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Original Articles

DRR1 promotes glioblastoma cell invasion and epithelial-mesenchymal transition via regulating AKT activation

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ABSTRACT

Metastatic invasion is the primary cause of treatment failure for GBM. EMT is one of the most important events in the invasion of GBM; therefore, understanding the molecular mechanisms of EMT is crucial for the treatment of GBM. In this study, high expression of DRR1 was identified to correlate with a shorter median overall and relapse-free survival. Loss-of-function assays using shDRR1 weakened the invasive potential of the GBM cell lines through regulation of EMT-markers. The expressions of *p*-AKT were significantly decreased after DRR-depletion in SHG44 and U373 cells. Moreover, the invasion was inhibited by the AKT inhibitor, MK-2206. The expression of Vimentin, N-cadherin, MMP-7, snail and slug was significantly inhibited by MK-2206, while the expression of E-cadherin was upregulated. Our results provide the first evidence that DRR1 is involved in GBM invasion and progression possibly through the induction of EMT activation by phosphorylation of AKT.

Introduction

Glioma, which arises from glial cells, accounts for 80% of all malignant brain tumors [1]. The World Health Organization categorizes gliomas into four grades, of which glioblastoma multiforme (GBM) (grade IV) is the most common malignant primary brain tumor in adults. Although optimal multimodality treatments that typically include surgery, radiation, and cytotoxic chemotherapy are available, recent clinical trials have reported a median survival of only 14–16 months with a 2-year survival rate of about 30% [2–4].

The highly invasive nature of GBM cells that extravasate into surrounding brain parenchyma is a major cause of treatment failure and tumor recurrence [5]. Yet the molecular mechanisms underlying its regulation remain elusive. Therefore, a deeper understanding of the GBM biology, including identification of their cells of origin and relevant molecular events leading to invasion, is of particular clinical importance to develop effective therapies for GBM patients.

Epithelial-mesenchymal transition (EMT) is a central process for normal embryonic development [6]. Recent evidence suggests that EMT is one of the most important molecular events in the invasion of tumors including GBM [7–9]. EMT can be a potential target for inhibiting the GBM invasion [10–12]. Therefore, understanding the molecular mechanism of EMT is crucial for the treatment of GBM.

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Downregulated renal cell carcinoma gene 1 (DRR1) is designated by Tohoku University cDNA clone A on chromosome 3 (TU3A) and also known as FAM107A [13]. DRR1 is considered as a candidate tumor suppressor gene because its expression is decreased in various types of cancer and its increased expression suppresses cancer cell proliferation and induces cell apoptosis [14–16]. DRR1 is expressed in the developing nervous system and downregulated during neuroblastoma carcinogenesis [17]. However, DRR1 was recently found to be highly expressed in the invasive component of GBM and thought to drive tumor invasion [18–20]. Therefore, DRR1 may have a dual function in the regulation of biological function, and thus may be important in the tumorigenesis and invasion of GBM.

In this study, we aimed to illustrate the correlation of DRR1 expression and the clinicopathological characteristics in human GBM and determine whether DRR1 is involved in the EMT of GBM, and furthermore, investigate the mechanism by which it influences EMT in GBM.

Materials and methods

Tissue samples and ethics statement

The study protocol and acquisition of tissue specimens were approved by the Ethical Committee of Shanghai Tenth People's Hospital, Tongji University School of Medicine (2017-Res-10). This study was registered with ClinicalTrials.gov (NCT03005132). Each participant provided written informed consent prior to initiation of study activities. We collected eight frozen GBM samples and paired normal brain tissues at the Shanghai Tenth People's Hospital and 102 paraffin-embedded tissues at the Affiliated Hospital of Jiangnan University from GBM patients undergoing surgical resection and classified according to the most recent WHO classification of central nervous tumors, which was independently confirmed by two experienced pathologists. Patients who died of diseases not directly related to GBM were excluded from this study. Patients' clinical information, such as age, sex, Karnofsky performance score (KPS) [21] and WHO grade was collected and stored in a database. Overall survival was calculated from the date of the initial surgical operation to death. Death of participants was ascertained by reporting from the family and verified by review of public records.

