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Original Investigation

14-3-3zeta Positive Cells Show More Tumorigenic Characters in Human Glioblastoma

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ABSTRACT

AIM: Expression of 14-3-3zeta is upregulated in many cancer types and plays an important role in tumorigenesis. Our previous studies have shown that 14-3-3zeta has a positive expression and is associated with a poor prognosis in patients with glioblastoma. In this study, we investigated whether 14-3-3zeta positive cells show more tumorigenic character and stronger chemotherapy resistance.

MATERIAL and METHODS: Six human glioblastoma cells lines were derived from the 6 patients with tumor, and the cells were sorted by 14-3-3zeta expression. The cell viability, invasion, tumorigenic ability and chemotherapy resistance were compared between the 14-3-3 positive and negative expression groups.

RESULTS: 14-3-3zeta positive cells displayed oncogenic properties, more tumorigenic character, high invasiveness, tumorsphere formation ability and resistance to temozolomide chemotherapy treatment.

CONCLUSION: Cells with 14-3-3zeta positive expression show more tumorigenic character and should be administered other treatments in the future.

KEYWORDS: 14-3-3zeta, Glioblastoma, Temozolomide, Tumorigenic

INTRODUCTION

Glioblastoma is a malignancy with a particularly poor prognosis because of its lack of response to current routine therapies. A combination of various treatment modalities, including surgical resection, radio-therapy and chemotherapy, is not sufficient in these cases to obtain ideal the treatment response and survival rate (28). The prospect of glioblastoma treatment is dismal. Therefore, underlying mechanisms of tumor formation should be actively investigated to improve the prognosis of patients with glioblastoma and to develop a more effective pathway.

The 14-3-3 protein family is one of regulatory factors that are involved in many pathways of tumorigenesis procedure, including apoptosis, cell cycle progression and mitogenic signals (9,22,25,29). Our previous study shows that general inhibition of 14-3-3 proteins or suppressing its expression with siRNA trigger apoptosis in glioblastoma cells (3). Moreover, the importance of 14-3-3zeta, one type of 14-3-3 proteins, has been emphasized on the formation and progression of many tumor types (2,5,8,10,14-16,18-21, 24,32). Additionally, we previously found that 14-3-3zeta is upregulated and related with the prognosis of glioblastoma patients (30). Niemantsverdriet et al. reported that 14-3-3zeta has oncogenic properties such as the downregulation of 14-3-3zeta sensitive cells to stress-induced apoptosis and increases apoptosis by eightfold in 14-3-3zeta downregulated cells (23). Similarly, inhibition or downregulation of 14-3-3zeta in many

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types of tumors suppresses tumor growth and enhances chemosensitivity (3,14,15,19). However, whether 14-3-3zeta positive glioblastoma cells show stronger tumor properties remains unclear. Accordingly, we investigated the cell viability, invasion, tumor formation and chemotherapy resistance of 14-3-3zeta positive cells from human glioblastoma tissue.

MATERIAL and METHODS

Patients

Intracranial tissue samples were obtained from 6 patients (male 3, female 3) with primary brain glioblastoma who underwent surgery at Wuhan Command General Hospital in 2014. All tumor tissues were acquired from primary tumor and defined as glioblastoma by the revised World Health Organization criteria for the central nervous system

The collection of tumor tissue from patients was approved by the Ethics Committee of Wuhan Command General Hospital and all patients signed informed written consent before the operating procedure (Wuhan, China).

Tumor cell culture and reagents

Human U251 glioma cells (originally obtained from Uppsala, Sweden) were maintained under conditions recommended by the ATCC. Six human glioblastoma primary cultures were established from the 6 patients with tumor. Briefly, tumor samples were dissociated to form a single cell suspension and plated in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, MO) containing 10% fetal bovine serum, supplemented with non-essential amino acid, 100 U/ml Penicillin/Streptomycin.

Fluorescence-activated cell sorting

Six established human glioblastoma primary cultures were incubated with 14-3-3zeta antibody (Santa Cruz, CA). After extensive washing, these cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, CA). These cells were trypsinized and washed with ice-cold FACS buffer. The cell suspension was then centrifuged, resuspended, and filtered through a 40um cell strainer to remove any clumping cells. The cells were sorted using a cell sorter (FACS, BD Bioscience, Belgium) on the basis of 14-3-3zeta expression and single cell viability. The sorted cells were confirmed under the fluorescence microscope.

