Expression of FAP-1 Correlates with Glioblastoma Proliferation, Migration and Treatment Resistance

Bo Li  
Jining Medical University

Yang Liu  
Xinjiang Medical University Affiliated First Hospital  
https://orcid.org/0000-0002-4426-4273

Shaya Mahati  
Xinjiang Medical University Affiliated First Hospital

Hu Qin  
Xinjiang Medical University Affiliated First Hospital

Aisha Maimaitili  
Xinjiang Medical University Affiliated First Hospital

Zengliang Wang (✉ wzl3ng@126.com)  
Department of Neurosurgery, The First Affiliated Hospital of Xinjiang Medical University (XJMU)  
https://orcid.org/0000-0002-8411-5678

Yongxin Wang  
Xinjiang Medical University Affiliated First Hospital

Research

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Abstract

**Background:** Glioblastoma (GBM) is the most severe type of brain cancer, with an extremely high mortality rate and the average survival time less than 15 months. Almost in all GBM cases, the residual tumor cells continue to divide uncontrollably, leading to tumor re-establishment, i.e., tumor recurrence, and ultimately death. Therefore, there is an urgent need to identify critical mediators of GBM progression and resistance to therapy. Fas-associated Phosphatase-1 (FAP-1, also known as PTPN13 or PTPL1) is a member of a large protein tyrosine phosphatase family. It has been shown that FAP-1 expression is downregulated in many types of cancers, which leads to unabated signaling and tumor progression and metastasis. The aim of this study is to evaluate the role of FAP-1 in GBM progression and resistance to therapy.

**Methods:** Cell viability assays, radiotherapy and temozolomide response assays, and Western blot were used to analysis of FAP-1 in GBM based on Oncomine.

**Results:** Our results showed that knockdown of FAP-1 enhanced viability and migration of GBM cell lines, and importantly, increased GBM cell resistance to radiotherapy and temozolomide.

**Conclusions:** FAP-1 is important for GBM cells, and may account for GBM resistance to therapy.

**Background**

It is estimated that approximately 2000 Australians are diagnosed with brain cancer and about 1500 Australians die from it in a year [1-3]. As the most serious form of brain cancer, Glioblastoma (GBM) is extremely lethal, with the average survival time less than 15 months [3]. Surgery is the most common method to remove the tumor, but residual tumor cells continue to divide uncontrollably in most cases, resulting in tumor recurrence and eventually patient death. Current treatment after surgery commonly includes the use of radiotherapy and chemotherapy drugs, such as temozolomide. However, these drugs and other anti-cancer agents have been proven to be ineffective in improving patient survival. Therefore, it is necessary to seek mediators that contribute to GBM progression and resistance to therapy. Our group focuses on two groups of proteins (pro-invasive proteins and phosphatases) that may play pivotal roles in promoting GBM progression and resistance to current treatment, and explores therapeutic agents that will prolong the survival time of patients with GBM. In this paper, experiments were carried out on one member of each group of proteins, namely the pro-invasive protein and Fas-associated Phosphatase-1 (FAP-1). FAP-1 (also known as PTPN13 or PTPL1) is a member of a large protein tyrosine phosphatase family, which plays a role in regulating cell signaling [4-9]. FAP-1 reduces signal transduction and gene expression through Fas and other signaling molecules [10-13]. Importantly, it has been demonstrated that FAP-1 expression is downregulated in many types of cancers, which gives rise to unabated signaling and tumor progression and metastasis [14-16]. Furthermore, very little has been established regarding the role of FAP-1 in GBM progression, recurrence and resistance to therapy. Therefore, this project aims at evaluating the role of FAP-1 in GBM progression and resistance to therapy.
Materials And Methods

Antibodies and Reagents

The polyclonal antibody directed against FAP-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-mouse Actin antibody was purchased from Sigma (St Louis, MO). Temozolomide was purchased from Roche. FAP-1 siRNA and control siRNA were purchased from Santa Cruz Biotechnology.

Cells and Cell Culture

U87MG and LN18 cells purchased from ATCC were maintained in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (FBS) (DKSH, Hallam, Victoria, Australia), 2mM glutamine, 100U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Frederick, MD), and incubated in a humidified atmosphere containing 10% CO₂ at 37°C.

SiRNA Knockdown

To assess the effects of FAP-1 knockdown, FAP-1 siRNA or control siRNA was transiently transfected into cells using metafectene™, and then the cells were seeded into appropriate tissue culture plates.

