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JNK Inhibition Modulates the Cytoskeleton, Hypoxia, and Neurogenesis on the Protein Level in Glioblastoma Cells and Astrocytes: An Immunofluorescence Study

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ABSTRACT

AIM: To evaluate the effects of c-Jun N-terminal kinase (JNK) inhibition and signal blocking on hypoxia (hypoxia-inducible factor 1-alpha (HIF-1α)), differentiation and neurogenesis (bone morphogenetic protein (BMP4)), and the cytoskeleton (F-actin) in glioblastoma multiforme cells (GBMCs).

MATERIAL and METHODS: We evaluated the differences between GBMCs and astrocytes in terms of the abovementioned parameters and assessed them with the aim of studying human GBMCs (U-87 MG) and astrocytes (SVG p12). The cells were exposed to different doses of the JNK inhibitor, SP600125, for 24, 48, and 72 hours. HIF-1α, BMP4, and F-actin expressions were evaluated using immunofluorescence image analysis.

RESULTS: The half-maximal inhibitory concentration value for SP600125 was determined to be 10 μM at 24 hours of exposure. After SP600125 administration, elevated levels of HIF-1α and BMP4 were detected in GBMCs and astrocytes. F-actin level only increased in GBMCs after SP600125 administration.

CONCLUSION: JNKs are important for cell proliferation, differentiation, survival, and death; thus, research on JNKs has become important for the treatment of many human diseases, especially brain tumors, Parkinson's disease, and Alzheimer's disease. The results of this study involving immunofluorescence techniques should be investigated and supported by studies that involve comprehensive molecular techniques.

KEYWORDS: c-Jun N-terminal kinases, Glioblastoma multiforme, HIF-1α, BMP4, F-actin

ABBREVIATIONS: GBM: Glioblastoma multiforme, **JNKs:** c-Jun N-terminal kinases, **MAPK:** Mitogen activated protein kinase, **JNK:** c-Jun N-terminal kinase, **HIF-1α:** Hypoxia-inducible factor 1-alpha, **BMP4:** Bone morphogenic protein 4; **GBMCs:** GBM cells, **BMPs:** Bone morphogenetic proteins, **Tgf-β:** Transforming growth factor beta, **ATCC:** American Type Culture Collection, **FBS:** fetal bovine serum, **EMEM:** Eagle's minimal essential medium, **CO2:** Carbon dioxide, **EDTA:** Ethylenediamine tetraacetic acid, **DAPI:** 4′,6-diamidino-2-phenylindole, **ml:** milliliter, **μM:** Micro molar, **h:** hours, **DMSO:** Dimethyl sulfoxide, IC50: Half-maximal inhibitory concentration, **BSA:** Bovine serum albumin, **min:** Minutes, **SAPK:** Stress-activated protein kinase

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common type of invasive primary brain tumor (7,8,35). Despite the use of modern surgical and clinical techniques and combined radiotherapy and chemotherapy treatments in neuro-oncology, the 1-year survival rate of patients with high-grade glioma is 46% (26), and patients with GBM have a median survival period of less than 15 months (29). Thus, it is imperative to fully clarify the molecular mechanisms underlying GBM biology and to develop new therapeutic agents.

There are three main types of c-Jun N-terminal kinases (JNK1, JNK2, and JNK3), and these kinases are members of the mitogen-activated protein kinase (MAPK) family, which plays a fundamental role in signal transduction in many biological processes (13,19). JNK1 and JNK2 are widely expressed in many tissues, whereas the expression of JNK3 is restricted to the brain, heart, and testes (18,34). JNK is a multifactorial kinase involved in many physiological and pathological processes, including cell growth, proliferation, differentiation, apoptosis, autophagy, survival, and inflammation (19,25,34). In addition, the MAPK signaling pathway plays a role in the pathogenesis of many diseases, including cancer, neurodegenerative diseases, and inflammatory diseases. It also plays an important role in the resistance to various drugs used in cancer treatment, such as cisplatin, irinotecan (used in colon cancer), and sorafenib (used in liver cancer) (18). SP600125 is an anthrapyrazolone compound that is commonly used as a JNK inhibitor in studies. Moreover, in some studies, SP600125 was investigated as an anticancer agent owing to its deoxyribose nucleic acid (DNA)-binding activity (19).

