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Research Article

Effect of temperature changes on the expression of cancer stem cell protein CD-44 and TAU protein in AMGM-5 cancer cell line: An immunocytochemistry study

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Abstract

Objectives: Glioblastoma multiforme (GBM) has long been one of the most common and particularly invasive malignant gliomas. High-grade gliomas are highly prone to relapse and associated with poor prognosis. This study tests the hypothesis that hyper-thermal conditions could influence TAU and CD-44 protein expression by increasing temperature in glioblastoma cancer cell line culture.

Methods: AMGM cancer cells were cultured and maintained under normal growth conditions, then separated into two groups: one group was cultured at 37°C, and the other at 40°C. After 24 hours of growth, cells underwent immunocytochemistry (ICC) to visualize the localization of TAU and CD-44 markers.

Results: The results show that fewer AMGM cells remained stable enough to grow at 40°C; these cells lost their fusiform shape and became spherical compared to cells grown under normal conditions. Additionally, an increase in microenvironmental temperature significantly affected TAU protein expression in the nucleus of AMGM cells, with a 71.4% increase at 40°C. In contrast, the expression of CD-44, typically expressed on the cell membrane of AMGM cells, decreased by 42.9% at 40°C.

Conclusion: Changes in the microenvironment may affect glioblastoma cell line development by influencing the cancer stem cell marker CD-44 and the microtubule-stabilizing protein TAU. These markers could serve as potential targets for the treatment and prevention of glioblastoma.

Keywords: AMGM cancer cell line, CD-44, hyper-thermal, TAU

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The tumor microenvironment (TME) refers to the conditions under which tumor cells grow, communicate, and die [1, 2]. In 2011, Ungefroren and his team defined TME as the context that controls tumorigenesis, including processes such as epithelial-mesenchymal transition (EMT), migration, invasion, metastasis, apoptosis, and chemotherapeutic drug resistance [3].

Various types of microenvironments are commonly used and identified in *in vitro* cell cultures, including thermal and serum conditions. Studies have shown that hyper-thermal treatment can enhance tumor shrinkage and decrease oxygen consumption [4]. Hyperthermia is one of the few promising strategies among alternative therapies for cancer treatment [5].

For years, gliomas have been recognized as highly heterogeneous tumors at the molecular level, with varying survival times, even among patients with the same grade [6]. According to the American Association of Neurological Sur-

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geons (2023), glioblastoma (grade IV glioma) is the highest-grade glioma, clinically representing the most common and aggressive primary brain tumor with poor patient survival rates. Experimental data suggest that the low survival rate may partially be due to the presence of glioma stem cells (GSCs) [7].

The quiescence of GSCs in their niches, effective DNA damage repair, drug transporter activity, and Notch signaling are factors contributing to therapy resistance [8]. To improve glioblastoma treatment outcomes, GSCs must be eradicated. Hyperthermia, particularly in combination with irradiation, is emerging as a promising therapeutic approach, as it appears that multiple DNA repair pathways in GSCs are sensitive to hyperthermia [9].

The cancer stem cell marker CD-44 is a transmembrane glycoprotein that functions as a hyaluronic acid receptor. CD-44 has been implicated in EMT and tumor invasion [10]. A recent review highlighted CD-44 as a predictor of chemotherapy resistance in mesenchymal-like glioma [11] and as a factor in GBM prognosis. CD-44 inhibition has been suggested as a therapeutic strategy for several malignant tumors [12].

Tau protein, also known as microtubule-associated protein (MAPT), was identified in 1986 as a protein that binds to and stabilizes microtubules [13]. Under normal physiological conditions, phosphorylation regulates Tau's binding to microtubules and other functions [14]. However, hyperphosphorylation of Tau protein leads to its aggregation and the formation of neurofibrillary tangles [15], which are key features in the development of Alzheimer's disease (AD). Tau pathology is correlated with neurodegeneration and AD progression [16, 17], making Tau phosphorylation a viable target for treating AD and other tauopathies, though no treatments currently exist [18].

