LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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MIRNA STUDIES OF TUMOR TISSUE AND BLOOD EXTRACELLULAR VESICLES FOR DIAGNOSIS AND PROGNOSIS OF GLIOMAS

Doctoral Dissertation
Natural Sciences,
Biology (N 010)

Kaunas, 2023

Dissertation has been prepared at the Laboratory of Molecular Neurooncology of Neuroscience Institute of Lithuanian University of Health Sciences during the period of 2018–2022.

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Dissertation will be defended at the open session of the Biology Research Council of Lithuanian University of Health Sciences at 3 p.m. on the 30th of August, 2023 in the auditorium A-202 of the Valley Centre for the Advanced Pharmaceutical and Health Technologies of Lithuanian University of Health Sciences.
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LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

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NAVIKINIO AUDINIO IR KRAUJO EKSTRALĄSTELINIŲ PŪSLELIŲ MIRNR TYRIMAI GLIOMŲ DIAGNOZEI IR PROGNOZEI

Daktaro disertacija
Gamtos mokslai,
biologija (N 010)

Kaunas, 2023

Disertacija rengta 2018–2022 metais Lietuvos sveikatos mokslų universiteto Neuromokslų instituto Molekulinės neuroonkologijos laboratorijoje.

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Disertacija bus ginama viešajame Biologijos mokslo krypties tarybos posėdyje 2023 m. rugpjūčio mėn. 30 d. 15.00 val. Lietuvos sveikatos mokslų universiteto Naujausių farmacijos bei sveikatos technologijų centre, A-202 auditorijoje.
Disertacijos gynimo vietos adresas: Sukilėlių pr. 13, LT-50162 Kaunas, Lietuva.

CONTENTS

ABBREVIATIONS

AGO2 – argonaute RISC catalytic component 2
ATRX – alpha thalassemia X-linked mental retardation
AUC – area under the curve
Bax/Bcl-2 – Bcl-2-associated X protein/B-cell lymphoma protein 2
BRCA1 – breast cancer gene 1
CDKN2A – cyclin dependent kinase inhibitor 2A
cDNA – copy-deoxyribonucleic acid
CIC – capicua transcriptional repressor
CNS – central nervous system
CRT – classification and regression trees
CT – computed tomography
DNA – deoxyribonucleic acid
EEG – electroencephalogram
EGFR – epidermal growth factor receptor
EOMES – eomesodermin
EORTC – European Organization for Research and Treatment of Cancer Core
EV – extracellular vesicle
FUBP1 – far upstream element binding protein 1
GAS1 – growth arrest specific 1
GB – glioblastoma
GTP – guanosine triphosphate
HOXA1 – homeobox A1
HRQOL – health-related quality of life
IDH – isocitrate dehydrogenase
IEIF4G2 – eukaryotic translation initiation factor 4 gamma 2
IGF2 – insulin growth factor 2
KPS – Karnofsky performance scale
LGG – low-grade glioma
LncRNAs – long non-coding RNAs
MGMT – O6-methylguanine-DNA methyltransferase
MIRN1-1 – micro RNA 1-1
MIRN1-2 – micro RNA 1-2
MiRNA – micro RNA
MRI – magnetic resonance imaging

MYT1L – myelin transcription factor 1-like
NAT – normal tissue adjacent to the tumor
NEC – not elsewhere classified
NOS – not otherwise specified
NOTCH1 – neurogenic locus notch homolog protein 1
PDCD4 – programmed cell death 4
Pri-miRNA – primary miRNA
PTEN – tensin homolog
QLQ – quality of life questionnaire
RE1 – repressor element 1
REST – RE1-silencing transcription factor
RISC – RNA-induced silencing complex
RNA – ribonucleic acid
RT-qPCR – reverse transcription-quantitative polymerase chain reaction
SHH – sonic hedgehog signaling molecule
SVM – support vector machines
TBR2 – T-box brain protein 2
TERT – telomerase reverse transcriptase
TLX – nuclear receptor subfamily 2 group E member 1
TMZ – temozolomide
TP53 – tumor protein p53
TPM1 – tropomyosin 1
TTN-AS1 – titin-antisense RNA1
UTR – untranslated region
WHO – World Health Organization
XRN2 – 5’-3’ exoribonuclease 2

INTRODUCTION

Gliomas are the most prevalent type of primary malignant brain tumors and are central nervous system (CNS) tumors that develop in the brain from glial cells. Glioblastoma accounts for almost 50 % of all malignant brain tumors and is the most common malignant CNS tumor histology. Just 6.8 % of glioblastoma patients survive longer than five years, and only 42.5 % survive longer than one year [1,2]. Mainly because brain cancer is detected late. Although, it is technically possible to identify asymptomatic glioma patients in the population using global Magnetic resonance imaging (MRI) scanning programs. This strategy, meanwhile, is very labor-intensive and not cost-effective [3,4].

