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Aleksandar Stepanović[1,2], Nina Petrović[3,4], Tatjana Arsenijević[1,2], Marina Nikitović[1,2,♣]

Correlation of microRNAs-10b/21/34a expression levels with *IDH1*-mutation status in patients with glioblastoma

Корелација нивоа експресије микроРНК-10б/21/34а мутационим статусом *IDH1* код болесника са глиобластомом

[1]University of Belgrade, Faculty of Medicine, Belgrade, Serbia;
[2]Institute for Oncology and Radiology of Serbia, Department of Radiation Oncology, Belgrade, Serbia;
[3]Institute for Oncology and Radiology of Serbia, Department of Experimental Oncology, Belgrade, Serbia;
[4]University of Belgrade, Vinča Institute of Nuclear Sciences – National Institute of the Republic of Serbia, Belgrade, Serbia;

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♣Correspondence to:
Marina NIKITOVIĆ
Pasterova 14, 11000 Belgrade, Serbia
E-mail: **marina.nikitovic@ncrc.ac.rs**

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SUMMARY

Introduction/Objective Isocitrate dehydrogenase mutations play a significant role in gliomagenesis. Specific microRNAs such as miR-10b/21 act as oncogenic microRNAs, while miR-34a acts as tumor suppressors in glioblastoma. Our study aimed to investigate the mutation status of IDH correlate with microRNAs-10b/21/34a expression levels in patients with glioblastoma.

Methods The study included 43 patients diagnosed with glioblastoma. We examined microRNA-10b, microRNA-21 and microRNA-34a expression levels in peripheral blood mononuclear cells after surgery and prior to concurrent radiotherapy with temozolomide, at the 15th and 30th fractions of radiotherapy with temozolomide. The data on IDH1 mutation status were gathered from medical history and histopathology.

Results Two groups were created to assess the association of microRNAs-10b/21/34a expression levels: glioblastoma *IDH1*-wild type and glioblastoma *IDH1*-mutant + Not Other Specified (NOS). The median microRNA-10b expression level before the initiation of concurrent radiotherapy with temozolomide was 130.44 (52.20 - 622.53) in the *IDH1*-wildtype glioblastoma group and 94.61 (2.13 - 816.89) in the *IDH1*-mutant + glioblastoma NOS group. The median microRNA-21 expression level was 57.16 (2.68 - 278.98) in the *IDH1*-wildtype glioblastoma group and 69.74 (4.60 - 825.43) in the *IDH1*-mutant + glioblastoma NOS group. The median microRNA-34a expression level was 13.52 (3.16 - 105.20) in the *IDH1*-wildtype glioblastoma group and 10.11 (1.00 - 210.55) in the *IDH1*-mutant + glioblastoma NOS group. The results showed no statistically significant difference in the expression levels of miR-10b/21/34a between the two groups ($p > 0.05$).

Conclusion Our results suggest that the IDH1 mutation status may not be a critical factor for altered expression of microRNAs-10b/21/34a in glioblastoma patients.

Keywords: glioblastoma; microRNA; IDH mutation

САЖЕТАК

Увод/Циљ Мутације изоцитрат дехидрогеназе (ИДХ) играју значајну улогу у глиомагенези. Поједине микро РНК као што су микро РНК-10б/21 делују као онкогене микро РНК, док микро РНК-34а делује као тумор супресор код глиобластома. Наша студија је имала за циљ да истражи потенцијалну корелацију статуса мутације ИДХ у са нивоима експресије микро РНК-10б/21/34а код пацијената са глиобластомом.

Методе Ова студија је обухватила 43 пацијента који су имали постављену дијагнозу глиобластома. Испитивани су нивое експресије микро РНК-10б, микро РНК-21 и микро РНК-34а у мононуклеарним ћелијама периферне крви након операције, односно пре почетка лечења радиотерапијом са конкомитантним темозоломидом, као и на 15. и 30. фракцији радиотерапије са конкомитантним темозоломидом. Подаци о статусу мутације ИДХ1 прикупљени су из историје болести и дефинитивног хистопатолошког налаза.

