

Ephrin-A1 is a negative regulator in glioma through down-regulation of EphA2 and FAK

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Received September 11, 2006; Accepted November 17, 2006

Abstract. Eph receptors, the largest receptor tyrosine kinases, and their ephrin ligands play important roles in axon guidance and cell migration during development of the nervous system. Recently, these molecules are also found involved in tumorigenesis of different kinds of cancers. In this study, we demonstrated that expression of ephrin-A1 was dramatically down-regulated in glioma cell lines and in primary gliomas compared to the matched normal tissues. Forced expression of ephrin-A1 attenuated cell migration, cell proliferation, and adhesion-independent growth in human glioma U251 cells. EphA2, a receptor for ephrin-A1 and an oncoprotein, was greatly decreased in ephrin-A1-transfected glioma cells. Overexpression of ephrin-A1 stimulated activation of EphA2 by phosphorylation and led to its degradation. Furthermore, focal adhesion kinase (FAK), a known downstream molecule of EphA2, was also down-regulated in the ephrin-A1 transfected cells. These results suggested that ephrin-A1 serves as a critical negative regulator in the tumorigenesis of gliomas by down-regulating EphA2 and FAK, which may provide potential valuable targets for therapeutic intervention.

Introduction

The Eph receptors, containing 14 distinct members, comprise the largest family of receptor tyrosine kinases with corresponding transmembrane ligands named ephrins (1). Both Eph receptors and ephrins can be classified as type A and B according to the sequence homology and binding specificity. A-ephrins (ephrin-A1-A6) are associated with the plasma membrane via a glycosylphosphatidylinositol (GPI) linker and preferentially bind to EphA receptors (EphA1-A8), whereas B-ephrins (ephrin-B1-B3) are trans-membrane proteins

that mainly interact with EphB receptors (EphB1-B6). Upon engagement of each other, these membrane-bound molecules can transduce intracellular signals, which have been demonstrated to regulate many biological processes, including axon guidance, neural crest cell migration, hindbrain segmentation, somite formation and vasculogenesis (2-4). There are also clues that the abnormalities of Eph/ephrin system are functionally involved in tumorigenesis. Brantley *et al* found that complementary expression of EphA2 in tumor blood vessel endothelium and ephrin-A1 in tumor cells resulted in pathogenic angiogenesis in tumors (5). It has been shown that EphA2 is highly expressed in different kinds of carcinomas, including breast, ovarian, gastric, pancreatic, prostate cancers, and gliomas, especially in some malignant tumors (6-17). Moreover, overexpression of EphA2 is proved to be sufficient to confer tumorigenic potential and malignant transformation on non-transformed breast cells *in vitro* (6,18). Contrarily, the usage of blocking antibody of EphA2 can partly relieve the progression and exacerbation of tumors (19). These results suggest that EphA2 might be an oncoprotein in tumorigenesis in several kinds of cancers (6,19,20).

Gliomas are malignant tumors known for their high mortality (21-23). As gliomas are often localized in the depths of the central nervous system (CNS) - brain or spinal cord - surgical resection or radiotherapy simultaneously imposes high risks on patients. Therefore, suppressing the malignant progression of gliomas rather than trying to remove the tumor tissues from CNS may be more practical and hopeful approaches to prolong the lives of patients suffering from these diseases.

In this aspect, it has been demonstrated that ephrin-A1, an effective ligand of EphA2, can negatively regulate the behavior of tumor cells and partially inhibit the progression of cancers by activating EphA2 expressed in tumor cells (24). For example, ephrin-A1-treatment of breast cancer cells remarkably slowed the tumor growth, decreased tumor cell viability in soft agar assays and prevented tumor formation in xenograft models (25,26). The growth of human gastric cancer cells can also be inhibited by exogenous ephrin-A1 (9). Although Ephs and ephrins play very important roles in the neural system, it is not clear whether the interaction of EphA2 and ephrin-A1 is involved in the development and progression of gliomas. In this study, we examined the

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Key words: ephrin-A1, EphA2, gliomas, FAK

expressions of both EphA2 and ephrin-A1 in clinical samples and six glioma cell lines and then investigated whether forced expression of ephrin-A1 could attenuate the neoplastic properties of glioma cells.

