Maher Kurdi*, Motaz M. Fadul, Bassam Addas, Eyad Faizo, Ahmed K. Bamaga, Taghreed Alsinani, Yousef Katib, Alaa Alkhotani, Amany A. Fathaddin, Alaa N. Turkistani, Ahmed A. Najjar, Saleh Baeesa, Fadi A. Toonsi, Majid Almansouri and Shadi Alkhayyat

Glioblastoma with *PRMT5* gene upregulation is a key target for tumor cell regression

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Abstract

Objectives: Protein Arginine Methyltransferase 5 (*PRMT5*) is an enzyme that regulates gene expression and protein function through arginine methylation. Its association with isocitrate dehydrogenase (IDH) mutation in Grade-4 astrocytoma was rarely investigated. Our aim was to aim to explore the association between IDH mutation and PRMT5 and its effect on tumor recurrence.

Methods: A retrospective cohort of 34 patients with Grade 4 astrocytoma has been tested for *PRMT5* expression using protein and gene expression arrays. The impact of IDH-mutation and *PRMT5* expression on tumor recurrence was explored.

Results: IDH-wildtype was detected in 13 tumors. *PRMT5* protein was highly expressed in 30 tumors and the expression was low in four tumors. *PRMT5* gene expression was upregulated in 33 tumors and downregulated in a single tumor case. Tumors with different *PRMT5* gene expressions and IDH mutation were found to have a significant statistical difference in recurrence-free interval (RFI) (p-value<0.001). IDH-wildtype glioblastoma with upregulated *PRMT5* gene or protein expression showed earlier tumor recurrence compared to IDH-mutant Grade 4 astrocytoma with upregulated PRMT5 expression.

Conclusions: The association between IDH mutation and *PRMT5* in IDH-mutant Grade 4 astrocytoma or IDH-wildtype glioblastoma is indirectly bidirectional. PRMT5 upregulation in glioblastoma can lead to increased cell proliferation and tumor regrowth.

Keywords: Grade 4 astrocytoma; isocitrate dehydrogenase (IDH) mutation; glioblastoma; Protein Arginine Methyltransferase 5 (PRMT5); recurrence

***Corresponding author: Dr. Maher Kurdi**, Department of Pathology, Faculty of Medicine, King Abdulaziz University, Rabigh, Saudi Arabia; and Neuromuscular Unit, King Fahad Medical Research Center, Jeddah, Saudi Arabia, E-mail: Ahkurdi@kau.edu.sa. https://orcid.org/0000-0002-8979-3849
Motaz M. Fadul, Department of Pathology, Faculty of Medicine, King Abdulaziz University, Rabigh, Saudi Arabia
Bassam Addas, Department of Surgery, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
Eyad Faizo, Department of Surgery, Faculty of Medicine, University of Tabuk, Tabuk, Saudi Arabia
Ahmed K. Bamaga, Department of Paediatric, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
Taghreed Alsinani, Department of Surgery, King Fahad General Hospital, Jeddah, Saudi Arabia
Yousef Katib, Department of Radiology, Faculty of Medicine, Taibah University, Madinah, Saudi Arabia
Alaa Alkhotani, Department of Pathology, College of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia
Amany A. Fathaddin, Department of Pathology, College of Medicine, King Saud University, Riyadh, Saudi Arabia
Alaa N. Turkistani and Saleh Baeesa, Department of Neurosciences, King Faisal Specialist Hospital and Research Center, Jeddah, Saudi Arabia
Ahmed A. Najjar, Department of Surgery, Faculty of Medicine, Taibah University, Jeddah, Saudi Arabia
Fadi A. Toonsi, Department of Radiology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
Majid Almansouri, Department of Clinical Biochemistry, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
Shadi Alkhayyat, Department of Internal Medicine, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

