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Relation of oncogenic microRNA-10b and microRNA-21 to glioblastoma size and localization

Повезаност онкогених микро РНК-10Б И микро РНК-21 са величином и локализацијом глиобластома

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Relation of oncogenic microRNA-10b and microRNA-21 to glioblastoma size and localization

Повезаност онкогених микро РНК-10Б И микро РНК-21 са величином и локализацијом глиобластома

SUMMARY

Introduction/Objective In glioblastoma, upregulation of oncogenic microRNA-10b and microRNA-21 is often found. Our study aimed to investigate whether there is a link between microRNA-10b/21 expression levels with tumor size and tumor localization.

Methods The research involved 43 patients diagnosed with glioblastoma. We analyzed the expression levels of microRNA-10b/21 post-surgery. The data on tumor size and tumor localization were obtained from magnetic resonance imaging.

Results The median expression level of microRNA-10b in patients with tumors < 4 cm was 214.86 (min-max: 2.13–816.89), while in patients with tumors ≥ 4 cm, the median expression level was 92.99 (min-max: 19.24–491.82). The median expression level of microRNA-21 in patients with tumors < 4 cm was 81.69 (min-max: 11.39–825.43), whereas in patients with tumors ≥ 4 cm, the median expression level was 40.84 (min-max: 2.68–278.98). Both, for microRNA-10b and microRNA-21 statistically significant difference was found for tumors smaller than < 4 cm (p = 0.027 and p = 0.047) compared to tumors ≥ 4 cm. There was no statistically significant difference in the expression levels of miR-10b (p = 0.675) and miR-21 (p = 0.183) in relation to tumor localization.

Conclusion Glioblastomas smaller than 4 cm have statistically significantly higher expression levels of microRNA-10b and microRNA-21 compared to glioblastomas equal to or larger than 4 cm. Although this result is unexpected, it could mean that microRNA expression levels dynamically change after surgery and according to altered microenvironment.

Keywords: glioblastoma; microRNA; tumor size

САЖЕТАК

Увод/Циљ Код глиобластома, често постоји усходна регулација онкогених микро РНК-10б и микро РНК-21. Наша студија је имала за циљ да истражи да ли постоји веза између нивоа експресије микро РНК-10б/21 са величином и локализацијом глиобластома.

Методе У истраживању су учествовала 43 пацијента са постављеном дијагнозом глиобластома. Анализирали смо нивое експресије микро РНК-10б/21 након оперативног лечења. Подаци о величини и локализацији глиобластома добијени су према налазу магнетне резонанце.

Резултати Медијана експресије микро РНК-10б код пацијената са туморима < 4 цм била је 214,86 (опсег: 2,13–816,89), док је код пацијената са туморима ≥ 4 цм, медијана експресије била 92,99 (опсег: 19,24–491,82). Медијана експресије микро РНК-21 код пацијената са туморима < 4 цм била је 81,69 (опсег: 11,39–825,43), док је код пацијената са туморима ≥ 4 цм, медијана експресије била 40,84 (опсег: 2,68–278,98). За микро РНК-10б и за микро РНК-21 нађена је статистички значајна разлика за туморе < 4 цм (p = 0,027 и p = 0,047) у поређењу са туморима ≥ 4 цм. Није било статистички значајне разлике у нивоима експресије микро РНК-10б (p = 0,675) и микро РНК-21 (p = 0,183) у односу на локализацију тумора.

Закључак Глиобластоми мањи од 4 цм имају статистички значајно веће нивое експресије микро РНК-10б и микро РНК-21 у поређењу са глиобластомима једнаким или већим од 4 цм. Иако је овај резултат неочекиван, он може упућивати на то да се нивои експресије микро РНК динамички мењају након операције и у складу са измењеним микроокружењем глиобластома.

Кључне речи: глиобластом; микро РНК; величина тумора

INTRODUCTION

Tumor histology, tumor size, grade, vascular invasion, stage and others clinicopathological and molecular features are often described as prognostic factors in patients with extracranial cancer [1, 2]. For patients diagnosed with malignant intracranial tumors such as glioblastoma, the most reliable prognostic factors are age, O6-methylguanine-DNA methyl-transferase (*MGMT)* promotor methylation, *IDH* mutation, extent of surgical resection and tumor location [3, 4]. Size

of the tumor in patients with glioblastoma has been shown also to be a possible prognostic factor for survival [3, 5, 6].