Cell viability assays

Cell viability was evaluated with Cell Titer 96 Aqueous Assay (Promega, WI). Cells (2×10^4 cells/well) in the wells of a 96-well plate were pre-incubated with glucose for 48 h. Next, 100 µl of MTT solution (Sigma-Aldrich Co., MO) was added to each well of the 96-well culture plate and incubated for 4 h at 37°C. The absorbance was analyzed at 490 nm using a microplate reader. The experiments were performed at least 3 times.

Cell invasion assay

QCM Collagen-based Cell Invasion Assay was conducted following the manufacturer's procedures (Chemicon, Millipore). 1×10^5 cells were seeded into the upper insert in serum-free medium. Outer wells were filled with DMEM containing 10% FBS. All cells were then incubated for 48 hours. These cells migrating to the lower surface of the membrane were stained and quantified by colorimetric measurement at 560 nm (Promega, CA). All experiments were performed in triplicate.

In vivo analyses

Male BALB/c nude mice aged six weeks were obtained from the Center of Laboratory Animal, Fourth Military Medical University, Xi'an, China. 14-3-3zeta + and - cells (5×10^5 cells) were subcutaneously injected after sorting. All animal experiments complied with the international guidelines for the care and treatment of laboratory animals. Tumor growth was measured with a caliper every week from the first week to the sixth week. Tumor volumes in mice were measured with a slide caliper and recorded using the formula: volume = $a \times b^2/2$, where a indicates the larger and b indicates the smaller of the two dimensions, respectively.

Bioluminescence assay

Bioluminescence assay experiments were performed according to previously described method (13). Briefly, after culturing pre-made spheroids for two days, various concentrations of TMZ were added to each chamber with a total volume of 100 ul medium. After several-days incubation of the spheroid with TMZ, the luciferin substrate was added and luciferase activity was then measured using a PerkinElmer Victor 3 Multilabel plate reader (PerkinElmer, CA). All experiments were performed more than 3 times.

RESULTS

14-3-3zeta+ cells show high cell viability

We explored whether 14-3-3zeta + and - expression would affect the characteristics of cell viability in human glioma. MTS assay were performed to evaluate cell viability. The viability of 14-3-3+ cells was higher than 14-3-3zeta- cells. These results suggest that 14-3-3zeta+ cells have higher viability in vitro (Figure 1).

14-3-3zeta+ subpopulation displays invasiveness

To investigate cell invasion of 14-3-3zeta sorted subpopulations, a collagen-based cell invasion assay was performed in vitro. The results showed that the 14-3-3zeta+ purified population was significantly more invasive than the 14-3-3zeta- control fraction (absorbance unit (AU) =0.62 and 0.35, respectively, (Figure 2).

14-3-3zeta+ cells are more tumorigenic in vivo

Various human glioma cell fractions were injected into mice at different concentrations to assess the vivo tumorigenicity of 14-3-3zeta+ cells. BALB/c Nude mice were injected subcutaneously with 14-3-3zeta- or 14-3-3zeta+ tumor cells. Tumor development was more efficient for 14-3-3zeta+ purified cells than for the other cell fractions (Figure 3A,B). Only 6×10^5 14-3-3zeta+ cells were shown to be able to expand and produce a complete tumor bulk after one week (2/6), while it

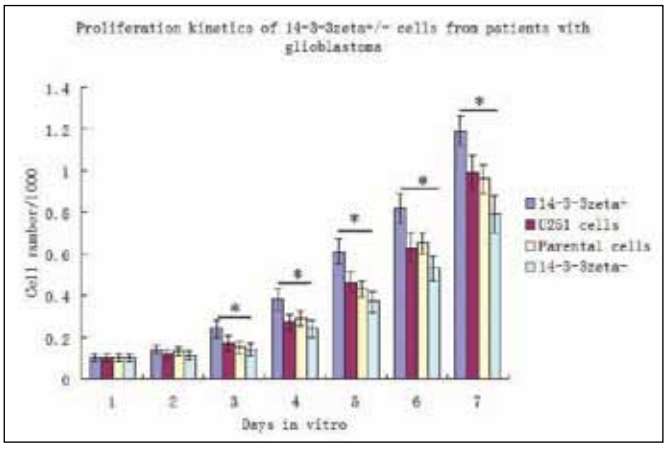

Figure 1: Cell viability measured by the MTS assay. U251 similar growth rate to parental cells, and 14-3-3zeta+ cells exhibited stronger growth rate. * represents higher 14-3-3zeta+ cell counts than 14-3-3zeta-, U251 cells and parental cells in the same cultured days. The results represent the mean±s.d. of three independent experiments (*P<0.05).

