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Effects of ß3-adrenergic receptor agonist on gene expression of leptin in glioblastoma

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Abstracts

LAB-CELL BIOLOGY AND SIGNALING

CB-01. THE EFFECT OF WT1 ON TUMORGENECITY, CELL PROLIFERATION, AND APOPTOSIS IN GLIOBLASTOMA

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Glioblastoma (GBM), one of the most frequently occurring malignancies in the central nervous system, still has a very poor prognosis. To improve the prognosis of GBM patients, various attempts have been made. Immunotherapy targeting Wilms' tumor 1 (WT1) has proved to be effective in GBM (Izumoto et al. 2008). However, the functional roles of WT1 in GBM have not been intensively studied. In this study, we aim to examine the functional roles of WT1 in GBM. We established WT1 shRNA knocked down GBM cell lines (U87 and U251) and examined the functional roles in vitro and in vivo. In cell-proliferation assays, we plated 3×10^4 cells in 12-well plates. On day 4 the numbers of the proliferated cells were $(39 \pm 7.8) \times 10^4$, $(19 \pm 3.5) \times 10^4$, $(17 \pm 6.5) \times 10^4$, and $(3.4 \pm 0.5) \times 10^4$ cells in the U87 control shRNA, U87 WT1 shRNA, U251 control shRNA, and U251 WT1 shRNA, respectively ($P < 0.05$). Furthermore, an Annexin V apoptosis assay showed the numbers of the apoptotic cells per 30 times magnified microscopic field were 2.5 ± 0.6, 15.3 ± 5.7, 3.0 ± 1.7, and 9.0 ± 1.0 cells in the U87 control shRNA, U87 WT1 shRNA, U251 control shRNA, and U251 WT1 shRNA, respectively ($P < 0.05$). In the in vivo experiment, U87 control shRNA and U87 WT1 shRNA cells were intracranially injected into newborn pups of immunodeficient mice. At day 30, all of the mice transplanted with U87MG control shRNA developed GBM, whereas none of the mice transplanted with U87MG WT1 shRNA developed GBM. These results suggest that WT1 is involved in GBM cell proliferation, apoptosis, and tumor formation.

CB-02. RESULTS OF IMMUNOHISTOCHEMICAL STAINING OF CELL-CYCLE REGULATORS—THE PREDICTION OF RECURRENCE-FREE SURVIVAL OF ANTERIOR PITUITARY ADENOMA

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To investigate the possible prognostic values of several cell-cycle regulators for the prediction of anterior pituitary adenoma (APA) recurrence after surgical resection, we immunohistochemically analyzed tumor samples obtained by surgical resection. The study population consisted of 156 patients with APA diagnosed from January 2000 to December 2009 at the Department of Neurosurgery at Samsung Changwon Hospital in Korea. By retrospectively reviewing medical records, we examined clinical manifestations associated with excess adenohypophysial hormone and biochemical study findings for serum adenohypophysial hormone. Immunohistochemical staining was performed on archived paraffin-embedded tissues obtained by surgical resection for adenohypophysial cells and cell-cycle regulatory proteins (p16, p15, p21, CDK 4 and 6, RB protein, and cyclin D1). Of the 156 adenomas, 55 (35.3%) recurred during the follow-up period (mean duration 58.5 months, range 24.2 to 118.9 months). There were 59 (37.8%) functional adenomas, 19 (12.2%) clinically silent adenomas, 57 (36.5%) true silent adenomas, and 21 (13.5%) null cell adenomas. Immunohistochemically, p16 stained positively in 79 samples (50.6%), p15 in 11 samples (7.1%), p21 in 14 samples (9.0%), CDK4 in 53 samples (34.0%), CDK6 in 5 samples (3.2%), RB protein in 57 samples (36.5%), and cyclin D1 in 74 samples (47.4%). Mean duration to recurrence after surgery was 24.8 months (range 3.4 to 80.6 months). Multivariate analysis using the Cox regression model showed that p16 positivity (HR of 1.62, 95% CI of 1.27-1.97; $P = 0.045$), Rb protein negativity (HR of 2.30, 95% CI of 1.68-2.92; $P = 0.007$), cyclin D1 negativity (HR of 2.16, 95% of 1.53-2.79; $P = 0.012$), functional adenoma (HR of 1.84, 95% CI of 1.42-2.26; $P = 0.029$), and null cell adenoma (HR of 2.26, 95% CI of 1.53-2.99; $P = 0.008$) predicted longer recurrence-free survival. Conclusively, our findings indicate that p16, RB protein, and cyclin D1 are associated with longer recurrence-free survival in APA.

CB-03. FIBULIN-3 PROMOTES GLIOMA GROWTH AND RESISTANCE THROUGH A NOVEL PARACRINE REGULATION OF NOTCH SIGNALING

Bin Hu, Hosung Sim, Nandhu Mohan, Paula Agudelo-Garcia, Gerard Nuovo, Susan Cole, and Mariano S. Viapiano; The Ohio State University, Columbus, OH, USA

Malignant gliomas are highly invasive and chemoresistant brain tumors with extremely poor prognosis. Glioma invasion is strongly associated with the resistance of these tumors to therapy, but the mechanisms that underlie this association are poorly understood. Targeting soluble factors triggering invasion and resistance could substantially affect the difficult-to-reach, infiltrative glioma cells that are a major source of recurrence. Fibulin-3, a matrix protein absent in normal brain tissue but upregulated in gliomas, promotes tumor invasion by unknown mechanisms. We show here that fibulin-3 is a novel soluble activator of Notch signaling that antagonizes DLL3, an autocrine inhibitor of Notch, and promotes tumor-cell survival and invasion in a Notch-dependent manner. Using a strategy for inducible knockdown, we demonstrate that controlled downregulation of fibulin-3 reduces Notch signaling and leads to increased apoptosis, reduced self-renewal of glioblastoma-initiating cells, and impaired growth and dispersion of intracranial tumors. Finally, we show that fibulin-3 expression correlates with expression levels of Notch-dependent genes (Hes1, Hes5) and is a marker of Notch activation in clinical gliomas. These results underscore a major role of the tumor extracellular matrix in regulating glioma invasion and resistance to apoptosis via activation of the key Notch pathway. More importantly, this is the first description of a noncanonical, soluble activator of Notch in a cancer model and a demonstration of how Notch signaling can be reduced by targeting tumor-specific accessible molecules in the tumor microenvironment.

CB-04. NF-κB INDUCED IL-6 ENSURES STAT3 ACTIVATION AND TUMOR AGGRESSIVENESS IN GLIOBLASTOMA

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Glioblastoma (GBM) is the most aggressive, neurologically destructive, and deadliest tumor of the CNS. Two signaling proteins, NF-κB and STAT3, have been linked to the mesenchymal genetic subtype, the most malignant subtype of GBM. Our lab has previously shown that both NF-κB and STAT3 are overly abundant and active in GBM and that both signaling pathways activate numerous pro-survival genes. In addition, the NF-κB and STAT3 pathways contribute to cellular communication within the tumor environment, activating additional non-tumor cells, particularly immune cells, that then suppress immune defense mechanisms and allow continued growth of the tumor. In this study, we have evaluated the ability of NF-κB and STAT3 to participate in a vicious cycle of cross-talk, which ultimately ensures that GBM tumors survive, proliferate, invade healthy tissue, and resist therapeutic efforts. In vitro, we demonstrated that TNF-α-induced activation of NF-κB is sufficient to induce interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) expression, activate STAT3, and elevate STAT3 target gene expression in cultured human and mouse glioma cell lines. Furthermore, TNF-α-induced STAT3 activation, as well as IL-6, LIF, and SOCS-3 expression are dependent on NF-κB p65 and require signaling through the JAK/gp130 complex. In vivo, we have determined that TNF-α-induced STAT3 activation occurs in other glioma sources, including human GBM xenograft tumors. Using pharmacological inhibition and shRNA techniques targeting NF-κB and STAT3, we are currently evaluating the effect of NF-κB and STAT3 inhibition on tumor growth and survival in subcutaneous and orthotopic intracranial mouse models of GBM. We believe that these studies will verify that pharmacological interventions to effectively inhibit the activity of both NF-κB and STAT3 will correlate with reduced GBM size and aggressiveness and will be imperative to develop more accurate pharmacological clinical interventions for patients with GBM tumors.

CB-05. GLIOBLASTOMA CELLS EXPRESSING EGFRVIII ARE MORE SENSITIVE TO CK2 INHIBITION

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Glioblastoma (GBM) is the most common and fatal primary brain tumor in humans. Since no cure exists for GBM, it is essential to develop new and better therapies to treat this disease. Previous research has shown that an oncogenic kinasecasein kinase 2 (CK2) may be a promising therapeutic target for GBMs. CK2 has enhanced expression or activity in a wide variety of cancers, including GBM, and it was demonstrated that inhibitors to CK2 regressed tumor growth in GBM xenograft mouse models. Through our own work, we demonstrated that GBM patients with a high expression of CK2 had a much worse prognosis than did patients with low levels. Currently, the mechanisms enabling enhanced expression or activity of CK2 are still unknown. Our studies demonstrated that a deletion mutant of the epithelial growth factor receptor (EGFRvIII) is involved in CK2-dependent tumorigenesis in GBM cell lines. We generated GBM cell lines (U87-MG, U138) that stably express EGFRvIII and showed that these cells are more sensitive to depletion of CK2. Initial studies showed that siRNAs specific to a particular CK2 subunit (CK2alpha) decreased GBM cell growth approximately by 50% in the control GBM cells. Interestingly, expression of EGFRvIII sensitized the GBM cells to growth arrest since cell growth was reduced 14%-20% with reduced expression of CK2alpha. In addition, inhibition of CK2alpha activity using commercially available inhibitors (TBB, TBBz) also reduced GBM cell growth (50%-66%), but we observed a more dramatic reduction (25%-50%) in EGFRvIII-overexpressing cells. We have also conducted some preliminary studies demonstrating that CK2 alpha kinase activity in vivo is enhanced by EGFRvIII expression. Our results suggest that EGFRvIII may play an important role in GBM tumorigenesis by regulating CK2 activity, and a combination treatment targeting both EGFRvIII and CK2alpha might be more efficacious than targeting each one individually.

CB-06. A NOVEL METHOD OF SONIC HEDGEHOG SIGNALING ACTIVATION IN THE CHEMORESISTANCE OF GLIOBLASTOMA: A LIGAND-INDEPENDENT MECHANISM

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Glioblastoma ultiforme (GBM) is the most common and lethal adult primary tumor of the central nervous system. Despite current chemotherapy with concomitant surgical resection and radiotherapy, GBM 5-year survival is approximately 3%. A better understanding of the cellular pathways in tumor proliferation and survival is required to develop new targeted treatments. Previously, the Sonic Hedgehog (SHH) pathway has been shown to be dysregulated in chemoresistant GBM. Pharmacological inhibition of SHH signaling with cyclopamine enhances GBM sensitivity to temozolomide (TMZ). The SHH receptor PTCH1 tonically represses signaling, and repression is removed by ligand binding followed by receptor-mediated endocytosis. Here we show SHH signaling in GBM is ligand-independent and is initiated by post-transcriptional regulation of PTCH1. MicroRNA (miR), small non-coding RNA molecules, regulate a number of cell processes such as development and oncogenesis. As far as we are aware, there is no report on microRNA regulation of PTCH1. Knockdown of Dicer, a Type III RNAase, which is required for miR synthesis, increased the cellular toxicity to TMZ, thus revealing the importance of miR in GBM chemoresistance. We have identified and characterized PTCH1 as a functional target of microRNA-9 (miR-9) in both non-neural and GBM cell lines. Overexpression of miR-9 in GBM cells results in downstream pathway activation and resistance to TMZ treatment. We also explored a number of mir-9/PTCH1-dependent mechanisms of chemoresistance and uncovered downstream upregulation of the ATP-binding cassette drug efflux transporters, MDR1 and ABCG2. Taken together, our data indicate a role for miR-9 in the acquisition of chemoresistance in GBMs and suggest that miR-9 may serve as a novel therapeutic target.

CB-07. MECHANISMS OF CHEMORESISTANCE IN CD133-EXPRESSING GLIOBLASTOMA CELLS: THE ROLE OF DRUG EFFLUX TRANSPORTERS

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Glioblastoma multiforme (GBM) is the most common and lethal adult primary tumor of the central nervous system. Despite current chemotherapy with concomitant surgical resection and radiotherapy, GBM 5-year survival is approximately 3%. A better understanding of the cellular pathways in tumor proliferation and survival is required to develop new targeted treatments. Although controversial, CD133 (Prominin-1) has been used as a cell-surface marker to enrich for cells exhibiting a primitive phenotype. We have isolated CD133-expressing cells from U87 and T98G GBM cell lines and confirmed their dose-dependent chemoresistance to temozolomide (TMZ). The mechanisms that underlie this chemoresistance are currently unknown. TMZ is an O6-methylguanyl alkylating agent, and resistance to TMZ may arise through a number of ways. We explored cell-cycle regulation in CD133-expressing GBM and have confirmed no difference in the cycling status of these cells. ATP-binding drug efflux pumps can bind to compounds such as TMZ and transport them into the extracellular space. MDR1 (ABCB1) and BCRP (ABCG2) are two such transporters known to be upregulated in GBM. Gene expression analysis confirms increased expression of both MDR1 and BCRP in CD133 GBM cells. Inhibition of MDR1 and BCRP by cyclosporin A (CyA) resulted in increased cell death when treated with TMZ. Knockdown by siRNA of both transporters confirmed their importance in GBM chemoresistance. Taken together, our data indicate that CD133 GBM resistance to TMZ is not caused by cell-cycle detention but rather by MDR1/ABCG2 upregulation.

