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MOLECULAR MARKERS IN PREDICTING THE OUTCOME OF DIFFUSE GLIOMA GRADE 4 TREATMENT

Aim. To assess the expression of *MGMT* (O^6-methylguanine-DNA methyltransferase) gene by *MGMT* RNA abundance and the presence of *IDH1/2* (isocitrate dehydrogenase) variants in glioblastoma (GBM) samples for predicting the efficacy of temozolomide (TMZ) treatment, recurrence risk, and patients' survival. **Materials and Methods.** The expression of the *MGMT* gene and the presence of *IDH1/2* variants were assessed by RT-PCR in tumor samples from 39 patients with histologically verified GBM or diffuse astrocytoma, grade 4. The number of *MGMT* RNA copies was determined by the calibration curves based on the pMA-RQ plasmid with the inserted *MGMT* gene. **Results.** The number of *MGMT* RNA copies in GMB samples varied broadly from 1.7 to 88,270.2 copies per 1000 cells. The patients with a low level of *MGMT* expression (<1000 copies) in tumors had a more favorable prognosis for the TMZ treatment compared to the patients with a high level of *MGMT* RNA abundance (>10,000 copies). Among the patients included in the study, a wild type of *IDH1/2* was detected in 36 cases, while 3 cases were IDH1 heterozygous. **Conclusion.** The level of *MGMT* expression is considered a significant factor for prognosing GMB patients' survival. Patients with a low level of *MGMT* expression are considered candidates for efficient therapy with alkylating agents.

Keywords: glioblastoma, *MGMT,* IDH1/2, temozolomide.

Glioblastoma (GBM) is the most common and dangerous brain tumor, accounting for 14.2% of all brain tumors and 50.9% of the malignant brain tumors [1]. This cancer is characterized by rapid growth, utmost invasiveness, and high heterogeneity of its molecular profiles, which significantly complicates the treatment.

The current standard therapy for GBM includes the maximal surgical resection of the tumor followed by radiotherapy and chemotherapy. The maximal surgical resection aims at reducing tumor burden, providing the time reserve for the appropriate adjuvant treatment, which is important for improving treatment outcome.

The survival of GBM patients improved due to the use of temozolomide (TMZ) for their treatment. This alkylating agent damages DNA in tumor cells resulting in their apoptosis. The TMZ efficacy depends largely on the activity of O^6-methylguanine-DNA methyltransferase (MGMT),

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which can reverse TMZ-induced DNA methylation. The assessment of the expression of the *MGMT* gene and the mutation status of the genes coding for enzymes isocitrate dehydrogenase (IDH) 1 and 2 is imperative for predicting the efficacy of TMZ therapy [2, 3]. Moreover, the current classification of CNS tumors (2016—2021) is based on the analysis of the molecular markers in tumor tissue [4, 5].

According to clinical studies, the methylation of the *MGMT* gene promoter is considered a favorable prognostic marker of the response to radio- and chemotherapy [6]. The high activity of *MGMT* in the unmethylated state results in reducing TMZ-induced DNA alkylation due to a more efficient DNA repair and, therefore, the treatment becomes less effective [7]. There are numerous techniques for the assessment of *MGMT* methylation, including a methylation-specific PCR (MSP), pyrosequencing, and microarrays. Although MSP is the most popular method for the analysis of the methylation of the *MGMT* gene promoter, it does not provide quantitative data on the extent of methylation [8]. However, merely dichotomous classification of the methylated vs. unmethylated status may be insufficient for the *MGMT* promoter evaluation, especially in cases of low or partial methylation. Several other techniques cover only a few CpGs in promoter regions that may be insufficient for predicting a response to TMZ [9]. Finally, there is a lack of consensus on which of the CpG sites and cut-off levels of their methylation are prognostically significant, and a nonlinear relationship between the extent of *MGMT* promoter methylation and survival in GBM has been suggested [10—13]. Therefore, further studies are required for a better understanding of the effects of *MGMT* methylation on the efficacy of TMZ therapy.

