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Research Article
To Explore the Mechanism and Experimental Verification of Brucine Inhibition of Glioblastoma Based on Network Pharmacology

[1]Shaoyan Xu, [2]Hairong Jiang and [3]Peng Zhang

[1]Department of Pathology, Zibo Municipal Hospital, 255400 Zibo, Shandong, China
[2]Education Office, The Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine, Beijing, China
[3]Neurology Ward, Zibo Municipal Hospital, 255400 Zibo, Shandong, China

Abstract

Background and Objective: Glioblastoma (GBM) is one of the deadliest cancers. However, due to the unclear pathogenesis of GBM, many drugs cannot be effective. Therefore, it is of great significance to develop new safe and effective therapeutic drugs and explore the regulatory mechanism of GBM. Brucine can inhibit tumor proliferation, but the mechanism of brucine on GBM has not been scientifically explained at the cellular and molecular levels. In this study, the inhibitory mechanism of Brucine on GBM was explored and verified by experiments. **Materials and Methods:** The common targets of Brucine and GBM are determined by constructing the Venn diagram of intersecting targets. Common targets were visualized through String databases and protein interaction networks (PPI) were constructed. The Hub genes were screened using Cytoscape 3.9.1 and gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were analyzed using DAVID online database. The inhibitory effect of Brucine on GBM U251 cells was observed *in vitro*. **Results:** Brucine and GBM had 40 intersection targets and the top 5 clustering networks were selected as the Hub targets, successively EGFR, AKT1, MTOR, HSP90AA1 and JUN. The targets mainly involve focal adhesion, cancer-related pathways, the main signaling pathways PI3K-Akt signaling pathway and VEGF signaling pathway. Brucine inhibited the proliferation of GBMU251 cells in a time- and dose-dependent manner. **Conclusion:** Brucine showed multi-target and multi-pathway inhibition on GBM and played an active pharmacological role in *in vitro* experiments, which can provide references for studying the mechanism of Brucine in GBM.

Key words: Brucine, Glioblastoma, network pharmacology, signal path, common target, kyoto encyclopedia of genes and genomes analysis, experimental verification

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Corresponding Author: Peng Zhang, Neurology Ward, Zibo Municipal Hospital, 139 Huan Gong Road, Linzi, 255400 Zibo, Shandong, China

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glioma is a primary tumor that mainly occurs in the central nervous system and is one of the most difficult cancers to cure in the world. The origin of glioma is the cells that themselves make up the brain[1-3]. Gliomas account for about 50% of all primary intracranial tumors and nearly 100,000 people are diagnosed with gliomas worldwide every year[4,5]. According to the characteristics of gliomas, different glial cell profiles (astrocytes, oligodendrocytes and ependymal cells) are distinguished and classified according to their similarities in immunohistochemistry and microscopic observation. According to the classification method of WHO, brain gliomas can be divided into four grades, Grades I~II belongs to Low-grade gliomas. LGGs), grade to belong to High grade gliomas (HGGs)[6]. The HGGs has a poor prognosis. The median overall survival (OS) of grade III is about 3 years, while the median OS of grade IV Glioblastoma, especially Glioblastoma (GBM), is about 15 months and the 5-year survival rate is less than 5%[7,8]. Since GBM has almost no boundary with normal brain tissue, it is difficult to completely remove it by surgery alone, which leads to a high probability of postoperative recurrence of patients. With current treatment methods, GBM is almost incurable and according to relevant statistics, the average life cycle of people with GBM is only about 14 months[9,10]. At present, the clinically known first-line drug for the treatment of glioma is Temozolomide (TMZ), but patients are prone to drug resistance[11]. Although surgery, radiation therapy and chemotherapy are used to treat Glioblastoma patients at the same time, they are still unable to cure patients or prolong their survival. Due to the existence of multiple subclonal populations and the high cell heterogeneity of GBM, the prognosis of glioma patients is poor and recurrence is inevitable. Therefore, it is essential to find and develop new effective chemotherapy drugs for the treatment of GBM.

