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Overexpression of FADD and Caspase-8 inhibits proliferation and promotes apoptosis of human glioblastoma cells

Hong-Bin Wang[a], Tao Li[a], Dong-Zhou Ma[a], Yan-Xin Ji[b], Hua Zhi[c,*]

[a] Department of Neurosurgery, Affiliated Hospital of Hebei University of Engineering, Handan 056029, PR China
[b] Department of Rehabilitation, Xingtai Hospital of Traditional Chinese Medicine, Xingtai 054000, PR China
[c] Department of Cardiology, Affiliated Hospital of Hebei University of Engineering, Handan 056029, PR China

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ABSTRACT

Introduction: The study aimed at exploring the effects involved in Fas-Associated protein with Death Domain (FADD) expression and cysteine-aspartic acid specific protease-8 (Caspase-8) in relation to the proliferation and apoptosis of human glioblastoma (GBM) cells.
Material and methods: 93 GBM tissues and 64 normal brain tissues were the central mediums used for the investigation of the study. Cultured human GBM SC189 cells were divided into separate groups including the blank negative control (NC), FADD and Caspase-8 groups. The mRNA and protein expressions of FADD and Caspase-8 in tissues and human glioblastoma (GBM) cells were detected using qRT-PCR and Western blotting techniques. Cell proliferation was tested by CCK-8. Flow cytometry was used for the measure of cell cycle and apoptosis rates.
Results: The mRNA and protein expressions of FADD and Caspase-8 in GBM tissues were less than the levels of expression displayed in normal brain tissues. Correlations between the expressions of FADD and Caspase-8 in GBM tissues were analyzed as being linked with the clinical grades of GBM patients. Patients in stage III + IV displayed lower expressions of FADD and Caspase-8 than patients in stage I + II. In comparison with the blank group, the FADD and Caspase-8 groups showed decreased proliferation rates of SHG44 cells and lower ratios of cells in the S phase and Bcl-2 expression. Greater ratios of cells in the G0/G1 stage as well as increased cell apoptosis and expressions of Caspase-8 and Bax were exhibited. The expression of FADD in the FADD group was higher than the blank group, however no significant differences in FADD expression was observed between the blank and Caspase-8 groups.
Conclusion: The data obtained during the study demonstrated that overexpression of FADD and Caspase-8 suppresses proliferation whilst promoting the apoptosis of human GBM cells.

1. Introduction

Glioblastoma (GBM) is widely renowned as being the most frequent and destructive malignant tumor of the brain. GBM is characterized by rapid metastasis, high heterogeneity, and diffusive and infiltrative growth in brain parenchyma. The tumor exhibits high rates of recurrence as well as having a poor prognosis [1,2]. Currently methods of treating GBM are limited to mainly surgical resection, radiotherapy, chemotherapy, immunotherapy and molecular targeted therapy [3]. GBM exhibits widespread molecular alterations as well as a highly-distorted epigenome [4]. At present various scientific literature and evidence supports notion held that microRNAs (miRNAs) promote tumor development through interaction with their target genes including GBM. Furthermore, many individual genes (as well as the aforementioned miRNAs) assigned to survival-related modules share a close relationship with the survival of GBM patients [2].

Fas-Associated protein along with Death Domain (FADD), also referred to as MORT1, is encoded by the *FADD* gene on the 11q13.3 region of the 11th chromosome in the human genome [5]. It consists of a C terminal death domain (DD) and an N terminal death effector domain (DED) [6] *FADD* is understood to be a necroptosis-regulating gene that accelerates inflammation. Patients suffering from relapsing remitting multiple sclerosis have displayed higher

Abbreviations: GBM, glioblastoma; NC, negative control; FADD, fas Associated protein with Death Domain; HRP, horse radish peroxidase; OD, optical density; FCM, flow cytometry; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; DR5, death receptor 5; DD, death domain; DISC, death-inducing signaling complex; DED, death effector domain; SDS-PAGE electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author at: Department of Cardiology, Affiliated Hospital of Hebei University of Engineering, No. 81, Congtai Road, Handan 056029, Hebei Province, PR China.
E-mail address: zhihuababy@yeah.net (H. Zhi).

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FADD levels of expression in comparison to healthy individuals [7]. Additionally, mutant cells lacking caspase-8 or FADD are resistant to cell death induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [8]. TRAIL is a cell apoptosis inducing ligand [9]. FADD combined with cysteine-aspartic acid specific protease-8 (Caspase-8) plays a significant role in the transmission of TRAIL-induced apoptosis signals [10]. Caspase-8 is a cysteine-aspartic acid specific protease encoded by the *CASP8* gene, whose sequential activation is essential in the execution-phase of cell apoptosis [11,12]. This study aimed to detect cell apoptosis in patients with GBM and measure the mRNA expression of FADD and Caspase-8 proteins amongst healthy brain tissues, GBM tissues, human cell lines, and human embryonic kidney HEK293 cells. Through various modes of analysis, the ability of FADD and Caspase-8 proteins to influence the proliferation and apoptosis rates in GBM cells was observed.

2. Materials and methods

2.1. Ethics statement

The study was conducted in full accordance with The Ethics Committee of the Institutional Review Board of the Affiliated Hospital of Hebei University of Engineering. Written informed consent documentation was obtained from all participants of the study.

