Published in final edited form as:

Mol Carcinog. 2012 February ; 51(2): 150–164. doi:10.1002/mc.20772.

ADAM17 promotes glioma cell malignant phenotype

Xuguang Zheng¹, Feng Jiang^{1,*}, Mark Katakowski¹, Yong Lu¹, and Michael Chopp^{1,2}

¹Department of Neurology, Henry Ford Hospital, Detroit, MI, USA

²Oakland University, Physics Department, Rochester, MI

Abstract

A disintegrin and metalloproteinase-17 (ADAM17) is involved in proteolytic ectodomain shedding of several membrane-bound growth factors and cytokines. The expression and activity of ADAM17 increase under some pathological conditions such as stroke and cancer. ADAM17 promotes neural progenitor cell migration and contributes to neurogenesis after stroke and breast cancer growth and invasion. In the present study, we sought to elucidate whether ADAM17 contributes to glioma progression. To this end, we examined the role of ADAM17 in the proliferation, invasion, and tube formation of U87 human glioma cells in vitro and tumor growth in vivo. Stable transfection of the U87 cell line with either a plasmid for over-expression of human ADAM17, or a siRNA to ADAM17 was employed in this study to establish high or low ADAM17 expression in glioma cells, respectively. For study of mechanism, the ADAM17 inhibitor TAPI-2 and the PI3K-AKT inhibitor LY294002 were used to counteract high ADAM17 expression and the activated PI3K-AKT pathway, respectively.

Proliferation of glioma cells were tested by thiazolyl blue tetrazolium bromide (MTT) assay, Bromodeoxyuridine incorporation assay, growth curve, and sulforhodamine B assay. Matrigel invasion assays were used to assess the ability of U87 cells to penetrate the extra-cellular matrix (ECM). A Matrigel tube formation assay was performed to test capillary tube formation ability. EGFR-PI3K-Akt pathway activation in U87 cells under different ADAM17 expression levels were tested by Western blot.

Our data show that ADAM17 promotes the U87 malignant phenotype by increased proliferation, invasion, angiogenesis and in vivo tumor growth. Tumor growth in nude mice was significantly inhibited by ADAM17 inhibitor and A17-shRNA in vivo transfection. TGF- α , VEGF secretion and VEGF expression was increased by ADAM17 and counteracted by ADAM17 siRNA, TAPI-2, and LY294002 in U87 cells. ADAM17 activated, whereas ADAM17 siRNA, TAPI-2, and LY294002 deactivated the EGFR-PI3K-AKT signal pathway, which correlated with U87 cell malignant phenotype changes.

This study suggests ADAM17 contributes to glioma progression through activation of the EGFR-PI3K-AKT signal pathway.

Keywords

ADAM17; TACE; Glioma; EGFR-PI3K-AKT; Proliferation; Invasion; Angiogenesis; Tumor growth; RNA interference

* please send all correspondence to: Feng Jiang, Pharm D, Henry Ford Hospital, Department of Neurology, Education & Research Building, Room # 3045, 2799 West Grand Boulevard, Detroit, MI 48202, USA. Tel: 1-313-916-3936, Fax: 1-313-916-1318, fengj@neuro.hfh.edu.

Introduction

A disintegrin and metalloproteinase (ADAM) family has been associated with the process of proteolytic ‘shedding’ of membrane-associated proteins and hence the rapid modulation of key cell signaling pathways in the tumor microenvironment. ADAM17 (a.k.a. TACE), is involved in the ectodomain shedding of multiple membrane-bound ligands and cytokines implicated in diverse biological processes, including growth and inflammation [1,2]. Currently, ADAM17 is best known as the primary secretase responsible for releasing the soluble form of tumor necrosis factor- α (TNF- α) from the plasma membrane [2]. However, the expression of ADAM17 is ubiquitous [3] and not limited to TNF- α -producing cells. ADAM17 also plays a pivotal role in development through the processing of numerous growth factors and growth factor receptors [4-7]. As a member of the ADAM family of transmembrane, multidomain zinc metalloproteinases [3,8], ADAM17 has protease activity against multiple substrates including TNF- α , transforming growth factor- α (TGF- α), TNF receptor, interleukin-6, L-selectin, and amyloid precursor protein (APP) [4-7,9]. ADAM17 has been associated with tumorigenesis [10] and tumor progression [11-13]. Proteolytic processing and the release of membrane proteins can function as a post-translational switch that regulates the activity of the cleaved substrate. This process, which is referred to as ‘protein ectodomain shedding’, may activate or inactivate the substrate protein, or substantially change its functional properties [14]. ADAM17 acts as a signaling scissor in the tumor microenvironment [11]. The expression and activity of ADAM17 increase under some pathological conditions such as stroke [15] and cancer [16]. ADAM17 promotes neural progenitor cell migration and contributes to stroke-induced neurogenesis and breast cancer progression [13]. Our study (unpublished data) indicates a significant increase in ADAM17 expression in glioma (~4.8 fold higher) compared to non-tumored human brain tissue, which suggest that ADAM17 may be a clinically relevant therapeutic target for glioma. We also checked the ADAM17 expression in The Cancer Genome Atlas (TCGA) expression database. 94% (399/424) GBMs show AgilentG4502A_07 log2 tumor/normal ratio greater than 0.5 (41.5% ADAM17 expression increase compared to normal brain tissue); 62% (261/424) GBMs show AgilentG4502A_07 log2 tumor/normal ratio greater than 1.0 (100% ADAM17 expression increase compared to normal brain tissue).

In glioma cells, we have demonstrated that a hypoxia-induced increase in ADAM17 proteolytic activity substantially contributes to invasiveness of brain tumor cells. [12] Recently, silencing of ADAM17 in human renal carcinoma cell lines was shown to diminish growth autonomy, tumor inflammation and tissue invasion in vivo. Of particular interest, ADAM17 has recently been identified as the primary sheddase for multiple epidermal growth factor receptor (EGFR) pro-ligands [17,18]. EGFR ligand-binding results in receptor self-dimerization, autophosphorylation and subsequent activation of downstream PI3K/AKT and Ras/MAPK/ERK signaling pathways [19-21]. EGFR is the prototype of a family of tyrosine kinases that participate in the control of differentiation, proliferation and cell survival, as well as in the development of tumors of epidermal origin [21-23]. Once occupied by ligands, EGFR dimerizes with another EGFR monomer or with one of three related receptors (ErbB2, ErbB3 or ErbB4) in order to exert its downstream effects. In fact, EGFR gene amplification, rearrangements, and over-expression are a particularly striking feature of glioma [24,25]. EGFR is commonly expressed at high levels in human glioma, and EGFR over-expression correlates with poor prognosis [16].

Our current study reports that ADAM17 promotes glioma cell proliferation, invasion, and angiogenesis in vitro, increases glioma cell growth in an animal model. ADAM17 increases shedding of EGFR ligands TGF- α and soluble VEGF, as well as activates the EGFR-PI3K-AKT pathway in U87 cells.

Materials and methods

Cell line and cell culture

The glioma cell line U87 was obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. The U87 cells were maintained in a humidified 37°C incubator with 5% CO₂, fed every 3 days with complete medium, and subcultured when confluence was reached.

Transfection of U87 cells with ADAM17 cDNA and ADAM-17 short hairpin RNA

The cDNA encoding ADAM17 was purchased from Origene and the pcDNA3.1 control vector was purchased from Invitrogen. ADAM17 shRNA was purchased from Santa Cruz. Transfections were done using a Nucleofector kit (Amaxa, Germany), according to the manufacturer's instructions. Briefly, 2×10^6 mature U87 cells were resuspended in Cell Line Nucleofector kit V solution (Amaxa), mixed with 2 µg cDNA, and pulsed as suggested by the manufacturer. Immediately after nucleofection, cells were transferred into wells containing culture medium (37°C). After 48 h, cells were selected using Geneticin (Invitrogen Life Technologies).