Tumor progression, metastasis, and invasion are strongly correlated with tumor hypoxia in the case of various types of tumors, such as cervical, head, neck, prostate, pancreas, and brain tumors (1). Hypoxia-inducible factor 1-alpha (HIF-1α) is the main transcriptional regulator responsible for the regulation of many genes in critical cellular events, in which hypoxia is involved in energy metabolism, apoptosis, and angiogenesis (12,25). HIF-1α is an important target in the development of novel cancer drugs. Studies in this area will lead to a better understanding of cancer biology and tumor behavior (16). Bone morphogenetic proteins (BMPs) are growth factors that belong to the transforming growth factor beta (Tgf-β) superfamily. BMP4 expression is associated with the invasion and migration of GBM cells (GMBCs), and it inhibits tumor growth; however, the underlying mechanism has not yet been fully elucidated in humans or animals (9,14,27,33). F-actin is a cell skeleton protein that performs the following functions: binds cell skeleton elements to each other; regulates cell shape changes, motility, and adhesion; regulates cell receptor activity; and binds the cell skeleton to various signaling pathways (28). The response of cells to cellular damage is regulated by MAPK signaling, and MAPK activation is required to stabilize the cell skeleton (3).

The aim of this study was to evaluate the effects of JNK inhibition and signal blocking on hypoxia (HIF-1α), differentiation and neurogenesis (BMP4), and the cell skeleton (F-actin) in GBMCs. and aimed to compare GBMCs and astrocytes using the same parameters.

MATERIAL and METHODS

Cell Culture

A human GBM cell line (U-87 MG American Type Culture Collection [ATCC] ® HTB-14™) and human astrocyte cell line (SVG p12 ATCC ® CRL-8621™) were obtained from ATCC. Cells were cultured in 10% fetal bovine serum (FBS) (Gibco 10270-106) containing Eagle's minimal essential medium (EMEM) (Sigma M4655) and were maintained at 37°C and 5% CO_2. They were detached from the surface with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Thermo 25300054) twice a week and were diluted with culture medium to 1 x 10^5 cells/ml for the experiments.

Determination of Drug Dose

The cells were diluted to 10^5 cells/ml and cultured in 6-well plates. SP600125 (BioVision 1669-5 USA) was dissolved in dimethyl sulfoxide (DMSO) and was administered at doses of 10, 20, and 40 μM 24 h after cell seeding. DMSO was used in the control group. Cell viability was controlled 24, 48, and 72 h after administration using a Muse® Cell Analyzer (Merck Millipore).

Experimental Groups

Group 1: Glioblastoma multiforme (GBM) (U-87 MG) cell line

Group 2: Human astrocyte cell line (SVG p12)

Group 3: JNK-inhibited GBM (U-87 MG)

Group 4: JNK-inhibited human astrocyte cell line (SVG p12)

The cells in these experimental groups were incubated for 24, 48, and 72 h following the administration of SP600125 and DMSO at doses of 10, 20, and 40 μM. The effects of the JNK inhibitor, SP600125, on the cells were investigated.

Analyses of Cell Viability

Cell viability was calculated in 100% DMSO. Logarithmic values of the administered doses were calculated. The log (drug dose) function based on the percentage of cell viability was calculated according to the obtained graphs Consequently, 10 μM at 24 h was determined to be the half-maximal inhibitory concentration (IC50).

