction between adult and childhood astrocytomas is an important one, given that patterns of genetic alterations in high-grade astrocytomas that arise in the two populations appear to be different, despite the tumors having histologically identical appearances. For instance, *TP53* mutations and *EGFR* amplification occur in ~50% of adult high-grade astrocytomas, but are much less frequently seen in pediatric high-grade astrocytomas.[13–15] These differences could have profound clinical implications for the translation of effective biologic target-driven therapeutics for high-grade astrocytomas in adults and children. Therefore, it is important to clearly define the unique genetic alterations that distinguish pediatric astrocytomas so the most appropriate therapeutic strategies may be designed.

In this study, we sought to identify the receptor tyrosine kinase and receptor tyrosine kinase-associated genes by monitoring their differential expression in low-grade and high-grade childhood astrocytomas, as a prerequisite for determining the best receptor tyrosine kinase candidates for future therapeutic targeting. To address this question, we first performed Affymetrix microarray gene profiling of primary untreated childhood non-brainstem astrocytomas to delineate the differentially expressed receptor tyrosine kinase-associated genes. We then selected the most differentially expressed receptor tyrosine kinase-associated genes for protein validation and in vitro functional testing as a way to gain further insight into the role of the identified receptor tyrosine kinase system in promoting the aggressive high-grade astrocytoma phenotype.

Materials and Methods

Tumor specimens and cells

Frozen tumor specimens were obtained from the Cooperative Human Tissue Network for the purpose of gene expression profiling, and paraffin-embedded tissue specimens were obtained from the pathology department of the Children's National Medical Center for the purpose of immunohistochemistry, with institutional review board approval. All samples were obtained from the primary tumor at the initial diagnostic biopsy and prior to any treatment. Specimens used for gene expression were immediately frozen in liquid nitrogen at the time of initial biopsy and then continuously stored at −80°C until used for array profiling. Frozen specimens consisted of 15 high-grade and 15 low-grade childhood astrocytomas. Specimens used for immunohistochemistry consisted of an independent set of 13 high-grade and 18 low-grade childhood astrocytomas. The astrocytoma cell lines T98, U87, U138, and CRL1718 were obtained from the American Type Culture Collection and grown at 5% CO_2, 37°C in Minimal Essential Medium and 10% fetal calf serum.

Gene expression profiling

Microarray gene expression profiling was performed as previously described.[16,17] In brief, total RNA was isolated from each snap-frozen astrocytoma and astrocytoma cell line using the TRIzol reagent method. RNA was converted into double-stranded complementary DNA using the SuperScript Choice system (Invitrogen, Carlsbad, CA). The primer used for the first-strand complementary DNA synthesis was an oligo(dT)24 primer containing a T7 RNA polymerase promoter (Genset, San Diego, CA). The double-stranded complementary DNA was purified by phenol-chloroform extraction using Phase Lock Gel system. Cleaned complementary DNA was used for in vitro transcription using the ENZO Bioarray RNA transcript labeling kit (Affymetrix, Santa Clara, CA). The biotin-labeled complementary RNA was then purified using the RNeasy kit (Qiagen, Valencia, CA) and the complementary RNA was fragmented to approximately 200 base pairs by alkaline treatment (200 mM Tris-acetate, Ph 8.2, 500 mM potassium acetate, 150 mM magnesium acetate). The integrity of each labeled complementary RNA was verified by hybridization to an Affymetrix TestChip2 array. Labeled complementary RNA was considered suitable if the

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ratio of the average intensity of the 3' to 5' ends of the β-actin gene is less than 3. Each verified complementary RNA was hybridized to the Affymetrix HU133A array (~30,000 characterized full-length gene sequences). Statistical analysis was performed using the Affymetrix software and permutation statistics, as previously described in detail.[16,17]

