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Immunohistochemical investigation of SOX2 expression in Iraqi patients with high-grade glioma

Hadeer Hashim Shams Uldeen[1], Abed Hassan Barraj[1], Sameer Hameed Hammadi[2]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
[2]Neurological Hospital, Baghdad, Iraq

Abed Hassan Barraj **ORCID ID:** 0000-0003-4466-5664

ABSTRACT

Background. Gliomas, particularly high-grade gliomas, are aggressive brain tumors with poor prognosis. Sex-determining region Y-box 2 (SOX2) is a transcription factor responsible for stem cell maintenance and differentiation and has been implicated in glioma progression and recurrence. The present study investigated SOX2 expression in Iraqi patients with high-grade glioma and assessed its potential as a diagnostic and prognostic biomarker.

Methods. Sixty-four paraffin-embedded samples from the Neurological Hospital in Baghdad were analyzed, including 34 high-grade glioma cases (21 males and 13 females) and 13 benign controls (10 males and 3 females). Immunohistochemical staining for SOX2 was performed, and staining levels were categorized using a scoring system. Statistical analyses were conducted to evaluate differences in SOX2 expression between genders and between patient and control groups.

Results. There were highly significant differences in SOX2 expression between patients and controls ($p \leq 0.01$). However, no significant difference was observed between male and female patients ($p \leq 0.01$). Additionally, there were significant differences in SOX2 expression distribution between genders, with p-values of 0.0022 for males and 0.0061 for females. The highest distribution percentages for males were at scores 2 and 3, with values of 38.10 and 47.62%, respectively. In contrast, females had distribution ratios only at scores 2 and 3, with values of 30.77 and 69.23%, respectively.

Conclusion. SOX2 is highly expressed in high-grade gliomas, supporting its potential as a diagnostic and prognostic marker. Future studies with larger cohorts and detailed sex-specific analyses are needed to elucidate SOX2's role in glioma pathogenesis and its potential as a therapeutic target.

Keywords: cancer, brain tumor, benign, glioma, SOX2, immunohistochemistry

INTRODUCTION

Cancer is one of the main causes of mortality globally. It represents a burden on any society and across the whole globe [1]. In 2018, there were 18.1 million new cancer cases diagnosed and 9.5 million cancer-related deaths worldwide, in 2020. The cases escalated to 19.3 million newly diagnosed cases, and it is estimated that by 2040, the number of new cancer cases per year will reach up to 30.2 million, and there have been over 16.4 million mortalities linked directly to cancer [2]. Globally, cancer is becoming a more serious hazard to public health. Since 1990, the incidence of cancer has increased in most countries due to causes, such as an aging population, a growing population, increased frequency of unhealthy behaviors, and certain risk factors, including smoking [1]. People who are exposed to ionizing radiation from both artificial and natural sources are at risk of developing malignant tumors [3]. Numerous intricate elements play a role in the genesis of cancer in humans, such as endogenous and exogenous induction, and genetic alterations that activate oncogenes through free radicals. The onset and progression of cancer correlate with the genetic alteration that activates oncogene by free radicals [4].

A brain tumor is an abnormal cell growth or mass in the brain that occurs because of the abnormal development of cells. It is one of the major reasons for death in adults around the globe [5]. Benign tumors, compared with their malignant counterparts, have

Corresponding author:
Abed Hassan Barraj
E-mail: abedhassan911@gmail.com

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much higher survival rates. They generally grow more slowly, have less invasion of surrounding nervous tissues, and are unlikely to metastasize. These tumors are generally treated with a combination of surgery, chemotherapy, and radiation, with surgical resection being the most common (first-line treatment) [6]. On the other hand, malignant brain tumors, are typically rapidly expanding and lack clear borders. Depending on their size and location in the brain, both types of brain tumors can produce symptoms that are similar to one another [7]. Proliferation and self-renewal ability are lost in most other tumor cells, resulting in tumor cells that are the phenotypic signature of the tumor [8]. The tumor microenvironment is es-sential in regulating the transdifferentiation of cancer cells [9].