Materials and methods

Cell culture conditions. Human astrocytoma cells A172, SHG-44, U87, U138, U251 and U343 were maintained in Dulbecco's modified Eagle's medium supplement with 10% FBS (Hyclone, Logan, UT) at 37°C in a humidified atmosphere containing 5% CO₂.

Antibodies and reagents. Anti-EphA2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-ephrin-A1 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and anti-β-actin antibody was from Sigma (St. Louis, MO), anti-FAK, FAK (pY397) were purchased from Transduction Laboratories, BD Biosciences (San Diego, CA).

Plasmid construction and cell transfection. The 618 bp coding sequence of ephrin-A1 was amplified from human brain cDNA library (Clontech, HL4500AK 1) using the primer: forward: 5'-cataagctccaccatggagttcctctggccctct-3', and reverse: 5'-aagctcgagacacggggttgccagcagca-3', the products were digested with the enzyme of HindIII and XhoI and cloned into pCDNA3.1. Monolayers of U251 cells were transfected with the pCDNA3.1-ephrin-A1 (U251/A1) and empty vector (U251/V), using lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cells were selected in the presence of 1 mg/ml G418 and eight positive clones were confirmed by Western blot assay. Two of the eight clones with ephrin-A1 forced expression were picked out for the next experiments, one clone (U251/A1-H) shows high expression of ephrin-A1 and the other (U251/A1-L) has relatively low expression of ephrin-A1.

Immunohistochemistry and immunofluorescent microscopy. Paraffin-embedded tissue blocks were sectioned onto slides and then deparaffinized in xylene. Sections were quenched with 3% hydrogen peroxide in methanol for 15 min, microwaved 5 min in H₂O, and blocked for 1 h with PBS containing 3% goat serum and 0.1% Triton X-100. Slides were incubated with anti-EphA2 or ephrin-A1 antibody (1:500 dilution) overnight at 4°C. The secondary antibody (HRP-conjugated or Alexa Fluor-conjugated) was applied at a 1:1000 dilution in PBS containing 1% goat serum. Sections were exposed to diaminobenzidine peroxidase substrate for 5 min for IHC. Fluorescence was monitored by inverted confocal laser microscopy (Carl Zeiss, New York, NY).

Western blot analysis. Western blot analyses were performed as described previously (27), and antibody binding was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography. The antibody specific for actin were probed to confirm equal sample loading.

Soft agar assay. For clonogenic assay, cells were plated into 24-well flat-bottomed plates using a two-layer soft agar system

with 1x10³ cells/well in a volume of 400 μl/well as described previously (28). After 20 days of incubation, the colonies were counted and measured. All of the experiments were done at least three times using triplicate plates per experimental point.

Wound healing assay to assess cell motility. U251/V and U251/A1 cells were allowed to grow to confluence. A linear wound was created by scraping the wells with a 200 μl pipette tip. The floating cells were removed by gentle washes with culture medium. The healing process was examined dynamically and recorded with an Olympus digital camera with microscope adapter.