Moreover, other epigenetic modifications affecting transcription activity may also be of importance, considering the molecular mechanisms of resistance to TMZ. For example, histone acetyltransferase (HAT) adds the acetyl group to histone, facilitating promoter deblocking, while histone deacetylase (HDAC) eliminates the acetyl growth, resulting in blocking the transcription of the corresponding genes [14]. The clinical trials of several inhibitors targeting the epigenetic mechanisms are ongoing with the aim of their possible use in GBM therapy in combination with TMZ [15].

A brief review of the factors affecting the efficacy of TMZ therapy points to the necessity of further optimization of methods for predicting its outcome. Gonzalez-Aponte et al. [16] demonstrated a correlation of in vitro TMZ sensitivity of glioma cells and MGMT activity with daily rhythms in both *MGMT* promoter methylation and *MGMT* mRNA abundance. The *MGMT* RNA levels may be influenced by the *MGMT* promoter methylation affecting transcription activity. Therefore, the analysis of *MGMT* RNA copy numbers may serve as an additional tool for assessing the resistance to TMZ therapy.

Furthermore, the methylation of DNA and *MGMT* promoter, in particular, depends on the status of IDH 1/2 (mutated vs unmutated). While the wild-type IDH converts isocitrate to α-ketoglutarate (α-KG), the mutant IDH catalyzes the conversion of α-KG to D2-hydroxyglutarate, the latter inhibits α-KG-dependent enzymes, including histone demethylases, which results in altering DNA methylation, including the methylation of *MGMT* promoter [17]. GMB patients with *IDH1* (R132H) and *IDH2* (R172) mutations have a more favorable prognosis of chemotherapy efficacy [18]. The wild-type *IDH* is detected in 90% of primary GBMs, whereas mutant *IDH1* is more common in secondary GBMs (diffuse astrocytoma grade 4 by the current classification) [19].

The aim of the study was to assess *MGMT* expression by calculating *MGMT* RNA copy numbers and the presence of the *IDH1/2* variants in GBM cells in relation to the data of the retrospective analysis of the one-year survival of GBM patients who received the standard treatment.

Materials and Methods

The study included 39 GBM patients who were treated at the State Institution "A.P. Romodanov Institute of Neurosurgery of the NAMS of Ukraine" in 2017—2019. An informed consent for collecting and using the data for research purposes was obtained from all the patients involved in the study. The research was approved by the Medical Ethical Committee of the Institute (report No. 3 of 06.06.2016 for research project "To Study Efficacy of Chemotherapeutic and Radiation Therapy of Malignant Intracerebral Tumors Accounting for their Molecular-genetic Features"). All procedures performed in the study were in accordance with the guidelines of the Declaration of Helsinki of 2000.

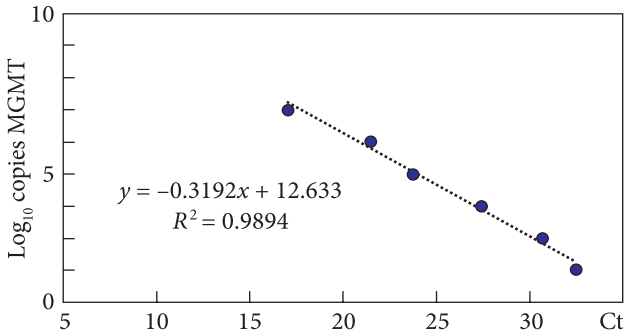

Fig. 1. Calibration equation for calculating *MGMT* copy number in samples of resected tumor tissue

The patients included in the study were at the age over 18 years, and their diagnosis of GBM or diffuse astrocytoma, anaplasia grade 4, was confirmed histologically according to the WHO classifications of 2016 and 2021 [4, 5].

The average age of the patients was 57.5 years (interquartile range 49—64; 23 males and 15 females). In all patients, the maximal safe surgical resection of the tumor was performed followed by the concomitant adjuvant radiochemotherapy and supportive chemotherapy with TMZ according to Stupp's protocol. The patients' survival was assessed from the day of the surgery.