Modified cell lines and inhibitors

Modified cell lines used in this study were stable transfection U87 glioma cells with ADAM17 cDNA plasmid (Origene) stable transfection (A17), U87 glioma cells with ADAM17 targeting shRNA plasmid stable transfection (A17-shRNA), and U87 glioma cells with vector control plasmid (control). The ADAM17 inhibitor TAPI-2 was purchased from Peptides International (Louisville, KY, USA) PI-3K phosphorylation inhibitor LY294002 was purchased from Sigma. The stable transfection was conducted by a Nucleofector kit (Amaxa, Germany) according to manufacturer's instructions.

α-secretase activity assay

ADAM17 possesses α-secretase activity [12,26]. ADAM17 cleaves APP at the α-secretase recognition site. TAPI-2 is an inhibitor of ADAM17 proteolysis. Thus an α-secretase activity kit (R&D system, Minneapolis, MN) was used to determine ADAM17 proteolytic activity under various treatments. U87 cells were incubated under normal conditions with or without the ADAM17 inhibitor or the PI-3K inhibitor for 24h. Cells were harvested and lysed with cell extraction buffer. The protein concentration of cell lysate was determined by using the BCA protein assay (Pierce, Rockford, IL). According to the manufacturer's instructions, 50µg of total protein per sample was used in the assay. Fluorescence was quantified using a Fusion Universal Multiplate reader (Packard Bioscience, CT), with excitation between 335-355nm and emitted light collected between 495-510nm. Data are shown as fluorescence intensity values.

Growth curve

1.5×10^4 cells were plated in each well of 24-well plates containing DMEM with FBS at a concentration of 10%. Every 24 hours medium was removed, adherent cells were trypsinized, and total adherent cells in each well were quantified using a hemacytometer. Cell counts of three wells per time point per group were averaged.

MTT assay for cell viability and proliferation

The effect of ADAM17 on glioma cell viability and proliferation was determined by MTT assay (thiazolyl blue tetrazolium bromide; Sigma). Briefly, U87 cells in an exponential

phase of growth were harvested and seeded in 96-well plates at a density of 10^4 cells per well, and cultured for 48h, 72h, and 96h at 37°C with 5% CO₂. Experimental groups were the same as those used for the growth curve experiments. At the end of the culture, medium in each well was substituted with 200 µL of fresh medium containing MTT (final concentration, 250µg/mL). Plates were then incubated for an additional 2h period at 37°C. Subsequently, the medium was carefully removed in such a way that no loosely adherent cells were removed. Cells containing the trapped MTT crystals were then solubilized in 200 µL DMSO at 37°C for 15 min. Absorbance was determined in a microtiter plate reader (EL340 Bio-TEK instruments, Hopkinton, MA) at 570 nm and subtracted from absorbance at 450 nm. All determinations were performed in quadruplicate and each experiment was repeated at least three times.

BrdU incorporation assay

Cells were plated in 96-well microculture plates (5×10^3 cells/well). After 48h of culture at 37°C with 5% CO₂, BrdU labeling reagent was added and cultured for a further 3-6h. Cells were washed twice in culture medium containing 10% serum and fixed in ethanol 70% (v/v) for 1 hour, then permeabilized with 1M HCl followed by subsequent washes with 0.1M sodium tetraborate and PBS. BrdU incorporation was elucidated by incubation with a mouse anti-BrdU mAb followed by incubation with a peroxidase-labeled rabbit anti-mouse polyclonal antibody. Peroxidase substrate with a volume of 100µl was added to each well for 30min. Absorbance was measured with a microplate reader at 405nm with a reference wavelength of 490nm. All determinations were performed six times and each experiment was repeated three times.

In vitro chemosensitivity/cytotoxicity assay

The sulforhodamine B (SRB) assay was performed to assess growth inhibition using a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with the dye SRB [20]. Briefly, 100 µL/well of cell suspensions (1×10^5 cells/mL) were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 h, the cells were treated with the extract by adding 100µL/well of each concentration in triplicate to obtain a final concentration of 5, 10, 20, 40, and 80µmol/L for the TAPI-2 or LY294002. The plates were incubated for 1 h (d 0) and 72 h (d 3) at 37°C. At the end of each exposure time, the medium was removed. The cells were fixed with 20% (w/v) trichloroacetic acid (TCA, Fluka, Buchs, Switzerland) at 4°C for 1 h, stained for 30 min with 0.4% (w/v) SRB (Sigma, St. Louis, MO, USA) dissolved in 1% acetic acid (Sigma) for 30 min, and washed four times with 1% acetic acid. The protein-bound dye was solubilized with 10 mmol/L Tris base, pH 10 (Sigma). The absorbance (OD) of each well was read on an ELISA plate reader (EL340 Bio-TEK instruments, Hopkinton, MA) at 492 nm. Percentage of cell survival was calculated using the formula: Percentage cell survival = $[(\text{OD test sample at d 3} - \text{OD d 0}) / (\text{OD control at d 3} - \text{OD d 0})] \times 100$.

In vitro invasion assay

Matrigel invasion assays were used to assess the ability of the glioma cells to penetrate the ECM in the presence or absence of ADAM17 or PI-3K inhibitors. Invasion of cells through Matrigel was determined using 24-well BD invasion chambers (8.0µm pore size with polycarbonate membrane; BD Biosciences, Cowley, UK) in accordance with the manufacturer's instructions with the following modifications [27]. BD invasion chambers were pre-hydrated with serum-free DMEM (500µl/well) for 2h of incubation at 37°C in 5% CO₂. After trypsinization, A17, A17-shRNA, and control cells were suspended in medium without serum (500µl) in a concentration of 1×10^5 cells/well and immediately placed onto the upper compartment of the plates. Subsequently, the lower compartment was filled with complete medium (750µl). The cells were incubated for 24 h. Following incubation, the non-

invading cells were removed from the upper surface of the membrane by wiping with cotton-tipped swabs. Cells on the lower surface of the membrane were stained with CellTracker Green (Molecular Probes, Eugene, OR) for 45 minutes and fixed in 4% paraformaldehyde. Five fields of adherent cells were randomly counted in each well under a fluorescent microscope at 4X magnification, and the results were numerically averaged and counted. The invasion index for each treatment was calculated by dividing the number of invading cells by the number of cells that invaded under control group. Invasion index was expressed as percentages of control values.

Capillary-like tube formation assay

Briefly, 0.1ml growth factor reduced Matrigel (Becton Dickinson) was added per well, and Mouse brain endothelial cells (MBECs) (2×10^4 cells) were added and incubated in: (1) regular cell culture medium (DMEM) for control, (2) cell culture medium from A17 cell culture; (3) cell culture medium from A17-shRNA cell culture; (4) cell culture medium from A17 cell culture with 20 μ mol/L TAPI-2; (5) cell culture medium from A17 cell culture with 20 μ mol/L LY294002 for 6 hours. All assays were performed in n=6/group. For quantitative measurements of capillary tube formation, Matrigel wells were digitized under a 4x objective (Olympus 1 $\times$ 71) for measurement of total tube length of capillary tube formation using the MCID image analysis system (Imaging Research, St. Catharines, Canada) at 6 hours [24]. Tracks of endothelial cells organized into networks of cellular cords (tubes) were counted and averaged in randomly selected 3 microscopic fields [25]. The total tube length was calculated by MCID software in each field. The tube formation index was expressed as tube length (mm) per mm² area.