Introduction

Post-translational modification (PTM) is essential to expand the cellular proteome, which can affect protein interaction, stability, and activity with other proteins on multiple sites. This PTM is mediated by a variety of enzymatic processes, including phosphorylation, hydroxylation, acetylation, and methylation [1]. Protein arginine methylation was characterized in 1964 by Hurwitz et al. [2]. They discovered that the enzyme arginine methyltransferase was responsible for adding a methyl group to the amino acid arginine in proteins. This modification plays an important role in the regulation of cellular processes, gene expression, homeostasis, and protein-protein interaction. The main type of protein arginine methyltransferases (*PRMT*) was *PRMT1*,

and it was followed by nine other members [3, 4]. *PRMT* family enzymes catalyze three distinct types of methylation. Type I *PRMTs*, including PRMT1, 2, 3, 4, 6 and 8, catalyze mono-methylarginines (MMA) and asymmetric dimethylarginines (ADMA) synthesis [5]. The type II *PRMTs* which include PRMT5 and 9, catalyze MMA and symmetric dimethylarginines (SDMA) synthesis. *PRMT7*, a type III *PRMT*, catalyzes only MMA [6, 7]. All *PRMT* family members, which have been found to be highly expressed in several solid tumors, are considered dominant regulators of arginine methylation [8]. Although *PRMT* mutations are infrequent in these tumors, increased *PRMT* gene expression has been identified as a predictor of poor prognosis [8].

A new type of PRM, known as *PRMT5*, was recently discovered in mammals [3]. The protein has two domains: an N-terminal domain, which adopts a triosephosphate isomerase structure and binds to methylosome protein 50 (MEP50) for full methyltransferase activity, and a C-terminal domain, which contains all methyltransferase motifs and is essential for plasma membrane association as a catalytic domain [9]. *PRMT5* is an epigenetic modifier that methylates histones to control gene expression. Specifically, it can methylate histone H2A at Arg3 to form H2AR3me2s and histone H3 at both Arg2 and Arg8 to form H3R2me2s and H3R8me2, respectively [10]. As H4R3me2s, *PRMT5* can also methylate histone H4 on Arg3 [11]. *PRMT5* functionally activates or suppresses gene expression by changing these residues in histone tails [4]. Studies over the last five decades demonstrated that *PRMT5* is an oncoprotein that regulates a variety of cellular processes involved in cancer development through several signaling pathways [12]. Epigenetically, upregulated *PRMT5* expression has been linked to a negative prognosis in numerous types of cancer [13, 14]. Expression levels in liver, lung, and breast cancers have been associated with large tumor size and advanced tumor grade [15–17].

The association between *PRMT5* and diffuse gliomas has been explored in scattered studies [18–21]. *PRMT5* expression varies in different grades of astrocytomas, either undetectable or very low in low-grade gliomas, whereas high-grade gliomas have the highest levels of expression [19]. A significant increase in *PRMT5* expression was seen in Grade 4 astrocytomas compared to normal brain tissue controls [20]. This expression negatively correlates with patient survival [21]. There is evidence to suggest that the growth of gliomas may be reliant on *PRMT5* expression, making it a potential new target for glioblastoma therapy. Otani et al. demonstrated that blocking *PRMT5* activity led to apoptosis in both differentiated and stem-like glioma tumor cells [22]. Moreover, Yan et al. demonstrated that *PRMT5* mediates the methylation of arginine in *P53* to control its activity [21]. Their findings implied that the significance of *P53* arginine methylation may vary between tumor types and that *PRMT5* could be an equally promising target in glioblastoma tumors where *P53* is often lost or mutated and linked to a negative prognosis. This is because the findings indicate that cell death triggered by *PRMT5* inhibition is unrelated to *P53* mutational status [21].

The exact mechanism of *PRMT5* upregulation in Grade 4 astrocytoma is not yet fully investigated. Upregulation of some transcriptional factors such as *c-Myc* or the down-regulation of miRNAs may contribute to increased *PRMT5* expression in glioblastoma [23, 24]. This increased expression can cause various effects on cellular processes, including alterations in gene expression, epigenetic modifications, and protein functions. Additionally, there was no sufficient evidence to observe the relationship between *PRMT5* and isocitrate dehydrogenase (IDH) mutation in gliomas. One study found that IDH-mutant Grade 4 astrocytoma has lower levels of *PRMT5* expression compared to IDH-wildtype glioblastoma, suggesting that *PRMT5* may be a target of IDH mutation [25]. Our research objective is to investigate the correlation between the expression of the PRMT5 gene and the presence of IDH mutation in Grade 4 astrocytoma. Additionally, we aim to assess the influence of this association on tumor growth and recurrence.