In recent years, natural products have played an important role in the field of cancer research. Brucine, also known as Brucine, Strychnine, Dafangba, Kuushi, etc., is a weakly alkaline indole alkaloid extracted from the traditional Chinese medicine plant Brucine, which is a kind of labor pain medicine used in Chinese medicine[12]. Recent studies have shown that this drug can produce anti-tumor effects on breast cancer, bone marrow tumors, etc., but its anti-tumor mechanism is not clear[13]. Other studies have shown that Brucine is a high-affinity ligand of G-quadruplex structure in the promoter region of c-Myb gene, which can stabilize the G-quadruplex structure and it is speculated that Brucine may play an anti-GBM role by inhibiting the expression of proto-oncogene c-Myb[14]. It has been reported that Brucine can inhibit the survival rate of U251 glioma cells and reduce the growth of xenograft tumors. However, no studies have been conducted to scientifically explain the mechanism of glioma cell death induced by this formula at the cellular and molecular levels. Network pharmacology is a systematic research method integrating bioinformatics, network biology, traditional pharmacology and other disciplines, which can reflect the relationship between drug composition and multi-target action and is an ideal prediction method before drug experiments. It provides a new idea for the research of the traditional Chinese medicine system and provides new scientific and technological support for clinical rational drug use and new drug development. Its research philosophy coincides with the holism of traditional Chinese medicine and has been widely applied to the research on the mechanism of action of ethnic medicine[15]. This study intends to use network pharmacology to analyze the potential mechanism of Brucine inhibiting GBM and preliminarily verify the predicted results of network pharmacology combined with cell experiments, to provide target components and targets for further research on specific mechanisms.

MATERIALS AND METHODS

Study area: The study was conducted in Zibo Municipal Hospital from April 2022 to May 2023.

Database website and software: Pubchem number according to the library[16]; Traditional Chinese medicine database system pharmacology and analysis platform (TCMSP,)[17]; Swiss number according to the library[18]; GeneCards database platform[19]; DAVID online database[20]; Online bioinformatics analysis platform[21].

Obtaining potential targets: Potential targets for related compounds not found in TCMSP can be supplemented by the Swiss database. Firstly, we searched the biological activity data of Brucine in Pubchem with the search term "Brucine" to obtain its 2D structure, SMILES number, etc. In the Swiss data, we selected the species "Homo sapiens". Input the SMILES number of strychnine retrieved from Puchem to obtain a potential target for the compound.

GBM target acquisition: In GeneCards database, "Glioblastoma" was used as the search term, GBM disease-related genes were obtained through screening relevance scores and the proteins encoded by their related genes were used as potential targets for drug therapy.

Acquisition of disease and drug intersection targets and construction of Protein-Protein Interaction (PPI) network: Using the Venn Diagram packet of R language (Version 4.0.3), the intersection of Brucine's target and GBM target was taken to find its potential target. Input the obtained action target into the STRING database, species select "*Homo sapiens*", set the hidden free point in the obtained image and the minimum interaction threshold is 0.4. Import the file "string_interactions.tsv" into Cytoscape software to generate the PPI network diagram.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis: Using DAVID online database[20] gene ontology (GO) enrichment analysis (including biological processes, cell components, molecular functions) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The GO enrichment analysis was used to draw bar graphs and KEGG channel enrichment analysis was used to draw bubble graphs, both of which were obtained on the online bioinformatics analysis platform[21].

Construct a component-target-disease-signaling pathway network: The common target and KEGG enrichment analysis results obtained above were introduced into Cytoscape 3.9.1, the data were visualized and the "Brucine-target-GBM-signal path" diagram was drawn.

Experimental verification

Drugs and reagents: Human Glioblastoma cell line U251 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences, China, No. RG130224.05. The RPMI Medium Modified Medium (Hy-Clone) and MEM (ATCC Modified) Medium (Prexcel), Fetal Bovine Serum (Gihco), Pancreatic enzyme (Biosharp Bio), Transwell Chamber (Corning Bio), Dimethyl sulfoxide (DMSO, Sigma Corporation), Matrix gum (Coming Bio Corporation), DEPC Water (Sigma Corporation), Cell eyele stainingKit, Annexin VAPC/PI apoptosis kit (Multi science Shanghai Biology Co. Ltd.), Annexin VAPC/PI Apoptosis Kit, GAPDH, PI3K, AKT, MMP9, CyclinD1, C-caspase9 (ABelonal Company), Brucine was purchased from Sichuan Jingzhu Tiancheng Pharmaceutical Technology Co. Ltd. (purity detected by HPLC is above 98%). BeyoClick™ EdU-488 cell proliferation detection kit was purchased from China Biyuntian Biological Company.

Instruments: The MCO18AIC carbon dioxide incubator (Japan Sanyo Electric Company), PH-3DMC inverted biological microscope purchased from Micro Vision Technology (Shenzhen) Co. Ltd., IMARK type enzyme marker (United States BIO-RAD Company).

Cell culture: Human Glioblastoma U251 cells and 143B cell lines were cultured in MEM medium and 1640 medium containing 10% fetal bovine serum and 1% penicillin streptomycin, respectively, in an incubator with a temperature of 37 and 5% CO saturation humidity. Cells with good growth state during logarithmic growth period were selected for the experiment. All experiments were repeated 3 times.

