ELK4 neutralization sensitizes glioblastoma to apoptosis through downregulation of the anti-apoptotic protein Mcl-1

Bryan W. Day†, Brett W. Stringer†, Mark D. Spanevello, Sara Charmsaz, Paul R. Jamieson, Kathleen S. Ensbey, Jacinta C. Carter, Joanne M. Cox, Vicky J. Ellis, Christopher L. Brown, David G. Walker, Po L. Inglis, Suzanne Allan, Brent A. Reynolds, Jason D. Licklitter, and Andrew W. Boyd

Brain Cancer & Leukaemia Foundation Research Units, Queensland Institute of Medical Research, Herston, 4029, Queensland, Australia (B.W.D., B.W.S., M.D.S., S.C., P.R.J., K.S.E., J.C.C., J.M.C., S.A., J.D.L., A.W.B.); Queensland Brain Institute, University of Queensland, St Lucia, 4067, Queensland, Australia (B.W.D., M.D.S., A.W.B.); The ESKITIS Institute for Cell and Molecular Therapies, Griffith University, Nathan, 4109, Queensland, Australia (V.J.E., C.L.B.); BrizBrain & Spine Research Foundation, Auchenflower, 4066, Queensland, Australia (D.G.W.); Cancer Services, Royal Brisbane and Women’s Hospital, Herston, 4029 Queensland, Australia (P.L.I., S.A.); McKnight Brain Institute, University of Florida, Gainesville, Florida, 32611, USA (B.A.R.); Department of Medicine, University of Queensland, Herston, 4029, Queensland, Australia (A.W.B.)

Glioma is the most common adult primary brain tumor. Its most malignant form, glioblastoma multiforme (GBM), is almost invariably fatal, due in part to the intrinsic resistance of GBM to radiation- and chemotherapy-induced apoptosis. We analyzed B-cell leukemia–2 (Bcl-2) anti-apoptotic proteins in GBM and found myeloid cell leukemia–1 (Mcl-1) to be the highest expressed in the majority of malignant gliomas. Mcl-1 was functionally important, as neutralization of Mcl-1 induced apoptosis and increased chemotherapy-induced apoptosis. To determine how Mcl-1 was regulated in glioma, we analyzed the promoter and identified a novel functional single nucleotide polymorphism in an uncharacterized E26 transformation-specific (ETS) binding site. We identified the ETS transcription factor ELK4 as a critical regulator of Mcl-1 in glioma, since ELK4 downregulation was shown to reduce Mcl-1 and increase sensitivity to apoptosis. Importantly the presence of the single nucleotide polymorphism, which ablated ELK4 binding in gliomas, was associated with lower Mcl-1 levels and a greater dependence on Bcl-xL. Furthermore, in vivo, ELK4 downregulation reduced tumor formation in glioblastoma xenograft models. The critical role of ELK4 in Mcl-1 expression and protection from apoptosis in glioma defines ELK4 as a novel potential therapeutic target for GBM.

Keywords: apoptosis, ELK4, glioblastoma, glioma, Mcl-1.

Treatment of glioblastoma multiforme (GBM), the most malignant primary adult brain tumor, involves surgical resection followed by radiation and chemotherapy. Therapy is almost never curative, due in part to the infiltrative nature of these tumors and their intrinsic resistance to radiation and chemotherapy. Even with optimal treatment, the median survival is less than 15 months, with only 10% of patients surviving 2 years without disease recurrence. This dismal situation highlights a pressing need to identify new therapeutic targets to improve patient outcome.

One important target for molecular therapy is the programmed cell death, or apoptotic, machinery of the cell. This is regulated by both pro-apoptotic and pro-survival proteins. One such pro-survival protein is Mcl-1, which is overexpressed in many cancers, including GBM, and plays a critical role in the regulation of apoptosis. In this study, we investigated the role of ELK4, an ETS transcription factor, in the regulation of Mcl-1 expression and sensitivity to apoptosis in glioma.
anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl/A1) B-cell leukemia–2 (Bcl-2) proteins. Imbalances between these underlie a number of neoplastic malignancies. The p53 tumor suppressor gene, which is frequently mutated in GBM, normally inhibits the pro-apoptotic BH3-only proteins Puma and Noxa, preventing apoptosis. Agents that mimic BH3 proteins therefore might trigger apoptosis in tumors, even those harboring p53 mutations. One such agent, ABT-737, which induces cell death in tumor cell lines, is a potent inhibitor of Bcl-2, Bcl-xL, and Bcl-w (Ki ≤ 1 nM) but has far lower affinity for myeloid cell leukemia–1 (Mcl-1) (Ki > 1 μM). Overexpression of Mcl-1 has been shown to attenuate ABT-737 sensitivity both in a mouse lymphoma model and in acute myeloid leukemia, whereas ABT-737 has been shown to induce apoptosis in human carcinoma cell lines when Mcl-1 was neutralized.