MicroRNA-10b (miR-10b) and microRNA-21 (miR-21) are amongst the most researched microRNAs in human oncology. They are often overexpressed in a spectrum of human cancer. MicroRNA-21 is frequently overexpressed in various cancers, including glioblastoma [7]. Due to the hypoxic conditions present in glioblastoma cells, an upregulation of miR-10b and miR-21 can be observed [8].

MicroRNA-10b is recognized as potent oncogenic microRNA (oncomiR) involved in regulation of cell cycle. Upregulation of microRNA-10b can promote tumor growth, invasion and migration [9]. Through diversiform gene regulation and signaling pathways, overexpression of microRNA-21 is shown to play an important role in oncogenesis and tumor metastasis, as well as in resistance to an oncologic treatment [10]. In glioblastoma, microRNA-21 is upregulated and thus, often linked with tumor pathogenesis, as well as to radioresistence and chemotherapy resistence [7]. Both, microRNAs-10b/21 are shown to be associated with clinical and pathologic features such as tumor size [11], disease stage, metastatic lymph nodes in extracranial tumors [12].

Inhibition of tumor growth and glioblastoma cell proliferation are proven in *in vitro* and *in vivo* experiments in gliomas [13] and glioblastoma [14] by knockdowning microRNAs, such as microRNA-10b [15] and microRNA-21 [13, 14]. However, the level of their expression in body fluids and the possible association with glioblastoma features, such as tumor size, and tumor location remains less clear.

Our study aimed to investigate whether there is an association between the expression levels of miR-10b and miR-21 and the tumor size and tumor localization of patients diagnosed with glioblastoma.

METHODS

This prospective cohort study followed 43 glioblastoma patients who had undergone surgery and were about to start treatment with Stupp's regimen [16]. The research focused on the levels of two specific miRNAs, miR-10b and miR-21, extracted from the patients' peripheral blood mononuclear cells (PBMCs). These patients were treated at the Clinic of Neurosurgery and the Neuro-Oncology Department at the University Clinical Center of Serbia, as well as the Institute for Oncology and Radiology of Serbia, starting in October 2017.

The study gathered clinical parameters, focusing on tumor size (less than 4 cm and 4 cm or larger) and tumor location (frontal lobe, temporal lobe, parietal lobe, occipital lobe, thalamus, or multilobar). Clinical data on tumor size and location were obtained from medical records, specifically Magnetic Resonance Imaging (MRI).

PBMCs were isolated from heparinized whole blood through centrifugation at 4°C using Histopaque-1077, following the manufacturer's instructions. miRNA molecules were extracted and purified from PBMCs using TRI Reagent, as per the manufacturer's protocol. The RNA samples were quantified using a BioSpec-nano spectrophotometer (Shimadzu corporation, Japan), with samples having an A260/280 ratio between 1.7 and 2.1 considered suitable for further analysis. To analyze miR-10b and miR-21, specific TaqMan® MicroRNA assays and the TaqMan® MicroRNA Reverse Transcription Kit were used. Starting with 10 ng of total RNA for reverse transcription, the cDNA was then amplified using TaqMan™ Universal Master Mix II on a 7500 Real-Time PCR System (Applies Biosystems, Foster City, California, USA). Relative quantities (RQ) were calculated using the comparative delta-delta Ct method, normalizing all samples to the endogenous control RNU6B and calibrating to the sample with the lowest RQ value.

Statistical analysis

To compare differences between two separate groups, we utilized the Mann-Whitney U test. For analyzing the correlation between miRNA expression levels and clinical parameters, we employed both Pearson and Spearman tests. Kruskal Wallis test is used for comparing three groups. The Log-Rank (Mantel-Cox) test was used to determine the significance of differences. All statistical analyses were performed using IBM SPSS Statistics version 22.

Ethics

All participants gave their informed consent, and the study complied with the ethical guidelines of the Declaration of Helsinki. The Ethical Research Committee at the Faculty of Medicine, University of Belgrade, reviewed and approved the study protocol under the reference number 1322/X-39.