Detection of cell proliferation ability: Human Glioblastoma U251 cells and 143B cells of the logarithmic growth stage were selected and inoculated into 96-well plates at a concentration of 6000 cells per well, with 6 parallel multiple pores in each group. Incubated in a constant temperature incubator until 90% density was reached in each well. After liquid change, Brucine dissolved by DMSO was added and treated with 0, 4, 8, 16, 32, 64, 128, 256 and 512 μmol/L of Brucine for 24, 48 and 72 hrs, respectively and then 0.5% MTT solution of 10 μL was added to each well. The supernatant was discarded and a DMSO solution of 150 μL/well was added and placed on a shaker for 30 min. The absorbance was measured at 560 mm by enzymoleter and the data were analyzed.

Human Glioblastoma U251 cells and 143B cells at logarithmic growth stage were cultured in a 24-well plate to grow to the required density. Brucine was used to treat the cells for 24 hrs and prepared EDU was added to the cells of the control group and the experimental group for incubation for 2-4 hrs and then fixed with neutral paraformaldehyde at room temperature for 15 min. Then, PBS was washed three times, the pre-prepared permeable solution was used at room temperature for 20 min and then the prepared click-iT reaction solution was added and incubated at room temperature for 30 min and after washing the washing solution, hoechst 33342 was added for nuclear staining and then photographs were taken under the microscope for observation and record.

Statistical methods: The IBM SPSS 29.0 was used for analysis and statistics and the result data were expressed as (Mean±SD). Independent sample t-test was used for analysis within 2 groups, one-way analysis of variance was used for multiple groups and LSD t-test was used for further pair comparison. The $p<0.05$ was considered statistically significant.

RESULTS

Screening of Brucine active ingredient targets: The 2D structure and SMILES number of Brucine are obtained by searching "Brucine" as the search term through Pubchem. Input the retrieved SMILES number "COC1=C (C=C2C (=C1) C34CCN5C3CC6C7C4N2C (=O) CC7OCC=C6C5) OC" into the SWISS database to obtain 100 Brucine potential targets. At the same time, the 2D structure predicted by the SWISS database (Fig. 1) was compared with that in Pubchem and the results were consistent.

Brucine and GBM intersect targets and PPI network construction: Using "Glioblastoma" as the search term, 7777 targets were identified in GeneCards and 2613 relevant targets were obtained by screening Relevance score>1. Brucine potential targets were mapped to GBM related targets, 40 intersection targets were obtained and Venny diagram was drawn (Fig. 2). Brucine's active ingredient-target network for GBM treatment was constructed (Fig. 3).

Determine the hub target and subnetwork clustering: Use Cytoscape 3.9.1 software network analysis plug-in to count nodes in the network diagram and analyze their connectivity according to the degree value. The greater the node degree, the more biological functions the node has in the network (Fig. 4a). The top 12 Hub targets are EGFR, AKT1, MTOR, HSP90AA1, JUN, SRC, ERBB2, PIK3CA, PARP1, GSK3B, KDR and JAK2 (Fig. 4b). Then, the target network is clustered through MCODE plug-in analysis and two sub-networks are obtained. According to the score, the top 5 clustering networks are EGFR, AKT1, MTOR, HSP90AA1 and JUN (Fig. 4c).

GO and KEGG enrichment analysis: By GO analysis, 254 biological processes (BP), 43 cell components (CC) and 58 molecular functions (MF) were obtained and the first 10 were screened for enrichment analysis. The BP mainly includes phosphorylation, protein phosphorylation, intracellular signal transduction and peptidyl-serine phosphorylation, regulation of the primary metabolic process, etc. The CC includes nucleus, cytosol, plasma membrane, cytoplasm, perinuclear

