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Association Between CD204-Expressed Tumor-Associated Macrophages and *MGMT*-Promoter Methylation in the Microenvironment of Grade 4 Astrocytomas

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Abstract

Background: Tumor-associated macrophages (TAMs) are principal immune cells in glioma microenvironment which support tumor growth and proliferation. Our aim in this study was to assess the relationship between CD204-expressed TAMs and O^6-methylguanine-DNA methyltransferase (*MGMT*)-promoter methylation in World Health Organization (WHO) grade 4 astrocytomas, and its impact on patient's clinical outcome.

Methods: The expression of $CD204^+$ TAMs was quantitively assessed on 45 samples of WHO grade 4 astrocytomas using immunohistochemistry. *MGMT*-promoter methylation was tested by methylation techniques. The relationship between TAMs, *MGMT*-promoter methylation, and recurrence-free interval (RFI) was statistically analyzed.

Results: There were 10 cases (22.2%) with isocitrate dehydrogenase (*IDH*)-mutant grade 4 astrocytoma and 35 cases (77.8%) with *IDH*-wildtype glioblastoma. *MGMT*-promotor was methylated in 18 cases (40%), unmethylated in 15 cases (33%), and the remaining 12 cases showed no *MGMT* status because of nucleic acid degradations. The expression of $CD204^+$ TAMs was high in 32 cases (71.7%) and low in 13 cases (28.8%). The relationship between *IDH1* mutation and $CD204^+$ TAM expression was insignificant ($P = 0.93$). However, the significant difference was found between *MGMT* methylation and $CD204^+$ TAMs expression ($P = 0.01$), in which $CD204^+$ TAMs were diffusely expressed in *MGMT*-methylated cases. There was no significant difference in RFI between $CD204^+$ TAMs expression, *MGMT*-promoter methylation and treatment modalities.

Conclusions: Grade 4 astrocytomas with diffusely expressed $CD204^+$ TAMs are usually associated with *MGMT*-promoter methylation. Although this association is unclear, $CD204^+$ TAMs may neutralize the effect of *MGMT*-DNA protein to loss its function, which contributes to tumor progression. This relationship had no significant impact on the patient's clinical outcome after different treatment modalities.

Keywords: Astrocytoma; Tumor-associated macrophages; CD204; *MGMT* methylation

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Introduction

Despite the palliative treatment of World Health Organization (WHO) grade 4 astrocytomas, it remains the deadliest cancer in the body with a median survival time of less than 18 months [1]. Because of this unfavorable prognosis, the need to explore new therapeutic approaches becomes crucial. According to the 2021 WHO classification of central nervous system (CNS) tumors and European Association of Neuro-oncology (EANO) guidelines, WHO grade 4 astrocytoma is classified into isocitrate dehydrogenase (*IDH*)-mutant and *IDH*-wildtype; however, *IDH*-wildtype astrocytoma is isolated for glioblastoma [2, 3].

The microenvironment of astrocytoma consists of different cellular lineages including tumor cells, immune cells and non-immune cells that infiltrate as tumor niches [4]. Tumor-associated macrophages (TAMs) are considered as essential immune

cells in this microenvirment, which assist and control tumor cells' proliferation and modulation [1, 4]. Nevertheless, the recruitment and the reprogramming between tumor cells and TAMs are still not well explained. Two types of TAMs were identified: 1) M1-polarized TAMs "anti-tumor" and 2) M2-polarized TAMs "anti-inflammatory". M2-polarized TAMs have an immune modulatory effect that stimulates tumor cells' proliferation and leads to formation of metastatic niches [5, 6]. When TAMs encircle tumor cells, they inhibit T-cell cytotoxic function, and consequently the tumor cells escape the immune system. This may cause TAMs aggregating in the microenvironment with no T-cell development [7]. Preclinical studies have suggested that these regulatory T cells (Tregs) are essential for promoting tumor immunosuppression parallelly with TAMs, by which they are strongly associated with vascular endothelial growth factor (VEGF) production [8, 9]. Tregs depletion significantly downregulates the expression of immune suppressive molecules, such as B7-H1 on TAM, and reduces tumor growth [8, 9].