We show that Mcl-1 is the most highly expressed anti-apoptotic Bcl-2 family member in high-grade glioma (n = 51). In addition, in analyzing the Mcl-1 promoter in GBM cells we identified a novel, functional GA single nucleotide polymorphism (SNP) in a consensus E26 transformation-specific (ETS) transcription factor binding site. The wild-type (WT) G form of the SNP actively binds nuclear proteins from GBM cells, whereas the A form does not. The SNP correlates with decreased promoter activity and lower Mcl-1 levels. Importantly, GBM cultures harboring the SNP showed increased sensitivity to ABT-737 and cisplatin treatment. Furthermore, apoptosis was observed in GBM cell lines and neurosphere lines without the ETS SNP when Mcl-1 was neutralized. We show that ELK4 is the dominant ETS domain transcription factor in GBM and binds to the identified Mcl-1 promoter ETS site. In addition, significant correlation (r = 0.76, P < .0001) between ELK4 and Mcl-1 mRNA in 51 glioma specimens was found. Downregulation of ELK4 by small-interfering (si)RNA resulted in loss of Mcl-1 expression and increased sensitivity to ABT-737 and cisplatin. Conversely, ELK4 overexpression increased Mcl-1 levels and increased resistance to higher concentrations of chemotherapy. Furthermore, in vivo downregulation of ELK4 reduced Mcl-1 levels and reduced tumor formation in both subcutaneous and intracranial glioma xenograft models. These findings demonstrate ELK4 to be a critical regulator of Mcl-1 and highlight both ELK4 and Mcl-1 as potential therapeutic targets in GBM.

Materials and Methods

Cell Culture

D series GBM cell lines were obtained from the Duke University Medical Center and U series from the American Type Culture Collection. Cell lines were cultured in Roswell Park Memorial Institute–1640 medium with 10% fetal bovine serum (JRH Biosciences) at 37°C in humidified air/5% CO2. Neurospheres were generated from a primary GBM sample (L1-NS) and cultured as described.

Clinical Specimens

Primary specimens were obtained from the Royal Brisbane and Women’s Hospital and the BrizBrain & Spine Research Foundation after informed consent from adult patients diagnosed with high-grade glioma (Table S1).

ELK4 cDNA Expression Construct

ELK4 cDNA was cloned into the plasmid elongation factor internal ribosome entry site (pEF-IREs)–puro6 mammalian expression vector.

Mcl-1 cDNA Expression Construct

Mcl-1 cDNA was cloned into the plRES2-DsRed-Express mammalian expression vector (JRH Biosciences).

Noxa Peptide Treatment

Mcl-1 was neutralized using a Noxa peptide as described.

Mouse Tumor Models

Cells were injected into 5-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. For subcutaneous experiments, animals were euthanized when tumors exceeded 1 cm in diameter. For intracranial experiments, animals were monitored for signs of tumor formation (rough coat, hunching, weight loss). Animals were culled and hematoxylin-and-eosin sections were prepared to identify tumors.

DNA Extraction

Genomic DNA was extracted as described.

Relative Quantitation by Real-time PCR

RNA was extracted using TRIzol (Invitrogen). First strand cDNA was synthesized using random hexamers and Superscript III (Invitrogen). Real-time PCR was carried out using SYBR (Synergy Brands) Green PCR Master Mix (Applied Biosystems). Cycling conditions were 15 min at 95°C and 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Full primer sequences are listed in the supplementary materials and methods (Table S2).

