CD9 modulates proliferation of human glioblastoma cells via epidermal growth factor receptor signaling

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Received March 8, 2014; Accepted January 2, 2015

DOI: 10.3892/mmr.2015.3466

Abstract. The tetraspanin CD9 has previously been shown to be involved in various cellular activities, including proliferation and migration. In addition, CD9 has been shown to be associated with epidermal growth factor receptor (EGFR). A common characteristic of glioblastoma multiforme histology is EGFR amplification, which affects signal transduction processes. The anti-proliferative effects of CD9 have been linked to EGFR signaling pathways, including phosphorylation of phosphoinositide-3-kinase (PI3K)/Akt and activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (Erk). The present study demonstrated that CD9 decreased the phosphorylation of EGFR at specific sites. In addition, CD9 attenuated EGFR signaling of PI3K/Akt and MAPK/Erk, which was associated with cell growth and proliferation. Conversely, small hairpin RNA-mediated knockdown of CD9 expression enhanced the activation of EGFR signal transduction pathways, including PI3K/Akt and MAPK/Erk. These results suggested that the mechanism underlying CD9-induced suppression of cell proliferation may involve the inhibition of phosphorylation of EGFR and the activity of PI3K/Akt and MAPK/Erk signaling pathways.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive form of brain cancer. GBM is a highly aggressive neuroepithelial tumor, which grows almost exclusively in neural tissue (1). The overall survival rate of patients with GBM has increased in the last decade due to the use of aggressive surgery combined with radiation, chemotherapy or biological therapy. However, malignant glioma cell proliferation and invasion remain the predominant factors affecting patient mortality (2,3). At the time of GBM diagnosis, in the majority of cases, the percentage of patients with a two-year survival rate is <30% (4). This severely malignant phenotype is at least partially due to acquired excess cell proliferation. A recent development in cancer research is the discovery of the tetraspanin proteins, which act as molecular facilitators of adaptors to organize a network of interactions among the cell surface molecules, known as the ‘tetraspanin web’. Members of the tetraspanin protein family are involved in regulation of the cell cycle as well as cell growth and proliferation. The mechanisms underlying the growth and proliferation of GBM cells remain to be elucidated; therefore, further study is required to improve therapeutic strategies.

CD9 is a cell surface glycoprotein of the tetraspanin protein family, which has a characteristic structure of four transmembrane domains and two extracellular loops. Tetraspanins can interact with other functional proteins, including growth factor receptors, intracellular signaling molecules and integrins, to form multiprotein complexes at the tetraspanin-enriched microdomain at the cell surface (5). Tetraspanins are involved in various biological processes, including cell survival, growth and migration. The tetraspanin CD9 was initially identified as a metastasis suppressor of solid tumors and was shown to be expressed in numerous types of cell, including platelets, pre-B cells and Schwann cells. Clinical and pathological findings have demonstrated that reduced CD9 gene expression is associated with poor prognoses in breast (6,7), colon (8) and lung cancer (9). Recent studies have suggested that CD9 may have the potential to regulate motility, through other transmembrane proteins (10,11). Furthermore, it has previously been reported that CD9 is directly associated with epidermal growth factor receptor (EGFR) and is able to destabilize surface expression of EGFR and consequently attenuate ligand-induced activation of the receptor (12).

A common characteristic of GBM histology is EGFR amplification, which affects signal transduction processes (13,14). EGFR signaling occurs through a complex network of intermediates, including phosphoinositide-3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase C-γ (15). The dysregulation of signal transduction processes affects various downstream biological processes.
associated with cell proliferation, migration, adhesion and invasion (16,17). Nakamura et al (18) previously reported that CD9 affects EGFR ligand binding activities of the membrane-bound forms of transforming growth factor-α and heparin-binding EGFr-like growth factor, which may contribute to human malignant glioma cell growth.

The present study aimed to present a novel mechanistic insight into the important role of tetraspanin CD9 in regulating the proliferation of human glioblastoma cells through epidermal growth factor receptor (EGFR) activity. The regulation of the expression and phosphorylation of EGFR and EGFR-induced signaling were examined in order to determined whether the function of CD9 was linked the inhibition of proliferation in human glioblastoma cells.