Studies on the effect of hyper/hypo-thermal conditions on cancer stem cell markers *in vitro* have suggested that hyperthermia at 46°C for 10 minutes can induce high levels of cancer cell death in pancreatic ductal adenocarcinoma compared to non-malignant cells [19].

The combined effect of chemotherapy and hyper-thermal treatment on cancer cell proliferation was explored in a 2014 study by Lee. Metformin alone, or in combination with hyperthermia, showed cytotoxicity against cancer stem cells (CD-44/CD24) in MCF-7 human breast cancer cells and MIA PaCa-2 human pancreatic cancer cells. The authors applied heating at 42°C for 1 hour, finding it partially toxic to cancer cells and CSCs and that it enhanced metformin's efficacy in reducing both cancer cells and CSCs [20]. Severe hyperthermia (45°C for 1 hour) was also found to reduce viability and induce apoptosis in MG-63 osteosarcoma cells, with increased activities of caspases 3/7, 4, and 12 after 72 hours at 37°C [21].

This study aims to determine the presence of TAU (MAPT) and CD-44 markers in glioblastoma cancer cell lines before and after exposure to hyper-thermal conditions at 40 °C for 24 hours using the immunocytochemistry (ICC) technique.

Materials and Methods

Cell culture maintenance and heat treatment

The glioblastoma AMGM cancer cell line was obtained from the Cell Bank Unit at the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) and cultured in RPMI-1640 (Sigma-Aldrich, Germany) supplemented with 10% calf bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cancer cell line falcons were passaged using Trypsin-EDTA (US Biological, USA) and incubated at 37°C.

To determine the expression of TAU and CD-44 in AMGM cells, cells were seeded at 4×10^5 cells per 60-mm dish in 3 mL of medium 24 hours before experiments at 37°C and 40°C. Each experiment was performed in triplicate and repeated twice (Fig. 1).

Immunostaining of TAU and CD-44 proteins

The primary antibodies used were mouse monoclonal anti-TAU (A-10, Abcam, 1:20 from 1 mg/mL) and anti-CD-44 (sc-9960, 1:20 from 1 mg/mL; Santacruz). Immunostaining was performed overnight with primary antibodies. Cells were incubated for 1 hour at room temperature in the dark with secondary conjugated antibodies (dilution 1:200 from 1.5 mg/mL; Pathinsitu). Slides were then covered with DPX mounting medium containing DAPI to counterstain the nuclei for CD-44 and the cytosol for TAU. A coverslip was applied, and the slides were analyzed under an Olympus light microscope at 400x magnification. Microscopy images were captured using a MICROS CAM 500 "PREMIUM" camera with Microvisible software.

Scoring

The number of stained cells was examined under microscopy and scored as follows: 0=no expression or stained cells; 1=5% of cells; 2=≥5% of cells; 3=≥25% of cells; 4=≥50% of cells; and 5=≥75% of cells.

Statistical analysis

Statistical comparisons between groups were conducted using the one-tailed Mann-Whitney U-test. A probability value of $p<0.05$ was considered statistically significant. Analyses were performed with SPSS-24 statistical software and Microsoft Excel.

Results

Maintenance and culturing of AMGM glioblastoma cell line

The AMGM glioblastoma cell line was initially established at the Iraqi Center for Cancer and Medical Genetics, Mustansiriyah University [22]. The specific staining criteria for the definition of primary glioblastoma cell lines and to check whether the glioblastoma-derived primary cell lines have similarities to glioblastoma tissue, we performed immunocytochemistry staining for markers commonly used in glioma diagnosis. We first observed the morphology and colony formation of cells