The relevant clinical data were collected, including the symptoms, preoperative functional capacity by Karnofsky index (KPS), the extent of the surgical intervention, and MRI data.

MGMT expression and *IDH1/2* variants were assessed in the samples of the resected tumor tissue. RNA was extracted using PureLink RNA Mini Kit (Applied Biosystems, USA) (5×10^5 cells per sample). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, USA). For assaying *MGMT* expression, TaqMan gene expression assay kit Hs01037698-m1 (Life Technologies, USA) was used. *IDH1* rs121913500 and *IDH2* rs121913503 variants were identified by RT PCR using a CFX96 RT PCR detection system (Bio-Rad, USA) with the allelic discrimination software.

The context sequences were as follows:

rs121913500

ACTACTTGATCCCCATAAGCATGA[C/T]GACCTATGATGATAGGTTTTACCCA;

rs121913503

GCCTACCTGGTCGCCATGGGCGTGC[T/C]TGCCAATGGTGATGGGCTTGGTCCA.

To analyze the *MGMT* expression, a calibration curve was plotted based on the pMA-RQ plasmid containing the 519 bp *MGMT* insert (Invitrogen, USA). All measurements were carried out in duplicates.

The serial dilutions containing 10^5, 10^4, 10^3, 10^2, and 10^1 *MGMT* molecules per 1 μL were prepared from 2.83347×10^{-6} μmol of *MGMT*-containing pMA-RQ plasmid corresponding to 1.71×10^{12} *MGMT* molecules. 10 μL of each dilution was added to the amplification reaction mixture. The plotted calibration curve (Fig. 1) was used for calculating the *MGMT* copy number per one thousand cells in the sample.

The primary end-point of the study was the association between one-year survival and *MGMT* expression level. The association between survival and risk factors was analyzed by the logistic regression model. The survival was assessed by the Kaplan — Meier method. The effect of different *MGMT* expression levels on the survival risk was assessed using a logrank test (for comparing CI of the survival curves for different groups in the study). The effect of independent predictors (covariates — age, sex) on the survival risk was assessed by a logrank test (to compare the Kaplan — Meier survival curves for the studied groups). $p \le 0.05$ values were considered significant. For statistical analysis, statistical software EZR v. 1.64 (graphical user interface for R statistical software version 4.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used [20].

Results and Discussion

The data from 38 of 39 GBM patients were analyzed to assess the possible association between the survival rate and *MGMT* expression (one patient died of thromboembolia 4 months after the beginning of the study).

Among tumor samples, 36 had a wild-type *IDH1*, and 3 cases with heterozygous *IDH1* mutations were detected. No *IDH2* variants were detected.

The number of *MGMT* RNA copies varied extensively within a range of 1.7—88270.2 copies per thousand cells (Fig. 2). Three groups of samples may be conventionally delineated: with a low expression (1—1,000 copies per thousand cells, 33.3% of the total cases); an elevated expression (1,000—10,000 copies per thousand cells, 28.2% of the total cases); and a high expression (>10,000 copies per thousand cells, 38.5% of the total cases).

The clinical significance of the findings was assessed based on the association between *MGMT* expression and one-year survival in the population