Materials and methods

Patients selection

This study was approved by the combined ethics committee between King Faisal Specialist Hospital and Research Center [CA-2020-06] in conjunction with King Abdulaziz University to use patient samples in this current research. The study involved 34 patients, in the period between 2017 and 2021, who were diagnosed with Grade 4 astrocytoma (Table 1). The histopathological diagnosis was established based on fifth edition of WHO classification of CNS tumors [26, 27].

All patients included in this study have received radiotherapy and Temozolomide (TMZ) chemotherapy after surgery [28] (Table 1). Patients' information was retrieved from the hospital archives and included the patient's age, sex, IDH-mutation status, treatment plan, and recurrence-free interval (RFI). RFI was estimated from the first day of surgical resection to the first day of tumor recurrence.

Tissue processing

Paraffin embedded tissue blocks from 34 cases were utilized to obtain 4 µm thick sections rolled tissue. The slides were used to assess protein expression via Immunohistochemistry (IHC) with anti-*PRMT5* antibody.

Table 1: The clinical and biological data of 34 tumors enrolled in our study.

Age	Sex	IDH status	IHC grading	ACTB Ct	GAPDH Ct	PRMT5 Ct	PRMT5 expression	CTX	RFI
80	Male	IDH-mutant	Diffuse	26.712	26.640	28.887	Upregulated	TMZ	600
67	Female	IDH-wildtype	Diffuse	32.656	29.961	35.013	Upregulated	TMZ	198
40	Male	IDH-mutant	Diffuse	30.684	27.271	34.997	Downregulated	TMZ +	1,400
44	Male	IDH-mutant	Diffuse	31.195	25.958	33.975	Upregulated	TMZ	177
56	Male	IDH-wildtype	Diffuse	31.938	27.298	33.867	Upregulated	TMZ	191
50	Female	IDH-wildtype	Diffuse	30.803	26.479	31.698	Upregulated	None	217
48	Male	IDH-mutant	Moderate	30.495	28.228	32.208	Upregulated	TMZ	747
51	Male	IDH-wildtype	Moderate	33.450	31.486	35.619	Upregulated	TMZ	180
31	Female	IDH-mutant	Diffuse	31.018	26.501	32.544	Upregulated	TMZ	359
60	Female	IDH-wildtype	Diffuse	33.198	27.299	33.911	Upregulated	TMZ	430
27	Female	IDH-mutant	Diffuse	35.828	32.589	37.702	Upregulated	TMZ	350
43	Male	IDH-mutant	Diffuse	33.230	29.435	34.878	Upregulated	TMZ	293
28	Male	IDH-mutant	Diffuse	33.592	29.404	35.475	Upregulated	TMZ	566
55	Male	IDH-wildtype	Diffuse	31.415	27.427	32.274	Upregulated	TMZ	190
69	Female	IDH-wildtype	Diffuse	31.823	28.840	33.469	Upregulated	TMZ	211
55	Male	IDH-mutant	Diffuse	30.803	26.479	31.698	Upregulated	None	1,128
61	Female	IDH-mutant	Diffuse	31.341	28.673	32.756	Upregulated	TMZ	339
44	Female	IDH-mutant	Diffuse	31.431	28.680	32.768	Upregulated	TMZ	623
38	Male	IDH-wildtype	Diffuse	32.795	30.107	33.256	Upregulated	TMZ	485
25	Male	IDH-mutant	Diffuse	35.779	32.587	38.387	Upregulated	TMZ	550
66	Female	IDH-wildtype	Diffuse	32.088	28.061	33.008	Upregulated	None	80
59	Female	IDH-wildtype	Diffuse	31.551	27.116	31.643	Upregulated	None	155
45	Male	IDH-mutant	Diffuse	34.666	26.032	30.802	Upregulated	TMZ +	684
51	Male	IDH-mutant	Diffuse	32.203	27.570	31.932	Upregulated	TMZ	762
31	Male	IDH-mutant	Diffuse	33.163	29.843	33.600	Upregulated	TMZ	548
24	Female	IDH-mutant	Diffuse	30.643	26.032	30.802	Upregulated	TMZ	229
59	Male	IDH-mutant	Diffuse	32.231	26.251	31.319	Upregulated	TMZ	461
78	Male	IDH-mutant	Diffuse	35.779	32.587	38.387	Upregulated	TMZ +	549
43	Female	IDH-mutant	Diffuse	30.777	26.633	30.588	Upregulated	TMZ	723
55	Female	IDH-wildtype	Diffuse	31.693	26.498	32.022	Upregulated	TMZ	208
59	Male	IDH-wildtype	Diffuse	31.946	27.113	31.079	Upregulated	None	455
51	Male	IDH-mutant	Moderate	34.794	31.737	34.256	Upregulated	TMZ	210
64	Male	IDH-wildtype	Moderate	33.875	30.914	34.345	Upregulated	TMZ	433
60	Male	IDH-mutant	Diffuse	31.205	25.597	31.226	Upregulated	TMZ	1,100