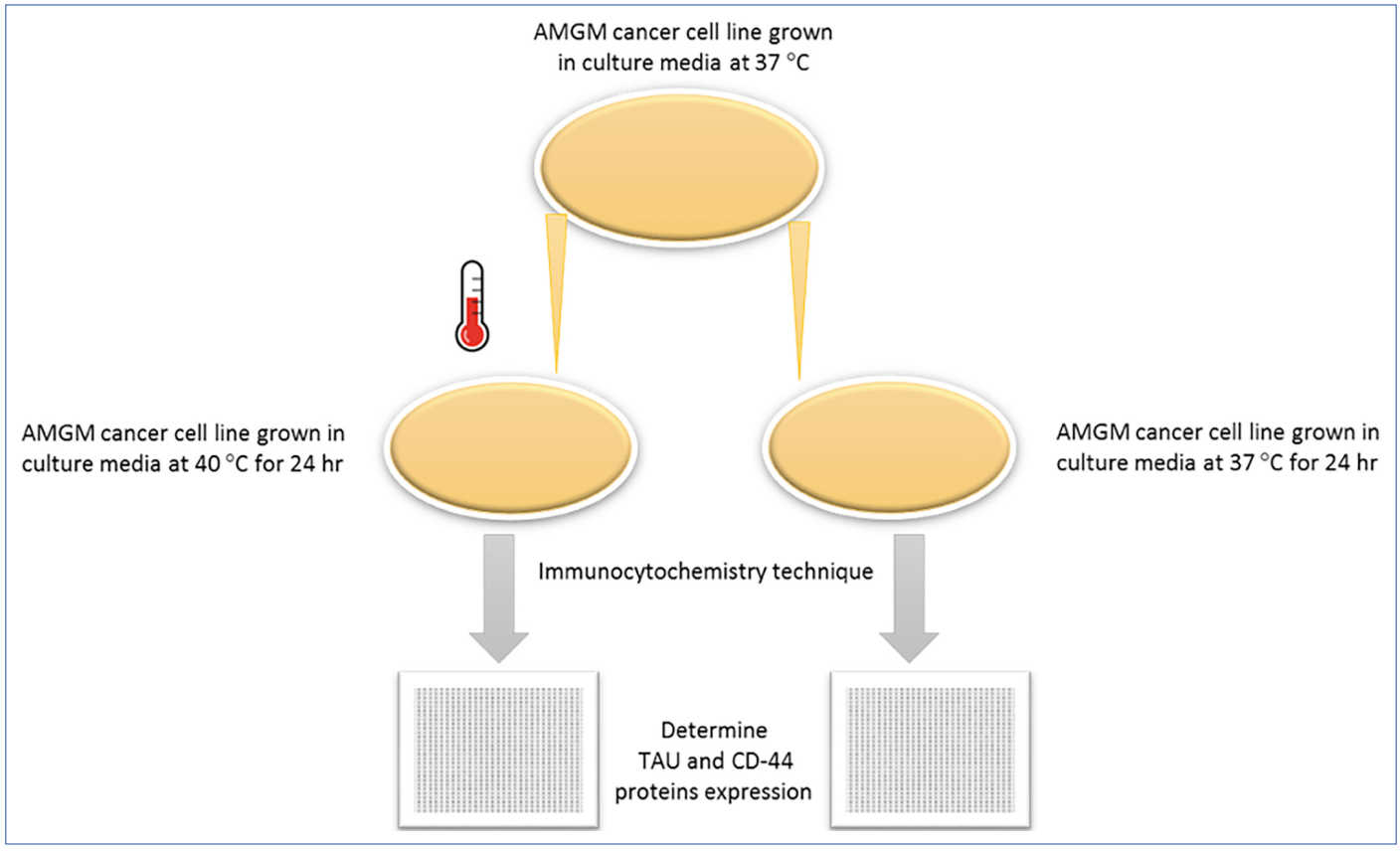

Figure 1. Summary determines the effect of incubation AMGM cancer cells under temperature (40°C) on TAU and CD-44 protein expression compared to normal incubation culture conditions (37°C).