2.2. Sample collection

Between October 2012 and October 2015 93 GBM tissues obtained from GBM patients at the Affiliated Hospital of Hebei University of Engineering. The 93 GBM tissues obtained were selected as the case group. The GBM tissues were confirmed as positive by means of surgery at the Department of Neurosurgery of the aforementioned hospital. All included patients were re-evaluated and again diagnosed, based on the World Health Organization (WHO) classification of tumors of the nervous system (2000) [13]. The control group consisted of 64 patients undergoing internal decompression due to severe craniocerebral injuries, as well as the normal brain tissues around the area of trauma. All patients included in the case group had no history of radiotherapy, chemotherapy or immunotherapy. Additionally, all participants in the study had no evidence of significant necrotic tumor tissues. Following hospitalization, all patients underwent an enhanced MRI examination of the head to verify the existence of GBM tissues. The case group consisted of 43 males and 50 females, with a mean age of 45.5 ± 11.5 years (age: 28–64 years). The control group consisted of 38 males and 26 females, with a mean age of 47.1 ± 8.5 years (age: 31–67 years). Both the GBM and normal tissues were obtained during surgery and immediately stored in liquid nitrogen.

2.3. Cell culture and grouping

The Human GBM cells (SC189, U251, SHG44) and human embryonic kidney cells (HEK293) were purchased from the Cell Center of Shanghai Institute for Biological Sciences. The cells were cultured in a DMEM culture medium (Corning Inc., Corning, NY, USA) containing 10% fetal calf serum (Gibico, Grand Island, NY, USA) in an incubator at 5% CO_2 with a saturated humidity temperature of 37 °C. The culture medium was changed every 3–5 days. The SHG44 cells in the logarithmic phase of growth were transfected and divided into the blank group, negative control (NC) group (transfected with empty plasmids), FADD group (transfected with overexpressed FADD plasmids) and Caspase-8 group (transfected with overexpressed Caspase-8 plasmids). Based on the sequences of *FADD* and *Caspase-8* genes in the Gen Bank, the cDNA primer sequences were set as follows: *FADD*: upstream: 5′-GCTAGCATGGACCCGTTCCTGGTGCTG-3′, downstream: 5′-TCAGGACGCTTCGGAGGT-3′; Caspase-8: upstream, 5′-TCAGTGCCATAGATGATGCCC-3′; downstream: 5′-AAGGGAACTTCAGACACCAGG-3′. The Bam H I and Xho I restriction-enzyme cleavage sites were inserted into the primers of FADD and Caspase-8 plasmids, were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). PCR amplification was conducted and purified FADD was cloned into the pEGFP-N1 vectors for the construction of overexpressed FADD and Caspase-8 plasmids.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from the frozen tissues. Cells in the logarithmic phase of growth were obtained using the Trizol method (Invitrogen, Carlsbad, CA, USA) and subsequently stored at a controlled temperature of −80 °C. The PrimeSeript® RT reagent Kit (Perfect Real Time, Takara Biotechnology Co., Ltd., Dalian, China) was utilized in order to reverse-transcribe the total RNA into cDNA which was then stored at −20 °C and reserved for further use. GAPDH was considered as the internal reference. The ABI 7500 quantitative PCR instrument (ABI, Austen, TX, USA) was used for qRT-PCR. The conditions of qRT-PCR were as follows: pre-denaturation for 5 min at 95 °C, denaturation for 30 s at 90 °C, annealing for 40 s at 60 °C and extension for 40 s at 72 °C, for a total of 40 cycles. Each experiment was conducted three times. Table 1 displays the primer sequences used in the PCR reactions. The $2^{-\triangle\triangle Ct}$ methods were used in order to calculate the relative expression of target gene to GAPDH.

2.5. Western blotting

Protein lysing solution (Beyotime Biotechnology, Shanghai, China) was added to the frozen tissues and cells in logarithmic phase of growth for the extraction of total protein content. The Bradford method (Thermo, USA) was employed to quantify the total protein extracted. The total protein (50 μg) was separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Following this the membrane was blocked using 5% slim milk in a shaking bed for 1 h, and was incubated with rabbit anti human monoclonal antibodies, including FADD, Caspase-8, B-cell lymphoma-2 (Bcl-2), BCL2-Associated X (Bax), GAPDH (Cell Signaling Technology, USA, 1:1000), at 4 °C overnight. The membrane was then washed by phosphate-buffered saline Tween (PBST) 3 times (5 min each time) and incubated for 2 h at 37 °C with horse radish peroxidase (HRP)-labeled goat anti rabbit secondary antibodies (Cell Signaling Technology, USA, 1:4000), and again washed by PBST. Luminol Reagent was mixed with Peroxide Solution (Millipore, USA) at a ratio of 1:1 for the purposes of photography and analysis. The

Table 1
The primer sequences for qRT-PCR.

Gene	Sequence
FADD	F: CCGCCATCCTTCACCAGA
	R: CAATCACTCATCAGC
Caspase-8	F: CCTCATCAATCGGCTGGAC
	R: ATGACCCTGTAGGCAGAAACC
GAPDH	F: AGAGGCAGGGATGTTCTG
	R: GACTCATGACCACAGTCCATGC

Note: qRT-PCR, quantitative real-time polymerase chain reaction; FADD, Fas-Associated protein with Death Domain; Caspase-8, cycstein-containing aspirate-specific protease-8;.