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Isolation of Cells and Exosomes from Glioblastoma Tissue to Investigate the Effects of Ascorbic Acid on the c-Myc, HIF-1α, and Lnc-SNHG16 Genes

Masoumeh Eliyasi Dashtaki[1], Alireza Tabibkhooei[2], Sepideh Parvizpour[3, 4], Ramin Soltani[1], Sorayya Ghasemi[5, 6*]

1. Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.

2. Department of Neurosurgery, Rasoul Akram Hospital, Iran University of Medical Sciences, Tehran, Iran.

3. Research Center for Pharmaceutical Nanotechnology, Biomedicine Institute, Tabriz University of Medical Sciences, Tabriz, Iran.

4. Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.

5. Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.

6. Cancer Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran.

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ABSTRACT

Glioblastoma multiforme (GBM) is incurable with routine treatments. Ascorbic acid (Asc) has antioxidant and anti-cancer properties. However, its specific anti-cancer mechanisms are only partially understood. In this study, the effect of Asc on the c-Myc, HIF-1α, and lnc-SNHG16 genes in GBM cells and their exosomes was investigated. Cells isolated from the tissue were characterized by the immunocytochemistry method ($GFAP^+$). The cell-doubling time was determined, and FBS-free medium supplemented with Asc (5 mM) was added to the cells. The extracted exosomes in the cell culture medium were scanned by electron microscopy, Zetasizer, and BCA assay. The expression of lnc-SNHG16 in the exosomes and c-Myc and HIF-1α in the treated and control cells was evaluated by real-time PCR. The interactions between Asc and the c-Myc and HIF-1α proteins were studied using the molecular docking method. The cells showed 90–100% $GFAP^+$ in passage 4, with a cell-doubling time of 4.8 days. Exosomal vesicles measuring 98.25–105.9 were observed. Zetasizer results showed a sharp pick at 90 nm. Protein quantitation showed 3.812 µg/ml protein in the exosomes. Lnc-SNHG16 expression was reduced ($P = 0.041$), and c-Myc was upregulated ($P = 0.002$). The expression of HIF-1α was not significant in the treated cells. Also, Asc was able to interact and affect c-Myc and HIF-1α. Asc exerts its effect by reducing lnc-SNHG16 expression in exosomes, upregulating c-Myc in GBM cells, and interacting with HIF-1α and c-Myc. Further research is necessary to achieve a full understanding of these findings.

Keywords: Glioblastoma tissue, primary cell culture, Ascorbic acid, c-Myc, HIF-1α, lnc-SNHG16

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*Corresponding: Sorayya Ghasemi
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E-mail: sorayya.ghasemi@gmail.com

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Introduction

The most common type of CNS tumor is glioblastoma multiforme (GBM), which presents a tremendous challenge because of its poor prognosis despite treatments, such as surgery, radiation, and chemotherapy [1]. Even with the development of alternative therapy modalities, the development of multidrug resistance (MDR) to traditional chemotherapy continues to be a significant factor contributing to the recalcitrance of GBM [2]. Amidst these challenges, ascorbic acid (Asc), a vital antioxidant, has been demonstrated to have potential therapeutic benefits by causing hydrogen peroxide-dependent cytotoxicity in cancer cells while protecting healthy cells [3]. Designing molecularly focused treatment strategies requires investigating the mechanisms of message transmission in glioblastoma stem cells (GSCs) and acquiring sufficient understanding of the associated processes. Extracellular vesicles (EVs) are double-membraned structures (150–300 nm) that are discharged from most cells into the extracellular environment, carrying a variety of biological components, including nucleic acids (mRNA, microRNA, and lncRNA). Actually, exosomes are in charge of communicating with other cells nearby, to alter the latter's performance. Long non-coding RNAs (lncRNAs) are one kind of compound found in exosomes [4]. Despite the widespread expression of lncRNAs in all cell types, abnormal patterns of expression, particularly in the setting of diseases like cancer, are of great importance [5]. The development of GBM has been associated with increased expression of SNHG16, an lncRNA generated by CSCs and detected within exosomes [6]. GBM is further complicated by the complex interactions between the oncogene c-Myc and the hypoxia-inducible factor 1-alpha (HIF-1α) gene. Asc's induction of HIF-1α degradation has significant effects on tumor cell survival in hypoxic environments [7]. An intriguing area of investigation is the interaction between c-Myc and HIF-1α and how it relates to the development of CSC characteristics [8, 9]. However, Asc's complex chemical pathways are yet to be fully explored. Therefore, the aim of the current investigation was to clarify how Asc affects doubling time, SNHG16 expression in exosomes released by CSCs, and c-Myc and HIF-1α expression in cultured GBM cells. We also explore the connections between Asc and the proteins c-Myc and HIF-1α *in silico*. We investigate the possibility of Asc-based therapies in GBM treatment by identifying these complex relationships.