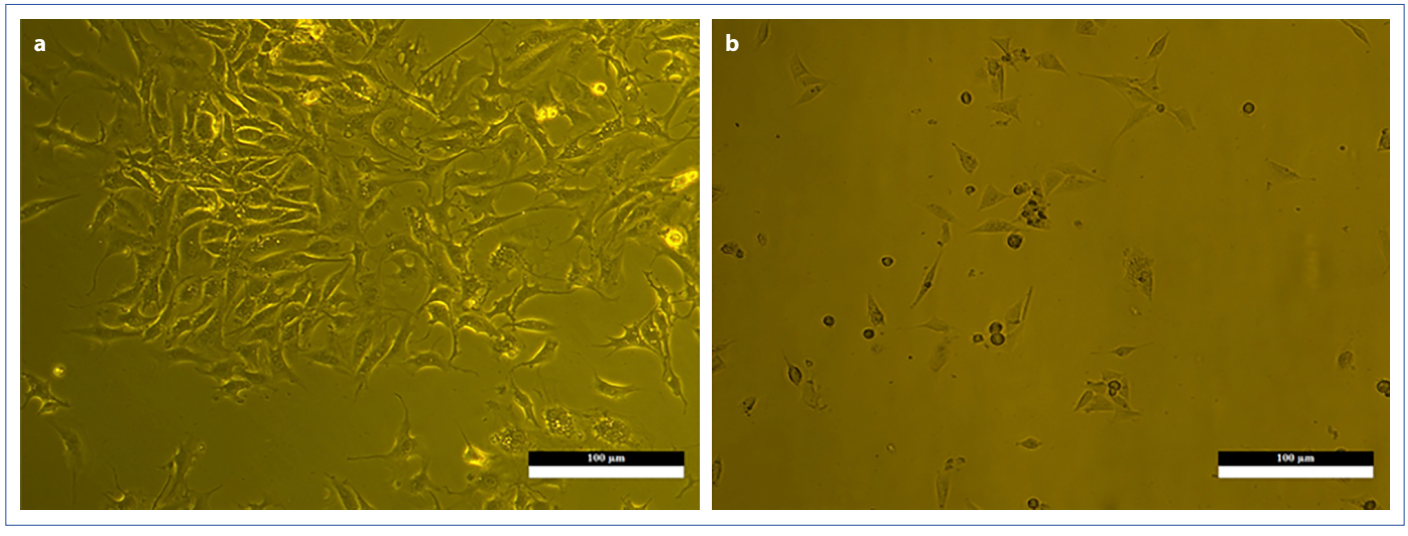

Figure 2. Growing AMGM cancer cell line culture at two different incubation temperatures: (a) 37°C and (b) 4°C under an inverted microscope. This shows how the glioblastoma cells were monolayer and had a fusiform at optimal incubation temperature, compared to cells cultured in high atmosphere temperature (40°C) the cells lose their unity and start to circle in shape an indication of apoptosis.

under both incubation conditions before staining (Fig. 2). Under standard incubation conditions, cells formed a single flattened layer, appeared elongated, and had a fusiform shape (Fig. 2a). In contrast, at 40°C, fewer cells retained the fusiform shape, and cell numbers were reduced (Fig. 2b).

CD-44 and MAPT staining: ICC

The results of Immunocytochemistry of both markers the CD-44 and MAPT in AMGM cancer cells cultured in two different incubation temperatures 37°C and 40°C were shown in Figures 3 and 4 respectively. In Figure 3a, TAU protein