Cell Viability Assays. Cells were plated in 96-well plates and allowed to adhere overnight. Triplicate wells were treated with varying concentrations of temozolomide with or without radiation for 3-7 days. Cells were then lysed and cell viability was determined using a commercially available Cell Titer-Glo kit (Promega) following the manufacturer’s instructions. Samples were read on a bioluminometer.

Western Blot Analysis

Cells were lysed with lysis buffer (containing 50mM Tris (pH 7.4), 150mM NaCl, 1% Triton-X-100, 50mM NaF, 2mM MgCl₂, 1mM Na₃VO₄ and protease inhibitor cocktail (Roche)) and clarified by centrifugation (13,000g for 15 min at 4°C). Proteins were then separated by SDS-PAGE (Invitrogen), blotted onto nitrocellulose and probed with the indicated primary antibodies. The signal was visualized using the ECL chemiluminescence detection kit (GE Healthcare, Rydelmere, N.S.W., and Australia) following incubation with appropriate secondary antibodies.

Migration Assays

Migration experiments with FAP-1 siRNA knockdown were carried out using xCELLigence RTCA DP instrument (Roche Diagnostics GmbH, Germany) as per the manufacturer’s instructions. Briefly, cells were
transfected with FAP-1 and control siRNA and allowed to adhere onto tissue culture plates for 2 days before they were classified and seeded into CIM16 plates for the migration assay. Migration was analyzed after 2-3 days of incubation in CIM16 plates.

Data mining using Oncomine. Gene expression of 16 phosphatases was analyzed using Oncomine 4.4.4.3 (www.oncomine.org, Compendia BioscienceTM, Ann Arbor, MI, USA, part of Life Technologies), an online tool that acquires 715 mRNA and copy number expression datasets from 86,733 cancer and normal tissue samples. These datasets are compiled from publically available cancer microarray data, which are processed according to the same criteria before being made available. We used the Oncomine compendium to study the expression profiles of 16 different phosphatases in brain cancer tissue versus their normal tissue counterparts by subjecting each dataset to threshold criteria for analysis. A p-value<0.05 and an mRNA expression fold change >1.4 were taken as the initial threshold criteria in this study. The fold change is classified as the difference between mRNA expression levels of genes, especially those of interest, in the cancer tissue and in the normal tissue. Based on the threshold criteria, Oncomine will then assign a gene rank percentile for each gene studied in a dataset. This figure is the percentage rank of your gene of interest calculated by comparing its p-value with the p-values of all the other genes within the same dataset. The gene expression data generated through Oncomine were log transformed and standard deviation was normalized to each array studied.

Statistical Analysis

Graph and statistical analysis was performed using Graph Pad Prism 6 (Graph Pad, San Diego, CA) or EXCEL. Statistical significance was determined by using ANOVA or Students’ T-tests.

Results

Oncomine Data Mining of FAP-1 in GBM

The focus of this study was to determine whether the mRNA expression of FAP-1 could predict the outcome of GBM patient through the public database Oncomine.

MRNA Expression of FAP-1 in GBM

The mRNA expression of FAP-1 in a number of cancer types was evaluated and compared with that in normal tissue (Fig. 1A). Six out of 9 studies showed that FAP-1 mRNA expression was upregulated in GBM cells, compared with normal brain cells (Fig. 1B-G). No difference was observed in FAP-1 mRNA expression in 3 studies (Fig 1H-J). The results suggested that FAP-1 expression might correlate with tumor development and progression, but it’s not conclusive.
Cell Viability after FAP-1 Knockdown

As FAP-1 over-expression might enhance tumor development, whether FAP-1 siRNA knockdown reduced cell viability was determined subsequently. Oncomine results showed that the high expression of FAP-1 might contribute to the progress of GBM. Cell activity detection is one of the most important experiments to measure the cell state, which can preliminarily determine whether FAP-1 silencing affects GBM cell activity. LN18 and U87MG cells were transfected with different concentrations of siRNA and cultured in a cell culture chamber for 7 days. Then, cells were measured with Cell Titer-Glo. The control group (0nm) had a relative survival rate. Statistical analysis was performed using ANOVA Minitab. The results in the figure are mean ±SEM (* P <0.02, ** P <0.01, *** P <0.001, **** P <0.0001). NS means no statistical significance. It was found that knockdown of FAP-1 in LN18 and U87MG cells enhanced cell viability (Fig. 2). However, ANOVA analysis revealed that only the transfection concentration of 6.25nm could achieve statistically significant results (p<0.001) (Fig. 2). The above findings suggested that FAP-1 knockdown could improve the activity of GBM cells in vitro in a concentration-independent manner. Repeated experiments failed to achieve the desired effect, which might be attributed to the activation of other related signaling pathways.