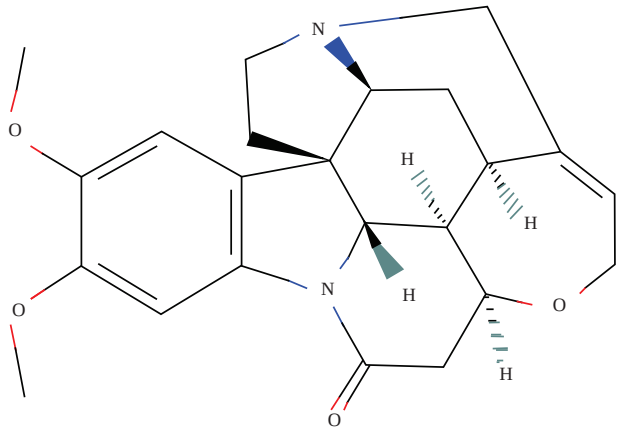

Fig. 1: Brucine 2D molecular structure

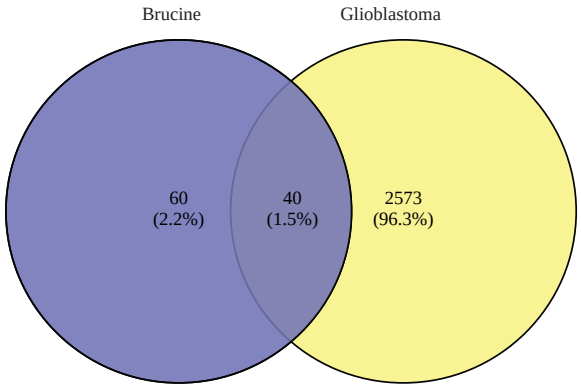

Fig. 2: Brucine and GBM share the target map

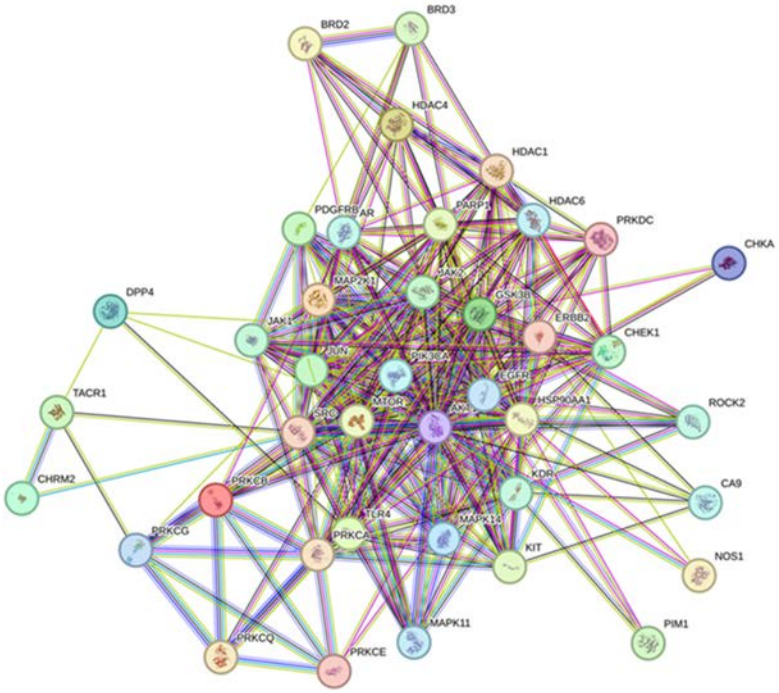

Fig. 3: Brucine active ingredient-target network diagram for anti-GBM

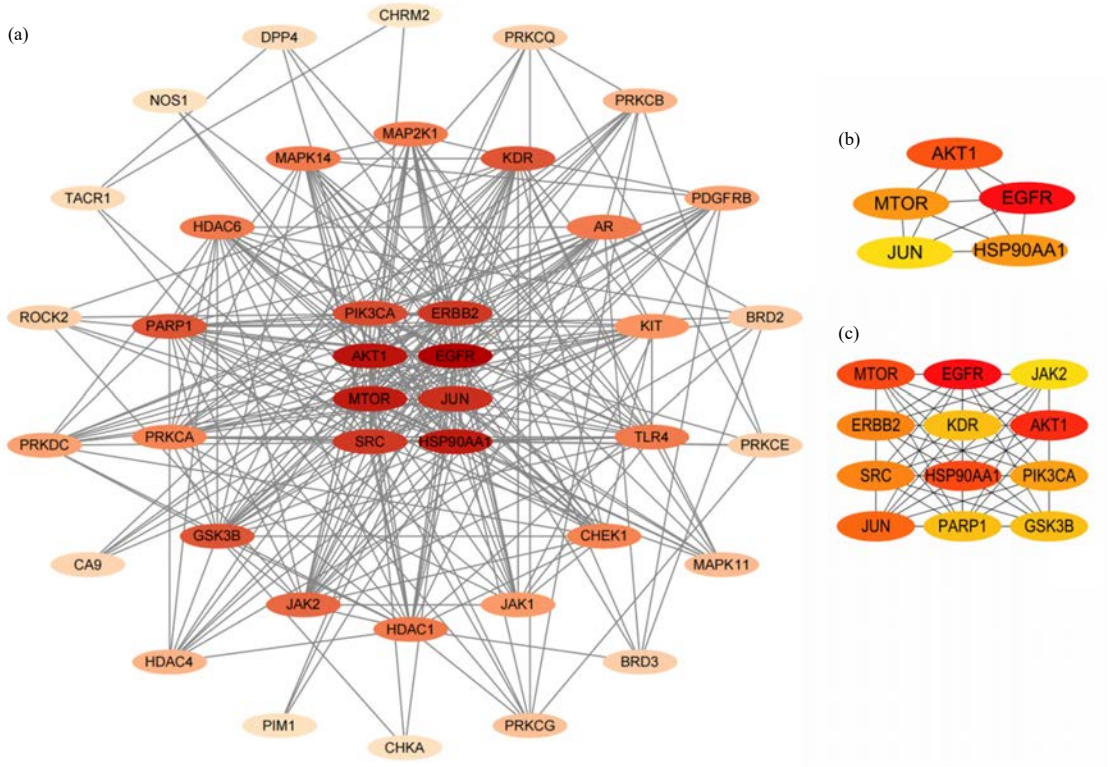

Fig. 4(a-c): Brucine PPI map of anti-GBM common target genes, (a) Node color response degree, (b) Top 5 targets with degree and (c) Top 12 targets with degree