Materials and methods

Cell line and transfection. The LN229 human glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA), 50 mg/ml streptomycin and 50 μ/ml penicillin (Gibco-BRL), in a humidified atmosphere containing 5% CO₂ at 37°C. The LN229 cells were stably transfected with a CD9 pcDNA3.1 plasmid (LN/CD9) or a control pcDNA3.1 plasmid (LN/cont). The coding region of CD9 cDNA was generated through reverse transcription quantitation-polymerase chain reaction (RT-qPCR) from LN229 human glioblastoma cells. The CD9 cDNA fragments were amplified by RT-qPCR, were digested with BamHI and EcoRI, and the purified cDNA fragments were ligated into the BamHI-EcoRI-digested pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA, USA). The primers used in the PCR were as follows: forward, 5'-CCGGATCCCATGCGCGTCAAGAGAAGGCA-3' and reverse, 5'-CGGAATTCCGCTAGACCATCTCGGCGTCC-3' (Takara Biotechnology Co., Ltd., Dalian, China). The construct was verified by sequencing. LN229 cell lines were transfected with a CD9 pcDNA 3.1 using the Lipofectamine 2000 reagent (Invitrogen Life Technologies). The LN229 cell lines were transfected with a CD9 pcDNA3.1 plasmid (Invitrogen Life Technologies) following selection in Zeocin (Invitrogen Life Technologies). A CD9 small hairpin (shRNA) plasmid (LN/shCD9) was constructed in order to generate CD9-depleted cells. The cells transfected with a negative control (NC) shRNA plasmid were considered control cells (shNC) and untransfected LN229 cells were considered the mock control cells. A CD9 shRNA and a negative control plasmid were constructed by GenePharma using pGPH1/Neo plasmid (GenePharma, Shanghai, China) followed by selection in G418 (Gibco BRL). The 8 nt siRNA directed against CD9 was 5'-UUCUUGCUAGAGUGCUUTT-3'. LN229 cells were transfected using Lipofectamine®2000 reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions.

Antibodies and reagents. Primary antibodies against the following antigens were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): Rabbit polyclonal CD9 immunoglobulin (IgG) (1:500; sc9148), goat polyclonal EGFR IgG (1:1,000; sc31157), goat polyclonal p-EGFR (Tyr-1173) IgG (1:500; sc12351), mouse monoclonal p-EGFR (Tyr-1066) IgG (1:500; sc81509), rabbit polyclonal p-Akt1/2/3 (Ser473) IgG (1:1,000; sc33437), rabbit polyclonal p-Akt1/2/3 (Thr308) IgG (1:1,000; sc135650), rabbit polyclonal Akt1/2/3 IgG (1:1,000; sc8312), goat polyclonal p-Erk1/2 (Thr202/Tyr204) IgG (1:1,000; sc16982), rabbit polyclonal Erk1/2 IgG (1:1,000; sc292838), goat polyclonal β-actin IgG (1:1,000; sc1616). The β-actin antibody was used as a loading control. The mRNA and protein expression levels of CD9 were determined by (A and C) western blot analysis and (B) reverse transcription polymerase chain reaction, respectively. shRNA, small hairpin RNA; NC, negative control.

Figure 1. Modulating CD9 expression in LN229 human glioblastoma cells. Cells were seeded into 12-well plates in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. LN229 cells were transfected with a CD9 pcDNA3.1 plasmid or a CD9 shRNA plasmid. (A) Cells were measured for overexpression of CD9 following transfection with pcDNA3.1/CD9 plasmid (LN/CD9) or an empty pcDNA3.1 plasmid (LN/cont). Untransfected LN229 cells were used as a control. CD9 protein expression levels were measured by immunoblotting with a CD9 antibody, and β-actin antibody was used as a loading control. Cells were also transfected with CD9 shRNA plasmid (LN/shCD9) or a shNC plasmid. Untransfected LN229 cells were used as a control. The mRNA and protein expression levels of CD9 were determined by (A and C) western blot analysis and (B) reverse transcription polymerase chain reaction, respectively. shRNA, small hairpin RNA; NC, negative control.