Резултати Направљене су две групе за процену корелације нивоа експресије микро РНК-10б/21/34а у односу на ИДХ1 мутациони статус: глиобластом ИДХ1-дивљи тип и глиобластом ИДХ1-мутант + *Not Other Specified* (*NOS*). Медијана експресије микроРНК-10б пре почетка конкоминатног лечења радиохемиотерапијом била је 130,44 (52,20–622,53) у групи ИДХ1-дивљег типа глиобластома и 94,61 (2,13–816,89) у групи ИДХ1-мутант + *NOS*. Медијана експресије микро РНК-21 била је 57,16 (2,68–278,98) у групи глиобластома ИДХ1 дивљег типа и 69,74 (4,6–825,43) у групи ИДХ1-мутант + *NOS*. Медијана експресије микро РНК-34а била је 13,52 (3,16–105,2) у групи глиобластома ИДХ1 дивљег типа и 10,11 (1–210,55) у групи ИДХ1-мутант + *NOS*. Није доказана статистички значајна разлика у нивоима експресије микро РНК-10б/21/34а између две посматране групе ($p > 0,05$).

Закључак. Наши резултати сугеришу да статус мутације ИДХ1 можда није кључни фактор за измењену експресију микроРНК-10б/21/34а код пацијената са глиобластомом.

Кључне речи: глиобластом; микро РНК; ИДХ мутација

INTRODUCTION

Over the past few years, a significant number of research has focused on the molecular and genetic profile of glioblastoma. This combined approach defined by histopathology, molecular

features, and genetic alterations in glioblastoma led to World Health Organization (WHO) classification changes in 2016 and then in 2021, and better understanding of tumor biology and clinical behavior of the disease [1, 2].

One of the most important features in the 2016 WHO classification of the brain tumors was inclusion of isocitrate dehydrogenase (IDH) mutation status in glioma classification. Since IDH1 is one of the most important enzymes for cell metabolism, alterations in IDH1 expression or gene mutations can impact enzyme activity and impair cellular metabolism [3]. IDH mutation is considered one of the initial occurrences in the development of astrocytomas and oligodendrogliomas [4]. In fact, research on the sequence of mutations in gliomas shows that IDH mutations occur even before *TP53* mutations in low-grade diffuse astrocytoma and secondary glioblastoma, but they are rare in primary glioblastoma [4]. According to the 2016 WHO classification, glioblastoma was divided into glioblastoma *IDH*-mutant, WHO grade IV, glioblastoma, *IDH*-wildtype WHO grade IV, and glioblastoma, Not Other Specified (NOS) WHO grade IV [1]. In 2021, in addition to other parameters for the classification of glioblastoma, any astrocytoma with wildtype *IDH*, is considered as glioblastoma, *IDH*-wildtype, Central Nervous System (CNS) WHO grade 4 [2].

MicroRNAs (miRNAs) are non-coding RNAs that play a critical role in gene expression regulation. They bind to incompletely complementary sequences on target messenger RNAs (mRNAs), leading to mRNA degradation or translation inhibition [5]. This mechanism of post-transcriptional regulation enables miRNAs to control various biological processes, such as development, differentiation, proliferation, and apoptosis, and they are one of the key regulators of cell metabolism [6]. The expression of microRNAs can be altered by various mutations or regulated through promoter methylation [5]. MiRNAs can directly or indirectly target different genes including *IDH*. Oppositely, *IDH* mutations after the production of 2-hydroxyglutarate (2-HG) can influence or alter the expression of various miRNAs and regulate tumor development in gliomas [7].

In glioblastoma, specific miRNAs may have significant impact on tumorogenesis, invasiveness and resistance to therapy. MiR-10b, miR-21 and miR-93 act as oncogenic miRNAs (oncomiRs), while miR-7, miR-34a and miR-128 act as tumor suppressors in glioblastoma, and they target multiple genes [8]. Research has shown that miRNAs can be found in extracellular fluids. As well, they are stable compared to cellular RNA, which is reason why they can serve as potential biomarkers for various diseases, including cancer [9].