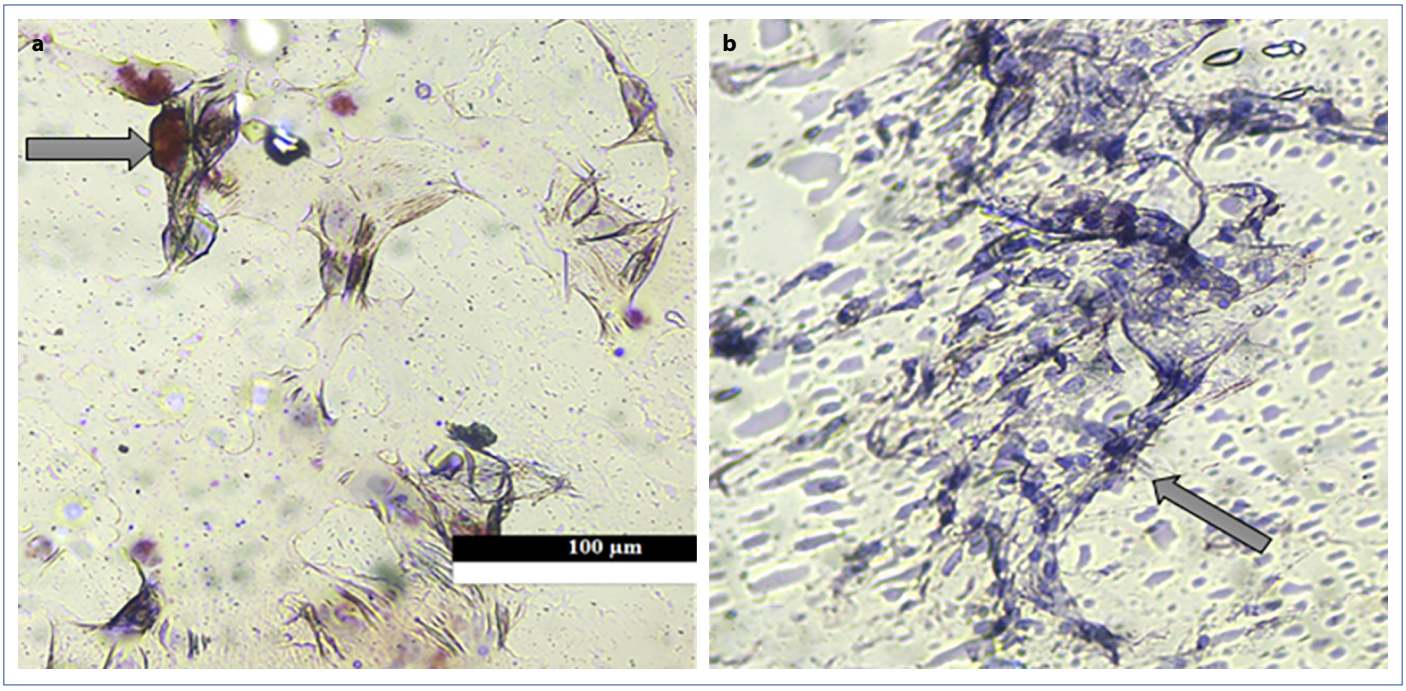

Figure 3. Immunocytochemistry staining of tau in glioblastoma cancer cell line. (a) Represents cells incubation culture at 37°C while (b) Represents culture cells at 40°C. the arrows show TAU protein expression in the nucleus of the glioblastoma cell line.