Some clinically reported data indicated that large numbers of TAM, such as CD163, CD204, and CD206, were associated with poor outcome in different types of cancers such as skin melanoma and carcinoma of breast, urinary bladder, ovary and lung [10]. Theses receptors are considered as crosstalk between cancer cells and TAMs for circulating tumor cells in the blood [11]. One of the recently explored markers of M2-polarized TAMs in the WHO grade 4 astrocytoma microenvironment is CD204 [12-14]. CD204 is involved in the process of tumor phagocytosis and the production of reactive oxygen species [12, 14]. Its expression in tumor microenvironment was found to be involved in the process of glioblastoma immunomodulatory system [15, 16].

Other than CD204-linked TAMs, several molecular biomarkers have been identified as prognosticators for patients with high-grade astrocytoma. Amongst these, O^6-methylguanine-DNA methyltransferase (*MGMT*)-promoter methylation is a biomarker for good clinical outcome [17, 18]. *MGMT* is a DNA repair protein that reverses alkylation at the O^6 position of guanine, thereby neutralizing the cytotoxic effects of temozolomide (TMZ) alkylating agent. A lack of *MGMT* repair contributes to the progression of cancers through the accumulation of DNA mutations [19, 20]. Because grade 4 astrocytomas started to develop resistant to TMZ treatment in *MGMT*-methylated tumors, the need to explore new therapeutic targets to increase sensitivity of tumor cells to TMZ becomes essential [21].

Although the relationship between the immune check point registry and *IDH1* mutation has been explored, the association between immune check point receptors, receptor blockers or current immunotherapies has never been investigated in correlation with *MGMT*-promoter methylation [22]. In our study, we investigated for the first time the relationship between the CD204-expressed TAMs and *MGMT* gene promoter methylation in the microenvironment of WHO grade 4 astrocytomas. We also explored the prognostic impact of these biomarkers on patient's clinical outcome after different treatment modalities.

Materials and Methods

This study has been approved by Biomedical Ethics Committee at King Abdulaziz University (HA-02-J-008) to authorize using patient samples in research, which complies with the guidelines of the "System of ethics of research" prepared by the King Abdulaziz City for Science and Technology and approved by Royal Decree No. M/59 on August 24, 2010.

Table 1. Demographic Data of 45 Cases of WHO Grade 4 Astrocytoma Enrolled in the Study

	Overall (n = 45)
Age	
< 55 years	10 (22%)
≥ 55 years	35 (78%)
IDH status	
IDH-wildtype	35 (77.8%)
IDH-mutant	10 (22.2%)
Genetic profile	
Unmethylated *MGMT*	15 (33.3%)
Methylated *MGMT*	18 (40%)
Unknown	12 (26.6%)
Tumor location	
Frontal	18 (40%)
Temporal	12 (26.6%)
Parietal	12 (26.6%)
Occipital	2 (4.4%)
Cerebellar	1 (2.2%)
$CD204^+$ TAMs expression	
High expression	32 (71.1%)
Low expression	13 (28.8%)
Adjuvant therapy	
Chemoradiotherapy	26 (57.7%)
Radiation	17 (37.7%)
None	2 (4.6%)

IDH: isocitrate dehydrogenase; *MGMT*: O^6-methylguanine-DNA methyltransferase; TAMs: tumor-associated macrophages; WHO: World Health Organization.

Patients sampling

Our study included 45 patients histologically diagnosed as WHO grade 4 astrocytoma after radical surgical resection, in the period between 2015 and 2018 (Table 1). Patients' information were collected from the hospital archives which included patient's age during diagnosis, gender, tumor location, and the results of $IDH1^{R132H}$ mutation and *MGMT* methylation. Recurrence-free interval (RFI) was estimated from the beginning of post-surgical therapy to the possible first day of recurrence. The entire cases included in this study were associated with tissue necrosis, microvascular proliferation, *ATRX* loss and intact 1p19q (Table 2). The histopathological diagnoses were re-evaluated based on 2021 WHO classification of CNS tumors [2, 3]. Standard radio-

therapy was given as a total dose of 60 Gy, and the post-surgical chemotherapy regime followed the Stupp's protocol [23]. TMZ was given at 150 - 200 mg/m^2 for 5 days for 6 - 12 cycles. All patients involved in this project died.

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tissue blocks and slides of 45 patients, diagnosed with WHO grade 4 astrocytomas, were collected. Sections stained with H&E, *ATRX* and *IDH1*^{R132H} were examined by consultant pathologist (MK) to reassess the histological diagnosis based on the 2021 WHO classification of CNS tumors (Table 2). Additional one slide of each 45 blocks was stained for anti-CD204 antibody.

