Astrocyte elevated gene-1 (AEG-1) interacts with Akt isoform 2 to control glioma growth, survival and pathogenesis

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Abstract:
The oncogene AEG-1 (MTDH) is highly expressed in glioblastoma multiforme (GBM) and many other types of cancer, where it activates multiple signaling pathways that drive proliferation, invasion, angiogenesis, chemoresistance, radioresistance and metastasis. AEG-1 activates the Akt signaling pathway and Akt and c-Myc are positive regulators of AEG-1 transcription, generating a positive feedback loop between AEG-1 and Akt in regulating tumorigenesis. Here we describe in GBM cells a direct interaction between an internal domain of AEG-1 and the PH domain of Akt2, a major driver in GBM. Expression and interaction of AEG-1 and Akt2 are elevated in GBM and contribute to tumor cell survival, proliferation and invasion. Clinically, in silico gene expression and immunohistochemical analyses of patient specimens showed that AEG-1 and Akt2 expression correlated with GBM progression and reduced patient survival. AEG-1-Akt2 interaction prolonged stabilization of Akt2 phosphorylation at S474, regulating downstream signaling cascades which enable cell proliferation and survival. Disrupting AEG-1-Akt2 interaction by competitive binding of the Akt2-PH domain led to reduced cell viability and invasion. When combined with AEG-1 silencing, conditional expression of Akt2-PH markedly increased survival in an orthotopic mouse model of human GBM. Our study uncovers a novel molecular mechanism by which AEG-1 augments glioma progression and offers a rationale to block AEG-1-Akt2 signaling function as a novel GBM treatment.
Introduction:

Glioma is the most common form of brain tumor (1). Approximately 34% of brain tumors are gliomas and 82% are malignant gliomas, graded as WHO III and IV (1). Despite significant improvements in neurosurgical approaches and chemotherapy/radiotherapy, grade IV glioblastoma (GBM) remains a cancer type with dire prognosis, primarily because GBM cells are highly invasive and are capable of infiltrating into surrounding normal brain tissue, resulting in failure to completely remove the tumor by surgery (2, 3). Consequently, glioma recurrence is a common event and recent research highlights a seminal role of radiation- and chemotherapy-resistant glioma stem cells as potential mediators of this recurrence (4, 5). Several studies have suggested that glioma cells are highly resistant to cytotoxic treatments and their dispersion may actually be triggered, in part, by these treatments (6, 7). In these contexts, defining appropriate targets for therapeutic intervention and approaches for delimiting invasion/spread and pathogenesis of GBM remain key priorities. Moreover, through comprehension of the mechanism(s) of glioma progression an appropriate path may become evident for developing effective therapeutic reagents and strategies for malignant glioma treatment.

Astrocyte elevated gene-1 (AEG-1) was first identified as an upregulated transcript in primary human fetal astrocytes infected with HIV-1 or treated with TNF-α and later characterized as a major oncogene highly upregulated in a broad array of cancers including GBM (8-10). Subsequently, using in vivo phage screening the mouse AEG-1 gene was cloned and named metadherin (MTDH) based on its pro-metastasis role in tumor cell homing to lung (11). AEG-1 is a multifunctional protein interacting with diverse partners in different cancer types and capable of promoting all hallmarks of cancer (9, 10, 12, 13). Through protein-protein interactions, AEG-1 functions as a key pathological gene regulating a variety of diseases (12).
Akt is a nodal point in a variety of cancer signaling pathways that ultimately regulates tumor cell fates including apoptosis, proliferation, survival, invasion, and cell cycling (14, 15). Akt signaling pathway is constitutively active in GBM (16, 17). Although Akt isoform distribution in gliomas has been investigated, limited functional studies were performed (18). Among the Akt isoforms, Akt2 is critical for glioma progression and resistance to chemotherapy and radiotherapy (19-21).

We reported previously that AEG-1 can activate Akt signaling pathway in immortalized human astrocytes and Akt and c-Myc positively regulate AEG-1 transcription, thereby establishing a positive feedback loop between AEG-1 and Akt in promoting tumorigenesis (22, 23). AEG-1-Akt signaling promotes cell proliferation, survival, angiogenesis and chemoresistance (24-26). However, the mechanism by which AEG-1 activates Akt remains unclear. We now demonstrate a novel interaction between AEG-1 and Akt2, in glioma, and document that this interaction is critical for downstream Akt signaling which regulates tumor cell survival, proliferation and invasion. Additionally, the interacting regions in both molecules have now been mapped, which provides a defined target site for developing small molecule drugs capable of disrupting this interaction that might provide potential novel therapeutics for GBM.