For early glioma identification, scalable and less intrusive technology is required. Micro RNA (miRNA) expression detection can be used for this purpose. MiRNAs are small non-coding ribonucleic acid (RNA) molecules that play important regulatory roles in gene expression [5]. In cancer, miRNAs can act as oncogenes or tumor suppressors, depending on the specific miRNA and the cellular context. Some miRNAs can promote cancer progression by targeting and suppressing tumor suppressor genes, while others can inhibit cancer growth by targeting and suppressing oncogenes [6]. Additionally, miRNAs can regulate other aspects of cancer biology, such as angiogenesis, invasion, and metastasis. Specific miRNAs that are dysregulated in gliomas have been identified by several studies, and it has been demonstrated that these miRNAs control important pathways involved in the progression of gliomas, including cell proliferation, invasion, and angiogenesis [7]. For instance, miR-124 is expressed at lower levels in higher-grade gliomas and is downregulated in gliomas relative to healthy brain tissue. MiR-124 targets genes involved in cell cycle regulation and proliferation, which studies have proven to suppress the proliferation and invasion of glioma cells [8] . On the other side of the specter is miR-21 – one of the most commonly upregulated miRNAs in gliomas. Studies have shown that miR-21 expression levels increase with higher-grade gliomas and are associated with poorer overall survival [9–12].

MiRNAs are generally stable and can also be contained in extracellular vesicles (EVs) before being transported to circulation [13,14]. EVs are small, membrane-bound particles that are released by various cell types, including cancer cells. They are classified into three main types: exosomes, microvesicles, and apoptotic bodies. EVs contain a variety of bioactive molecules, including proteins, lipids, and nucleic acids, such as miRNAs. They play a crucial role in intercellular communication, as they can transfer their contents to

neighboring or distant cells, modulating various cellular processes, such as differentiation, proliferation, and immune response. In cancer, EVs can promote tumor growth and progression by transferring oncogenic molecules, such as miRNAs, and by modulating the tumor microenvironment [15,16]. To track the development of gliomas, EVs can be isolated, and their miRNAs quantified.

Recent studies have explored the potential of EV-associated miRNAs as biomarkers for glioma diagnosis and prognosis. For example, EV-associated miR-10b has been shown to be a potential biomarker for glioma diagnosis, while EV-associated miR-21 and miR-221 have been linked to poor prognosis in glioma patients [17–21]. However, there is a still lack of consistent data in the field of miRNAs of extracellular vesicles for creating of a diagnostic test for glioma with a minimally desired 95 % specificity and sensitivity.

Aim and objectives

Aim:

The aim of this study was to determine a set of diagnostic and prognostic miRNAs for glioma progression and outcome.

Objectives:

1. To assess the association between glioma grade and miRNA expression in a) tumor tissue and b) extracellular vesicles of the blood
2. To evaluate the connection between tumoral and extracellular miRNA expression
3. To assess the prognostic potential of analyzed miRNAs according to standard molecular markers of gliomas – *MGMT* gene promoter methylation and *IDH* gene mutations
4. To determine the relationship of the miRNA expression profile with the clinical outcome of the glioma patients
5. Based on the miRNA expression, *MGMT* gene promoter methylation status, *IDH* gene mutations data, and clinical characteristics of patients – create an algorithm that allows patients to be divided into survival prognostic groups

The novelty and relevance of the study

This study expands the knowledge of miRNA expression in the tissue and blood's extracellular vesicles (EVs) of patients with different glioma grades. In addition, this study, for the first time analyzed Lithuanian glioblastoma cohort

for the miRNA expression in tumor tissue and the same patients' EVs of blood serum. Also, for the first time, glioma patients' quality of functioning was evaluated against tumoral and extracellular miRNA expression for prognostic indications. Furthermore, this study provides insights into a set of specific miRNAs suitable for further investigation for the development of minimally invasive diagnostic and prognostic tests for glioma and glioblastoma. The data presented in this study can serve as a starting point for future studies of the bioactivity of miRNAs in glioma.