Real time reverse transcription polymerase chain reaction (real-time RT-PCR)

Quantitative RT-PCR was performed using real-time PCR with the SYBR Green reporter. Cell cultures were washed in PBS and RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). OD 260/280 ratio OD was used to determine RNA purity and 260nm was used to determine RNA yield. RNA was subsequently reverse transcribed to cDNA with SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed afterward. Primer (Invitrogen, Carlsbad, CA) concentrations (10nM) were optimized before use. SYBR Green PCR master kit was used with the appropriate concentrations (10nM) of forward and reverse primers in a total volume of 20 μ l. PCR reactions contained 1 μ l cDNA. Optimization was performed of each gene-specific primer prior to the experiment to confirm that 10nM primer concentrations did not produce nonspecific primer-dimer amplification signals in no-template control wells. Quantitative RT-PCR was performed on as ABI 7000 PCR instrument (PE Applied Biosystems, Foster City, CA) by using three-stage program parameters provided by the manufacturer as follows; 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during RT-PCR. Each sample was tested in triplicate with quantitative RT-PCR, and samples obtained from three independent experiments were used for analysis of relative gene expression. The following primers for real-time PCR were designed using Primer Premier 5 software. ADAM17: Forward 5'-ACT CTG AGG ACA GTT AAC CAA ACC-3'; Reverse 5'-AGT AAA AGG AGC CAA TAC CAC AAG -3'. VEGF: Forward 5'-AGTCCAACATCACCATGCAG-3'; Reverse 5'-CAA GGC CCA CAG GGA TTT-3'. TBP: Forward 5'-TGC ACA GGA GCC AAG AGT GAA-3'; Reverse 5'-CAC ATC ACA GCT CCC CAC CA-3'. TBP was used as the internal control.

Western blot

Cultures were rinsed with PBS and proteins were extracted in 500µl RIPA lysis buffer. Equal amounts of protein, as determined using the BCA (bicinchoninic acid) protocol (Pierce, Rockford, IL), were loaded on 10% Bis-Tris Gels (Invitrogen, Carlsbad, CA) after being denatured. The proteins were then transferred to Invitrogen PVDF membranes (Invitrogen, Carlsbad, CA). The membranes were blocked at RT for 1h with 5% BSA in TBS-T (10mM Tris-HCl, pH 7.6 and 150mM NaCl, 0.1% Tween-20). Afterward the membranes were incubated with primary antibodies against ADAM17 (Abcam, Cambridge, MA), EGFR (Santa Cruz, Santa Cruz, CA), p-EGFR (Santa Cruz, Santa Cruz, CA), AKT (cell signaling, Danvers, MA), p-AKT (cell signaling, Danvers, MA) or VEGF (Santa Cruz, Santa Cruz, CA) in 3% BSA at 4 °C overnight. The membranes were washed with TBS-T and incubated for 1h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA). Following washing, the immunoblots were detected using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL). The experiment was repeated in triplicate. β -actin was used as the internal control. The densities of bands were analyzed by the Gelpro 4.5 program.

ELISA for VEGF and TGF- α in cell culture supernatants

VEGF and TGF- α concentration in supernatants of the cultured U87 cells was determined using commercial human VEGF ELISA kit according to the manufacturer's instructions (R&D System, Minneapolis, MN, USA).

Human glioma cells implantation in nude mice

Nude mice (Nu/Nu Athymic, Charles River Breeding, MA) with body weight of 15-25g were anesthetized with ketamine (80mg/kg) and xylazine (13mg/kg) administered intramuscularly (i.m.). Once fixed in a stereotaxic device, the scalp was retracted and the cranium exposed, and a 3-4mm incision was made directly down the midline. Using a drill, a 2mm craniotomy was made on the right hemisphere anterior to the coronal suture. A 10µl Hamilton syringe was positioned to inject tumor cells intra-parenchymally into the right hemisphere-2.5mm depth, 1.5mm to the right and 1.0mm anterior to the bregma. 5×10^5 U87 cells were injected intracerebrally in a 5µl volume. The craniotomy was covered with a film of polyvinyl chloride glued to the surrounding intact bone and the incision was closed with 4-0 silk suture.

Treatment of ADAM17 inhibitor TAPI-2 on U87 brain tumor growth in vivo

To determine whether inhibition of ADAM17 activity would alter tumor progression in vivo. 5×10^5 U87 cells were injected stereotactically at a depth of 2.5mm, at 1.5mm to the right of midline and 1.0mm anterior to the bregma of nude mice brain. Using a brain pump, 10mM at 0.5µl/h TAPI-2 was infused into the mouse brain at 7 days after surgery and continuing for 7 consecutive days. Tumor volume was evaluated in 6µm paraffin sections in the tumor region stained with H&E. During sacrifice, mice were perfused via the left ventricle with heparinized saline followed by 4% paraformaldehyde. Tumor-bearing brain was processed, placed in paraffin, and subsequently five equally spaced sections (6µm thick) were obtained from each of the 1mm thick blocks encompassing the tumor. The sections were stained with H&E for microscopic analysis.

Plasmid/polyethylenimine formulations

150 mM of polyethylenimine (PEI) in monomer nitrogen residues was prepared in 5% glucose for plasmid delivery. An N/P ratio (number of nitrogen residues of in vivo-jetPEI™ per DNA phosphate) of 10 was chosen to reach the ionic balance of the complexes. For each injection, 5µg of plasmid DNA was prepared to obtain a final concentration of 5% glucose

with total volume of 5 μ l. Meanwhile, a 0.8 μ l of in vivo-jetPEI™ was diluted to 5% glucose (final concentration) with the same volume as plasmid DNA. A PEI/plasmid DNA mixture was prepared by slowly adding PEI to DNA solution and gently vortex to thoroughly mix. The solution was incubated at room temperature for 15 minutes before use.

PEI-mediated transfection of A17-shRNA into tumor-bearing nude mice

U87 (5×10^5) was implanted into the brain of nude mice. Employing PEI (in vivo-jetPEI, PolyPlus-Transfection, France) to deliver a vector encoding for ADAM17-targeting shRNA, we tested the therapeutic effect of ADAM17 inhibiting therapy in an in vivo model of glioma. Two doses of 5 μ g ADAM17-targeting shRNA were administered intratumorally via PEI. PEI/A17-shRNA was administered at days 5 and 7 post-tumor implantation. Each dose carried 5 μ g of A17-shRNA-encoding complexes, and at 10 μ l, had an N/P ratio (ionic balance) of 10. This N/P ratio is considered optimal as per the manufacturer's instructions. The protocol of intra-tumor injection is the same as for tumor implantation. Tumor animals treated with scrambled control shRNA as well as saline vehicle were employed as control groups. During sacrifice, mice were perfused via the left ventricle with heparinized saline followed by 4% paraformaldehyde. Tumor-bearing brain was processed, placed in paraffin, and subsequently five equally spaced sections (6 μ m thick) were obtained from each of the 1mm thick blocks encompassing the tumor. The sections were stained with H&E for microscopic analysis.

Statistics

Data are presented as mean $\pm$ SD. Significance between the two groups was examined by a two-tailed Student's t-test. A value of $P < 0.05$ was considered significant. Statistical significance among three or more groups was analyzed by one-way ANOVA using SPSS 11.0 software (Chicago, IL, US). A value of $P < 0.05$ was considered significant. Data are presented as mean $\pm$ SD.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Results

ADAM17 expression and activity are altered by cDNA or shRNA transfection

To determine ADAM17 expression level in U87 with ADAM17 cDNA or shRNA transfection, real-time PCR analysis of ADAM17 was measured in ADAM17 cDNA (A17), A17-shRNA, and control U87 cells. Three cDNA transfected and three A17-shRNA transfected U87 clones were selected. cDNA transfection increased ADAM17 mRNA expression by 3.99 ± 0.24 - 4.25 ± 0.15 fold and A17-shRNA decreased ADAM17 mRNA expression by $64.3 \pm 2.2\%$ - $76.2 \pm 3.5\%$ compared to control (Fig. 1A). One high ADAM17 expressing cell line from cDNA and one low ADAM17 expressing cell line A17-shRNA transfection were selected (A17 and A17-shRNA) for alpha-secretase activity after treatment with TAPI-2 and LY294002.