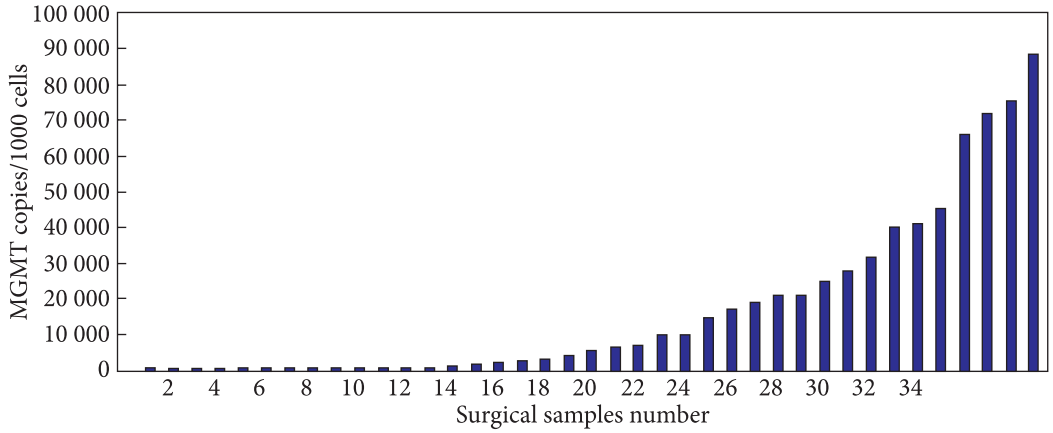

Fig. 2. Number of *MGMT* RNA copies in GMB surgical samples

under study — the primary end-point. The ROC curve was computed for predicting the risk of death within one year (Fig. 3). The data of the univariate analysis for predicting this risk are given in the Table. The age, gender, and *MGMT* expression were analyzed as such risk factors.

According to the data presented, there was no association between the risk of death within one year and the age or gender of the patient. Nevertheless, this risk increased with the increased *MGMT* expression (OR = 1.11; 95% CI 1.03—1.19).

Survival curves constructed using the Kaplan — Meier method with a logrank test further demonstrated a marked difference in OS between the groups with different *MGMT* expression levels (Fig. 4). The one-year survival rate of the patients with the lower (<10,000 per 1,000 cells) *MGMT* RNA copy number in GBM cells was higher compared to the patients with the higher (≥10,000 per 1,000 cells) *MGMT* RNA copy number (95.7 ± 4,3% vs 26.7 ± 11.4%; $p < 0.001$). The average survival of the patients with a low *MGMT* RNA copy number in GBM cells was 23 months (95% CI 16—35 months) compared to 10 months (95% CI 7—11 months) in patients with a high copy number in GBM cells.

The *MGMT* expression, which usually correlates negatively with the *MGMT* promoter methylation, is considered a decisive factor for GBM cell sensitivity to TMZ, since the transfer of methyl groups from O^6-methyl guanine in DNA to the *MGMT* molecule reverses the alkylation-induced DNA damage, thus reducing the TMZ efficacy. High levels of *MGMT* mRNA are, in most cases, associated with the resistance to TMZ. Nevertheless, the mechanisms of TMZ resistance may be more sophisticated as comprising some other factors be-

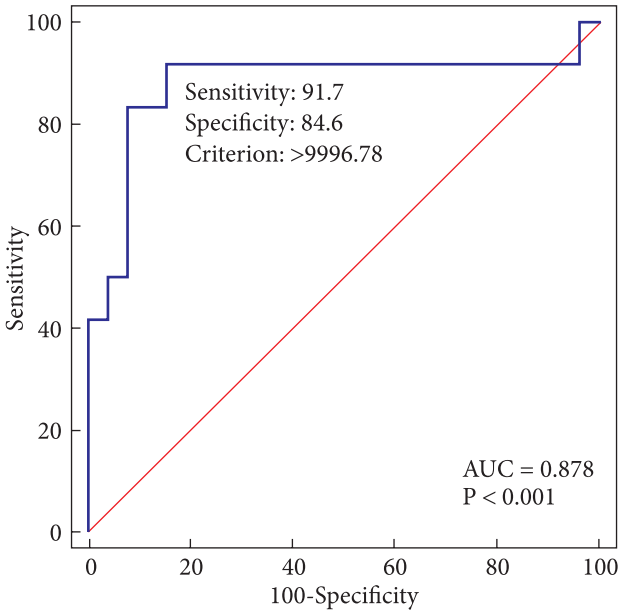

Fig. 3. ROC curve of the model performance for predicting the risk of death within one year depending on the *MGMT* expression. The optimal cut-off point (9996.78 copies per 1,000 cells) was selected according to Youden's index. With such cut-off, the sensitivity of the model is 91.7% (95% CI 61.5—99.8%), specificity — 84.6% (95% CI 65.1—95.6%), prognostic significance for the positive value — 73.3% (95% CI 52.4—87.3%), prognostic significance for the negative value — 95.7 % (95% CI 77.0—99.3%).