Immunofluorescence Staining

After an appropriate incubation time, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. The cells were incubated for 10 min in 0.25% Triton-X100 in order to increase cell membrane permeability. For blocking, the cells were incubated in 1% bovine serum albumin (BSA) for 1.5 h at room temperature. Primary antibodies against HIF-1α (Bioss bs-0737R, 1/100 dilution) and BMP4 (ThermoFisher PA5-27288, 1/100 dilution) were prepared in 1% BSA and incubated overnight. The cells were incubated for 1 h with a secondary antibody (Alexa Fluor® 488, 1/200 dilution) for HIF-1α and BMP4 staining. F-actin (Abcam, ab176753,

1/1000 dilution) staining was performed through incubation for 1 h at room temperature. The samples were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (ab104139) and were inspected under a microscope (Olympus Cell Sens Entry). The cells were analyzed using the ImageJ (Image analysis software, National Institutes of Health, Bethesda, MD) software.

Statistical Analysis

Immunofluorescence images were analyzed using the open-source image-processing program, Fiji/ImageJ. The HIF-1α, BMP4, and F-actin intensity analysis and cell major length analysis were performed using JMP Pro software (version 13.0; SAS Institute Inc., 2017). The distribution of data was elucidated using the Shapiro-Wilk W test, and all three protein intensities were not normally distributed. Therefore, the data were analyzed through nonparametric comparisons for each pair using the Wilcoxon method. The threshold for statistical difference was set at 0.05. (7) The data values are listed in Table I.

RESULTS

HIF-1α Immunofluorescence Staining

There was a significant increase in HIF-1α protein expression in the JNK-inhibited GBMCs compared with in the control GBMCs ($p<0.05$). Similarly, the inhibition of JNK led to a significant increase in HIF-1α protein expression in the astrocytes compared with in the control astrocytes ($p<0.05$). Remarkably, the cells in the GBM group exhibited a significantly more intense HIF-1α staining pattern than the astrocytes ($p<0.05$) (Figures 1 and 4).

BMP4 Immunofluorescence Staining

The expression of BMP4 was higher in the JNK-inhibited GBMCs than in the control GBMCs ($p<0.05$). JNK inhibition increased the expression of BMP4 in the astrocytes to levels greater than those in the control astrocytes ($p<0.05$). The cells in the GBM group exhibited a significantly more intense staining pattern than the astrocytes ($p<0.05$) (Figures 2 and 4).

F-actin Immunofluorescence Staining

There was a significant increase in F-actin intensity following JNK inhibition in the GBMCs compared with in the control GBMCs ($p<0.05$). However, the expression of F-actin in the astrocytes was lower following JNK inhibition ($p<0.05$). The cytoskeleton (F-actin) in the astrocyte group exhibited significantly more intense staining than that in the GBMCs ($p<0.05$) (Figures 3 and 4). According to the actin values, the astrocyte length was the same with and without drug administration (Figure 4). Contrarily, GBM cell length increased, which was compatible with the immunofluorescence results (Figure 4).

DISCUSSION

GBM is a rapidly growing brain tumor with very high invasive ability, low prognosis, and high mortality rate (2). Although surgical resection, radiotherapy, and gene therapies are used to treat the most malignant types of astrocytic tumors, resistance to chemotherapy and radiotherapy remains a major problem (6,20,35). It is intended that new treatment agents will be developed that will decrease tumor growth and prolong survival time to over 15 months (20). In this study, we treated human GBMCs and astrocytes with the JNK inhibitor, SP600125.

The MAPK signaling pathway plays an important role in cancer cell proliferation and metastasis, and the activation of the JNK signaling pathway has been observed to be high in cancer cells (18). The MAPK pathway, including the JNK signaling pathway, effectively inhibits cell growth and regulates the cell cycle (2). In leukemia cell lines, increases in cell size and cell numbers were observed in the G2/M phase of the cell cycle after JNK inhibition (24). Studies have shown that the inhibition of the JNK signaling pathway increases the antitumor effect, while the use of SP600125 reduces cell viability (23). Another study that used SP600125 showed that JNK inhibition reduced autophagy and increased apoptosis; however, the mechanism is not fully understood (20). The JNK signaling pathway is