Outline

The dissertation is structured into 3 studies:
1) A search of potential miRNAs for glioma prognostic and diagnostic.
2) MiRNA analysis in glioma post-surgical tissue.
3) MiRNA analysis in EVs of blood serum from glioma patients.

For a quicker reference, simplified graphical and textual side-by-side micro-summaries of this thesis are represented in Figures 1 and 2.

The combination of these studies contributes to a better understanding of glioma's molecular biology and miRNA association with glioma grade, post-surgical survival time, and quality of life of glioma patients. The results from Study II and Study III have been published in *International Journal of Molecular Sciences*, and *Cancers* peer-reviewed journals.

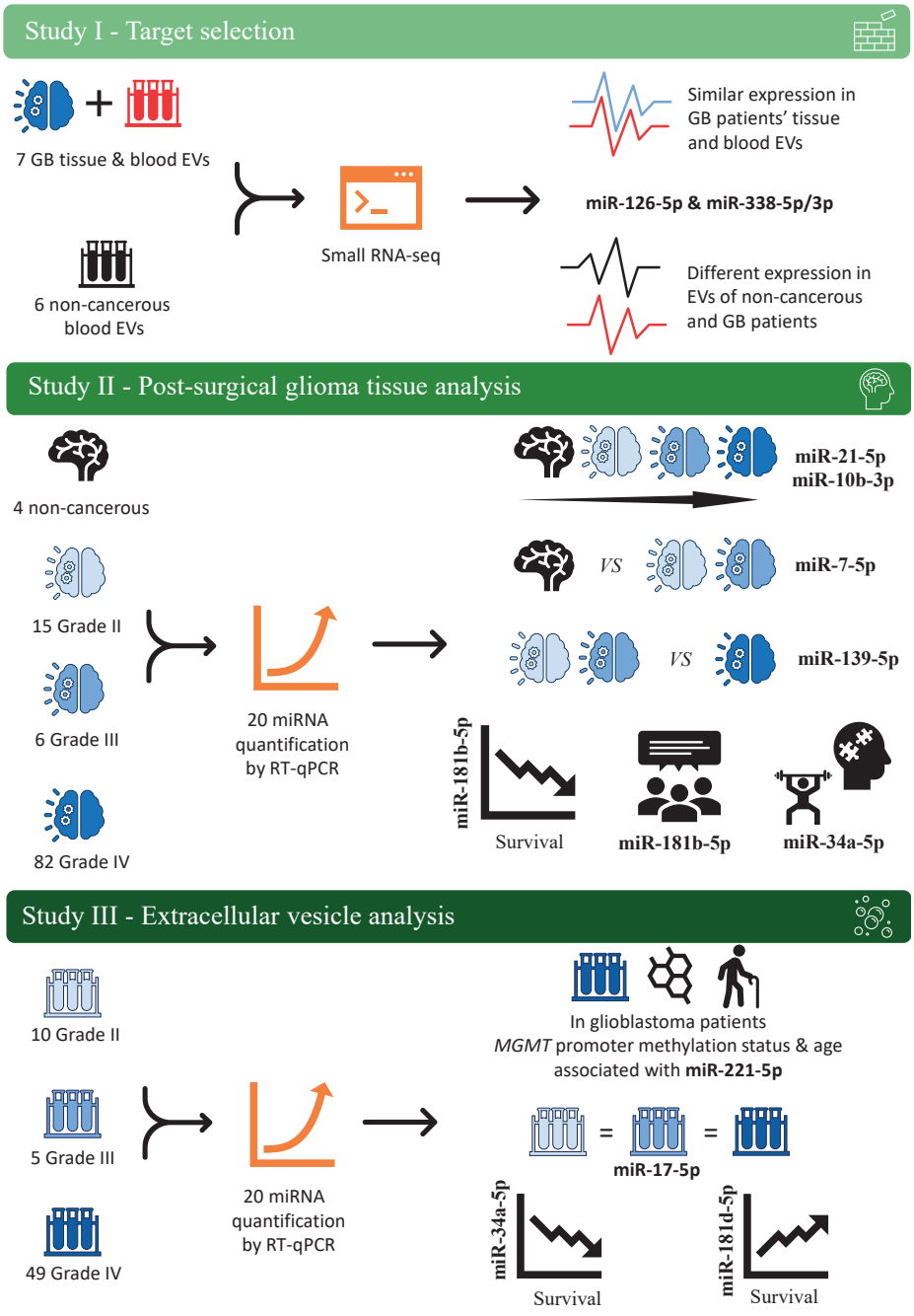

Fig. 1. *Graphical outline of the thesis*

Graphical summarization of the thesis. Illustrations provide a quick reference to the samples, main methods, and main results of this thesis. Textual summarization explains a less intuitive parts of the graphical summarization.