ADAM17 activity was measured with an α -secretase activity kit (Fig. 1B). ADAM17 cDNA transfected U87 cells showed a significantly higher ($131.6 \pm 10.9\%$) α -secretase activity than control. Compared to control, A17-shRNA transfection significantly decreased cell lysate α -secretase activity by $63.1 \pm 7.1\%$. Our results indicate that expression of ADAM17 substantially contributed to α -secretase activity of U87 glioma cell lines. Additionally, we found TAPI-2 and LY294002 decreased α -secretase activity in ADAM17 high expressing cell line by $51.3 \pm 1.9\%$ ($p < 0.01$) and $11.1 \pm 4.1\%$ ($p < 0.05$), respectively. These results suggest

that ADAM17 expression and proteolytic activity were successfully regulated by cDNA, A17-shRNA, and/or inhibitors.

ADAM17 increases cell growth in U87 and HF66 glioma cells

U87 cells transfected with ADAM17 cDNA (A17), cells transfected with ADAM17 shRNA (A17-shRNA), and control (vector transfection) U87 were harvested and replated at a density of 2×10^4 cells/well in 24 well plates. Also, A17 cells were treated with LY294002 (PI-3K phosphorylation inhibitor) or TAPI-2 (ADAM17 inhibitor), respectively. The total cell number was quantified daily with a hemacytometer. Our data showed that high expression of A17 significantly increased the growth rate of U87 cells, with a 18.1 ± 6.4 - $39.4 \pm 7.2\%$ increase at different time points compared to control, evident after 3 days of culture ($P < 0.01$). Loss of ADAM17 expression significantly decreased the growth rate of U87 cells, with an 11.1 ± 6.7 - $19.2 \pm 4.4\%$ decrease at different time points (after 3 days) compared to control U87 cells ($P < 0.05$). Additionally, three days after culture, TAPI-2 and LY294002 significantly decreased the growth rate of A17 cells with 10.7 ± 5.4 - $32.2 \pm 5.2\%$ and 16.5 ± 5.7 - $35.7 \pm 5.6\%$ (after 4 days), respectively (Fig 2A). In another glioma cell line, i.e. HF66, TAPI-2 and LY294002 significantly decreased the growth rate of HF66 cells with 13.9 ± 4.1 - $24.6 \pm 6.7\%$ and 13.1 ± 3.6 - $25.7 \pm 5.9\%$ (after 3 days), respectively (Fig 2D).

Glioma cell viability is regulated by ADAM17

The MTT assay was employed to test expansion of U87 glioma cells. Cell viability was measured at 48h, 72h, and 96h of culture, respectively. ADAM17 cDNA significantly increased cell viability by $49.0 \pm 10.4\%$, $52.8 \pm 13.3\%$, and $62.0 \pm 8.5\%$, at 48h, 72h, and 96h, respectively, compared to control vector transfection. ADAM17-targeting shRNA transfection significantly decreased cell viability by $23.3 \pm 5.4\%$ and $24.4 \pm 7.9\%$ at 72h, and 96h, compared to control transfection. TAPI-2 treatment significantly decreased U87 cell viability by $12.6 \pm 3.1\%$ and $19.6 \pm 6.1\%$ at 72h and 96h compared to A17. LY294002 significantly decreased U87 cells viability by $30.8 \pm 2.4\%$, $50.0 \pm 4.1\%$, and $50.9 \pm 3.8\%$ at 48h, 72h, and 96h, respectively, compared to A17 (Fig 2B). A similar assay was conducted on HF66 cells, TAPI-2 treatment significantly decreased HF66 cell viability by $29.2 \pm 8.2\%$, $28.2 \pm 5.7\%$ and $49.4 \pm 8.0\%$ at 48h, 72h and 96h respectively. LY294002 significantly decreased U87 cells viability by $30.6 \pm 8.5\%$, $29.0 \pm 10.5\%$, and $44.5 \pm 4.7\%$ at 48h, 72h, and 96h, respectively, (Fig 2E).

ADAM17 promotes glioma cell proliferation

BrdU incorporation assay was employed to further test glioma cell proliferation. Two cDNA transfected and two A17-shRNA transfected U87 clones were tested. After 48 hours of culture, A17 cells showed a $34.6 \pm 4.7\%$ and $38.3 \pm 7.5\%$ increase in BrdU incorporation compared to control. A17-shRNA transfected cells showed a $8.9 \pm 4.0\%$ and $12.5 \pm 4.0\%$ decrease in BrdU incorporation compared to control. At the same time point, TAPI-2 and LY294002 significantly inhibited BrdU incorporation in A17 cells by $18.8 \pm 4.7\%$ - $21.9 \pm 1.3\%$ and $27.3 \pm 3.1\%$ - $29.3 \pm 0.8\%$, respectively. Fig 2C shows one A17 and one A17-shRNA clone treated with TAPI-2 and LY294002. We also tested whether TAPI-2 and LY294002 decrease cell proliferation in HF66 cells. Our data showed TAPI-2 and LY294002 significantly suppressed BrdU incorporation in HF66 cells by $32.2 \pm 8.4\%$ and $36.6 \pm 4.7\%$, respectively (Fig 2F).

ADAM17 high-expression U87 cell line is more sensitive to ADAM17 and AKT inhibitors

A17, A17-shRNA, and control cell lines were employed in the SRB cytotoxicity assay. The cells were treated with TAPI-2 or LY294002 at final concentrations of 5, 10, 20, 40, and 80 $\mu\text{mol/L}$. TAPI-2 and LY294002 inhibited the three cell lines growth at different levels.

Compared to the inhibitory effect in the control cell line, both TAPI-2 and LY294002 showed a significantly greater inhibition rate in A17 cells ($P<0.01$ at 4 doses), but showed a significantly lower inhibition rate in A17-shRNA cells ($P<0.01$) at all five doses. In this study, A17 appears more sensitive to ADAM17 reduction and A17-shRNA is less sensitive to ADAM17 reduction (Fig. 3). In vitro cytotoxicity assay based on ADAM17 or PI-3K inhibition proved conversely that the level of ADAM17 and PI-3K activation contribute to the high growth rate in A17 or low growth rate in A17-shRNA U87 glioma cells.

ADAM17 activity is related to glioma cells invasiveness

Three cDNA transfected and three A17-shRNA transfected U87 clones were tested using an invasion assay. Results obtained using the 24h in vitro invasion assay revealed that the invasiveness of A17 cells was 1.96 ± 0.13 - 2.13 ± 0.23 fold higher than control cells, and the invasiveness of ADAM17 shRNA transfected U87 cells significantly decreased by $42.5\pm3.5\%$ - $47.9\pm3.8\%$ compared to control cells. ADAM17 inhibitor TAPI-2 significantly decreased invasiveness in U87 cells by $50.5\pm9.2\%$. Fig 4 showed an A17 and a A17-shRNA clone and U87 cells treated with TAPI-2. The in vitro invasion assay indicated that the invasiveness of U87 glioma cell lines are related to ADAM17 expression levels and reduction in ADAM17 activity decreased the invasiveness in U87 cells

ADAM17 over expression induces tube formation in cultured endothelial cells

Three cDNA transfected and three A17-shRNA transfected U87 clones were tested by tube formation assay [13]. Culture medium from ADAM17 cDNA transfected cells increased mouse brain endothelial cells (MBECs) capillary tube formation by 2.27 ± 0.14 - 2.49 ± 0.10 fold compared to control medium. While TAPI-2 and LY294002 decreased capillary tube formation by $55.8\pm1.8\%$ - $75.2\pm2.5\%$ and $60.1\pm2.2\%$ - $62.6\pm1.8\%$, respectively, in A17 medium cultured MBECs. A17-shRNA medium showed a $23.9\pm4.2\%$ - $33.7\pm5.1\%$ decrease in MBECs capillary tube formation compared to control medium. Fig.5 shows an A17 and a A17-shRNA clone treated with TAPI-2 and LY294002. We then tested the tube formation in MBECs with conditioned media from U87 and TAPI-2 treated U87 culture. Compared to control medium, U87 conditioned medium increased the tube formation in MBECs, and TAPI-2 treated U87 medium counteract significantly decreased tube formation by $31.2\pm3.3\%$, when compared to media from U87. These data indicate that ADAM17 in glioma cells induces angiogenesis and that the PI-3K signal pathway mediates ADAM17-induced tube formation.