Protein Analysis

Immunoblots and immunohistochemistry were performed as described. Complete immunoblots are shown in Figure S6.
Luciferase Reporter Assay

The Mcl-1 promoter was cloned into pGL2-Basic (Promega). Reporter assays were conducted using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were performed with end-labeled double stranded oligonucleotides using GBM nuclear extracts as described.15

Chromatin Immunoprecipitation Assay

ChIP assays were performed as described (http://genomics.ucdavis.edu/farnham/protocols/chips.html). Primer sequences and antibodies were used as in the supplementary materials and methods.

siRNA/shRNA Gene Knockdown

Mcl-1 and ELK4 small hairpin (sh)RNA was cloned into pSuperior.neo+gfp (Oligoengine). siRNA Stealth DuoPak (cat # 1293609) (Invitrogen) and shRNA sequences are listed in the supplementary materials and methods (Table S1).

Annexin V Staining

Apoptosis was determined by Annexin V cell staining as described.16

ETS SNP Assignment

The novel −116G>A Mcl-1 promoter SNP has Single Nucleotide Polymorphism Database accession number ss107739141.

Statistical Analysis

Student’s t-test determined the probability of difference; \( P < .05 \) was considered significant. All statistical tests were 2-sided. The correlation coefficient was determined using a Spearman rank.

Results

Mcl-1 is the Dominant Anti-apoptotic Bcl-2–related Protein in High-grade Glioma

Expression of anti-apoptotic Bcl-2 family members was assessed in a series of resected gliomas (tumor type and World Health Organization grade listed in Table S1). Expression of Mcl-1, Bcl-xL, Bcl-2 (L12), Bcl-w, Bcl-2, and A1 mRNA (as well as the pro-apoptotic Mcl-1 short isoform) was determined by quantitative PCR (qPCR) in 51 resected brain tumors (~75% GBM), as well as in normal brain (Fig. 1A). While the total expression was variable from tumor to tumor, the expression profile was characterized by substantial levels of Mcl-1 and to a lesser degree Bcl-xL and Bcl-2 (L12) in glioma tissues. In this study 74% (38/51) of samples expressed high levels of Mcl-1 compared with normal total brain RNA, which showed only low levels. Bcl-xL was also significantly elevated in a proportion of samples but was greater than Mcl-1 in only 2 cases. Mcl-1 and Bcl-xL protein levels were also evaluated in 4 common GBM cell lines (Fig. 1B). Notably, 3 of the 4 lines tested expressed Mcl-1 (U87, U118, and D645, but not U373). All 4 lines expressed Bcl-xL, with U87 and D645 expressing high levels. Mcl-1 and Bcl-xL levels also were found to be high in a series of tumor-derived primary GBM early passaged lines (Fig. S1). Immunocytochemical analysis of Mcl-1 in U87, U118, D645, and U373 indicated that Mcl-1 protein expression paralleled the mRNA expression pattern (Fig. 1C). Taken together, this expression profile suggested a potentially significant role of Mcl-1 and, to a lesser extent, Bcl-xL in high-grade glioma survival.

Identification of a Novel SNP in an ETS Site in the Mcl-1 Promoter

Targeted sequencing of genomic DNA from GBM tissue samples and normal control DNA identified a previously unknown SNP (dbSNP, ss107739141) in the Mcl-1 promoter in high-grade glioma. This G>A transversion was located in a consensus ETS transcription factor binding site located 116 bp upstream of the major transcription start site (Fig. 2A). The −116G>A SNP was identified in the GBM cell lines U251 and U373 and in 2/75 normal control peripheral blood mononuclear cell samples tested. The SNP was not identified in any of 37 GBM/astrocytoma tumor samples tested, consistent with the low frequency in normal samples.

Notably, the cell lines U251 and U373, which harbored the ETS SNP, had the lowest Mcl-1 mRNA expression of 12 GBM cell lines tested (Fig. 2B). To investigate whether the SNP might affect Mcl-1 transcription, the −116G>A substitution was investigated by reporter gene assay in 5 GBM cell lines (Fig. 2C). The −116G>A SNP resulted in statistically significant reductions \( (P < .01) \) in Mcl-1 promoter activity (U87, 36%; U118, 50%; U251, 42%; D645, 53%; and U373, 35%) compared with the WT promoter.