IDH, Isocitrate dehydrogenase; IHC, Immunohistochemistry; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ACTB, Active B; PRMT5, Protein Arginine Methyltransferase 5; RE, Relative expression, CTX, Chemotherapy; RFI, Recurrence free-interval.

RNA extraction was performed to assess *PRMT5* gene expression through Real-Time Polymerase-Chain Reaction (RT-PCR).

Protein expression measurement using IHC for anti-*PRMT5* and IDH1 antibodies

Anti-*PRMT5* antibody (rabbit monoclonal, Cat# EPR5772, Abcam, Cambridge, UK) was used on the 34 sections in IHC. The assay was processed through a GX-automated stainer from Ventana (Tucson, AZ, USA) Using an Ultra-View detection Kit from Ventana. The protocol involved deparaffinization using EZ preparation solution at 75 °C, followed by heat pre-treatment in a cellular medium for 60 min. This was further followed by an optimum incubation of 20 min at 75 °C after adjusting the antibody using a dilution of 1:200.

Anti-*PRMT5* was used to identify tumor cells (TC) in the tumor microenvironment. Each section was examined using a light microscope (DM500 Leica, Germany) examined at ×10 high-power field (HPF) and a focal non-necrotic area with anti-*PRMT5* expression was re-examined ×25 HPF. Cells with anti-PRMT5 expression were considered as *PRMT5* positive while the total cells were defined as cells with expressed *PRMT5* and non-stained cells. The labelling index (LI) of *PRMT5* expression at each examined area was evaluated using the following equation:

$$\text{Labelling Index } (\%) = \frac{\text{PRMT5} + \text{TC}}{\text{Total cells}} \times 100$$

$IDH1^{R132H}$ (monoclonal mouse antibody, clone H09, Abcam, UK) was retrospectively assessed during tumor diagnosis and grading, using an automated stainer from Ventana (Tucson, USA). Sections in which >10 % of tumor cells were positively stained were defined as IDH1-mutant.

Three expression patterns were defined: no expression: 0 %; minimal expression: <20 %; moderate expression: 20–60 %; diffuse expression: >60 %. No tumors were found to have a minimal *PRMT5* expression. Therefore, tumors with moderate expression were

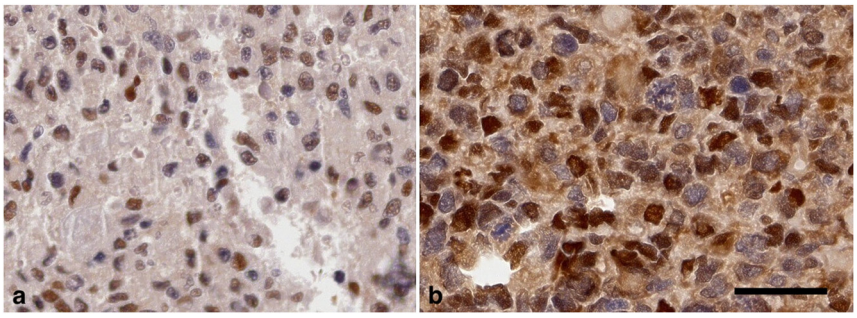

Figure 1: Anti-*PRMT5* stains tumor cells in the microenvironment using IHC. (a) Low expression (b) high expression. Magnification (×25)=100 µm on scale bar.

categorized as having low expression, while those with diffuse expression were categorized as having high expression (Figure 1). IDH1 mutation has been previously assessed using the IHC technique.