CB-08. NF1-DEFICIENT GLIOBLASTOMAS ACQUIRE MEK INHIBITOR RESISTANCE THROUGH RECEPTOR TYROSINE KINASE OR PI3K/MTOR UPREGULATION

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The Ras pathway is aberrantly activated in most glioblastomas (GBM) and signals downstream through RAF/MEK/ERK and the PI3K pathways to promote growth and survival. In the mesenchymal subset of glioblastomas, Ras activation is associated with mutations and/or deletions of NF1, a negative regulator of Ras signaling. We previously identified a subset of NF1-deficient GBM that was especially sensitive to the single agent MEK inhibitors PD0325901 or AZD6244, suggesting that NF1-deficient GBMs may be ideal candidates for MEK inhibitor-based trials. However, prolonged exposure to targeted therapies ultimately leads to tumor relapse. Understanding the mechanisms of acquired resistance can help identify novel therapies and may also help us better understand the mechanisms of intrinsic resistance. To investigate the mechanisms of acquired resistance in NF1-deficient GBM, we chronically treated MEK inhibitor-sensitive LN229 and U373 GBM cells with 1 μM PD0325901 for 4-8 weeks, isolated resistant colonies, and expanded them as clonal populations. LN229 MEK inhibitor-resistant clones demonstrated increased levels of the receptor tyrosine kinases (RTKs) phospho-EGFR and phospho-PDGFRβ, which were associated both with increased levels of the RAF/MEK/ERK effectors phospho-ERK and cyclin D1 and with sensitization to combination treatment with erlotinib and imatinib (RTK inhibitors of EGFR and PDGFR, respectively). By contrast, U373 MEK inhibitor-resistant clones did not demonstrate upregulation of RTK signaling but instead demonstrated increased levels of the PI3K/mTOR pathway effectors phospho-AKT, phospho-S6, and phospho-4EBP1 and sensitization to PI-103, a dual PI3K/mTOR inhibitor. Together these data suggest that NF1-deficient GBM acquire MEK inhibitor resistance through multiple mechanisms, including upregulation of RTK signaling that leads to reactivation of the target pathway or through activation of alternative pathways, such as PI3K/mTOR. Future therapies designed at inhibiting upstream RTK signaling or that target PI3K/mTOR signaling may be useful for treating NF1-deficient GBM patients who relapse upon prolonged MEK inhibitor therapy.

CB-09. MICRORNA 768-3P TARGETS EXPRESSION OF KRAS AND PAK1 AND IS DOWNREGULATED IN HUMAN GLIOBLASTOMA

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Glioblastoma multiforme (GBM) is the most lethal primary brain tumor. Dysregulation of microRNA (miRNA) expression occurs in GBM and may drive tumor growth and recurrence. We have identified miR-768-3p as one of the most downregulated miRNAs using a microarray analysis of miRNA dysregulation in GBM stem cells. We verified miRNA expression using Taqman qPCR in the microarray samples and further validated that this miRNA is downregulated in human GBM as compared with normal brain tissue. Indeed, in seven of twelve GBM specimens, 768-3p was undetectable. Given that the biological relevance of miR-768-3p is totally unknown, we evaluated potential targets. Targetscan and miRbase suggested KRAS and PAK1 as potential target mRNAs. KRAS and PAK1 interact in the same cell-migration pathway and both proteins have been proposed to contribute to GBM growth and invasion. qPCR and immunoblot analysis of KRAS and PAK1 in 768-3p-treated glioma cells indicated that both targets were likely regulated by miR-768-3p. Forced expression of miR-768-3p in

GBM cell lines resulted in decreased cell numbers and viability in GBM but not in normal astrocytes. Migration of miR-768-3p-expressing GBM cells toward serum was decreased by approximately 50% in several GBM lines, an expected result given the antagonism of KRAS and PAK1 expression by miR-768-3p. The relevance of miR-768-3p as a tumor suppressor will be further investigated in future studies.

CB-10. NOVEL NF-KAPPAB DECOY OLIGONUCLEOTIDE DERIVED FROM MGMT ENHANCER INDUCES AN ANTINEOPLASTIC EFFECT IN HUMAN CANCER CELLS

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INTRODUCTION: Alkylating agents show efficacy in the treatment of various tumors including gliomas. High cellular levels of O(6)-methylguanine-DNA-methyltransferase (MGMT) affect the cytoxicity of alkylating chemotherapeutics because it confers the efficient repair of DNA alkylation; thus, MGMT is an ideal pharmocological target. Two pseudosubstrates, O6-benzylguanine (O6-BG) and O6-(4-bromothenyl)-guanine, that aimed to reduce MGMT activity had low efficacy because the combined treatment induced a profound hematologic toxicity and the MGMT levels rapidly recovered. Accordingly, the aim of the present study was to develop a chemopotentiation treatment that will effectively and specifically reduce MGMT expression in tumors without an increase in toxicity in normal tissue. RESULTS: We previously described two NF-kappaB binding sites within the MGMT enhancer and proved the pivotal role of kappaB1 site in MGMT expression. We assumed that interference with the binding of NF-kappaB to MGMT enhancer will attenuate MGMT expression and consequently will reverse MGMT-induced chemoresistance. For that purpose, we designed locked nucleic acid modified decoy oligonucleotides (LMODNs) corresponding to the specific sequence of MGMT-kappaB1. We demonstrated in the HEK293T cell line that MGMT-kB1 LMODNs interfere with the binding of NF-kappaB to the MGMT enhancer. The treatment effect of these LMODNs was studied in vitro in three human cancer cell lines. MGMT-kB1-LMODN concentrations of 2 nM for T98G or 1 uM for MCF-7 and ACHN lines significantly augmented ($P < 0.05$) temozolomide-induced cell killing by 2, 4.8, and 2.1 times, respectively. Furthermore, MGMT-kB1-LMODN monotherapy in concentrations of 4 nM for T98G or 1 uM for MCF-7 and ACHN induced 70%, 79%, and 68% cell killing. CONCLUSIONS: Our results suggest that MGMT-kB1-LMODN may provide a novel strategy for treating human cancers, and it should be evaluated as a monotherapy or in combination with alkylating agents.

CB-11. TGFα IS THE MAJOR TARGET GENE OF THE CONSISTENTLY UNDEREXPRESSED LARGE CLUSTER OF 42 MICRORNAS LOCATED ON CHROMOSOME 14Q32.31 IN GLIOMAS

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BACKGROUND: We previously demonstrated that in gliomas a cluster of 42 microRNAs (miRNAs) from chromosome 14q32.31 are consistently underexpressed. We hypothesized that this region operates as a tumor suppressor and that dysregulation of the related miRNAs may lead to abnormal expression of their targets, resulting in tumorigenesis. AIM: We aimed to identify the target genes of the miRNAs from the 14q32.31 region and their related oncogenic functions. METHODS: Four bioinformatic algorithms (PITA, TargetScan, PicTar, and miRanda) served to identify the potential target genes of miRNAs from this cluster. Three miRNAs from three different regions on 14q32.31 (miR-323-3p, miR-433, and miR-369-3p) were introduced into mouse and human glioma cell lines, and we evaluated their effect on tumor proliferation and migration. RESULTS: TGFα was selected for further analysis out of the identified candidates because 71% of the miRNAs from this cluster were predicted to target this oncogene. Minor overexpression (approximately 10-fold) of the 2 miRNAs 323-3p and 369-3p significantly reduced the proliferation and migration rate of the human and mouse glioma cell lines, whereas miR-433 had no obvious effect. Overexpression of miR-323-3p and miR-369-3p in the U87 cell line decreased TGFα expression by 70% and 50%, respectively, whereas overexpression of miR-433 had no effect. The ability of these miRNAs to reduce TGFα protein expression was validated by Western blot analysis. CONCLUSIONS: Our study indicates that the large miRNA cluster in chromosome 14q32.31 may function as a tumor-suppressor gene through mechanisms that inhibit tumor cell growth and migration. Our results also suggest that this miRNA cluster suppresses tumors via post-transcriptional regulation of TGFα, as 71% of the underexpressed miRNA from this region targeted the TGFα oncogene. These findings support previous observations that overexpression of TGFα in gliomas augments features of tumor proliferation and migration.

CB-12. PREDICTED SIGNALING PATHWAYS INVOLVED IN THE PATHOGENESIS OF MENINGIOMAS AND EGFL6 AS A POTENTIAL NOVEL SERUM BIOMARKER FOR BENIGN MENINGIOMAS

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PURPOSE: In this study, we investigated the possible signaling pathways involved in the tumorigenesis of benign and anaplastic meningiomas. We also found evidence that EGFL6 serves as a potential novel serum biomarker for benign meningiomas. EXPERIMENTAL DESIGN: Differential gene-expression profiles between meningiomas and brain arachnoidal tissue were established through microarray analysis. KEGG pathway analysis was performed to identify potential signaling pathways that may be involved in the pathogenesis of meningiomas. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the differentially expressed genes in the KEGG pathways. EGFL6 mRNA levels were determined in brain arachnoidal tissue, meningiomas, and other tumors by qRT-PCR. Serum EGFL6 levels were measured via ELISA in healthy people and patients with various tumors. RESULTS: Fibroblastic meningiomas exhibited upregulated PI3K/Akt and TGFβ signaling pathways and accelerated the G1/S progression cell cycle. Focal adhesion and ECM-receptor interaction pathways were activated in anaplastic meningiomas. The EGFL6 gene was overexpressed in benign meningiomas and ovarian cancers but found in low levels in all other tumors. We also found high levels of EGFL6 protein in serum samples of patients with benign meningiomas (675 pg/mL) and ovarian cancers (617 pg/mL). Healthy people and patients with all other tumors, however, had very low levels of serum EGFL6. CONCLUSIONS: Our results suggest that activation of PI3K/Akt and integrin-mediated signaling pathways are involved in the pathogenesis of benign and anaplastic meningiomas, respectively. These findings also suggest that EGFL6 might serve as a novel serum biomarker for benign meningiomas.

CB-013. MITOSIS INTERFERENCE OF CANCER CELLS BY NOVOTTF-100A CAUSES DECREASED CELLULAR VIABILITY

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Cancer cell death from conventional cytotoxic chemotherapy and radiation is a result of triggering the cell-cycle checkpoint, with p53 playing a prominent role in orchestrating this effect. Unlike traditional approaches, the NovoTTF-100A device, which has been approved for the treatment of recurrent glioblastomas, kills tumor cells by a novel mechanism that interferes with their proper progression through mitosis. Our research reveals that the tumor-treating field (TTField) affects processes proximal to the metaphase-to-anaphase transition, leading to cell-cycle arrest in anaphase and eventually to cell death. Tissue culture cells exposed to the TTField during mitosis exhibit a variety of outcomes, including cell arrest in anaphase, cytokinesis defects with cytokinetic furrow regression, and slippage out of mitosis without division. Time-lapsed movies of cells stained with DRAQ5 revealed membrane blebbing and oscillation, together with failed segregation of sister chromatids from the metaphase plate, both of which were initiated near the time of metaphase-to-anaphase transition. Immunofluorescence of cells after short-term TTField treatment during mitosis showed disordered separation of chromosomes from metaphase plates accompanied by lagging chromosomes, dispersion of chromosomes from the metaphase plate, chromosomes decondensation in the absence of cytokinesis, and daughter cells with asymmetric chromosomal segregation. Flow cytometry also revealed the presence of cells with abnormal DNA content. In contrast to the p53 mutant cell line MDA-MB-231, U87 cells with wild-type p53 exhibited increased Annexin V binding after brief TTField exposure during mitosis, suggesting that TTField-induced cellular damage is able to trigger p53-dependent cell death in cells that escape killing during mitosis. These data show that the TTField causes cellular damage during mitosis and raises the hypothesis that the differential responses seen in patients are likely rooted in the underlying genetic alterations of the tumors. This study was supported in part by NovoCure, Inc. and A Reason To Ride research fund.

CB-14. VHL AND PPARα ARE RESPONSIBLE FOR MIR-21-REGULATED EGFR/AKT SIGNALING IN THREE "PTEN" DIFFERENT EXPRESSION STATUS GLIOMA CELL LINES

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The epidermal growth factor receptor (EGFR) is frequently amplified, overexpressed, or mutated in human glioblastomas. MicroRNA-21 (miR-21), shown to target several tumor suppressor genes, functions as an oncogene. In our previous study, we demonstrated that miR-21 could regulate EGFR/AKT signaling independent of PTEN status, though PTEN is a direct target of miR-21. In the present study, we demonstrated that VHL and PPARα are direct targets of miR-21 and that miR-21 regulates EGFR/AKT signaling partly through the VHL/β-catenin and PPARα/AP-1 pathways. The lentivirus-delivered miR-21 inhibitor impairs the in vitro colony forming and invasion in three PTEN different expression status glioma cell lines. We used a luciferase activity assay to identify VHL and PPARα as two direct targets of miR-21. A Top/Fop luciferase acivity experiment showed that miR-21 could regulate EGFR/AKT signaling through the VHL/β-catenin pathway. In miR-21-downregulated glioma cell lines, PPARα siRNA could restore the expression of EGFR and AKT. Our findings suggest that miR-21 regulates EGFR/AKT signaling by targeting VHL and PPARα.

CB-15. GINSENOSIDE RG3-INDUCED APOPTOSIS IN THE HUMAN GLIOBLASTOMA U87MG CELLS THROUGH THE MEK SIGNALING PATHWAY AND REACTIVE OXYGEN SPECIES

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OBJECTIVE: Ginsenoside might be an effective substance for cancer prevention. The major active components in red ginseng represent diverse kinds of ginsenosides, including ginsenoside Rg3, Rg5, and Rk1, and each of these ginsenosides has different pharmacological activities. Among them, ginsenoside Rg3, a kind of chemical extracted from Korean red ginseng (Panaxginseng C.A. Meyer), has been reported to control anti-cancer activities through inhibiting angiogenesis and the proliferation of various kinds of cancer cells. However, the effect of ginsenoside Rg3 and its molecular mechanism in glioblastoma multiforme (GBM) is still unclear. In the present study, we investigated the effect of ginsenoside Rg3 on a human GBM cell line and its regulating signaling molecular mechanisms. MATERIALS AND METHODS: U87MG cells (a human GBM cell line) were treated with ginsenoside Rg3, and we measured cell viability using an MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay and a trypan blue exclusion assay. The apoptotic characteristics were detected by TUNEL [Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling] assay, flow cytometry analysis, and Western blotting. The molecular mechanisms were evaluated through various pre-treated inhibitors (MAPKs inhibitors, general caspase inhibitors, and antioxidants). RESULTS: Ginsenoside Rg3 inhibited U87MG cell viability in a dose- and time-dependent manner. The apoptotic effect was determined based on the positive stained cells by TUNEL assay, immunocytochemistry, and flow cytochemistry. Ginsenoside Rg3-induced apoptosis was associated with the MEK signaling pathway and reactive oxygen species. CONCLUSION: These results might suggest that ginsenoside Rg3 induces apoptosis in U87MG cells and that its effect is controlled by the MEK signaling pathway and reactive oxygen species. Therefore, ginsenoside Rg3 could be an innovative chemotherapeutic agent against malignant glioma.