Results

The expressions of EphA2 and ephrin-A1 in the glioma cell lines and glioma samples from patients. We first examined the expression patterns of EphA2 and ephrin-A1 in six glioma cell lines (A172, SHG-44, U87, U138, U251, U343). Western blot analysis showed that all these glioma cells exhibited constitutive expression of EphA2, but the expression of ephrin-A1 was hardly detected in the cell lines (Fig. 3A). We further employed immunohistochemistry to examine the expressions of EphA2 and ephrin-A1 in primary gliomas and their neighboring normal counterparts from glioma patients. Our results clearly show that EphA2 was expressed in glioma as well as normal brain tissues (Fig. 1A and B). Moreover, EphA2 in normal brain tissues was regularly distributed along process-like structures. In glioma tissues, however, the signals of EphA2 were promiscuously dispersed and more intense, which inferred that the expression of EphA2 might accompany the malignant proliferation of glioma cells. Interestingly, we also detected apparent expression of ephrin-A1 in normal brain tissue around the focus of glioma, but its expression in glioma tissue was remarkably decreased (Fig. 1C and D). Double immunofluorescence staining of EphA2 and ephrin-A1 clearly displayed that the expressions of the two molecules were exactly co-localized in normal brain tissues (Fig. 2A-D, 2A'-D'). In contrast, only EphA2 was dominantly expressed in glioma regions and almost no expression of ephrin-A1 was observed around the signals of EphA2 (Fig. 2E-H, 2E'-H'). The aberrant disappearance of ephrin-A1 seemed to be a significant event in glioma tissues. Altogether, our data both *in vivo* and *in vitro* revealed down-regulation of ephrin-A1 in gliomas, which hinted that ephrin-A1 might serve as a tumor suppressor in normal glial cells and its dramatic decrease might be involved in the glioma tumorigenesis.

Forced expression of ephrin-A1 in U251 cells altered cell morphology as well as cell proliferation. To identify the potential inhibitive effects of ephrin-A1 in glioma cells, we transfected U251 cells with a construct containing human full-length ephrin-A1 cDNA or empty vectors (U251/V) in parallel as control. Eight ephrin-A1 stable cell lines (U251/A1) were obtained and confirmed by Western blotting of ephrin-A1 antibodies (data not shown). Two of stably ephrin-A1-transfected clones were used for further experiments, one of them having high expression of ephrin-A1 (U251/A1-H) and the

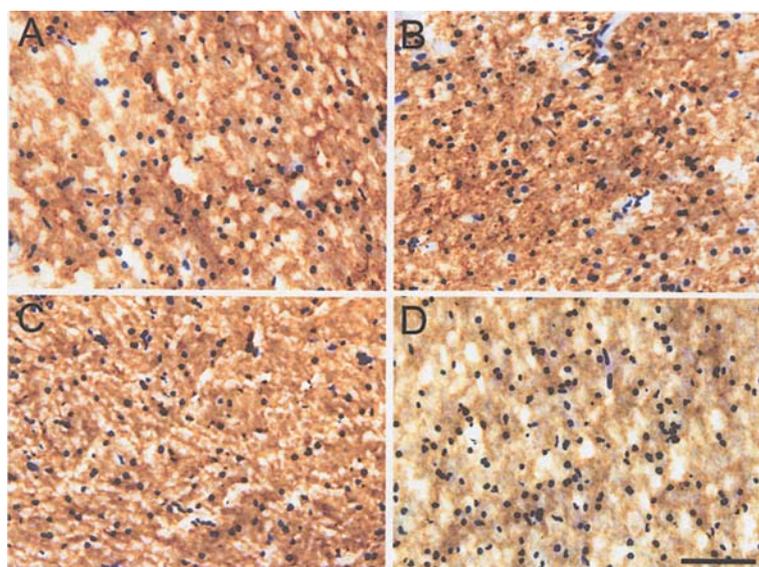


Figure 1. Immunohistochemistry of EphA2 and ephrin-A1 protein in glioma area and neighboring normal brain tissue. Immunostaining displays clear EphA2 signals (brown) in region of glioma focus (B) and its neighboring brain tissue (A) that is mainly composed of relatively normal cells. Notably, EphA2 is regularly distributed along process-like structures in normal region of brain and assumes typical astrocyte-morphology (A). In glioma tissue, however, the expression of EphA2 seems to be promiscuously dispersed with more intense immunoreaction (B). Immunohistochemistry also shows intense signals of ephrin-A1 (brown) in normal area around the focus of glioma (C), but both intensity and density of ephrin-A1 signals are remarkably decreased in glioma tissue (D). Counterstained with Hematoxylin (blue). Scale bar, 100 μ m.