Materials and methods

Isolation and culture of GBM cells

The ethics code (IR.SKUMS.REC.1401.014) was obtained from the Ethics Committee of Shahrekord University of Medical Sciences before the sampling, and written consent was obtained from the patient. GBM tumor tissue (grade 4) was obtained from Rasoul Akram Hospital in Tehran and immediately placed in phosphate-buffered saline (PBS; pH 7.4; BioIdea) containing 3% penicillin/ streptomycin (10,000 units/mL penicillin and 10 mg/mL streptomycin in normal saline; Pen/ Strpt; BioIdea) and transferred to the laboratory. The tissue sample was transported on ice until it reached the laboratory. Tumor cells were isolated according to the following steps: two tissue washing steps were performed in PBS containing 3% penicillin/ streptomycin, and the blood vessels were removed from the tumor tissue. Then, the tissue was cut into small pieces in the PBS. Afterward, trypsin-EDTA (0.25% trypsin, 1 mM; BioIdea) was used for the enzymatic digestion of tissues for 10 min at 37 °C. After a milky solution was produced, a culture medium containing

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fetal bovine serum (FBS) was used to neutralize the enzyme. Then, the solution was centrifuged at 1300 rpm for 10 min, and the pellet was cultured in Dulbecco's Modified Eagle's Medium with 1000 mg/mL glucose (DMEM/F12; BioIdea) medium containing 15% FBS and 1% Pen/Strpt. After 24 h, the culture medium was replaced, and the dead cells and excess tissues were removed. The culture medium was changed every two days until the cells reached 80% confluency. Then the cells were passaged into new flasks.

GBM cell identification and characterization

Immunocytochemistry (ICC) was used to confirm the nature of the isolated cells. After washing the cells at passage 3 with PBS, the cells were fixed in a 4% paraformaldehyde solution for 30 minutes. Then, the solution was removed, and the cells were washed. The permeability step was performed with Triton-x100 (Sigma-Aldrich st. Louis, MO, USA). Following the washing step, the cells were incubated for one hour at 37 °C with the primary antibody for GFAP (MAD-000716QD). The suppression step was done with H2O2. After the washing, the secondary antibody was added to the cells at room temperature for one hour. The cells were washed, and HRP (Sigma-Aldrich), conjugated to streptavidin, was added to the cells. After washing, DAB (ab94665, Abcam, Cambridge, UK) was added to the cells for 10 minutes, and then the cells were rinsed and evaluated under a microscope.

Determining the doubling time

First, 3000 cells per well were seeded in 96-well plates. After an overnight incubation period, the tumor cells were treated for 1 hour with 5 mM Asc [3]. Asc-free cells were considered the control group. Then, the cells were washed with PBS, and fresh medium was added to the cells. After that, the cells were trypsinized for 24, 48, 72, 96, and 120 h and were counted using a homocytometer slide. Then, the doubling time of the cells was calculated.