region of cytoplasm, etc. MF includes protein kinase activity, ATP binding, protein serine/threonine kinase activity, enzyme binding, protein tyrosine kinase activity, etc. (Fig. 5a). A total of 153 pathways were obtained by KEGG enrichment pathway analysis and the top 20 signaling pathways with the highest correlation were selected (Fig. 5b). It mainly involves focal

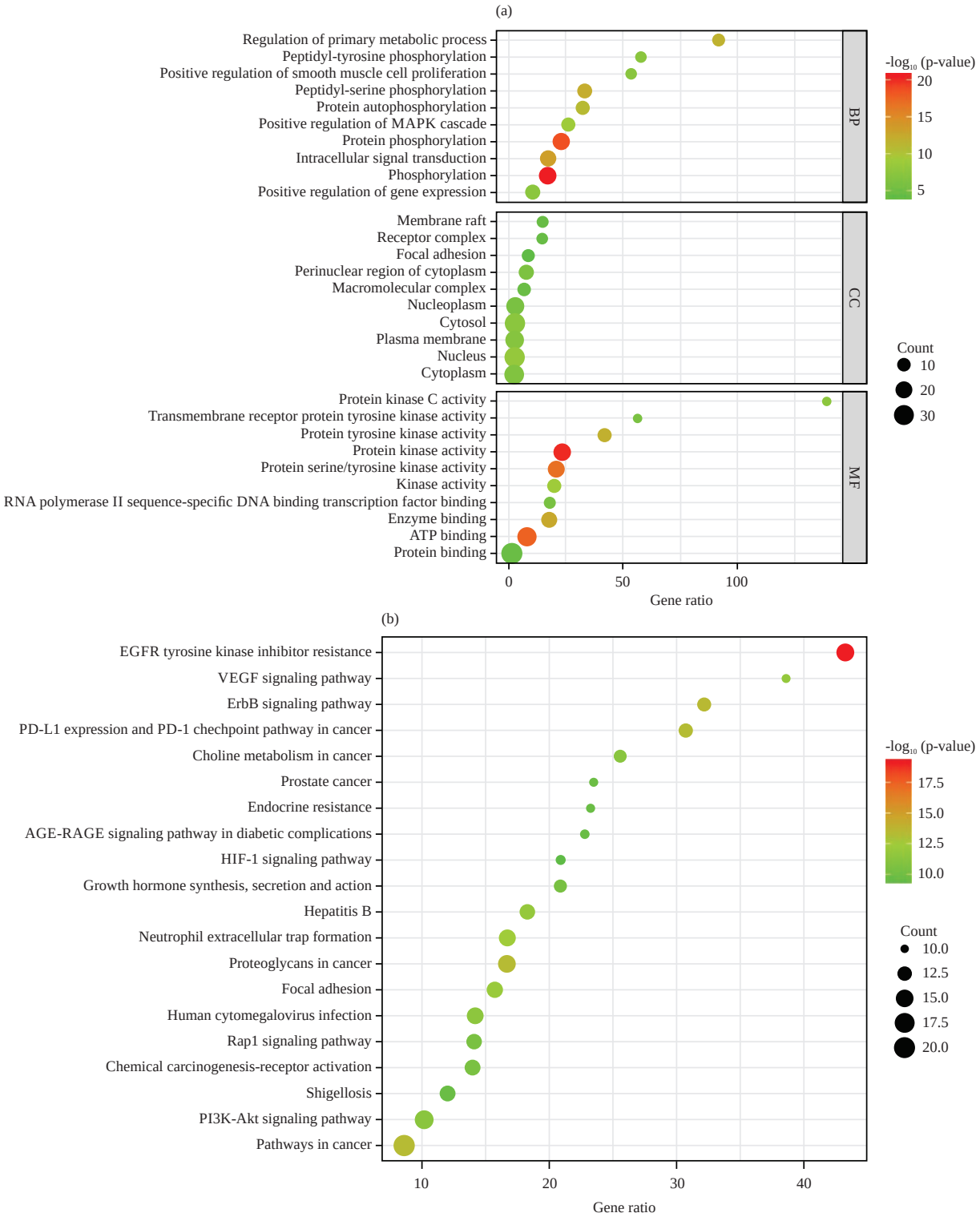

Fig. 5(a-b): GO and KEGG enrichment analysis of intersection targets, (a) GO analysis and (b) KEGG analysis