Heart and cerebrovascular diseases are the two main causes of death in Iraq. In 2010, Iraq developed a national cancer control plan aligned with the World Health Organization's (WHO's) cancer control strategy recommendations. However, the country's medical resources and healthcare services have significantly declined due to successive wars, local conflicts, and population displacement in recent decades [10]. Approximately 2 to 8% of all cancer cases are thought to be caused by occupational hazards [11]. Gliomas are brain tumors of the brain-supporting tissue. The course of treatment for low-grade gliomas is still up for debate, although options include simple follow-up major surgery, chemotherapy, or none [12]. Astrocytomas, oligodendrogliomas, and ependymomas are the three main forms of glial tumors. Gliomas are categorized histologically based on their similarity to several glial cell types [13]. Gliomas can be classified as low-grade gliomas (LGG), which do not cause much damage or high-grade gliomas (HGG), which are more damaging and infiltrating. Due to the variable shape, size, and location of tumors, detection can be challenging [14]. The incidence of gliomas, particularly high-grade gliomas, increases with age [15]. More than half of all brain tumors are caused by gliomas, which are the most common primary tumors of the central nervous system [16].

The family of transcriptional regulators known as sex-determining region Y (SRY) box (SOX) factors is responsible for important functions during embryonic development and maintaining stem cells in adult tissues [17]. Sex-determining region Y-box 2 (SOX2) has been implicated in various development and proliferative processes in different organs. Numerous severe clinical disorders are associated with their involvement in the etiology and later events of carcinogenesis, like tumor invasion and metastasis [18]. The abnormal expression of SOX2 is thoroughly connected to the occurrence and development of most types of tumors [19]. SOX2 has been implicated in maintaining the stem cell-like properties of glioma cells (GSCs), which are thought to drive tumor growth and recurrence, and often express SOX2 at high levels. SOX2 contributes to the self-renewal capacity of GSCs, allowing them to propagate and maintain the tumor. It promotes the migration and invasion of glioma cells into surrounding healthy brain tissue, which is a critical aspect of tumor aggressiveness [20, 21].

Detecting antigens in cells of a tissue section is achieved through the immunohistochemistry (IHC) technique using monoclonal and polyclonal antibodies [22]. Immunohistochemistry employs monoclonal and polyclonal antibodies to determine the distribution and localization of biomarkers and proteins, expressed differently in different parts of a biological tissue in healthy and diseased parts [23]. Immunohistochemistry also plays a key role in many areas of pathology including oncologic pathology and neuropathology. It is distinct from other laboratory procedures because it does not damage the histological architecture and can evaluate the expression pattern of a molecule in a microenvironment [24]. The increased uncontrolled expression of specific tumor antigens in certain cancers makes it commonly used in cancer diagnosis [25].

The present study aimed to investigate the expression of SOX2 in high-grade gliomas of Iraqi patients using immunohistochemical techniques, and to evaluate its potential role as a diagnostic and prognostic biomarker in glioma progression.

METHOD

Collection of specimens

This study was conducted on 64 fixed paraffin-embedded samples selected randomly from Neurological Hospital in Baghdad. Thirty-four (34) patients were diagnosed with high-grade glioma (21 males and 13 females) with a mean age of 44.94 ± 2.99 years, while 13 benign cases (10 males and 3 females) served as controls. The samples were collected between December 2022 and July 2023, with the age range of all participants spanning from 13 to 70 years.

Ethics approval and consent to participate

Ethical permission was obtained from the Iraqi Ministry of Health. The research was approved by the Ethical Committee (Approval No.: CSE-C0922l0096/). All clinic pathological information including age, gender, type of tumor, and glioma grade were taken from the patient's reports.

Immunohistochemical analysis of specimens

Immunohistochemical analysis was conducted on the specimens to investigate SOX2 expression using adapted methods from Jackson and Blythe [26]