Immunohistochemistry

Immunohistochemistry staining of the tumors was performed as previously described.[16,17] In brief, 5-μm formalin-fixed paraffin-embedded tumor tissues were cut and immunohistochemistry was performed using the avidin-biotin complex method, with the DAKO Target Retrieval Solution (DakoCytomation, Carpinteria, CA) used for antigen unmasking according to the manufacturer's protocol. Primary antibodies used were goat polyclonal anti-human IGFBP2 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-human IGF-I (clone 1IGF01, NeoMarkers, Fremont, CA), and goat polyclonal anti-human IGF-IR (R&D Systems, Minneapolis, MN) at 15 μg/mL. Secondary antibodies were diluted with 1.5% bovine serum albumin in phosphate-buffered saline as follows: 1:500 goat anti-mouse (Santa Cruz); and 1:300 rabbit anti-goat (Santa Cruz). Section staining was photographed on a conventional light microscope at 200× power. Normal brain tissue was used as background control. Two investigators (M.R.S. and T.J.M.) independently reviewed the specimens, and assigned a grading of the staining using a 0 to 4+ scoring as follows: 0, absent staining; 1+, weak; 2+, intermediate; 3+, intermediate-strong; and 4+, intense positive staining. Fischer's exact test was used to determine significance of differential protein expression between high-grade and low-grade tumors based on the absence or presence of positive staining of any score between the two classes.

Migration assay

Fibronectin-mediated cell migration was tested using the Chemicon QCM-FN Quantitative Cell Migration assay (Chemicon International, Temecula, CA). Four astrocytoma cell lines, CRL1718, T98, U87MG, and U138, were tested. Cells were grown to 80% confluency and then starved in serum-free Minimal Essential Medium for 24 hours prior to re-suspension (1×10^6 cells/mL) in serum-free Minimal Essential Medium with 5% bovine serum albumen for 1 hour. Cells were washed to remove endogenous insulin-like growth factors (IGFs) and insulin-like growth factor-binding proteins (IGFBPs) and incubated at 37°C for 30 minutes with recombinant IGF-I (10 ng/mL, R&D Systems) or IGF-II (10 ng/mL, R&D Systems), with or without recombinant IGFBP2 (5 ng/mL to 50 ng/mL, R&D Systems). Untreated cells were used as the baseline controls. After incubation, cells were washed and re-suspended in aliquots of 2×10^5 cells in 200 μL of serum-free Minimal Essential Medium and plated into the upper fibronectinor bovine serum albumen-coated (negative control) Boyden chambers. The lower chambers contained 300 μL of serum-free Minimal Essential Medium with IGFBP2 (100 ng/mL) to establish a chemotactic gradient. The cells were incubated for 3 hours at 37°C and then cells on the upper side of the membrane were removed with a cotton swab and cells migrating to the lower side of the membrane were fixed and stained with Diff-Quick solution kit (Chemicon). Stained migrating cells were eluted with an extracting solution according to manufacturer's instructions (Chemicon). Two 50 μL aliquots of eluant were placed into a micro titer-plate well and optical density was measured by a Thermomax microplate reader (Molecular Devices) at 550 nm. Using a standardized migration rate of 130 cells/mm^2, equivalent to an optical density at 550nm of 0.270 (per Chemicon protocol), we obtained the final cell counts by averaging the two optical density measurements after subtraction of the bovine serum albumen negative control measurements (background). Assays were repeated twice. Statistical significance was calculated using a Student *t* test.

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Microarray analysis of IGFBP2-stimulated astrocytoma cells

T98 and U87 cells (1×10^6) were grown to 70% confluency in T75 flasks and then starved in serum-free Minimal Essential Medium overnight. Cells were then stimulated with IGFBP2 (50 ng/mL), IGF-I (10 ng/mL), or negative control serum-free Minimal Essential Medium for 24 hours and incubated in 5% CO_2 at 37°C. The cells from each flask were then collected and frozen at four time-points: 0, 1, 8, and 24 hours post-stimulation. All cells were frozen in phosphate-buffered saline at −80°C until used for gene profiling. Expression profiling of stimulated astrocytoma cells was performed as described above using the HU133A Affymetrix array. The assay was repeated three separate times and the Affymetrix software was used to perform the statistical analysis.