Immunohistochemistry (IHC) protocol

Anti-CD204 antibody (Rabbit polyclonal, Abcam, Cat# 217843,) directed against human antibody, was used in the IHC assay of the entire 45 FFPE sections. The procedure was done by using a Ventana detection Kit (Ultra-View) that was processed in GX automated immunostainer from Ventana (Tuscon, AZ, USA). The protocol comprised of deparaffinization with EZ Prep at 75 °C, heat pre-treatment in a cellular medium for 60 min followed by an optimum incubation for 20 min at 37 °C. The antibody was adjusted using a dilution of 1:300. The slides were counterstained with hematoxylin II and bluing reagent for 30 min. The positive control was histological sections containing macrophages.

IHC assessment

Quantitative assessment of CD204 expression in WHO grade 4 astrocytomas

Anti-CD204 stains TAMs in the microenvironment of astrocytoma. Each histological section was screened at low power field (× 10) using light microscopy (Digital Tele-Path Technology using Grundium *Ocus* × 40, Finland) and focal non-necrotic area was elected to manually count the cells at high magnification (× 40). Cells expressing anti-CD204 were considered as CD204-positive (CD204$^+$ TAMs). The total cells were defined as cells with both stained TAMs and non-stained TAMs. The cell that did not express CD204 included neoplastic astrocytic cells, lymphocytes, and other types of neurological cells. The labelling index (LI) was assessed through the following equation:

$$\text{Labelling Index (\%)} = \frac{\text{CD204}^+ \text{ stained TAMs}}{\text{Total cells}} \times 100.$$

Two staining patterns were defined: high expression and low expression, based on the density of the staining. The assessment of CD204 expression matches to what has been described by Kurdi et al protocol [15]. The expression was considered high when CD204$^+$ TAMs were expressed in more than 40% of the cellular microenvironment. The expression was considered low when CD204$^+$ TAMs were expressed in less than 40% of the cellular microenvironment (Fig. 1a-d).

Assessment of IDH1^{R132H} expression in WHO grade 4 astrocytomas

Sections in which > 10% of neoplastic glial cells positively stained with *IDH1*^{R132H} were defined as *IDH1*-mutant [24]. Tumors with negative *IDH1* staining were not DNA-sequenced due to the redundant amount of the tissue.

Assessment of *MGMT*-promoter methylation

MGMT gene promoter methylation was assessed by using one of the two different methods: methylation specific-polymerase chain reaction (MS-PCR) and pyrosequencing using Qiagen. The techniques were chosen based on the institution's protocol. Both techniques started with DNA extraction using FFPE kit. DNA concentration and purity were assessed using a NanoDrop spectrophotometer.

For first method (MS-PCR), DNA concentration was standardized to 60 ng and transformed using EpiTect bisulfate Kit from Qiagen. The forward and reverse primers were targeted to the methylated and unmethylated exon of the human *MGMT* gene which matches the protocol described by Esteller et al [21] (Table 3). Thermal cycling started at 95 °C for 2 min followed by 40 - 45 cycles of half minute and half minute at 52 °C and 72 °C. The PCR products were visualized using gel electrophoresis. Samples with both methylated and non-methylated products were recorded as *MGMT*-methylation positive.

For second method (pyrosequencing), *MGMT* Pyro Kit from Qiagen was utilized to evaluate the methylation at four CpG sites on human *MGMT* gene. After DNA extraction and optimization, the Therascreen *MGMT*-PyroKit and PyroMark sequencer were both employed to evaluate the *MGMT* methylation. The control was built-in as a positive control for sequencing reaction. This procedure matches the protocol described by Pangopoulos et al [25].

Statistical analysis

To explore the relationship between CD204-expressed TAMs, *MGMT*-promoter methylation, and *IDH1*^{R132H} mutation, the analyses were processed using a Fisher's exact test. Kaplan-Meier curve (KMC) was used to compare the distribution of RFI among WHO grade 4 astrocytoma cases with different CD204$^+$ TAMs expressions. A P-value of < 0.05 was considered statistically significant. All statistical analyses in this study were performed using IBM SPSS1 ver. 24 (SPSS Inc., Chicago, IL, USA).

