

PROGNOSTIC VALUE OF COMBINED IMMUNOHISTOCHEMICAL EXPRESSION OF BMI1 AND EZH2 IN ASTROCYTOMA

*Lobna Abdelaziz Abdelsalam, Yehia Elalfy Ali, Kamal Ahmed Elkashishy, Eman Taher Nour Eldeen
Department of Pathology, Faculty of Medicine, Zagazig University, Egypt*

Corresponding author:

Lobna Abdelaziz Abdelsalam,
e.mail:dr.lobna2013@yahoo.com

ABSTRACT

Background: Although current improvements in surgery, chemotherapy and radiotherapy, the still survival of astrocytoma's patient is poor. It is assumed that the combined expression of BMI1 and EZH2 may be associated with malignant transformation of astrocytomas and also it may reveal the biological aggressiveness of this disease.

Aim of work: assessment the value of the combined immunohistochemical expression of BMI1 and EZH2 and their correlation with the clinicopathological characters and prognosis in astrocytoma patient.

Subjects & Methods: BMI1 and EZH2 expressions were evaluated using immunohistochemical staining in 70 patients 40 cases with astrocytomas and 30 cases of non-neoplastic brain tissue. The relationship between their expressions and clinicopathological factors were analyzed.

Results: A significant difference ($P < 0.002$) between expression of BMI1 and EZH2 in astrocytoma compared to corresponding non-neoplastic brain tissue. A significant association was found between high expression of BMI1 and EZH2 and WHO high grades in astrocytomas. No statistically significant association was found between BMI1 or EZH2 with gender of patients, age at diagnosis, site and size of tumor ($P > 0.05$). The spearman correlation analysis revealed a significant positive association between BMI1 and EZH2 expressions ($r = 0.311$; $P = 0.05$) revealing direct relationship between BMI1 and EZH2.

Conclusions: BMI1 and EZH2 were involved in astrocytoma malignant transformation and poor prognosis in astrocytoma particularly glioblastoma.

Keywords: BMI1, EZH2, astrocytoma, PcG, glioblastoma and GSCs.

INTRODUCTION

Although current improvements in surgery, chemotherapy, and radiotherapy, still the survival of astrocytoma's patient is low. Astrocytic tumors include; pilocytic astrocytoma [World Health Organization (WHO) grade I but pilomyxoid type is grade II], subependymal giant cell astrocytoma [WHO grade I] diffuse astrocytoma [(fibrillary, gemistocytic, and protoplasmic types), pleomorphic xanthoastrocytoma, WHO grade II], anaplastic astrocytoma [WHO grade III] and glioblastoma (giant cell glioblastoma, gliosarcoma and gliomatosis cerebri) [WHO grade IV] ^[1]. The prognosis of a given fibrillary astrocytic neoplasm is a complex function of both clinical and morphologic variables ^[1]. Particularly, the prognosis of glioblastoma

multiform (GBM) patients has not changed considerably for decades, revealing median survival less than one year ^[2]. GBM comprises functional groups of cells called glioblastoma stem-like cells (GSCs) that are radioresistant and chemoresistant and are involved in tumor recurrence ^[3]. Glioblastoma stem-like cells exist in a niche around arterioles that protects these cells from therapy by maintaining a somewhat hypoxic environment ^[4].

PcG Proteins role in cancer

Polycomb group (PcG) proteins are considered as transcriptional inhibitors, regulating many vital cellular physiological and developmental processes and playing essential roles in carcinogenesis ^[5]. Dysregulation of polycomb group (PcG) proteins (including BMI1 and

EZH2) are closely related to the glioblastoma stem-like cells maintenance and tumorigenicity [3]. PcG proteins suppress gene expression selectively by development of multi-subunit complexes called polycomb repressive complexes (PRCs), regulating chromatin organization, maintaining it transcriptionally inactive [5]. The PRCs essentially comprise PRC1 (including BMI1) and PRC2 (including EZH2). Both PRC1 and PRC2 make covalent post-translational histone alterations [6]. Abnormal PcG expression or mutations falsely activate proto-oncogenes, resulting in loss of cell character, with sustained ability of proliferation, resistance to apoptosis mechanisms, and escape of cellular senescence programs, increased migratory and invasive ability, cancer stem cells maintenance and generation [7]. Numerous PcG proteins reveal diverse expression in malignant tissue in comparison to conforming non neoplastic tissue [8]. Not only abnormal levels of PcG proteins are found in human cancers but also many missense mutations and chromosomal translocations [9]. BMI1 and EZH2 furthermore inhibit tumor suppressor genes like phosphatase and tensin homolog (PTEN), consequently activating the phosphoinositide 3-kinase (PI3K)-Akt-glycogen synthase kinase 3 β (GSK3 β) pathway and promoting the EMT (epithelial-mesenchymal transition) and invasion [10]. Abnormal expression of EZH2 leads to genomic instability and consequent malignant transformation [11].