Western blot of IGFBP- stimulated astrocytoma cells

T98 and U87 cells (1×10^6) were grown to 70% confluency in T75 flasks and then starved in serum-free Minimal Essential Medium overnight. Cells were then stimulated with IGFBP2 (5 ng/mL to 50 ng/mL), IGF-I (1 ng/mL to 10 ng/mL), or negative control serum-free Minimal Essential Medium for 24 hours and cells from each flask were collected in lysis buffer at 4 time-points: 24, 48, 72, and 96 hours post-stimulation for subsequent Western blot analysis. In brief, whole cell lysate protein concentrations were determined using Bradford dye-binding assay (Bio-Rad, Grand Prairie, TX). Approximately 15 μg of protein was equally loaded on 7.5% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were incubated with 0.5 μg/mL rabbit polyclonal anti-human DNA-PKcs antibody (Oncogene Research Products, Cambridge, MA) overnight at 4°C, washed, and then secondary antibody was added using goat anti-rabbit immunoglobulin G horseradish peroxidase (HRP)-linked (Cell Signaling Technology, Beverly, MA) diluted 1:3000 (3 μL in 10 mL of blocking buffer) and incubated for 1 hour at room temperature. Blots were incubated in LumiGLO (Cell Signaling Technology, Beverly, MA) reagent for 5 minutes, and then exposed. Protein signal strengths were quantitated by densitometric analysis using software from Scion Corporation (Frederick, MD) and then values were converted to the relative ratio of DNA-PKcs:actin. The assay was performed three separate times. Statistical significance was calculated using a Student's *t* test.

Results

Gene expression profiling of childhood astrocytomas

To determine differential expression of specific receptor tyrosine kinase and receptor tyrosine kinase-associated genes in pediatric low-grade and high-grade astrocytomas, we performed Affymetrix microarray gene expression profiling (~30,000 gene sequences) of 30 primary non-brainstem astrocytomas (15 high-grade, 15 low-grade) obtained at the time of diagnosis and prior to treatment. Genes were ranked according to *P* value and the top 50 differentially expressed genes were screened for the presence of known receptor tyrosine kinase or receptor tyrosine kinase-associated genes. In order of increasing *P* value, three receptor tyrosine kinase/receptor tyrosine kinase-associated genes were significantly upregulated in high-grade astrocytomas compared with low-grade astrocytomas: 1) insulin-like growth factor-binding protein 2 (*IGFBP2*), 2) *EGFR*, and 3) ephrin receptor B4 (*EPHB4*); and six receptor tyrosine kinase/receptor tyrosine kinase-associated genes were significantly down regulated in high-grade astrocytomas compared with low-grade astrocytomas: 1) insulin-like growth factor I (*IGF-I*), 2) fibroblast growth factor 1 (*FGF1*), 3) fibroblast growth factor 2 (*FGF2*), 4) granulocytemonocyte colony stimulating factor receptor (*GMCSFR*), 5) brain-derived neurotrophic factor receptor (*TRKB*), and 6) granulocyte colony stimulating factor receptor (*GCSFR*). The results of the statistically significant differentially expressed receptor tyrosine kinase and receptor tyrosine kinase-associated genes, defined as genes with threshold P value < .01, are summarized in Table 1.

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Validation of IGFBP2 protein overexpression by high-grade astrocytomas

IGFBP2 was selected for protein validation and further investigation because of its highly significant differential messenger RNA overexpression (P = .0006). An independent set of 31 paraffin-embedded astrocytomas was used for IGFBP2 protein expression validation by immunohistochemistry. Nine of 13 (69%) high-grade astrocytomas had positive staining, whereas only 5 of 18 (28%) low-grade astrocytomas had positive staining (P = .027, Fisher exact test). IGFBP2 protein expression was detected diffusely in high-grade tumor cells throughout the tumor sections and intense staining was noted in focal clusters of cells within sections. Positive staining did not appear to be associated with areas of necrosis, vascularity, or high cell density. Although some low-grade tumors had detectable diffuse IGFBP2 expression, intense staining of IGFBP2 in focal clusters of cells was only observed in high-grade tumors (Figure 1). IGF-I protein expression was relatively decreased in high-grade tumors, while IGF-IR protein was similarly expressed between the tumor classes (results not shown), consistent with the microarray data results. IGFBP2 protein was not detected in normal brain. Table 2 summarizes the immunohistochemistry results.

Effect of IGFBP2 stimulation on astrocytoma cell migration

The IGF system has been reported to influence tumor cell migration and astrocytoma invasion.[18–21] To determine whether IGFBP2 directly modulates the migration of astrocytoma cells, we tested four astrocytoma cell lines stimulated with IGF-I or IGF-II (both 10 ng/mL), with or without IGFBP2 (50 ng/mL) treatment, and compared the migration rates obtained after stimulation using an in vitro fibronectin-coated Boyden chamber migration assay. Fibronectin coating was used because of previous reports suggesting that IGF facilitates cell migration on fibronectin by interaction with the fibronectin-binding integrins.[22–24] Incubation with increasing concentrations of IGFBP2 alone (5 ng/mL to 50 ng/mL) did not affect the migration of astrocytoma cells on fibronectin compared with baseline untreated control cells (data not shown). Compared with controls, IGF-I and IGF-II modestly increased cell migration in a consistent fashion in all four cell lines tested, whereas IGFBP2 only minimally inhibited IGF-I-mediated migration of astrocytoma cells (Figure 2). IGFBP-2 had no effect on IGF-II-mediated migration. IGFBP2 stimulation, with or without IGF-I or IGF-II, also had no effect on the growth of any of the cells tested, as measured by cell counts, under the same conditions (data not shown).