Table I: Statistical Data Corresponding to Protein Intensities and Length Values. The Mean Values, Standard Deviations, and Number of Cells are Presented

		Control			JNK inhibition		
		Mean	**SD**	**n**	**Mean**	**SD**	**n**
Hif1a	GBM	5059.504	1490.468	223	5967.77	1962.833	133
	Astrocyte	2740.63	812.5454	244	3471.301	971.4254	163
Bmp4	GBM	1598.139	487.1633	134	2287.295	756.1611	180
	Astrocyte	1340.226	365.065	92	1886.83	667.7457	85
F-actin	GBM	698.6633	299.876	143	4860.908	2587.44	25
	Astrocyte	15011.48	4647.681	90	9023.714	3445.573	69
Lenght	GBM	71.84904	32.47049	157	109.8142	41.18411	25
	Astrocyte	128.3085	46.59256	101	121.097	37.26492	69

associated with increased apoptosis following treatment with anticancer drugs. Wang et al. showed that although JNK inhibition enhances the antiapoptotic effect of proteasome inhibitors in pancreatic cancer cells, it also increases the apoptotic response in renal cancer cells (31). The inhibitory effect on JNK1, JNK2, and JNK3 in terms of adenosine triphosphate (ATP) was competitive and fully reversible. In Parkinson's disease studies, JNK inhibition has been shown to be clinically useful for dealing with cell death, infections, stroke, ischemia-reperfusion injuries, and chronic neural cell death, as it plays a pivotal role in cell proliferation, survival, and differentiation. JNK, a proto-oncogene, is effective in regulating cell growth and proliferation (3,5,30). In cases of epilepsy, astrocytes exhibit proliferation and hypertrophy due to JNK activation, as well as an increase in inflammatory cytokines due to an excessive increase in JNK (32).