To determine whether the −116G>A SNP affected DNA binding by nuclear protein(s), EMSAs were performed. These revealed complexes formed between an Mcl-1 WT promoter probe (nucleotides −124 to −105) and nuclear extracts from each of 4 GBM cell lines (arrowed in Fig. 2D) that were not detected, or only weakly detected (U118), with a −116G>A SNP probe. Cold competition EMSA with U118 nuclear extracts demonstrated that these complexes were specific (Fig. 2D). Taken together, these data demonstrate that the −116G>A SNP specifically ablates DNA binding by nuclear protein(s) to the Mcl-1 promoter ETS site in vitro.
ELK4 is the Dominant ETS Family Member in High-grade Glioma

Given the identification of a functional SNP within a consensus ETS binding site in the Mcl-1 promoter, we sought to determine whether this site bound an ETS family transcription factor in GBM. To begin, we surveyed ETS family member expression in primary GBM tumor samples and common GBM cell lines. Twenty-seven ETS family members were investigated, of which 12 showed detectable expression (Fig. 3A). Notably, 4 of the 5 GBM tumor samples and 4 of the 5 GBM cell lines expressed substantial levels of ELK4 compared with other ETS family members.

To determine whether ELK4 bound the Mcl-1 promoter ETS site, EMSA supershift and chromatin immunoprecipitation (ChIP) assays were conducted. In the presence of an ELK4-specific antibody, 2 supershifted complexes (arrowed in Fig. 3B) were detected with nuclear extracts from 4 GBM cell lines. The appearances of these bands correlated with a decrease in intensity of the primary band in all samples. This prompted us to use ChIP assays to determine whether ELK4 bound the endogenous Mcl-1 promoter (Fig. 3C). ELK4 binding to the Mcl-1 promoter was detected using 2 ELK4 antibodies. Greater immunoprecipitation was observed with the H-167 antibody, which binds ELK4 farthest from its ETS DNA binding domain. The H-167 antibody was selected for testing by qPCR in the U118 line (Fig. 3D) and a four cycle threshold difference between the antibody and control was found. Together these results show that ELK4 binds to the Mcl-1 promoter ETS site in GBM cells.

ELK4 is a Key Regulator of Mcl-1 Expression in High-grade Glioma

To further investigate the relationship between ELK4 and Mcl-1 expression in high-grade glioma, we used qPCR to determine the correlation between ELK4 and Mcl-1 mRNA in 51 high-grade glioma samples (Fig. 4A). This showed a highly significant positive correlation (Spearman $r = 0.76$, $P < .0001$). Sequence analysis determined that none of the glioma samples tested contained the $-116G>A$ SNP. Interrogation of the larger The Cancer Genome Atlas database of 424 GBM specimens further confirmed the positive correlation between ELK4 and Mcl-1 expression in GBM (Spearman $r = 0.14$, $P < .002$).
We also compared the level of ELK4 and Mcl-1 mRNA in 3 GBM cell lines, 2 of which contained the −116G>A SNP. Significant and comparable ELK4 expression was detected in all 3 lines. However, U251 and U373, both of which are heterozygous for the −116G>A (ETS) SNP, expressed significantly less Mcl-1 than U87. Indeed, Mcl-1 expression was in fact significantly lower from the remaining WT Mcl-1 allele.

To further determine whether ELK4 was the critical ETS family member regulating Mcl-1 expression in GBM, we used siRNA to downregulate ELK4 in 2 GBM cell lines (Fig. 4C). There was substantial
Mcl-1 is a Critical Survival Determinant in GBM Cultures

To investigate the potential role of Mcl-1 in GBM resistance to chemotherapy-induced apoptosis, 5 GBM cell lines were treated with either cisplatin (cisdiamine dichloroplatinum (II) cisplatinum), ABT-737, or both agents combined (Fig. 5A). U87, U118, and D645 lines were largely resistant to these treatments; however, the low Mcl-1 –116G>A ETS SNP lines, U251 and U373, showed greater sensitivity ($P < .05$) to both ABT-737 and cisplatin as well as to these agents combined. To confirm that Mcl-1 was conferring a survival advantage, we inhibited Mcl-1 function using a Noxa peptide previously shown to neutralize Mcl-114 (notably A1, the other target of Noxa, is not significantly expressed in glioma, Fig. 1). U87 cells were treated with Noxa or control peptide alone and in combination with ABT-737 or cisplatin (Fig. 5B). Results show increased apoptosis ($>20\%$) ($P < .05$) following Noxa treatment compared with the control peptide. Increased apoptosis also occurred with Noxa treatment in the presence of ABT-737 or cisplatin treatment, though the additional increases observed were modest.