Study I - Target selection

- **Samples**:
  - Non-cancerous blood samples – 6
  - Paired tissue & blood samples from glioblstoma (GB) patients – 7
- **Methods**:
  - Small RNA extraction from extracellular vesicles (EVs) of blood serum ("*exoEasy*" kit)
  - Small RNA extraction from tissue ("*mirVana*" kit)
  - Ligation-free library preparation for smallRNA-seq ("*CATS Small RNA-seq*" kit)
  - Illumina sequencing (*MiSeq*, SE50, ~2.5M reads/sample)
- **Main results**:
  - miR-126-5p, miR-338-5p/3p most similarly expressed in GB tissue and EVs but differentially expressed between EVs of non-cancerous and GB patients

Study II - Post-surgical glioma tissue analysis

- **Samples**:
  - Non-cancerous brain tissue – 4
  - Glioma tissue: Grade II – 15; Grade III – 6; Grade IV (glioblastoma) – 82
- **Methods**:
  - miRNA selection from Study I and the literature: reference – 4; onco – 10; onco-suppressive – 4
  - Small RNA extraction from tissue ("*mirVana*" kit)
  - miRNA quantification using RT-qPCR ("*TaqMan Advanced miRNA*" cDNA synthesis kit and quantification assays)
- **Main results**:
  - Gradually higher expression of miR-21-5p and miR-10b-3p detected in higher grades of glioma
  - Highest difference between non-cancerous and low-grade glioma (LGG) tissue groups: miR-7-5p
  - Highest difference between LGG and GB tissue groups: miR-139-5p
  - Longer survival time for GB patients was associated with a low miR-181b-5p tumoral expression
  - Higher expression of miR-181b-5p correlated with GB patients' better social functioning
  - Higher expression of miR-34a-5p correlated with better total & physical functioning and overall performance, according to KPS, in GB male patients
  - Lower expression of miR-21-5p and miR-148a-3p correlated with bigger glioblastoma volume

Study III - Extracellular vesicle analysis

- **Samples**:
  - Blood serum from glioma patients: Grade II – 10; Grade III – 5; Grade IV (glioblastoma) – 49
- **Methods**:
  - Small RNA extraction from extracellular vesicles (EVs) of blood serum ("*exoEasy*" kit)
  - miRNA quantification using RT-qPCR ("*TaqMan Advanced miRNA*" kits)
- **Main results**:
  - Combination of GB patients' MGMT promoter methylation status and age group was associated with the expression of an extracellular miR-221-5p
  - miR-17-5p had the most stable extracellular expression in all grades of glioma
  - Low extracellular levels of miR-34a-5p was associated with a longer GB patients' survival
  - An increase of an extracellular miR-181d-5p expression was estimated to have the highest effect on reducing the hazard of dying
  - Higher expression of an extracellular miR-181d-5p correlated with worse physical and emotional functioning in glioblastoma patients

Fig 2. *Textual outline of the thesis*

Textual summarization of the thesis explaining less intuitive parts of the graphical summarization.

1. LITERATURE REVIEW

1.1. Epidemiology of CNS cancers and gliomas

Cancer is one of the leading causes of death worldwide, accounting for almost 10 million deaths, of which 251,329 or 2.8 per 100,000 were brain or central nervous system cancer-related deaths in 2020. The incidence of brain CNS cancer was 308,102 or 3.5 per 100,000 in 2020, according to the Global Cancer Observatory data [22]. CNS tumors in the brain that arise from glial cells are called gliomas and are the most common type among primary malignant brain tumors. Glioma-type tumors include high-grade glioblastomas and low-grade gliomas, such as astrocytoma and oligodendrogliomas [2]. The most frequent malignant CNS tumor histopathology was glioblastoma, with 50 % of all malignant tumors. It was estimated that 26,670 new cases of malignant CNS tumors were diagnosed in the United States in 2022 [1]. According to the Central Brain Tumor Registry of the United States statistical report 2015–2019, glioblastoma (GB) was the most frequent cancer type, 59.2 % of all CNS gliomas [1]. In the Lithuanian population, cancer incidence in 2020 was 17,073, and the age-standardized incidence rate (world) was 293.4. Also, in 2020 there were 8,168 deaths, and the age-standardized mortality rate (World) was 122.1. Brain and CNS cancer comprised 366 new cases, 2.1 % of all cancer cases, with 259 new deaths, 3.2 % of all deaths [22]. Glioma is not one of the most frequent tumor types. However, it is associated with a poor prognosis. The 5-year survival rate for GB is 5.6 %, while the 10-year only reaches 0.71 % of diagnosed and treated patients [23]. The glioblastoma incidence rate is 1.6 times higher in males than in females, and it is higher in the white race than in any other [24].