and O'Hurley et al. [27], with kits provided by My BioSource Company (ab 97959), following the manufacturer's instructions. The first step involved dewaxing and rehydration. The slides were placed in an oven at 62°C to remove the excess wax from the slices. The tissue was subsequently dewaxed by immersing slides in xylene (3-5 minutes) and rehydrated by soaking in decreasing ethanol concentrations (90% ethanol for 5 minutes, 70% for 5-3 minutes, 50% ethanol for 5 minutes, succeeded by a 5-minute dip in distilled water). In the second step, the slides were treated with endogenous hydrogen peroxide and protein-blocking agents for 10 minutes, followed by a 5-minute incubation with a ready-to-use hydrogen peroxide solution to inhibit endogenous peroxidase activity. Following that, a protein block was applied to the tissue slices to block the non-specific protein and incubated for 10 minutes before being rinsed twice in phosphate-buffered saline (PBS) for 5 minutes. In the third step, the primary antibody SOX2 (ab97959), diluted 1:100 (10 µl antibody + 990 µl PBS), was applied to the sections and incubated for 1 hour. The slides were washed thrice with PBS for 10 minutes each before further incubation. The primary antibody was extracted, and the slides were washed in PBS for 5 minutes for all antibodies.

Step 4 involved incubating the secondary antibodies after washing the slides to remove the main antibodies. After 20 minutes of incubation at 25°C, the second antibodies were applied to the slides, which were then rinsed four times with PBS. The sections were treated with goat anti-rabbit horseradish peroxidase (HRP)-conjugate for 10-20 minutes before being rinsed with PBS twice for 5 minutes each. In the fifth step, the diaminobenzidine (DAB) chromogenic reaction was visualized using a peroxidase substrate. The DAB reagents from the DAB kit were made immediately before use by mixing one drop of chromogen for every 49 drops of a particular chromogen diluent (DAB substrate). The slides were then incubated for 5-10 minutes, or until the brown color appeared, before being washed twice in PBS for five minutes each. The final step included counterstaining, dehydration, clearing, and mounting of slides. The slides were counterstained for 10 seconds in hematoxylin to stain nuclei, followed by a one-minute wash with running water. The slides were dehydrated by dripping them in increasing ethanol concentrations (30% ethanol for 30 seconds, 95% ethanol for 30 seconds, and 100% ethanol for 30 seconds). The slides were then cleaned by soaking in xylene for 2 minutes. Super mount was applied, and a cover slip was gently placed over the slides, ensuring no bubbles remained. The slides were left to dry overnight before examination. Brown-stained nuclei for SOX2 indicate a positive IHC reaction. The intensity of the staining and the percentage of positive tumor cells were assessed blindly and examined histopathologically. A two-headed microscope was used to measure the intensity of the stain and scored numerically from 0 to 3, with 0 representing negative, 1 representing light, 2 representing moderate, and 3 representing intense or strong.

Scoring system

Staining levels were assessed using scoring methods for evaluating SOX2 protein immuno-histochemistry. The proportion of immune-positive cells among all tumor cells was categorized as follows: 0: <10%; 1: 10–50%; 2: 50–90%; 3: >90% [28]. Histopathologists interpreted the im-munohistochemistry results to evaluate tumor and histological grades.

Statistical analysis

To detect the different effects in the two groups (patient and control), a statistical package for social sciences (SPSS; 2019) was used for the statistical analysis. To compare means, the LSD (Least Significant Difference) and T-test were used, while the Chi-square test was employed to compare percentages. Statistical significance was assessed at probability levels of 0.05 and 0.01.

$$\chi 2 = \Sigma ((O - E)2)/E$$

$\chi 2$: Chi-square; Σ: Summation; O: Observed no.; E: Expected no. [29]

RESULT

Effect of gender on SOX2 expression in control and patient groups

The results of immunohistochemistry staining for the SOX2 marker (Table 1 and Figure 1) showed that a highly significant difference ($p \leq 0.01$) was observed in the expression of SOX2 among males and females with high-grade glioma with values of 3.33 ± 0.159 and 3.69 ± 0.133, respectively, compared with the control (1.00 ± 0.0). Statistical analysis indicated no significant difference ($p \leq 0.01$) between males and females with glioma.