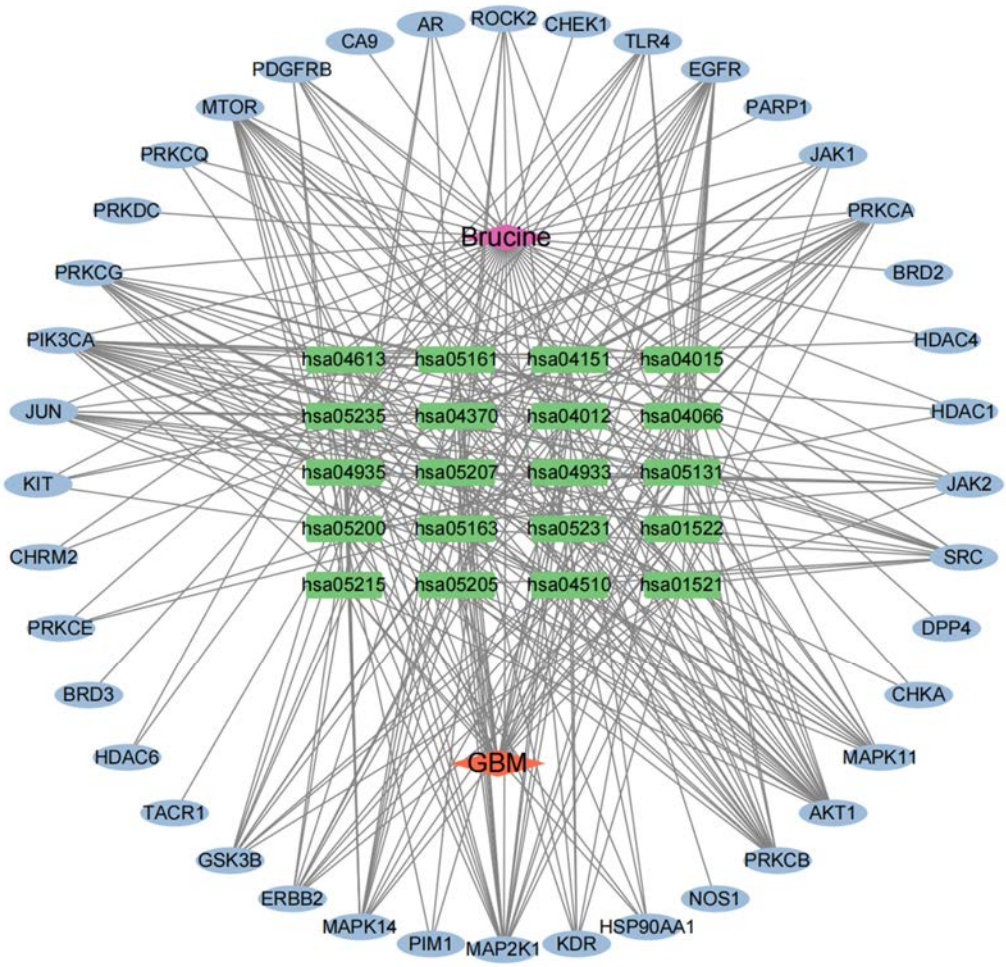

Fig. 6: Brucine-network diagram of target-disease-signaling pathway interactions

adhesion, cancer-related pathways, the main signaling pathways PI3K-Akt signaling pathway and VEGF signaling pathway. It is suggested that Brucine may play an anti-GBM role by intervening in the above biological processes and participating in the regulation of GBM generation and transfer.

Brucine-target-disease-signaling pathway network model construction: Brucine and 40 molecule-disease common targets of GBM were used to draw the network diagram of "component-target-disease-signaling pathway" interaction via Cytoscape v3.9.1 software (Fig. 6).

Brucine inhibited the proliferation of human Glioblastoma U251 cells and 143 B cells: The cell activity maps of 143B and human Glioblastoma U251 cells treated with Brucine at different concentrations (Fig. 7a-b) and their corresponding IC_{50} values (Fig. 7c-d) were detected by MTT assay. It was found that Brucine could inhibit the proliferation of GBMU251 cells in a time- and dose-dependent manner.