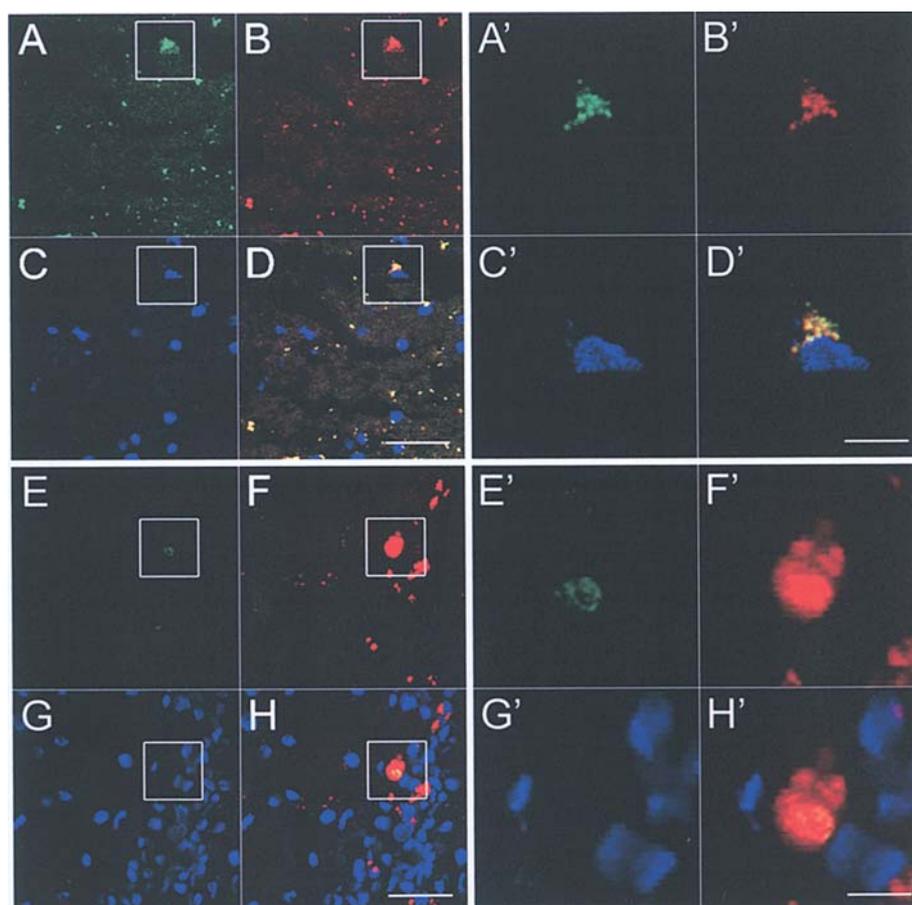


Figure 2. Immunofluorescent staining of EphA2 and ephrin-A1 in glioma area and neighboring normal brain tissue. Confocal images of immunofluorescence labeling identify the expressions of ephrin-A1 (A and its local higher magnification A', green) and EphA2 (B and its local higher magnification B', red) in normal brain tissue and the merged result indicated that the two proteins are well co-localized (D and its local higher magnification D', yellow). In glioma tissue, the signals of ephrin-A1 are dramatically decreased (E and its local higher magnification E', green) though the expression of EphA2 is still obvious (F and its local higher magnification F', red). Merged figure (H and its local higher magnification H') shows that almost no ephrin-A1 is co-localized with EphA2. Counterstained with Hoechst 33342 (C, C', D, D', G, G', H and H', blue). Scale bar, 100 μ m (A-H); 25 μ m (A'-H').