**Method and materials:**

**Cell cultures**

Human glioma cell lines, U87 and U251, were obtained from ATCC and cultured with DMEM containing 10% fetal bovine serum. VG2, 4 and 6 primary glioma neurospheres were isolated
from GBM tumor samples provided by VCU TDAAC. The tumor cells were isolated following the described protocol (27) and cultured with glioma stem cell media for no more than 5 passages. All cell lines were authenticated within 6 months using the “CellCheck™” service provided by the Research Animal Diagnostic Laboratory (IDEXX BioResearch, Columbia MO).

**Co-immunoprecipitation (Co-IP)**

Pierce Protein G conjugated Agarose (Thermo Scientific, Waltham, MA) was used in Co-IP experiments. Corresponding mouse or rabbit IgG (Jackson Immuno, West Grove, PA) were used as controls. Horseradish peroxidase-conjugated secondary antibody with light-chain IgG (mouse/rabbit) was used to eliminate potential overlapping heavy-chain background in all IP experiments.

**Analysis of TCGA Glioma Genome-wide Expression Datasets**

Genome-wide expression datasets for glioma were downloaded from The Cancer Genome Atlas (TCGA) (28). The level 3 (processed) datasets, along with accompanying clinical information, were obtained through the UCSC Cancer Genomics Browser (29). Data processing steps, using the Gene-E program (Broad Institute, Cambridge, MA) started with inverse log (base 2) transformation, and quantile normalization of expression values, then annotation of samples with matching clinical records. Further statistical analyses were conducted using the JMP Pro 10 statistical program (SAS, Cary, NC).

**Tissue microarray (TMA), immunohistochemistry, immunofluorescence and confocal imaging**
GL801 glioma TMA slides from US Biomax were used for immunohistochemistry with standard protocol as described (30). The sample information including sample number and grade are listed in detail in supplementary Table 2. 20x images were taken with a Nikon microscope system. AEG-1 and Akt2 staining was scored blindly by 4 independent investigators. Correlations between staining for both proteins were analyzed with Prism 5 software. All 63x oil images were taken with Zeiss LSM 700 confocal laser scanning microscope and analyzed.

**Quantitative Akt phosphorylation assay:**

U87 glioma cells were seeded (300,000 cells per dish) in 35-mm culture dishes and infected with control, AEG-1, or AEG-1-siRNA adenoviruses (50 MOI). LY294002 (4µM) was added 48 hours after infection, time 0. The samples were collected by flash freezing the dishes at 15’, 30’, 1h, 2h, and 3h. Total lysates were prepared on ice with phosphatase inhibitor cocktail (Roche). Western blots were analyzed by GelPro to measure total density of defined bands. Graph results were normalized using tAkt2 and Actin.

**Intracranial implant of VG2 cells in mice and survival experiments**

VG2 cells were established with lentivirus transduction to express AEG-1-shRNA and Akt2-PH domain (with a lentivirus Tet-on expression system) in 4 different combinations: Ctrl-shRNA/Ctrl (C/C), AEG-1-shRNA/Ctrl (A/C), Ctrl-shRNA/Akt2-PH (C/A), and AEG-1-shRNA/Akt2-PH (A/A). Using stereotaxic frame these VG2 cells (10,000 cells in 2 µl PBS) were intracranially injected into athymic nude mouse brains with 6 animals in each group. The coordination of injection is 2.5-mm lateral, 0.4-mm frontal of bregma and 3.5-mm deep from skull. The animals were continuously fed with Doxycycline water (1 mg/ml) starting from the
fourth day after tumor cell implants to induce Akt2-PH expression. Animals of each group were monitored until they reached the point of euthanization according to the VCU IACUC approved protocol. Survival data was collected and analyzed with Prism 5.

Statistics

All in vitro experiments were repeated at least in triplicate with 3 independent replicates. Animal number N=5 was used in survival tests. Statistical analyses were calculated using One-way and Two-way ANOVA for grouped samples, followed by the Bonferroni post hoc test; and two–tail student T test which was used for comparison between control and treatment groups. *P<0.05, **P< 0.01 and ***P<0.001 with mean ± SD are shown in the figures.

Results:

**AEG-1 interacts with Akt2 and expression correlates in glioma specimens**

Since AEG-1 activates Akt signaling we determined whether AEG-1 directly interacts with Akt. Co-IP analysis using anti-AEG-1 antibody effectively pulled down Akt in U87 cells (Figure 1A, left panel). We overexpressed HA-tagged AEG-1 in U87, U251 and in a primary glioma (VG2) neurosphere (Figure 1A). Immunoprecipitation with anti-HA antibody successfully pulled down AEG-1 and endogenous Akt in all of these cells indicating an AEG-1/Akt protein complex in glioma cells.

Akt has three isoforms, which play different functional roles in tumor cells (18, 31, 32). Akt isoform expression was analyzed in VG2, VG4, and VG6 primary human glioma tumor samples versus normal brain (Figure 1B) and in U87 and U251 glioma cells versus primary human fetal
astrocytes (PHFA) (Figure 1C). Akt1 and 2 were upregulated in all glioma samples and cell lines compared to controls. Akt3 was down regulated in GBM cell lines and samples (VG2 and VG6) except in VG4, a grade II astrocytoma. These results are consistent with Akt isoform distribution in gliomas as previously reported (18).