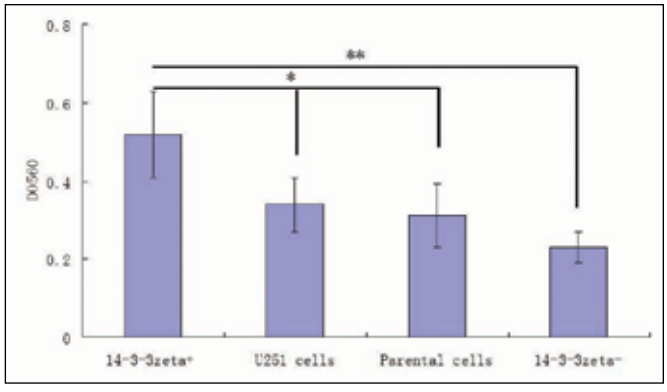

Figure 2: Invasiveness of different cell fractions measured using a collagen-based invasion kit (relative DO measured at 560nm). * indicates that 14-3-3zeta+ cell has more invasion ability than U251 cell, 14-3-3zeta- and parental cells. The results represent the mean±s.d. of three independent experiments (*P<0.05, **P<0.01).

takes more than two weeks to observe formation of a single tumor bulk from 2×10^6 14-3-3zeta- purified cells (1/6).

It also was observed that 50% of the mice inoculated with 14-3-3zeta+ purified cells had bone invasion of tumor cells, however, in the 14-3-3zeta- group, no mice had bone invasion of tumor cells.

14-3-3zeta+ cells attenuate sensitivity to TMZ in glioma cells

We then tested the response of tumor cell to TMZ in the presence or absence of 14-3-3zeta+ cells. Bioluminescence based quantification demonstrated that 14-3-3zeta+ cells attenuated the response of glioma cells to TMZ at the concentration of 20-300 uM (Figure 4).

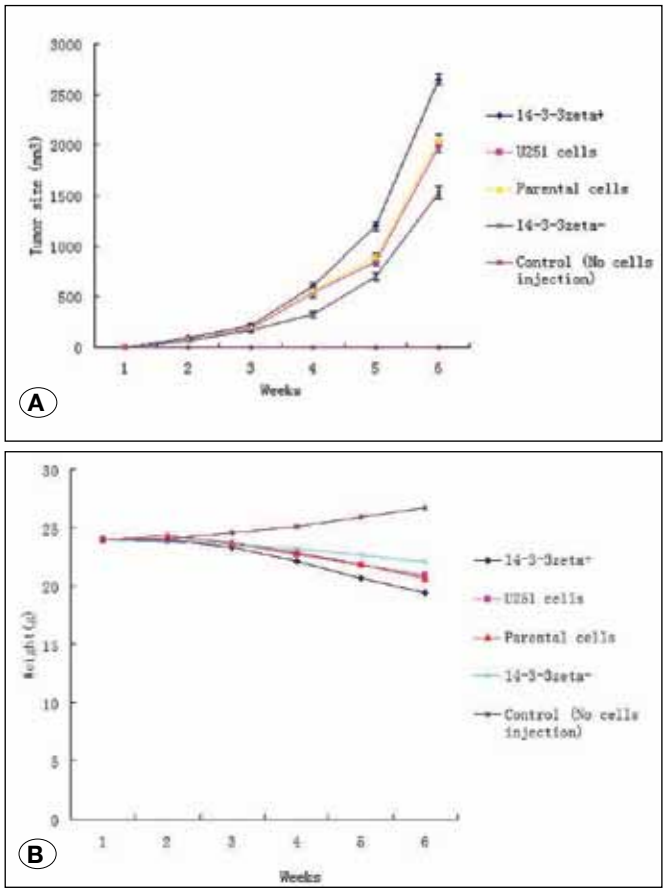

Figure 3: 14-3-3zeta+ cells are more tumorigenic in vivo than 14-3-3zeta- cells. **A)** Evaluation of mouse weight after cell inoculation. **B)** Tumor size evaluation after inoculations of parental cell, U251 cell, 14-3-3zeta+ and – cells..]