1.2. The World Health Organization glioma classification

The first official World Health Organization (WHO) CNS tumors classification edition was established in 1979 and was primarily based on morphological parameters [25]. The second edition came out a decade later with a major update to include immunohistochemistry analysis [26]. In 2002 the third edition of WHO tumors classification for nervous system tumors was introduced that included descriptions of each entity's epidemiological, radiological, pathological, and other features [27]. After that, the WHO 2007 glioma classification was still based on tumor histology, resulting in inconsistent patient survival rates within grades. Tumors were classified based on histological criteria by the general appearance of the tissue: astrocytoma, oligodendroglioma and mixed oligoastrocytoma. Tumors were graded II-IV.

However, in recent years the understanding of molecular alterations extended, and genetic risk factors have been identified.

The WHO 2016 classification system incorporated tumor morphology and molecular alterations and resulted in five glioma subtypes with reduced misclassification and more consistent patient outcomes with each subtype [28,29]. Also, during the past decade, new risk factors and rare mutations were identified [30–32]. The WHO 2016 classification included tumor morphology, isocitrate dehydrogenase (*IDH*) mutation, and 1p19q co-deletion status for diffuse glioma in adults [29]. Therefore, five types of glioma were determined: glioblastoma, *IDH*-wild type; glioblastoma, *IDH*-mutant; diffuse or anaplastic astrocytoma, *IDH*-wild type; diffuse or anaplastic astrocytoma, *IDH*-mutant; and oligodendroglioma or anaplastic oligodendroglioma, *IDH*-mutant and 1p19q co-deleted. Glioblastoma, *IDH* wild type, had the highest age at diagnosis (median 59 years) and the worst prognosis (median overall survival 1.2 years). Glioblastoma, *IDH*-mutant, was associated with younger age at diagnosis (median 38 years) and better prognosis (median overall survival 3.6 years). Astrocytoma, *IDH*-wild type, was associated with a median age of diagnosis of 52 years and median overall survival of 1.9 years. Astrocytoma, *IDH*-mutant, was associated with the lowest median age at diagnosis of 36 years and median overall survival of 9.3 years. Oligodendroglioma, *IDH*-mutant and 1p19q co-deleted were associated with a median age at diagnosis of 44 years and the longest median overall survival of 17.5 years [33,34]. It was estimated that in 2019, 71 % of newly diagnosed gliomas would be glioblastoma, *IDH*-wild type; 7 % – Glioblastoma, *IDH*-mutant; 5 % – Astrocytoma, *IDH*-wild type; 12 % – Astrocytoma, *IDH*-mutant; 5 % – Oligodendroglioma, *IDH*-mutant and 1p19q co-deleted [28]. Diffuse astrocytoma and *IDH*-wild type tumors were associated with variable outcomes [35]. This group includes tumors with good prognoses, such as glial-neural tumors and pilocytic astrocytoma, and tumors with poor prognoses similar to GB but lacking histological confirmation to be assigned as one [28]. Therefore it was suggested to change the characterization of this group and update it to reflect such disparities [35]. A sixth group was based only on histological features when molecular marker information was unavailable [28]. Moreover, a seventh group was known as diffuse midline glioma H3 K27M-mutant (that is, with a histone 3 Lys27Met amino acid substitution). It was recognized as a separate group because, histologically, it can be classified as a low-grade tumor, however, the clinical outcome is unfavorable [36]. Therefore, the updated WHO classification identified diffuse glioma tumors arising in the midline (spinal cord, thalamus, brainstem, cerebellum) and harboring the H3 K27M mutation as a separate group [29,37].