We next performed Co-IP of endogenous AEG-1 with all three isoforms of Akt in U87 and VG2 cells. Anti-AEG-1 antibody successfully pulled down Akt2, but not the other two isoforms (Figure 1D). Because of low levels of Akt3 in glioma cells, we performed Co-IP of AEG-1 with overexpressed Akt3 in U87 cells, and the result ruled out interaction between these two molecules (Supplementary Figure 1). These findings demonstrated that AEG-1 specifically interacts with Akt2. Double immunofluorescence analysis documented that AEG-1 (green) and Akt2 (red) co-localized (yellow in merged image) predominantly in the peri-nuclear region (Fig. 1E). Both AEG-1 and Akt2 are capable of translocating to different cellular compartments and play distinct functional roles (33-35). Further detailed sub-cellular analysis of AEG-1 and Akt2 interaction is warranted.

We analyzed the clinical relevance of AEG-1 and Akt2 in GBM using TCGA database. A total of 372 glioma patients were divided into Akt2_low or Akt2_high based on Akt2 levels below or above the median, respectively (Supplementary Table 1). The resulting Kaplan-Meir plot clearly indicated that high Akt2 level is associated with poor survival ($p< 0.0001$ in both log-rank and Wilcoxon statistical tests) (Figure 2A, left panel). However, when the patients were further classified according to AEG-1 expression (similar to Akt2 level), thus creating 4 patient subgroups, it is evident that patients belonging to the Akt2_low/AEG-1_low subgroup had the best clinical outcome (followed by the Akt2_low/AEG-1_high subgroup) (Figure 2A, right panel). In the same data set, subgroups of Akt3 strikingly showed opposite results while Akt1
subgroups only showed narrowed separation when compared to Akt2 (Supplementary Figure 2). These results indicate a strong correlation between AEG-1 and Akt2 levels with survival of GBM patients.

Next, we checked AEG-1 and Akt2 expressions in a glioma tissue microarray (TMA) by immunohistochemistry. Low levels of AEG-1 and Akt2 were detected in normal brain samples (Figure 2B). Both AEG-1 and Akt2 expressions were progressively elevated in glioma samples from grade I to grade IV versus normal brain and a high correlation between AEG-1 and Akt2 levels were observed in these samples ($r^2=0.9221$) (Supplementary Figure 3 and Supplementary Table 2). Collectively these findings clearly suggest that interaction between AEG-1 and Akt2 is clinically relevant and further support our hypothesis that AEG-1-Akt interaction plays a germane role in glioma pathogenesis.

We mapped interaction regions of AEG-1 and Akt employing HA-tagged deletion constructs (Figure 3A), in Co-IP analysis. Anti-HA antibody successfully pulled down endogenous AEG-1 upon co-transfection of Akt deletion mutants, $\Delta$120-2 ($\Delta$2), $\Delta$120-3 ($\Delta$3), and $\Delta$120-4 ($\Delta$4), but not $\Delta$PH (Figure 3B, left panel). In comparison, N2, N4, N6 and C2 deletion mutants of AEG-1 interacted with endogenous Akt2 while C4 (a.a. 289-582 deleted) failed to interact with endogenous Akt2 (Figure 3B, right panel). These results support the conclusion that relevant interaction domains between AEG-1 and Akt2 are located at 290-404 a.a. in AEG-1 and 1-118 a.a. in Akt (PH domain). To confirm the specificity of AEG-1 to Akt2, we overexpressed PH domains from the 3 Akt isoforms with a Myc tag at the NH$_2$-terminus and performed co-IP with anti-AEG-1 antibody. Only the Akt2 PH domain interacted with AEG-1 (Figure 4A). Our domain mapping provides clues for potentially developing inhibitory compounds that may block
the interaction between these two important molecules. However, defining precise local interaction motifs and structures are important and require further investigation.

**AEG-1-Akt2 interaction regulates Akt signaling and function**

Based on domain mapping results, we hypothesized that the Akt2-PH domain might disrupt endogenous AEG-1-Akt2 interaction by competitively binding to AEG-1. To address this possibility, we performed competitive binding of Akt2-PH to AEG-1 by determining the amount of Akt2 immunoprecipitated with anti-AEG-1 complex. When Akt2-PH was expressed at increasing concentrations it dramatically reduced the magnitude of AEG-1-Akt2 interaction (Figure 4B), which was associated with decreasing phosphorylation levels of pAkt2-S474 (Figure 4C).