CB-16. STRESS-REGULATED EXPRESSION OF MIR-451 CONTROLS THE PROLIFERATION/MIGRATION DICHOTOMY OF GLIOBLASTOMA IN VITRO AND IN VIVO AFFECTING RESPONSE TO RADIATION AND CHEMTHERAPY

Daisuke Ogawa, Jakub Godlewski, Agnieszka Bronisz, and E. Antonio Chiocca; The Ohio State University, Columbus, OH, USA

In rapidly growing tumors such as glioblastoma (GBM), where glucose availability may fluctuate, cells must engage adaptive strategies to survive periods of metabolic stress. We previously identified a pathway mediated by microRNA 451 (miR-451) that not only allows rapid proliferation under favorable conditions (high glucose) but also is essential for survival and cell migration under conditions of glucose starvation through modulating the activity of the LKB1/AMPK pathway. Based on our in silico analysis, we selected two candidates for promoting/inhibiting miR-451 expression: Oct1 and PPARγ. By a siRNA approach, we found that Oct1 (known to increase anaerobic glucose metabolism, thus promoting tumor glucose-dependency) promotes miR-451 expression, whereas PPARγ, whose agonists are known anti-neoplastic compounds, inhibited miR-451 expression. We have also demonstrated negative feedback between miR-451 and PPARγ expression. Our results show that although miR-451 is essential for tumor progression, it can also render tumor cells less capable of survival under conditions of stress. In fact, when treated with temozolomide or radiation, tumors overexpressing miR-451 exhibit higher mortality than do. We can also show that compound C (an AMPK inhibitor), which induces miR-451 expression, sensitizes glioma cells to radiation and chemotherapy. These results suggest the usefulness of miR-451 for boosting therapy effectiveness. Intriguingly, oncogene kinase EphB2, which has pro-invasive and anti-proliferative actions in glioma, is a putative target of miR-451. We have shown that miR-451 decreases the EphB2 mRNA level, suggesting that EphB2 is another pathway that explains the observed phenotype. Finally, we also tested the relationship between miR-451 and glucose in vivo. We have established a hyperglycemia model in rats and performed qPCR on brain extracts. miR-451 expression was significantly elevated in hyperglycemic than in normoglycemic brain tissue. Furthermore, global miR expression analysis revealed that miR-451 was 1 of only 12 significantly upregulated microRNAs. Our preliminary findings clearly show the importance of miR-451 in glioma biology.

CB-17. IDENTIFICATION OF GENES INVOLVED IN BRAIN METASTASIS OF THE MOST COMMON CANCERS

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Approximately 40% of patients with systemic cancer will develop central nervous system (CNS) metastasis. In adults, metastases to the brain arise most commonly from primary tumors of the lung, breast, skin (melanoma), and gastrointestinal tract. Current systemic treatments have failed to prevent the development of CNS metastasis of these malignancies. CNS involvement is almost always fatal, even in the cases in which the primary tumor is under control. Therefore, preventing the development of CNS metastasis would be a great step forward in the treatment of patients with potentially disseminating or disseminated tumors. So far, no therapeutic agents preventing tumor cells from colonizing the brain are available. Our study aims to identify gene expression patterns of various primary tumors involved in the formation of cerebral metastasis and to determine if similar genes are operative. To this end, we compared the gene expression profiles of primary tumors from patients suffering from a particular cancer type and cerebral metastases with the profiles of primary tumors in comparable stages from patients who did not have brain metastases. To investigate whether the identified genes in the primary tumors are still active in the metastases, we also compared the gene expression profiles of cerebral metastases from various primary tumors. This strategy yielded several differentially expressed genes. To validate them, we silenced the genes in the cell lines and tested their effect in an in vitro blood brain barrier (BBB) model.

CB-18. MULTI-CYCLE MULTIPLEXED IN SITU IMMUNOFLUORESCENCE ANALYSIS OF 18 BIOMARKERS ON A SINGLE FFPE GLIOBLASTOMA TISSUE SECTION

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Glioblastoma multiforme (GBM) is a highly heterogeneous disease at both the cellular and the molecular level. A better understanding and characterization of this inherent heterogeneity could improve diagnostics for determining prognosis and therapy response. We have developed a multi-cycle multiplex technology that allows in situ analysis of multiple proteins on a single FFPE section. Software tools have been developed to quantify and visualize each protein in the context of the overall tissue morphology. In the current study, we show an example of sequential staining of 18 biomarkers on a single section of tissue microarray constructed from 100 GBM patients. Markers included EGFR, PTEN, Glut1, signaling molecules in PI3K/AKT and TGF-beta pathways, and biomarkers for vessels. The expression pattern of each marker was analyzed, and significant heterogeneity was observed: some markers had a binary profile (eg, EGFR), and others had a continuous pattern (eg, PTEN). Co-localization of these markers was visualized and demonstrated on single cell/subcellular levels. Further analysis will determine associations with clinical outcome. In summary, we demonstrate the powerful technology that allows multiple biomarkers to be analyzed on a single GBM tissue section, which enables complete characterization

of biomarkers with limited tissue samples. More importantly, it is a valuable tool to study the interplay of multiple pathways in GBM pathogenesis, leading to a greater understanding of tumor heterogeneity and to an identification of biosignatures for improved tumor diagnosis and prognosis.

CB-19. ENDOGENOUS DOWNREGULATION OF DRUG RESISTANCE GENE MGMT BY MICRORNA

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O6-methylguanine-DNA methyltransferase (MGMT) is related to the resistance of aggressive glioblastoma (GBM) to DNA-alkylating agent temozolomide (TMZ) therapy. In this study, we searched for microRNAs that modulate MGMT expression and give better sensitivity to TMZ. Out of tens of candidates generated by several binding site prediction programs, we selected 6 microRNAs that have a complementary seed region with 3′-UTR of MGMT. One out of 6 microRNAs downregulated more than 40% MGMT mRNA and protein expression levels in both the T98G glioma cell line and the primary GBM cell line (GBM30). MicroRNA-transfected T98G cells showed increased sensitivity to TMZ as did U87MG, a cell line that does not express MGMT, and the T98G cells transfected with siRNA of MGMT. MNude mice with microRNA-expressing GBM30 transplants showed better survival with injection of TMZ in vivo. Thus, this endogenous mechanism of suppressing the drug-resistant gene MGMT will increase chemosensitivity to TMZ. Our results suggest a direct interaction between microRNA and MGMT and describe the function of that microRNA in drug resistance. Our findings suggest a new target to enhance the efficacy of chemotherapy.

CB-20. HYPOXIA INDUCES CELL PROLIFERATION, EXPRESSION OF ANGIOGENIC FACTORS, AND PRIMITIVE NEURONAL MARKERS IN NORMAL GLIAL CELLS

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Cancer-associated fibroblasts (CAFs) have been shown to foster tumor growth in several solid cancers. In the brain, the stroma is mainly composed of glial cells such as astrocytes and oligodendrocytes whose role in brain tumor-stroma interactions is poorly characterized. We previously identified a unique gene-expression profile of tumor-associated glial cells (TAGs) by fluorescence activated cell sorting (FACS) of glial cells from GFP/Nod Scid mice engrafted with human glioblastoma biopsies. Although little is known about the mechanisms mediating these expression changes, hypoxia is a prominent feature of most malignant brain tumors. Thus, we investigated how hypoxia affected the phenotype of glial cells from normal mouse brains. Briefly, mouse brain tissue was enzymatically digested to obtain single-cell suspensions, and then glial cells were isolated by FACS with simultaneous removal of immune cells (CD11b+) and endothelial cells (CD31+). These cells were then seeded at a density of 50 000 cells/well in a 48-well plate and incubated under either normoxic (21% oxygen) or hypoxic (0.5% oxygen) conditions for 7 days. Notably, glial cells upregulated Sox2, HIF-2a, Nestin, and Vimentin in hypoxic not normoxic conditions. In addition glial cells also upregulated angiogenic factors such as VEGF, Angiopoietin 2, and FGF2. Furthermore, cell-cycle analysis revealed a significantly higher fraction of cycling cells in hypoxia than in normoxia. These changes strongly resembled the changes observed in tumor-associated glial cells, suggesting that hypoxia is a major contributor to the phenotype of tumor-associated glial cells. Ongoing work aims at further characterizing the TAG population to reveal its cell composition and to address the role of various subpopulations of glial host cells.

CB-21. β-CATENIN SIGNALING INITIATES ASTROCYTE ACTIVATION AND CONTRIBUTES TO THE PATHOGENESIS OF GLIOMAS

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Astrocytes, the most abundant cells in the central nervous system (CNS), respond to a variety of stress and pathologic conditions through a process known as astrocyte activation. Dysregulation of the molecular pathways involved in this process is thought to lead to an aggressive cellular state associated with neoplasia. We investigated whether a comparable correlation exists between the response of astrocytes to injury and the malignant phenotype of gliomas. We found that loss of contact inhibition plays a critical role in the initiation and regulation of reactive astrocytes during wound healing. Specifically, interruption and destabilization of the cadherin-catenin complexes at the cell membrane lead to nuclear translocation of β-catenin and a subset of characteristic changes associated with cell activation. We further identified similar expression changes and dysregulated signaling pathways in gliomas and glioblastoma-derived stem cells. β-catenin signaling is critical for cell proliferation since inhibition of β-catenin diminished activation of both normal astrocytes and the malignant phenotype of gliomas. These findings provide a unique mechanism of astrocyte activation and shed light on the pathogenic mechanism of glioma, which may help to develop diagnostic and therapeutic targets.

CB-22. FEASIBILITY OF AN INTRAMOLECULAR COMPLEMENTATION STRATEGY FOR SPLIT-REPORTER GENE IMAGING OF DRUGGABLE PROTEIN MISFOLDING IN BRAIN CANCER

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INTRODUCTION: Misfolded proteins may lead to brain cancer through loss-of-function phenotype for VHL and NF2, through dominant-negative inactivation of p53, or through constitutive activation of Src family kinases. We preliminarily demonstrate a new generalizable application of a protein-fragment complementation assay based on a split bioluminescence reporter (synthetic humanized *Renilla* luciferase [hRluc]) for potential future imaging of protein (mis)folding in living subjects. We hypothesized that the normal conformational changes in protein folding, which result in close approximation of amino and carboxy termini, can be used to drive intramolecular complementation of correctly oriented chimeric split imaging reporters in a strategy to pre-clinically image and quantify effects of new drugs on protein misfolding in brain cancer animal models. METHODS AND RESULTS: We used split hRluc to indirectly record the folding of 3 test proteins: the mutant (sr39) HSV1 thymidine kinase (HSV1-TK), firefly luciferase, and enhanced green fluorescent protein (EGFP) in mammalian cells. We first engineered a mutant EGFP (mEGFP) that deliberately misfolds, and then we constructed the following expression vectors: NhRluc—sr39TK—ChRluc, ChRluc—sr39TK—NhRluc, NhRluc—EGFP—ChRluc, NhRluc—mEGFP—ChRluc, and NhRluc—Fluc—ChRluc. We transiently transfected 293T cells and assayed them for in vitro complemented hRluc and, separately, folded protein activities using luminometry, or TK uptake assay of cell lysates, or fluorescence microscopy of EGFP labeled cells. Co-transfected NhRluc—TK and TK—ChRluc were used as positive controls. Activity of hRluc in RLU/microgram protein/minute was NhRluc—TK plus TK—ChRluc (82.9 ± 8.8), ChRluc—sr39TK—NhRluc (1.7 ± 0.1), NhRluc—mEGFP—ChRluc (1.6 ± 0.1), NhRluc—sr39TK—ChRluc (124.3 ± 21.2), NhRluc—EGFP—ChRluc (90.5 ± 5.8), and NhRluc—Fluc—ChRluc (14.9 ± 2.0). CONCLUSIONS: Our preliminary in vitro and cell culture experiments to detect protein folding are encouraging. Further studies are necessary to optimize this strategy prior to animal imaging. The future ability to detect, locate, and quantify protein misfolding in vivo and to develop new targeted therapies will have important implications for a wide variety of research endeavors in drug discovery and molecular neuro-oncology.

CB-23. THE DEVELOPMENTAL LINEAGE MARKER PAX2 IS EXPRESSED IN CNS HEMANGIOBLASTOMAS IN VON HIPPEL-LINDAU DISEASE

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INTRODUCTION: Von Hippel-Lindau (VHL) disease is a neoplasia syndrome resulting from a germline mutation in the VHL gene, followed by a "second hit" in the wild-type allele. Affected individuals may develop hemangioblastomas (HBs) in the cerebellum, brainstem, spinal cord, and retina, as well as tumors of the inner ear and kidney. HBs, the most common manifestation of VHL disease, are benign tumors classified as "of unknown histogenesis." Expression of HB and other developmental markers (eg, SCL or Notch) indicates that HBs arise from pluripotent embryonic cells. Paired box protein 2 (PAX2) is a transcription factor essential for proper lineage commitment during the development of the hindbrain, spinal cord, eye, inner ear, and kidneys, all of which correlate with the distribution of VHL tumors. During early development, co-expression of the HB marker

SCL and PAX2 has been described. PAX2 is upregulated in renal cell carcinoma (RCC) in VHL disease and is upregulated by loss of pVHL in vitro. In the current study, we hypothesized that PAX2 is expressed in CNS HBs in VHL disease. METHODS: Twenty CNS HBs from the cerebellum, brain stem, and spinal cord were obtained at surgery. Immunohistochemistry, immunofluorescence, and Western blots were performed. RESULTS. Nuclear staining for PAX2 was observed in all 20 HBs. PAX2-positive cells also stained for inhibin and NSE, markers used to confirm the HB tumor cell identity. These cells did not stain for CD10, ruling out metastatic RCC in these samples. Western blots confirmed the presence of PAX2 in HB. CONCLUSION: CNS HBs in VHL disease express PAX2. Perpetuation of PAX2 signaling may be a common feature of tumors in VHL disease. The presence of PAX2 in HBs supports the hypothesis that these tumors originate from a subset of pluripotent embryonic cells that are unable to differentiate normally without the VHL gene.