BMI1 role in cancer

BMI1 overexpression played a vital role in several types of cancer, for example bladder, skin, prostate, breast, ovarian, colorectal and mantle cell lymphomas [12]. BMI1 inhibits Myc-induced apoptosis by suppression of the cyclin-dependent kinase inhibitor locus, encoding ARF and INK4 proteins, both of limit proliferation of cells in response to abnormal mitogenic signaling [13]. BMI1 overexpression expects poor prognosis and advanced disease in numerous human cancers [8]. The expression of BMI1 is frequently linked to aggressive and stem cell-like chemoresistant cancers as

prostate cancer [14], head and neck cancer [15], pancreatic adenocarcinoma [16]. However, BMI1 overexpression may not associated with a poor prognosis for all types of cancer, as BMI1 high expression are related to a improved survival in breast cancer, malignant melanoma and endometrial carcinomas patients [8]. BMI1 high expression predicts metastases in precancerous tissues and biopsies, thus acting as an invaluable diagnostic tool as in precancerous gastrointestinal tract lesions [17]. BMI1 overexpression is related to dysplastic transformation of cells in oral carcinogenesis, essential for cancer cell maintainance and proliferation [18]. In renal carcinoma, BMI1 expression is correlated inversely with carcinoma grade, acting as a marker of differentiation, which is lost in carcinomas of high grade [19]. BMI1, regarding progression of cancer, depends on the type of tumor cell so it necessity to be considered when developing targeted new plans for therapy [8].

EZH2 role in cancer

EZH2 is not expressed normally in healthy adults but it is only present in actively dividing cells, as those active during fetal development [20]. EZH2 is expressed in a many human cancers, associating with tumor aggressiveness through all types of cancer [8]. EZH2 is upregulated in multiple cancers including breast [21], and human prostate cancer, especially hormone-refractory prostate cancers [22]. Somatic EZH2 deletions and mutations, inhibiting the activity of EZH2 methyltransferase are present in follicular lymphomas, B cell lymphomas, myeloproliferative and myelodysplastic disorders [23]. In glioblastoma multiform, EZH2 hinders differentiation and activates genes regulating cell cycle progression, cell proliferation, and cell migration [24]. EZH2 is supposed to involve the senescence program in malignant cells owing to the transcriptional suppression of the INK4b-ARF-INK4a locus [13].

MATERIALS AND METHODS

Patients and tissue specimens

Seventy paraffin blocks of brain tissue were collected from the archives of the department of pathology, faculty of medicine, Zagazig university and Teiba lab. In the period from January 2005 to December 2014. Approval is obtained for performing the study and ethical committee in the faculty is put in consideration. The studied specimens included 40 cases of astrocytoma were classified as 5 cases pilocytic astrocytomas WHO I and 10 cases diffuse astrocytomas WHO II, 12 cases anaplastic astrocytomas WHO III, and 13 cases glioblastomas WHO IV. None of the patients received chemotherapy or radiotherapy before biopsy. 30 control samples of non-neoplastic brain tissue included inflammatory, brain edema, normal brain tissue adjacent to cystic lesions or neoplasm. The clinicopathologic data of age, sex, size and site were collected from patient files and reviewed. Consecutive 4 μ m thick sections from each block was evaluated histopathologically using (H&E) stain to confirm the diagnosis. Serial sections from the same blocks are submitted for immunohistochemical staining for BMI1 and EZH2. The corresponding hematoxylin and eosin (H&E) stained slides of each case were reviewed according to diagnostic criteria of the WHO 2007 Classification of tumors of the Central Nervous System and the 40 cases of astrocytoma were graded according to Louis et al. ^[1], into astrocytomas WHO grade I and astrocytomas WHO grade II, and astrocytomas WHO grade III, and glioblastomas WHO grade IV.