To further substantiate the observed apoptosis when Mcl-1 was inhibited, we also used shRNA to downregulate Mcl-1 in U87 and U373 cells (Fig. 5C). Mcl-1 down-regulation induced apoptosis in U87 (29\%), whereas the already low Mcl-1 expression line, U373, was only mildly affected (Fig. 5C).

Overexpression of ELK4, resulting in elevated Mcl-1, was anticipated to confer increased survival in GBM cells. To investigate this, we overexpressed ELK4 in U118 cells. As expected, this resulted in increased Mcl-1 expression (shown in Fig. 4C). U118 control cells and ELK4 overexpressing cells were treated with cisplatin. Results show increased protection from apoptosis when ELK4 was overexpressed (17\% apoptosis) ($P < .05$) relative to the control cells (25\% apoptosis), highlighting the protective effect of elevated ELK4 and hence Mcl-1 against increased concentrations of chemotherapy.

A primary derived GBM tumorsphere line (L1-NS) was also tested for inhibition of Mcl-1 cultured under neurosphere conditions. Results show markedly increased apoptosis ($>50\%$) ($P < .05$) following Noxa peptide treatment compared with control (Fig. 5B). Once again, only modest increases in apoptosis accompanied the addition of ABT-737 or cisplatin treatment.

The longer-term effects of ABT-737 and cisplatin on tumor initiating cells were investigated using the neurosphere assay.18 L1-NS was serially passaged 7 times, and the total theoretical cell number calculated (Fig. 5S). L1-NS control cultures generated $1 \times 10^9$ cells over 7 weeks; this was reduced to $1.2 \times 10^8$ cells with ABT-737 treatment. Cisplatin had a greater effect, with no cells remaining after 7 weeks of treatment. Notably, no cells remained after 2 weeks of treatment when these agents were combined. Importantly, these agents were able to prevent extensive self-renewal and...
generation of a large number of progeny in primary GBM neurospheres.

ELK4 Downregulation Sensitizes GBM Cells to Apoptosis and Reduces in vivo Tumor Formation

To determine whether stable reduction of ELK4 would increase GBM sensitivity to ABT-737 or cisplatin treatment, the more effective ELK4 siRNA sequence #2 was cloned into the pSuperior shRNA vector and stable U87 clones generated. Following stable knockdown of ELK4, U87 cells were treated with ABT-737, cisplatin, or ABT-737 + cisplatin (Fig. 5D). As expected, stable downregulation of ELK4 sensitized these cells to both agents. Combined treatment with ABT-737 + cisplatin (1 μM) or cisplatin (10 μM) increased apoptosis >20% and >30%, respectively (P < .05). To confirm that increased apoptosis was indeed mediated by Mcl-1, the stable ELK4 shRNA knockdowns were rescued by overexpression of Mcl-1 (Fig. 5D). When ELK4 shRNA Mcl-1 rescued cells were again challenged with ABT-737 or cisplatin,
apoptosis returned to normal levels, as observed for cells expressing control shRNA Mcl-1.

We also investigated the consequence of reduced ELK4 expression on tumor formation using a U87 subcutaneous and U118 intracranial murine xenograft model. Two U87 ELK4 shRNA clones were tested for subcutaneous tumor formation in NOD/SCID mice. In experiment #1 (clone #1) (Fig. 6A), a reduction in tumor formation was observed, with a median survival of 27 days for the control shRNA versus 40 days for the ELK4 shRNA ($P = .006$). In experiment #2 (clone #2), a smaller number of cells was injected. In this experiment, the mice injected with control shRNA had a longer median survival of 67 days, in keeping with the reduced inoculum. Significantly, however, no tumor formation was observed in the animals in which ELK4 was downregulated after 5 months (141 days) ($P = .004$). At autopsy, a small lesion was found in 2 of the ELK4 shRNA animals at the site of injection, whereas the remaining 3 animals were free of tumors. This very marked difference was investigated further. Quantitative PCR was conducted to determine the level of ELK4 and Mcl-1 knockdown in vivo (Fig. 6C). This showed that while clone #1 had a relatively