CB-24. YES-ASSOCIATED PROTEIN 1 AND ITS ONCOGENIC FUNCTION IN MENINGIOMAS

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The deregulation of the Hippo pathway has been implicated in several human cancers and seems to alter the adequate balance of cell proliferation and apoptosis, promoting tumorigenesis. The core components of this signaling pathway comprise a kinase cascade that culminates with the phosphorylation and inhibition of Yes-associated protein 1 (YAP1). Phospho-YAP1 is retained in the cytoplasm, but in the absence of Hippo signaling, YAP1 translocates to the nucleus, associates with co-activators TEAD1-4, and functions as a transcriptional factor promoting the expression of key target genes. Loss of the NF2 tumor-suppressor gene is the most common genetic alteration in meningiomas, and the NF2 gene product Merlin acts upstream of the Hippo pathway. In this study we show that primary meningioma tumors have high nuclear expression of YAP1. In meningioma cells, Merlin expression is associated with phosphorylation of YAP1. Using a siRNA transient knockdown of YAP1 in NF2 mutant meningioma cells, we show that suppressing YAP1 impaired cell proliferation and migration. Conversely, YAP1 overexpression strongly augmented cell proliferation and anchorage-independent growth and restricted cisplatin-induced apoptosis. In addition, expression of YAP1 in nontransformed arachnoidal cells led to the development of tumors in nude mice. Together, these findings suggest that, in meningiomas, deregulation of the Hippo pathway is largely observed in primary tumors and that YAP1 functions as an oncogene that promotes meningioma tumorigenesis.

CB-25. UNRAVELLING ACQUIRED RESISTANCE IN GLIOBLASTOMA

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Despite undeniable progress in the care and treatment of primary brain tumors, for most patients diagnosed with glioblastoma (GBM), prognosis is poor, and few survive more than 3 years. GBM is highly refractory to the standard treatment involving maximal surgical resection followed by treatment with the alkylating agent temozolomide (TMZ) combined with radiotherapy (RT) (chemoradiotherapy). However, essentially all tumors recur after this initial therapy. Local tumor recurrence, occurring 2-3 cm from the original resection cavity (the area exposed to radiation treatment) is frequently observed. Relapsed GBMs are typically unmanageable, with a median survival after recurrence of only a few months. Urgent attention to understanding this intrinsic resistance and the molecular changes occurring during treatment is required. Previous studies have highlighted the role of MSH6 inactivation in mediating the acquired resistance of GBM; however; very few samples were included. In our study, we also found loss of MSH6 protein expression in recurrent post-temozolomide GBM. In addition, we demonstrated the frequent loss of other mismatch repair (MMR) enzymes, namely MSH2 and MLH1. Interestingly, we also identified loss of all MMR proteins in treatment-naive GBM, indicating that this loss may not be acquired from temozolomide exposure. To better understand the mechanisms leading to MMR loss, we screened specimens for mutations in MSH6. We also tested specimens for MLH1 promoter methylation and the regulation of microRNA 21 (miR-21) and miR-155, which have never been investigated in GBM before. In addition, we modeled MMR inactivation in vitro by knocking down the expression of each MMR enzyme, and we examined the survival of GBM cells under the cytotoxicity of temozolomide. Overall, our results indicate that the acquired resistance in GBM is a very complex pathological process, and, in addition to MSH6, MSH2 and MLH1 are also important players.

CB-26. ACQUIRED RESISTANCE TO ERLOTINIB (TARCEVA®) IN AN EGFR- AMPLIFIED GLIOMA MODEL: A CRITICAL ROLE FOR EGFRVIII AND HISTONE ACETYLATION

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Resistance to the epithelial growth factor receptor (EGFR) tyrosine-kinase inhibitor (TKI) erlotinib is commonly observed in glioma. We analyzed the BS153 glioblastoma cell line, an EGFR- amplified, EGFRvIII-positive in vitro and in vivo model system, to identify responsible mechanisms, for the ability of BS153 cells to develop a resistant cell line BS153resE in the presence of erlotinib. Erlotinib resistance was associated with a strong upregulation of the EGFRvIII protein in BS153resE cells, suggesting that a quantitative increase in the constitutive activation of this oncogenic receptor variant represents a cardinal mechanism by which cells counteract EGFR TKI. Nevertheless, resistance to erlotinib was transient, as withdrawal led to regained sensitivity to EGF, which was absent in chronically treated cells. Genomic amplification levels of the EGFR gene remained unchanged in BS153resE cells compared with the parental cell line as determined by quantitative PCR (39.5-fold +/− 4.9 vs 38.3-fold +/− 6.1). In vivo, BS-153 cells formed highly invasive tumors with an unusual growth pattern, displaying numerous satellites distant from the initial injection site. The erlotinib-resistant phenotype led to delayed onset of tumor growth as well as prolonged overall survival of mice injected with these cells, whereas the unusual morphology of the experimental tumors remained unchanged. Knockdown of EGFRvIII in BS153resE cells using EGFRvIII-specific shRNA largely restored the sensitivity of the cells to erlotinib. Alternatively, targeting downstream mediators of EGFR signaling, especially phosphoinositide-3-kinase (PI3K), as well as inhibition of histone deacetylases caused a dose-dependent decrease in cell viability in BS153resE cells, thus overcoming resistance to the TKI. In conclusion, erlotinib resistance is associated with upregulation of the oncogenic EGFRvIII variant in BS153 cells. Strategies to overcome resistance include preventing a compensatory increase in EGFRvIII, targeting downstream effectors of EGFR signaling such as PI3K, or modulating chromatin remodeling events by interfering with histone deacetylases.

CB-27. ACTB AND SDHA ARE SUITABLE ENDOGENOUS REFERENCE GENES UNDER DIFFERENT TREATMENT REGIMENS FOR GENE EXPRESSION STUDIES IN HUMAN ASTROCYTOMAS USING QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTION-POLYMERASE CHAIN REACTION

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Quantitative real-time reverse-transcription PCR (qPCR) is frequently used as a research tool in experimental oncology. This qPCR analysis was performed to test a panel of the six most suitable reference genes identified by other studies as representing different physiological pathways (ACTB, GAPDH, POLR2A, RPL13A, SDHA, and TBP) in all common glioma groups: diffuse astrocytoma WHO grade II, anaplastic astrocytoma WHO grade III, and secondary glioblastoma WHO grade IV with and without chemotherapy, primary glioblastoma, recurrent glioblastoma, and gliomas before and after radiation. Moreover, the expression stability was tested during the longitudinal course of the disease in eight patients. Evaluation of the expression levels of the six target genes showed that ACTB, GAPDH, and RPL13A are expressed at higher levels than SDHA, POLR2A, and TBP. The candidate genes were not differentially expressed between primary and secondary glioblastomas. Furthermore, they remained stable before and after radiotherapy and/or chemotherapy. Only ACTB, GAPDH, and RPL13A showed significantly different expression levels between astrocytoma grade II and primary glioblastoma; however, these groups are unlikely to be compared. Therefore, they are adequate references for glioblastoma gene expression studies. Using NormFinder software to compare different glioma entities, we found that SDHA and ACTB are the most stable reference genes. Our data revealed the lowest intragroup variation in SDHA and the highest in RPL13A. In summary, ACTB and SDHA exhibited the best stability values and showed the lowest intergroup expression variability.

CB-28. MAST CELLS ARE TUMOR-DERIVED IN VON HIPPEL-LINDAU DISEASE CNS HEMANGIOBLASTOMAS

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INTRODUCTION: Von Hippel-Lindau (VHL) disease is a tumor syndrome resulting from an inherited mutation in the VHL gene followed by a mutation in the other allele. Central nervous system (CNS) hemangioblastomas (HBs) of the cerebellum, brainstem, and spinal cord are a major manifestation of this disease. HBs are benign tumors comprising two predominant cell types: tumor stromal cells and vascular cells. Several reports have also noted abundant mast cells in HBs, although the significance and source of these cells remain unknown. HBs likely arise from pluripotent embryonic hemangioblasts, and some of these tumor cells have the capacity to differentiate in vitro into erythrocytic, granulocytic, and endothelial progenitor cells. We hypothesized that mast cells in HBs are derived from VHL-deficient tumor cells. METHODS: HB surgical specimens were frozen, sectioned, and stained for either mast cell tryptase or inhibin to visualize mast and tumor stromal cells, respectively. Each cell type exhibited a distinct morphology, allowing selective microdissection. Lymphocytes were harvested from the peripheral blood from the same patient. For each patient, DNA was extracted from the 3 cell types (tumor, mast, and lymphocyte) and analyzed for loss of heterozygosity (LOH) by polymerase chain reaction (PCR) using primers that flank the VHL gene. RESULTS: In all four VHL patients, tumor stromal cells and mast cells exhibited an imbalance of the VHL alleles, indicating LOH in the VHL gene. Peripheral blood lymphocytes did not exhibit LOH. CONCLUSION: Mast cells in HBs can arise from VHL-deficient tumor cells rather than migrating into the tumor from the periphery. Previous results have observed the same phenomenon regarding areas of extramedullary hematopoiesis in HBs. These data suggest that VHL-deficient HB stromal cells are pluripotent embryonic cells and that some of these tumor cells have the capacity to differentiate along multiple hematopoietic lineages in vivo.

CB-29. DUAL ROLE OF MRK IN GLIOBLASTOMA INVASION AND RADIO-RESISTANCE

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Glioblastoma multiforme (GBM), the most aggressive tumor of the central nervous system, has poor prognosis both because of diffuse infiltration of tumor cells in the normal parenchyma and resistance to radiation treatment and chemotherapy. We found that MRK, a stress-activated MAP3K protein kinase, is overexpressed in GBM compared with normal brain tissue, evident in microarray expression data and confirmed by immunohistochemical analysis. We have shown that MRK can be activated by at least two distinct signaling inputs: MRK kinase activity is stimulated by ionizing radiation (IR) downstream of NBS1 and ATM in a pathway that leads to activation of Chk2 and cell-cycle arrest. We also showed that downregulation of MRK by RNA interference sensitizes SNB19 and U87 GBM cells to IR. In addition, MRK functions as an effector of RhoC in a pathway that is initiated by lysophosphatidic acid (LPA), a motogenic factor that is overproduced in GBM tumors. We also showed that MRK depletion impairs LPA-stimulated invasion in GBM cells. To examine the role of MRK in GBM in vivo, we used an orthotopic xenograft model. Human GBM explant cells (GBM6) were maintained in nude mice as flank tumors, transduced in vitro with lentiviruses expressing control or MRK shRNA, and subsequently implanted into the brains of nude mice. Control mice had a median survival of 47 days, whereas downregulation of MRK increased survival by 14 days. In a parallel experiment, mice were treated with 20-Gy radiation in 10 fractions over 5 days. This radiation treatment on its own increased survival by only 7 days; in contrast, downregulation of MRK had a strongly synergistic effect, extending survival by more than 35 days. In conclusion, these data validate MRK as a novel target for GBM therapy.

CB-30. DEPLETION OF CA2 + /CAM-STIMULATED PHOSPHODIESTERASE 1C (PDE1C) ATTENUATES PROLIFERATION, MIGRATION, AND INVASION OF ADULT HIGH-GRADE ASTROCYTOMA CELLS

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Cyclic nucleotides (cAMP and cGMP) are critical intracellular second messengers involved in the transduction of a diverse array of stimuli. The catabolism of cyclic nucleotides is mediated by phosphodiesterases (PDEs). PDEs have a putative role in controlling the cell cycle and invasion. We previously showed that genomic amplification of PDE1C in approximately 90% of high-grade astrocytoma (HGA) cultures using array CGH analysis. These data suggested that PDE1C could be involved in the development of HGA and could have potential as a novel therapeutic target. In the current study, in a panel of 15 short-term HGA cultures (with known PDE1C copy numbers), PDE1C expression levels were determined by QPCR and correlated with aCGH data. In 6 of these cultures, PDE1C was depleted using RNAi. PDE1C-expressing (PD+) and nonexpressing (PD-) cells were employed both to study intracellular levels of cAMP and cGMP and to assess their capacity to proliferate, migrate, and invade. PDE1C-specific RNAi resulted in almost 90% depletion of PDE1C mRNA levels. We detected approximately a 43% reduction in proliferation of PD- cells. In addition, PD- cells showed less migratory and invasive capacity [almost a 45% reduction]. We noted that these effects were associated with an accumulation of intracellular cAMP and cGMP levels. Comparison of whole genome expression analysis (Affymetrics HG-U133 plus2) between PD- and PD+ cells was carried out for 2 cultures (IN1472 and IN1760). A fold change in expression of 2 or more and a *P* value of less than 0.05 were considered significant. Differentially expressed genes that were common between IN1472 PD + /PD- and IN1760 PD + /PD- cells included APOE, HSD11B1, MMP1, and ST6GALNAC5, alongside other genes that affected similar cellular pathways. These genes are involved in processes affecting metabolism, proliferation, migration, and invasion of cells. Therefore, PDE1C has the potential to be a novel therapeutic target in HGA.

CB-31. RADIATION-INDUCED HIF-1α INDUCES RADIORESISTANCE AND ENHANCES THE MIGRATION ABILITIES IN GLIOBLASTOMA CELLS THROUGH THE PTEN-SRC-HIF-1α SIGNALING AXIS

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PURPOSE/OBJECTIVE: Glioblastoma is one of the most radioresistant tumors, and hypoxic regions are commonly observed in it. Hypoxia inducible factor-1α (HIF-1α), a molecular marker of hypoxia, is expressed in glioblastoma and is associated with poor survival. Recent studies have shown that HIF-1α is induced by irradiation even under normoxic conditions, although the mechanism and the role are still unclear. We investigated the association between radiation-induced HIF-1α and its role in glioblastoma cells. MATERIALS/METHODS: We used a shRNA transfection approach to knock down HIF-1α expression in two established cell lines (U87 and LN18). A clonogenic survival assay and an MTS assay were used to evaluate cell survival and radioresistance after irradiation. The migration and invasion ability were evaluated by a wound-healing assay and a matrigel invasion chamber assay. The protein expressions were analyzed with Western blots. RESULTS: HIF-1α was increased in irradiated LN18 and U87 and knocked down by shRNA. Knockdown of HIF-1α decreased the radioresistance significantly in LN18 but only slightly in U87. Knockdown of HIF-1α also decreased migration and invasion abilities in both of the cell lines. Knockdown of HIF-1α increased the expression of PTEN in LN18 (wild-type PTEN) but not in U87 (mutant PTEN). The pSrc (Tyr-416) was less observed in HIF-1α knockdown LN18 than in normal LN18. CONCLUSIONS: These results suggest that radiation-induced HIF-1α is associated with radioresistance and radiation-induced migration and invasion abilities in glioblastoma cells. The degree of the reduction of radioresistance was different between the two cell lines. The tumor suppressor gene PTEN and oncogene Src might be associated with this mechanism.