Results

Our study included 45 patients diagnosed as WHO grade 4 as-

Table 2. Patients' Data Enrolled in This Study

Age	Gender	Location	*IDH1*	*ATRX*	CD204	*MGMT*	Adjuvant	CT	RFI
50	Male	Frontal	*IDH*-m	Loss	High	Methylated	RT	None	670
66	Male	Frontal	*IDH*-m	Loss	Low	Methylated	CT + RT	TMZ	1,034
71	Male	Parietal	*IDH*-m	Loss	High	Methylated	CT + RT	TMZ	440
31	Female	Parietal	*IDH*-m	Loss	High	Unknown	RT	None	670
64	Female	Parietal	*IDH*-m	Loss	High	Unmethylated	CT + RT	TMZ+	731
57	Male	Parietal	*IDH*-m	Loss	Low	Methylated	CT + RT	TMZ+	1,096
63	Male	Cerebellar	*IDH*-m	Loss	High	Unknown	CT + RT	TMZ+	1,123
72	Female	Frontal	*IDH*-m	Loss	Low	Unmethylated	RT	None	643
69	Female	Parietal	*IDH*-m	Loss	High	Methylated	RT	None	638
58	Female	Temporal	*IDH*-m	Loss	High	Unmethylated	CT + RT	TMZ	801
19	Male	Frontal	*IDH*-w	Loss	Low	Unknown	RT	None	330
58	Male	Frontal	*IDH*-w	Loss	Low	Unmethylated	RT	None	530
28	Female	Parietal	*IDH*-w	Loss	Low	Unmethylated	CT + RT	TMZ	330
22	Male	Temporal	*IDH*-w	Loss	High	Unknown	RT	None	250
63	Male	Frontal	*IDH*-w	Loss	High	Unmethylated	RT	None	430
68	Male	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ	1,016
59	Male	Temporal	*IDH*-w	Loss	High	Unmethylated	RT	None	293
73	Female	Parietal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	156
76	Female	Frontal	*IDH*-w	Loss	High	Unknown	CT + RT	TMZ	150
46	Female	Occipital	*IDH*-w	Loss	Low	Unmethylated	RT	None	183
63	Female	Temporal	*IDH*-w	Loss	High	Unknown	CT + RT	TMZ	194
82	Male	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	340
57	Female	Parietal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	260
10	Male	Temporal	*IDH*-w	Loss	Low	Methylated	None	None	92
42	Male	Frontal	*IDH*-w	Loss	High	Unknown	CT + RT	TMZ	306
59	Female	Occipital	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	826
64	Male	Frontal	*IDH*-w	Loss	High	Methylated	RT	None	273
63	Male	Temporal	*IDH*-w	Loss	Low	Unmethylated	RT	None	550
47	Male	Temporal	*IDH*-w	Loss	High	Unknown	RT	None	141
62	Female	Frontal	*IDH*-w	Loss	Low	Unmethylated	RT	None	90
69	Male	Temporal	*IDH*-w	Loss	High	Unknown	None	None	114
59	Female	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ	0
17	Male	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	460
68	Male	Temporal	*IDH*-w	Loss	High	Unmethylated	CT + RT	TMZ	311
61	Female	Temporal	*IDH*-w	Loss	Low	Unmethylated	CT + RT	TMZ+	853
56	Male	Parietal	*IDH*-w	Loss	High	Unmethylated	CT + RT	TMZ	174
55	Male	Frontal	*IDH*-w	Loss	High	Unknown	CT + RT	TMZ+	200
70	Male	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ+	730
76	Male	Frontal	*IDH*-w	Loss	Low	Unmethylated	CT + RT	TMZ	169
61	Male	Temporal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ	191
76	Male	Parietal	*IDH*-w	Loss	High	Unknown	RT	None	128
62	Male	Temporal	*IDH*-w	Loss	Low	Unmethylated	CT + RT	TMZ	555
73	Female	Parietal	*IDH*-w	Loss	High	Unknown	RT	None	195
57	Female	Frontal	*IDH*-w	Loss	High	Methylated	CT + RT	TMZ	288
81	Male	Parietal	*IDH*-w	Loss	High	Methylated	RT	None	59

Data include age, gender, tumor location, *IDH1* status, *MGMT*-promoter methylation, $CD204^+$ TAM expression, treatment modalities, and RFI. All the enrolled cases were WHO grade 4 astrocytomas that presented with necrosis, microvascular proliferations, *ATRX* loss and intact 1p19q. *IDH*-w: *IDH*-wildtype; *IDH*-m: *IDH*-mutant; *MGMT*: O^6-methylguanine-DNA methyltransferase; RT: radiotherapy; CT: chemotherapy; TMZ: temozolomide; RFI: recurrence-free interval.