DISCUSSION

The GBM has the highest degree of malignancy, the most aggressive and the worst prognosis, invasive growth of glioma and the pathological characteristics of unclear boundaries. Moreover, GBM grows in important functional areas of the brain, making it impossible to completely remove tumor cells by current surgical methods and other methods, which are important reasons for the recurrence of this tumor[22]. The common biological feature of malignant tumors is uncontrolled growth, the main molecular mechanism of which is cell cycle disorder, resulting in excessive cell proliferation and reduced apoptosis. Therefore, inhibition of tumor cell proliferation has become one of the important means of tumor therapy. The anti-tumor effects of traditional

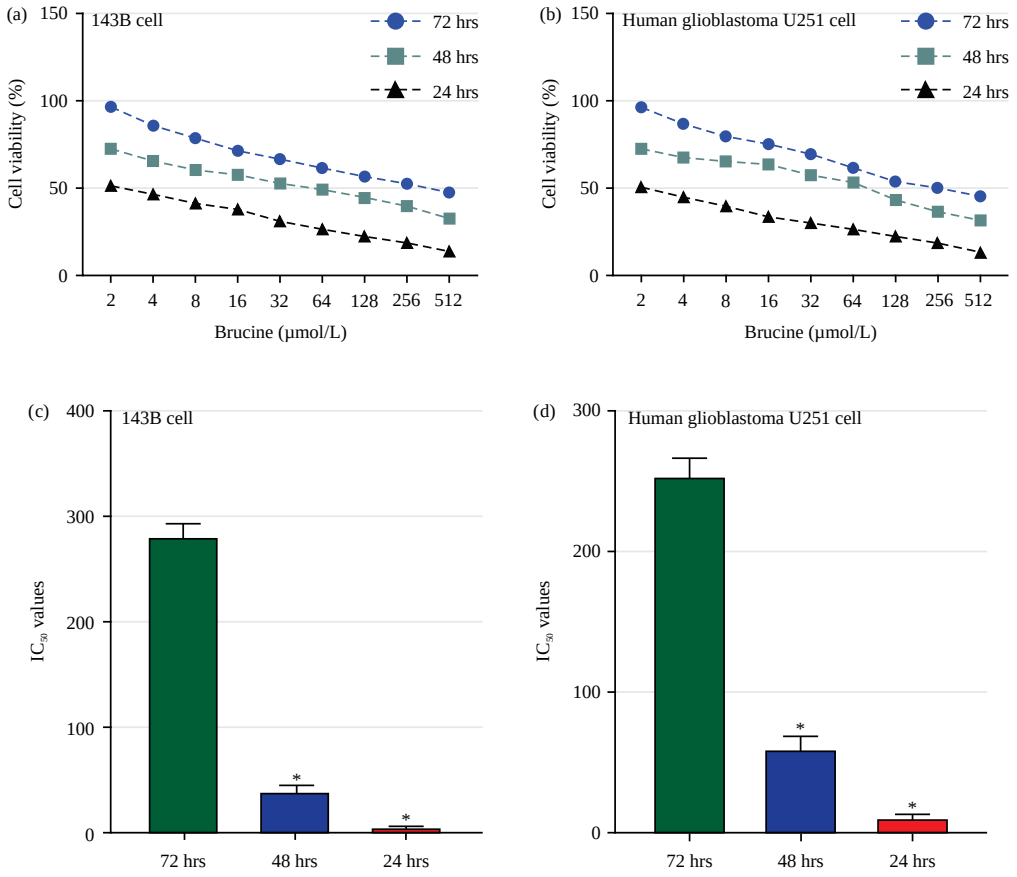

Fig. 7(a-d): Brucine inhibited the proliferation of human Glioblastoma U251 cells and 143B cells with (a) Brucine treated with different concentrations of 143B cells, (b) Brucine treated with different concentrations of human Glioblastoma U251 cells, (c) IC_{50} values of 143B cells treated with different concentrations of Brucine and (d) IC_{50} values of U251 cells treated with different concentrations of Brucine

Mean±SD and n = 3

Chinese medicines (TCM), whether single or combined, have been concerned for a long time and the screening of low-toxicity effective anti-tumor drugs and the mechanism of action of TCM to inhibit the proliferation of malignant tumors and induce tumor cell apoptosis have become the focus of current research[23,24].

At present, toxic Chinese medicines account for a large proportion of the anti-cancer drugs developed from natural medicines, such as toad, cantharides, camptotheca, podophylloides, etc., which are all effective anti-cancer drugs developed from toxic Chinese medicines[25,26]. *Strychnos nux-vomica* L. It is a dry and mature seed of the Strychoniaceae plant, which is bitter, warm and highly toxic and has the function of clearing collages, relieving pain, dispersing knots and reducing swelling[12]. Current studies[13,27-31] have found Brucine has anti-tumor effects, involving liver cancer, colon cancer, lung cancer, kidney cancer, breast cancer and so on. The inhibitory mechanisms of Brucine on various tumor cells are different and the related mechanisms have been studied. Many studies have found that it is related to apoptosis and metastasis. According to Deng *et al.*[32], Brucine can induce intracellular Ca^{+} release through the JNK-Fas pathway, thereby inducing apoptosis of HepG2 cells. Brucine can also inhibit inflammation and cell proliferation and promote apoptosis of cervical cancer cells (ME-180) by regulating PI3K/AKT/mTOR signaling pathway[33].