TABLE 1. Effect of gender on the expression of SOX2 in control and patient groups

Gender	Mean ± SE		p-value
Male	1.00 ± 0.00	3.33 ± 0.159	0.0001**
Female	1.00 ± 0.00	3.69 ± 0.133	0.0001**
p-value	1.00 NS	0.285 NS	---

***p ≤ 0.01; NS: Non-significant*

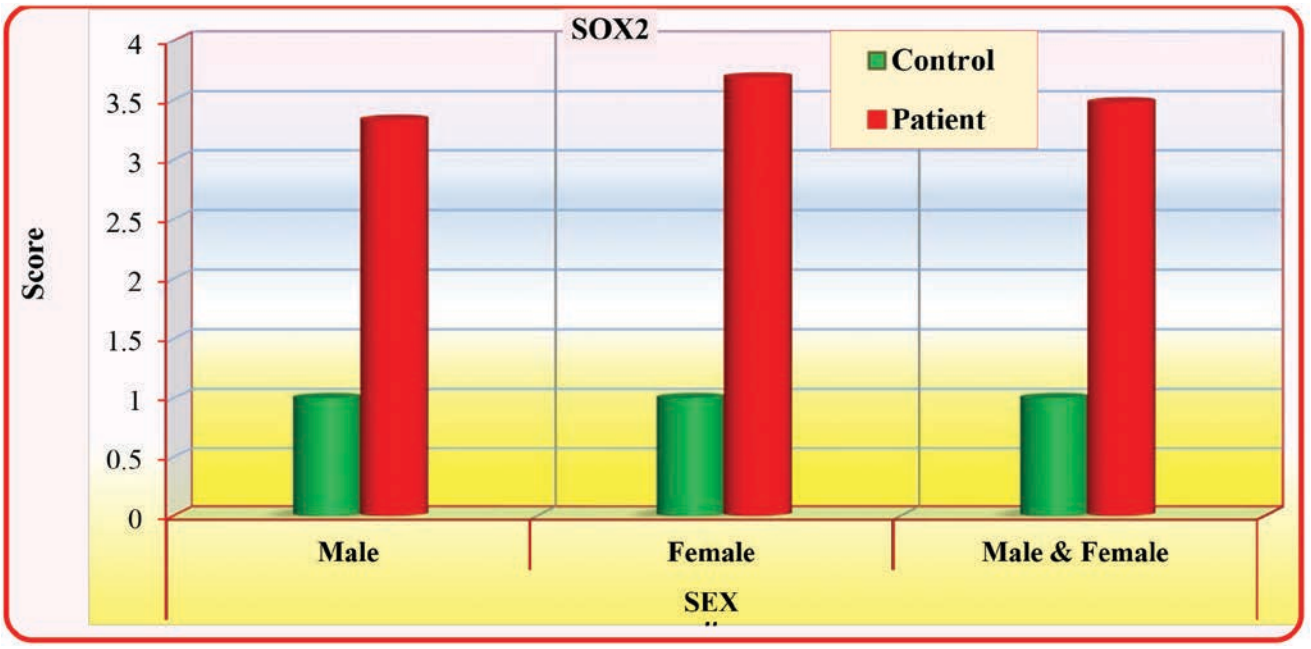

FIGURE 1. Expression of SOX2 marker in patient and control groups according to gender

Immunohistochemical scoring of SOX2 as a marker for glioma in male and female patients

The results (Table 2 and Figure 2) of immunohistochemistry for the SOX2 marker showed that there was a highly significant difference ($p \leq 0.01$) in the distribution of males and females with high-grade glioma p-values (0.0022 for males and 0.0061 for females). The highest distribution percentages for males were at scores 2 and 3, with values of 38.10% and 47.62%, respectively, while 14.29% of males were at score 1. For females, the distribution was observed only at

TABLE 2. Distribution of patient samples by SOX2 score and gender

SOX2 score	Male n =21		Female n =13	
	No	%	No	%
0	0	0.00	0	0.00
1	3	14.29	0	0.00
2	8	38.10	4	30.77
3	10	47.62	9	69.23
p-value	--	0.0022**	--	0.0061**