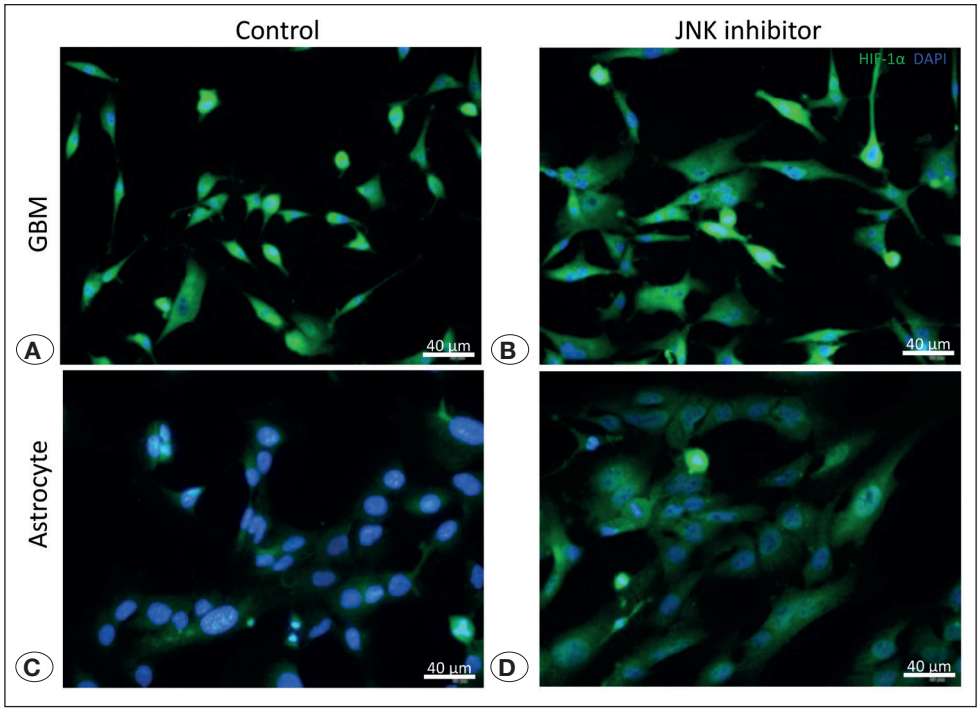

Figure 1: Immunofluorescence staining of HIF-1α.
A) Control human GBM cells,
B) JNK-inhibited human GBM cells,
C) Control human astrocytes,
D) JNK-inhibited human astrocytes.
After JNK inhibition, the level of HIF-1α protein intensity in GBM cells and astrocytes was elevated. On comparing the control cell groups, GBM cells exhibited higher intensity than astrocytes.
Abbreviations: HIF-1α, hypoxia-inducible factor 1-alpha; GBM, glioblastoma multiforme; JNK, c-Jun N-terminal kinase.

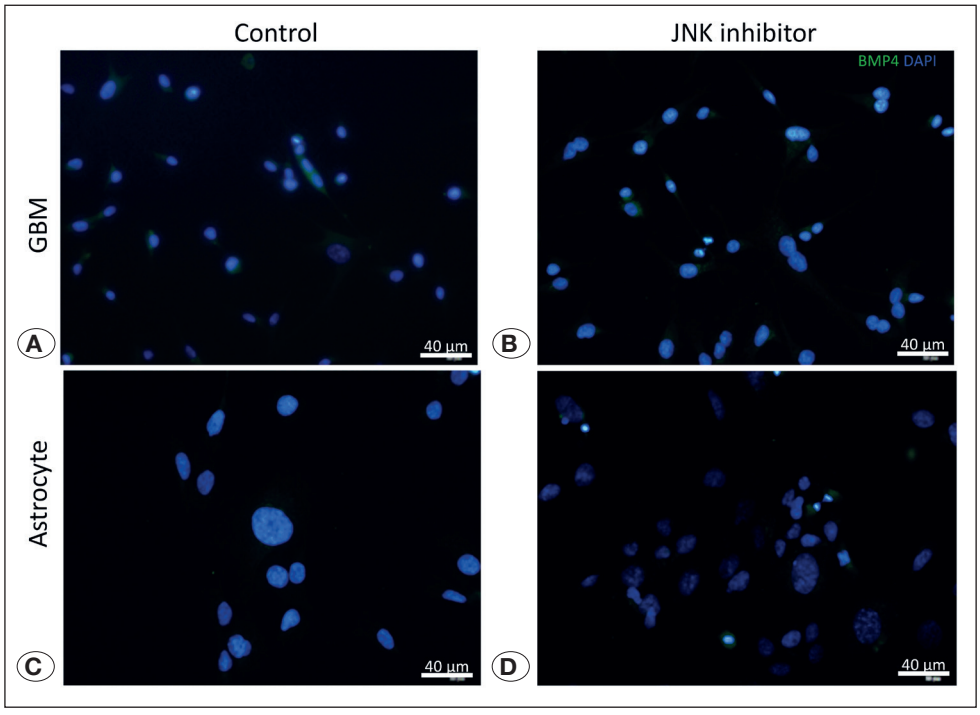

Figure 2: Immunofluorescence staining of BMP4 DAPI
A) Control human GBM cells,
B) JNK-inhibited human GBM cells,
C) Control human astrocytes,
D) JNK-inhibited human astrocytes.
After JNK inhibition, the level of BMP-4 protein intensity in GBM cells and astrocytes was elevated. On comparing the control cell groups, GBM cells showed higher intensity than astrocytes.
Abbreviations: BMP4, bone morphogenic protein 4; DAPI, 4′,6-diamidino-2-phenylindole; GBM, glioblastoma multiforme; JNK, c-Jun N-terminal kinase.