Exosome extraction and characterization

After the cells reached 80% confluency, the culture medium was exchanged, and an FBS-free medium supplemented with Asc (5 mM) was added to the cells. After 16 hours, the cell culture medium was collected in a falcon tube and stored at -80 °C until use. Exosome extraction was performed according to kit instructions. In summary, the collected medium was centrifuged at 3000 rpm for 20 minutes to remove cellular debris. After that, solution A from the kit was added to the resulting supernatant at a 5:1 ratio, and the falcon was vortexed for 5 minutes before being incubated overnight at 4 °C. The falcon was then vortexed for one minute before being centrifuged for 40 minutes at 3000 rpm at 4 °C. The Kit's solution B was used to dissolve the exosome pellet. The exosomes were stored at -20 °C until they were used. The cultured cells without FBS and Asc were taken as the control group. Exosome characterization methods include the BCA assay (cytomatingene), electron microscopy (ZEISS), and Zetasizer.

RNA extraction, cDNA synthesis, and real-time PCR

Primer3 web-based server and Integrated DNA Technologies (IDT) (Coralville, IA, USA) designed the primers for real-time PCR analysis of c-Myc, HIF-1α, lnc-SNHG16, and B-Actin (as an endogenous control). Their specificity was tested with Primer-BLAST on the NCBI genome browser. The used primer sequences are listed in Table 1. RNA was extracted from the treated and control cells and exosomes by RNX™-Plus (Sinaclon, Iran). After confirming the quantity of RNAs using a nanodrop device (Thermo Scientific™ NanoDrop 2000), cDNA synthesis was done by a cDNA synthesis kit (YTA, Iran) according to manufacturer protocol. Finally, the expression of lnc-SNHG16 in the extracted exosomes and c-Myc and HIF-1α in the

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treated and control groups was evaluated by real-time PCR on a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia) using SYBR Green Master Mix (YTA, Iran). The $2^{-\Delta\Delta ct}$ method was used to analyze the results.

Protein and ligand retrieval

The amino acid sequence of the target protein was retrieved from the UniProt database. The protein structure was modeled using the SWISS-MODEL online server. The drug structure was retrieved from PubChem and saved in SDF format.

Molecular docking analysis

A molecular docking study is generally employed to identify the intermolecular interactions between a macromolecule and a small molecule. To this end, AutoDock Vina was used to determine the best intermolecular interaction between the target proteins and the drug molecule based on the binding energy (kcal/mol). The binding interaction of the protein-ligand complex was investigated using BIOVIA Discovery Studio Visualizer v19.1.0.18287.

Statistical analysis

All data were presented as mean ± SD. All statistical analyses were performed using GraphPad Prism v. 8.4.3. Statistical tests were performed via the student's *t*-test and Mann-Whitney *U* test, and $P < 0.05$ was considered to be significant. All cell manipulation tests were performed in triplicate.

Results

GBM cell characterization

Isolated cells adhere to the flasks within 12–24 h. The star-shaped cells form a monolayer that is composed of astrocyte cells. The cells were at confluence within 14 days. GFAP, an astrocyte cytoskeleton protein, was used to distinguish astrocytes from other cell types in the central nervous system. Figure 1 shows that 90–100% of GFAP-positive cells were present in passage 4.