RESULTS

In this study, we examined the correlation between the expression levels of microRNA-10b and microRNA-21 and the data on tumor size and tumor location in 43 patients with glioblastoma, using 43 samples for each microRNA molecule.

Most patients had a tumor located in temporal lobe (34.9%), while both a multifocal tumor and thalamic tumor had 2.3% of the patients. Tumor ≥ 4 cm had 62.8% of the patients. Observed clinical features were presented in Table 1.

The association between miR-10b and miR-21expression levels and tumor size (tumors less than 4 cm and tumors equal to or greater than 4 cm) was investigated. The median expression level of microRNA-10b in patients with tumors smaller than 4 cm was 214.86 (range: 2.13–816.89), while in patients with tumors 4 cm or larger, the median expression level was 92.99 (range: 19.24–491.82). For miR-10b, a statistically significant difference was found for tumors smaller than 4 cm ($p = 0.027$). The median expression level of microRNA-21 in patients with tumors smaller than 4 cm was 81.69 (range: 11.39–825.43), whereas in patients with tumors 4 cm or larger, the median expression level was 40.84 (range: 2.68–278.98). A statistically significant difference was also found for miR-21 in tumors smaller than 4 cm ($p = 0.047$) compared to tumors 4 cm or larger.

Due to the unfavorable ratio of the number of different tumor localizations (frontal lobe–14 patients, temporal lobe–15 patients, parietal lobe–10 patients, occipital lobe–2 patients, multilobar tumor–1 patient, thalamus–1 patient) and potential predictors, it was not possible to make a comparison for each localization separately. Instead, according to data from the literature, three groups were created to compare tumor localization with miR-10b and miR-21 expression levels: tumors in the frontal lobe (frontal), tumors in the temporal lobe (temporal), and tumors in other lobes (Figures 1, 2). The results showed that there was no statistically significant difference in the expression levels of miR-10b ($p = 0.675$) and miR-21 ($p = 0.183$) in relation to tumor localization (Table 2) (Figures 1, 2).

DISCUSSION

Our study aimed to determine whether there is a link between the expression levels of miR-10b and miR-21 and clinicopathological factors such as tumor size and localization.

Scientific studies on glioblastoma often classify tumors into two categories based on size: those measuring less than 4 cm and those measuring 4 cm or greater. However, the rationale for

selecting this specific size as a benchmark is not clearly established. The debate on this matter frequently involves questions about whether 4 cm represents the size at which tumors are most commonly diagnosed—namely, when patients exhibit symptoms prompting diagnostic procedures or whether this threshold impacts the extent of surgical resection, the success of adjuvant therapies, and the overall prognosis of the disease. Upon review of the literature and the data presented, we have decided to utilize a tumor size of 4 cm as the reference value in this study. Statistical significance was found only for the expression levels of miR-10b and miR-21 in tumors smaller than 4 cm, which was unexpected. We had a hypothesis that the expression levels of miR-10b and miR-21 would be statistically significant in tumors larger than 4 cm.

MicroRNA-21 is recognized as one of the most potent oncogenes, playing a pivotal role in carcinogenesis, metastatic potential, and disease relapse [17]. This observation led us to hypothesize that larger tumors at the time of disease presentation may exhibit higher expression levels of microRNA-21. Supporting this hypothesis, we noted that overexpression of microRNA-21 has been found in breast tumor tissue, and using a miR-21 inhibitor, known as antimiR-21, inhibited tumor cell growth both in vitro and in vivo [18]. Furthermore, microRNA-21 is also one of the most upregulated microRNAs in glioblastoma, and the studies demonstrated that knocking down miR-21 in glioblastoma cells resulted in reduced cell growth [19]. On the other hand, miR-10b is regarded as a highly oncogenic microRNA, with its overexpression observed in glioblastoma, influencing tumorigenesis, or gliomagenesis [20]. Ji et al. investigated the association of miR-10b expression level and prognosis in patients with glioma. They found out that there is a higher expression level of miR-10b in glioma patients compared to normal brain parenchyma [21]. As well, upregulation of miR-10b in glioma was correlated with higher glioma grade and larger tumor size [21]. Regarding glioblastoma size and microRNA expression, Siegal et al. found out that in the group of patients with glioblastoma who were treated with bevacizumab, there was a significant negative correlation between miR-10b and miR-21 levels and changes in tumor diameters [22]. The authors revealed that they used the serum for the determination of expression levels of specific microRNAs. Moderately analogous to the previously mentioned study, in our study, we investigated microRNA expression levels from patients' plasma and measured tumor size by MRI, as well.