IMMUNOHISTOCHEMISTRY

Immunohistochemical stain was carried out using the indirect streptavidin-biotin immunoperoxidase staining technique with the following primary antibodies used were used anti BMI1 rabbit polyclonal antibody (#ab

219469, 1:200 dilution; USBiological, USA) and Anti EZH2 mouse monoclonal antibody (Clone No. 144CT2.1.1.5, #ab 030954, 1:50 dilution; USBiological, USA). 30 cases of non-neoplastic brain tissue were used as control. Normal testicular tissue in EZH2 and human breast carcinoma for testing BMI1 were used as positive control. Negative controls were employed using antibody diluent in PBS buffer instead of primary antibodies and subsequently stained with the positive controls. Normal, Positive and negative controls were stained at the same staining setting with the studied cases. The percentage scoring of immunoreactive tumor cells was calculated as follows: 0 (0%), 1 (1–10%), 2 (11–50%) and 3 (>50%). The staining intensity was divided into following: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). According to Wu et al. ^[25], final immunoreactivity scores (IRS) were calculated by multiplying the percentage and the intensity scores for each case then selection the median of IRS for BMI1 or EZH2 expression level cutoff points. This method measured the following values as standards for the high expression group: BMI1 \geq 6 and EZH2 \geq 5.

STATISTICAL ANALYSIS

Data was analyzed by Statistical Package of Social Science (SPSS), software version 22.0 (SPSS Inc., 2013). Categorical data (nominal or ordinal data) were presented by the frequency and percentage. The chi-square test of association is used for correlation between two categorical variables. Spearman's correlation is used as a nonparametric measure of the strength and direction of correlation between two variables measured on at least an ordinal scale. Cohen's kappa (κ): measures agreement for categorical scales when exist two raters. $P \leq 0.05$ was considered to indicate significance.

RESULTS

Table 1: BMI1 and EZH2 expression in astrocytoma and (Control) samples:

	Groups		(X ²)*	P-value
	Astrocytoma	Control		
BMI1 IRS expression				
Low (n, %)	17(42.5%)	28(93.3%)	19.29	< 0.001
High (n, %)	23(57.5%)	2(6.7%)		
EZH2 IRS expression				
Low (n, %)	21(52.5%)	26(86.7%)	9.071	0.002
High (n, %)	19(47.5%)	4(13.3%)		

*Chi-square test

Table 2: Associations of BMI1 and EZH2 expression in human astrocytoma tissues with different clinicopathological features:

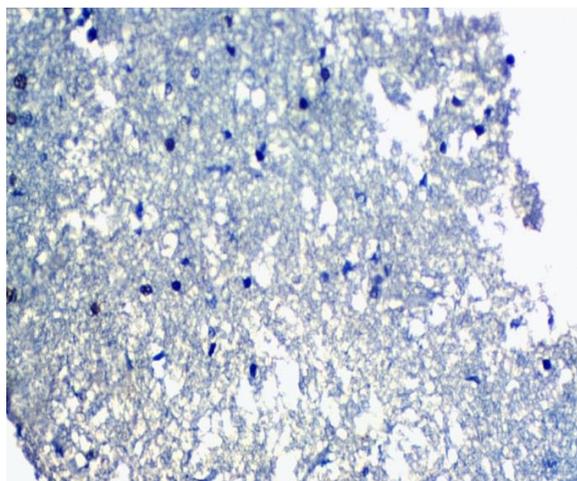
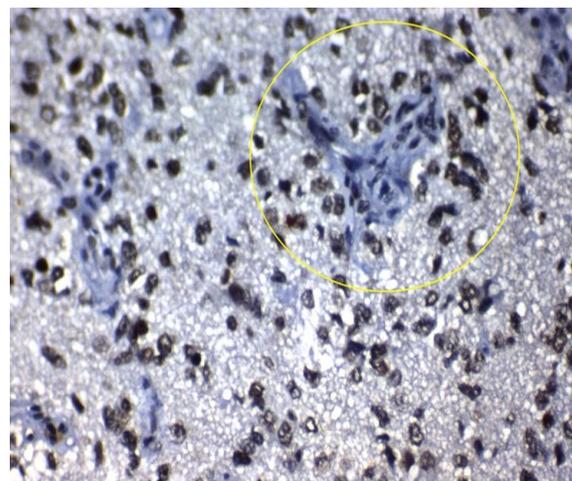
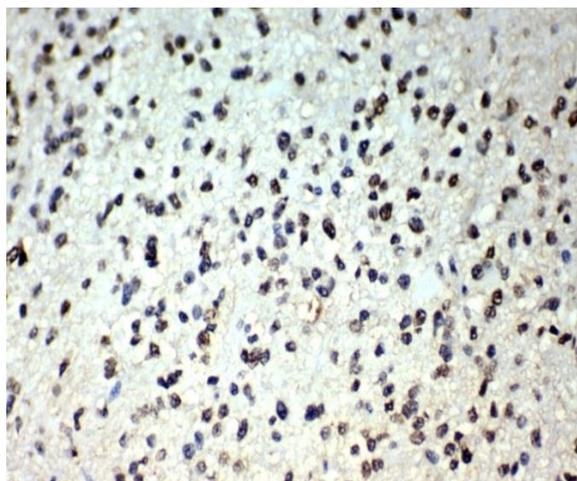
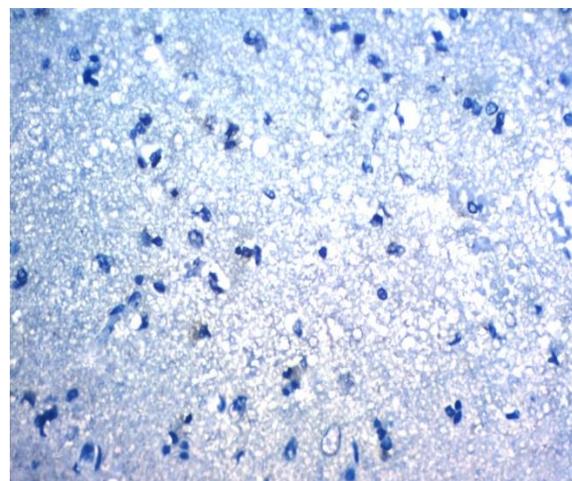
Clinicopathological features	No. of cases	BMI1 expression		(X ²)*	P	EZH2 expression		(X ²)	P
		High (N, %)	Low (N, %)			High (n, %)	Low (n, %)		
WHO grade									
I	5	1(20.0%)	4(80.0%)	14.047	0.003	0(0.0%)	5(100.0%)	12.63	0.005
II	10	2(20.0%)	8(80.0%)			2(20.0%)	8(80.0%)		
III	12	9(75.0%)	3(25.0%)			7(58.3%)	5(41.7%)		
IV	13	11(84.6%)	2(15.4%)			10(76.9%)	3(23.1%)		
Age									
≤50	22	11(50.0%)	11(50.0%)	1.125	NS	9(40.9%)	13(59.1%)	0.852	NS
>50	18	12(66.7%)	6(33.3%)			10(55.6%)	8(44.4%)		
Gender									
Male	22	11(50.0%)	11(50.0%)	1.125	NS	11(50.0%)	11(50.0%)	0.123	NS
Female	18	12(66.7%)	6(33.3%)			8(44.4%)	10(55.56%)		
Size (cm)									
≤5	16	7(43.75%)	9(56.25%)	2.06	NS	7(43.75%)	9(56.25%)	0.15	NS
>5	24	16(66.7%)	8(33.3%)			12(50.0%)	12(50.0%)		
Site									
Supratentorial	35	23(65.7%)	12(34.3%)	7.73	NS	19(54.3%)	16(45.7%)	5.17	NS
Infratentorial	3	0(00%)	3(100%)			0(00%)	3(100%)		
Intraventricular	2	0(00%)	2(100%)			0(00%)	2(100%)		

Note: "NS" refers to the difference without statistical significance.

Table 3: Correlation between BMI1 IRS and EZH2 IRS:

Variable		BMI1 IRS
EZH2 IRS	r*	0.311
	P	0.05

*r: Spearman's rho correlation

**Figure1:** normal brain tissue shows negative BMI1 nuclear expression (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).**Figure3:** grade IV astrocytoma shows strong BMI1 nuclear expression with neoplastic cells concentrated around endothelial proliferations negative BMI1 nuclear expression (yellow circle) (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).**Figure2:** grade II astrocytoma shows moderate BMI1 nuclear expression (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).**Figure4:** non-neoplastic brain tissue shows negative EZH2 nuclear expression (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).