RNA extraction and complementary cDNA synthesis

RNA was extracted from 34 tumor samples and two controls for testing housekeeping genes: Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) and Actin Beta (*ACTB*) and targeting gene (*PRMT5*). RNeasy Kit from QIAGEN (Venlo, Netherlands) was used in RNA extraction. Each sample was deparaffinized at the Eppendorf tube after vertexing with 99 % xylene. Samples were centrifuged and the pellet was washed with 100 % ethanol to ensure the samples were completely clean of xylene. The samples were dried off in a dry bath for 15 min. Buffer Protein Kinase (Buffer PK) (Haven Scientific, KAUST, Thuwal, Saudi Arabia) was mixed thoroughly with the pellet and followed with optimum repetitive incubations at 56 °C for 15 min; followed by incubation at 80 °C for 15 and 3 min on ice. The tubes were centrifuged to pellet insoluble tissue debris and the supernatant was mixed with DNase Buffer at room temperature. Buffer was vortexed with the sample and 100 % ethanol was added and mixed by pipetting. The remaining lysate-ethanol mixture was added to the spin column, centrifuged, and the flow-through was discarded. RNA extraction buffer (Buffer RPE) was added to the spin, centrifuged, and the flow-through was discarded. The dry spin column was transferred to an RNase-free tube and 30 µL RNase-free water was added and centrifuged for 3 min. The sample was used from the RNA-containing eluates for spectrophotometric analysis. A spectrophotometer was used to measure the absorbance of the nucleic acid sample at specific wavelengths of light. The two most commonly used wavelengths were 260 and 280 nm. The absorbance at 260 nm was used to determine the concentration of nucleic acids in the sample. This measurement is based on the principle that nucleic acids absorb UV light at this wavelength. By comparing the value of 260 nm to a standard curve or using specific equations, the concentration of the nucleic acid was determined.

The cDNA was synthesized using the cDNA Reverse Transcription (RT) Kit (Applied Biosystems, Waltham, USA) according to the manufacturer's protocol. Briefly, a master mix was prepared with 1 µL RT Buffer, 0.4 µL dNTP Mix (100 mM), 1 µL RT Random Primers, and 1 µL MultiScribe™ Reverse Transcriptase (Applied Biosystems, Waltham, USA) was mixed with 70 ng of RNA, and the final volume was adjusted to 10 µL with RNase-free water. For samples whose concentration was <10 ng/µL, the maximum volume of RNA was added (7.1 µL). After cDNA synthesis, 170 µL RNase-free water was added.

Table 2: The designed primer sequences used for PRMT5 gene.

Primer	Sequence	Amplicon size
Forward	5′-GAGTATCCGTCCAGAGACTCAC-3′	81
Reverse	5′-ACCGTTATGGGCTGCTTAATAG-3′	81

Gene sequencing using Real-Time Polymerase Chain Reaction (RT-PCR)

The primers for the targeting gene (*PRMT5*) and two reference housekeeping genes (*GAPDH* and *ACTB*), were designed (Haven Scientific, KAUST, Thuwal, Saudi Arabia). The following primer sequences for PRMT5 were used in Table 2.

RT-PCR was performed using the EverGreen Universal PCR Master Mix (Cat#PCR5505, Haven Scientific, KAUST, Thuwal, Saudi Arabia) in triplicate reactions. The synthesized cDNA was mixed with a small volume of each oligo for a final PCR reaction. Plates were sealed with an adhesive seal. Two replicates of threshold cycle (C_T) values were used for both genes. The C_T mean and standard deviation for the reference genes (*GAPDH* and *ACTB*), as well as the target gene (PRMT5), were analysed based on the RT-PCR data and for $\Delta\Delta C_T$ and ΔC_T. The relative quantification (Rq) and the fold change (FC) were also estimated to assess the gene expression. The data results are summarized in Table 1.