CB-32. DETECTION OF "ONCOMETABOLITE" 2-HYDROXYGLUTARATE BY MAGNETIC RESONANCE ANALYSIS AS A BIOMARKER OF IDH1/2 MUTATIONS IN GLIOMA

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PURPOSE: We aimed to develop a magnetic resonance (MR)-based methodology for detecting 2-Hydroxyglutarate (2HG) as a biomarker for isocitrate dehydrogenase (IDH)1/2 mutations in human gliomas. EXPERIMENTAL DESIGN: We used a study set of 65 human glioma specimens, representative of the different tissue histologies and grades, and 10

non-tumoral brain samples. The mutation status of hotspot codons in IDH1/2 genes was determined by sequencing and immunohistochemistry. Metabolic profiles of tissue samples were analyzed by solid-state high-resolution nuclear magnetic resonance (NMR). The pure 2HG compound was initially used to identify the 2HG-specific spin-system and proton resonances using one- (1D) and two-dimensional (2D) correlation spectroscopic (COSY) methods. Then, using the 2HG-specific cross-peak pattern, the signals of endogenous 2HG from the complex NMR spectra of the 75 tissue samples were identified and subsequently quantified using the signal integrals in 1D spectra. RESULTS: Somatic mutations in IDH1/2 were detected in 37 (57%) of the 65 tumors analyzed, all of which showed elevated levels of 2HG (1-11 mM) by NMR. The levels of 2HG in the 28 mutation-negative tumors were at ($n = 1$) or below ($n = 27$) the quantification limit (0.1 mM) but were not detectable in all 10 non-tumoral controls. The sensitivity and specificity of detecting 2HG in IDH1/2 mutation-bearing samples by the 2D COSY reached 96% and 95.2%, respectively. The overall accuracy of identifying IDH1/2 mutations by NMR using 2HG as a biomarker was 97.8%. CONCLUSIONS: This study demonstrates the feasibility of using MR detection of 2HG for identifying IDH1/2 mutation-positive brain tumors and opens up the possibility of developing analogous noninvasive MR-based imaging and spectroscopy for detecting 2HG directly in brain tumor patients.

CB-33. EPHA2/EPHRINA1 SYSTEM IN CONJUNCTION WITH TKS5 REGULATES INVADOPODIA FORMATION IN GLIOBLASTOMA CELLS

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EphA2 receptors are overexpressed in glioblastoma (GBM) cells but not in normal brain tissue. EphrinA1 binds to and downregulates EphA2 receptors, decreasing cell migration and invasion. Invadopodia are electron-dense, actin-rich protrusions that bind to and proteolytically degrade extracellular matrix (ECM) proteins. In this study, we explored the role of EphA2/ephrinA1 complexes in invadopodia formation and matrix degradation. We also investigated the activation of Tks5, a substrate of the protooncogene Src and an adaptor protein, which is overexpressed in GBM and is linked to patient survival. Invadopodia formation and matrix degradation were assessed by an in situ gelatin zymography assay combined with fluorescence microscopy. GBM cells form invadopodia-like structures that degrade the ECM; the Snb-19 cell line exhibits the greatest degradation activity. Expression of the EphA2 receptor was examined by immunoblot analysis using parental, vector control, and ephrinA1-expressing U-251 cells, and A-172, G48a, and Snb-19 GBM cells. Src-transformed NIH3T3 (Src3T3) cells were used as a positive control. Unexpectedly, immunoblot analysis showed low levels of EphA2 among the cells that most degraded the ECM, such as Snb19 cells, and high levels among cells that least degraded the ECM, such as G48a cells. Accordingly, diminishing levels of EphA2, either by knockdown or ligand activation, evoked more prominent gelatin-degrading invadopodia activity. Interestingly, we observed opposite changes in Tks5 relative to the EphA2 receptor. Our results suggest a significant involvement of the EphA2/ephrinA1 system in invadopodia function based on an inverse correlation between matrix degradation and EphA2 expression. This inverse correlation is also seen between EphA2 and Tks5, suggesting interdependence of these two signaling pathways. These findings have potentially important implications concerning the role of the EphA2/ephrinA1 complex and Tks5 in brain tumor migration and invasion.

CB-35. ECTOPIC OVEREXPRESSION OF MIR145 COMPROMISES THE INVASIVE ABILITY OF MALIGNANT GLIOMAS BY MODULATING NEDD9

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Recent results define microRNA 145 (miR145) as a tumor suppressor implicated in proliferation, invasion, and stemness of cancer stem cells (Zhu, 2011). We confirm that miR145 is strongly downregulated in glioblastoma (GB) specimens and in corresponding GB stem-like cells (GSCs) compared with normal brain tissue ($P < 0.0001$) and with low-grade gliomas (LGGs; $P < 0.03$). After comparing the results of a microarray analysis performed on GSCs overexpressing miR145 (miRover-GSCs) withEmpty-GSCs, we identified relevant differences in gene-expression profiles, including genes involved in proliferation, invasion, and stemness. We focused on HEF1/Cas-L/NEDD9, a scaffold protein involved in invasion in several types of cancer (O'Neill, 2007). We confirmed by RT-PCR a significant down-modulation of NEDD9 expression in miRover-GSCs ($P = 0.0001$). Moreover, NEDD9 is upregulated in GB and in GSCs compared with LGGs ($P = 0.015$). About 50% of LGGs express higher levels of NEDD9 than normal brain tissue (2.3 ± 0.8), and progression-free survival (PFS) of these patients was shorter than that of patients with a lower expression of NEDD9 (0.8 ± 0.3; median PFS 41 vs 82 months, respectively, $P = 0.04$). We also observed that miR145 overexpression in GSCs affects tumor development after intracranial injection in nude mice. Mice injected with miRover-GSCs survived significantly longer than mice injected with Empty-GSCs ($P = 0.03$). Notably, histological characterization and quantification showed that NEDD9 is significantly downregulated in gliomas from miRover-GSCs compared with controls ($P = 4.9$ E-07). In conclusion, our results show an important involvement of miR145 in regulating GB invasion and a potential role of NEDD9 as a biomarker for glioma progression.

CB-36. THE EFFECTS OF CD44 SILENCING ON CYTOSKELETAL PROTEINS: A POSSIBLE MECHANISM OF CD44'S INVOLVEMENT IN GLIOMA CELL INVASION

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The complex machinery of cellular invasion involves key players such as cell-adhesion molecules (CAMs), the extracellular matrix (ECM), and the cytoskeletal components. CD44, a CAM, is overexpressed in glioma cells and promotes invasion through its interaction with hyaluronic acid (HA) and other ECM components. To further identify potential mechanisms involved in CD44's promotion of glioma invasion, we examined the expression of several cytoskeletal-associated proteins. Here, we report the possible association of CD44 with the cyoskeleton dynamics in glioblastoma (GBM) cells. SNB-19, a grade IV GBM, was used to investigate the effects of CD44 silencing on F-actin, Vimentin, GFAP, and microtubules through immunocytochemistry (ICC), flow cytometry, and Western blotting. Variation in morphology and distribution of CD44 and the cytoskeleton-linked proteins were demonstrated by ICC. CD44 was well distributed on the surface of wild-type SNB-19 cells, with higher expression at the peripheral edges, suggesting a potential link between CD44 and the cytoskeleton. siRNA CD44-treated cells showed rather faint staining and rounded cells devoid of any processes. Disorganized ruffles of F-actin were particularly detected in knockdown cells. The consequent decline in the above-mentioned protein levels following siRNA CD44 treatment was observed by Western blotting. Significant decreases in the expression levels in knockdown cells was confirmed by flow cytometry. There was a marked decline in the expression levels of CD44 from 98.44 % to 74.13%, of F-actin from 99.77% to 70.35%, of microtubules from 99.50% to 76.54%, of GFAP from 92.28 % to 72.84%, and of vimentin from 97.32% to 68.61% in SNB-19 wild-type compared with siRNA CD44-silenced cells ($P < 0.05$). We previously demonstrated that CD44 influences glioma cell invasion. The results from the present study provide information on glioma cell morphology and cytoskeletal-associated proteins that are differentially expressed when CD44 is silenced. Current studies are underway to identify signaling pathways associated with CD44 and glioma cell invasion.

CB-37. TEMOZOLOMIDE ACTIVATES UPSTREAM REGULATORY KINASES OF NA+-K+-2CL− COTRANSPORTER ISOFORM 1 IN GLIOBLASTOMA CANCER CELLS

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Na^+-K^+-$2Cl^-$cotransporter isoform 1 (NKCC1) plays a role in glioma migration. We recently reported that NKCC1 is the primary cell volume regulatory protein to maintain K^+, Cl^-, and volume homeostasis against temozolomide (TMZ)-induced apoptotic volume decrease and to promote survival of glioma cells (GCs). However, it is not well understood how NKCC1 is regulated in GCs after TMZ treatment. With-no-lysine kinases 1 (WNK1) is a known upstream regulator of NKCC1, while Ste20-related proline alanine-rich kinase (SPAK) and oxidative stress-responsive kinase-1 (OSR1) are likely links between WNK1 and NKCC1 to regulate volume homeostasis. Here, we further investigated the role of WNK1-mediated signaling transduction pathways in regulation of NKCC1 in response to TMZ treatment. Exposure of GCs to TMZ triggered a concurrent activation of NKCC1, SPAK, OSR1, and WNK1 (detected with an increased protein

phosphorylation). Downregulation of WNK1 by small interfering RNA (siRNA) in GCs nearly stopped the TMZ-mediated activation of NKCC1 and partially inhibited the activation of SPAK and OSR1. These findings clearly demonstrated that WNK1 is a dominant upstream regulatory kinase of NKCC1 in response to TMZ-mediated apoptosis in GCs. It remains to be determined whether WNK1 directly regulates NKCC1 or acts through SPAK/OSR1 pathways.

CB-38. UNEXPECTED NUCLEAR SUBLOCALIZATION OF THE TYROSINE KINASE RECEPTOR TIE2 INDUCES RADIORESISTANCE OF MALIGNANT GLIOMAS

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Striking radioresistance of malignant gliomas accounts for the inevitable recurrence and the subsequent poor prognosis associated with this disease. The development of new therapeutic strategies requires identifying the key molecular pathways that regulate the resistant phenotype of malignant gliomas. Previous work from our laboratory showed that the tyrosine kinase receptor Tie2 is expressed in brain tumor stem cells. Current knowledge supports a significant role of this stem cell population in the recurrence and relapse of malignant gliomas after radiotherapy. Here, we report that ionizing irradiation (IR) of mice bearing brain tumor stem cells-derived intracranial xenografts results in the nuclear translocation of Tie2, otherwise a membrane receptor. Immunoflurorescence studies using confocal microscopy and subcellular fractionation followed by Western blot analysis showed that upon IR, Tie2 is hyperphosphorylated and localizes as a full-length protein into the nucleus. Nuclear Tie2 binds γH2AX, one of the key DNA repair protein complexes, as part of the DNA-repair foci, as assessed by co-immunoprecipitation and confocal microscopy. Of clinical significance, the presence of Tie2 in the nucleus is associated with radioresistance, as observed by cell viability and clonal assays, using isogenic cell lines and brain tumor stem cells. This phenomenon seems to be ligand-dependent, as increased levels of Angiopoietin1 (Ang1) are observed after ionizing radiation. In addition, blocking the Ang1/Tie2 interaction by using siRNA against Tie2 or soluble Tie2 as a decoy receptor results in radiosensitization of the malignant glioma cells. Similarly, the use of a Tie2 chemical inhibitor reverts their radioresistance. Collectively, our data present solid evidence of the trafficking of a tyrosine kinase receptor Tie2 into the nucleus, where it exerts a new role in the DNA repair mechanism and in the radioresistance of brain tumor stem cells. These results encourage the design of Tie2-targeting combinational therapies, such as Tie2 inhibitors and radiotherapy, for patients with glioblastomas.

CB-39. EFFECTS OF β3-ADRENERGIC RECEPTOR AGONIST ON GENE EXPRESSION OF LEPTIN IN GLIOBLASTOMA

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In the 25 years since temozolomide entered phase I clinical trials, few new primary or adjuvant therapies have been developed for the treatment of glioblastoma multiforme (GBM) tumors. Our laboratory has been exploring novel methods for the treatment of GBMs. Recent studies indicate that the expression of the hormone leptin and its receptor (OBR) increases in gliomas and positively correlates with the malignancy of the tumor. Interestingly, ß3-adrenergic receptor agonists are known to decrease leptin expression in adipocytes but have not been examined in GBM cells. We hypothesized that β3-adrenergic agonists downregulate the expression of leptin and its receptor. In our studies, the relative expression of leptin, OBR, and ß3 adrenergic receptor were measured in the GBM cell lines T98G and LN229 using quantitative real-time PCR. Gene expression levels in untreated cells were compared with those of cells treated with 400 ng/mL of a selective ß3 agonist (BRL 37344). The results indicate that the relative expression of leptin in the ß3 agonist-treated cells was reduced 27-43% when compared with controls (T98: 0.73, [0.59-0.89]; LN229: 0.57 [0.55-0.59]). Similarly, expression of the leptin receptor was reduced 31-39% when compared with controls (T98: 0.69 [0.63-0.75]; LN229: 0.63 [0.56-0.71]). These results suggest that the use of ß3-adrenergic agonists may be beneficial for patients with GBM tumors by downregulating tumor-derived leptin and leptin signaling through the leptin receptor.