Under hypoxic conditions, HIF-1α accumulates in cells, thereby avoiding ubiquitination and proteasomal degradation. HIF-1α induces the expression of certain genes that participate in cancer development and progression of cell survival and proliferation. The increase in HIF-1α under hypoxic conditions is considered a cell response for reducing cell death (10). Hypoxia is an important negative prognostic factor in cancer treatment and tumor behavior in various types of cancer, including brain cancers. HIF-1α, which plays a role in angiogenesis, is important for the development of new anticancer therapies. Thus, focusing on HIF-1α will facilitate a better understanding of tumor biology and behavior. Increased hypoxia is important for tumor progression, metastasis, and drug resistance (1,16). Studies in GBM patients indicate that the elevation of hypoxia markers is associated with poor prognosis; hypoxic conditions protect GBM stem cells and

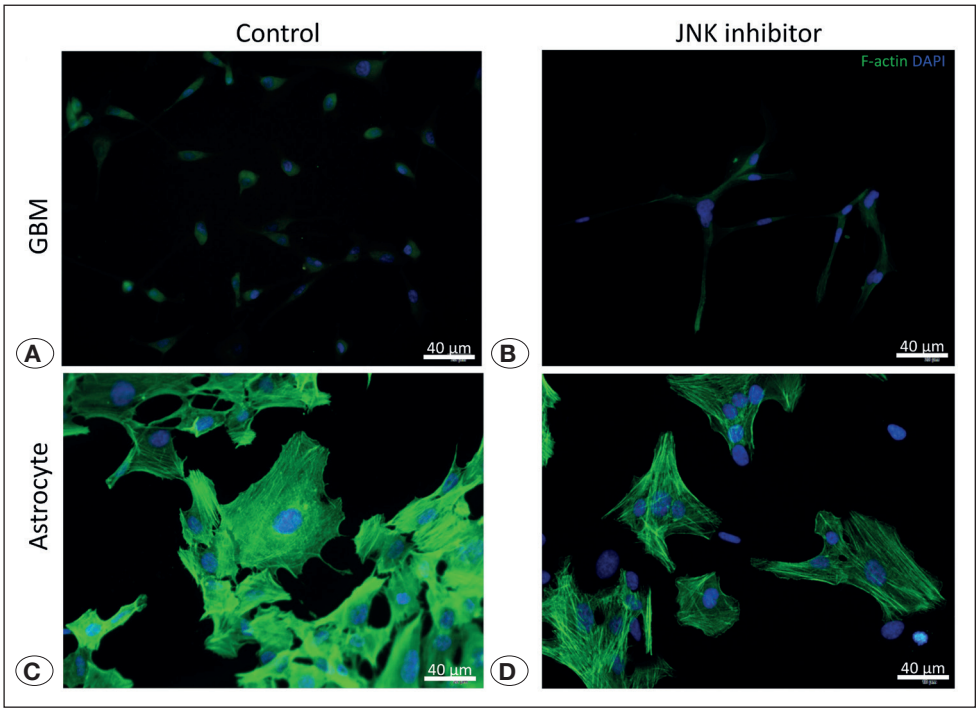

Figure 3: Immunofluorescence staining of F-actin.
A) Control human GBM cells,
B) JNK-inhibited human GBM cells,
C) Control human astrocytes,
D) JNK-inhibited human astrocytes.
After JNK inhibition, the level of F-actin protein intensity in astrocytes decreased in contrast to the increase in GBM cells. On comparing the control cell groups, astrocytes showed higher intensity than GBM cells. **GBM:** Glioblastoma multiforme, **JNK:** c-Jun N-terminal kinase.