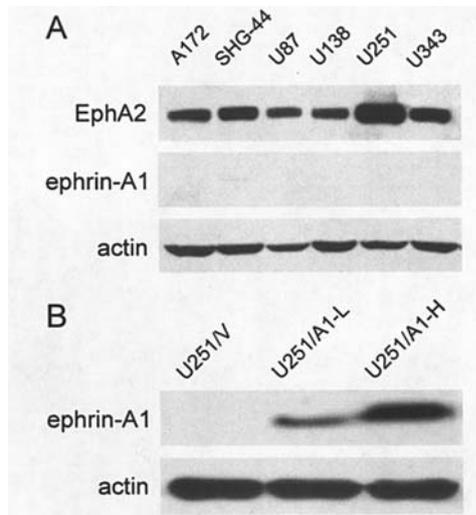


Figure 3. Expression of EphA2 and ephrin-A1 in glioma cells. A, EphA2 is constitutively expressed in all six glioma cell lines tested shown by Western blotting. In contrast, the expression of ephrin-A1 is barely detected in these glioma cell lines. B, U251 cells are transfected with vector containing full-length human ephrin-A1 (U251/A1) or empty vector (U251/V). Two stably ephrin-A1-transfected U251 cell clones were chosen for further studies; one of them has relatively lower expression of ephrin-A1 (U251/A1-L) and the other has higher expression of ephrin-A1 (U251/A1-H). Actin is used as an internal standard to determine the loading amounts of proteins in each sample.

other having relatively low expression (U251/A1-L) (Fig. 3B). Forced expression of ephrin-A1 dramatically altered the cell morphology of gliomas. During culture in standard conditions, adhered U251/V showed fibroid morphology (Fig. 4A), which indicated wide-type U251. In the same conditions, however, most of cells transfected with ephrin-A1 (U251/A1) adopted rounded morphology (Fig. 4B and C). To test the effects of expression of ephrin-A1 on cell growth, MTT assay was used to measure the cell proliferation of U251/A1-H, U251/A1-L and U251/V cells. The results showed that the proliferation of U251/A1 cells was inhibited compared with that of U251/V cells (Fig. 4D).

Glioma cells overexpressing ephrin-A1 form fewer colonies in soft agar. In general, normal cells must grow in appropriate adhesion to extracellular matrix and can not form colonies when cultured in soft agar; whereas malignant cancer cells can often survive in an adhesion-independent manner and acquire the ability to form colonies in soft agar, which is a useful parameter for evaluating the malignant potential of tumor cells *in vitro* (29). We investigated, therefore, the effects of ephrin-A1 overexpression on adhesion-independent growth of glioma cells in soft agar. The U251/V cells had strong ability of adhesion-independent growth and formed many colonies in soft agar (Fig. 4E and H). In contrast, the efficiency of colony formation in soft agar was significantly inhibited in two ephrin-A1-transfected cell lines: the colonies of U251/A1-L cells were reduced to 10% of the U251/V cells (Fig. 4F and H) and almost no colonies of U251/A1-H cells were detected (Fig. 4G and H). These results revealed that overexpression of ephrin-A1 inhibited dramatically the adhesion-independent growth of glioma cells in an ephrin-A1 dosage-dependent manner.