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Fig. 6. ELK4 downregulation reduces tumorigenicity in vivo. (A) Kaplan–Meier survival analysis shows significant survival extension of ELK4 shRNA U87 GBM cells vs control shRNA. Two independent clones were tested. For clone #1 (experiment #1, $2 \times 10^6$ cells injected subcutaneously), ELK4 shRNA median survival was 40 days vs control 27 days ($P = .006, n = 7$). For clone #2 (experiment #2, $1 \times 10^6$ cells injected subcutaneously), ELK4 shRNA median survival was 141 days (experiment terminated) vs control 67 days ($P = .004, n = 5$). (B) qPCR expression data showing reduction of both ELK4 and Mcl-1 mRNA levels in both experiment #1 and experiment #2. Knockdown of ELK4 and Mcl-1 correlated with increased survival in vivo. (C) Aperio images of U87 subcutaneous xenograft tumors showing tumor morphology (hematoxylin and eosin), apoptosis (cleaved caspase-3), and proliferation (Ki67). Necrotic and apoptotic cells were observed in ELK4 shRNA treated tumors. (D) Kaplan–Meier survival analysis shows significant survival extension of ELK4 shRNA U118 GBM cells vs control shRNA using an intracranial xenograft model. ELK4 shRNA median survival was 38 days vs control 28 days ($P = .01, n = 6$).
modest reduction in ELK4 mRNA (34%), which reduced Mcl-1 to equivalent levels (30%), a more significant ELK4 knockdown was achieved in experiment #2 (48%), which had a more dramatic effect on Mcl-1 mRNA levels (80% reduction). Thus, tumor formation appeared to correlate with both the level of ELK4 and Mcl-1 knockdown. In addition, we prepared hematoxylin-and-eosin tumor sections and measured levels of apoptosis and proliferation (Fig. 6C). In both control animal cohorts, tumor cells were viable with prominent angiogenesis. ELK4-downregulated tumors, however, lacked blood vessel formation, with prominent areas of necrosis and apoptosis, as detected using cleaved caspase-3. Only mild changes in proliferation between the 2 groups was observed using Ki67 staining. Given the unique microenvironment of the brain, we also assessed knockdown mediated by ELK4 shRNA using the U118 GBM cell line with an intracranial xenograft model. Similar to subcutaneous results, a reduction in tumor formation was observed, with a median survival of 28 days for control shRNA compared with 38 days for ELK4 shRNA–transfected cells \( (P = .01) \).

**Discussion**

GBM is one of the most lethal of all human cancers, and despite recent advances in therapy, the outlook has improved very little. Identifying and targeting key gene products offers a rational approach to therapy, and recent work suggests that glioblastoma patients in particular may benefit from molecularly targeted therapies.¹⁹ Here we identify two potential GBM therapeutic targets: the anti-apoptotic protein Mcl-1 and, as our results show, a critical regulator of its expression, the ETS family transcription factor ELK4. Importantly, ETS family members are implicated in the regulation of Bcl-2 family anti-apoptotic proteins.²⁰ FLI1 blocks apoptosis in primary erythroblasts by regulation of Bcl-2, whereas Rel ETS family members have been shown to directly regulate Bcl-xL, promoting survival.²¹,²² Moreover, it has been shown that serum responsive factor and ELK1 act in concert to mediate the expression of Mcl-1 in hematopoietic cells.²³

Elevated expression of Bcl-2 family anti-apoptotic proteins in tumors can confer a survival advantage on cancers and contribute to the resistance of malignant cells to conventional cytotoxic therapy. Here we show Mcl-1 to be highly expressed in a significant majority of both human primary gliomas and GBM cultures. This observation, based on 51 samples, is supported by interrogation of the National Cancer Institute Rembrandt database, albeit using an alternative analytical method. Of 405 glioma specimens, 205 show upregulation (>2-fold) and 92 show intermediate expression of Mcl-1. Furthermore, a significant difference in survival \( (P = .0417) \) was shown between the upregulated and intermediate populations.