To understand the functional roles of AEG-1-Akt interaction on cell survival under stress, we subjected U87-MG (p53wt) and U251 (p53mut) cells, expressing Akt2-PH, AEG-1 or both, to serum starvation or TMZ treatment. In 1% FBS, AEG-1 overexpression enhanced colony formation ability in soft agar (Figure 4D). Significantly fewer colonies developed in cells expressing Akt2-PH. Cells expressing both AEG-1 and Akt2-PH, but not Akt1-PH, showed significant reductions in colony number compared to AEG-1 alone (Figure 4D). AEG-1 overexpression significantly improved overall cell viability in U87 and U251 cells upon TMZ treatment (Figure 4E). In contrast, Akt2-PH, but not Akt1-PH, significantly decreased cell viability by itself, and also reduced the pro-survival effects of AEG-1 when treated with TMZ (Figure 4E). These results indicate that AEG-1/Akt2 interaction is important for glioma cell survival under stress, which might be blocked by Akt2-PH, and this effect is p53-independent.

To distinguish between Akt1 and Akt2, which are frequently overexpressed in GBM, we tested
cell invasion and proliferation assays using either overexpression of the relevant PH domain or by knocking down of Akt1 or Akt2 (Supplementary Figure 4A and B). Interestingly overexpression of PH domain or Akt isoform specific RNAi abrogated cell proliferation, but AEG-1 failed to reverse the inhibitory effect (Supplementary Figure 5). However, Akt2-PH, but not Akt1-PH, abrogated pro-invasive function of AEG-1 (Figure 4F). Similar findings were observed with Akt2-siRNA, but not Akt1-siRNA (Figure 4G). These results strongly suggest that AEG-1-Akt2 interaction plays an important functional role in glioma invasion.

Classical Akt activation requires PI3K-dependent phosphorylation at the T308 site by PDK1 followed by maximal activation at the S473/474 site phosphorylated by mTORC2. Blocking of PI3K-Akt signaling by LY294002 has been shown to reduce Akt phosphorylation at both S473 and T308 sites (36). To define how AEG-1 regulates Akt activation we overexpressed or knocked down AEG-1 and checked the phosphorylation status of Akt2-S474 in a time-dependent manner. We first measured phosphorylation of Akt at S473 and T308 sites (Supplementary Figure 6). Overexpression of AEG-1 prolonged, while knockdown of AEG-1 reduced phosphorylation of Akt-S473 versus control (Supplementary Figure 6). No difference in total p-Akt-308 was observed among control, AEG-1 OE and AEG-1 KD groups (Supplementary Figure 6). We further studied the dynamics of p-Akt2-S474. Semi-quantitative analysis showed that overexpression of AEG-1 prolonged phosphorylation of Akt2-S474, and knockdown of AEG-1 reduced initial Akt phosphorylation and resulted in a shorter half-life of p-Akt2-S474 compared to control (Figure 5A). A linear fit analysis of Western blot gel quantification indicated a greater effect of AEG-1 on phospho-S474 measured with 1/slope (smaller number equals slower decay and vice versa) (Figure 5B). In contrast, the dephosphorylation dynamics of
p-Akt1-S473 was minimally changed when using specific phospho-Akt1 antibody (Supplementary Figure 9).

Since AEG-1 is required to stabilize phosphorylation of Akt at S473/474, we investigated the effects of disrupting AEG-1-Akt interaction by Akt2-PH on Akt and its downstream effectors. Disruption of the interaction down-regulated phosphorylation of S474, but not T308, on Akt. Phospho-GSK3β and phospho-BAD, substrates of Akt, and CyclinD1 levels were increased upon AEG-1 overexpression, which was inhibited upon co-expression of Akt2-PH (Figure 5C).

Insulin is an established upstream activator of Akt through IGFR-IRS (37). To further explore the mechanism of AEG-1-Akt signaling, we examined Akt signaling upon insulin stimulation when AEG-1 expression was knocked down with siRNA. We also checked the effect of blocking Akt activation by PTEN or LY294002 when AEG-1 was overexpressed. AEG-1 KD successfully blocked insulin-induced elevation of Akt2-S474 phosphorylation (Figure 5D). In contrast, AEG-1 overexpression retained S474 phosphorylation when PTEN and LY294002 inhibited PI3K-Akt activation. Noticeably, Akt downstream signaling p-GSK3β and CyclinD1 levels also changed corresponding to phosphorylation of Akt2-S474 (Figure 5C-E). Moreover, AEG-1 overexpression significantly rescued growth inhibition caused by LY294002 (Figure 5F). Collectively, these data demonstrate that AEG-1-Akt2 interaction is important for glioma proliferation, survival, and invasion. Overexpression of AEG-1 or loss of AEG-1 in glioma can lead to changes in phosphorylation of Akt2-S474, thus resulting in downstream signaling activation or inhibition, respectively.