CB-40. GLYCOSYLATION IS CRITICAL FOR EPHRINA1 PRODUCTION AND EPHA2 ACTIVATION

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INTRODUCTION: EphrinA1, a member of the ephrin family of ligands that interacts with the Eph-related tyrosine kinase receptors, specifically binds the EphA2 receptor that is highly overexpressed in gliobastoma (GBM) but not in normal adult brain tissue. This binding induces a receptor-mediated internalization and downregulation and subsequently suppresses tumors-, making EphrinA1 an attractive target for the development of new anti-tumor therapeutics. In the crystal structure, EphrinA1, in complex with EphA2, was shown to be glycosylated on Asparagine 26; however, no data available confirm the presence or explains the importance of this glycosylation. We have thus initiated a study to investigate the role of glycosylation in EphrinA1 biological activity. METHODS: 6xHis-tag ephrinA1 and EphrinA1-Fc were produced in Sf9 insect cells using the baculovirus expression system. Proteins were purified by proteinG and Ni-NTA affinity chromatography, using FPLC. EphrinA1 activity was evaluated in U-251 MG GBM cells by examining cell morphology and EphA2 receptor downregulation. Enzymatic deglycosylation with PNGaseF and ENDO H was performed as recommended by the manufacturer. RESULTS: Enzymatic deglycosylation with PNGaseF and ENDO H decreased the molecular weight of EphrinA1 as detected by SDS-PAGE. Western blot analysis confirmed this observation of EphrinA1 obtained from PNGaseF-treated U-251[EphrinA1 +] conditioned media. Unglycosylated mutants of EphrinA1 (EphrinA1-N26A/N26Q/N26D) overexpressed in U-251 cells were almost completely retained by cells and minimally released into the media when compared with wild-type EphrinA1. U-251 cells treated with deglycosylated EphrinA1 (Deg-EphrinA1), in the presence of PNGaseF, lost almost all their cell-rounding and EphA2 downregulating abilities compared with the naturally glycosylated EphrinA1. CONCLUSIONS: Glycosylation is essential for proper production of EphrinA1 and its release from cells and is possibly involved in EphA2 receptor activation. This observation was surprising, but it has important implications for the understanding of the ligand-receptor dynamics and for the future production of novel EphrinA1-based, EphA2-targeted therapeutics.

CB-41. GALECTIN-3 SELECTIVELY KILLS BRAIN TUMOR CELLS THROUGH N-GLYCAN DEPENDENT BINDING OF β1 INTEGRIN AND INDUCTION OF APOPTOSIS

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Galectin-3 (Gal3), the only chimeric family member of the galectin family, consists of a collagen-like N-terminal region and a C-terminal carbohydrate recognition domain (CRD), which has binding affinity for galactose and N-acetyllactosamine. Gal3 is secreted through a Golgi-independent pathway. We found that extracellular Gal3 can specifically induce apoptosis in a variety of tumor cell types (including malignant glioma and cancers of the breast, colon, prostate, and lung), while normal cells (fibroblast and endothelial) were unaffected. Further, overexpression of secreted Gal3 in tumor cells significantly inhibited in vivo s.c. and i.c. glioma growth. We found that Gal3-mediated tumor cell killing was mediated through caspase-9-dependent apoptotic induction, involved an increase in Bax levels and a decrease in Survivin levels, and was neutralized with lactose, a Gal3-CRD-binding ligand. Gal3-induced tumor-specific apoptosis was mediated through Gal3-β1 integrin interaction at the cell surface, detected by a variety of experimental approaches (GST-Gal3 pulldown assay, cell membrane-specific Gal3-β1 integrin co-immunoprecipitation, and β1 integrin siRNA). We hypothesized that Gal3 interacts with β1 integrin through specific glycans on its extracellular domain. We confirmed that treatment of N-glycosylation inhibitor (kifunensine) neutralized Gal3-β1 integrin interaction, whereas the O-glycosylation inhibitor (benzyl-O-N-acetyl-D-galactosamide) had no effect. N-acetylglucosaminyltransferase V (MGAT5) is responsible for branching N-acetylglucosamine in β 1,6-linkage to the α-mannose residue of N-linked oligosaccharides to form tetra-antennary glycans and is highly expressed in various tumor cells. Overexpression of MGAT5 increased the sensitivity of Gal3-mediated apoptosis in normal cells (human foreskin fibroblast-1, HFF-1, and 293 cells), and shRNA-mediated neutralization of MGAT5 in glioblastoma cells (LN229) reduced Gal3-induced apoptosis. We also confirmed via GST-Gal3 pulldown assays that MGAT5 modulation affected Gal3-β1 integrin interaction. These data support a model in which Gal3 selectively kills tumor cells through its interaction with aberrantly N-glycosylated β1 integrin and consequently induces apoptosis. This study provides the foundation for the development of more selective and safe tumor therapeutics.

CB-42. CLINICAL SIGNIFICANCE OF KALLIKREIN 6 IN GLIOBLASTOMA MULTIFORME

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Kallikreins have prognostic value in specific malignancies, but few studies have addressed their clinical significance in glioblastoma (GBM). Kallikrein 6 (KLK6) is potentially highly relevant to GBM, since it is upregulated at sites of CNS pathology and was recently linked to reactive astrogliosis (Scarisbrick et al., 2012). To determine the clinical significance of KLK6 to GBM, we investigated the association between patient survival and levels of KLK6-immunoreactivity (IR) in 60 grade IV astrocytoma patient tumor specimens. Also, we evaluated the effects of elevated KLK6 on the response of GBM cell lines (U251, SF767) to cytotoxic agents. A range of KLK6 expression levels was observed across grade IV tumors; we found that higher levels indicated a poor prognosis of patient survival (univariable analysis, $P = 0.02$). GBM patients with KLK6-IR scores below 10 had a median survival of 408 days and patients with scores at 10 or above had a median survival of 276 days (HR = 2.36, 95% CI = 1.19-4.68, univariable analysis $P = 0.01$). The prognostic significance of KLK6-IR remained after adjusting for gender and Eastern Cooperative Oncology Group performance scores. Our results suggest a mechanism by which elevated KLK6 reduces patient survival: KLK6 promotes resistance of GBM cell lines to cell death induced by staurosporine, cisplatin, or to radiation or temozolomide alone, or in combination. The ability of KLK6 to elicit pro-survival effects was shown to depend at least in part on activation of the thrombin receptor protease activated receptor 1 (PAR1). These results suggest KLK6 and PAR1 as targets for the development of therapies to enhance the sensitivity of GBM to cell death-inducing agents.

CB-43. MICRORNA 145 IS DOWNREGULATED IN GLIAL TUMORS AND DECREASES GLIOMA CELL MIGRATION AND INVASION BY TARGETING CTGF

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Glioblastoma (GBM), the most common and aggressive type of malignant glioma, is characterized by increased invasion and by resistance to apoptosis. Despite intensive therapeutic strategies, the median survival of GBM patients remains dismal. MicroRNAs (miR), small 20-22-long nucleotide-coding RNAs, regulate tumor progression, migration, and invasion via targeting specific genes in tumor cells. miR-145 is a putative tumor suppressor; however, its expression and function in glioma cells have not been yet reported. We found that the expression of the miR-145/143 cluster was significantly lower in GBM and in glioma cells and glioma stem cells (GSCs). Analysis of miR-145 and miR-143 expression in TCGA demonstrated an inverse correlation with patient survival. Transfection of glioma cells with miR-145 mimic or a lentivirus vector expressing pre-miR-145 significantly decreased the migration of glioma cells and the self renewal of GSCs. miR-145 targeted the expression of Oct4 and Sox2 in GSCs. In addition, we identified CTGF as a novel target of miR-145 in glioma cells; transfection of the cells with this miRNA decreased the expression of CTGF as determined by Western blot analysis, qRT-PCR, and luciferase activity of a CTGF 3′-UTR reporter plasmid. Moreover, overexpression of a CTGF plasmid lacking the 3′-UTR or a recombinant CTGF protein restored the inhibitory effect of miR-145 on cell migration and invasion. Similarly, silencing CTGF decreased the migration and invasion of glioma cells, the expression of SPARC, and the phosphorylation of FAK. These results demonstrate that miR-145 is downregulated in GBM, and its low expression predicts poor patient prognosis. In addition, miR-145 regulates glioma cell migration and GSC self-renewal by targeting CTGF, Sox2, and Oct4, respectively. Therefore, miR-145 is an attractive therapeutic target as an anti-invasive treatment for the eradication of GSCs and for the treatment of malignant gliomas.

CB-44. EXPRESSION OF β-CATENIN IN BLOOD VESSELS OF CNS HEMANGIOBLASTOMAS IN VON HIPPEL-LINDAU DISEASE

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INTRODUCTION: Von Hippel-Lindau (VHL) disease is an autosomal dominant neoplasia syndrome resulting from a germline mutation in the VHL gene and a subsequent mutation in the second allele. Hemangioblastomas (HBs) and renal cell carcinoma (RCC) are the most common tumors in VHL disease. HBs are benign tumors comprising two major cell types, tumor stromal cells and abundant vasculature. Loss of VHL protein function leads to upregulation of hypoxia inducible factors (HIF) and downstream targets (eg, vascular endothelial growth factor [VEGF]). PVHL has also been shown to stabilize Jade-1, a protein that targets β-catenin for degradation. β-Catenin is a key signaling molecule that regulates development, proliferation, and differentiation. Dysregulation of this pathway has been implicated in the oncogenesis of multiple neoplasms, including RCC. We hypothesized that in the absence of pVHL, Jade-1 is downregulated, and β-catenin is upregulated in HBs. METHODS: Frozen and formalin-fixed paraffin-embedded surgical HB specimens from VHL patients were used for immunohistochemical, immunofluorescent, and Western blot analysis of β-catenin and Jade-1. RESULTS: By immunohistochemistry, Jade-1 was not observed in HB stromal cells (VHL-negative) but was observed in larger blood vessels. However, in 19/20 specimens, β-catenin was weak or absent in HB stromal cells but strongly expressed in the cytoplasm and nuclei of both small and large blood vessels. Western blot analysis minimally detected, Jade-1 but clearly detected β-catenin. CONCLUSION: Despite the absence of Jade-1 in HB stromal cells, β-catenin is not generally upregulated in these cells. The strong presence of β-catenin in the vasculature of HBs is consistent with the requirement for β-catenin in CNS angiogenesis and is likely the result of VEGF-induced angiogenesis. However, a subset of Jade-1-negative and β-catenin-positive smaller blood vessels may represent vascular cells that are VHL-negative and arise from the tumor rather than from reactive angiogenesis.

CB-45. P75 NEUROTROPHIN RECEPTOR IS REQUIRED FOR GLIOMA INVASION AND PROLIFERATION

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More than 70% of all brain tumors are malignant gliomas. Although how gliomas arise is not certain, isolation and characterization of CD133+ brain tumor initiating cells (BTICs) from primary tumors suggest that gliomas arise from stem cell-like neoplastic clones. Previous studies from our laboratory have demonstrated that the p75 neurotrophin receptor (p75NTR) is overexpressed in gliomas, that most invasive glioma cells express high levels of p75NTR, and that expression of p75NTR is sufficient to convert noninvasive gliomas to highly invasive tumors when xenografted into immunodeficient mice (Johnston et al., 2007). In contrast, siRNA knockdown of p75NTR reduces glioma migration (Johnston et al., 2007). More recently, we have showed that p75NTR is also involved in invasion of BTICs in vivo (Wang et al., 2008). These results are particularly stimulating in light of the fact that p75NTR is also highly overexpressed in aggressive, malignant melanoma tumors (Berger et al., 2012), and neurotrophin binding to p75NTR on cultured melanoma cells increases their invasiveness in vitro (Herrmann et al., 1993) and promotes their survival (Marchetti et al., 2004). p75NTR is a multifunctional protein that regulates neurite outgrowth, myelin formation, and cell survival and death (Barker 2004), as well as proliferation and differentiation of neuronal and non-neuronal cells (Cattaneo and McKay, 1990; Moscatelli et al., 2009). Here, we demonstrate that p75NTR and its ligand NGF are produced in BTICs isolated from glioblastomas. Downregulation of p75NTR expression by siRNA decreased proliferation of BTICs and their differentiation into astrocytes. Further, specific activation of p75NTR by NGF induced receptor proteolysis and increased proliferation and differentiation of BTICs. Our data suggest targeting p75NTR as a unique therapeutic strategy for gliomas. Future research is aimed at investigating the mechanisms involved in p75NTR-mediated glioma proliferation and invasion.

CB-46. A NOVEL ROLE FOR NFIA AS A CRITICAL REGULATOR OF NFKB IN GLIOMA GROWTH

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INTRODUCTION: Gliomas (astrocytomas or oligodendrocytomas) are the most common primary CNS tumors in humans and are thought to exhibit aberrant differentiation. Recent evidence showed that nuclear factor I A (NFIA), an important regulator in glial development, is highly expressed in astrocytomas is associated with survival, suggesting that NFIA may have a role in astrocytoma biology. METHODS/RESULTS: To investigate the role of NFIA in glioma, we first examined the growth of glioma cells using gain-of-function (overexpression) and loss-of-function (stable shRNA). We found that NFIA overexpression accelerated growth of glioma cells,

whereas knockdown of NFIA decreased their growth. Further, knockdown of NFIA in the glioma cells induced apoptosis, as evidenced by cleavage of PARP, caspase-8, caspase-9, and by increased caspase-3 activity. Given not only the emerging role of NFkB in evading apoptosis by suppressing caspase-8 and in activating anti-apoptotic signaling but also the function of NFIA as a transcription factor, we reasoned that regulation of tumor growth by NFIA may be mediated through the NFkB pathway. We first examined the effect of NFIA on expression of NFkB and its effectors. Indeed, knockdown of NFIA reduced expression of NFkB, TRAF1, and cIAPs, whereas NFIA overexpression augmented their expression. The induction of NFkB by NFIA was required for protecting cells from apoptosis since inhibition of NFkB (siRNA or NFkB inhibitor) in NFIA-overexpressing cells increased caspase-3 activity to a similar level as that of the vector control in glioma cells, suggesting that NFkB is a major mechanism governing NFIA-mediated inhibition of apoptosis. CONCLUSIONS: This is the first evidence for a functional molecular link between NFIA and NFkB in glioma biology. Further understanding of molecular mechanisms of the NFIA-NFkB axis in glioma will facilitate our understanding of the emerging role of NFIA in cancer, which may guide future development of therapy by targeting relevant pathways.