ADAM17 increases VEGF in mRNA level and protein expression, and soluble VEGF release in U87 cells

Real-time PCR analysis was employed to measure VEGF expression level in glioma cells A17, A17-shRNA, and control. VEGF expression showed a significant 2.46 ± 0.07 fold ($P<0.01$) increase in A17 cells, and $37.9\pm3.7\%$ decrease in A17-shRNA cells, compared to control cells. LY294002 and TAPI-2 decreased VEGF mRNA expression $33.6\pm6.3\%$ ($P<0.01$) and $43.7\pm5.2\%$ ($P<0.01$), respectively (Fig.5). These results indicate ADAM17 proteolytic activity is related to VEGF expression. VEGF protein showed the same trend as mRNA level in all experimental groups (Fig. 9). High ADAM17 expression significantly increased VEGF expression by 2.66 ($P<0.01$) fold in A17, and low ADAM17 expression decreased VEGF expression by 32.6 % in A17-shRNA cells, respectively, compared to control cells. LY294002 and TAPI-2 significantly decreased VEGF expression by 68.7% and 66.0%, respectively, in A17 cells after 24h treatment.

A17, A17-shRNA, control U87 cells were cultured under 37°C with 5% CO_2 for 24h to attach to the bottom of the culture plate, respectively. Twenty-four hours later, medium in 24-well plate was removed and adherent cells were rinsed by PBS. Serum free medium was

then added to the culture plate for an additional 24h in culture. TAPI-2 and LY294002 were employed in A17 cells to test the effects of ADAM17 or PI-3K inhibition on VEGF release. Compared to control U87 cells, A17 increased VEGF release to supernatant by 2.57 fold ($P<0.01$), while A17-shRNA showed significantly less VEGF release ($31.0\pm5.3\%$, $p<0.05$). Both LY294002 and TAPI-2 significantly suppressed soluble VEGF release in A17 culture supernatant by $52.2\pm1.9\%$ ($p<0.01$) and $50.5\pm7.2\%$ ($p<0.01$, Fig. 5), respectively.

ADAM17 promotes U87 glioma growth in nude mice

ADAM17-over-expressing and ADAM17-incompetent glioma cells as well as control glioma cells transfected with vector plasmid, 1) control, 2) A17, 3) A17-shRNA were injected stereotactically at a depth of 2.5mm, at 1.5mm to the right of midline and 1.0mm anterior to the bregma. Animals were sacrificed at 3 weeks after tumor implantation. Tumor-bearing brain was processed, placed in paraffin, and subsequently seven equally spaced sections ($6\mu\text{m}$ thick) were obtained from each of the 1mm thick blocks encompassing the tumor. The sections were stained with H&E for light microscopic examination and analysis. A17 showed significant increase in tumor volume ($10.13\pm2.00\text{mm}^3$ vs. $8.15\pm1.19\text{mm}^3$, $P<0.05$) in nude mice compared to control glioma cells, and A17-shRNA transfected cells showed a significantly lower tumor volume ($5.82\pm1.88\text{mm}^3$) compared to their parent cells transfected with vector only ($P<0.05$) (Fig.6)

The ADAM17 inhibitor TAPI-2 reduces U87 brain tumor volume in vivo

As ADAM17 activity contributes to glioma invasion and proliferation in vitro, we sought to determine whether inhibition of ADAM17 activity would alter tumor progression in vivo. 5×10^5 U87 cells were injected stereotactically at a depth of 2.5mm, at 1.5mm to the right of the midline and 1.0mm anterior to the bregma of nude mice brain. Using a brain pump, 10mM at $0.5\mu\text{l/h}$ TAPI-2 was infused into the mouse brain at 7 days after surgery and continuing for 7 consecutive days. At 3 weeks after tumor implantation, animals were sacrificed. Tumor volume was evaluated in $6\mu\text{m}$ paraffin sections in the tumor region stained with H&E. TAPI-2 treated U87 bearing nude mice showed a significant decrease in tumor size ($6.73\pm2.47\text{mm}^3$) compared to untreated animals ($12.11\pm2.76\text{mm}^3$) (Fig.7). These data indicate ADAM17 significantly contributes to glioma xenograph growth, and suggests inhibitors of ADAM17 may have therapeutic anti-tumor effects.

PEI-mediated transfection of ADAM17-targeting siRNA reduces U87 glioma growth in nude mice

Employing Polyethylenimine (PEI) cationic lipids (in vivo-jetPEI, PolyPlus-Transfection, Illkirch, France), we delivered a plasmid encoding for ADAM17-targeting small interfering shRNA to test the therapeutic effect of ADAM17 inhibiting therapy in an in vivo model of glioma. Animals were sacrificed 3 weeks after tumor implantation for tumor volume evaluation. Two doses of $5\mu\text{g}$ ADAM17-targeting shRNA ($10\mu\text{l}$) administered intratumorally via PEI at 5 and 7 days post-implantation significantly inhibited U87 tumor growth in a nude mouse model ($4.18\pm1.65\text{mm}^3$ vs. $9.51\pm2.19\text{mm}^3$, $P<0.05$). (Fig.8) Equivalent treatment with a PEI-delivered scrambled shRNA control did not significantly affect tumor growth. These data suggested that ADAM17 is a viable target for anti-tumor therapy. In addition, our data indicate use of PEI with siRNAs designed to silence ADAM17 is an effective means to deliver ADAM17-targeting gene therapy.

ADAM17 over expression increases EGFR ligand TGF- α release

A17, A17-shRNA, control U87 cells were cultured at 37°C with 5% CO_2 for 24h to attach to the bottom of the culture plate. 24h later, medium in 24-well plate was removed and adherent cells were rinsed by PBS. Serum free medium was then added to the culture plate

for an additional 24h culture. TAPI-2 and LY294002 were employed in A17 cells to test the effects of ADAM17 or PI-3K inhibition on TGF- α release. Compared to control U87 cells, A17 had more TGF- α ($64.3 \pm 8.4\%$ increase) released to supernatant ($P < 0.01$), while A17-shRNA showed significantly less ($55.0 \pm 6.9\%$) TGF- α release ($p < 0.01$). TAPI-2 significantly suppressed soluble TGF- α release in A17 culture supernatant by $44.1 \pm 11.0\%$ ($p < 0.01$). (Fig. 9A)

ADAM17 over expression activates EGFR-PI3K-AKT signaling pathway in U87

p-EGFR and p-AKT were measured by Western blot to evaluate EGFR activation of the PI3K-AKT pathway after transfection of ADAM17 cDNA or A17-shRNA. The effects of TAPI-2 and LY294002 on EGFR-AKT activation were also tested in A17 cells. p-EGFR and p-AKT expression showed significant 2.30 ($P < 0.01$) and 3.14 ($P < 0.01$) fold increases in A17 and 46.7% and 60.5% decrease in A17-shRNA cells, respectively, as compared to control. Expression of EGFR and AKT was not significantly affected by the level of ADAM17. In A17 cells, LY294002 significantly decreased p-AKT expression by 33.7%, but with no effect on AKT expression. TAPI-2 significantly decreased p-EGFR and p-AKT expression by 40.8% and 82.8%, respectively, but with no effect on EGFR and AKT expression. (Fig. 9B)

Discussion

ADAMs are best known as ectodomain sheddases, a function of their metalloprotease domains. This family belongs to the superfamily of Zn dependent metalloproteinases [2,28]. Several proteolytically active ADAMs cleave growth factors or cell surface proteins related to tumor progression. The over expression of ADAM17 was found in tissues at different pathologic conditions, such as tumor hypoxia [12], or external stimulation, such as UVA exposure [29] and photodynamic therapy [30].