**Combination of Akt2-PH overexpression and AEG-1 KD decreases glioma cell proliferation and prolongs animal survival**
To examine the effect of inhibition of AEG-1-Akt2 interaction in vitro and in vivo, we combined KD of AEG-1 with conditional overexpression of Akt2-PH, in VG2 primary glioma neurospheres. We chose this low passage primary GBM cell line because it is more closely associated with the clinical state. In order to disrupt AEG-1-Akt2 interactions, we stably knocked down AEG-1 and conditionally overexpressed Akt2-PH with a lentivirus Tet-on expressing system. Four different subgroups of cells in combinations were used: Ctrl-shRNA/Ctrl, AEG-1-shRNA/Ctrl, Ctrl-shRNA/Akt2-PH, AEG-1-shRNA/Akt2-PH. Overexpression of Akt2-PH domain was induced by doxycycline (Dox) in the culture media. Western blotting analysis showed that phospho-Akt2-S474 level was dramatically reduced in the presence of AEG-1-shRNA and/or Akt2-PH (Figure 6A). A significant inhibition of cell growth was observed when AEG-1 was knocked down or Akt2-PH was over expressed following induction by Dox, and the combination resulted in a more profound inhibitory effect (Figure 6B). Exclusion of Dox did not cause any inhibition in cell proliferation.

We performed orthotopic injection of aforementioned 4 subgroups of VG2 cells into athymic nude mice brains and subsequently induced Akt2-PH expression through Dox-containing water (Figure 6C). The control group of animals started to die 15-days after intracranial tumor implant. AEG-1 KD accrued a 5-day improvement in median survival. In comparison, AEG-1 KD plus Akt2-PH expression significantly extended median survival to 42.5-days (***p< 0.0001). This finding strengthens our hypothesis that targeting AEG-1 and inhibiting AEG-1-Akt2 interaction might be an effective therapeutic approach for GBM.

**Discussion:**
Our previous studies demonstrated a positive feedback loop involving AEG-1-Akt signaling that regulates cell proliferation, survival, angiogenesis, chemoresistance and invasion (9, 10). However, the details of how AEG-1 signals to Akt remain unclear. Here we report that AEG-1 specifically interacts with Akt2 and not with Akt1 or Akt3 isoforms in GBM. Moreover, this interaction plays a decisive role in glioma cell biology. Our results demonstrate a high correlation between expression of AEG-1 and Akt2 in clinical glioma samples and suggest that AEG-1-Akt2 signaling is more dominant in high-grade glioma, GBM. Our results also suggest that various Akt isoforms may play distinct roles in gliomas. Akt3 is known to play a more significant role in triple negative breast cancer (38) and in our study we found that VG4 low-grade glioma shows higher Akt3 expression. Interestingly, our results also demonstrate that disruption of AEG-1-Akt2 interaction reduced AEG-1 pro-invasive function, which is consistent with a previous report that Akt2 promotes breast cancer cell migration and invasion, whereas Akt1 has an opposite effect (32). A recent study shows that Akt2, but not Akt1, is necessary for PTEN deficient prostate tumor cell survival (39). AEG-1 is localized in different compartments of the cancer cell (40). When both Akt-S473/474 and T308 sites are phosphorylated, Akt is fully activated and translocates from the cytoplasm into the nucleus (33, 34). Interestingly, AEG-1 and Akt2 interaction occurs in the peri-nuclear region of glioma cells. We show that AEG-1 prolongs Akt phosphorylation at the S473/474 site. Collectively, our findings suggest that AEG-1 might temporally regulate Akt activity and localization through phosphorylation of the S473/474 site. AEG-1 interacts with the Akt-PH domain, which is also the interaction domain that recruits Akt to cell membranes by PIP3 (15). Most likely, this interaction happens after release of PIP3 binding, because AEG-1 has no direct impact on phospho-T308, which is the initial step of Akt activation at the cell membrane.
Our experiments support the importance of AEG-1-Akt2 signaling in glioma survival, proliferation and invasion. Akt2 is essential for glioma survival while knocking down Akt1 did not affect cell survival or proliferation (18). Our domain mapping results confirm that Akt2-PH domain is responsible for interacting with AEG-1 in glioma. Although protein sequence alignment indicates an overall high homology among human Akt isoforms, it is striking that there is about 35% amino acid sequence difference among the PH domains of Akt isoforms (Supplementary Figure 8). Results from functional assays indicate that AEG-1-Akt2 signaling is more important for glioma cell invasion. AEG-1 failed to rescue inhibition of cell proliferation by both Akt1-PH and Akt2-PH indicating possible modulation through interruption of Akt-PH-PIP3 lipids interaction. As a regulator, AEG-1 might interact with multiple proteins, which allow it to bind and change dynamics of Akt2-PH protein-lipid and protein-protein interactions. Hence, these differences might explain the binding specificity of AEG-1 to Akt2 rather than to the other Akt isoforms. The discovery of direct interaction between AEG-1 and Akt2 reveal the mechanism of previously reported activation of AEG-1-Akt signaling. It also provides valuable information to potentially develop anti-cancer strategies to target malignant gliomas harboring overactive Akt2.