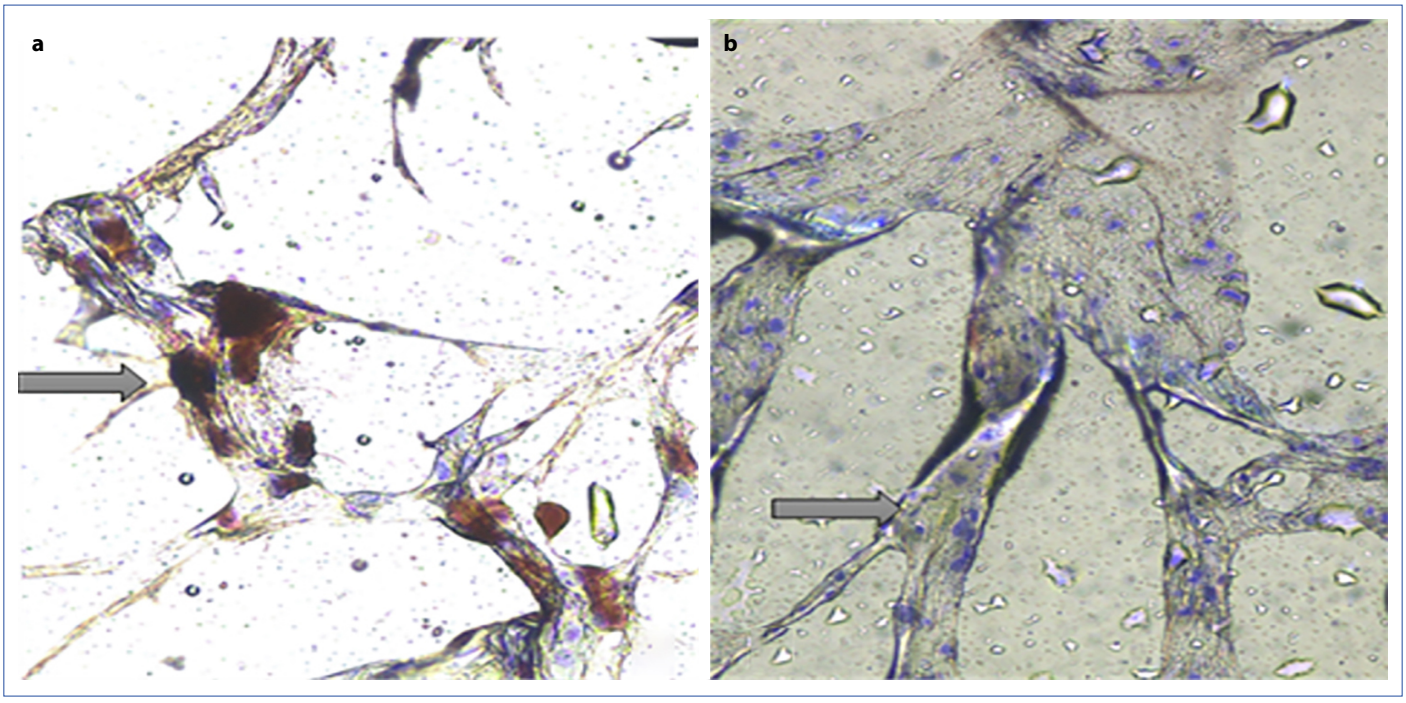

Figure 4. Immunocytochemistry staining of CD-44 in glioblastoma cancer cell line. (a) Represents cells incubation culture at 37°C while (b) Represents culture cells at 40°C. the arrows show CD-44 protein in the nucleus of the glioblastoma cell line.

was poorly expressed in the nucleus of glioblastoma cancer cells growing at 37°C compared to B- expressed highly when growing at 40°C.

Next, in Figure 4c, CD-44 protein was expressed in the cell membrane of glioblastoma cancer cells growing at 37°C compared to D- expressed highly when growing at 40°C.

Tables 1 and 2 show the scoring of both TAU and CD-44 proteins in the growing glioblastoma AMGM cancer cell line in two different culture conditions 37°C and 40°C respectively. In Table 1, TAU protein expression was affected by increasing the temperature where the scoring was highly +3 in 71.4 % at 40°C significantly compared to

Table 1. Tau marker expression in selected AMGM cell line while cultures in both 37°C and 40°C respectively

AMGM cells cultured conditions	Tau 1+ (%)	Tau 2+ (%)	Tau 3+ (%)	p
37°C	-	57	42.9	0.001
40°C	28.6	-	71.4	

Table 2. CD-44 marker expression in selected AMGM cell line while cultures in both 37°C and 40°C respectively

Cells cultured conditions	CD-44 1+ (%)	CD-44 2+ (%)	CD-44 3+ (%)	p
37°C	-	-	100	0.02
40°C	28.6	28.6	42.9	

growing the cells at 37°C the score +3 was determined in 42.9% of the cells with p<0.001.

