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Expression and significance of ALDH1A1, CD44, and OCT3/4 stem cell markers in glioblastoma tissues: an immunohistochemical study in Iraqi patients

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Abstract

Cancers of the brain and nervous system are among the top five most common malignancies affecting both men and women in Iraq. Improvements in diagnostic techniques alongside increased medical awareness have facilitated earlier detection, thereby potentially improving patient outcomes. Cancer stem cells (CSCs) have been recognized as key players in the initiation, progression, and recurrence of tumors, including glioblastoma, the most aggressive form of brain cancer. These CSCs are characterized by specific markers that contribute to tumor growth, resistance to therapy, and poor prognosis. In this study, we collected 26 glioma tissue samples from Iraqi patients and classified them according to tumor grade. Using immunohistochemical methods, we investigated the expression patterns of three important CSC markers—ALDH1A1, CD44, and OCT3/4—across different glioblastoma grades. Our findings demonstrated a significant upregulation of cytoplasmic ALDH1A1 and membrane-bound CD44 in higher-grade tumors (grades III and IV), with P-values of less than 0.0174 and 0.0013, respectively. Additionally, nuclear OCT3/4 expression was markedly increased in these advanced tumor grades ($P < 0.05$), suggesting a role in tumor aggressiveness and stemness. These data provide compelling evidence that ALDH1A1, CD44, and OCT3/4 are not only involved in glioblastoma progression but may also serve as useful prognostic biomarkers. Furthermore, their elevated presence in more malignant tumors highlights their potential as targets for novel therapeutic interventions aimed at improving treatment efficacy and patient survival. This study thus contributes valuable insights into the molecular landscape of glioblastoma in the Iraqi population and sets a foundation for future research in targeted cancer therapy.

Keywords: ALDH1A1, CD44, OCT3/4, Brain tumor, Cancer stem cells.

1. Introduction

Neoplasms of the brain and central nervous system (CNS) represent a diverse group of tumors within two types of primary and metastatic brain tumors [1]. The percentage of glioblastoma to malignant brain tumors was recorded at 49%, and 30% are diffusely infiltrating lower-grade gliomas. Other malignant brain tumors include primary central nervous system lymphoma (7%), malignant forms of ependymomas (3%) and meningiomas (2%) [2].

Brain and other nervous system cancer is the 10th leading cause of death for men and women. In the United States 2023 registry, it was estimated that 24,810 adults (14,280 men and 10,530 women) were diagnosed with primary cancerous tumors of the brain and spinal cord. [3]

In Iraq, the distribution of cancer incidence in the brain and other central nervous systems comes in the 4th rank (6.2%) after breast, lung, and colorectal cancer, according to the Annual report of the Iraqi Cancer Registry 2020 [4]. The percentages were (19, 7, and 6.9%), respectively.

Public awareness regarding brain cancer needs to be raised in the Iraqi and worldwide cultures to find more effective preventive and treatment modalities. Due to the highly invasive and malignant nature of GBM, the treatment remains challenging, with a high recurrence rate. [5, 6]

The strategies of current cancer treatment involving radiation or chemotherapeutic drugs were based upon the model of cancer and target rapidly dividing cells, leaving the quiescent and highly chemo-resistant cancer stem cell markers (CSCs) behind. This highlights the drastic need to develop new drugs and treatment strategies that can also target CSCs. Novel diagnostic, prognostic, and predictive biomarkers and therapeutic targets in tumor cells are important for optimizing the choice and efficacy of therapies [7, 8], improving the accuracy of diagnosis and prediction of therapeutic responses and overall survival of patients.

It was identified that the known potential biomarkers in cancer cells include stem cell-like markers: CD133, aldehyde dehydrogenase ALDH1A1, CD44, and CD24. Besides, different pluripotency-associated transcription factors: Oct3/4, Nanog, Sox2, and Myc, might also be suggested as molecular biomarkers to predict metastases, treatment resistance, and patient relapse [9, 10].

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The study of [11] determined the possible resistance mechanisms to CD44-targeted drug delivery through increased activity of the drug efflux channels, along with reversal of drug effect via increased ALDH1A1 activity and downregulation of CD44 expression.

The study [12] indicated a mechanism by which colon cancer progression through soluble factors released by tumor-associated dendritic cells (TADCs) is responsible for increasing cancer stem cell (CSC) properties indicated by increasing CD44 and ALDH1A1, cell mobility, and epithelial-to-mesenchymal transition (EMT) by increasing N-cadherin and Vimentin.