CB-47. NEUROTROPHINS PROMOTE THE GROWTH OF GLIOMA-INTIATING CELLS VIA TRK RECEPTORS

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Glioblastoma multiforme (GBM) is a highly invasive disease that is refractory to current treatments. Previous work by this lab has identified and characterized the role of the neurotrophin receptor p75NTR in promoting neurotrophin-dependent glioma cell invasion. Neurotrophins are a group of growth factors that signal through the p75NTR receptor (TNFR family) and Trk receptors (receptor tyrosine kinases) to regulate diverse functions such as neuronal survival and cell motility. Here, we have further investigated the role of p75NTR and Trk receptors in mediating the response of patient-derived gliomasphere cultures (commonly referred to as brain tumor initiating cells [BTICs]) to neurotrophins. Neurotrophin receptor and ligand expression was analyzed in a panel of BTICs by RT-PCR, Western blotting, and FACS. Growth assays were performed to measure the response of BTIC cultures to different neurotrophin ligands, and signaling pathways downstream of tyrosine kinase receptors were examined. Function of neurotrophin receptors was investigated by chemical inhibition and RNAi. BTIC cultures express a diverse repertoire of neurotrophin receptors, and the expression of specific Trk receptors (TrkA, TrkB, and TrkC) correlates with the response of each BTIC line to specific neurotrophin ligands (NGF, BDNF, and NT3). In particular, BDNF and NT3 promote the growth of BTICs and stimulate MAPK and PI3K pathway activity. Inhibiting Trk receptor function by pharmacological therapeutics or by lentiviral-mediated shRNA knockdown renders BTICs unresponsive to neurotrophins in terms of growth promotion and MAPK stimulation, whereas inhibition of p75NTR has no effect on these phenotypes. In addition, growth stimulation by neurotrophins can compensate for inhibition of EGF-stimulated growth by erlotonib.

CB-48. NOTCH LIGANDS, LATERAL INHIBITION, AND STEM-LIKE GLIOBLASTOMA CELLS

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Cancer stem cells are thought to be critical for long-term growth and therapeutic resistance of glioblastoma (GBM). The Notch pathway helps to maintain this poorly differentiated subpopulation, but the mechanisms of Notch induction are still poorly understood. The "lateral inhibition" model, a central concept in Notch signaling, explains how Notch signaling can drive two initially identical progenitor cells to adopt different fates. Jagged or Delta-like ligands initiate this process by binding to Notch receptors on adjacent cells, causing both induction of Notch signaling in the neighbor and suppression of pathway activity in the ligand-expressing cell. Although Notch is known to be a critical player in stem-like GBM cells, and ligand expression and juxtacrine signaling have been demonstrated in these tumors, it is not yet known whether lateral inhibition occurs. To test this, we engineered a model system in which isogenic cells expressing low and high levels of the Jagged1 ligand are co-cultured; we then performed flow-based separation and determined the Notch pathway activity in each respective population. In the GBM neurosphere lines HSR-GBM1 and 040821, the co-culture induced expression of Notch targets Hes5 and Hey1 in Jagged1-low "signal receiving" cells but inhibited expression of these targets in Jagged1-overexpressing "signal sending" isogenic cells. The ligand intracellular domain (ICD) appears to play a role in downregulating Notch signaling in these "signal sending" cells. In support of this observation, expression of Jag1 or Dll1 ligand ICD significantly reduced Notch target transcript levels and decreased GBM clonogenic growth potential. Furthermore, we found that ligand ICD localizes to the nucleus, suggesting that the observed effects may be mediated by transcriptional regulation. Our data suggest conservation of a developmental mechanism regulating Notch activation in cancer and a novel role for the ligand ICD that could help treat Notch-dependent tumors.

CB-49. DNA HYPERMETHYLATION AND 1P LOSS SILENCE NHE-1 IN OLIGODENDROGLIOMA

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Oligodendroglioma is characterized by mutations of IDH and CIC, 1p/19q loss, and slow growth. We found that NHE-1 on 1p is silenced in oligodendrogliomas secondary to IDH-associated hyper-methylation and 1p allelic loss. Silencing lowers intracellular pH and attenuates acid load recovery in oligodendroglioma cells. Others have shown that rapid tumor growth cannot occur without NHE-1-mediated neutralization of the acidosis generated by the Warburg glycolytic shift. Our findings show for the first time that the pH regulator NHE-1 can be silenced in a human cancer and also suggest that pH deregulation may contribute to the distinctive biology of human oligodendroglioma.

CB-50. HETEROGENEITY OF RESISTANCE TO EPITHELIAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS IN GLIOBLASTOMA INVOLVES PI3K/AKT PATHWAY-DEPENDENT AND -INDEPENDENT MECHANISMS

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Epithelial growth factor receptor (EGFR)-targeted therapies, such as the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib, have had limited success for the treatment of glioblastoma (GBM). In an effort to model and characterize mechanisms of resistance to EGFR TKIs in GBM, TKI-sensitive ink4a/arf -/- astrocytes overexpressing the constitutively active mutant ΔEGFR (or EGFRvIII) were seeded in soft agar in the presence of gefitinib or erlotinib. Following initial response to the drug as measured by inhibition of colony formation, the dose was escalated and TKI-resistant colonies were isolated to generate clonal cell lines. TKI-resistance was verified in vitro and in vivo using an intracranial model. In TKI-resistant cell lines, gefitinib inhibited ΔEGFR phosphorylation, indicating the ability of the drug to effectively inhibit kinase activity. Interestingly, gefitinib suppressed Akt phosphorylation in parental cells and 4 out of 7 resistant cell lines. The remaining 3 lines presented reduced PTEN expression concomitant with elevated and sustained PI3K/Akt pathway signaling. Furthermore, TKI-resistance was reversible upon drug removal in cells having no change in PTEN expression and was irreversible in those cells with decreased PTEN. There were also common features amongst the resistant cell lines: an inability to undergo PARP cleavage in the presence of gefitinib and a dramatic elevation of autophagy. TKI re-sensitization was accompanied by significant reduction in autophagy, whereas cells with irreversible TKI-resistance maintained high levels of autophagy. Importantly, this model system has allowed for the isolation of resistant clones that are phenotypically distinct from one another, thereby demonstrating a potential for intratumoral resistance heterogeneity. These findings also suggest that reversibility of TKI-resistance following drug removal may depend on the resistance mechanism, which has potential clinical implications. Moreover, the existence of common features involving resistance to apoptosis and autophagy suggests a potential therapeutic opportunity that may apply despite apparent differences in molecular mechanism.

CB-51. NEUROKININ-1 RECEPTOR: A POTENTIAL TARGET TO INHIBIT PEDIATRIC GLIOBLASTOMAS

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The neurokinin-1 receptor (NK1R), a G protein-coupled receptor that mediates the effects of substance P (SP), plays a key role in the growth of adult glioblastomas (GBM). Two forms of NK1R are known, a full-length form of

407 amino acids and a truncated form that ends at amino acid 311 (NK1RΔ311). Truncated NK1RΔ311 has been shown to be oncogenic, and we have found it present in every primary adult GBM we have tested, albeit at different levels. However, despite the evidence supporting NK1R as a good target for inhibition of adult GBM, to date, the activity of NK1R in pediatric GBM has not been examined. As a first step toward this area of research, we recently examined the expression of NK1R and NK1RΔ311 in 8 pediatric GBM xenografts that were established at Duke's Pediatric Brain Tumor Foundation Institute. We found that both NK1R and NK1RΔ311 are expressed in all 8 pediatric GBM xenografts. To determine whether NK1R plays a role in the growth of pediatric GBM, we examined NK1R expression and function in a pediatric GBM cell line (D2368 MG), which was established from a pediatric GBM. Real-time quantitative PCR analysis of RNA isolated from D2368 MG cells indicate that these cells mainly express the truncated form of NK1R. Stimulating these cells with SP causes little or no increase in Akt phosphorylation while simultaneously increasing Erk1/2 phosphorylation. On the other hand, blocking NK1R with L703, 606 (an NK1R antagonist) decreases the phosphorylation of both Akt and Erk1/2. Incubation of D2368 MG cells with L703,606 for 6 days reduces the cell number by over 60%. Taken together, our study provides the first evidence that NK1R may play a role in the growth of pediatric GBM and represents a novel target to inhibit these tumors.

CB-52. BLOCKADE OF NDRG4 INDUCES P53 AND BAX MEDIATED APOPTOSIS IN HUMAN MENINGIOMA CELL LINES

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Meningiomas are the most commonly occurring intracranial tumors and account for approximately 20-30% of central nervous system tumors. Patients whose tumors recur after surgery and radiation therapy have limited therapeutic options. It has also been reported recently that radiation triggers DNA repair, cell survival, and cell proliferation, and reduces apoptosis via the induction of cellular protective mechanisms. We have recently identified the overexpression of N-Myc downregulated gene 4, NDRG4, protein in aggressive meningiomas. We have demonstrated that NDRG4 downregulation results in decreased cell viability, migration, and invasive properties. In the present study we investigated the role of NDRG4 in cell proliferation and mitochondrial-mediated apoptosis. We utilized shRNA-containing lentiviral plasmids to downregulate NDRG4 mRNA and protein expression in two high-grade meningioma cancer cell lines, IOMM-Lee and CH157MN. In follow up to our prior studies on the effects of NDRG4 gene silencing in meningioma, we performed DNA laddering and Annexin V/APC flow cytometry assays to demonstrate that the predominant form of cell death is apoptosis. We show that apoptosis caused by transduction of lentiviral shNDRG4 involves Bcl-2, Bcl-xL inhibition, cytochrome c release from mitochondria, and caspase-9 and caspase-3 activation followed by PARP I cleavage. After we treated cells with LVshNDRG4, we detected upregulation of p53 and Bax, downregulation of Bcl-2, and inhibition of inhibitory apoptotic molecules survivin and XIAP. Flow cytometry analysis revealed changes in mitochondrial membrane potential ($\Delta\psi$), which was observed by JC-1 membrane permeabilization as a differential mitochondrial mass, and the percentage of mitochondrial $\Delta\psi_m$ damage was measured using a lipophilic, cationic, fluorescent probe (DiOC6). Sub-cellular distribution of Bax and cytochrome c indicated mitochondrial-mediated apoptosis following depletion of NDRG4. In conclusion, this study demonstrates that NDRG4 gene silencing induces Bax activation and subsequently initiates mitochondrial-mediated apoptosis. This therapeutic strategy may have promise in the management of meningiomas.

CB-53. AN IN VIVO LOSS OF FUNCTION SCREEN FOR GENES INVOLVED IN EPIDERMAL GROWTH FACTOR RECEPTOR VIII-INDEPENDENT GLIOMA GROWTH

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Amplification and mutation of the epidermal growth factor receptor (EGFR) gene are common genetic hallmarks of glioblastoma (GBM). The most common mutation is an in-frame deletion of exons 2-7 of EGFR, resulting in a constitutively active variant, EGFRvIII/ΔEGFR. EGFR and ΔEGFR-specific tyrosine kinase inhibitors (TKIs) are currently in clinical trials for the treatment of GBM; however, their efficacy has been limited owing to both upfront and acquired drug resistance. Using a genetic model of tetracycline-regulated ΔEGFR expression, we previously found that while this receptor is essential for the maintenance of glioma growth in vivo, similar to a clinical situation of acquired resistance, some tumors eventually regained aggressive growth after a significant period of stasis. Surprisingly, these breakthrough tumors persisted despite sustained suppression of ΔEGFR, indicating that blocking these receptors alone may not sufficiently translate into a clinical benefit for GBM patients. Microarray gene expression profiling of cell lines generated from these breakthrough tumors revealed a number of genes that were significantly upregulated compared with ΔEGFR-dependent cell lines. Using qPCR technology, we confirmed that 84% (16/19) of the genes revealed in the microarray analyses are indeed upregulated and potentially reflect a descriptive genetic signature of acquired ΔEGFR independence. To test this hypothesis, we established a non-biased loss-of-function in vivo screen to identify which of these genes alone or in combination are most essential to overcome dependence on ΔEGFR signaling. Genetic screens in mouse models have been shown to be highly effective in identifying cooperating cancer genes. Given that the genetic profiles generated for each individual ΔEGFR-independent cell line are not identical, results from these studies will shed light on genetic mechanisms of resistance to EGFR inhibition and may reveal novel putative targets for therapeutic development.

CB-54. ANALYSIS OF THE EPTHELIAL GROWTH FACTOR RECEPTOR VIII-INDUCED TRANSCRIPTOME IN RAT GLIOMAS AND HUMAN GLIOBLASTOMAS

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Epithelial growth factor receptor (EGFR) vIII, a constitutively active truncated mutant of EGFR, has been shown to increase neoplastic transformation and tumorigenicity in a variety of tumors including glioblastoma (GBMs). However, transcriptional mediators of EGFRvIII have not been fully elucidated. In the present study, we analyzed 52 primary human GBMs and a 9L.EGFRvIII rat brain tumor model to identify EGFRvIII-specific gene signatures. Gene expression analyses of 9L.EGFRvIII tumors demonstrated increased expression of approximately 1498 gene probes when compared with control tumors ($P = 0.05$). DAVID enrichment analyses revealed nine clusters of genes that mediate increased transformation, invasion, glycolysis, and hypoxia in 9L.EGFRvIII tumors. Comparative evaluation of gene expression profiles from rat tumors and primary human GBMs revealed nine novel genes (ckap4, lrp5, fat3, slc7a1, cdk6, socs2, aqp1, spry2, and aebp1) that were significantly upregulated in EGFRvIII-expressing tumors. In the present study, we also correlated our transcriptome data to the TCGA database to define important genes mediating EGFRvIII signaling in GBMs. Collectively, our data not only present a comprehensive EGFRvIII-specific gene signature profile using a rat glioma model and human GBMs but also characterize potential targets to inhibit EGFRvIII-mediated transforming phenotypes in malignant gliomas.