Migration of GBM after FAP-1 Knockdown

The xCELLigence system could accurately measure and quantitate cell migration through real-time impedance analysis. Compared with alternate migration assays, it was non-invasive and analyzed cells in their natural state, capable of measuring the dynamic kinetics of cell migration throughout the entire experiment process. A total of 5000 U87MG cells and 40000 LN18 cells in each well were used. 70h was chosen as the end-point to prevent proliferation from affecting cell measurements. After FAP-1 knockdown, the cell migration activity in U87MG cells declined substantially, but the difference of decrease in the cell migration activity between U87MG and LN18 cells was not significant. At the 70 h, compared with the control group, the migration rates of LN18 and U87MG cells in the knockdown group were approximately 33% and 42% respectively higher than that in the control group (Figures 3A and 3B) (both P<0.001). It indicated that FAP-1 might regulate migration through inhibitory pathways.

Resistance to Radiotherapy and Temozolomide after FAP-1 Knockdown

In order to detect whether knockdown of FAP-1 can enhance the treatment resistance of GBM cells, LN18/U87MG cells were divided into different groups. One group was treated with different concentrations of temozolomide (0, 125µM, 250µM, 500µM), while the other groups were transfected with different concentrations of siRNA (0, 6.25nM, 12.5nM, 25nM). All groups were then treated with radiotherapy at different doses (0, 1.25Gy, 2.5 Gy), and cell activity (cell viability) was tested. The results were analyzed by one-way ANOVA. It was found that the cell viability of the radiotherapy group and
temozolomide group was significantly different from that of the transfection group in the LN18 group (P=0.002), while the difference of the cell viability was not significant between the radiotherapy group and the temozolomide group. In the U87MG group, the cell viability of the temozolomide group was significantly different from that of the transfection group and the radiotherapy group (P<0.001), and significant differences were also observed between the radiotherapy group and the transfection group, as well as between the temozolomide group and the radiotherapy group (P<0.001). Importantly, our results also showed that FAP-1 reduction led to increased resistance of both LN18 and U87MG cells to radiotherapy and temozolomide (Fig. 4), although FAP-1 might depend on the expression of non-essential genes.

Discussion

GBM is the most malignant form of all brain and central nervous system tumors with an overall survival of less than 15 months after diagnosis [17]. Due to the high aggressiveness and invasiveness, it is impossible to completely resect GBM, invariably leading to tumor recurrence and patient mortality. Receptor tyrosine kinases and downstream signaling substrates have been studied extensively in GBM progression and resistance to therapy. However, a less studied, yet equally important super-family of proteins are the ever growing sub-families of phosphatases that regulate these pro-oncogenic kinases [18-20]. Several phosphatases have been shown to be involved in GBM progression. However, the analysis of mRNA expression in a large set of phosphatases has not been performed extensively.

In this paper, the publicly accessible database, Oncomine, was used for analysis. We evaluated the mRNA expression levels of 16 well-studied phosphatases and found that the mRNA expression of only 2 phosphatases (CDC25B and PTPRN13) were enhanced in GBM tumor tissue, compared with that in the control brain tissue. Interestingly, another 7 phosphatases were inconclusive, while their expression clearly did not show any difference between GBM cells and normal brain cells. Strikingly, according to our data, all studies examined displayed that phosphatase expression in GBM tissue was either not different from or significantly enhanced than that in normal tissue. No study examined in our Oncomine searching reported a decrease in mRNA expression levels. This is somewhat surprising as most phosphatases are established to de-phosphorylate kinases, many of which can promote tumor progression.

Our data therefore suggested a few scenarios or discussion points different from the current dogma that phosphatase expression inhibited tumor growth and progression. Firstly, the phosphatase expression may not be able to greatly reduce kinase activity so that tumor growth can be suppressed. Secondly, other kinases may continue to promote tumor growth despite the “shutdown” of kinases by the specific phosphatases we have evaluated, and therefore tumor growth is unabated. Finally, mRNA expression may not be directly related to protein expression, and post-transcriptional modification results in no significant change in phosphatase protein expression. This perhaps explains why our siRNA knockdown results were contrary to what we expected with Oncomine data.
The results showed that knockdown of FAP-1 in both LN18 and U87MG GBM cells led to enhanced migration, indicating that FAP-1 might play a role in inhibiting migration. However, according to the extensive Oncomine data analysis, the over-expression of FAP-1 mRNA seemed to favor GBM development rather than prevent tumor growth as we initially expected, suggesting that despite increased mRNA expression, a translation into over-expression of proteins might not have taken place. The knockdown experiments were done in merely one cell line, but conclusive inferences could be only made after numerous cell lines and primary cell lines were experimented. In addition, whether FAP-1 played a role in cell viability of enhanced invasion, a common clinical feature of GBM, was yet to be determined.