Since the mutations in *IDH* play a role in gliomagenesis, and miRNAs-10b/21 act as oncomiRs and miR-34a act as tumor supressors in glioblastoma, we aimed to investigate does mutation status of *IDH* correlate with miRNA-10b/21/34a expression levels in patients with high-grade gliomas (glioblastoma). Understanding of this insufficiently understood mechanism of feedback and regulation between *IDH* mutation and miRNAs can gain additional valuable insights into the differing biological behaviours of *IDH*-mutant versus *IDH*-wildtype gliomas and possible have therapeutic implications.

METHODS

This study examined miR-10b, miR-21 and miR-34a levels in peripheral blood mononuclear cells (PBMCs) from 43 glioblastoma patients. The 2016 World Health Organization Classification of Tumors of the Central Nervous System is used. Blood samples were taken post-surgery and prior to treatment with concurrent radiotherapy and chemotherapy with temozolomide, and at the 15^{th} and 30^{th} fraction of radiotherapy with concurrent temozolomide. Conducted at the Clinic of Neurosurgery, University Clinical Center of Serbia, and the Institute for Oncology and Radiology of Serbia since October 2017, the study adhered to the ethical guidelines of the Declaration of Helsinki. The study protocol received approval from the Ethical Research Committee of the Faculty of Medicine, University of Belgrade (approval number 1322/X-39).

After surgery, patients received radiotherapy (RT) combined with temozolomide (TMZ), followed by adjuvant TMZ. RT began 4-6 weeks post-surgery, with 30 fractions of 2 Gy each, totalling 60 Gy, using either 3D conformal or VMAT technique (**Figure 1**). Concomitant therapy included 75 mg/m² TMZ daily during RT.

The data on IDH1 mutation status were gathered from medical history, histopathology and immunohistochemistry confirmation.

PBMCs were isolated from heparinized blood using Histopaque-1077, and total RNA containing miRNAs was extracted using TRI Reagent. RNA quality was assessed using a BioSpec-nano spectrophotometer, ensuring an A260/280 ratio of 1.7 to 2.1. Specific TaqMan® assays were employed to analyze miR-10b, miR-21 and miR-34a expression. The comparative delta-delta Ct method was used to calculate relative quantitiy values, normalizing to RNU6B and calibrating against the sample with the lowest RQ value.

Statistical analysis

The Mann-Whitney U test is used to compare differences between two independent groups. For the analysis of the correlation of the level of expression of miRNA and *IDH* mutation status, Pearson and Spearman test were used. Log-Rank (Mantel-Cox test) was used to examine the significance of the difference. All statistical analyses were conducted using IBM SPSS Statistics 22.

RESULTS

Due to the unfavourable ratio of the number of outcomes to potential predictors, it was not possible to make comparisons among the three groups based on IDH1 status (22 patients had glioblastoma *IDH1*-wild type, two patients had glioblastoma *IDH1*-mutant, and 19 patients had glioblastoma NOS).

According to data from the literature on the prognostic significance of the mutation's presence or absence, two groups were created to assess the association of miR-10b/21/34a expression levels: glioblastoma *IDH1*-wild type and glioblastoma *IDH1*-mutant + NOS.

The median microRNA-10b expression level post-surgery and before the initiation of concomitant radiotherapy with temozolomide was 130.44 (52.20 - 622.53) in the *IDH1*-wildtype glioblastoma group and 94.61 (2.13 - 816.89) in the *IDH1*-mutant + glioblastoma NOS group.

The median microRNA-21 expression level was 57.16 (2.68 - 278.98) in the *IDH1*-wildtype glioblastoma group and 69.74 (4.60 - 825.43) in the *IDH1*-mutant + glioblastoma NOS group.