Protein extraction and analysis by nano-LC-MS/MS

Paired eight GBM and normal brain tissues were cut into small pieces (about 1 mm^3) and rinsed in PBS to remove blood. Then tissues were homogenized in 4% SDS and 0.1 M DTT in 0.1 M Tris-HCl, pH7.6 solution on ice, sonicated 10 times (80 w, working 10 s, suspending 15 s), incubated for 5 min at 95 °C. The protein concentrations of clarified lysates were determined using fluorescence assay. A 200 μg of clarified lysates were proteolysed on 10 kDa filters (PALL Life Sciences, USA) using a Filter-Aided Sample Preparation (FASP) method. The peptide samples were then desalted onto a solid-phase extraction cartridge (Empore 7 mm/3 ml). The lyophilized peptide mixture was re-suspended in water with 0.1% formic acid (v/v) and its content was estimated by UV light spectral density at 280 nm, then 3 μg of the digest peptides were analyzed by nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) on LTQ Orbitrap Velos Pro mass spectrometer [22]. Raw data was processed by Maxquant software (1.3) and then used for database and spectral library searching using Andromeda peptide search engines. The Maxquant peptide and protein quantification results files were imported into Perseus software (version 1.5.1.6) for further analysis [23]. All of the MS proteomics data have been deposited to iProX (http://www.iprox.org/index) and can be accessed with the accession IPX00084901.

Bioinformatics analysis

The expression levels of genes were investigated in paired GBM tissue samples based on GEO datasets (GSE45921, GSE51146 and GSE4412) using the NCBI Platform (http://www.ncbi.nlm.nih.gov/) and TCGA datasets (TCGA_GBM_Exp_U133a) from UCSC Genome Browser (https://genome-cancer.ucsc.edu/). Hierarchical clustering was performed using the multiple experiment viewer (MeV) 4.7.1 software (http://www.tm4.org/mev/).

Immunohistochemistry

GBM paraffin sections were cut into 4-μm thick sections, then added onto poly-lysine coated slides and incubated at 65 °C overnight. The incubated slides were then deparaffinized in xylene and rehydrated with graded alcohol. Next, antigens were retrieved using citrate buffer (pH 6.0) and the slides were rinsed in Tris-buffered saline (TBS), and incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. Slides were incubated overnight at 4 °C in monoclonal primary antibody (Novus, Littleton, CO, USA) solution at 1:200 dilution. Finally, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin secondary antibody, and color was developed using the DAB Horseradish Peroxidase Color Development Kit (Maixin Co., Fuzhou, China).

Construction of SHG44 and U373 DRR1-knockdown cells

Human GBM-derived SHG44 and U373 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM media (Invitrogen, Carlsbad, USA) and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell culture was conducted at 37 °C in a humidified 5% CO2 incubator. All the cell lines were confirmed as mycoplasma free by mycoplasma PCR tests. DRR1-knockdown cell lines were generated using short hairpin RNAs and retroviral transduction [24]. The distal C-terminal sequence (GCTCTCTCTCTTCGCCGGCCAATGCGGCA) was used to produce the short hairpin loop. SHG44 and U373 cell lines were cultured in high-glucose DMEM supplemented with 10% FBS and penicillin-streptomycin antibiotic mixture.

RNA extraction and real-time quantitative reverse transcription PCR

According to the manufacturer's guidelines, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA quantity was determined using NanoDrop ND-1000 spectrophotometer and the integrity of RNA was measured by gel electrophoresis. cDNA was synthesized from total RNA (1 μg), and quantitative PCR reactions were performed with the Taqman Universal PCR Kit (Life Technologies). GAPDH was used as the internal control, and the $2^{-\Delta\Delta CT}$ method was used to analyze the expression levels of genes [25].

Western blot analysis

Samples were lysed by incubation in a buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 10 mM NaF, 10 mM Na4P2O7, 0.4 mM Na3VO4, and protease inhibitors) for 30 min on ice. After centrifugation, proteins (30 μg) were separated by 10% polyacrylamide gel electrophoresis containing 0.1% SDS and electrophoretically transferred to nitrocellulose membranes. After blocking with 3% nonfat milk in