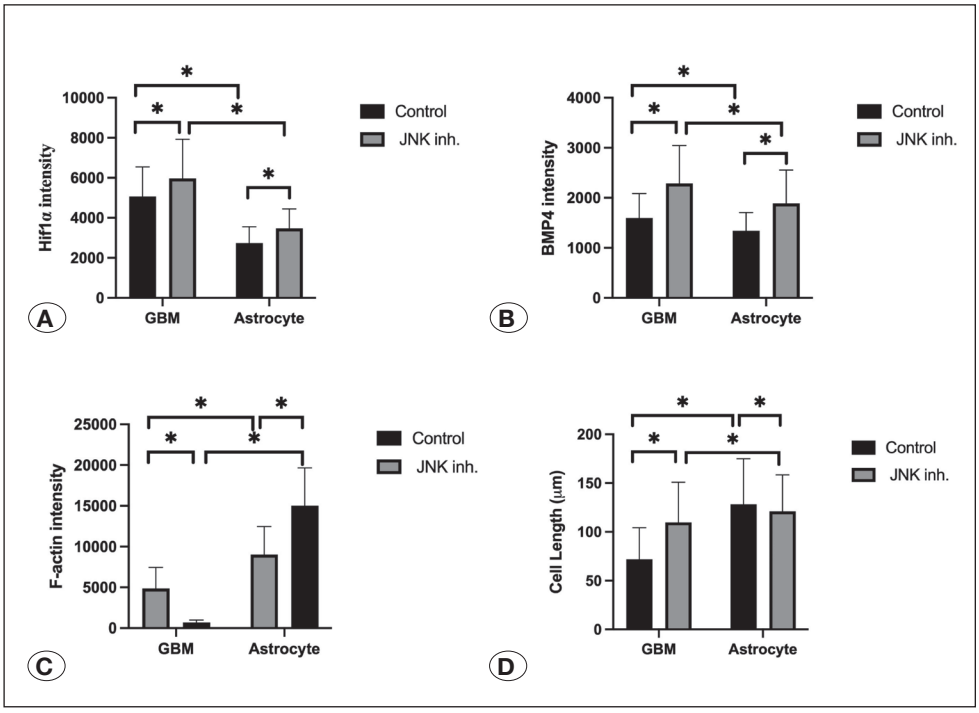

Figure 4: Protein intensity levels and cell length of GBM cells and astrocytes in the absence and presence of the JNK inhibitor
The graph shows the mean protein intensity levels of **A)** HIF-1α, **B)** BMP4, and **C)** F-actin per cell. **D)** The lengths of the cells were compared in the absence and presence of the JNK inhibitor. **GBM:** Glioblastoma multiforme, **JNK:** c-Jun N-terminal kinase, **HIF-1α:** Hypoxia-inducible factor 1-alpha, **BMP4:** Bone morphogenic protein 4.

lead to reprogramming in the direction of cancer stem cells (1). There is limited information on the efficacy of c-JNK in regulating HIF-1α. Some studies have shown that c-JNK promotes the activation of HIF-1α, whereas others have shown that HIF-1α is not phosphorylated by c-JNK (17). In this study, an increase in HIF-1α levels was detected in the GBMC and astrocyte groups after the administration of SP600125. As expected, the GBM control cells had higher HIF-1α levels than the astrocytes, indicating their survival success and malignant behavioral capacities. The increase in HIF-1α protein expression levels in GBMCs via JNK inhibition indicates that this protein should be considered as a target in treatment planning, as it may be one of the sources of cancer recurrence and drug resistance.

Recent studies have demonstrated the role of BMP in the pathogenesis of cancer. BMP4, one of the fifteen subtypes of BMP, plays an important role in cancer pathogenesis and neuron and astrocyte differentiation during the development of nervous system stem cells. BMP4 expression increases in gastric, hepatocellular, and colorectal cancers but decreases in meningioma and pituitary tumors. In addition, BMP4 treatment or overexpression has been shown to decrease cell growth in basal cell carcinomas and myeloma and in breast, gastric, lung, and pancreatic cancers (4).

Studies on GBM have shown that BMP4 inhibits the growth of glioma cells (14,33). Previous studies have shown that BMP4 expression in GBM is low and that low BMP4 levels increase the proliferation and tumor-forming capacity of GBMCs (21,22). Overexpression of BMP4 has been shown to suppress tumor development, invasion, and size (27,33). In this study, an increase in BMP4 expression levels was detected in the GBM groups after the administration of SP600125 in human GBMCs and astrocytes. The levels of BMP4, which is effective for astrocytic differentiation, were found to be lower in mature astrocytes. Increased BMP4 levels decrease the proliferation and tumor-forming capacity of GBM cells. The detection of increased BMP4 expression following JNK inhibition highlights the importance of this mechanism in cancer chemotherapy.