The median microRNA-34a expression level was 13.52 (3.16 - 105.20) in the *IDH1*-wildtype glioblastoma group and 10.11 (1.00 - 210.55) in the *IDH1*-mutant + glioblastoma NOS group. A complete overview of microRNA-10b/21/34a expression levels in relation to IDH1 mutation status is provided in **Table 1**.

The results showed no statistically significant difference in the expression levels of miR-10b/21/34a between the two groups ($p > 0.05$).

DISCUSSION

Given the established role of IDH mutations in glioblastoma and the overexpression of certain oncomiRs and tumor suppressor miRNAs in glioblastoma cells, we investigated the potential

association between miR-10b/21/34a expression levels in peripheral blood mononuclear cells and IDH1 mutation status.

We hypothesized that expression levels of miR-10b, miR-21 and miR-34a would positively or negatively correlate with IDH1-mutation status in glioblastoma. Ji et al. found that expression levels of miR-10b progressively rise with the advancement of WHO grades [10]. Considering that miR-21 is a potent oncogene overexpressed in glioblastoma and that glioblastoma cells depend on miR-10b (with the ablation of the miR-10 gene being lethal for these cells) [11], we expected significantly higher expression of miR-10b/21 in IDH1-wild type glioblastoma group than in *IDH1*-mutant + NOS group. However, our study did not find a statistically significant association between miR-10b/21 expression levels and IDH1 mutation status. On the other hand, Wang et al. proposed IDH1 mutation-specific microRNA signature. Precisely, in glioblastoma samples, the expression levels of 23 miRNAs varied by more than 1.5-fold between those with mutant *IDH1* and those with wild-type *IDH1*, respectively. One of the microRNAs with aberrant expression in *IDH1* mutation glioblastoma is miR-34a. Similar to miR-10b/21 and IDH1-wild type, we did not find significantly higher levels in the IDH1-mutant + NOS group compared to the IDH1-wild type group. To check if there is a change in expression levels of miR-10b/21/34a during radiotherapy with temozolomide in terms of IDH1 mutation status, we have investigated and compared expression levels at the 15^{th} and 30^{th} fractions of RT with temozolomide, but we did not find statistical significance, as well.

In low-grade glioma (LGG), IDH1/2 mutation status significantly influences miRNA expression [12]. The researchers developed a four-miRNA-based classifier (including miR-10b, miR-130b, miR-1304, and miR-302b) that effectively differentiated between high and low risk for poor prognosis in IDH1/2-mutant LGG [12]. As well, one study revealed a trio of miRNAs (miR-1-3p, miR-26a-1-3p, and miR-487b-3p) that showed differential expression in the serum of glioma patients, dependent on their IDH mutation status [13]. The expression and release of these miRNAs were lower in IDH-wild type glioma cells compared to IDH-mutant cells [13].

Taking into account the previous data from the literature, we tried to understand the results we got and why they did not completely match the results from the literature. Despite some differences in the study's design, to the best of our knowledge, direct studies linking miR-10b/21/34a and *IDH1* mutation status are limited. However, in the abstract published in 2013, Silber et al. indicate that IDH mutations in gliomas lead to the repression of miR-34a, which is associated with enhanced platelet-derived growth factor (PDGF) (PDGF signaling) (14). Their findings

suggest that miR-34a plays a crucial role in the cellular changes induced by IDH mutations, impacting tumor progression and potential therapeutic strategies [14].