Akt isoforms are highly conserved domain structures with different sequence variations and deletions. They play wide-ranging roles in cancer biology (18, 41). We demonstrate that Akt2 rather than the other two Akt isoforms is more abundant in high-grade gliomas and interacts with AEG-1. Furthermore, disrupting AEG-1–Akt2 interaction reduced cell proliferation and reverted TMZ resistance (Hu et al. unpublished data) which are consistent with a previous report that indicated that knockdown of Akt2 decreased cell proliferation and increased chemo-sensitivity in glioma cells (19). Transgenic Akt2 knockout animals are viable but show metabolic defects with
impaired insulin-stimulated glucose transport/uptake (42). Our finding that AEG-1-Akt2 interaction regulates GSK3β phosphorylation correlates with the phenotype of cellular deficiency in glucose uptake. Akt2 is also involved in cancer cell invasion and metastasis (41). Our results confirmed that disruption of the AEG-1-Akt2 interaction reduces cell invasion, which is consistent with a previous report indicating that Akt2 promotes cell migration and invasion in other cancers (43, 44). Cytoplasmic localization of AEG-1 is important for enhanced cancer cell survival (35). It is possible that AEG-1-Akt2 interaction in the cytoplasm may enforce pro-survival signaling in glioma cells.

Previous studies suggest that AEG-1 regulates cancer cell proliferation and promotes glioma survival through cross talk with different signaling pathways (40, 45). AEG-1 interacts with the p65 subunit of NF-κB resulting in enhanced cancer cell invasion and migration by increasing MMPs (46). We explored the potential crosstalk between AEG-1-Akt2 and NF-κB signaling, by using CAPE (Caffeic Acid Phenethylster) blocking IKK. CAPE weakly affected AEG-1-Akt2 signaling (Supplementary Figure 7) suggesting that NF-κB activation by AEG-1 is independent of Akt activation. Akt plays a pivotal role in tumor cell survival through direct phosphorylation of the pro-apoptotic protein BAD. Phosphorylation levels of BAD were regulated by AEG-1-Akt2 interaction and affected glioma survival under different stress conditions (Figures 4D, 4E and 5C). These results suggest that AEG-1-Akt2 complex protects glioma cells from classical intrinsic mitochondrial apoptosis pathway. Additionally, tumor cell proliferation is controlled by AEG-1-Akt2 via GSK3β-CyclinD1 cascade. Consistent with earlier studies, the present study shows that a combination of AEG-1 KD with Akt2-PH overexpression dramatically inhibits survival, again confirming that AEG-1 plays a significant role in tumor cell proliferation and survival. AEG-1 was reported to have a functional role in chemoresistance through MDR in
hepatocellular carcinoma (25) and our previous studies showed that AEG-1 could activate AMPK and induce protective autophagy in cancer cells (47, 48). Even though our in vivo experiments suggest better survival following combination of AEG-1 KD and Akt2-PH overexpression, we cannot rule out the possibility that AEG-1 might modulate other pro-survival signaling pathways, such as p38, EGFR or Wnt signaling, to affect glioma cell survival. Nonetheless, we demonstrate here that AEG-1-Akt2 is a critical protein-protein signaling complex in glioma. This interaction may also occur in other cancer contexts in which both of these molecules are highly upregulated. Accordingly, it is worth exploring further this connection in other cancers.

Based on our interaction domain mapping, we hypothesize that it may be possible to synthesize small molecule compounds to block AEG-1 and Akt2 interactions. Escape from conventional chemo-radiotherapy and development of recurrence remain major hurdles for effectively treating gliomas in the clinic. It is unlikely that simply targeting AEG-1 or Akt2 alone will offer long lasting efficacy in genetically diverse malignant gliomas. As such, small molecule inhibitors designed to disrupt AEG-1-Akt2 interactions in combination with conventional treatment modalities might provide an optimal strategy to cure this dismal disease.

In summary, we document a direct interaction between AEG-1 and Akt2, two important cancer drivers, in malignant gliomas. The association between elevated expression of these two molecules and poor glioma patient survival has been confirmed using TCGA database information and this correlation in expression also exists when performing immunohistochemistry. We further elucidated the molecular mechanism of AEG-1-Akt2 signaling and demonstrate that this interaction plays an important functional role in glioma biology (Figure 6D). In these contexts, our results expand our understanding of the mechanism
of AEG-1-Akt signaling in glioma and suggest a novel potential anti-tumor strategy that could use a combination of therapeutic drugs blocking the AEG-1-Akt2 association with conventional therapies to treat malignant gliomas. Considering the now documented important role of AEG-1 in many diverse cancers (49, 50), defining ways of inhibiting the functions of this oncogene holds significant potential to impact on cancer phenotype with broad therapeutic applications.