Statistical analysis

The McNamar test was used to identify the expression sensitivity and specificity of *PRMT5* between two diagnostic differentiation methods. The log-rank test was used to compare the recurrence distributions among the groups. Kaplan–Meier curves (KMC) were used to compare the distribution of RFI among cases with different *PRMT5* expressions and IDH mutational status. A p-value of <0.05 was considered statistically significant. All statistical analyses in this study were performed using IBM SPSS Version 24 (SPSS Inc., Armonk, NY, USA).

Results

Patients age range between 24 and 80 years (mean 50.2). IDH-wildtype was detected in 13 (38.2 %) tumors. *PRMT5* protein was highly expressed in 30 (88.2 %) tumors and low expressed in 4 (11.8 %) tumors. *PRMT5* gene expression was

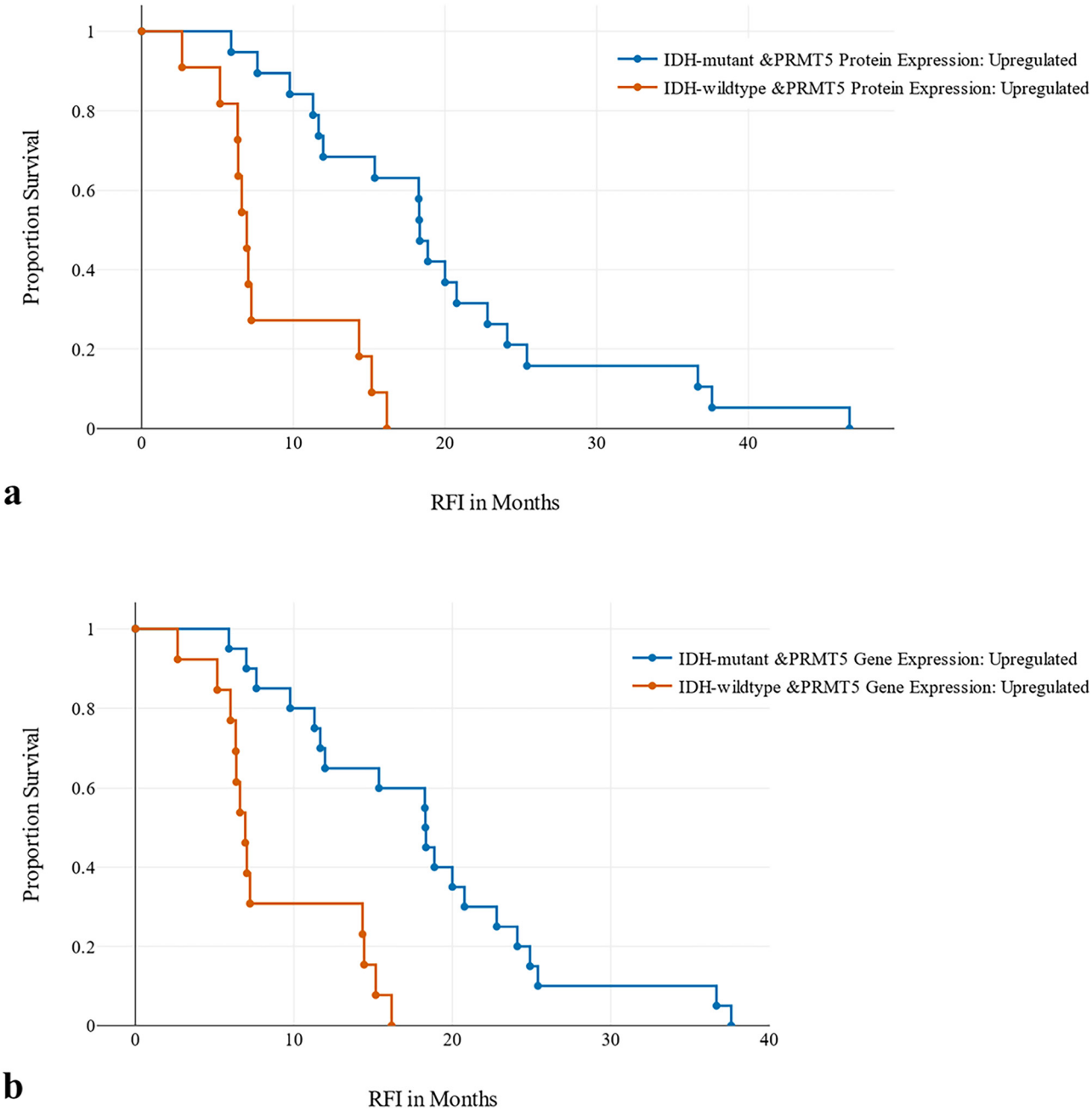

Figure 2: The impact of *PRMT5* expression on RFI. (a) The impact of *PRMT5* protein expression and IDH mutation on RFI; (b) the impact of *PRMT5* gene expression and IDH mutation on RFI.

upregulated in 33 (97.1 %) tumors and downregulated in single tumor cases (2.9 %). The mean RFI is 15.5 months (standard deviation: 10.2). There was no major difference in diagnostic consistency between the two testing methods used for *PRMT5* expression. Testing *PRMT5* expression with RT-PCR was 87 % sensitive compared to IHC protein expression. The overall accuracy between the two testing methods was 85.3 %.

There was a significant statistical difference in RFI among tumors with different *PRMT5* gene regulations and IDH mutations (p-value<0.001). IDH-wildtype tumors with upregulated *PRMT5* gene or protein expression showed earlier tumor recurrence compared to IDH-mutant tumor with upregulated *PRMT5* expression (Figure 2).

Discussion

PRMT5 regulates the expression of genes involved in cell proliferation, cell cycle regulation, and DNA damage