The fifth, most recent WHO 2021 classification, was introduced at the end of 2021 [27]. One of the main changes was grading. Firstly, it was changed to Arabic numerals instead of Roman numerals. Also, now grading is done within tumor type. Therefore, grade 4 does not mean the same prognosis for different tumor types, although it is the worst within a specific tumor type. Finally, the official usage of CNS tumors should be "CNS WHO grade 4" instead of "WHO grade 4" as it can differ from other types of cancer. Other changes include NOS (not otherwise specified) and NEC (not elsewhere classified), and the main difference is that for NOS that molecular testing required for classification is not available. In contrast, molecular testing was performed for NEC, but the results were inconclusive. Adult-type gliomas are classified into one of three groups by morphology and molecular features: astrocytoma, *IDH*-mutant; Glioblastoma, *IDH*-wildtype; Oligodendroglioma, *IDH*-mutant, and 1p/19q-codeleted [38]. *IDH*-wildtype gliomas are now defined and graded. Now morphologic features are considered, but tumors without *IDH* mutation, a gain of chromosome 7 and loss of chromosome 10, epidermal growth factor receptor (*EGFR*) amplification or telomerase reverse transcriptase (*TERT*) promoter mutations are called glioblastoma and graded as CNS WHO grade 4 [35]. Astrocytoma, *IDH*-mutant, is defined by a mutation in *IDH1* or *IDH*2 genes and occurs in younger adults; patients usually present with seizures and have tumor protein p53 (*TP53*) alterations, and around 90 % of supratentorial *IDH*-mutant astrocytoma also have alpha thalassemia X-linked mental retardation (*ATRX*) mutations [37,39,40]. Astrocytoma, *IDH*-mutant, is graded from 2 to 4 based on various parameters such as mitotic activity, necrosis, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) deletion and microvascular proliferation [41]. Also, in the current CNS WHO 2021 classification, grade 4 astrocytoma is no longer considered glioblastoma [42]. Oligodendroglioma is defined by the whole arm 1p/19q co-deletion and *IDH* mutation, but also the majority have *TERT* promoter mutations, alterations in capicua transcriptional repressor *(CIC),* far upstream element binding protein 1 (*FUBP1),* neurogenic locus notch homolog protein 1 (*NOTCH1)* genes. The grading did not change from the 2016 classification and included grades 2–3 [38].

1.3. Symptoms, diagnosis, and prognosis of glioma

Adult brain tumors have vague early symptoms. Usually, these are the signs caused by elevated intracranial pressure and present as headaches that are worse lying down; they can also be accompanied by vomiting or visual disturbances. Symptoms can also progress over time, and combinations of symptoms, like headaches combined with cognitive decline, weakness, or

a personality change, can occur. Seizures could also indicate brain tumors. Patients who exhibit signs of elevated intracranial pressure or seizures are referred for an electroencephalogram (EEG), a contrast-enhanced computed tomography (CT), or MRI [43,44]. If scan results suggest a brain tumor, the patient is referred to a multidisciplinary neuro-oncology team for further investigation and treatment. It usually begins with a biopsy or maximal surgical resection. After that, if the tumor is high grade, for example, glioblastoma, the Karnofsky performance status is evaluated. If the score is equal to or higher than 70, radiotherapy and temozolomide are the standard treatment options in doses based on the patient's age group. If the patient's Karnofsky performance status is less than 70, but the patient is younger than 70 years old, different treatment options and combinations, including radiotherapy, temozolomide, and supportive care, are considered. However, if the patient is 70 years old or older, only the best supportive care is usually offered. The median survival for adults under 70 who do not receive therapy is roughly 3-4.5 months [45,46]. With or without radiotherapy, a biopsy operation followed by chemotherapy raises the median survival to about 8–10 months. The median survival duration is increased to about 15–16 months with surgery and chemotherapy, and the associated survival rates are 27–31 % at two years and 7–10 % at five years [45–47]. The median survival time for elderly patients receiving the finest supportive care alone is predicted to be less than 4 months [48]. Hypo-fractionated radiotherapy plus chemotherapy has a greater median survival of 7–9 months compared to radiation alone in individuals over 65 who have undergone biopsy or resection. Including adjuvant chemotherapy does not enhance the quality of life in this group, despite evidence of a survival advantage [49].

Since GB is an incurable disease, patients should receive the proper counseling regarding the potential adverse effects of treatments on quality of life and any potential survival benefits. This is especially important for elderly patients and patients with poor performance status, with a particularly poor prognosis [43]. If the tumor is lower-grade glioma, surgery is still a standard treatment for low-grade gliomas, although choices are more complicated than with higher-grade tumors. The choice to perform surgery is simple if a patient has uncontrolled seizures that are undeniably tumor symptoms. However, many asymptomatic low-grade tumors are unintentionally found when imaging is performed for other conditions such as trauma, migraines, or vertigo. The decision to operate may be more difficult if the tumor is asymptomatic or in the eloquent cortex. Clear guidelines on the function of surgery have not yet been established since it is challenging to acquire controlled data in the context of overall extended life and numerous competing demands, such as seizure management and functional preservation. Also, radiotherapy and

chemotherapy are considered for each case [50]. Despite receiving the best possible care from surgery, radiation, and chemotherapy, even the subgroup of oligodendrogliomas with the best prognosis among *IDH*-mutant 1p19q co-deleted oligodendrogliomas will eventually develop into malignant gliomas. Randomized studies have shown that the 5-year overall and progression-free survival rates for diffuse low-grade gliomas range from 58 % to 72 % and 37 % to 55 %, respectively [51,52] .