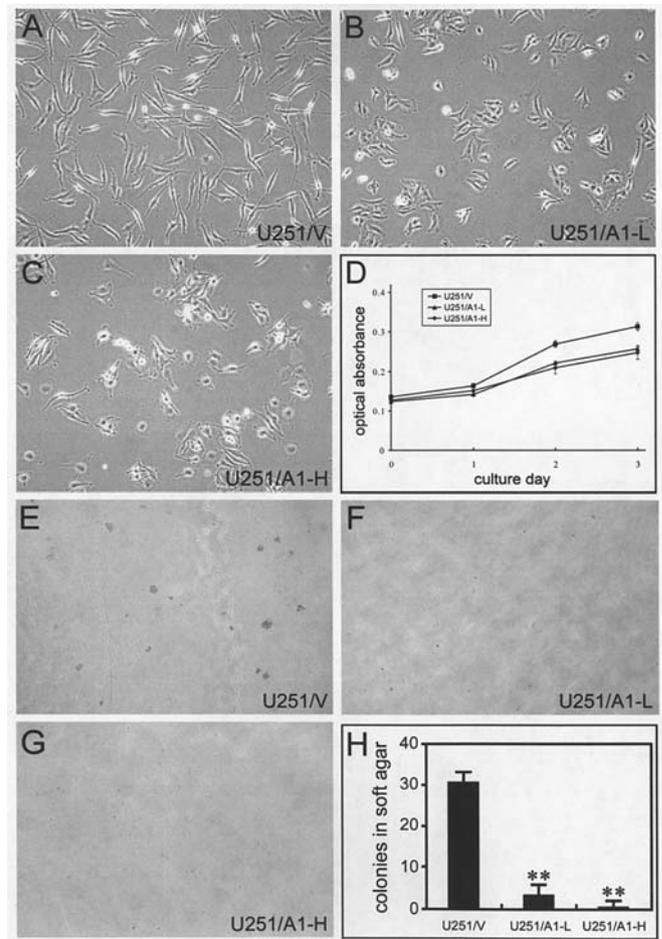


Figure 4. The alterations of cell morphology and growth ability in U251/A1 cells. Compared with U251/V cells (A), which assume spindly appearance, most of stably ephrin-A1-expressed cells are relatively rounded (B and C). Additionally, the statistic analysis of cell number by MTT (D) displays that the cell proliferation of ephrin-A1-transfected cells seems to be slower than that of U251/V. In soft agar culture, U251/V cells have the ability to form obvious colonies (E, >20 cells as a colony). U251/A1-H and U251/A1-L, however, only form smaller colonies and the number of colonies is also greatly reduced (F and G) compared to U251/V controls (E). The results (H) indicate that the colony formation of U251/A1-H and U251/A1-L in soft agar is statistically significant against U251/V controls and moreover, this ability seems to be inversely related with ephrin-A1 expression level. ** $P < 0.01$ vs. U251/V, Student's t-test.

Forced expression of ephrin-A1 slows down migration of glioma cells. As glioma cells often possess migrating tendency, we studied the role of ephrin-A1 on migration of glioma cells *in vitro* by wound-healing assay, a method that is widely used to examine cell migration. When a wound was introduced into a monolayer of U251/V, cells progressively migrated into the wound and almost filled the area by 96 h (Fig. 5A, C, E and G). In the same conditions, however, the migration of U251/A1 was notably inhibited compared with U251/V and only a few cells appeared in the wound zone within 96 h after wounding (Fig. 5B, D, F and H). Hence, ephrin-A1 might also be a negative regulator in the process of glioma cell migration.

The down-regulation of EphA2 and FAK following ephrin-A1 transfection. Since EphA2 is the receptor of ephrin-A1, we further investigated whether the expression of EphA2 in glioma