The cytoskeleton protein, F-actin, determines cell shape, motility, and proliferation and all other biological processes, but its effectiveness is still being investigated (11,28). Actin activates the stress-activated protein kinase (SAPK) pathway and JNK signaling pathway, which are components of the MAPK signaling pathway, within the cell's signaling mechanisms when cell skeletal elements experience disruption and mechanical stress. Densham et al. demonstrated that JNK activation following cell skeletal degradation arrests the cell cycle. The activation of the JNK signaling pathway is associated with F-actin and has been shown to regulate actin polymerization and remodeling within the cell (11). Jung et al. showed that JNK activation plays an active role in actin stability and migration in cancer cells and neurons (15). In this study, after SP600125 administration, actin expression was found to increase in the GBMCs, while it was found to decrease in the astrocytes. The increased actin density, indicating an active cytoskeletal organization in astrocytes—the most dominant cell in the central nervous system and main cell in the blood-brain barrier—was as expected, and a decrease in F-actin levels was observed after drug administration. This may lead to potential drug side effects when used during cancer chemotherapy. The increase in F-actin density in the GBMCs after the inhibition of an effective pathway, such as the JNK pathway involved in actin stabilization control, demonstrates that these cells can preserve their malignant cytoskeletal properties through various mechanisms and that they can exhibit resistance to chemotherapeutic agents. These findings together with those of future studies elucidating secondary resistance mechanisms can aid in planning effective combination treatments targeting cancer cells.

Apoptosis is induced by various stresses in cells. JNKs within cells are signaling pathways that act as stress-activated pro-apoptotic elements. Owing to these effects, they play a role in the response of tumor cells to various chemotherapeutics. Depending on the tumor cell type and chemotherapeutics used, specific types of JNKs may exhibit both pro-apoptotic and anti-apoptotic behaviors. Heasley and Han reported that there was an increase in lung tumors in rats deficient in JNK1 and JNK2 and that half of the human brain tumors exhibited a JNK3 mutation (13). It is known that c-Jun induces the development of hepatocellular carcinoma in the early stages and inhibits androgen receptors in prostate cancers. In light of these data, the proto-oncogenic and tumor-suppressor effects of JNKs in various cancer types should be investigated, and new treatment protocols should be developed.

GBM is a highly invasive and extremely malignant brain tumor that needs to be investigated histopathologically in order to develop new and effective treatment methods. JNKs are important for cell proliferation, differentiation, survival, and death, and research on JNK has become important for the treatment of many human diseases, including brain cancer, Parkinson's disease, and Alzheimer's disease. The results of this study show that there was an increase in BMP4, F-actin, and HIF-1α expression after JNK inhibition. While it has been observed that JNK inhibition may have an anticancer effect on GBMCs, especially since it increases BMP4 expression, it has been pointed out that there may be an insufficient treatment response in relation to the increase in HIF-1α and F-actin expression. When considering JNK inhibition as a therapeutic approach, side effects related to the cytoskeleton should also be considered. In addition, future studies that include secondary pathways and combined drug therapies should be planned in order to prevent drug resistance and, consequently, recurrence. The results of this immunofluorescence study should be investigated and supported by further studies using comprehensive molecular techniques, as these techniques can be more effective for understanding the characteristics of cancer cell biology.

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AUTHORSHIP CONTRIBUTION

Study conception and design: CK, HA
Data collection: BO, DCK
Analysis and interpretation of results: BO, SU
Draft manuscript preparation: SU, CK
Critical revision of the article: HA
All authors (CK, SU, DCK, BO, HA) reviewed the results and approved the final version of the manuscript.

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