IGFBP2 stimulation induces astrocytoma DNA-PK gene expression

To elucidate whether IGFBP2 plays a functional role in modulating astrocytoma gene expression that may promote the high-grade phenotype independent of the IGF system, we performed serial microarray gene expression profiling, using the Affymetrix U133 array (~30,000 characterized gene sequences), of serum-depleted T98 and U87 cells stimulated with IGFBP2, IGF-I, or Minimal Essential Medium alone (negative baseline control). At 1 hour, only one transcript, DNA-dependent protein kinase catalytic subunit (*DNA-PKcs*), was consistently upregulated by stimulation with IGFBP2 but not affected by IGF-I, compared with the Minimal Essential Medium-stimulated baseline negative control cells in all three separate experiments and in both cells. On the basis of this unexpected finding, we reanalyzed the initial tumor profiling results and noted that *DNA-PKcs* was also significantly upregulated in high-grade astrocytomas in comparison with low-grade astrocytomas (*P* = .0008, Table 3). Only one gene, protein tyrosine kinase 9 (*PTK9*), was differentially downregulated at 1 hour after IGFBP2 stimulation of both cells; however, *PTK9* was not found differentially expressed by the tumors. At all other time points tested, no other oncogenic kinases were consistently differentially expressed in comparison with baseline control cells in an IGF-independent manner in both cells.

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IGFBP2 stimulation induces astrocytoma DNA-PK protein expression

To validate that IGFBP2 stimulation induces astrocytoma cell DNA-PKcs expression independent of the IGF system, we stimulated T98 cells with increasing concentrations of IGFBP2 and over different time points and compared DNA-PKcs expression, as measured by Western blot of whole cell lysates, with that of cells stimulated with IGF-I. IGFBP2 stimulation induced protein expression of DNA-PKcs in both a dose- and time-dependent manner, while IGF-I had no significant effect on the protein expression of DNA-PKcs (Figure 3). Similar results were observed in U87 cells (not shown).

Discussion

We show for the first time that IGFBP2 and *DNA-PKcs* are highly overexpressed by high-grade childhood astrocytomas at the protein and transcriptional level, respectively. Moreover, in comparison to all other receptor tyrosine kinase or receptor tyrosine kinase-associated genes screened, we identified *IGFBP2* as most overexpressed in high-grade astrocytomas, followed by *EGFR* and *EPHB4*. In fact, these three genes were the only receptor tyrosine kinase-associated genes differentially overexpressed with P value < .01, thereby implicating *IGFBP2*, *EGFR*, and *EPHB4* as the major receptor tyrosine kinase-associated genes characterizing the malignant phenotype of childhood astrocytoma. Lastly, we show that IGFBP2 directly induces DNA-PKcs messenger RNA and protein expression in a dose- and time-dependent manner, independent of IGF, thus demonstrating a novel function for IGFBP2 in astrocytoma cells that could have important clinical implications.

High-grade pediatric astrocytomas overexpress EGFR, a potent mitogenic receptor.[13] Our results confirm this finding and also show that EGFR is significantly differentially expressed. Expression of the ephrin receptor, EPHB4, which has not been shown before in childhood astrocytomas, is an important regulator of angiogenesis.[25] Elevated concentrations of IGFBP2 have been reported in the cerebrospinal fluid of children with high-grade brain tumors compared with peripheral tumors.[26] We previously demonstrated, in a separate cohort of pediatric astrocytomas, the overexpression of *IGFBP2* in high-grade astrocytomas in an analysis that was restricted to 133 angiogenesis-associated genes.[16] Surprisingly, our analysis of ~30,000 genes in this larger cohort of pediatric astrocytomas showed that *IGFBP2* maintained its significance as the most discriminating gene for malignant astrocytoma among receptor tyrosine kinase-associated genes. In adult glioblastoma, *IGFBP2* is similarly overexpressed and expression correlates directly with tumor grade.[27,28] In diffuse astrocytomas in adults, IGFBP2 was the most distinct progression-related change found, correlating with progression and poor patient survival.[29] Another study similarly reported that *IGFBP2* discriminated primary from nonprimary glioblastoma in adults and that *IGFBP2* was coexpressed with vascular endothelial growth factor (*VEGF*) in areas of tumor necrosis.[30] More recently, a significant positive correlation was demonstrated between expression of *IGFBP2*-matrix metalloproteinase-2 (*MMP2*) and *IGFBP2-VEGF* in adult glioblastoma, but not anaplastic astrocytoma.[31] Together, these findings suggest a potential important role for IGFBP2 in astrocytoma invasion and angiogenesis.