CB-55. DECADRON TREATMENT INHIBITS GLIOMA CELL PROLIFERATION IN VIVO

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Glioblastoma multiforme (GBM) is the most common and malignant primary brain neoplasm. Standard therapy for GBM is surgical resection followed by radiation therapy (RT) and temozolomide. Additionally, the corticosteroid dexamethasone (Dex) is frequently used for symptomatic improvement prior to surgical resection and, in many cases, for maintenance through post-operative radiation therapy. Several studies have investigated the role of Dex in symptom management using various glioma models, but the results have been inconsistent. The purpose of this study was to investigate the effect of Dex on the PDGF-driven RCAS/t-va model of glioma and to elucidate possible mechanisms of action. Symptomatic mice were treated with Dex at 10 mg/kg for 3 days, and tissue was collected for immunohistochemical and microarray analysis. We found that the majority of the differentially expressed genes were downregulated by Dex and were involved in cell-cycle progression and proliferation, which was confirmed by PCNA and Ki67 immunohistochemistry. Interestingly, treating primary mouse glioma cultures in vitro with escalating concentrations of DEX did not decrease proliferation, suggesting that the anti-proliferative phenotype observed in vivo might be mediated via the tumor microenvironment. When these genes were investigated in the TCGA data set, we found that high expression of the

genes downregulated by Dex predicts a significantly longer survival compared with tumors with lower levels. In summary, our data suggest that Dex inhibits tumor cell proliferation, potentially through tumor microenvironment interaction. TCGA analysis suggests that downregulation of our Dex-regulated gene set may have an adverse effect on survival. It is possible that if Dex decreases tumor cell proliferation, it may also decrease the efficacy of antineoplastic therapy that is most toxic to proliferating cells. This study underscores the importance of defining the mechanism of how Dex affects tumor and stromal cells in glioma.

CB-56. SIGNALING NETWORK-BASED ANALYSES OF SONIC HEDGEHOG PATHWAY COMPONENTS: PREDICTIONS AND POSSIBILITIES

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Medulloblastomas show considerable dependance on the Sonic Hedgehog (Shh) pathway. Since cellular functions result from coordinated networks involving protein-protein networks, it is imperative to explore different events cooperating with Shh entities in order to develop novel targets for medulloblastoma therapy. The Yes associated protein (YAP), a component of the Hippo pathway, has been previously established as a downstream target of the Shh pathway (Fernandez et al., 2011). We developed several interesting gene targets featured in the networks using YAP overexpression in CGNPs. A concurrent upregulation of IGF2 and H19, both imprinted genes, was observed. Possible links between the Shh/Hippo pathways and IGF2 signaling also featured in the networks developed in our study. Important links to lipid metabolism, a process highly active in medulloblastomas and essential for the proliferation of CGNPs, were also predicted. The study includes results of signaling interactions between the Shh/Hippo pathways and entities from other relevant pathways that together play a role in medulloblastoma development and sustenance.

CB-57. PHOSPHORYLATION OF DOCK180Y722 BY SRC FAMILY KINASES MEDIATES EGFRVIII-DRIVEN GLIOBLASTOMA TUMORIGENESIS

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Glioblastoma, the most common primary malignant cancer of the brain, is characterized by rapid tumor growth and infiltration of tumor cells throughout the brain. These traits cause glioblastoma to be highly resistant to current therapies, resulting in poor prognosis. While aberrant oncogenic signaling driven by signature genetic alterations such as epithelial growth factor receptor (EGFR) gene amplification and mutation, plays a major role in glioblastoma pathogenesis, the responsible downstream mechanisms remain less clear. Here, we report that EGFRvIII (also known as ΔEGFR and de2-7EGFR), a constitutively active EGFR mutant that is frequently co-overexpressed with EGFR in human glioblastoma, promotes tumorigenesis through Src family kinase (SFK)-dependent phosphorylation of Dock180, a guanine nucleotide exchange factor for Rac1. EGFRvIII induces phosphorylation of Dock180 at tyrosine residue 722 (Dock180Y722) and stimulates Rac1-signaling, glioblastoma cell survival, and migration. Consistent with this causal relationship, siRNA knockdown of Dock180 or expression of a Dock180Y722F mutant inhibits each of these EGFRvIII-stimulated activities. The SFKs, Src, Fyn, and Lyn, induce phosphorylation of Dock180Y722, and inhibiting these SFKs by pharmacological therapeutics or shRNA depletion markedly attenuates EGFRvIII-induced phosphorylation of Dock180Y722, Rac1 activity, and glioblastoma cell migration. Lastly, phosphorylated Dock180Y722 is co-expressed with EGFRvIII and phosphorylated SrcY418 in clinical specimens; this co-expression correlates with an extremely poor survival in glioblastoma patients. These results suggest that targeting the SFK-p-Dock180Y722-Rac1 signaling pathway may offer a novel therapeutic strategy for glioblastomas with EGFRvIII overexpression.

CB-58. EPITHELIAL MESENCHYMAL TRANSITION AS A THERAPEUTIC TARGET IN GLIOMA

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Epithelial mesenchymal transition (EMT) is felt to drive carcinoma metastasis through activating cell invasion, intravasation, and promotion of stem cell phenotypes. We previously showed that TWIST1, a central driver of EMT-related invasion in carcinoma, is upregulated in malignant gliomas and promotes human glioma cell invasion in vivo and glioblastoma (GBM) stem cell growth and self-renewal in vitro. Combined with the emerging recognition of the importance of mesenchymal phenotypes in glioma, these observations support our hypothesis that targeting EMT-associated genes like TWIST1 may have a unique therapeutic benefit for glioma patients. To address this possibility, we studied the effect of inhibiting TWIST1 and its putative target gene Periostin (POSTN), also a known regulator of carcinoma EMT, in human GBM stem cell xenograft models. Using stable expression of gene-specific shRNAs, we found that 80% knockdown of TWIST1 in the GBM8 stem cell line and 90% knockdown of POSTN in GBM4 stem cells resulted in nearly a complete eradication of tumor cells, whereas a 50% knockdown resulted in prolongation of median survivals from 75 days to 100 days. Unlike GBM8, POSTN is expressed and regulated by TWIST1 in GBM4. Therefore, we tested the effect of POSTN knockdown alone and found that a 90% knockdown of POSTN in GBM4 dramatically phenocopied the effect of TWIST1 inhibition in GBM8. TWIST1 and POSTN inhibition also corresponded with cell line-specific decreases in stem cell-marker expression (CD133) and functional indicators of stem cell activity (self-renewal and sphere formation). These results indicated that inhibition of EMT-associated genes TWIST1 and POSTN dramatically reduce glioma growth, possibly through effects on stem cell function. We conclude that EMT is not only a useful framework for conceptualizing glioma biology but also a potentially productive resource for identifying new molecular targets for GBM invasion and stem cell function.

CB-59. BCL-2 FAMILY MEMBER MCL-1 REGULATES HYPOXIA-INDUCED CELL DEATH THROUGH BLOCKING BH3-ONLY MEMBER BNIP3 FUNCTION IN MALIGNANT GLIOMA CELLS

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Brain tumors that have areas of low oxygen (hypoxia) are resistant to chemotherapy. We investigated proteins found in malignant glioma cells that regulate hypoxia-induced cell death. We have shown that the Bcl-2 family member Mcl-1 protects cells from hypoxia-induced cell death, whereas BNIP3 (pro-cell death) regulates hypoxia-induced cell death. However, even though BNIP3 expression leads to cell death, it is associated with a poor prognosis for patients with solid tumors, and we investigated this dichotomy by determining whether the function of BNIP3 changes when it is expressed in tumor cells with Mcl-1. In the current study, we have shown that normal mouse astrocytes are more resistant to hypoxia-induced cell death when compared with knockout mouse astrocytes that lack BNIP3 expression. However, when BNIP3 expression was knocked down in U373 glioma cells, they were more resistant to hypoxia-induced cell death. Under hypoxic conditions, Mcl-1 expression decreased, whereas BNIP3 expression increased. Overexpression of Mcl-1 effectively blocked both hypoxia and BNIP3-induced cell death. Furthermore, treatment with epidermal growth factor (EGF) increased Mcl-1 expression and blocked both hypoxia and BNIP3-induced cell death. Knockdown of Mcl-1 expression blocked EGF protection against BNIP3 and hypoxia-induced cell death. Expression of mutated EGF receptor (EGFRVIII) in U373 cells also increased Mcl-1 expression, even under hypoxic conditions. EGFRVIII-expressing tumor xenografts grown in SCID mice showed increased Mcl-1 and BNIP3 expression, correlating with hypoxia regions in these tumors. Thus, Mcl-1 expression reduces hypoxia and BNIP3-induced cell death in glioma cells, a process mediated by EGF signaling. Understanding these pathways could lead to more targeted therapies that reduce intrinsic drug resistance in glioblastoma.

CB-60. CYTOMEGALOVIRUS ENHANCES GLIOBLASTOMA PROLIFERATION VIA STAT3 ACTIVATION

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Recently, several groups have demonstrated the presence of cytomegalovirus (CMV) protein and DNA in a large majority of glioblastomas (GBMs), though the role of CMV in tumors remains controversial. Although CMV is capable of activating several oncogenic pathways, it has never been shown to be a transforming virus. Because of this, we hypothesized that CMV infection can modify the rate of gliomagenesis. To test this hypothesis, we perinatally infected Mut3 (GFAP-cre; Nf1loxP/+; Trp53+/−) mice that develop spontaneous gliomas at an adult age with mouse CMV (MCMV). After viral inoculation, mice exhibited infection throughout the body, including the brain. MCMV-infected Mut3 mice succumbed to glioma significantly earlier than Mock-infected Mut3 mice. To validate our results, we used an additional syngeneic model of intracranial mouse GBM. Wild type mice were perinatally MCMV- or Mock-infected and injected intracranially with mouse GBM cells as adults. MCMV-infected mice with intracranial tumors died significantly earlier than Mock-infected mice. Analysis of pre-tumorigenic Mut3 mice revealed a significant increase of pSTAT3 in the subventricular zone, a hypothesized origin of GBMs. Additionally, MCMV-infected mice with intracranial syngeneic GBMs expressed higher pSTAT3 in tumors. Since pSTAT3 mediates proliferation in GBMs, we measured PCNA, a marker of proliferation, in our tumors. MCMV-infected intracranial tumors contained a significantly higher number of PCNA-positive cells, suggesting a higher rate of proliferation. To investigate CMV in human tumors, we infected patient-derived GBM neurospheres in vitro with human CMV (HCMV). Infection activated STAT3 and significantly increased proliferation. Additionally, HCMV-infected neurospheres injected into flanks of nude mice grew faster than Mock-infected tumors. Addition of Stattic, a STAT3 inhibitor, abrogated proliferation of HCMV-infected neurospheres, showing that the proliferation advantage garnered by HCMV occurred via STAT3 activation. Taken together, our data suggest that CMV in gliomas accelerate GBM progression by STAT3 signaling and increase proliferation.

CB-61. ENHANCING ONCOLYTIC HSV-1 THERAPY BY HDAC6 INHIBITION IN GLIOBLASTOMA

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Novel strategies, including oncolytic viral (OV) therapy using conditionally replicating viruses, have shown promise in various neurological tumors, but some glioma cells resistant/evade the lytic effect of the OV. We have found that, right after infection and entry into a glioma cell, incoming oncolytic HSV (oHSV) are transported via microtubules (MTs) to autophagosomes and lysosomes, inducing an innate antiviral signal within the cancer cell. This appears to be a host defense pathway, and inhibition of one particular HDAC (HDAC6) suppresses these host defense pathways, thus improving oHSV1 replication in glioma. To prove that HDAC6 mediates antiviral effects, we have employed genetic approaches, including shRNA knockdown and overexpression of HDAC6. Since HDAC6 deacetylates alpha-tubulin and alters MT dynamics, we attempted oHSV infection in cells overexpressing acetyl-mimic and -deficient forms of tubulin. Our experimental results show that inhibition of HDAC6 suppresses the lysosomal degradation of incoming oHSV during infection. In addition to MT-mediated defensive pathway, we found that HDAC6 also modulated interferon-stimulated genes (ISGs), patterns, and expression upon oHSV infection, suggesting a novel role for HDAC6 in antiviral immune responses. Using biochemical approaches, we discovered that HDAC6 physically interacts with one of the ISGs after IFN-treatment. The roles of HDAC6 in antiviral response and post-entry oHSV1 trafficking are highly novel, and manipulating HDAC6 activity could improve oHSV1 replication in vitro and in vivo in order to increase therapeutic efficacy in GBM.

CB-62. REVERSAL OF MIRNA-125B-TRIGGERED HUMAN GLIAL CELL PROLIFERATION USING ANTI-MIRNA (AM; ANTAGOMIR) STRATEGIES

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A conserved class of soluble, 22 nucleotide, single-stranded non-coding ribonucleic acids known as microRNAs (miRNAs) bind to the 3′ un-translated region (3′-UTR) of their target messenger RNAs (mRNAs), thereby regulating specific gene expression. The total number of known miRNAs is currently about 2000; however, human brain cells are specifically enriched in a significantly smaller subclass of about 40 miRNAs that include a high abundance miRNA-125b. Levels of human miRNA-125b, transcribed from multiple genes at chr 11q24 and chr 21q21, are significantly upregulated in glioma and glioblastoma tissues, in cultured human glioma and glioblastoma cell lines, and in interleukin-6 (IL-6)-stressed normal human astroglial (HAG) cells. IL-6 treatment triggers HAG cell proliferation. Upregulated miRNAs downregulate their mRNA targets and hence downregulate selective gene expression. Here we report that miRNA-125b, when added to cultured HAG cells, induces astrogliosis and increases markers for senescence, including nuclear atrophy and increased cytoplasmic-to-nuclear ratios. Anti-miRNA-125b (AM-125b) strategies robustly inhibit these triggered effects. We further demonstrate a strong positive correlation between miRNA-125b abundance in biopsied human glioma and glioblastoma tissues and the astroglial cell markers glial fibrillary acidic protein (GFAP) and vimentin. Taken together, these results suggest that miRNA-125b contributes to the proliferation and accelerated senescence of HAG cells and that anti-miRNA-125b (AM-125b; antagomir-125b) based strategies may be clinically useful in treating cellular proliferative diseases that involve astroglial cells. This study was supported in part by the Translational Research Initiative (LSUHSC-NO), an Alzheimer Association IIRG Award, an NIH NIA AG18031, and an NIH NIA AG038834.