***p ≤ 0.01*

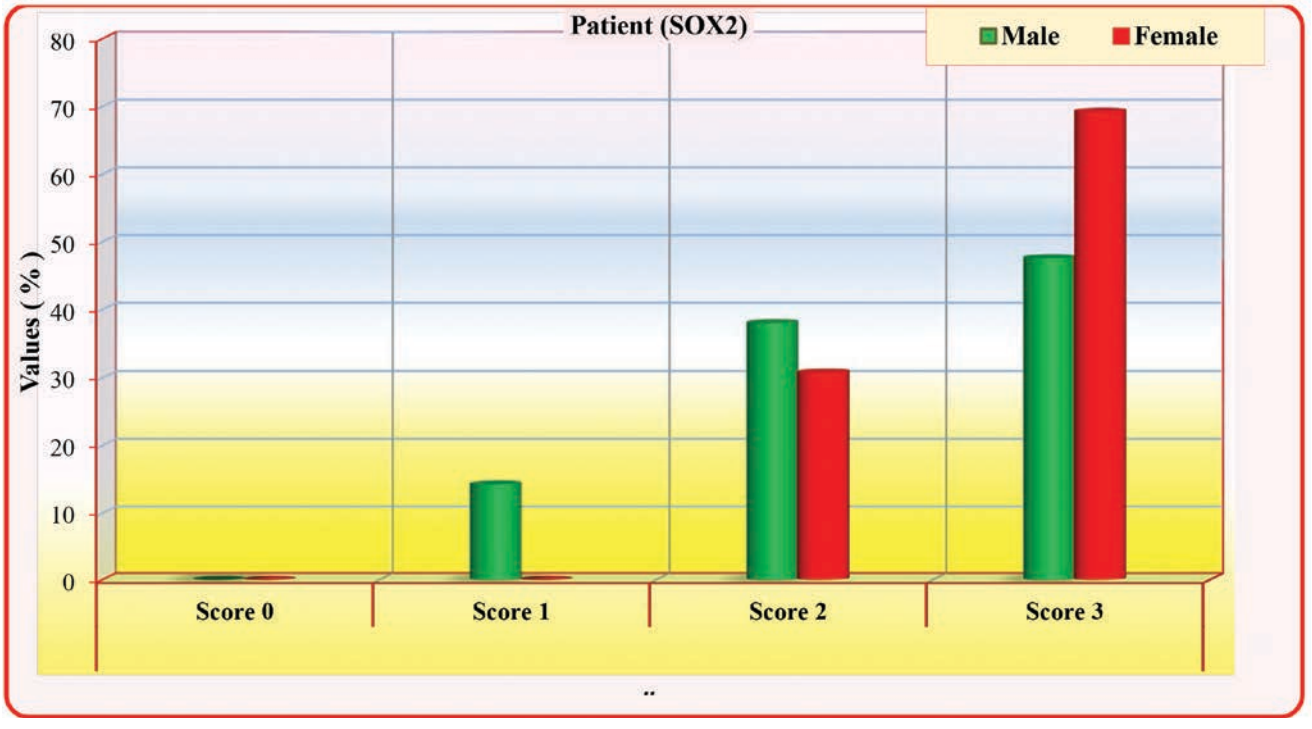

FIGURE 2. SOX2 expression scores in glioma according to gender

scores 2 and 3, with values of 30.77% and 69.23%, respectively. Notably, females had a higher distribution rate. SOX2 antibody expression was elevated due to its interaction with the antigen in patient samples, as depicted in Figures 3, 4, and 5. These figures illustrate the immunological reaction of the SOX2 antibody at scores 1, 2, and 3. The results were categorized into negative, mild, moderate, and strong, based on a scoring system that assesses the proportion of immune-positive cells relative to the total number of tumor cells. The scoring system was divided into four categories: 0: <10%; 1: 10–50%; 2: 50–90%; 3: >90% [30].

DISCUSSION

The significant differences between the patient and control groups suggest that the SOX2 marker is highly expressed in cancer cells. This observation aligns with the findings of Mansouri et al. [31], who reported a positive correlation between SOX2 expression and the malignancy grade of brain tumors, which is consistent with the results of the present study. The elevated SOX2 expression in the patient group supports the diagnosis of high-grade glioma, as it is known to be highly expressed in gliomas, particularly in glioblastoma, the most aggressive and common type of primary brain tumor in adults, as noted in several studies, including Lathia et al. [32]. The study showed that the expression of SOX2 is associated with the stemness of glioma cells, contributing to their self-renewal capacity and resistance to therapy. According to a study by Ding et al. [33], SOX2 is involved in multiple stages of embryonic development, a factor that promotes tumor growth and metastasis by preserving cancer cells' stemness. In addition, SOX2 also regulates the proliferation, apoptosis, invasion, migration, and drug resistance of cancer cells.