Also, the importance of OCT3/4 in cancer was determined in the review [13] that described several mechanisms suggested for this octamer: positive correlations between OCT4 expression in cancer stem cells and chemotherapy resistance, tumorigenicity, metastasis, and clinical prognosis.

Understanding the interactions between cancer stem cell markers and brain tumor development is important to provide prospective targets for therapeutic strategies and selective biomarkers. This study aimed to determine the correlation of cancer stem cell markers: CD44, ALDH1A1, and OCT3/4 in different stages of brain tumors in Iraqi patients.

2. Materials and methods

2.1. Ethical considerations

This study was performed in agreement with the Declaration of Helsinki and in accordance with the ethical committee of the Iraqi Center for Cancer and Medical Genetics Research / Mustansiriyah University / Baghdad / Iraq (25/2019). Written informed consent was required for the present study.

2.2. Type of samples and reasons for selection

For this study, 26 samples were recruited to investigate the expression level of cancer stem cell markers ALDH1A1, CD44, and OCT3/4 in different brain cancer stages. Glioblastoma samples from 26 patients (18 men and 8 women) who underwent tumor resection at the Neurological Science Hospital, Ministry of Health and Environment, Baghdad, Iraq. The age range was 9 to 57 years, including 15 females and 11 males. Six tissue samples were benign, while the remaining samples were graded from G I to G IV according to the pathologist's assessment, as detailed in Table 1. The tumor grade of the tissues was determined according to the WHO criteria by the pathologist in the hospital. All tissue samples were micro-dissected to exclude tissue areas presenting necrosis or not matching GBM diagnostics.

Table 1. Patients' clinicopathology features.

Variables	n	Percentage (%)
Median age: (range) (years)		45 (9-57)
Histological cell type		
Benign	6	23.07
Astrocystic glioblastoma	7	26.92
Glioblastoma	13	50
TNM stage		
I	4	15.38
II	3	11.53
III	8	30.76
IV	11	40.30
Grade G		
1	3	11.53
2	5	19.23
3	7	26.92
4	11	40.30

2.3. Inclusion criteria

All samples of glioblastoma have different stages during the study period. Volunteers of all ages and sexes were selected in the inclusion criteria.

2.4. Exclusion criteria

Post and prior-chemotherapy treatment samples, old tissue, and other glioma tissues were excluded from this study.

2.5. Immunohistochemistry technique

The primary antibodies used were anti-ALDH1 (A1334-33W, US-bio, USA), anti-CD44 (Sc-9960L, USA), and anti-OCT3/4 (Sc-5279, USA). Immunohistochemistry was performed on a paraffin-embedded human tumor tissue, diagnosed as glioblastoma grade I-IV according to the WHO classification. The tissue was sectioned 4-µm thick and de-paraffinized. To unmask epitopes, the tissue was boiled in citrate buffer (pH 6.0) for 20 minutes. After washing in TRIS buffer (pH 7.6) for 5 minutes, an endogenous peroxidase quench was done in 3% H_2O_2 for 15 minutes at room temperature followed by blocking with Avidin /Biotin (Vector) for 15 minutes each. To block nonspecific binding sites, 5% goat serum (Normal, Dako) diluted in Antibody solution was used. The anti-ALDH, anti-CD44, and anti-OCT3/4 antibodies were diluted in Antibody solution (1:50). Antibody incubation was done at 4°C using 200 µL per slide overnight. Detection was performed with ImmunoCruz LSAB staining System, Peroxidase/DAB (sc-2050, USA), and Rabbit/Mouse. Slides were incubated for 15 minutes in a humidified chamber at room temperature with biotinylated secondary antibody and then with streptavidin peroxidase antibody for 15 minutes. Slides were incubated with DAB chromogen working solution for 4 minutes. Counterstaining was done with hematoxylin. Dehydrated and mounted with water-free mounting medium (DPX), then analyzed by Olympus light microscope at 400x. Microscopy images were captured using a MICROS CAM 500 “PREMIUM” camera and included Micro-Visible software.

2.6. Evaluation of staining

For ALDH1A1 and CD44 expression analysis, cytoplasmic, membranous and intra-glandular debris staining was taken as positive staining, and nuclear localization staining for Oct 3/4 expression. The cells were categorized based on their positive staining, with zero (indicating no staining), and scores of 1 (weak positive staining), 2 (moderate positive staining), and 3 (strong positive staining). For qualitative analysis, the positive stain intensity was classified into four categories: none, weak, moderate, and strong expression. Scores of zero and 1 were considered low expression, whereas scores of 2 and 3 were grouped as high expression [14].