ADAM17 sheds a variety of important cell surface molecules, including cytokines (e.g. tumor necrosis factor- α , growth factors (e.g. epidermal growth factor family members) and adhesion molecules (e.g. L1, selectins, and vascular cell adhesion molecule) [31]. Several ligands released by ADAM17 play key roles in cell proliferation and invasion.

In our studies, we employed a U87 human glioma cell line to investigate the effect of ADAM17 on glioma proliferation, invasion, and angiogenesis in vitro. High or low ADAM17 expression was established by cDNA or siRNA transfection. To test U87 cell viability and proliferation, we employed the MTT assay, BrdU incorporation assay, and a growth curve assay. Our data demonstrate that ADAM17 promotes U87 cell growth, viability, and proliferation. Reduction of ADAM17 by shRNA or ADAM17 inhibitor TAPI-2 significantly decreased tumor cell proliferation. To assess the sensitivity of ADAM17 high or low expressing U87 cells to the ADAM17 inhibitor TAPI-2, a sulforhodamine B (SRB) in vitro chemosensitivity/cytotoxicity assay was employed. A17, A17-shRNA, and control cells show differential sensitivity to specific ADAM17 withdrawal. When compared to control, A17 is more sensitive to TAPI-2 at 72h. With each dose from 5 to 80 $\mu\text{mol/L}$, the A17 survival rate is lower than control. A17-shRNA resulted in a higher survival rate under different doses compared to control. All in vitro proliferation assays employed lead to the conclusion that ADAM17 contributes to U87 cell proliferation.

We used Matrigel invasion assays to assess the ability of the glioma cells to penetrate the ECM. Our data show that the invasion potential of U87 cells is increased by high ADAM17 expression (A17), and decreased by ADAM17 shRNA. Additionally, we demonstrate that TAPI-2 treatment significantly diminishes the enhanced invasive ability of U87 cells.

Angiogenesis is essential for embryo development, tissue repair, inflammatory diseases, and tumor growth and metastasis. The angiogenesis process includes dissolution of the basement membrane of the vessel, migration and proliferation of endothelial cells, formation of a new vessel lumen and vessel branches. Tumor growth and metastasis require angiogenesis when the tumor reaches 1-2 mm in diameter [32, 33]. Tumor angiogenesis can be triggered by extracellular signals such as growth factors and by genetic alterations such as activation of oncogenes. EGFR-PI3K- Akt involvement in regulating angiogenesis was demonstrated in vivo [34, 35]. PI3K/AKT signaling is important for regulating HIF-1 α and VEGF expression [27,35]. VEGF is one of the mediators of PI3K-induced oncogenic signals for tumor growth and angiogenesis [34] PI3K is activated by upstream activators such as TGF- α , via activation of receptor tyrosine kinases. PI3K induces Akt activation, which increases the expression of hypoxia-inducible factor 1 (HIF-1). HIF-1 increases VEGF expression and then activates VEGF and VEGFR in an autocrine loop to regulate tumor angiogenesis[32].

To address the angiogenic function of ADAM17 on glioma, we further tested VEGF expression in U87 cells and angiogenesis in cultured endothelial cells with culture supernatant from U87 cells. Gene expression analysis indicated that ADAM17 proteolysis contributes to VEGF expression in mRNA, protein, and functional form levels. Inhibition of Akt phosphorylation reduced VEGF expression indicating that ADAM17 increases VEGF expression through the Akt signaling pathway, consistent with other findings [35]. As VEGF mediates tumor angiogenesis, an in vitro tube formation assay was then employed to assess the effect of ADAM17 on angiogenesis. Our results indicate that ADAM17 induced capillary-like tube formation. This tube formation can be counteracted by ADAM17 inhibition or Akt dephosphorylation. Increased VEGF expression may be one of the downstream mechanisms through which ADAM17 promotes angiogenesis. Some other factors may also be involved in this process. Due to the complexity of how ADAM17 might influence angiogenesis, further investigation of PI3K-AKT or other signal pathways is warranted.

Our in vivo data suggest ADAM17 is a positive regulator of glioma progression of tumor xenographs in vivo. Stable transfection of U87 cell lines with either a plasmid for over-expression of human ADAM17, or a siRNA to ADAM17 enabled us to selectively isolate the contribution of ADAM17 to the malignant phenotype. ADAM17 expression increased the rate of tumor growth in U87-A17 glioma-bearing animals compared to those from the parent tumor control. A decrease of tumor volume was showed from U87-A17-shRNA tumor animals compared to control animals, which provided additional evidence supporting the hypothesis that increased ADAM17 activity promotes glioma progression.

Despite conventional therapies, glioma has a mean survival of only one year [36]. In light of the fact that ADAM17 plays a central role in tumor cell growth, we therefore, investigated if ADAM17 is an effective therapeutic target in the treatment of human glioma. Our data demonstrate ADAM17-targeting PEI/A17-shRNA (ADAM17-targeting RNA interference) significantly reduced the tumor intracranial U87 tumor growth, compared to saline vehicle or scrambled shRNA control. The ADAM17 inhibitor TAPI-2 suppresses U87 brain tumor growth in vivo. TAPI-2 treated U87 bearing nude mice showed a significant decrease in tumor size compared to non-treated animals. These data indicate ADAM17 activity significantly contributes to glioma xenograph growth, and suggest inhibitors of ADAM17 may have therapeutic anti-tumor effects.

Experiments were performed to reveal the primary mechanism by which ADAM17 increased U87 cell proliferation and invasion. ADAM17 is a primary sheddase for multiple EGFR pro-ligands such as HB-EGF and TGF α [17,37,38]. We investigated the EGFR signaling as a primary downstream mechanism by which ADAM17 proteolysis contributes

to glioma cell invasion and proliferation. EGFR is the first receptor tyrosine kinase to be identified [39] and it is amplified and overexpressed in tumors of many tissues. EGFR and its downstream signaling pathway is a key regulator of cell proliferation and it is frequently deregulated in cancer [40, 41]. The activity of the pathway can be increased by overproduction of ligands, resulting in hyperstimulation of the receptor. EGFR can be activated by its ligands including EGF, TGF- α , amphiregulin and betacellulin [19,42], of which ADAM17 is a primary shedding protease [43]. EGFR ligand binding results in receptor self dimerization, autophosphorylation and subsequent activation of downstream PI3K/AKT and Ras/MAPK pathways, which contribute to the malignant phenotype [19-21]. Thus, we investigated the contribution of ADAM17 proteolysis to these signal transduction pathways. Our results with ADAM17 cDNA transfection indicate EGFR and Akt phosphorylation are dependent upon ADAM17 proteolysis, via TGF- α , suggesting that stimulation of PI-3K/AKT activity is an important signal transduction pathway of ADAM17-induced tumor proliferation and invasion. Specific ablation of ADAM17 using shRNA reduced shedding of the ligands such as TGF- α and decreases EGFR and Akt phosphorylation and finally reverts the malignant phenotype of U87 cells. Targeting ADAM17 with the inhibitor TAPI-2 also reduces the high malignancy in A17 cells.

We further investigated the contribution of EGFR/ PI3K/AKT pathway to the ADAM17 induced proliferation and invasion of glioma cells after transfection with an ADAM17 cDNA. ADAM17 exhibited a significant increase in U87 cell proliferation and invasion as well as EGFR/ PI3K/AKT activation in vitro. Specific suppression of EGFR/PI3K/AKT pathway by inhibitor LY294002 reduces the high proliferation and invasion characteristics in ADAM17-expressing cells. These data further suggest that ADAM17 contributes to tumor progression through activation of EGFR/PI3K/AKT pathway.