sides *MGMT* expression. Perazzoli et al. [21] demonstrated that the modulation of *MGMT* expression in GBM cell lines in vitro resulted in the corresponding changes in TMZ IC_{50}, while in some GBM cell lines with a low *MGMT* expression, there was no direct correlation between *MGMT* expression level and TMZ sensitivity. Similarly, when the relationship between MGMT protein expression and tumor response to TMZ was studied in GBM xenografts, in some of them the increased MGMT

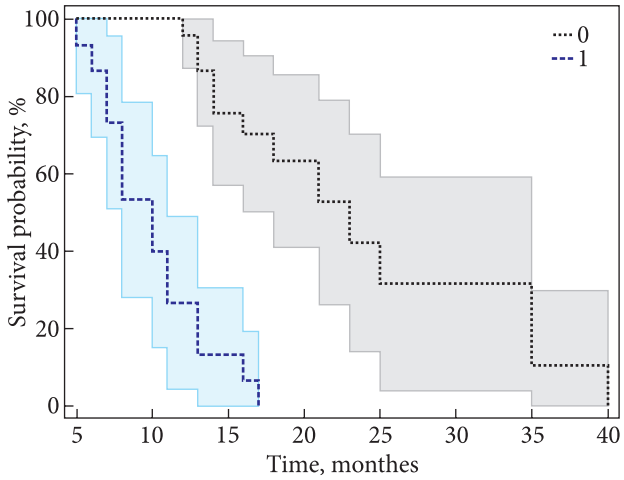

Fig. 4. Kaplan — Meier survival curves for patients with a low (<10,000 per 1,000 cells) (curve 0) or high (≥10,000 per 1,000 cells) (curve 1) *MGMT* RNA copy number in GBM cells. Cut-off was selected according to Youden's index

expression was not paralleled with the increased TMZ resistance [22]. Nevertheless, the quantitative assessment of *MGMT* mRNA in most studies demonstrates that the substances sensitizing cells to TMZ decrease *MGMT* expression [23].

In our study, the one-year survival rate of the patients with a relatively low *MGMT* RNA copy number in GBM cells was higher compared to the patients with a high *MGMT* RNA copy number. The model accounting for Youden's index inferred in our study allows for correctly identifying the patients at high risk of death within one year (sensitivity of 91.7% and specificity of 84.6%). The survival analysis also demonstrates the longer survival median following TMZ treatment in the group of patients with the lower *MGMT* RNA copy number in GBM cells.

In our nonrandomized clinical study, the standard treatment of GBM patients by the Stupp protocol resulted in the more favorable survival indices in cases with the lower *MGMT* RNA copy number in GBM cells. Our study also proved the expedience of the assessment of *MGMT* expression based on the quantification of *MGMT* RNA copy number using the calibration curve plotted with reference to the plasmid containing the standard number of *MGMT* RNA copies. Such an approach allows us to improve the assessment of *MGMT* expression for predicting sensitivity to TMZ treatment.

Therefore, the assessment of *MGMT* mRNA expression based on the quantification of MGMT mRNA copy numbers may be an effective tool for predicting the death risk depending on the *MGMT* mRNA expression level, which may be a defining factor for the individual treatment strategy.

Recent findings indicate that the *IDH* mutation and *MGMT* promoter methylation status correlate with the response to TMZ therapy. Millward et al. [24] demonstrated that a combination of an *MGMT* promoter and *IDH1* mutation is associated with a more prolonged survival of GBM patients following chemoradiotherapy. A favorable role of *IDH*1/2 mutations in combination with a methylated *MGMT* promoter in the outcome of the treatment of patients with malignant glioma was also noticed in the study of Pandith et al. [25].