In Table 2, CD-44 protein expression was affected by increasing the temperature where the scoring highly +3 in 100 % of the glioblastoma AMGM cell line at 37°C significantly compared to growing the cells at 40°C the score of +3 was determined in 42.9% of the cells with p<0.02.

Discussion

Glioma is known for its high heterogeneity, influenced by genetic, epigenetic, and tumor microenvironmental factors. This heterogeneity is linked to the adaptive response, treatment resistance, and overall behavior of brain tumors [23]. Understanding the mechanisms underlying this heterogeneity is critical for advancing glioma diagnosis, treatment, and potentially prevention.

This study aimed to explore whether changes in the microenvironment affect the development of glioblastoma in the AMGM cell line by examining the expression of TAU protein and the cancer stem cell marker CD-44. The microenvironmental change tested here was an increase in incubation temperature, with AMGM cells cultured at 37°C and 40°C. Immunohistochemistry staining revealed that elevated temperature increased TAU protein expression while decreasing CD-44 levels. A limitation of our study was the lack of a normal cell line for comparison, as initially planned.

According to a study by Lim et al. [24], glioblastoma cell aggressiveness increases with tumor growth and migration. These cells secrete various molecules, including soluble CD-44 and adhesion molecules, into the extracellular matrix, which can induce neuronal degeneration by activating TAU protein.

TAU protein is encoded by the MAPT gene on chromosome 17 and is primarily expressed in neuronal axons. It normally facilitates the polymerization and stabilization of microtubules [25]. TAU is extensively post-translationally modified, and in Alzheimer's disease (AD), it detaches from microtubules and aggregates to form plaques [26].

Recent research has shown that TAU depletion significantly inhibits *in vitro* spheroid growth of the glioblastoma cell line U87-MG and reduces 2D cell proliferation [27]. A correlation between TAU expression and heat shock proteins has also been documented, with HSP70, a chaperone protein, mediating the ubiquitinylation of aberrant TAU species for selective elimination [28].

Another recent study utilizing RNA sequencing data suggested that CD-44 could serve as a biomarker for M2 tumor-associated macrophages (TAMs), promoting immune suppression and glioma progression in the tumor microenvironment [29]. Johansson et al. [30] demonstrated that CD-44 is activated under hypoxic conditions by interacting with HIF-2α to enhance hypoxia in glioma stem cells. They proposed that blocking CD-44-ligand interactions through antibodies, inhibiting external cleavage of CD-44, or using gamma-secretase inhibitors to inhibit internal cleavage could mitigate these effects.

A separate transcriptomic study noted that a reduction in CD-44 levels in U251MG glioblastoma cells suppressed cell growth and induced cellular senescence. The authors proposed that CD-44 could serve as a marker for hypoxia, glycolysis, and anti-tumor immune responses [31].

In summary, our study demonstrates that an elevated microenvironmental temperature of 40°C affects the expression of the cancer stem cell marker CD-44 and increases TAU protein expression in glioblastoma AMGM cancer cell cultures.

Conclusion

In conclusion, this preliminary study found that hyperthermia at 40°C increased TAU protein and decreased CD-44 expression in the AMGM glioblastoma cancer cell line. These findings suggest that controlling the cellular environment's temperature could be a potential therapeutic approach for glioblastoma. Further studies examining chronic or repeated mild hyperthermia exposure in glioma mouse models are required to assess its effects on cancer progression and related diseases.

Ethics Committee Approval: The local Institutional Review Board deemed the study exempt from review.

Authorship Contributions: Concept – Z.S.A., N.A.M.; Design – Z.S.A., N.A.M.; Supervision – Z.S.A., N.A.M.; Data collection &/or processing – Z.S.A., N.A.M.; Analysis and/or interpretation – N.A.M., N.M.K.; Literature search – Z.S.A.; Writing – Z.S.A.; Critical review – Z.S.A., F.S.S.

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