2.7. Statistical analysis

The difference between different grades of tissues of brain benign and metastatic tumors was analyzed using the program GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as mean, median, and standard deviation. ANOVA one-way Kruskal-Wallis test statistic was utilized to determine the value of the expression levels between benign and metastatic brain cancer tissues. A P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Immunohistochemically staining of paraffin sections from 26 resected GBMs was performed to confirm ALDH1A1, CD44, and OCT3/4 expression in different cancer stages tissues.

3.2 Immunohistochemistry shows ALDH1 expression in different grades of glioblastoma tissues.

Immuno-expression of ALDH1A1was predominantly found in the cytoplasm. Varying degrees of ALDH1A1expression were found in 20 tumor samples, and 6 specimens were negative. Interestingly, ALDH1A1 was significantly ($P<0.0174$) expressed in tumor cells was mainly observed in the GIII–IV of and rarely in GI and GII tumors (Figures 1 and 2).

3.3 Immunohistochemistry shows CD-44 expression in different grades of glioblastoma tissues.

On the other hand, CD44 staining was detected in the cell membrane, although cytoplasmic staining was also observed in some cases. Immunostaining of CD44 in stage III and stage IV glioblastoma tissues showed a significant increase ($P<0.0013$) in tumor cells as compared with stage I and stage II cancer cells (Figures 3 and 4).

3.4. Immunohistochemistry shows OCT3/4 expression in different grades of glioblastoma tissues.

Immuno-expression of OCT3/4 was found in the nucleus and, to a lesser extent, in the cytoplasm. OCT3/4

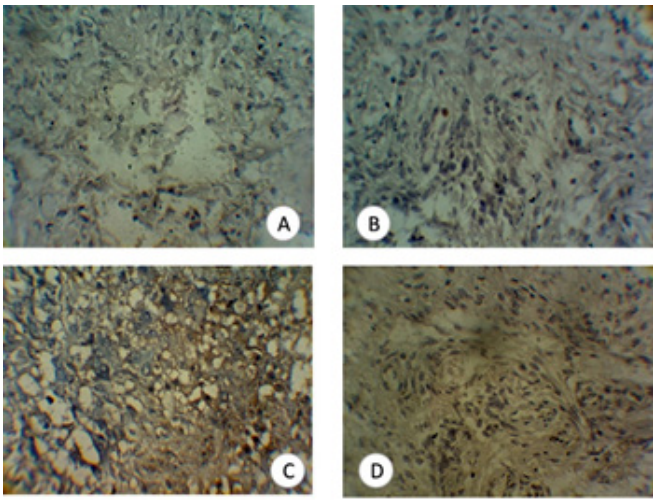

Fig. 1. Immunohistochemical staining showing tumor cells with ALDH1A1 in different tumor stages. (A) Stage I glioblastoma cells with low ALDH1A1 expression level. In GII (b), tumor necrosis, pseudo-palisades, and ALDH1A1cells can be seen. (C) Staining of ALDH1A1in GIII tumor cells with positive expression. (D) High ALDH1A1 expression in stage IV cancer cells.

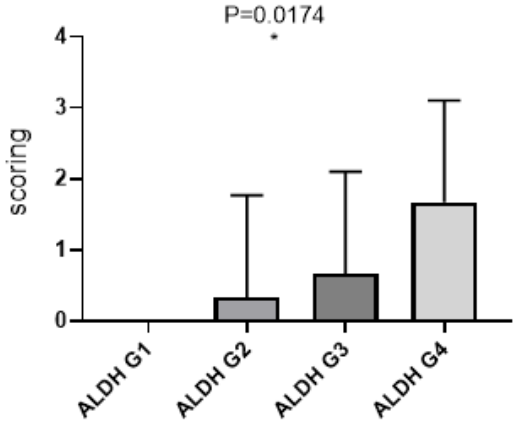

Fig. 2. Immunohistochemistry scoring results of ALDH1A1expression in different grades of glioblastoma tissues, n=26. G1: negative, G2: low expressed ALDH1A1protein, G3: moderate and G4: high expression.