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References


Figure Legends

Figure 1. AEG-1 interacts with Akt in multiple glioma cells. A) Left panel: endogenous AEG-1 and Akt were immunoprecipitated in U87 cells with AEG-1 antibody. Akt was detected with rabbit pan-Akt antibody and rabbit IgG was used as control. Right panels: HA-tagged AEG-1 was overexpressed in U87, U251 and in a primary GBM neurosphere VG2. Immunoprecipitation was performed with anti-HA antibody and detected with anti-HA and anti-Akt antibodies. Bottom panel: input Akt proteins were detected with pan-Akt antibody, which represents 10% of
total loading. B) AEG-1 and Akt2 are upregulated in human glioma samples. Total lysates of VG2, VG4, and VG6 flash frozen clinical glioma samples were used to compare with a normal brain sample. C) Protein levels of AEG-1 and Akt isoforms in glioma cells were compared with primary human fetal astrocytes by Western blotting. D) Co-immunoprecipitation analysis of endogenous AEG-1 and Akt isoforms in U87 and VG2 total cell lysate with light-chain IgG rabbit secondary antibody used for detection. E) Immunofluorescence followed by confocal microscopy imaging of AEG-1 and Akt2 in multiple glioma cells. Bar =10 µm.

**Figure 2.** A) Kaplan-Meir survival of GBM patients (n=372) from TCGA datasets. Patients with Akt2 levels below or above the median were classified as Akt2_low or Akt2_high, respectively, in the left panel. Then the two groups were further divided into AEG-1_high and AEG-1_low in the right panel. Specific number of each group is listed in the tables below (p< 0.0001 in both log-rank and Wilcoxon statistical tests). B) Expressions of AEG-1 and Akt2 positively correlated with glioma progression. AEG-1 and Akt2 were detected by immunohistochemistry with respective antibodies in a human glioma tissue microarray. Images were taken with 20x objective. Bar= 50 µm.

**Figure 3.** Domain mapping of AEG-1 and Akt2 interaction. A) Schematic diagrams of AEG-1 and Akt2 full length (FL) and deletion constructs. B) Overexpressed Akt2 (upper left) and AEG-1 (upper right) in glioma cell total lysates were used in Co-IP with HA antibody. Pull down of endogenous AEG-1 (middle left) and Akt2 (middle right) were detected by immunoblotting.
**Figure 4.** Disruption of AEG-1-Akt2 interaction reduces glioma invasion and survival. A) Co-IP of AEG-1 and PH domains of Akt1, 2, and 3 in U87 cell lysates. U87 cells were transfected with constructs expressing Myc-tagged PH domains of Akt1, 2 and 3. Immunoprecipitation was performed with anti-AEG-1 antibody and immunoblotting was performed with anti-AEG-1 and anti-Myc antibodies. Rabbit IgG was used as control. Input protein detected with anti-Myc represents 10% of total. B) U87 cells were transfected with 0, 3 and 6 μg of Akt2-PH-Myc plasmid DNA along with AEG-1-HA. Immunoprecipitation was performed with anti-HA antibody. Pull down of endogenous Akt2 was detected by immunoblotting with anti-Akt2 antibody. C) U87 cell overexpressing increasing amount of Akt2-PH as shown in the second top panel. Endogenous AEG-1, Akt2, p-Akt2-S474, Akt1 and p-Akt1-S473 were detected with appropriate antibodies. D) Colony formation assay in soft agar: U87 and U251 cells were transfected with the indicated constructs and cultured in soft agar with DMEM containing 1%FBS. Calcein-AM stained live colonies were imaged and quantified. E) U87 and U251 cells (2,000/well) overexpressing the indicated constructs were treated with 200 μM of Temozolomide and DMSO as control for 48 hours with 1% FBS. F and G) Representative images and quantification of U87 *in vitro* invasion assay upon transfection of the indicated constructs. Two-way ANOVA showed significance at *P<0.05, ** P< 0.01, and ***P<0.001 with mean ± SD.

**Figure 5.** AEG-1 enhances the half-life of phospho-Akt2-S474. A) U87 with either AEG-1 overexpression (OE) or knock down (KD) was treated with 2 μM LY294002. Cell lysates were collected over time. B) Phospho-Akt2-S474 at each time point was quantified with *GelPro*
Analyzer software and normalized with Akt2 and actin. Linear regression analysis was performed and displayed as different lines with shapes. C-E: AEG-1 augments downstream Akt signaling. C) Western blotting of the indicated proteins was performed in U87 overexpressing Akt2-PH-Myc, AEG-1 or both proteins. D) AEG-1 expression affects insulin-stimulated and PTEN-inhibited Akt signaling. AEG-1 was knocked down with RNAi in U87 and treated with 100 ng/ml insulin for 30 minutes. In another set, U87 were transfected with vector control or AEG-1, with and without PTEN. Phospho-Akt, phospho-GSK3β, and Cyclin D1 levels were analyzed. E) LY294002 induces a significant reduction in the level of p-Akt2-S474, and its downstream effectors p-GSK3β and Cyclin D1, which were significantly reverted by overexpression of AEG-1. F) Overexpression of AEG-1 rescues U87 from growth inhibitory effects of LY294002. LY294002 was added at day 1 and day 3 at 2 μM final concentration. Two-way ANOVA analysis showed significance at ***P<0.001 with mean ±SD.