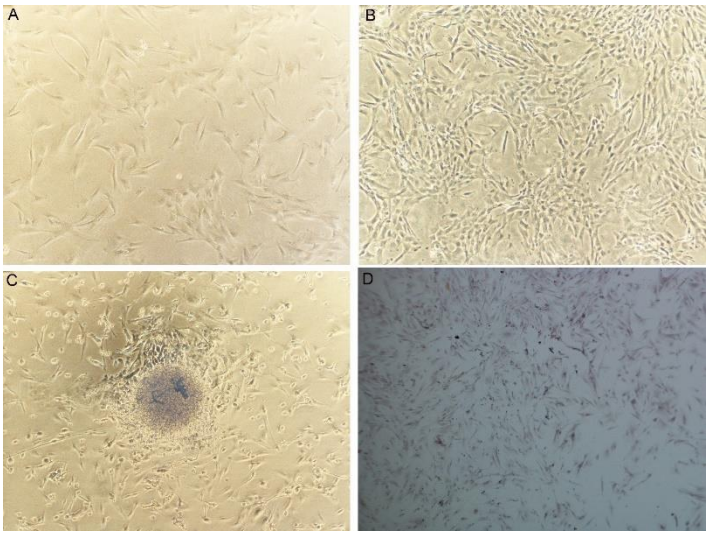

Fig.1. Microscopic images depicting GBM tumor cell isolation and subsequent characterization; **A. Isolated** GBM tumor cells observed after 10 days. **B.** Passage 2 of the isolated cells. **C.** Observation on day 13 of the isolated cells. **D.** Immunocytochemistry (ICC) results. In (**D**), the immunocytochemistry (ICC) assay highlights GFAP-positive cells (brown staining). (Microscope: Nikon; TS100; Magnification: 100X).

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GBM cell doubling time

The growth rate and doubling time of isolated GBM cells were evaluated based on the following formula: (https://www.doubling-time.com/compute.php) Doubling time = duration log (2) / log (final number of cells) log (initial number of cells). The doubling time of the GBM cells was 4.8 days (Figure 2).

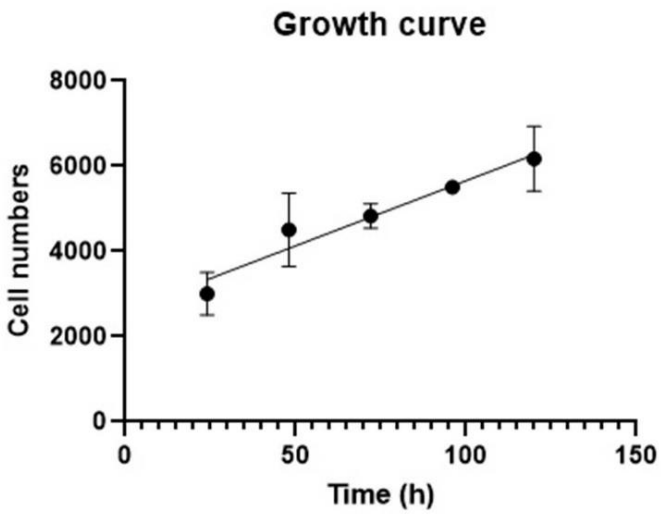

Fig.2. The growth curve of isolated GBM cells. The doubling time of isolated GBM cells was evaluated based on the following formula: (https://www.doubling-time.com/compute.php) Doubling time = duration log (2)/log (final number of cells) log (initial number of cells). The doubling time of the GBM cells was 4.8 days.

Characterization of GBM cell exosomes

The verification of isolated exosomes was done by scanning electron microscopy (SEM), Zetasizer, and BCA assays. Protein quantitation by the BCA assay showed 3.812 µg/ml protein in the isolated exosomes and indicated the presence of exosomes. Scanning electron microscopy revealed spherical vesicles ranging from 98.25 nm to 105.9 nm in diameter. Comprehensive validation of isolated exosomes through rigorous analysis of size distribution is depicted using Zetasizer data, showcasing a pronounced peak at 90 nm (Figure 3).

Real-time PCR results

Lnc-SNHG16 expression was reduced in the treatment group ($P = 0.041$). Also, c-Myc was upregulated in the treated group ($P = 0.002$). The expression change of HIF-1α was not statistically significant (Figure 4).

Molecular docking analysis

The amino acid sequences of HIF1A (A8MYV6) and C-MYC (Q6LBK7) were retrieved from the UniProt database. The SWISS-MODEL online server was used to build the protein models. The Asc structure was obtained with the PubChem CID (54670067) from the PubChem drug database and saved in SDF format.