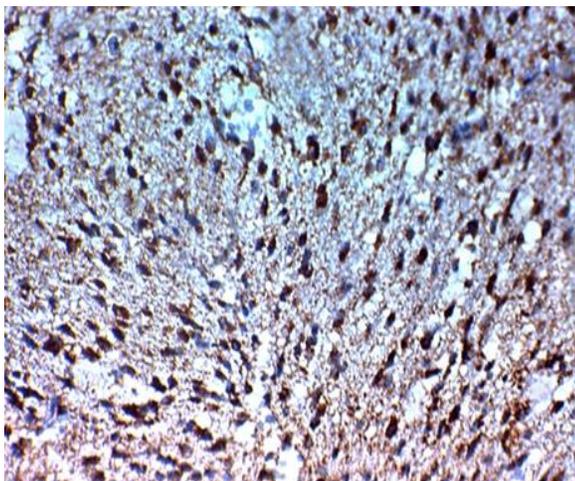


Figure5: grade II astrocytoma shows moderate EZH2 nuclear expression (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).

DISCUSSION

Astrocytic tumors in Egypt constituted about (54.9%) of all Central Nervous System tumors, in study by national cancer institute (NCI) included 810 cases of CNS tumors. They recorded according to WHO classification, 4th edition 2007, by histology. The most frequently reported malignant CNS neoplasms were fibrillary astrocytomas which accounts for (22.6%). glioblastoma multiform (GBM), the most aggressive brain neoplasm, alone represented (14.3%) of all CNS tumors [26].

GBM is an incurable disease with only 15 months a median survival. Based on the 2013 Central Brain Tumor Registry of the United States (CBTRUS) 2006–2010 report, the average annual incidence rate (IR) of GBM is 3.19 per 100,000 populations. This is the highest incidence rate among CNS tumors with malignant behavior [27]. GBM includes functional groups of cells termed glioblastoma stem-like cells (GSCs), which are radioresistant and chemoresistant and ultimately result in tumor recurrence. Dysregulation of PcG (BMI1 and EZH2) is closely linked to the GSC maintenance and tumorigenicity [3]. This study depends on theory of cancer stem cell prognostic role and aimed at immunohistochemical evaluation of BMI1 and

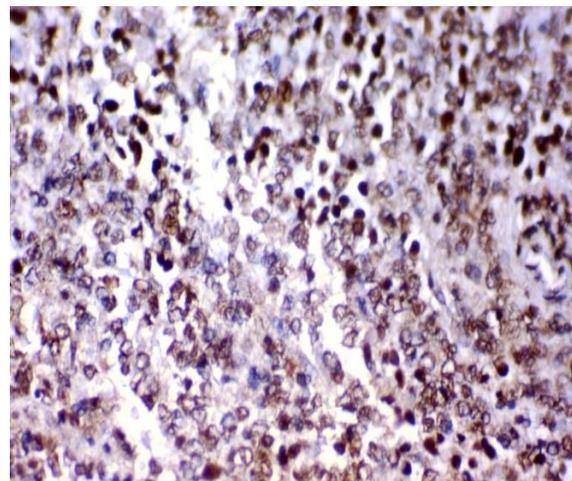


Figure6: grade III astrocytoma shows strong EZH2 nuclear expression (Immunoperoxidase staining, DAB chromogen, Mayer's hematoxylin counter stain, original magnification X400).

EZH2 and their correlation with prognosis and the clinicopathological characters in astrocytoma (particularly in glioblastoma multiform) patients.

BMI1 and EZH2 expression in astrocytoma and non-neoplastic brain tissue:

Current study demonstrated a highly significant difference ($P < 0.001$) between BMI1 expression in astrocytoma cases and in non-neoplastic brain tissue (Control) as BMI1 showed high expression (23/40) (57.5 %) in astrocytoma cases versus only (2/30) (6.7%) in control. In agreement with our study, Wu et al. [25], reported that 86 cases (67.19%) were regarded as BMI1 group with high expression. The level of BMI1 protein expression was significantly higher in glioma tissues than those in equivalent nonneoplastic brain tissues ($P < 0.001$). With regard to involvement of BMI1 in tumor progression, several reports have employed by Bruggeman et al. [28], who concluded that BMI1 has been implicated in the pathogenesis of brain tumors such as gliomas. In addition Häyry et al. [29], explored the genetic status and the correspondent expression patterns of BMI1 in a series of 100 low- and high-grade primary and recurrent gliomas suggesting that BMI1 possibly contributes to brain tumor pathogenesis.