To study the inhibitory effect of Brucine on GBM and reveal its potential molecular mechanism, the method of network pharmacology was adopted in this study. In this study, it is found that in Brucine's GBM PPI network, EGFR, AKT1, MTOR, HSP90AA1, JUN, SRC, ERBB2, PIK3CA, PARP1, GSK3B, KDR and JAK2 are the first 12 targets based on node Degree value. These proteins are thought to be Hub targets and may play an important role in Brucine's inhibition of GBM. To predict the potential mechanism of Brucine inhibiting GBM, GO enrichment and KEGG analysis were performed on

40 potential Brucine targets. The results showed that the above targets were mainly involved in phosphorylation, intracellular signal transduction, regulation of primary metabolic process and other biological processes. It is closely related to the inhibition of the nucleus, cytosol, plasma membrane, cytoplasm, perinuclear region of cytoplasm and other signaling pathways. The relevant signaling pathways include focal adhesion, PI3K-Akt signaling pathway and VEGF signaling pathway. The VEGF signaling pathway plays an irreplaceable role in the process of angiogenesis. Mu *et al.*[34] found that CALM2 enhances the polarization of macrophages by regulating the VEGF and HFEI-1 signaling pathway, thus promoting the proliferation and migration of vascular endothelial cells in tumor tissues. The PI3K-AKT signaling pathway is an intracellular signal transduction pathway that promotes metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals. As a key pathway, bioactivity related to tumorigenesis is affected by the PI3K/Akt pathway[35]. By regulating MMPs, the activated PI3K/Akt pathway regulates tumor angiogenesis and metastasis[36]. Seshadri's[33] study found that Brucine inhibited the proliferation of inflammatory cells and promoted apoptosis by down-regulating PI3K/AKT/mTOR pathway.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase encoded by protein tyrosine kinase-2 gene, which is one of the adhesion plaque complex family. Growth factors or integrins can promote rapid phosphorylation of FAK at 397 site, enhancing cell activity[37]. Importantly, because FAK translates signals from upstream RTK into roles in downstream signaling pathways[38], it often acts as a signaling hub to control molecular networks and mediate various cellular activities in tumor cells. Phosphorylation of FAK can promote the formation of adhesion plaques, promote tumor EMT and invasion and metastasis and activation of FAK can trigger the activation of SRC, resulting in activation of downstream substrates, thereby regulating several signaling pathways such as PI3KAkt, MAPK and STAT3 and promoting tumor invasion and metastasis[39]. Studies of Wang *et al.*[40] and Che *et al.*[41] showed that by inhibiting the expression of FAK, it was found to mediate the inhibition of downstream PI3K-Akt signal and the expression of specific tumor markers. The above studies suggest that under the premise of paying attention to PI3K-Akt, FAK as an upstream target may play a role in inhibiting GBM.

In this study, through *in vitro* cell experiments, we showed that in the TMM cell proliferation activity experiment, MTT experiment detected the cell activity of 143B and human Glioblastoma U251 cells treated with different concentrations of Brucine and found that the cell activity of human Glioblastoma U251 cells decreased significantly with the increase of Brucine concentration. It was suggested that Brucine could inhibit the proliferation of GBM cells in a time-dependent and dose-dependent manner. There are still some limitations in this study. In this paper, the role of Brucine in GBM is discussed only at the level of network pharmacology, but the current network information technology is not perfect and the accuracy and real-time update of the database need to be improved. Therefore, the results obtained in this study need to be verified in terms of pharmacodynamics and the inhibitory effect of Brucine on GBM needs to be further explored, so as to clarify the multi-target and multi-pathway efficacy of Brucine in inhibiting GBM.

CONCLUSION

In this study, Brucine component-target-GBM correlation pathway network was constructed and analyzed the mechanism of Brucine inhibiting GBM by network pharmacology. The results showed that Brucine showed multi-target and multi-pathway inhibition on GBM and played an active pharmacological role in vitro experiments, which provided references for further research on the mechanism of Brucine in GBM.

SIGNIFICANCE STATEMENT

In this study, we explored the inhibitory mechanism of Brucine on Glioblastoma and carried out experimental verification. It is found that Brucine and GBM have 40 intersection targets and the top 5 clustering networks are selected as the Hub targets, EGFR, AKT1, MTOR, HSP90AA1 and JUN. The targets mainly involve focal adhesion, cancer-related pathways, the main signaling pathways PI3K-Akt signaling pathway and the VEGF signaling pathway. Brucine inhibited the proliferation of GBMU251 cells in a time- and dose-dependent manner. Therefore, we speculated that Brucine might inhibit the proliferation of Glioblastoma through signaling pathways such as the PI3K-Akt signaling pathway and the VEGF signaling pathway.

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