MiRNAs play a vital role in complex regulatory networks that connect numerous genes and pathways, and their expression can be influenced by a variety of factors, making it difficult to establish a direct correlation with a specific mutation such as *IDH1*-mutation. Glioblastomas are highly heterogeneous tumors, meaning that different regions of the same tumor can have varying genetic and epigenetic profiles. The presence of an IDH1 mutation may trigger compensatory mechanisms within the tumor cells, which could mitigate the impact of the mutation on miRNA expression. Also, although IDH mutations may be the earliest steps in glioma genesis, it is highly likely that other simultaneous or subsequent molecular events are required for further tumor progression, primarily during the transformation of low-grade gliomas into high-grade gliomas [15]. The glioma microenvironment, various immune cells, stromal elements and the cytoskeleton can trigger pathways and alter miRNA expression. It is important to emphasize that in our study, we collected samples for microRNA analysis after surgery and prior to starting RT, which may impact our findings. Additionally, the precision of these results might not match those obtained directly from glioblastoma or cerebrospinal fluid (CSF) samples. Nevertheless, even with complete resection, in glioblastoma, there can be no real complete removal of all tumor cells due to its infiltrative behaviour [16]. The tumor cells are considered to be located or migrated in the surrounding brain parenchyma after surgery [17], as well as glioma stem cells responsible for recurrence [18], which suggests that residual tumor cells can still express a spectrum of miRNAs.

It's also worth mentioning that miR-10b and miR-21 are not the only significant microRNAs in gliomagenesis. There is a spectrum of microRNAs with potential roles as oncomiRs or tumor suppressors. For example, Sippl et al. suggested that miR-181a2 may serve as a prognostic marker for certain patients with *IDH1*-wild type glioblastoma [19]. Given that miR-181a2 regulates IDH1 expression in adipose tissue and considering the impact of *IDH1* mutation on glioblastoma's clinical course and biological behaviour, the researchers investigated the possible influence of miR-181a2 expression levels on IDH expression, the clinical course, and prognosis of GB patients [19]. More precisely, their findings suggest that low expression of miR-181a2 may positively influence the survival of glioblastoma patients through IDH1 regulation (19). In LGG, Bondarev et al. implied that certain miRNAs, such as miR-182, miR-455, and miR-891a, were generally increased in IDH-mutant gliomas, which are associated with a negative prognosis [20].

Besides the previously mentioned blood sampling after surgery we did in the study, another difficulty that can be another limitation of the study is the number of the patients included in the study. Increasing the number of patients and samples for microRNA analysis could potentially show a different result. In our study, patients have been classified according to the 2016 World Health Organization Classification of Tumors of the Central Nervous System, which in this particular study should not represent a obstacle. Despite a slight difference in prognosis, glioblastoma and diffuse astrocytoma grade 4 have low survival rates, indicating that this design of the study could be applied to high-grade gliomas as well.

Results from this study are one of the results of the doctoral dissertation of the first author, and represents a continuous work in the field of translational research in the field of radiobiology [21] and continuation of the previous published work on miRNAs in glioblastoma [22].

CONCLUSION

Our study did not confirm the significant correlation of microRNAs-10b/21/34a with IDH1 mutation status. Based on the results, it can be concluded that the expression levels of microRNAs miR-10b, miR-21, and miR-34a do not significantly differ between glioblastoma patients with *IDH1*-wild type and those with *IDH1*-mutant + NOS. These results suggest that the IDH1 mutation status may not be a critical factor for altered expression of microRNAs-10b/21/34a in glioblastoma patients. However, further research is encouraged. Identifying a possible association between specific miRNAs and IDH1 mutation status and other clinical and pathological parameters could refine our understanding of high-grade gliomas.

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Conflict of interest: None declared.