Acknowledgments

This work was supported by NIH grant RO1 CA12944

References

1. Borrell-Pages M, Rojo F, Albanell J, Baselga J, Arribas J. TACE is required for the activation of the EGFR by TGF- α in tumors. *Embo J*. 2003; 22(5):1114–1124. [PubMed: 12606576]
2. Black RA. Tumor necrosis factor- α converting enzyme. *Int J Biochem Cell Biol*. 2002; 34(1):1–5. [PubMed: 11733179]
3. Black RA, Rauch CT, Kozlosky CJ, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature*. 1997; 385(6618):729–733. [PubMed: 9034190]
4. Buxbaum JD, Liu KN, Luo Y, et al. Evidence that tumor necrosis factor α converting enzyme is involved in regulated α -secretase cleavage of the Alzheimer amyloid protein precursor. *J Biol Chem*. 1998; 273(43):27765–27767. [PubMed: 9774383]
5. Zatovicova M, Sedlakova O, Svastova E, et al. Ectodomain shedding of the hypoxia-induced carbonic anhydrase IX is a metalloprotease-dependent process regulated by TACE/ADAM17. *Br J Cancer*. 2005; 93(11):1267–1276. [PubMed: 16278664]
6. Crowe PD, Walter BN, Mohler KM, Otten-Evans C, Black RA, Ware CF. A metalloprotease inhibitor blocks shedding of the 80-kD TNF receptor and TNF processing in T lymphocytes. *J Exp Med*. 1995; 181(3):1205–1210. [PubMed: 7869036]
7. Mullberg J, Durie FH, Otten-Evans C, et al. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. *J Immunol*. 1995; 155(11):5198–5205. [PubMed: 7594530]
8. Moss ML, Jin SL, Milla ME, et al. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- α . *Nature*. 1997; 385(6618):733–736. [PubMed: 9034191]
9. Bennett TA, Lynam EB, Sklar LA, Rogelj S. Hydroxamate-based metalloprotease inhibitor blocks shedding of L-selectin adhesion molecule from leukocytes: functional consequences for neutrophil aggregation. *J Immunol*. 1996; 156(9):3093–3097. [PubMed: 8617927]

10. Katakowski M, Jiang F, Zheng X, Gutierrez JA, Szalad A, Chopp M. Tumorigenicity of cortical astrocyte cell line induced by the protease ADAM17. *Cancer Sci.* 2009; 100(9):1597–1604. [PubMed: 19515085]
11. Murphy G. The ADAMs: signalling scissors in the tumour microenvironment. *Nat Rev Cancer.* 2008
12. Zheng X, Jiang F, Katakowski M, et al. Inhibition of ADAM17 reduces hypoxia-induced brain tumor cell invasiveness. *Cancer Sci.* 2007; 98(5):674–684. [PubMed: 17355261]
13. Zheng X, Jiang F, Katakowski M, Zhang ZG, Lu QE, Chopp M. ADAM17 promotes breast cancer cell malignant phenotype through EGFR-PI3K-AKT activation. *Cancer Biol Ther.* 2009; 8(11): 1045–1054. [PubMed: 19395875]
14. Blobel CP. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol.* 2005; 6(1):32–43. [PubMed: 15688065]
15. Katakowski M, Chen J, Zhang ZG, Santra M, Wang Y, Chopp M. Stroke-induced subventricular zone proliferation is promoted by tumor necrosis factor-alpha-converting enzyme protease activity. *J Cereb Blood Flow Metab.* 2007; 27(4):669–678. [PubMed: 16926839]
16. Mineo JF, Quintin-Roue I, Lucas B, Buburusan V, Besson G. Glioblastomas: clinical study and search for prognostic factors. *Neurochirurgie.* 2002; 48(6):500–509. [PubMed: 12595806]
17. Lee DC, Sunnarborg SW, Hinkle CL, et al. TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase. *Ann N Y Acad Sci.* 2003; 995:22–38. [PubMed: 12814936]
18. Fan H, Derynck R. Ectodomain shedding of TGF- α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *Embo J.* 1999; 18(24):6962–6972. [PubMed: 10601018]
19. Wells A. EGF receptor. *Int J Biochem Cell Biol.* 1999; 31(6):637–643. [PubMed: 10404636]
20. Lal A, Glazer CA, Martinson HM, et al. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res.* 2002; 62(12):3335–3339. [PubMed: 12067969]
21. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev.* 1999; 13(22):2905–2927. [PubMed: 10579998]
22. Maher EA, Furnari FB, Bachoo RM, et al. Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* 2001; 15(11):1311–1333. [PubMed: 11390353]
23. Wechsler-Reya R, Scott MP. The developmental biology of brain tumors. *Annu Rev Neurosci.* 2001; 24:385–428. [PubMed: 11283316]
24. Collins VP. Gene amplification in human gliomas. *Glia.* 1995; 15(3):289–296. [PubMed: 8586464]
25. Schwachheimer K, Huang S, Cavenee WK. EGFR gene amplification--rearrangement in human glioblastomas. *Int J Cancer.* 1995; 62(2):145–148. [PubMed: 7622287]
26. Allinson TM, Parkin ET, Turner AJ, Hooper NM. ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res.* 2003; 74(3):342–352. [PubMed: 14598310]
27. Skinner HD, Zheng JZ, Fang J, Agani F, Jiang BH. Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1 α , HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J Biol Chem.* 2004; 279(44):45643–45651. [PubMed: 15337760]
28. Primakoff P, Myles DG. The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet.* 2000; 16(2):83–87. [PubMed: 10652535]
29. He YY, Council SE, Feng L, Chignell CF. UVA-induced cell cycle progression is mediated by a disintegrin and metalloprotease/epidermal growth factor receptor/AKT/Cyclin D1 pathways in keratinocytes. *Cancer Res.* 2008; 68(10):3752–3758. [PubMed: 18483258]
30. Zheng X, Jiang F, Katakowski M, et al. Sensitization of cerebral tissue in nude mice with photodynamic therapy induces ADAM17/TACE and promotes glioma cell invasion. *Cancer Lett.* 2008; 265(2):177–187. [PubMed: 18358600]
31. Ding X, Yang LY, Huang GW, Wang W, Lu WQ. ADAM17 mRNA expression and pathological features of hepatocellular carcinoma. *World J Gastroenterol.* 2004; 10(18):2735–2739. [PubMed: 15309730]

32. Jiang BH, Liu LZ. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta*. 2008; 1784(1):150–158. [PubMed: 17964232]
33. Folkman J. Angiogenesis. *Annu Rev Med*. 2006; 57:1–18. [PubMed: 16409133]
34. Xia C, Meng Q, Cao Z, Shi X, Jiang BH. Regulation of angiogenesis and tumor growth by p110 alpha and AKT1 via VEGF expression. *J Cell Physiol*. 2006; 209(1):56–66. [PubMed: 16775835]
35. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A*. 2000; 97(4):1749–1753. [PubMed: 10677529]
36. Shand N, Weber F, Mariani L, et al. A phase 1-2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group. *Hum Gene Ther*. 1999; 10(14):2325–2335. [PubMed: 10515452]
37. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res*. 2004; 64(6):1943–1950. [PubMed: 15026328]
38. Ruhe JE, Streit S, Hart S, Ullrich A. EGFR signaling leads to downregulation of PTP-LAR via TACE-mediated proteolytic processing. *Cell Signal*. 2006; 18(9):1515–1527. [PubMed: 16478662]
39. Ullrich A, Coussens L, Hayflick JS, et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*. 1984; 309(5967):418–425. [PubMed: 6328312]
40. Kenny PA. Three-dimensional extracellular matrix culture models of EGFR signalling and drug response. *Biochem Soc Trans*. 2007; 35(Pt 4):665–668. [PubMed: 17635116]
41. Kenny PA. TACE: a new target in epidermal growth factor receptor dependent tumors. *Differentiation*. 2007; 75(9):800–808. [PubMed: 17608729]
42. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene*. 2000; 19(56):6550–6565. [PubMed: 11426640]
43. Sahin U, Weskamp G, Kelly K, et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol*. 2004; 164(5):769–779. [PubMed: 14993236]