Glioblastoma is an infiltrative tumor, and glioma stem cells could be in the area of the cavity or in the remaining tumor after partial resection, which could be responsible for different microRNA expression levels. Nevertheless, for accurate interpretation of microRNA expression levels and their association with tumor size, it could be essential to measure microRNA levels

before any surgical treatment and compare them not only to MRI size of the tumor, but the actual size of the tumor after surgery. In our study, we measured these levels after surgery and before the start of radiotherapy with temozolomide, which could affect the results. However, when it comes to glioblastomas, which are known for their heterogeneous pathological features, including varying foci and sizes of necrosis, smaller tumors might show less pronounced necrotic areas. This means that the microenvironment of the glioblastoma and the surrounding tissue, as well as intracellular and intercellular communication, might not be completely disrupted. That might imply that even smaller tumors might show microRNA overexpression and exhibit a higher proliferative capacity. In most cases, for extracranial tumors, the proportion of tumor necrosis is often associated with tumor size [23]. Regarding glioblastoma, the interpretation of the connection between tumor size and tumor necrosis size may be difficult. Some studies revealed that even small glioblastoma can have various extents of necrosis, and conversely [24]. Moreover, due to the complexity of the glioblastoma microenvironment in terms of the possibility of glioblastoma and glioblastoma stem cells reprogramming its microenvironment [25], different microenvironment may affect microRNA expression, especially after surgery. When interpreting microRNA expression levels, it's important to consider how microRNAs are released, the ability of cells to release them, and what tissue is used for the determination of their expression levels. Although the exact mechanism of microRNA release isn't fully understood, the data suggests that microRNAs are released from both apoptotic bodies and viable tumor cells. Intercellular communication involving microRNAs occurs through exosomes, extracellular vesicles, and other pathways [26]. It also should be borne in mind that in the human brain, the only cells that could express miR-10b are the human brain microvascular endothelial cells [27], which could affect the results, as well.

Bearing in mind the data from the literature on the different expressions of specific microRNAs in the corresponding parts of the brain, we investigated whether there is a difference between the expression level of miR-10b and miR-21 in relation to the localization of the tumor. However, we did not find statistical significance in the expression level of miR-10b and miR-21 between tumors in the frontal lobe, temporal lobe and tumors in other localizations. To the best of our knowledge, we have not found a study that compared the expression level of microRNA-10b and miR-21 in relation to the localization of glioblastoma in different brain lobes. However, we found another research on a similar topic. Among other results, Ozdogan et al. did not find a significant correlation between the expression level of miR-221 and glioma localization in the brain [28].

We considered that the main limitations of our study were collecting samples after surgery and tumor size measurements that were noted only in the MRI. On the other hand, this limitation could also lead to more research on the different dynamic changes in microRNA profiles after surgery and the possible influence of microenvironment or treatment on the tumor size and expression profile of microRNAs, respectively.

Given that peripheral blood mononuclear cells (PBMCs) are a minimally invasive and easily accessible source of microRNAs (miRNAs), we isolated miRNAs from the PBMCs of patients with glioblastoma in our study. Nevertheless, some studies acknowledge that miRNA values may differ between PBMCs and whole blood [29]. The results from our study would be even more precise if the miRNA values isolated from PBMCs were compared with those from whole blood or even tumor tissue.

Results from this paper are part of the doctoral dissertation of the second author of the manuscript, and part of the translational research from the radiobiology team of our institute [30].

CONCLUSION

Tumors smaller than 4 cm have statistically significantly higher expression levels of microRNA-10b and microRNA-21 compared to glioblastomas equal to or larger than 4 cm. Although this result is unexpected, it could mean that microRNA expression levels dynamically change after surgery and according to altered microenvironment. There was no statistical significance in the expression level of miR-10b and miR-21 between tumors in the frontal lobe, temporal lobe and tumors in other localizations.