RT-PCR. Total RNA was extracted from the cells using TRIzol® (Invitrogen Life Technologies), and 1 μg total RNA was reverse-transcribed using a cDNA Synthesis kit (Invitrogen Life Technologies) with random hexamers. The PCR cycling parameters were set as follows: 30 cycles of 40 sec at 94°C, 40 sec at 60°C and 60 sec at 72°C. The identity of the PCR products was confirmed through sequencing by BGI Technologies Co. (Shenzhen, China) using T7 primer. The relative quantitative analysis was normalized to the endogenous
control GAPDH. The following primer sequences were used: GAPDH forward, TACCTATGCGAGTGTGCTGTTG; reverse, CCACTCCTGGTCCTCCTAGA; CD9 forward, TGCATCTGTATTCCAGCGCCA; reverse, CCAGCCTCGTCCCGTAGA; GAPDH forward, TACTTATGCCGATGTCGTTGT and reverse, CTCAGGGATGCTTACGCA; CD9 forward, TGCCTACGATGGCTGCTGTTGT and reverse, CTCAGGGATGCTTACGCA. The following primer sequences were used: GAPDH forward, TACTTATGCCGATGTCGTTGT and reverse, CTCAGGGATGCTTACGCA; CD9 forward, TGCATCTGTATTCCAGCGCCA and reverse, CTCAGGGATGCTTACGCA (Takara Biotechnology Co., Inc.) (19).

Flow cytometry. Cell cycle distribution was analyzed using flow cytometry with propidium iodide (PI) staining. Briefly, 1.5x10^5 cells transfected with CD9 and negative control plasmids for four days were seeded in 6-cm dishes and cultured for two days at 37°C. The cells were then treated with the desired agents as described in the figure legends, in serum-free medium overnight. The medium was refreshed and the cells were stimulated with 50 ng/ml EGF for 10 min at room temperature. The cells were then harvested and lysed in 200 µl radioimmunoprecipitation lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 25 mM 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate and 50 mM NaF; Sigma-Aldrich), supplemented with 1 mM Na_3VO_4, 1 mM dithiothreitol, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail per well. 10 µl inhibitor cocktail (Sigma-Aldrich) was added to 1 ml lysis buffer, all reagents described above were purchased from Sangon Biotech Technogene Ltd. (Shanghai, China). The lysate was determined using a bicinchoninic acid protein assay kit (Beyotime, Jiangsu, China). Heat-denatured protein samples (20 µg per lane) were separated using 12% SDS-PAGE (Invitrogen Life Technologies) and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The non-specific binding sites on the PVDF membranes were blocked with 5% nonfat dry milk in tris-buffered saline with 0.1% Tween-20. The target protein bands in the PVDF membranes were blocked with 5% nonfat dry milk in tris-buffered saline with 0.1% Tween-20. The target protein bands in the PVDF membranes were revealed by immunoblotting with primary antibodies, followed by an incubation with the species-specific HRP-conjugated secondary antibodies. Signal bands were detected using enhanced chemiluminescence reagents (GE Healthcare Life Sciences, Little Chalfont, UK). β-actin was used as a housekeeping antibody, to verify equal amounts of protein were loaded in all of the lanes.

Statistical analysis. All of the experiments were repeated at least three times and consistently yielded similar results. The data were analyzed by GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons between groups were performed using a one-way analysis of variance. P<0.01 was considered to indicate a statistically
significant difference between values. Values are expressed as the mean ± standard error of the mean.

**Results**

**CD9 expression in LN229 cells.** The CD9 gene was constructed into a pcDNA3.1 vector and transfected into LN229 cells (LN/CD9). The protein expression levels of CD9 were measured after 48 hours by western blotting. The protein expression levels of CD9 were significantly enhanced in the LN229 cells transfected with the pcDNA 3.1/CD9 vector, as compared with the cells transfected with an empty pcDNA3.1 vector (Fig. 1A). The LN229 cells were also transfected with CD9 shRNA plasmids in order to suppress CD9 expression (LN/shCD9). RT-PCR and western blot analyses indicated that the expression levels of endogenous CD9 were stably decreased by ~70% (Fig. 1B and C).