The best intermolecular framework formed between the HIF1A and C-MYC proteins and Asc was identified through the molecular docking study using AutoDock Vina. The results indicated that the binding affinity of C-MYC (-5.9 Kcal/mol) is better than that of HIF1A (-4.6 Kcal/mol), and Asc can interact with and affect both of these proteins. Figure 5 shows the details of these interactions.

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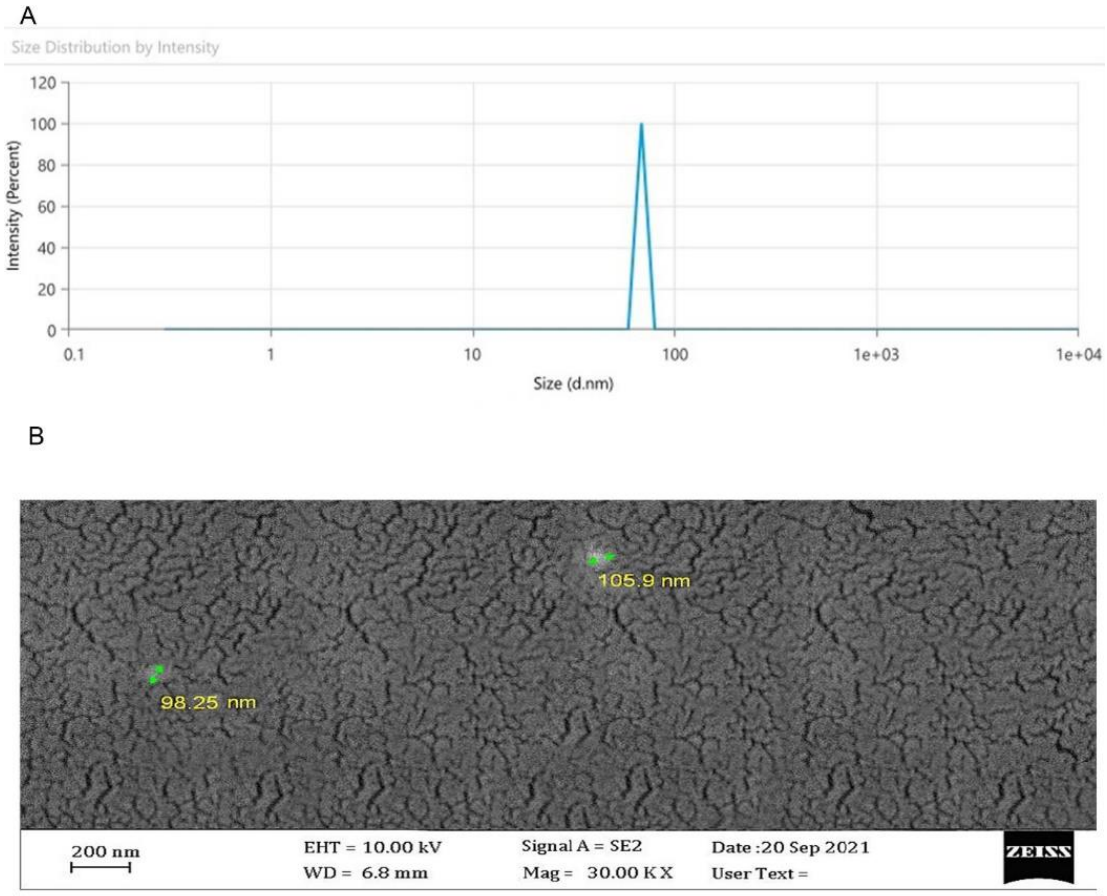

Fig.3. Verification of isolated exosomes A. Zetasizer analysis revealed a distinct peak at 90 nm, indicative of exosome size distribution. (Method: dynamic light scattering) B. SEM image showcasing spherical vesicles within the size range of 98.25–105.9 nm (Microscope: electron microscopy; ZEISS).