On the other hand, SOX2 regulates many pathways involved in glioma progression, including pathways related to cell proliferation, differentiation, and invasion. Its overexpression can lead to the activation of oncogenes and suppression of tumor suppressor genes, thereby promoting tumor genesis [34]. SOX2 expression was significantly higher in malignant glioma samples, while its expression in non-malignant tissues was negligible. This is consistent with the findings of Schmitz et al. [35], who reported that the SOX2 marker was overexpressed in most glioblastoma samples, while its expression in normal brain and other non-malignant tissues was nearly negligible. The present study also found no significant difference in SOX2 expression between females and males with high-grade glioma, consistent with the findings of Suvà et al. [38], who reported no sex-related differences in SOX2 expression. However, they emphasized the importance of confirming its role in the pathogenesis of high-grade glioma, which may be influenced by sex-specific factors. Most studies on SOX2 expression in glioma focus on its role in tumor progression and prognosis, rather than comparing expression specifically between males and females. However, evidence suggests potential differences in SOX2 expression related to glioma biology and clinical outcomes, which could indirectly relate to gender differences.

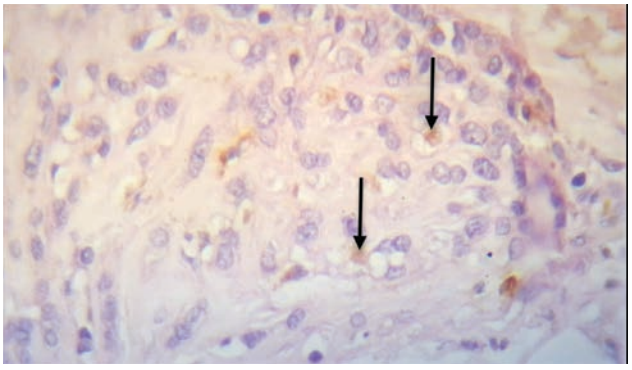

FIGURE 3. A cross-section of the brain of a glioma (grade IV) patient with SOX2 by immunohistochemistry, showing brown nuclear stain in mild intensity (40X). Score 1: black arrows

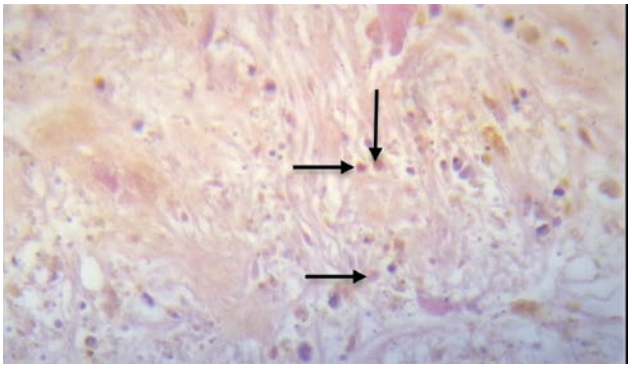

FIGURE 4. A cross-section of the brain with glioma (grade IV) patient with SOX2 by immunohistochemistry, showing brown nuclear stain in moderate intensity (40X). Score 2: black arrows

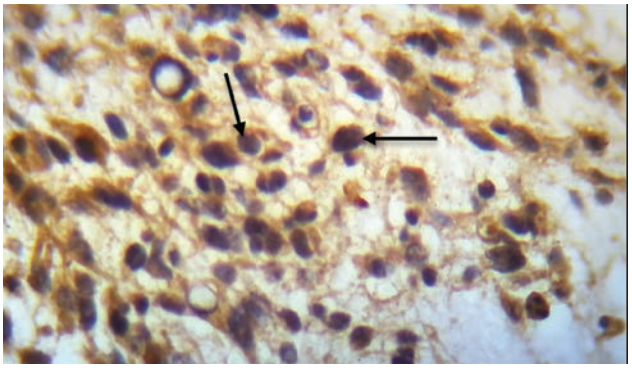

FIGURE 5. A cross-section of the brain of a glioma (grade IV) patient with SOX2 by immunohistochemistry, showing brown nuclear stain in strong intensity (40X). Score 3: black arrows