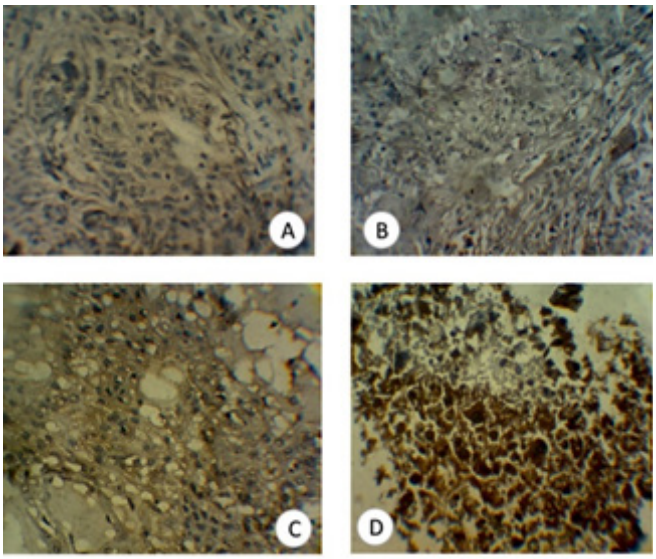

Fig. 3. Immunohistochemical staining showing tumor cells with CD44 in different glioblastoma tumor stages. (A) Stage I of glioblastoma cells with low CD44 expression level. In GII (b), tumor cells showed low CD44 expression level. (C) Staining of the GIII glioblastoma cell line showed positive CD44 expression. (D) High CD44 expression levels in stage IV glioblastoma cancer cells.

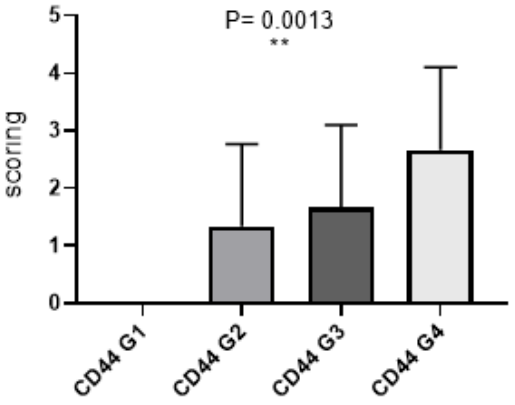

Fig. 4. Immunohistochemistry scoring results of CD44 expression in different grades of glioblastoma tissues, n=26. G1: negative G2: low expressed CD44 protein, G3: moderate and G4 high CD44 expression levels.

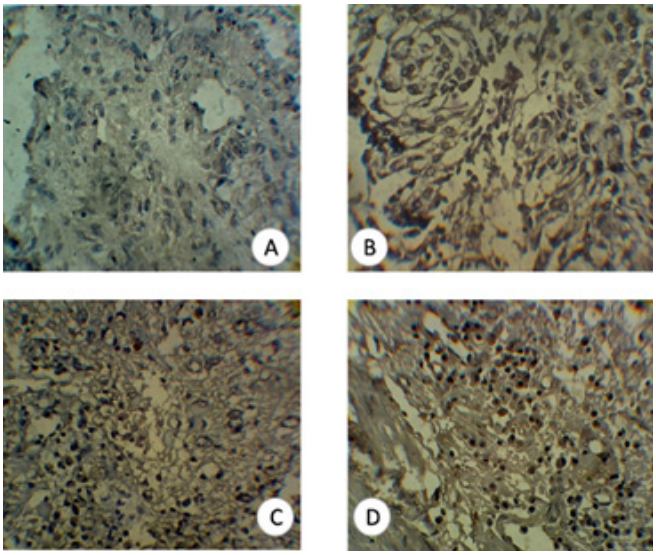

Fig. 5. Immunohistochemical staining showing OCT3/4 expression level in tumor cells in different glioblastoma tumor stages. (A) Stage I glioblastoma cells with absent OCT3/4 expression level. In GII (b) cells of glioblastoma showed low OCT3/4 expression levels. (C) Immunostaining of GIII glioblastoma cell line showed a positive OCT3/4 expression level. (D) High OCT3/4 expression levels in stage IV glioblastoma cancer cells.

showed a highly significant (P<0.05) expression level in advanced cancer stages GIII and GIV as compared with early cancer stages GI and GII, respectively (Figures 5 and 6).

3.5. Comparison between cancer stem cell markers in different grades of glioblastoma tissues.

As presented in Figure 7, there were highly significant differences (P<0.0001) in cancer stem cell markers ALDH1A1, CD44, and OCT3/4 between different stages of glioblastoma. The expression of these cancer stem cell markers was highly present in GIII and GIV stages of the tumor.