Considering all the functions microRNAs have in normal cells and tumor cells, further research on glioblastoma microRNA profiles is needed. Elucidation of the mechanisms of gliomagenesis and tumor growth in relation to the expression profile of specific microRNAs is important in the future for developing potential diagnostic methods using liquid biopsies or new therapeutic strategies.

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Conflict of interest: None declared.

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Table 1. Data on tumor location and tumor size of patients with glioblastoma

Tumor location	**No (%)**
Frontal lobe	14 (32.6%)
Temporal lobe	15 (34.9%)
Parietal lobe	10 (23.3%)
Occipital lobe	2 (4.7%)
Multifocal tumor	1 (2.3%)
Thalamic tumor	1 (2.3%)
Tumor size	**No (%)**
< 4 cm	16 (37.2%)
≥ 4 cm	27 (62.8%)

Table 2. MicroRNA-10b and microRNA-21 expression levels and tumor localization

MicroRNA	Median (range)	p-value
microRNA-10b		
Frontal lobe	153.23 (2.13–622.53)	0.675
Temporal lobe	92.99 (41.18–816.89)	
Other lobes	102.18 (30.09–465.62)	
microRNA-21		
Frontal lobe	81.69 (11.42–825.43)	0.183
Temporal lobe	53.48 (2.68–706.23)	
Other lobes	35.70 (5.82–278.98)	