**Overexpression of CD9 inhibits EGF-stimulated LN229 cell proliferation EGF and affects the phosphorylation of EGFR.** To elucidate whether CD9 is able to inhibit cell growth by affecting cell cycle progression, the cell cycle distribution was assessed in the LN229 cells by flow cytometry. The cell population in the LN/CD9 group displayed a significant increase in the proportion of cells in G0/G1 phase, and a significant decrease in the number of cells in the S phase, as compared with the control cells (Fig. 2A and B). These results suggested that CD9 may inhibit the proliferation of LN229 cells via cell cycle regulation. To investigate the molecular mechanisms underlying how CD9 affects EGF-stimulated cell growth, the expression and phosphorylation status of EGFR was determined by western blotting. Increased CD9 expression significantly reduced EGF-stimulated phosphorylation of EGFR at Y1173 and Y1086 without significantly affecting EGFR expression levels (Fig. 3A), while downregulation of
CD9 elevated EGF-stimulated phosphorylation of EGFR at Y1173 and Y1086 (Fig. 3B).

**Effects of inhibiting the PI3K/Akt and MAPK/Erk pathways.** To investigate how CD9 modulated LN229 cell proliferation and growth regulation via the PI3K/Akt and MAPK/Erk pathways, the EGF-stimulated LN229 cells were transfected with an empty pcDNA3.1 vector and were treated with LY294002 and U0126, respectively. Inhibition of the PI3K/Akt and MAPK/Erk pathways, using LY294002 and U0126, suppressed EGF-stimulated proliferation (Fig. 2C-E). These results suggested that the PI3K/Akt and MAPK/Erk pathways may be involved in the modulation of LN229 cell growth regulation in vitro.

**CD9 attenuates EGFR signaling of PI3K/Akt and MAPK/Erk pathways.** Based on previous results, it was hypothesized that PI3K/Akt and MAPK/Erk pathways may be associated with glioma cell proliferation. To further investigate the mechanisms underlying the suppressive effects of CD9 on EGF-stimulated growth in vitro, these two signaling pathways, which are essential for the modulation of tumor cell growth, were measured by western blot analysis. Overexpression or suppression of CD9 directly affected the activation of the PI3K/Akt and MAPK/Erk pathways. CD9 negatively affected EGFR-mediated activation of Akt and Erk (Fig. 4). In the EGF-treated LN229 cells overexpressing CD9, the inhibitory effect of CD9 on phosphorylation of Akt at Ser473 was enhanced, and the opposite effect was observed on the phosphorylation of Akt at Ser473 and Erk at Erk1 and 2 following knockdown of CD9 (Fig. 4A and B). However, alterations in the cellular content of CD9 did not affect the level of EGF-induced phosphorylation of Akt at Thr308. These results indicated that the PI3K/Akt and MAPK/Erk signaling pathways may have an important role in CD9-regulated cell proliferation.

**Discussion**

The present study demonstrated that CD9 decreased the phosphorylation of EGFR at specific sites. CD9 attenuated EGFR signaling of PI3K/Akt and MAPK/Erk, which are associated with cell growth and proliferation. Conversely, shRNA-mediated knockdown of CD9 expression enhanced the activation of the EGFR signal transduction pathway, including PI3K/Akt and MAPK/Erk, which enhanced cell proliferation. This activation was blocked by treatment with PI3K and MAPK inhibitors.