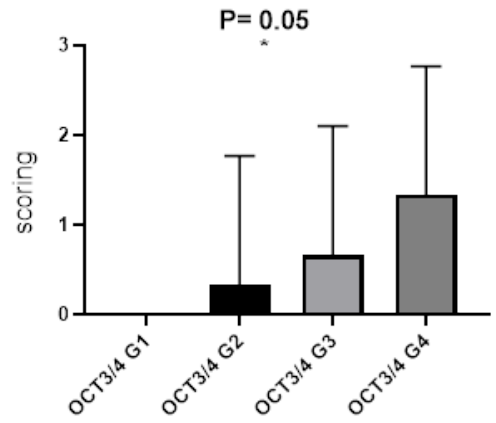

Fig. 6. Immunohistochemistry scoring results of OCT3/4 expression in different grades of glioblastoma tissues, n=26. G1: negative, G2: low expressed OCT3/4 protein, G3: moderate, and G4: high expression. Comparison between cancer stem cell markers in different grades of glioblastoma tissues .

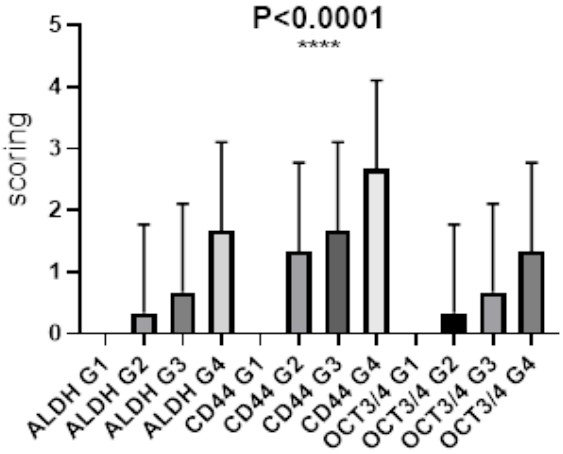

Fig. 7. Immunohistochemistry scoring results of cancer stem cell markers expression in different grades of glioblastoma tissues, n=26.

4. Discussion

Brain cancer accounts for approximately 1.4 % of all cancers and is responsible for about 2.3 % of all cancer-related deaths. Glioblastoma and meningioma are the most common subtypes, accounting for over 80 % of brain tumor cases [6].

The involvement of ADH in ethanol oxidation in the brain cannot be excluded because of the data concerning the translation of ALDH1A1 in the brain tissue [7]. Acetaldehyde formed in the brain is then oxidized by aldehyde dehydrogenase [8]. However, our study found that the activity of ALDH1A1 was significantly higher in cancer tissues, stage IV and stage III, than in primary brain tissues, stage I and II. The activity of ALDH1A1was not different between the two tissues.

Aldehyde molecules are considered intermediates of different anabolic, catabolic, and synthetic pathways generated in response to biotic and abiotic environmental stresses. Although aldehydes are indispensable to developmental and growth processes, excessive amounts of aldehydes interfere with metabolism and become toxic, so their unbalanced levels must be regulated within the cells.

As ALDH1A1 belongs to a large family of ALDH, it plays pivotal role in detoxification processing and participates in the pathophysiology of several cancer types. Previous studies illustrated that elevated expression levels of ALDH1A1 are connected with invasion, metastasis, chemo-radio resistance, and poor overcoming of different tumor types [15,16]. In addition to its action in the detoxification process, ALDH1A1 acts as a cancer stem cell marker, and its elevated expression level is associated with cancer stem cell self-renewal and differentiation. These cells showed high carcinogenic activity with increased tumor invasion. In this study, our results showed that ALDH1A1 expression levels in glioblastoma cells were increased with tumor grade [17, 18].

The malignancy of the brain tumor may be due to its histological subtype, the origin of the cells and also to the location of the tumor. The point of this study was to investigate the possibility of ALDH1A1 activity relationships to a variety of tumor locations [9].

The level of CD44 gene expression has been connected with different types of solid tumors, such as breast cancer [19], lung cancer [20], ovarian carcinoma [21], and non-small cell lung cancer [22]. The effect of elevated levels of CD44 expression in glioma tumors has been an

argumentative issue [22, 23]. Some previous studies have suggested that high CD44 expression level was associated with worse prognosis and poor overall survival in patients with glioblastoma tumors. In contrast, the other studies elucidate no prominent link between high CD44 expression levels and the patient's clinical features or even overall survival. Previous studies illustrated that the elevated CD44 tumor expression level was associated with poor overall survival in Low-grade glioma patients, but not for patients with glioblastoma [24-25]. In our study, we found that the expression levels of CD44 in glioblastoma were increased in different stages of glioblastoma tumors.