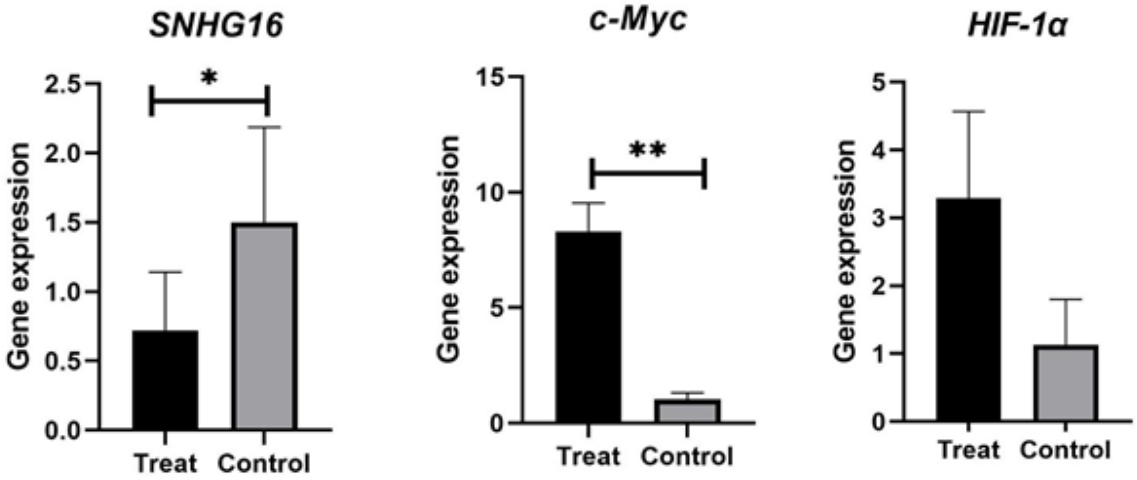

Fig.4. The gene expression results. A. Lnc-SNHG16 was downregulated in the treated group (P = 0.041). B. c-Myc was upregulated in the treated group (P = 0.002). C. The expression change of HIF-1α was not statistically significant. The reference gene is β-Actin; Treated: GBM cells cultured with 5 mM Asc; Control: GBM tumor cells cultured in Asc-free medium.

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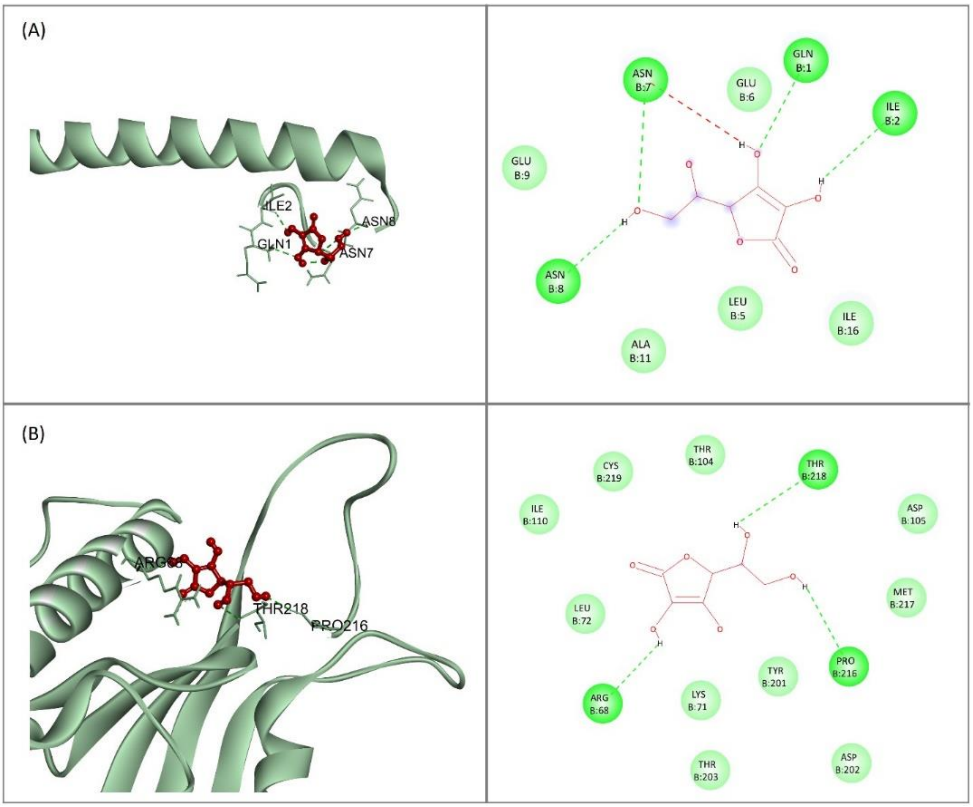