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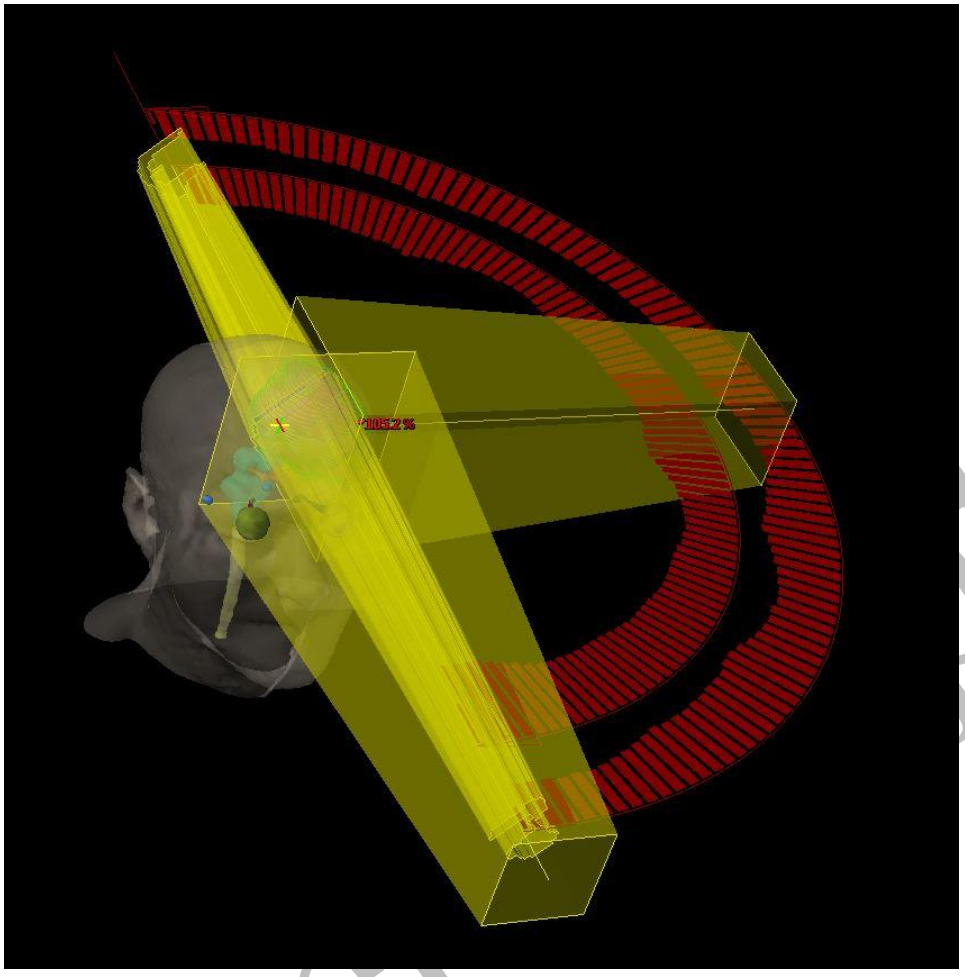

Figure 1. Volumetric modulated arc therapy (VMAT) technique of radiotherapy in a patient with glioblastoma (Institute for Oncology and Radiology of Serbia)

Table 1. Correlation between miR-10b/21/34a expression levels and IDH1 mutation status

miR	*IDH1*-wild type GB	*IDH1*-mutant + NOS GB	p-value
miR-10b prior RT median (min-max)	130.44 (52.2–622.53)	94.61 (2.13–816.89)	0.234
miR-10b 15f + TMZ median (min-max)	83.35 (16.03–433.53)	100.7 (1–922.88)	0.451
miR-10b 30f + TMZ median (min-max)	131.75 (1.47–493.53)	102.96 (2.32–2751.5)	0.560
miR-21 prior RT median (min-max)	57.16 (2.68–278.98)	69.74 (4.6–825.43)	0.903
miR-21 15f + TMZ median (min-max)	30.53 (2.79–542.32)	70.57 (4.37–960.07)	0.451
miR-21 30f + TMZ median (min-max)	60.56 (1–410.72)	62.03 (3.11–1940.21)	0.981
miR-34a prior RT median (min-max)	13.52 (3.16–105.2)	10.11 (1–210.55)	0.662
miR-34a 15f + TMZ median (min-max)	34.48 (3.48–198.64)	41.93 (4.04–352.38)	0.504
miR-34a 30f +TMZ median (min-max)	51.42 (2.94–363.04)	88.52 (3.71–871.28)	0.644

f – fraction; miR – microRNA; IDH – isocitrate dehydrogenase; GB – glioblastoma; NOS – not otherwise specified; RT – radiotherapy; TMZ – temozolomide