1.4. Quality of life

Because gliomas are heterogeneous and GB remains an incurable condition, it's critical to enhance patients' quality of life and anticipate their ability to function post-surgery or other selected treatments. The Karnofsky Performance Scale (KPS) was created by Karnofsky and Burchenal to assess performance status and was initially reported in 1949. The scale, which assesses patients according to their activity level and medical needs, was initially developed for patients with systemic malignancies [53]. Patients are scored into categories from 100 (normal with no signs or symptoms of disease) to 0 (dead) see Table 1.4.1 [54]. There are issues with using this scale in patients with malignant brain tumors, even though it has been shown to be generally valid and efficient for cancer patients. Evaluation of preoperative KPS scores and their relationships with results are the foundation of previously published publications that describe KPS scores as predictors of prognosis in patients with gliomas [55–59]. However, individuals with GB are especially susceptible to symptoms like seizures, abrupt changes in mental status, and focal neurologic impairments that may make it difficult to accurately characterize their overall performance status [60]. With surgery and medication control for edema and seizures, many of these symptoms may disappear quickly. Alternately, some GB patients who were previously high-functioning could experience surgical complications that cause significant postoperative impairments and lower their performance status. This contributes to a decline in the overall survival [61,62].

The European Organization for Research and Treatment of Cancer Core (EORTC) has established a questionnaire for the evaluation of the quality of life of cancer patients (QLQ C30) [63]. In this questionnaire, the quality of life is estimated through the physical, psychological, and social functioning of the patient. The 30-item questionnaire includes a global health status scale, three symptom scales (fatigue, pain, and nausea), five functional domains (physical, role, cognitive, emotional, and social), as well as several other single-item assessments. EORTC QLQ C30 is a reliable and valid tool for measuring the impact of glioma and its treatments on patients' physical,

social, and emotional well-being. It can also help to identify symptoms and functional impairments that are important to patients but may not be captured by clinical tests [64].

Table 1.4.1. *Description of Karnofsky Performance Status values*

Karnofsky Performance Status	Description
100	No signs or symptoms of disease
90	Normal activity with few signs of symptoms
80	Normal activity with some difficulty
70	Cannot conduct normal activity but still cares for self
60	Requires occasional help to care for self
50	Requires frequent help to care for self
40	Disabled
30	Severely disabled; hospital admission may be indicated
20	Hospital admission and treatment required
10	Moribund
0	Dead

Several studies have figured out the role of brain miRNAs and psychological disorders, like major depressive disorder, bipolar depression, or schizophrenia [65,66]. For instance, the down-regulation of hsa-miR-34a-5p was associated with unipolar and bipolar depression [67], and hsa-miR-181b-5p has been reported to be up-regulated in schizophrenia patients [68]. Despite these links between miRNA and psychological disorders, there is still a lack of knowledge on the effect on miRNA to the quality of life of glioma patients. In addition, the quality of the survival time may sometimes be more important to glioma patients than the time of survival. Therefore, these factors make postoperative performance status more clinically relevant to the care of a specific patient and potentially more accurate as a prediction of patient prognosis [54].

1.5. MiRNAs biogenesis

MiRNAs are short non-coding RNA molecules in length of 20–23 nucleotides. According to the first and most used miRNA register – miRbase (release 22.1), humans have 2656 unique mature miRNAs [69]. These small molecules are also found in plants, animals, some viruses and are involved in RNA silencing and post-transcriptional regulation of gene expression [70].