Fig.5. Three-dimensional (left) and two-dimensional (right) images of protein-ligand interactions involving the model proteins C-MYC (A) and HIF1A (B) and the Asc PubChem CID (54670067).

Discussion

Medicinal ascorbate (plasma levels> 10 mM) has shown potential as a cancer drug with selective toxicity to cancer cells [10]. It exploits the altered cancer cell metabolism to induce vulnerability, as validated by preclinical and clinical studies, although the associated signaling pathways remain unexplored.

In this study, GBM cells were isolated. Preliminary investigations, including cell characterization and the doubling time of these cells, were conducted. After cultivation and treatment with Asc, the exosomes of both groups (treated and control) were extracted, and gene expression studies were performed for exosomes and cells.

Then, the expression of the c-Myc and HIF-1α genes and lnc-SNHG16 in the GBM primary culture was evaluated by real-time PCR after treatment with Asc. As a result of treatment with Asc, there were changes in c-Myc in the cells and lnc-SNHG16 in the exosomes secreted from these cells. Furthermore, the interaction of c-Myc and HIF-1α proteins with Asc was detected and confirmed *in silico*.

Parallel with these results, preclinical research and clinical trials have shown that Asc is a feasible, selectively toxic, tolerable, and potentially effective chemical for treating GBM and NSCLC [11]. In patients with ovarian and pancreatic cancer, medicinal doses of Asc combined with chemotherapy medications have been proposed as a viable anticancer treatment, with tolerability comparable to or better than chemotherapy alone [12, 13]. Our research shows no observable influence on HIF-1α expression, despite certain findings

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suggesting a connection between higher Asc and lower HIF-1α activity [14]. However, docking interactions imply that protein function may be altered.

In the regulation of CSCs via Notch, Wnt/β-catenin, and hedgehog pathways, c-Myc is a key player [15]. As a result, c-Myc is one of the genes that should be targeted to regulate these pathways in CSCs. However, we found in this experiment that Asc can alter the amount of c-Myc in GBM cells and even inhibit the function of this protein.

Although our results showed that the expression of HIF-1α was unchanged by Asc, its docking interaction with Asc can influence its function. Therefore, it can be said that Asc is effective in this way.

The ROS production of some anticancer drugs was inhibited by Asc. In contrast, antioxidants, some of which have been used as dietary supplements among the general population, showed varying effects[16]. The positive outcomes seen in a clinical phase I study combining Asc with radiation and temozolomide (TMZ) are suggestive of the potential for this interaction to enhance therapeutic outcomes [17].

Finally, we suggest protein tests, estimates of safe doses, and analyses of interactions in the human system be conducted to further confirm and develop the findings this study. Our findings imply that although Asc may not directly affect the expression of genes relevant to hypoxia and CSC, its docking can possibly impact protein function, rendering it effective cancer treatment.

In conclusion, this study emphasizes ascorbic acid's (Asc) potential as a treatment for GBM. The study shows its influence on significant molecular pathways within GBM cells, causing alterations in lnc-SNHG16 and c-Myc expression and potential interactions with HIF-1α and c-Myc. These results point to Asc's ability to have a significant impact on GBM biology. Further clinical investigations are necessary to confirm these findings and assess whether including Asc in GBM treatment plans is practical.

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