Abbreviations

ADAM17	A disintegrin and metalloproteinase-17
BrdU	Bromodeoxyuridine
SRB	Sulforhodamine B
ECM	Extra Cellular Matrix
TNF-α	Tumor necrosis factor- α
MBECs	Mouse brain endothelial cells

Fig 1. ADAM17 expression and activity changed by cDNA or shRNA transfection

Three cDNA transfected and three A17-shRNA transfected U87 clones were selected. cDNA transfection increased ADAM17 mRNA expression by 3.99 ± 0.24 - 4.25 ± 0.15 fold and shRNA decreased ADAM17 mRNA expression by $64.3 \pm 2.2\%$ - $76.2 \pm 3.5\%$ compared to control (A). One high ADAM17 expressing cell line from cDNA and one low ADAM17 expressing cell line A17-shRNA transfection were selected (A17 and A17-shRNA) for alpha-secretase activity with treatment of TAPI-2 and LY294002. A17 cells showed $131.6 \pm 10.9\%$ increase and A17-shRNA showed $63.1 \pm 7.1\%$ decrease in cell lysate alpha-secretase activity. TAPI-2 and LY294002 significantly decrease alpha-secretase activity in A17 cells by $51.3 \pm 1.9\%$ ($p < 0.01$) and $11.1 \pm 4.1\%$ ($p < 0.05$) respectively (B). **, $P < 0.01$ vs Control; #, $P < 0.05$ vs A17; ##, $P < 0.01$ vs A17

Fig.2. ADAM17 promotes glioma cell growth, viability, and proliferation in vitro

High expression increased glioma proliferation. Reduction of ADAM17 by shRNA or inhibitor decreased tumor cells proliferation. PI-3K inhibitor decreases glioma cells growth, viability, and proliferation in ADAM17 high expressing cells. (A) growth curve; (B) MTT assay at 48h, 72h, and 96h; (C) BrdU incorporation assay at 48h. ADAM17 and PI-3K inhibitor decreases glioma HF66 cells growth, viability, and proliferation. (D) growth curve; (E) MTT assay at 48h, 72h, and 96h; (F) BrdU incorporation assay at 48h. *, $P < 0.01$ vs Control.

Fig.3. ADAM17 increases the sensitivity of U87 cells to TAPI-2 or LY294002

A17, A17-shRNA, and control cell lines were treated with TAPI-2 (A) or LY294002 (B) in SRB cytotoxicity assay with final concentrations of 5, 10, 20, 40, and 80 $\mu\text{mol/L}$. TAPI-2 and LY294002 inhibited all the three cell lines growth in different levels. Compared to the inhibitory effect in control cell line, both TAPI-2 and LY294002 showed a significantly larger inhibition rate in A17 cells ($P < 0.01$ at 4 doses except 80 $\mu\text{mol/L}$) but showed a significantly less inhibition rate in A17-shRNA cells ($P < 0.01$) at all 5 different doses.

Fig.4. ADAM17 activity is correlated to glioma cell invasiveness in vitro

24h in vitro invasion assay revealed that the invasiveness of A17 cells was 1.96 ± 0.13 fold higher than control cells, and the invasiveness of A17-shRNA significantly decreased by $44.9 \pm 8.9\%$ compared to control cells. While ADAM17 inhibitor TAPI-2 significantly decreased the U87 cells invasiveness by $50.5 \pm 9.2\%$. In vitro invasion assay showed that the invasiveness of U87 glioma cell line is related to ADAM17 expression levels. Reduction in ADAM17 decreased the invasiveness of U87 cells. **, $P < 0.01$ vs Control

Fig. 5. ADAM17 in glioma cells promotes tube formation in MBECs

Culture medium from A17 cells promoted MBECs capillary tube formation by 2.27 ± 0.14 - 2.49 ± 0.10 fold compared to control medium. Medium from TAPI-2 or LY294002 treated A17 cells decreased capillary tube formation by $55.8 \pm 1.8\%$ - $75.2 \pm 2.5\%$ and $60.1 \pm 2.2\%$ - $62.6 \pm 1.8\%$, respectively, compared to A17. A17-shRNA medium showed $23.9 \pm 4.2\%$ - $33.7 \pm 5.1\%$ decrease in MBECs capillary tube formation compared to control. Compared to control medium, U87 conditioned medium increased the tube formation in MBECs, and TAPI-2 treated U87 medium significantly decreased tube formation by $31.2 \pm 3.3\%$, when compared to U87. High ADAM17 expression significantly increased VEGF expression. While A17-shRNA, TAPI-2, and LY294002 significantly decreased VEGF expression in A17 cells after 24h treatment. VEGF expression is correlated to ADAM17 proteolysis in U87. Soluble VEGF release is significantly correlated to ADAM17

proteolysis in cell culture supernatants. *, $P < 0.05$ vs Control; **, $P < 0.01$ vs Control; ##, $P < 0.01$ vs A17

Fig. 6. The expression level of ADAM17 is related to U87 glioma cells growth in nude mice A17 showed significant increase in tumor volume ($10.13 \pm 2.00 \text{ mm}^3$ vs. $8.15 \pm 1.19 \text{ mm}^3$, $P < 0.05$) in nude mice compared to control glioma cells, and A17-shRNA transfected cells showed significant lower growth rate ($5.82 \pm 1.88 \text{ mm}^3$) compared to their parent cells transfected with vector only ($P < 0.05$). $n = 7$ nude mice/group for control and A17, $n = 6$ mice/group for A17-shRNA. * $P < 0.01$, compared to control. Bar = 500 μm .

Fig. 7. TAPI-2 reduces U87 brain tumor growth

TAPI-2 treated U87 bearing nude mice showed significant decrease in tumor size ($6.73 \pm 2.47 \text{ mm}^3$) compared to untreated animals ($12.11 \pm 2.76 \text{ mm}^3$) $n=6$ nude mice/group

** $P < 0.01$, compared to control. Bar = 500 μm .

Fig. 8. PEI-delivered ADAM17-targeting shRNA reduces growth of U87 xenographs in a nude mouse model of brain tumor

(A-C) H ematoxylin and eosin stained brain sections from control (A), scrambled shRNA treated (B), and ADAM17-targeting siRNA treated (C) mice, 21 days post-implantation. Bar = 500 μ m. (D) Quantitative data of tumor volumes within treatment groups (n=7 mice per group, *P<0.01 compared to control and scramble shRNA).

Fig. 9.

(A) TGF- α concentration in U87 cell supernatants with different ADAM17 expression levels and treated with TAPI-2 and LY294002. ELISA Kit was employed to test TGF- α release under different ADAM17 expression levels. Compared to control U87 cells, A17 had more TGF- α ($64.3 \pm 8.4\%$ increase) released to supernatant, while A17-shRNA showed a significant $55.0 \pm 6.9\%$ less TGF- α release. TAPI-2 significantly suppressed soluble TGF- α release in A17 culture supernatant by $44.1 \pm 11.0\%$. LY294002 had no significant effect on TGF- α release in A17 cells. **, $P < 0.01$ vs Control; ##, $P < 0.01$ vs A17 (B) Effect of ADAM17 cDNA or A17-shRNA transfection on EGFR -AKT activation and VEGF expression in U87 cells. p-EGFR and p-AKT expression showed a significant increases in A17 and a significant decrease in A17-shRNA cells. Expression of EGFR or AKT was not significantly affected by ADAM17 level. In A17 cells, LY294002 and TAPI-2 significantly decreased p-EGFR and p-AKT expression, but had no effect on EGFR and AKT expression. VEGF protein showed the same trend as EGFR-AKT activation in all experimental groups.