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Oncogenic DIRAS3 promotes malignant phenotypes of glioma by activating EGFR-AKT signaling

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ABSTRACT

Epidermal growth factor receptor (EGFR)-Akt signaling cascade activation plays a pivotal role in gliomas malignant phenotype, especially in Classical and Mesenchymal subtype gliomas. However, the molecules and mechanisms underlying regulate and maintain the activation of EGFR-AKT signaling remains unclear. Previously reports showed that DIRAS3 inhibits cell proliferation and induces autophagy in ovarian, breast, lung and prostate cancers, which is heterozygosity loss or down-regulated in aforementioned cancers and functionally as a tumor suppressor, whereas the role of DIRAS3 in glioma is still veiled. Here, in this study, we investigated the biological function and role of DIRAS3 in gliomas, and found that DIRAS3 is up-regulated in gliomas and is positively correlated with poor prognosis of glioma patients, meanwhile, over-expressed DIRAS3 promotes glioma cells proliferation and invasion. Further mechanistic study showed that the expression level of DIRAS3 in Classical and Mesenchymal subtype GBMs is higher, and over-expression of DIRAS3 promotes EGFR-AKT signaling activation at the downstream of EGFR and increases AKT phosphorylation, meanwhile suppression of AKT by MK-2206 reverses the tumor promoting function of DIRAS3. Taken together, these findings reveal a novel oncogenic role of DIRAS3 in the development and progression of glioma, which suggest that DIRAS3 could serve as a potential diagnostic marker and a promising therapeutic target of gliomas.

1. Introduction

Glioma, the most common malignant primary brain tumors in adults, represents one of the most aggressive and lethal cancer types [1]. Despite advances in treatments and targeted drugs during past decades, the prognosis of glioma patients remains poor, with the median survival time approximately 12–14 months [1,2]. The extremely poor prognosis of patients with gliomas is largely due to the high tendency of tumor growth and invasiveness, which leads to severe structural and functional damage to the surrounding brain tissue, incomplete surgical resection, and high frequency of tumor recurrence [1–3]. However, the underlying molecular mechanisms of the aggressive malignant phenotype of gliomas remain largely unknown.

Epidermal growth factor receptor (EGFR) amplification and mutations are major drivers promoting glioma tumor growth and invasion through persistent activation of signaling networks [4]. Mechanistically, EGFR drives tumorigenesis primarily through activation of AKT signaling, thereby stimulating cancer cell proliferation, survival and invasiveness [5,6]. However, the mechanisms that regulate EGFR-AKT signaling downstream cascades activity in gliomas are not well known.

GTP-binding protein Di-Ras3 (DIRAS3), a member of the Ras superfamily, is down-regulated in ovarian, breast, lung, prostate cancers and hepatocellular carcinoma. And reexpression of DIRAS3 suppresses tumor growth, induces autophagy and promotes tumor dormancy in aforementioned cancer types [7–15]. Notably, the role of DIRAS3 in gliomas was barely known. Interestingly, we found that DIRAS3 was overexpressed at the mRNA level and protein level in gliomas. And overexpression of DIRAS3 promoted proliferation and invasiveness of gliomas. Moreover, we found that the mRNA levels of DIRAS3 were higher in Classical and Mesenchymal subtypes than that in Proneural subtype gliomas. Furthermore, Western Blot showed that DIRAS3 activates EGFR-AKT signaling at the downstream of EGFR and promotes AKT phosphorylation,

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meanwhile treatment of AKT inhibitor MK-2206 reverses the oncogenic effect of DIRAS3. Importantly, upregulated DIRAS3 level was positively correlated with poor prognosis of glioma patients. These findings, thus, reveal a novel oncogenic role of DIRAS3 in the development and progression of glioma.

2. Materials and methods

2.1. Cell culture

Glioma cell lines, LN382T and SNB19, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. All cell lines were authenticated by short tandem repeat fingerprinting at Hunan Xiangya Judicial Identification Center (Changsha, China). AKT inhibitor MK-2206 (Selleck Chemicals) was used to treat glioma cell lines at the concentration of 5 μM.

2.2. Human tissue samples

A cohort of 8 paired primary glioma tissue and adjacent tissue surgical samples, without radiotherapy or chemotherapy, was obtained from The Second Xiangya Hospital of Central South University. All participants in this study signed an informed consent for samples to be used for research purposes and this study was approved by the Ethics Committee of The Second Xiangya Hospital of Central South University.

2.3. Plasmid transfection

We constructed the lentiviral plasmid to overexpress DIRAS3 by using the pSIN-EF2-Puro plasmid (Addgene), and the empty pSIN-EF2-Puro vector as negative control. The lentiviral particles were produced by co-transfecting the empty vector or DIRAS3 plasmid, with the packaging plasmids psPAX2 and pMG2.G (Addgene), into 293T cells with Lipofectamine 3000 (Invitrogen), described previously [16].