The IGF system, composed of two peptide ligands (IGF-I and IGF-II), six high-affinity IGF-binding proteins (IGFBP1 to IGFBP6), and two IGF receptors (IGF-IR and IGF-IIR), has mitogenic and anti-apoptotic effects on malignant cells.[32] IGF-I and IGF-II appear to exert their mitogenic signaling through IGF-IR.[32] IGFBPs tightly regulate the biological actions of IGFs by sequestering IGFs away from their receptors and targeting for degradation.[32] Overexpression of *IGF-I*, *IGF-II*, and their respective receptors has been shown in adult astrocytomas, and expression correlates with histologic grade.[33,34] In contrast, our study did not find differential expression of *IGF-II*, *IGF-IR*, or *IGF-IIR*, indicating that the biology of

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the IGF system in astrocytomas in children may be distinct from that in adults. Moreover, we show that *IGF-I* is downregulated in high-grade astrocytomas, suggesting that the regulation of *IGFBP2* expression, and consequently its function, is independent of the IGF system in these tumors. This differs from normal neurodevelopment, whereby IGF-I and IGFBP2 expression is temporally synchronized, peaking together during postnatal glia maturation.[35] Thus, it appears that the IGF system is dysregulated in childhood malignant astrocytomas. Because of our data demonstrating overexpression of IGFBP2 on the messenger RNA and protein level, we explored the possibility of IGF-independent functions for IGFBP2 in promoting the aggressive behavior of malignant astrocytomas.

While accumulating evidence demonstrates that IGFBP2 is also overexpressed in non-central nervous system malignancies,[36,37] its functional role in tumor progression remains unclear. Glioblastoma cells stably transfected with *IGFBP2* demonstrated enhanced invasion and activation of invasion-promoting genes such as *MMP2*.[20] However, in vascular smooth muscle cells, exogenous IGFBP2 inhibited IGF-I-stimulated migration and DNA synthesis.[38] A more recent report showed that integrin $\alpha v \beta 3$ and IGFBP2 act cooperatively to reduce breast cancer cell migration.[39] In contrast to these reports, we found no direct effect of IGFBP2 stimulation alone on astrocytoma cell migration and only minimal inhibition of IGF-I-mediated (but not IGF-II-mediated) migration on fibronectin. Moreover, our data of astrocytoma cells exposed to IGFBP2 did not support a role for IGFBP2-mediated gene expression promoting growth, invasion, or migration.

Evidence suggests that IGFBP2 may have IGF-independent effects.[32] In support of this, we show for the first time that messenger RNA and protein expression of DNA-PKcs is induced by IGFBP2 stimulation of astrocytoma cells, yet is unaffected by IGF-I stimulation. DNA-PKcs is a nuclear serine/threonine kinase that plays a crucial role in repairing double-strand breaks secondary to radiation- and chemotherapy-induced damage, and overexpression of DNA-PKcs activity in tumor cells confers resistance to killing by ionizing radiation.[40–42] Interestingly, a recent report demonstrated that blockade of radiation-induced import of EGFR to the nucleus abolished DNA-PK activation, suggesting that EGFR expression may also be crucial to DNA-PK repair function.[43] DNA-PK also controls *c-myc* and *MAGE-A* gene transcription and phosphorylates key proteins regulating cell cycle and apoptosis, including p53, transcription factor Sp1, myc, replication protein A, and RNA polymerase I.[44–46] Our data confirm that *DNA-PKcs* is differentially overexpressed in high-grade tumors. Expression of IGFBP2 is regulated by the hypoxia-inducible transcription factors, HIF-1α and HIF-2α.[32] We have previously shown that HIF-2α is also significantly upregulated in high-grade childhood astrocytomas.[16] Because hypoxic tumor areas are relatively radiation resistant, we speculate that one way high-grade astrocytomas may acquire radiation resistance is through HIF-mediated IGFBP2 expression, which in turn promotes DNA-PKcs expression that is activated by EGFR nuclear import.