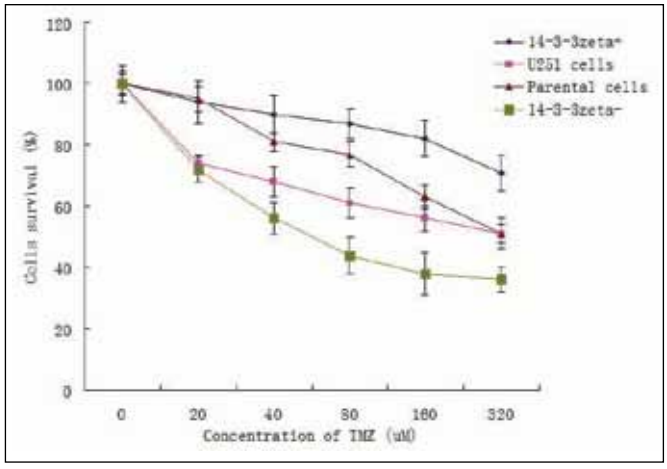

Figure 4: Chemotherapy sensitivity of TMZ to 14-3-3zeta+/- cells from glioblastoma tissue. 14-3-3zeta+ cells show stronger TMZ-resistance, especially with the high drug concentration.

DISCUSSION

Tumor tissues have been found to contain heterogeneous cell populations (1). They should be treated differently by special molecular status for a good outcome, such as HER-2 positive expression in human breast cancer with Herceptin chemotherapy (27) and MGMT inactivation in human glioblastoma with TMZ therapy (11). 14-3-3zeta expression is upregulated in various types of malignancies, including prostate cancer, breast cancer, lung carcinoma and hepatocellular carcinoma (15,17,18,20). A high cell percentage of 14-3-3zeta positive expression was found in these tumor tissues, especially in human glioblastoma tissue (30). Therefore, to investigate whether 14-3-3zeta+ cells show more tumorigenic characters than 14-3-3zeta- cells is very important for the next 14-3-3zeta-targeting therapy. Moreover, 14-3-3zeta has positive expression in cancer stem cells and regulates neurogenesis and differentiation of neuronal progenitor cells. Its expression levels in CD133+ cell were more upregulated than CD133- cells (14). It seems that 14-3-3zeta+ cells represent more tumor cells, including cancer stem cells, and especially cancer stem cells with CD133+ expression.

In this study, we confirmed that glioblastoma bulks consisted of a heterogeneous population in which two clear cell subpopulations were maintained by 14-3-3zeta expression. 14-3-3zeta+ cells showed higher cell viability, stronger invasion, chemotherapy resistance with TMZ, more tumorigenesis compared with 14-3-3zeta- cells. This observation was also consistent with the previous studies showing that silencing of 14-3-3zeta increases Bax activation (15,23). Why were 14-3-3zeta+ cells more tumorigenic than 14-3-3zeta- cells? There are some possible potential molecular pathways. A previous study shows that 14-3-3zeta affects many tumorigenic pathways and factors, including ErbB2, miR221, Hsp27, Wnt, STAT3 and PI3K pathways, during tumor onset, cell proliferation, progression and malignant transformation (7,13, 17,18,22,26,31,32). Knockdown of 14-3-3zeta enhanced chemosensitivity and radiosensitivity in several types of tumor, including CD133+ cancer stem cells (6,12,14). These results shows that 14-3-3zeta participates in malignant transformation of various tumor types. 14-3-3zeta is upregulated and related with the prognosis of glioblastoma patients (30). Additionally, in human brain glioma, the relationship of 14-3-3zeta, HIF-1a and VEGF has been established (4). In our study, 14-3-3zeta+ cells show stronger invasion and tumorigenesis abilities in vitro and in vivo. These results suggest 14-3-3zeta+ cells give rise to more tumorigenicity and other treatment should be used for these cells.

CONCLUSION

Increasing evidence as well as our data indicates that 14-3-3zeta overexpression is significantly associated with disease initiation and progression, recurrence and resistance to chemotherapy in cancer patients. 14-3-3zeta+ cells from glioblastoma bulks have stronger survival, growth, invasion and chemotherapy resistance ability. Treatment targeting 14-3-3zeta+ cells may be more accurate and effective in future studies.

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