Modern life has left little untouched, unlike the past when people consumed what they grew in their

yards. Excessive use of pesticides and genetic modification in animal products contribute large amounts of free radicals thus increasing risk factors for cancer. The dramatic population growth, particularly in the developing world, has led to a massive increase in the demand for animal products, often sourced from genetically modified organisms. This, coupled with the rising consumption of fast food, has become a hallmark of the modern era. As a result, cancers of all types are at higher rates nowadays. The gradation is due to the difference in intensity of the immune reaction between the marker and the grade of the tumor. The present study found that high SOX2 expression is associated with higher tumor grade, consistent with the findings of Gangemi et al. [37], who reported a correlation between elevated SOX2 expression and higher tumor grades in glioma, including glio-blastoma (grade IV). The findings of the present study align with those of Annovazzi et al. [38], who demonstrated a positive correlation between SOX2 expression and malignancy grade in glioma, identifying the hypercellular and hyperproliferative regions of glioblastoma as having the highest SOX2 expression. On the other hand, Yu et al. [28] demonstrated that a decrease in SOX2 expression in high-grade gliomas may occur due to chemotherapy, radiation therapy, or a combination of both. Additionally, studies such as that by Garros-Regulez et al. [17] have concluded that SOX2 expression plays a potential role in glioma biology across various grades, including low-grade gliomas, and is detectable in both low-grade and high-grade gliomas, suggesting its involvement throughout different stages of glioma development. Several factors contribute to the high expression of SOX2 in glioblastoma at score 3, including its association with biological and clinical factors. SOX2 is linked to the stem cell-like characteristics of glioma stem cells (GSCs), which are crucial for tumor initiation, recurrence, aggression, and resistance to treatment [32]. Conversely, Suvà et al. [38] suggested that high SOX2 expression in high-grade gliomas regulates cellular plasticity, enabling glioma cells to transition between different states. This phenotypic plasticity contributes to re-sistance to chemotherapy and radiation.

Previous studies have confirmed the role of SOX2 in tumor formation, where it is overexpressed in high-grade gliomas and is associated with the maintenance of glioma stem-like cells which are a subpopulation of tumor cells with stem cell-like properties that contribute to tumor initiation and recurrence [39]. Many studies have reported that SOX2 regulates the stemness and self-renewal of cancer stem cells (CSCs). Given that CSCs share the self-renewing capacity with normal stem cells, it is not surprising that SOX2 is frequently associated with regulating these processes in CSCs. A recent study has underscored the role of SOX2 in glioblastoma progression and recurrence. Several research groups demonstrated that silencing SOX2 in GBM tumor-initiating cells significantly reduced their proliferative, migratory, invasive, and tumorigenic potential, while also causing them to remain in the G0/G1 phase of the cell cycle. This highlights the crucial role of SOX2 in the progression and recurrence of glioblastoma [40]. Finally, high expression of SOX2 holds up a red flag in each glioma case representing poor survival high rate of recurrence, and resistance to therapy. All these make SOX2 a perfect target for designing new therapeutic approaches for glioma depending on the wide range of research needed for this mission. In the future, it is rec-ommended to use molecular techniques, such as polymerase chain reaction (PCR), to measure and investigate SOX2 expression in patients with glioma. This approach has been successfully used in various medical fields for disease diagnosis and infection detection [41-54].

Study limitation

A significant limitation of the study was the small sample size of glioma cases. Despite being a long-recognized disease, the causes and treatment of glioma remain poorly understood.

CONCLUSION

The present study did not observe a difference in SOX2 expression between females and males. However, confirming its role in the pathogenesis of high-grade glioma is crucial, as sex-specific factors may play a role. A dedicated study comparing these aspects would be necessary to provide direct evidence on whether SOX2 expression differs between genders. Such research would require a large patient cohort with comprehensive clinical and molecular data, including sex-specific analyses.

Authors' contributions:
All authors contributed to the study's conception and design. Material preparation, data collection, laboratory investigations, and analysis were carried out by Hadeer Hashim Shams Uldeen. The study idea, design, and supervision were provided by Abed Hassan Barraj and Sameer Hameed Hammadi. All authors reviewed and commented on previous versions of the manuscript and approved the final version.

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