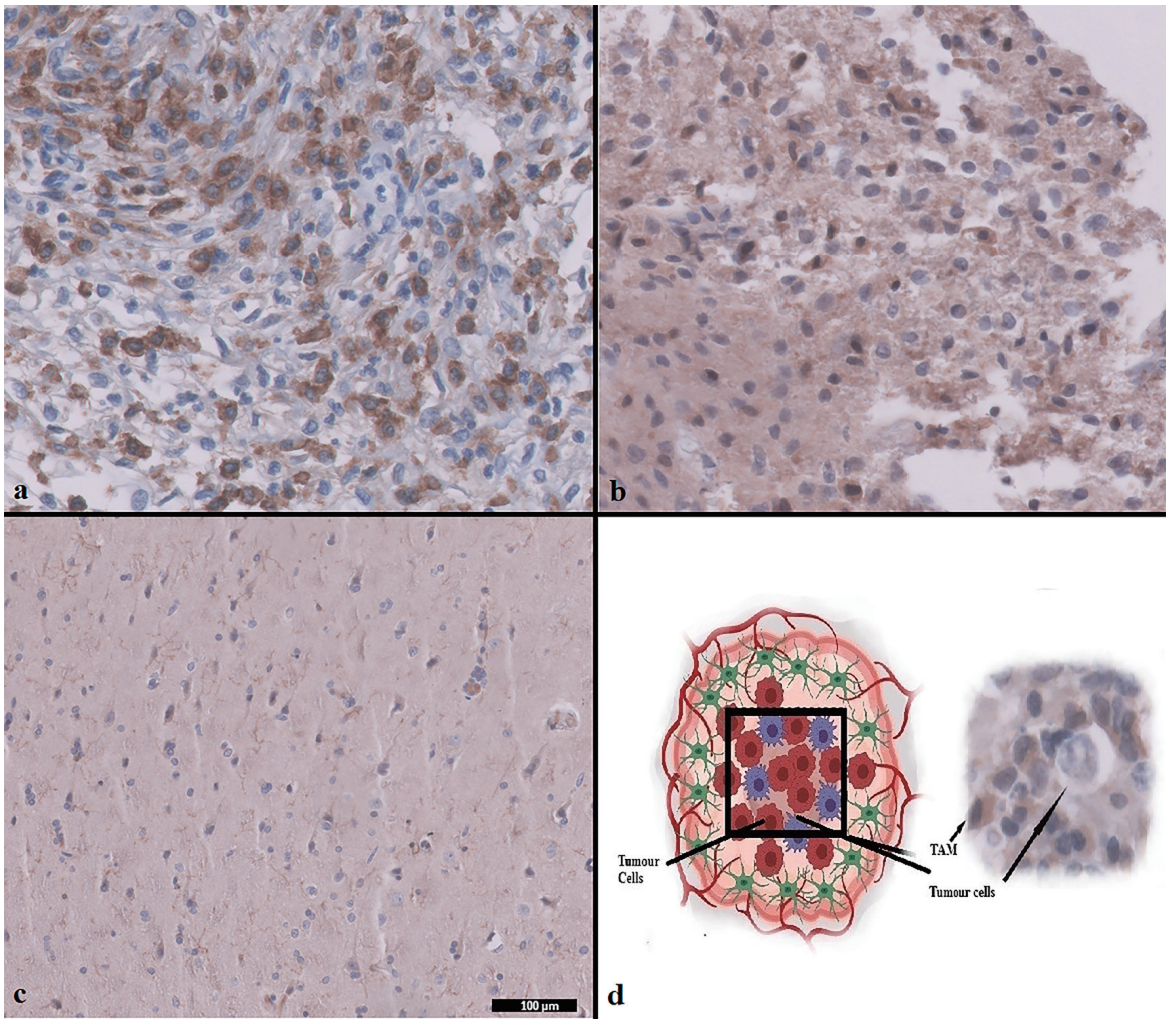

Figure 1. CD204$^+$ TAMs expression in WHO grade 4 astrocytoma using IHC. (a) CD204$^+$ TAMs high expression. (b) CD204$^+$ TAMs low expression. (c) Normal brain control. (d) Diagram shows the relationship between TAMs and tumor cells. Magnification (× 100 µm). TAMs: tumor-associated macrophages; WHO: World Health Organization; IHC: immunohistochemistry.

trocytoma based on 2021 WHO classification of CNS tumors. Around 22% (n = 10) of cases were less than 55 years and 78% (n = 35) of the cases were more than 55 years (Tables 1 and 2). Ten samples showed *IDH1* mutation and referred as *IDH*-mutant astrocytomas, while 35 samples showed no *IDH1* mutation and referred as glioblastoma. *MGMT* was methylated in 18 cases (40%) and 15 cases (33%) had unmethylated *MGMT*-promoter. For the remaining 12 samples, *MGMT* methylation status could not be determined due to nuclei acid degradation.