The tetraspanin CD9 was initially characterized as a cell surface antigen on lymphohemopoietic cells, platelets, eosinophils, and pre-B cells (20,21). CD9 is also normally expressed in mature oligodendrocytes and Schwann cells, and is implicated in neurite outgrowth and myelination (22,23). Recently, numerous clinical studies have demonstrated that CD9 expression is inversely correlated with patient survival and metastatic potential in various types of human cancer (6-9,24). Conversely, Kawashima et al (25) demonstrated that among the neuroepithelial tumors; high-grade astrocytic tumors, including glioblastomas, had higher immunoreactivity of CD9, as compared with low-grade cerebral astrocytomas, thus suggesting that CD9 expression in astrocytic tumors may be correlated with their malignancy (25). Tetraspanin members, such as CD82, can associate with EGFR and attenuate EGFR signaling by accelerating EGFR internalization, and redistribute EGFR into distinct cellular compartments following endocytosis. This process significantly impacts the early events associated with receptor activation, including ligand binding, dimerization and re-localization of activated receptors into clathrin-coated pits (26,27). For numerous reasons, the present study hypothesized that CD9 may affect glioma tumor biology. To fully understand the roles of CD9 on glioma tumor biology, CD9 expression was modulated in the LN229 human glioblastoma cell line, and it was determined whether CD9 was able to drive cell proliferation. Numerous studies have suggested that CD9 provides a bridge among cell surface proteins, including growth factor receptors, to generate functional complexes involved in cell proliferation, division migration and apoptosis (28,29). To explore the precise roles and mechanistic action of CD9 on the proliferation of glioblastomas, the present study focused on the association between CD9 and EGFR, and the EGFR signals mediated by CD9 (30). CD9 was shown to decrease the phosphorylation of particular tyrosine residues and reduce the EGFR signals, which usually mediate the proliferation and survival of glioblastoma.

The EGFR is the prototypical member of the ErbB/EGFR family, which is a primary contributor to glioblastoma initiation and progression. To clarify the role of the association between EGFR and CD9, CD9 content was altered in LN229 cells by transfection with CD9 and shRNA-CD9. The results demonstrated that CD9 inhibited EGF-stimulated phosphorylation at Y1173 and Y1086. EGFR phosphorylation leads to the recruitment of complex effector proteins through recognition and binding of Src homology 2 and phosphotyrosine binding domains on the effector proteins, to phosphotyrosine motifs on the receptor (31-33). Consequently, various downstream signaling cascades, including the PI3K and MAPK are activated.

The results of the present study demonstrated that inhibitors of PI3K or MAPK blocked the proliferation of LN229 cells, which indicates that the PI3K/Akt and MAPK/Erk pathways are important for cell growth and survival (34,35). The effects of CD9 were then determined on the activity of these two signaling pathways stimulated by EGF. The results suggested that CD9 negatively regulated EGF-stimulated activation of PI3K/Akt phosphorylation at Ser473 and MAPK phosphorylation, but did not affect PI3K/Akt phosphorylation at Thr308. Flow cytometric analyses confirmed that CD9 had negligible effects on EGF-stimulated cell proliferation. Phosphorylation of PI3K/Akt by phosphoinositide-dependent protein kinase 1 at Thr308 and at Ser473 by the mammalian target of rapamycin complex 2 is required for full kinase activity (36). The results of the present study suggested that Akt Ser473 phosphorylation contributes to CD9-mediated cell proliferation in response to EGF. In addition, CD9 affects the MAPK/Erk signaling pathway. Activation of the MAPK/Erk pathway is triggered by growth factor receptor-bound protein 2 binding directly to the receptor at Y1068, and indirectly through Src homology domain-containing adaptor protein C binding at Y1173 and Y1148 (37). Initial experiments demonstrated that CD9 suppressed EGF-stimulated EGFR phosphorylation at Y1173 and...
and Y1086. Therefore, it may be hypothesized that CD9 can regulate the phosphorylation of Y1173 and Y1086 residues on EGFR, which is responsible for the EGFR-induced activation of PI3K/Akt and MAPK/Erk. The changes to the levels of phosphorylation at the Y1173 and Y1086 residues were consistent with those of the cellular content of CD9.

In conclusion, upregulated CD9 expression in LN229 cells inhibited cell proliferation, decreased EGFR-induced phosphorylation of PI3K/Akt and MAPK/Erk. Conversely, knockdown of CD9 expression resulted in attenuation of EGFR-induced activation of PI3K/Akt and MAPK/Erk, and enhanced the EGFR-induced phosphorylation of EGFR at Y1173 and Y1086. These results suggested that the mechanism by which CD9 affects EGFR signaling appears rather complex and requires further study.

References