In vitro experiments found that FAP-1 knockout could improve GBM cell activity and enhance the migration of GBM cells. Temozolomide and radiation experiments confirmed that FAP-1 reduction led to resistance of GBM cells to radiotherapy and temozolomide.

To our knowledge, this experiment analyzed a wide range of GBM-associated protein phosphatases using the Oncomine database for the first time in the world. It provided an important basis for future big data analysis on the relationship between the gene and tumor progression [21-23]. Our results indicated that the knockdown of FAP-1 could enhance cell viability and migration of GBM cell lines, and more importantly, increase the resistance of GBM cells to radiotherapy and temozolomide. This study provided a new perspective for progression and postoperative treatment mechanism of GBM. FAP-1 might be a new biological target. In addition, in order to clarify the relationship between GBM and FAP-1, more complete studies that considered more measurement results of GBM tumor cells, assessed the relevance between recurrent GBM tissue survival and FAP-1 expression should be carried out.

Nonetheless, we have performed an extensive analysis on the correlation between GBM formation and the mRNA expression of 16 different phosphatases. To the best of our knowledge, this extensive analysis has not been done for all these phosphatases in the GBM setting. Our data have represented an ideal starting point for assessment of the true value of these phosphatases in blocking (or enhancing) GBM growth and development.

Declarations

Ethics approval and consent to participate:

Written informed consents were obtained from all patients; and, this study conformed to the principles of the Declaration of Helsinki was approved by the investigational review board of the First Affiliated Hospital of Xinjiang Medical University.

Consent for publication:

Not applicable.
**Availability of data and material**

Please contact corresponding author for data requests.

**Conflicts of interest**

The authors declare that they have no competing interests.

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**Authors' contributions**

ZW has full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: ZW, BL, YW, AM, HQ, SM, YL; acquisition of data: ZW, BL, YL, SM; analysis and interpretation of data: BL, YL, AM, HQ, YW; drafting of the manuscript: YL, BL, SM; critical revision of the manuscript for important intellectual content: YL, HQ, AM; administrative, technical, or material support: ZW, BL, YW, AM, HQ, SM; study supervision: YW. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

A: The mRNA expression of PTPN13/FAP-1 in a number of cancer types and in normal tissue. B-G: Six out of 9 studies showed enhanced PTPN13/FAP-1 mRNA expression. H-J: Three studies showed no difference of PTPN13/FAP-1 mRNA expression.
SiRNA knockdown is successful in each of LN18/ U87MG cell lines; with the increase of the knockdown level, we observed an enhanced trend in cell viability for all cell lines (P<0.001). Cell viability was measured using Cell Titer-Glo and represented as the value relative to that of the control group (0nM). Statistical analysis was performed in minitab using one way ANOVA. Graphs represent mean ± SEM * P<0.02, ** P<0.01, *** P<0.001 and **** P<0.0001. NS represents no statistical significance.
Real-time analysis showed that FAP-1 regulated migration. LN18 and U87MG cells were seeded into the serum-free media in the upper chamber of the CIM-16 plate at a density of 40,000 and 5000 cells/well, respectively. 10% FBS serum acted as the chemo-attractant in the bottom chamber. Migration was measured after 70h incubation and data were expressed as values relative to those of the control group. Statistical analysis was performed in minitab using linear regression models. FAP-1 knockdown was significant P<0.001 for all datasets. Results showed that cell migration was enhanced in LN18 and U87MG after FAP-1 siRNA knockdown (green: control, blue: FAP-1 siRNA 12.5nM)
Figure 4

FAP-1 reduction led to increased resistance to radiotherapy and temozolomide. LN18 and U87MG cells were seeded at a density of 250 cells/well in 96-well plates. 2 days later, cells were irradiated and treated with different concentrations of temozolomide. Cell viability was measured 3 days later. Statistical significance was assessed using minitab ANOVA analysis for interactions between variables. In LN18 cells: All variables were independent predictors of cell viability P<0.001. A significant interaction was present in irradiation, temozolomide and siRNA (P=0.002). In U87MG cells: irradiation, siRNA and temozolomide states were all significant P<0.001.