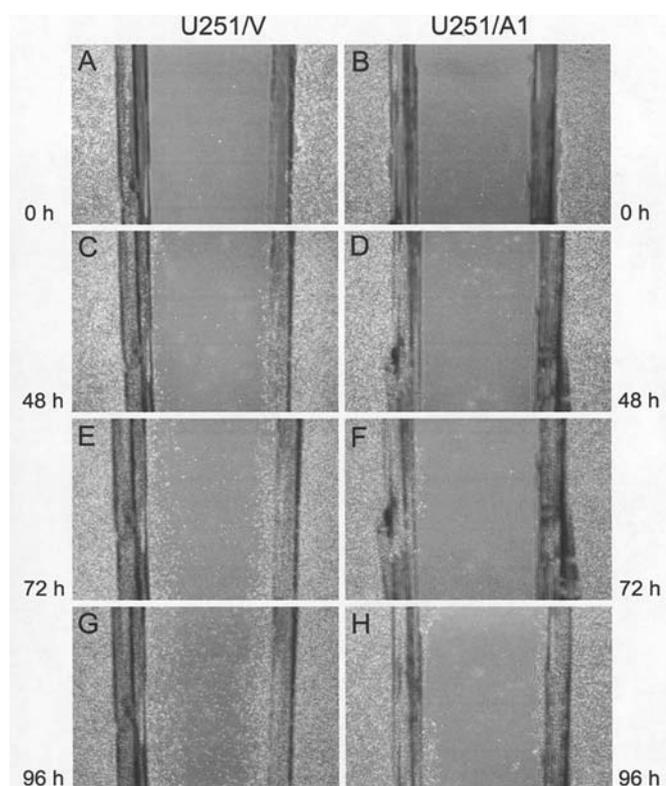


Figure 5. The wound-healing assay of U251/A1 cells. The wound is made using sterile pipette in monolayer of U251/V (A) or U251/A1 cells (B), respectively, and the scrapes are confirmed clear under a microscope. At 48 h after wounding, a few of U251/V cells have migrated through the edges of the wound (C), but still no U251/A1 cells are found in the scraped area (D). At 72 following the wounding, the migrated U251/V cells were observed in the wound region (E) and further increased by 96 h (G). In the same conditions, however, the migration of U251/A1 cells is notably inhibited compared with U251/V controls and only a few cells appear in the wound zone within 96 h after wounding (F and H). This result suggests that ephrin-A1 may be a key negative regulator in the process of cell migration.

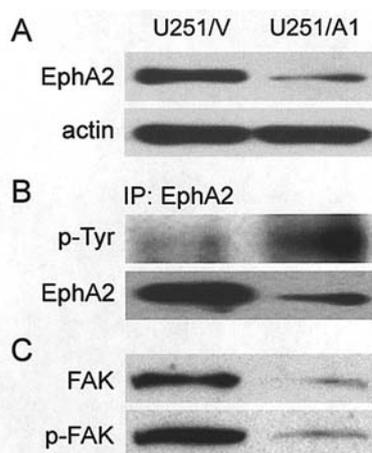


Figure 6. The expressions of EphA2 and focal adhesion kinase (FAK) in U251/A1 cells. A, Western result shows that the level of EphA2 is remarkably decreased in U251/A1 cells compared with U251/V. Actin is used as an internal standard to determine the loading amounts of proteins in each sample. B, Immunoprecipitation with excess anti-EphA2/protein-A complex further identifies the down-regulation of EphA2 in U251/A1 cells, but blotting using anti-tyrosine antibody (p-Tyr) proves that the phosphorylation of EphA2 is obviously elevated in U251/A1 cells in comparison with U251/V. C, Western blotting indicates that both protein expression and phosphorylation level of FAK in U251/A1 cells are dramatically lower than those in U251/V cells.

cells was also influenced due to the forced expression of ephrin-A1. Western result showed that the level of EphA2 in U251/A1 cells was remarkably decreased with the overexpression of ephrin-A1 (Fig. 6A). However, the phosphorylation level of EphA2 was elevated in U251/A1 cells compared with that in U251/V cells (Fig. 6B). It seemed that the activation of EphA2 by ephrin-A1 actually led to the down-regulation of EphA2.

We also examined the expression of focal adhesion kinase (FAK), which is considered a downstream factor of EphA2 (10,30,31). As expected, the level of FAK was concomitantly down-regulated and phosphorylated FAK, which possesses more activity than non-phosphorylated FAK, was simultaneously decreased (Fig. 6C).

Discussion

The major finding of our present study is that ephrin-A1 served as a critical negative regulator in the tumorigenesis of gliomas by down-regulating EphA2 and FAK. We found that expression of ephrin-A1 was nearly undetectable in all six glioma cell lines tested, and down-regulated in primary glioma tissues compared to the normal tissues. To further examine the effects of ephrin-A1 on glioma cells, we transfected the tumor cells with vectors expressing full-length human ephrin-A1. We demonstrated that forced expression of ephrin-A1 greatly attenuated cell migration, cell proliferation, as well as adhesion-independent growth of glioma cells. We also observed by Western blotting that the increase of EphA2 phosphorylation and decrease of total EphA2 following ephrin-A1 transfection, suggesting that introduced ephrin-A1 could effectively bind and activate endogenous EphA2 in glioma cells and lead to the degradation of EphA2. This activation of EphA2 led to the internalization and degradation of endogenous EphA2. Additionally, both FAK and phosphorylated FAK were found to be markedly reduced in ephrin-A1-transfected glioma cells.