Also current study demonstrated a highly significant difference ($P=0.002$) between EZH2 expression in astrocytoma cases and in non-neoplastic brain tissue (Control) as EZH2 showed high expression (19/40) (47.5%) in astrocytoma cases versus only (4/30) (13.3%) in control. As regard EZH2 role in tumor development, our results supported by similar reports by Wu et al. who investigated that 80 cases (62.50%) that have EZH2 high expression level were significantly higher in glioma tissues than those in equivalent nonneoplastic brain tissues ($P < 0.001$). In addition Ding and Kleer^[30], concluded that increased expression of EZH2 in normal breast tissue disposes to subsequent development of cancer, also similar results by Zhang et al.^[24], and Ahani et al.^[31]. In glioblastomas, who demonstrated that EZH2 inhibited differentiation and activated genes, regulating cell cycle progression, cell proliferation and cell migration.

So our study demonstrated that the deregulation of BMI1 and EZH2 correlating with astrocytoma development and pathogenesis. Our results matched with Abdouh et al.^[32], who shown that BMI1 and EZH2 were related to GBM tumor growth and needed to maintain renewal of cancer initiating stem cell.

BMI1 and EZH2 expression with clinicopathological features of astrocytoma:

This study proved a highly significant association ($P=0.003$) between low expression of BMI1 and low grades (grade I & grade II) as (80%) of the cases show low expression, also, a statistically significant association was found between high expression of BMI1 and high grades (grade III & glioblastoma) with percentage of (75.0%) & (84.6%) respectively. Several reports varied in explaining BMI1 correlation to tumor grade and prognosis. Our results agreed with Wu et al.^[25], who reported that upregulation of BMI1 was significantly correlated with advanced WHO grades ($P < 0.001$) and concluded that BMI1 expression ($P = 0.008$) was poor prognostic factor in glioma patients. In addition Farivar et al.^[33], also concluded that BMI1 high expression is correlated with lower survival and

could be used as a strong molecular marker of prognosis in pediatric brain tumors.

In contrary to other studies by Cenci et al.^[34], who revealed that BMI1 high expression was favourable for the patient survival contributed that to possible proapoptotic role of BMI1 protein in primary glioblastoma. On the other hand, Wei et al.^[35], explained the unpredictable relationship between BMI1 expression and the prognosis to its role in DNA repair. Acceptable explanation by Wang et al.^[8], stated that high level of BMI1 expression was dependent on the cell type and not necessary to associate with a poor prognosis for all types of cancer. For example, high levels of BMI1 were related to a better overall survival in patients with breast cancer, malignant melanoma and endometrial carcinomas. Therefore, these results are necessary to be considered when developing targeted new plans for therapy. In current study, there was a significant association ($P=0.005$) between low expression of EZH2 and low grades (grade I & grade II) with percentage of (100.0%) & (80.0%) respectively, also, a significant association between high expression of EZH2 and high grades (grade III & glioblastoma) as (58.3%) & (76.9%) respectively, suggesting relation to tumor aggressiveness. Many studies demonstrated this EZH2 relation to tumor aggressiveness; according to Wu et al.^[25], study showed upregulation of EZH2 protein was significantly associated ($P < 0.001$) with advanced WHO grades in glioma patients and concluded that expression of BMI1 and EZH2 ($P = 0.008$), were prognostic factors for overall survival in glioma patients. In addition Wang et al.^[8], revealed that EZH2 expression correlated with tumor aggressiveness across all cancer types and Li et al.^[36], showed that the most frequently overexpressed PcG gene in GBMs was EZH2 in (98.6%) and suggested that EZH2 a hopeful therapeutic target in GBMs because of the extremely high frequency of overexpression. Also similar results were obtained by Suvà et al.^[37] and Orzan et al.^[38]. As regard other clinicopathologic characteristics of astrocytoma (age at diagnosis,

gender of patients, site and size of tumor), the current study found no statistically significant association ($P > 0.05$) with BMI1 or EZH2. Our results agreed with Wu et al.^[25], who concluded no statistically significant association of with age at diagnosis and gender of patients was found.

Finally the current study showed that expressions of BMI1 and EZH2 were correlated positively with the astrocytoma grades, suggested that both were considered as poor prognostic factors. This was agreement with Wu et al.^[25], who prove that expression of BMI1 and EZH2 ($P = 0.008$), were prognostic factors for overall survival in glioma patients.