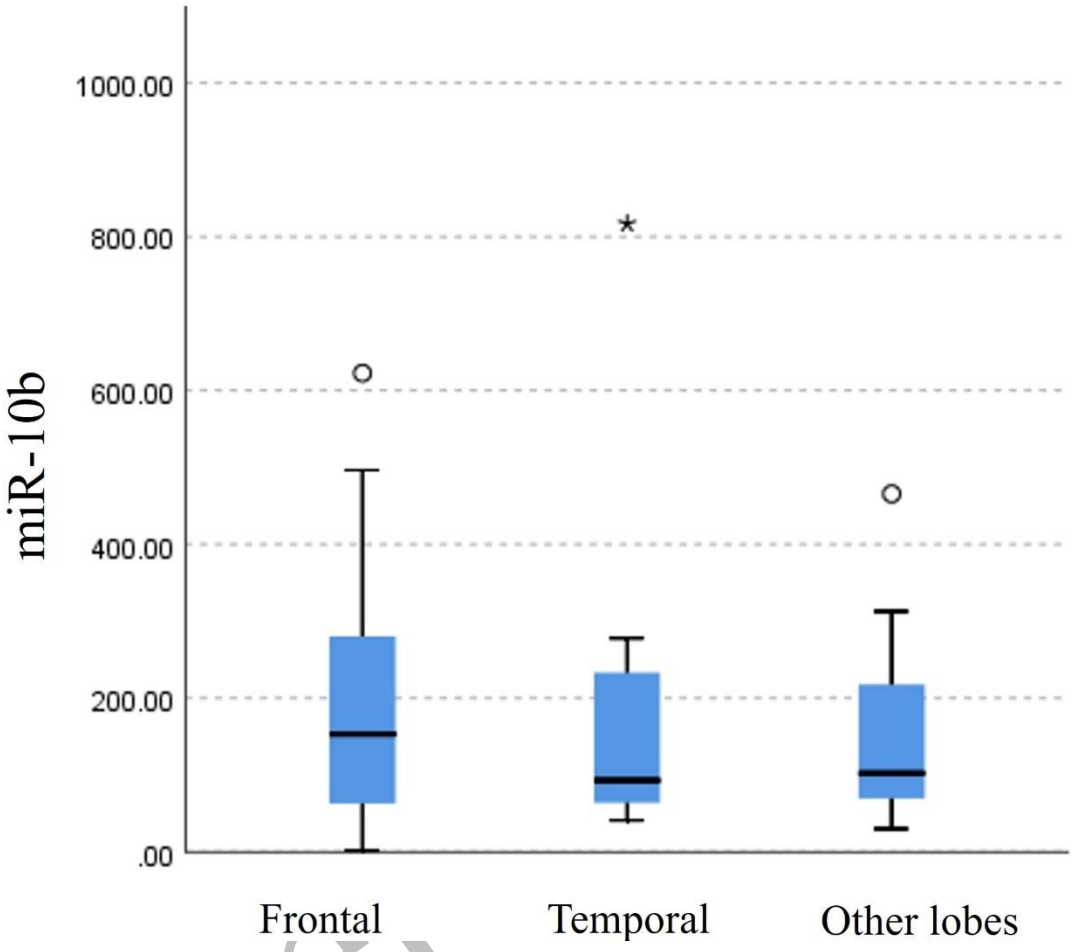

Figure 1. Comparison of median expression levels of microRNA-10b in the frontal, temporal, and other lobes where glioblastoma is diagnosed

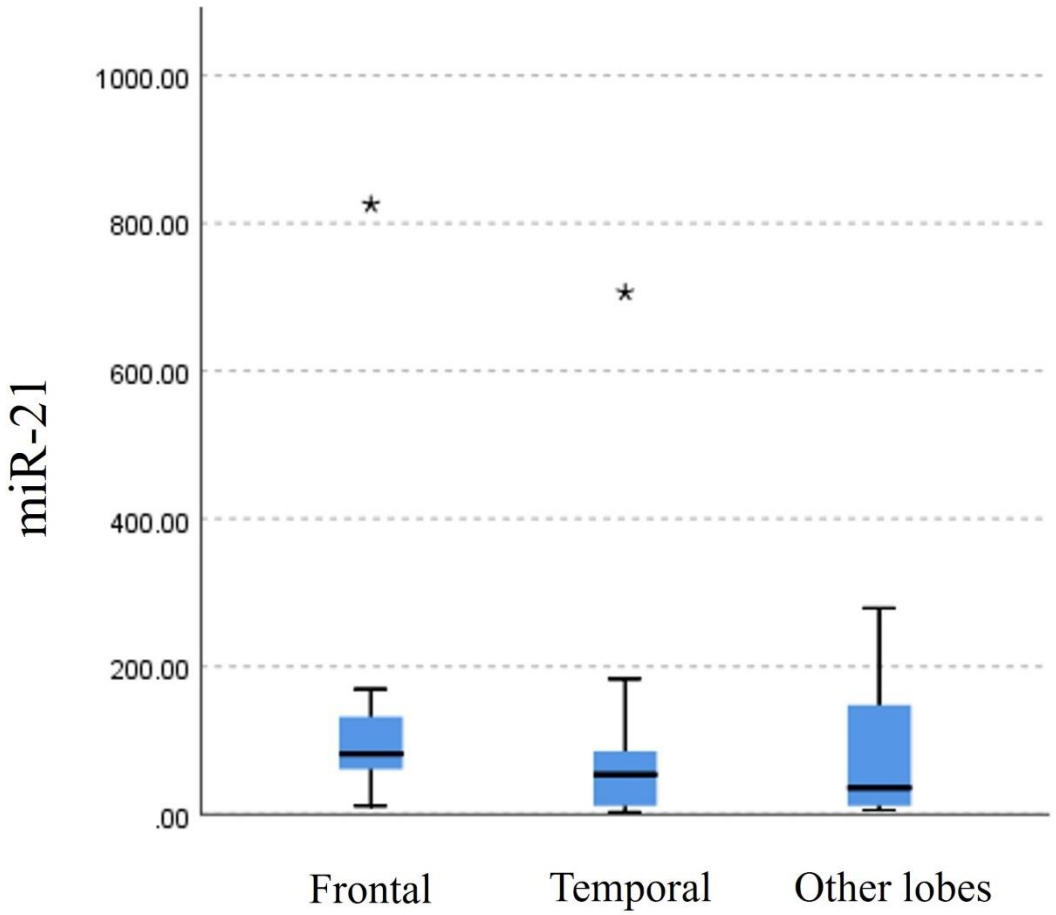

Figure 2. Comparison of median expression levels of microRNA-21 in the frontal, temporal, and other lobes where glioblastoma is diagnosed