The expression of CD204$^+$ TAMs was evaluated in the whole 45 patients (Tables 1and 2). Around 57% (n = 26) of patients received combined radiotherapy and chemotherapy, 37% patients (n = 17) received radiotherapy, and two patients were reluctant to receive any adjuvant because of their comorbidities. Around 57% (n = 15) patients received TMZ alone, and the remaining 42% (n = 11) patients received TMZ and other adjuvant chemotherapies such as bevacizumab, irinotecan, lomustine, or etoposide. The median RFI was 1 year and 2 months (Table 2).

Table 3. Primers for MS-PCR Used for the Assessment of *MGMT*-Promoter Methylation

Primer	Sequence
MSP-*MGMT*-MetF	5′-TTTCGACGTTCGTAGGTTTTCGC-3′
MSP-*MGMT*-MetR	5′-GCACTCTTCCGAAAACGAAACG-3′
MSP-*MGMT*-UnMetF	5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′
MSP-*MGMT*-UnMetR	5′-AACTCCACACTCTTCCAAAAACAAAACA-3′

MS-PCR: methylation specific-polymerase chain reaction; *MGMT*: O^6-methylguanine-DNA methyltransferase.

Table 4. Relationship Between CD204+ TAMs and *MGMT*-Promoter Methylation

CD204+ TAMs	*MGMT*-promoter profile		P-value
	Unmethylated, n (%)	Methylated, n (%)	
High expression	6 (28.5%)	15 (71.6%)	0.01
Low expression	9 (75%)	3 (25%)	

MGMT: O^6-methylguanine-DNA methyltransferase; TAMs: tumor-associated macrophages.

Relationship between CD204+ TAMs and *MGMT*-promoter methylation

The relationship between *MGMT*-promoter methylation status and CD204+ TAMs expression status was statistically significant (P = 0.01) (Table 4). Approximately, 71.6% (n = 15) of WHO grade 4 astrocytoma cases with high CD204+ TAMs expression were associated with *MGMT*-promoter methylation, while 28.5% (n = 6) of the cases were found in astrocytoma with unmethylated *MGMT*-promoter. Cases with low expression of CD204+ TAM (75%, n = 9) was associated with unmethylated *MGMT*-promoter. Hence, CD204+ TAMs increase when *MGMT*-promoter is methylated.

Relationship between CD204+ TAMs and *IDH* mutation

The relationship between *IDH* mutation and CD204+ TAMs expression was statistically insignificant (P = 0.93) (Table 5). Nevertheless, CD204+ TAMs were overexpressed in *IDH*-wildtype cases (78.2%) more than *IDH1*-mutant cases (21.8%).

Relationship between CD204+ TAMs, *MGMT*-promoter methylation, with the type of treatment modalities and RFI

There was no significant difference statistically found in the recurrence interval between CD204+ TAMs (high and low expression) and *MGMT*-promoter methylation (P = 0.95 and P = 0.09) (Fig. 2a, b) (Table 6). Furthermore, no statistically significant relationship was also identified in the RFI between CD204+ TAMs expression (mainly high expression), *MGMT*-promoter methylation and the treatment modalities or specific chemotherapeutic agents respectively (P = 0.06 and P = 0.9) (Fig. 2c, d).