Association between BMI1 and EZH2 expression:

The current study showed a significant ($P = 0.049$) overall a fair agreement between the results of BMI1 and EZH2 expression was (65%), with kappa (κ) = 0.305. The spearman correlation analysis showed a significant positive correlation between BMI1 expression and EZH2 expression ($r = 0.311$; $P = 0.05$) revealing direct relationship between BMI1 and EZH2. These results supported by one study of Wu et al.^[25], proved such correlation between BMI1 and EZH2 overexpression in gliomas with spearman correlation analysis ($r = 0.56$; $P < 0.001$). In addition Hyland et al.^[39], established that EZH2 may be one of the PcG proteins essential for BMI1 recruitment to the PcG bodies, suggesting the closed relationship between BMI1 and EZH2

CONCLUSION

Deregulations of BMI1 and EZH2 have a role in astrocytoma development and pathogenesis, revealed higher expression in astrocytomas compared to non-neoplastic brain tissue. BMI1 and EZH2 are considered poor prognostic factors in astrocytoma depending on their positive correlation with the astrocytoma grades. No statistically significant association between the clinicopathologic characteristics of astrocytoma (age at diagnosis, gender of patients, site and size of tumor) and BMI1 or EZH2 expression. There is a positive correlation between BMI1 expression and

EZH2 expression in astrocytoma. In the future, BMI1 and EZH2 will be considered as promising therapeutic targets in GBMs.

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القيمة التكهنية للإظهار المناعي الهستوكيميائي المشترك لـ BMI1 و EZH2 في أورام الخلايا النجمية

بالرغم من التطورات الحديثة في الجراحة، والعلاج الإشعاعي، والكيميائي، إلا أن متوسط بقاء مرضى الأورام النجمية لا يزال ضعيفا ولم يطرأ تغيير كبير على مدى عقود. وبالتالي، فمن الضروري أن نفهم الخلفية الجينية والعمليات الجزيئية المسببة لهذه الأورام من أجل الكشف عن الآليات الكامنة وراء بدء وتطور المرض، وفتح أبواب جديدة لتطوير الاستراتيجيات التشخيصية والعلاجية. مجموعة البروتينات Polycomb (PCG)، تلعب دورا هاما في تنظيم مختلف العمليات الفسيولوجية والمرضية، بما في ذلك تكون الورم وتشمل هذه المجموعة بروتينات BMI1، و EZH2. دراسات سابقة أشارت إلى أن ظهور EZH2 هو علامة على المرض المتقدم والمنتشر في العديد من الأورام، وقد أثبتت الدراسات الحديثة أن EZH2 مرتبطا بظهور BMI1 مما يشير إلى وجود علاقة بينهما في التحول الخبيث في الأورام النجمية، وأنه قد يعكس أيضا العدوانية البيولوجية لهذا المرض. تهدف الرسالة إلى تقييم إظهار BMI1 و EZH2 بالطرق المناعية النسيجية الكيميائية في حالات الأورام النجمية ومقارنتها بالعوامل السريرية والنسجية. شملت الدراسة الحالية تجميع سبعون حالة أرشيفية منهم اربعون حالة للأورام النجمية وثلاثون حالة أخرى لنسيج المخ غير الورمي، تمت الدراسة في قسم الباثولوجي بكلية طب الزقازيق واثبتت الدراسة قائمه وجود دور لبروتينات BMI1 و EZH2 في التحول الخبيث في الأورام النجمية مقارنة مع أنسجة المخ غير الورمية، وأنها علامات تكهنيه تعكس أيضا العدوانية البيولوجية لهذا المرض استنادا على ارتباط ظهورها الإيجابي بتدرج تخبث الورم. بينما استنتجت الدراسة الحاليه عدم وجود ارتباط ذو دلالة إحصائية بين (العمر عند التشخيص، وجنس المرضى، وموقع وحجم الورم) من الخلايا النجمية وظهور بروتينات BMI1 or EZH2. أشارت الدراسة قائمه ان هناك علاقة طرديه بين ظهور بروتينات BMI1 و EZH2 يستخلص منه ارتباطهما ببعضهما في دورهم في اورام الخلايا النجمية. اوصت الدراسة اعتبار BMI1 و EZH2 أهدافا علاجية في المستقبل.