Since CD44 is considered a cell membrane glycoprotein, CD44 engages in different cellular processes, encompassing cell motility, proliferation, metastasis, angiogenesis, and apoptosis [25]. A previous study illustrated that a comparison between normal brain tissue and glioblastoma tumor cells showed that the CD44 expression levels of human glioblastoma cells were significantly elevated and reduced CD44 expression improved the invasion and the metastasis of glioblastoma tumor cells. Meanwhile, CD44 expression levels may be used to identify and characterize tumor cells with stemness and help to improve prognostic evaluation and cancer therapy.

Although CD133 is the most commonly used cell surface marker, other markers, such as integrin α6, have been proposed to segregate CSCs and NSTCs. CD15/SSEA-1 and CD44 markers have been suggested as possible markers concerning specific subgroups of GBM [26]. These markers have utility but must be approached with caution. Each can mark a different number of cells, consistent with a high false-positive rate. Due to the current limitations in the functional assays defining CSCs, false-positive markers are sometimes claimed to be superior to functional identification; still, biomarkers lack significant utility in discovery studies, which benefit from greater specificity. Additionally, likely, no marker will ever be uniformly informative for CSCs because most tissue types contain multiple populations of stem cells expressing different markers due to the inherent adaptability of cancer cells.

According to its function in self-renewal and differentiation in different tumor types, the high expression level of OCT3/4 is related to various manifestations of tumor cells, including invasion, metastasis, and resistance to chemoradiotherapy. On the other hand, high OCT3/4 markers and other genes such as NANGO and SOX2 link to worse clinical consequences, including aggressiveness, reduced overall survival rate, and drug resistance [27].

Accumulating evidence illustrated that OCT3/4, as a transcription factor, plays a key role in the self-renewal and pluripotency of cancer stem cells, tumorigenicity, and chemoradiotherapy resistance. The presence of such biological markers has been linked to a poorer prognosis in various solid tumors [28, 29]. Conversely, reduced expression of OCT3/4 has been associated with a lower likelihood of vascular invasion. Furthermore, there appears to be a significant correlation between decreased expression of OCT3/4 and a reduced incidence of metastasis to other organs or lymph node involvement. Nevertheless, studies demonstrated that OCT3/4 expression increased in tumor tissues across various solid tumors. [30, 31].

Previous studies showed that OCT3/4 expression in colon cancer and normal colon tissue revealed no significant statistical correlation between OCT3/4 expression and the incidence of cancer [32]. Meanwhile, the study illustrated a strong association between OCT3/4 expression levels with poor prognosis and distant recurrence in patients under chemotherapy regimens [33]. According to Amini et al, an increased expression of OCT3/4 in adult patients with colon cancer is linked with more advanced stages and a poorer prognosis [34]. Metastasis is a complex process involving converting epithelial cells to mesenchymal cells, resulting in the formation of epithelial-mesenchymal cells [35]. High OCT3/4 expression levels play an important role in this process rules in the aggressive and progressive behavior of cancer cells [36].

In this study, the level of ALDH1A1, CD44, and OCT3/4 expression was explored by using an immunohistochemical technique. The results of this study revealed that the expression level of these markers was significantly elevated in tumor cells compared to normal cells. Furthermore, this study showed that significantly increased ALDH1A1, CD44, and OCT3/4 expression levels were elevated in different stages of tumor cells at the protein level. In conclusion, the results imply that these markers could serve as reliable indicators for detecting cancer stem cells and could be an attractive target for advanced antitumor therapies against human malignant glioma.

This study employed immunohistochemistry to evaluate the expression of cancer stem cell markers ALDH1A1, CD44, and OCT3/4 in glioblastoma tissues. The results demonstrated a significant increase in the expression levels of these markers across different glioblastoma stages, with the highest expression observed in stages III and IV. Immunohistochemistry proved to be a reliable method for identifying these specific cancer stem cell markers in glioblastoma. To the best of our knowledge, this is the first study of its kind conducted in Iraq, providing a valuable foundation for future research aimed at improving diagnosis, prognosis, and the development of targeted therapies in glioblastoma treatment.

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Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
The ethics approved statement, according to Mustansiriyah University law, is on using human samples in research only.

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Zaynab S. Abdulghany, Noah A Mahmood: Research design and supervision; Firas S. Salah and Noah A Mahmood: Perform all laboratory procedures; Zaynab S. Abdulghany and Kara R. read and revised the manuscript; Noah A Mahmood and Zaynab S. Abdulghany performed the data analysis

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