In summary, we conclude that IGFBP2 is highly differentially overexpressed by malignant childhood astrocytomas and that a novel function of IGFBP2, independent of the IGF system, is to induce DNA-PKcs expression. Thus, together with EGFR-mediated activation of DNA-PKcs, IGFBP2 may serve to protect astrocytomas from therapeutic killing, thereby contributing to the overall malignant potential of these cells. Further studies are needed to confirm these mechanistic associations and to delineate how IGFBP2 upregulates DNA-PKcs. Ultimately, these studies may help define IGFBP2 as a novel target for therapeutic intervention in high-grade childhood astrocytomas.

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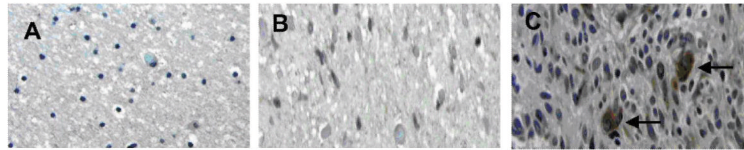

Figure 1.
Validation of IGFBP2 differential protein expression in childhood astrocytomas by immunohistochemistry. Representative images demonstrate A) absence of IGFBP2 staining in normal brain, B) weak, diffuse IGFBP2 staining in low-grade astrocytoma, and C) strong, diffuse IGFBP2 staining in high-grade astrocytoma; arrows indicate focal areas of intense IGFBP2 positive staining. Photographed by light microscopy at 200×

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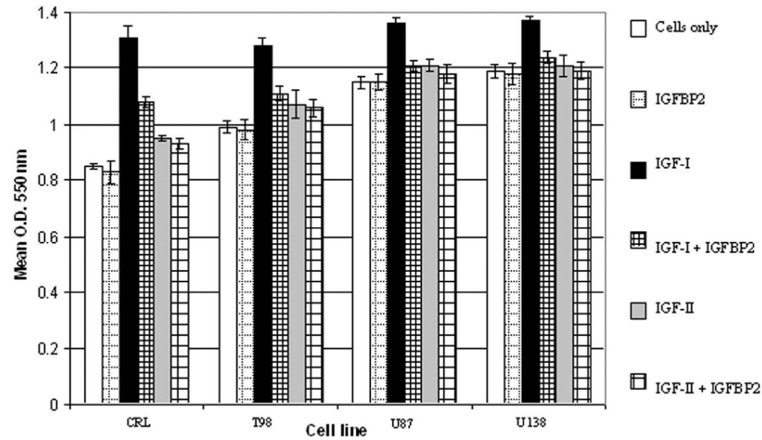

Figure 2.

IGFBP2 modulation of IGF-mediated astrocytoma cell migration. Transwell migration assay of astrocytoma cells CRL, T98, U87, and U138 on fibronectin stimulated with IGFBP2 alone (50 ng/mL) or IGF-I or IGF-II (10 ng/mL), with or without IGFBP2 (50 ng/mL), for 30 minutes. IGFBP2 minimally inhibited IGF-I-mediated but not IGF-II- mediated cell migration. Increasing concentrations of IGFBP2 alone (5 ng/mL to 50 ng/mL) had no effect on migration over controls (not shown). Optical density (OD) values are the average of three separate experiments and error bars indicate standard deviation. Statistical significance was calculated using a Student *t* test

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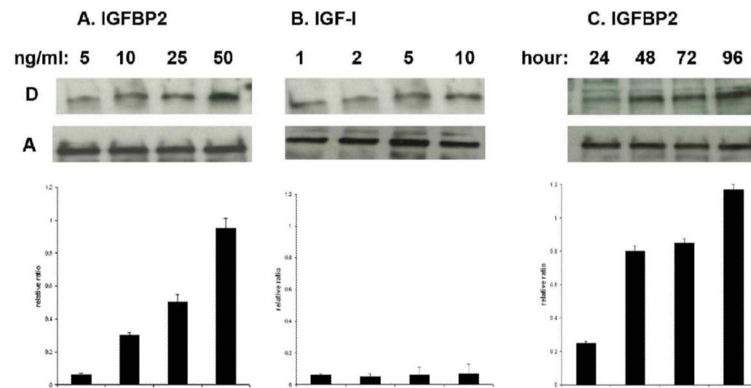