response. It also promotes the growth of cancer cells, which are thought to be responsible for tumor recurrence and resistance to treatment. *PRMT5* has also been identified as a potential therapeutic target in many cancers [4]. Targeting *PRMT5* with specific inhibitors can lead to the suppression of glioma cell growth and the restoration of the immune response. This approach showed promising results in some preclinical models and is currently being tested in clinical trials as a potential treatment for Grade 4 astrocytoma [18].

The exact mechanisms by which *PRMT5* is overexpressed in Grade 4 astrocytoma are not fully understood. One possible mechanism is the upregulation of transcription factors that directly or indirectly activate *PRMT5* expression [23]. Jin et al. found that the transcription factor *E2F1*, a key regulator of the G1 to S phase transition in the cell cycle involved in DNA replication and cell proliferation, can stimulate *PRMT5* expression by binding to its promoter [24]. Another possible mechanism is the dysregulation of microRNAs (miRNAs) that target *PRMT5* mRNA. In glioblastoma, the expression of these miRNAs is often downregulated, leading to increased *PRMT5* expression [28].

Insufficient evidence exists to establish a direct correlation between PRMT5 and IDH mutation in gliomas. It is also unclear if IDH mutation or its products would affect *PRMT5* gene activity. Our results revealed that most of the glioblastomas associated with upregulated PRMT5 had earlier tumor recurrence. It means that IDH is more directly linked to tumor recurrence than *PRMT5* (Figure 2). Suvà et al. found that IDH-mutant Grade 4 astrocytoma has lower levels of PRMT5 expression compared to IDH-wildtype glioblastoma, suggesting that *PRMT5* may be a downstream target of IDH mutation [25]. Additionally, IDH-mutant tumors have a distinct DNA methylation signature, which can lead to the dysregulation of genes involved in cell differentiation and proliferation.