Importantly, inhibition of Mcl-1 sensitized GBM cell lines to apoptosis and increased sensitivity to ABT-737 or cisplatin. The dominant role of Mcl-1 in glioma is further supported by the ineffectiveness of ABT-737 alone, a small molecular weight inhibitor of Bcl-2, Bcl-xl, and Bcl-w, but not of Mcl-1.

Given the unique dependence of glioma on Mcl-1 and its very high expression in these tumors, we investigated the molecular basis for elevated Mcl-1 expression in GBM. Genomic analysis of the Mcl-1 promoter revealed that guanine-cytosine–rich insertions of 6 bp and 18 bp, as reported previously in chronic lymphocytic leukemia (CLL), were common to both GBM tissue samples and in normal control samples. Although a previous study²⁴ suggested that these insertions resulted in increased promoter activity in leukemic cells, we found no significant increases in promoter activity in GBM cells (Fig. S4). Furthermore, no correlation was found between Mcl-1 mRNA expression and the presence of insertions in GBM, consistent with more recent findings in CLL²⁵,²⁶ Taken together, these data suggest that these insertions are irrelevant to glioma biology.

More significantly, though, in the course of this analysis, we identified a novel SNP (–116G>A) in the Mcl-1 promoter in GBM, the presence of which resulted in decreased promoter activity. Two GBM cell lines, identified as being heterozygous for the –116G>A SNP, also exhibited reduced Mcl-1 levels. Significantly, this SNP occurs in a potential ETS transcription factor binding site that is bound by ELK4 in GBM cells. These results provide strong evidence that ELK4 is a key regulator of both Mcl-1 promoter activity and Mcl-1 expression in GBM and is therefore a potential target for therapies aimed at downregulating Mcl-1 and enhancing sensitivity to both ABT-737 and cisplatin treatment.

To assess whether ELK4 regulates expression of Mcl-1 in tumors other than glioma, mRNA expression analysis is now under way. Interestingly, preliminary findings show a highly significant positive correlation between ELK4 and Mcl-1 mRNA in kidney, lung, thyroid, and colon tumors (Fig. S5). These findings, while requiring further follow-up, are extremely positive and suggest that ELK4 could be a potential target to inhibit Mcl-1 in a variety of human tumors.

Both ELK4 and Mcl-1 are identified in this study as potential targets for therapeutic intervention in high-grade gliomas. Indeed, Mcl-1 has been targeted by others using a variety of conventional and experimental agents to enhance the efficacy of ABT-737 in diverse human tumor cells, including leukemia (U937 and OCI-AML3), cervical (HeLa), breast (MCF-7), and small cell lung carcinomas (NCIH196 and NCI-H146).²⁷,²⁸ What then might be the advantage in targeting ELK4 in GBM? Consideration of the respective phenotypes of Mcl-1 and ELK4 knockout mice suggest it could be associated with fewer side effects than targeting Mcl-1 directly. Mcl-1 knockout mice, for example, die at early embryonic stages,²⁹ with Mcl-1 critical for lymphocyte and hematopoietic stem cell survival.³⁰,³¹ Conditional deletion of Mcl-1 in mice also shows it to be essential for neutrophil survival,³² whereas inhibition of Mcl-1 expression by
anti-sense oligonucleotides results in human macrophage apoptosis. In contrast, ELK4-deficient mice are viable with no gross physical abnormalities, although some immune suppression was noted with a reduction in single-positive thymocytes and peripheral T cell numbers. Given this milder phenotype, targeting ELK4 may provide a more attractive target for downregulating Mcl-1 and sensitizing GBM to apoptosis. The in vitro and in vivo experiments provide proof-of-principle that suppression of ELK4 can indeed significantly compromise glioma cell survival and tumorigenicity.

In summary, we provide evidence that elevated Mcl-1 expression is a key factor providing protection from apoptosis in GBM. Elevated expression of Mcl-1 in GBM in turn depends critically on expression of ELK4, suggesting that ELK4-mediated Mcl-1 overexpression is a key step in the oncogenic pathway leading to GBM. Both ELK4 and Mcl-1 accordingly are identified as potential targets for therapeutic intervention in GBM. The novel, functional Mcl-1 promoter ETS SNP, although identified at low frequencies, clearly reduces Mcl-1 expression and may prove a useful prognostic marker for GBM survival outcome or a marker that determines treatment strategies.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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