Figure 3.
IGFBP2 induces DNA-PKcs protein expression in a dose- and time-dependent manner. Western blot of T98 whole cell lysates for DNA-PKcs after 24-hour stimulation with A) IGFBP2 (5 ng/mL to 50 ng/mL), B) IGF-I (1 ng/mL to 10 ng/mL), and C) IGFBP2 (10 ng/mL) measured at 24 hours to 96 hours post-stimulation. Bar graphs show the relative ratio of DNA-PKcs:actin that was used to determine the relative change in DNA-PKcs expression after stimulation with IGFBP-2 and IGF-I. IGF-I had no effect on DNA-PKcs induction over time and similar results were observed with IGFBP-2 stimulation of U87 cells (not shown). Statistical significance was calculated using a Student *t* test. D indicates DNA-PKcs, A indicates actin control

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Table 1

Differentially Expressed ($P < .01$) Receptor-Tyrosine Kinase-Associated Genes in Childhood Astrocytomas

Gene Name	Affymetrix Probe ID	Genbank ID	Average Fold Change	*P* Value[a]
Increased in HGA				
IGFBP2	40422_at	X16302	14.06	.0006
EPHB4	1351_at	U07695	11.96	.0022
EGFR	1537_at	X00588	54.25	.0031
Decreased in HGA				
TRKB	36042_at	X75958	4.97	.0006
IGF-I	38737_at	X57025	2.20	.0008
FGF1	996_at	X59065	3.96	.0015
FGF2	1593_at	J04513	1.90	.0020
GMCSFR	33665_s_at	M73832	9.08	.0043
GCSFR	34223_at	M59818	1.18	.0092

Abbreviations: *EGFR*, epidermal growth factor receptor; *EPHB4*, ephrin receptor B4; *FGF1*, fibroblast growth factor 1; *FGF2*, fibroblast growth factor 2; *GCSFR*, granulocyte colony-stimulating factor receptor; *GMCSFR*, granulocyte macrophage colony-stimulating factor receptor; HGA, high-grade astrocytoma.; *IGF-I*, insulin-like growth factor I; *IGFBP2*, insulin-like growth factor binding protein 2; *TRKB*, tyrosine receptor kinase B.

[a] Significance in differential gene expression between tumor classes was determined using permutational *t* test statistics.

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Table 2

Immunohistochemistry Results for IGFBP2 in 13 High-Grade and 18 Low-Grade Childhood Astrocytomas

	High-Grade Astrocytoma	Low-Grade Astrocytoma
1.	++++[a]	++++
2.	++++	++++
3.	++++	++
4.	+++	+
5.	+++	+
6.	++	–
7.	++	–
8.	+	–
9.	+	–
10.	–	–
11.	–	–
12.	–	–
13.	–	–
14.	ND	–
15.	ND	–
16.	ND	–
17.	ND	–
18.	ND	–
Total positive	9/13	5/18
% positive	67%	27%
***P* value** [b]	.027	

[a]Staining positive detection score denoted as absent (–), weak (+), intermediate (++), intermediate-strong (+++), intense (++++), or not done (ND).

[b]Significance of differences in positive/negative staining between tumor classes was determined using a Fisher exact test.

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Table 3

Differentially Expressed Kinase Genes ($P < .01$) in Astrocytoma Cells Following IGFBP2 Stimulation for 1 h and in High-Grade Astrocytomas

Gene Name	GenBank ID	Average Fold Change[a]	*P* Value[b]
Astrocytoma cells + IGFBP2			
DNA-PKcs (increased)	U47077	1.2	<.01
PTK9 (decreased)	U02680	0.4	<.01
High-grade astrocytomas			
DNA-PKcs (increased)	U47077	2.37	.0008
PTK9 (no change)	U02680	NC[c]	NS[c]

Abbreviations: *DNA-PKcs*, DNA-dependent protein kinase catalytic subunit; *PTK9*, protein tyrosine kinase 9.

[a]Data presented in log base 2.

[b]Significance in differential gene expression between experimental classes (test and controls) and tumor classes was determined using permutational *t* test statistics.

[c]NC, no change; NS, not significant.