PRMT5 has been shown to play a role in the regulation of DNA methylation, and it is possible that its dysregulation in IDH wild-type gliomas contributes to the altered methylation patterns observed in these tumors [29]. We believe that IDH mutation in Grade 4 astrocytomas with *PRMT5* upregulation may lead to decreased cell proliferation and increased differentiation, suggesting that *PRMT5* may be involved in maintaining the stem-like state of glioma cells. On the other hand, absent IDH mutation may lead to tumor re-growth and progression.

The relationship between hydroxyglutarate (2-HG), a product of IDH, and *PRMT5* in glioma is complex. 2-HG is a structural analog of alpha-ketoglutarate (αKG), which is a cofactor for several enzymes involved in epigenetic regulation, including *PRMT5*. 2-HG can compete with αKG for binding to *PRMT5* and other enzymes, effectively inhibiting their activity [30]. In addition to these direct effects on enzyme activity, 2-HG can also alter the availability of substrates for epigenetic regulation, such as histones, by affecting metabolic pathways in the cell. For example, 2-HG has been shown to inhibit the activity of demethylases, enzymes that remove methylation marks from histones, leading to alterations in the epigenetic landscape that contribute to gliomagenesis [10, 30].

It seems that the interaction between 2-HG and *PRMT5* is bidirectional, with *PRMT5* activity modulating 2-HG levels in gliomas. 2-HG usually accumulates in cells with IDH mutations, which can inhibit the activity of several enzymes involved in epigenetic regulation, including *PRMT5*. However, epigenetic dysregulation may occur when 2-HG is not released, and this can cause *PRMT5* overexpression [10]. In the end, the exact mechanisms underlying the relationship between *PRMT5* and IDH mutation in Grade 4 astrocytoma require further research.

PRMT5 inhibitors are a class of compounds that specifically target and inhibit the activity of the *PRMT5* enzyme. In IDH-mutant WHO Grade-4 astrocytoma or glioblastoma, *PRMT5* inhibitors have gained attention as potential therapeutic agents due to their ability to disrupt the aberrant PRMT5-mediated processes that contribute to tumor growth and progression [31, 32]. Preclinical studies have shown promising results regarding the use of *PRMT5* inhibitors in glioblastoma. These inhibitors have been found to suppress the growth of glioblastoma cells, and induce cell cycle arrest, and apoptosis. Additionally, *PRMT5* inhibitors have demonstrated the ability to inhibit the migration and invasion of glioblastoma cells, which are crucial processes involved in tumor metastasis. Based on our findings, we believe that *PRMT5* inhibitor would make beneficial anti-tumor activity on IDH-wildtype glioblastoma with *PRMT5* upregulation than downregulated tumors. *PRMT5* inhibitors have also been shown to sensitize glioblastoma cells to other treatment modalities, such as radiation therapy and chemotherapy [32]. This suggests that combining *PRMT5* inhibitors with existing therapies may enhance their effectiveness and improve patient outcomes. Further research and clinical trials are needed to evaluate the safety, efficacy, and potential side effects of these inhibitors in glioblastoma patients.

One acknowledged limitation of our study is the relatively low number of samples analyzed. However, it is important to note that our study aimed to examine the relationship between IDH and PRMT5 in Grade 4 astrocytoma.

Conclusions

IDH mutation directly affects the mechanism of *PRMT5* expression in Grade 4 astrocytomas. IDH-wildtype

glioblastoma with *PRMT5* upregulation can accelerate tumor re-growth, implying that IDH and *PRMT5* have a bidirectional mechanism.

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Research ethics: This study was performed in line with the principles of the Declaration of Helsinki. The approval was granted by Biomedical Ethics Committee at King Faisal Specialist Hospital and Research Center [CA-2020-06] and King Abdulaziz University to authorize using Patient Samples in Research.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Author contributions: All authors contributed to the study conception and design. **MK** wrote the conceptualization and study design. **MK** and **MF** performed the genetic analysis. **BA, EF, AB, TAS, YK, AAK, AF, FT, MAL, SAL, AT, AN, and SB** provided clinical and data information as well as tissue samples. **MK** and **AAK** performed IHC and histological interpretation. **MF** help to perform the statistical analysis. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: No conflict of interest.

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Data availability: The data that support the findings of this study are available from the corresponding author **MK** upon request.

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