Figure 6. Effect of AEG-1 knock down and temozolomide treatment on glioma survival. A) Western blotting indicates reduction of AEG-1 and doxycycline induced Akt2-PH protein levels in stable VG2 compared with control cells. Phosphorylation of Akt2-S474 was detected with specific antibody. B) Cell proliferation assay with 6 subgroups of VG2 treated with or without doxycycline (200 μg/ml): Ctrl-shRNA/Ctrl (C/C), AEG-1-shRNA/Ctrl (A/C), Ctrl-shRNA/Akt2-PH (no Dox) (C/A-), AEG-1-shRNA/Akt2-PH (no Dox) (A/A-), Ctrl-shRNA/Akt2-PH (+ Dox) (C/A+), and AEG-1-shRNA/Akt2-PH (+ Dox) (A/A+). Nonlinear fit curve was used for each group and One-way ANOVA indicate significant difference compared to Ctrl-shRNA + DMSO, *P<0.05. C) Kaplan-Meier survival curve of athymic nude mice intracranially injected with 4
subgroups of VG2 cells as described in materials and methods. Median Survival: C/C = 16 days, A/C = 21 days, C/A = 27 days, A/A = 42.5 days. D) Schematic model of AEG-1-Akt2 signaling in glioma.
Figure 1

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Figure 2

A

![Graph A: Fraction Surviving vs. Follow-up (days)]

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Log rank, Wilcoxon: $p < 0.0001$

B

![Images B: AEG-1 and Akt2 expression across different groups (NB, G I, G II, G III, G IV)]
Figure 3

A  
AEG-1 deletion mutants

AEG-1 FL

1-101     N2     1-232     N4

1-290     N6

C2

C4

Akt deletion mutants

PH Kinase HD FL

1-118     Kinase HD ΔPH

PH 119-240 Kinase HD Δ120-2

PH     241-360 HD Δ120-3

PH     Kinase 361-480 Δ120-4

B

Input AEG-1

IP: HA IB: HA

IP: HA IB: AEG-1

IP: HA IB: Akt2

Input Akt2
Figure 4

A. IP: IgG
AEG-1
1-PH 2-PH 3-PH
CoIP
Input

B. IP: HA
IB:
CoIP
AEG-1
Akt2
Akt2-PH
Myc
Input
Akt2
Akt2-PH
Myc

C. Akt2-PH
IB:
AEG-1
Myc
p-Akt2-S474
Akt2
p-Akt1-S473
Akt1
Actin

D. Glioma Cell Soft Agar Colony Formation

E. Glioma cell viability after TMZ treatment

F. Vector
Akt2-PH
Akt2-PH+AEG-1
Akt1-PH
Akt1-PH+AEG-1
AEG-1 OE

G. Control
Akt2-siRNA
Akt2-siRNA+AEG-1
Akt1-siRNA
Akt1-siRNA+AEG-1
AEG-1 OE
Figure 5

A

+LY294002: 0' 15' 30' 1h 2h 3h

Control

AEG-1 OE

AEG-1 KD

Akt2

Actin

B

Ctrl 1/slope=-194.5
AEG1 siRNA 1/slope=-177.7
AEG1 OE 1/slope=-206.5

Relative fold changes

Time (minutes)

C

D

E

F

U87 Proliferation Assay

Fold Change to CH1+DMO

|

Cdh1+OEMO
| Cth+LY294002
| AEG1+LY294002

Days

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Figure 6

AEG-1-shRNA: – + – +
Akt2-PH: – – – +

(A) Western blot analysis of AEG-1, Akt2-PH, p-Akt2, Akt2, and Actin in VG2 cells.

(B) VG2 Cell Proliferation Assay

(C) Survival proportions

Days after tumor cell implantation

Survival proportions

Days after tumor cell implantation

*** P<0.0001

(D) Diagram illustrating the role of AEG-1, Akt2, PI3K, and other signaling pathways in VG2 cell survival, invasion, and proliferation.
Astrocyte elevated gene-1 (AEG-1) interacts with Akt isoform 2 to control glioma growth, survival and pathogenesis

Bin Hu, Luni Emdad, Manny D. Bacolod, et al.

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