Discussion

Several molecular biomarkers have been identified as prognosticators in high-grade astrocytomas. Amongst these, genetic biomarkers such as *IDH* mutation and *MGMT* gene promoter methylation were considered essential targets for patients' treatment and prognosis [17, 18]. In the tumor microenvironment, TAMs and tumor-infiltrating lymphocytes (TILs) are considered as central immune-modulatory cells that are distributed as tumor niches where treatment-resistant is localized. M2-polarized TAMs, one of the subclasses of TAMs, behave as an immunomodulator to stimulate tumor growth or suppress tumoricidal effect of TILs [5]. One of the recently explored TAMs receptor is CD204, a macrophage scavenger receptor 1 (MSR1) [12, 14]. Kurdi et al found that CD204 is highly expressed in glioblastoma patients and associated with a reduced expression of CD4+ TILs in the tumor microenvironment [15]. They also found that insignificant relationship between *IDH* mutation and CD204+ TAMs expression [15]. Nonetheless, the association of CD204+ TAMs and *MGMT*-promoter methylation was significantly explored in our results. We found that around 71% of WHO grade 4 astrocytomas with elevated expression of CD204+ TAMs were associated with *MGMT*-promoter methylation. Consequently, CD204+ TAMs in WHO grade 4 astrocytomas become dense when *MGMT*-promoter is methylated. This relationship has never been broadly explicated in the literatures. The only explanation here is that CD204+ TAMs may neutralize the effect of *MGMT*-DNA protein to loss its function which contributes into the progression of cancers. Nevertheless, we revealed that the relationship between CD204+ TAMs and *MGMT*-promoter methylation had no significant impact on tumor recurrence (Fig. 2a, b). In excess, this association also had no significant impact on RFI amid all types of treatment modalities (Fig. 2c, d). This irrelevance might be related to the limited number of samples in our study.

Because TAMs encircle cancer cells and inhibits T-cell cytotoxic function, tumor cells will escape the immune system with less tumor cells killed by TILs. Indeed, the immune check point targets, anti-CD204 receptor, will be an effective immunomodulator that can prevent TAMs role, evolute TILs and increase sensitization of glioma cells to chemotherapeutic agents. Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-*

Table 5. Relationship Between CD204+ TAMs and *IDH* Mutation

CD204+ TAMs	*IDH* mutation		P-value
	IDH-mutant, n (%)	*IDH*-wildtype, n (%)	
High expression	7 (21.8%)	25 (78.2%)	0.93
Low expression	3 (23.1%)	10 (76.9%)	

IDH: isocitrate dehydrogenase; TAMs: tumor-associated macrophages.