In our study, 36 of 39 GBM samples (92.3%) contained a wild-type *IDH1/2,* and only 3 cases were *IDH1* heterozygous. However, according to the literature, the mutant *IDH1/2* status can be found in less than 10% of GBM, predominantly secondary glioblastomas (diffuse astrocytoma anaplasia grade 4 by the recent classification) [26]. As a rule, such a mutated status is associated with a better survival [27, 28]. Yang et al. [29] consider wild-type *IDH1* GBMs as more aggressive and invasive. In contrast, astrocytomas anaplasia grade 4 with *IDH1* mutation resemble less anaplastic gliomas and have more prolonged remission even with possibly lesser sensitivity to TMZ. Therefore, the multimodal

Univariate logistic regression analysis for predicting the risk of death within one year

Factors		Model coefficient, b ± m	Significance of OR difference from 1, *p*	OR (95% CI)
Sex	Women	Reference		
	Men	0.13 ± 0.71	0.851	–
Age, years		0.023 ± 0.034	0.512	–
MGMT expression per one cell		0.10 ± 0.04	**0.004**	**1.11 (1.03–1.19)**

Notes: OR — odds ratio; CI — confidence interval, b — coefficient of the logistic regression model; ± m — standard error of the coefficient of the logistic regression model.

approach is clinically appropriate considering the combination of radiotherapy and TMZ.

A low number of GBM cases with *IDH* mutations in our study did not allow us to analyze whether these patients have better survival in the context of our study. Nevertheless, all three patients with a mutated *IDH* status survived one year from the beginning of the observation and are still alive (remission duration 13, 16, and 20 months at the moment of compiling the findings for publication.

The analysis of the *IDH* mutation status in combination with *MGMT* mRNA expression as a stratification factor seems to be expedient for the strategy of the treatment of patients with GBM and diffuse astrocytoma, anaplasia grade 4 [28]. This approach allows for making more reasonable decisions on scheduling TMZ in a treatment protocol for each GBM patient. The level of *MGMT* expression in the postoperative tumor samples should be assessed as an independent prognostic factor for GMB patients in more extended cohorts.

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МОЛЕКУЛЯРНІ МАРКЕРИ В ПРОГНОЗУВАННІ РЕЗУЛЬТАТІВ ЛІКУВАННЯ ХВОРИХ НА ДИФУЗНУ ГЛІОМУ IV СТУПЕНЮ АНАПЛАЗІЇ

Мета. Визначення експресії гена *MGMT* та генотипів *IDH1/2* у зразках пухлин гліобластоми (ГБМ) для прогнозування ефективності хіміотерапії темозоломідом (ТМЗ), ризику рецидиву та виживаності пацієнтів. **Матеріали та методи.** Експресію *MGMT* та генотипів *IDH1/2* визначали в зразках ГБМ або дифузної астроцитоми 4 ст. анаплазії від 39 прооперованих хворих методом ЗТ-ПЛР. Кількість копій мРНК *MGMT* визначали за допомогою калібрувальних кривих на основі плазміди pMA-RQ, до якої було вбудовано фрагмент гена *MGMT*. **Результати.** Дослідження показало широкий діапазон експресії *MGMT* у зразках пухлин від 1,7 до 88270,2 копій на 1000 клітин. Виявлено, що пацієнти з низьким рівнем експресії *MGMT* (1—1000 копій) мають кращий прогноз для лікування ТМЗ, тоді як пацієнти з високим рівнем (>10000 копій) мають нижчу ефективність терапії. Серед досліджуваних зразків пухлин 36 мали генотип дикого типу *IDH1/2*, а три були гетерозиготами *IDH1*. Аналіз показав, що рівень експресії *MGMT* є значущим фактором прогнозу виживаності пацієнтів. **Висновки.** Результати підтверджують важливість кількісного аналізу експресії *MGMT* для прогнозування ефективності хіміотерапії ТМЗ в пацієнтів із ГБМ. Пацієнти з низьким рівнем експресії *MGMT* є кандидатами на ефективну терапію алкілуючими агентами.

Ключові слова: гліобластома, *MGMT*, *IDH1/2*, темозоломід.