Early studies have reported that FAK is regulated by EphA2 and might be a downstream molecule of EphA2. In prostate and pancreatic cancer cells, for example, activation of EphA2 by exogenous ephrin-A1 can suppress phosphorylation level of FAK (10,30). Therefore, the decrease of FAK might be a result of EphA2 degradation induced by ephrin-A1. The engagement of extrinsic ephrin-A1 with intrinsic EphA2 and the subsequent dephosphorylation of FAK in prostate cancer cells also induced cell morphological change (30) as in gliomas observed in this study. Moreover, the decrease of FAK was established to be necessary for cell morphological transformation of rounded phenotype (32,33). FAK was found to participate in regulating cell proliferation of gliomas as well. For example, astrocytoma cells overexpressing FAK formed larger tumors in nude mice with an increased rate of cell proliferation than tumors derived from the parental cell line (34). Conversely, inhibition of FAK expression inhibited the growth of human carcinoma cells into tumors in nude mice (35). Therefore, ephrin-A1 overexpressed in glioma cells might suppress cell proliferation by EphA2 activation-mediated down-regulation of FAK pathway.

FAK signaling is also an important component of adhesion-independent growth of cancer cells. The overexpression of

FAK in human malignant astrocytoma cells and some other cells increased their adhesion-independent growth in soft agar (34,36). In contrast, the attenuation of FAK expression resulted in a loss of adhesion-independent growth of non-small cell lung cancer cells (37). These studies suggest that endogenous FAK is required for cancer cell viability and adhesion-independent growth and thus the weakened adhesion-independent growth of U251/A1 might just be caused by ephrin-A1-induced FAK reduction.

Furthermore, the ability of tumor cells to migrate is demonstrated to be associated with increased FAK expression. The expression of FAK in human malignant astrocytic tumor cells increased their migration (34). In contrast, inhibiting FAK expression and signaling reduced motility of adenocarcinoma cells (38). The activation of endogenous EphA2 by ephrin-A1 was also found to suppress the activity of FAK and integrin (30), and thus inhibits integrin-mediated cell migration. Our result, herein, proved the decrease of FAK and phosphorylated FAK by overexpression of ephrin A1, which might explain the attenuated migration of U251/A1 as observed by us.

Because ephrin-A1 can inhibit the tumorigenesis in several kinds of carcinomas by activating EphA2, it might be applied as clinical target for drug development in future for cancer therapy. Using mice as a model, Noblitt *et al* demonstrated that introduced ephrin-A1 into breast cancer cells could successfully inhibit the formation of breast tumors following activation of endogenous EphA2 (25). In addition, recent investigation revealed that human adenoviral vectors, which were engineered to express secreted-forms of ephrin-A1, could effectively infect human breast cancer cells and prevent subsequent tumor growth in therapeutic modeling (39). Our results further show the inhibited efficacy of ephrin-A1 on gliomas accompanied by the down-regulation of EphA2 and FAK, which provided supplements for the functions of ephrin-A1 in carcinomas and implied a potential strategy for the therapy of intractable gliomas. However, much deeper studies on the signal cascades of ephrin-A1-EphA2 in the glioma cells are still needed.

Acknowledgements

This work was supported by the National Science Foundation of China (No. 30370690 and 30470847 for D. Xie), Chinese Academy of Sciences (One Hundred Talents Program; grant KSCX-YW-R-73 for D. Xie). China Postdoctoral Science Foundation (Y. Wang), and Chinese Academy of Sciences K.C. Wong Post-doctoral Fellowships (Y. Wang).

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