2.4. Western blot analysis

Western blot analysis was performed consistent with our previous work [16]. Cultured cells were lysed with cold RIPA buffer containing protease and phosphatase inhibitors, equal amounts of protein (40μg/cell line) were separated using 12% (w/v) SDS-PAGE, and protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Roche). PVDF membrane was subsequently incubated in PBST with 5% non-fat milk for 1 h at room temperature, and incubated with respective primary antibodies (DIRAS3 antibody, ThermoFisher, PA5-55898; EGFR antibody, Abcam, ab52894; EGFR (phospho Y1068) antibody, Abcam, ab40815; AKT antibody, Abcam, ab8805; AKT (phospho T308) antibody, Abcam, ab38449; AKT (phospho S473) antibody, CST, 9271S; GSK3 beta antibody, Abcam, ab32391; GSK3 beta (phospho S9) antibody, Abcam, ab75814; GAPDH antibody, Abcam, ab8245) overnight at 4 °C. These were subsequently incubated with anti-rabbit secondary antibody (Abcam) for 1 h at 37 °C. Immune complexes were detected using the ECL Western Blotting Kit (Millipore).

2.5. Colony formation assay

Cells were plated in 6-well plates (1×10^3 cells per well) and cultured in DMEM supplemented with 10% FBS for 7 days. The colonies were stained with 1% crystal violet for 5 min after 30 s fixation with 4% formaldehyde.

2.6. Transwell assay

Cells (2×10^4) to be tested were plated on the top side of polycarbonate Transwell filter with Matrigel (Corning) in the upper chamber of the BioCoat Invasion Chambers (BD) and incubated at 37 °C for 22 h, followed by removal of cells inside the upper chamber with cotton swabs. Migrated and invaded cells on the lower membrane surface were fixed in 1% araformaldehyde, stained with hematoxylin, and counted. Cell counts were expressed as the mean number of cells per field of view. Three independent experiments were performed, and the data are presented as mean ± standard deviation.

2.7. MTT assays

Cells were plated in 96-well plates at a density of 1×10^3 cells in 100 μL medium, per well. After incubation for 0 Day, 1 Day, 2 Days, 3 Days, 4 Days, 5 Days, 6 Days, add 50 μL of MTT solution into each well and incubate the plate at 37 °C for 3 h. After incubation, add 150 μL of MTT solvent into each well. Wrap plate in foil and shake on an orbital shaker for 15 min. Occasionally, pipetting of the liquid may be required to fully dissolve the MTT formazan. Read absorbance at OD = 490 nm. Three independent experiments were performed, and the data are presented as mean ± standard deviation.

2.8. Statistical analysis

All experiments were repeated at least three times, and data was expressed as the mean ± SD. Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Prism Inc.), *t*-test was utilized to compare two independent groups, and one-way analysis of variance (ANOVA) for multiple group comparisons. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. DIRAS3 is up-regulated in gliomas and is correlated with poor prognosis of glioma patients

To elucidate the alteration of DIRAS3 expression in various types of cancer, we initially analyzed DIRAS3 mRNA levels in Cancer Cell Line Encyclopedia (CCLE) and found that DIRAS3 levels were relatively lower in ovarian, breast, lung, prostate cancer cell lines as previously reported, however in glioma cell lines, the mRNA level of DIRAS3 was the highest (Fig. 1A). Meanwhile, analyses of DIRAS mRNA levels in The Cancer Genome Atlas Glioblastoma (TCGA GBM) dataset and Rembrandt Glioblastoma dataset showed that DIRAS3 were significantly upregulated in GBM cohort as compared to adjacent normal tissues ($P < 0.01$, respectively; Fig. 1B). Furthermore, Western Blot analysis of 8 paired glioma and adjacent normal tissues also confirmed that DIRAS3 was overexpressed at the protein level in gliomas (Fig. 1C). To further address the potential role of DIRAS3 in gliomas, we analyzed the DIRAS3 expression profiles of glioma patients for whom overall survival (OS) data were available, and the results showed that patients stratified by a median cutoff of DIRAS3 expression with higher DIRAS3 expression (>median) had shorter OS than those expressing lower levels of DIRAS3 in TCGA GBM dataset and Rembrandt Glioblastoma dataset (log-rank test, $P < 0.001$ and $P = 0.003$; Wilcoxon test, $P < 0.001$ and $P = 0.003$, respectively; Fig. 1D). These data indicate that DIRAS3 is upregulated in gliomas and high level of DIRAS3 is associated with poor prognosis of glioma patients.

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