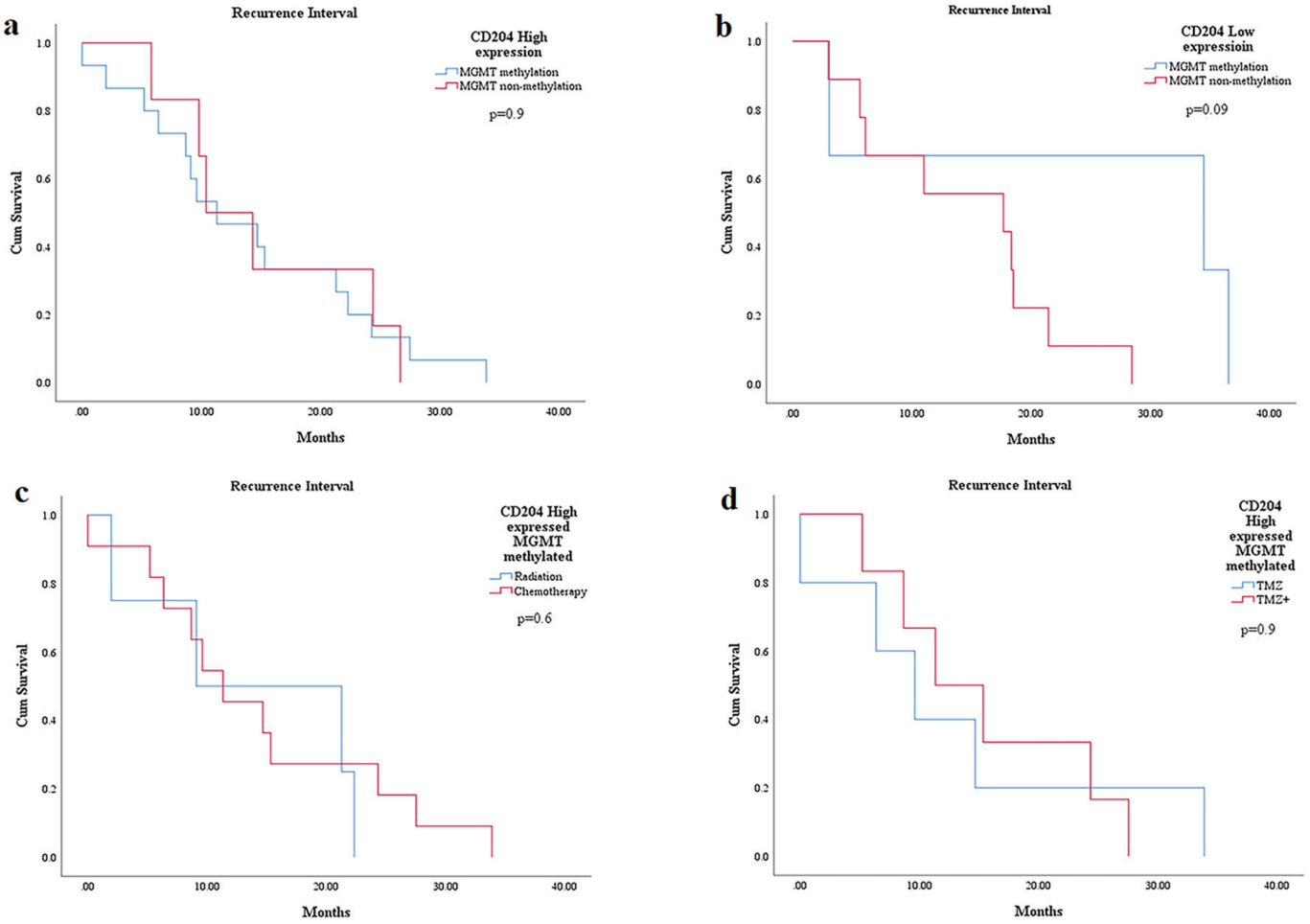

Figure 2. The relationship between CD204$^+$ TAMs expression and *MGMT*-promoter methylation with RFI in WHO grade 4 astrocytoma patients. KM graphs show no statistically significant difference in RFI between CD204$^+$ TAMs expression and *MGMT*-promoter methylation using different treatment modalities. TAMs: tumor-associated macrophages; *MGMT*: O^6-methylguanine-DNA methyltransferase; RFI: recurrence-free interval; WHO: World Health Organization; KM: Kaplan-Meier.

Table 6. Relationship Between CD204$^+$ TAMs, *MGMT* methylation and RFI

	Mean				Median		
	Estimate	Standard error	95% confidence interval		Estimate	Standard error	95% confidence interval
			Lower bound	Upper bound			Lower bound
RI							
CD204 high							
MGMT methylation	423.133	76.467	273.258	573.009	340.000	107.558	129.186
Non-methylation	456.667	103.673	253.468	659.865	311.000	83.895	146.566
Overall	432.714	60.851	313.447	551.982	340.000	104.517	135.147
RFI							
CD204 low							
MGMT methylation	740.667	324.827	104.006	1,377.327	1,034.000	769.140	0.000
Non-methylation	433.667	84.896	267.270	600.063	530.000	298.142	0.000
Overall	510.417	101.655	311.172	709.661	530.000	190.526	156.570

MGMT: O^6-methylguanine-DNA methyltransferase; TAMs: tumor-associated macrophages; RI: recurrence interval; RFI: recurrence-free interval.

4), programmed cell death-1 receptor (*PD-1*), and T-cell inhibitory receptor (*TIM-3*) were all found to perform suppressor effect by interacting with their receptors on tumor cells or TAMs [22, 26]. The influence of these check point modulators has never been studied in high-grade astrocytomas with *MGMT*-promoter methylation.

One limitation that should be admitted in our research is that the whole number of analyzed samples is relatively low. Despite this limitation, this is the first study that correlates *MGMT*- promoter methylation and CD204 biomarkers in WHO grade 4 astrocytomas.

Conclusions

Our study emphasized that the expression of $CD204^+$ TAMs in WHO grade 4 astrocytomas increases when *MGMT*-promoter is methylated. $CD204^+$ TAMs may also neutralize the effect of *MGMT*-DNA protein to loss its function, which contributes to tumor progression. This mechanism targets a key approach to suppress TAMs to increase tumor cells sensitivity to chemotherapeutic agents.

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Financial Disclosure

None to declare.

Conflict of Interest

None to declare.

Informed Consent

Informed consent was obtained.

Author Contributions

MK: idea, writing, study design and data, and histological analysis; YK: data provider, writing, and analysis; EF: data entry, tissue collection, and writing; BB: writing and editing; AK: study design, writing, and editing; SK: writing and editing; AN: writing and editing; RM: statistical analysis and histological analysis; TH: writing and editing; BA: writing and editing; KB: writing and editing; SH: interpreted data and histological revision. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon request.

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