In order to function in an RNA silencing manner, miRNAs have to undergo a series of complex biological maturation processes (Fig. 1.5.1) [71]. MiRNA biogenesis begins with transcription of its coding gene by RNA polymerase II, which generates a long, in some cases up to 1000 base pairs in length,

primary miRNA (pri-miRNA) transcript. The pri-miRNA contains a stem-loop structure which is processed into a functional miRNA. Next, still in the nucleus, the pri-miRNA is recognized and cleaved by a complex of proteins known as the Microprocessor, which is composed of the RNase III enzyme Drosha and the cofactor DGCR8. The Microprocessor recognizes the stem-loop structure of the pri-miRNA and cleaves it, releasing a hairpin-shaped precursor miRNA (pre-miRNA) that is about 70-100 nucleotides long. The pre-miRNA is then exported from the nucleus to the cytoplasm by the exportin-5/Ran-GTPase complex. Once in the cytoplasm, the pre-miRNA is cleaved by another RNase III enzyme called Dicer, which removes the loop of the hairpin and generates a short double-stranded RNA molecule that is 20-23 nucleotides long. This double-stranded RNA is composed of two complementary RNA strands, the 5' and 3' ends of which have two-nucleotide overhangs. One of the RNA strands in the double-stranded miRNA precursor is chosen as the guide strand, which is incorporated into the RNA-induced silencing complex (RISC) to target specific mRNA molecules. The choice of the guide strand is determined by the relative thermodynamic stability of the two ends of the double-stranded RNA. However, in some cases, the passenger strand can also be loaded into RISC and function as a miRNA, particularly if it has unique features that allow it to be incorporated into the complex. This phenomenon is known as "passenger strand loading" and has been observed in certain miRNA-miRNA pairs. Nevertheless, it is generally believed that most passenger strands are rapidly degraded by cellular machinery such as the 5'-3' exoribonuclease 2 (XRN2) or the RISC itself. This degradation helps to prevent the accumulation of non-functional or potentially harmful RNA molecules in the cell [72]. Despite that, the guide strand is typically the miRNA strand with the lower free energy at its 5' end. The guide strand of the miRNA-RISC complex binds to complementary sequences in the 3' untranslated region (UTR) of target mRNA molecules, leading to either translational repression or mRNA degradation, depending on the degree of complementarity between the miRNA and its target mRNA. In general, a near-perfect complementary of miRNA and its mRNA target will lead to mRNA degradation initiation, and a more mismatched nucleotide complementary of miRNA-mRNA, will cause the translational inhibition of the targeted mRNA [70,73].

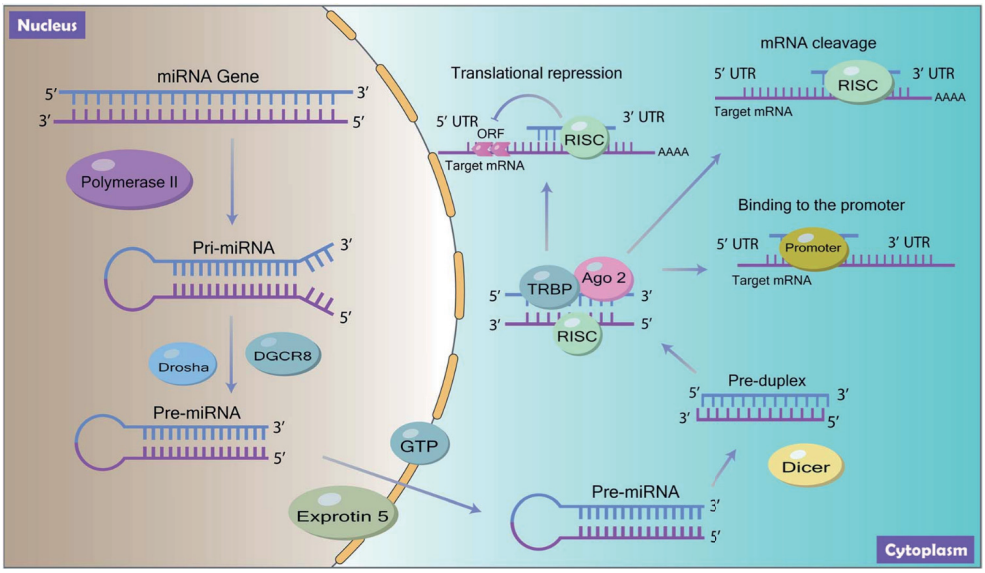

Fig. 1.5.1. *Steps of miRNA biogenesis and major functions*

Drawn by Zixiang Wu, Airong Qian et al. for the publication titled "MicroRNAs and long noncoding RNAs: new regulators in cell fate determination of mesenchymal stem cells". Reproduced from Ref. https://doi.org/10.1039/C9RA06563F with permission from the Royal Society of Chemistry

1.6. MiRNA function

MiRNAs can modulate the expression of genes and are involved in many cellular processes such as development, differentiation, apoptosis, and metabolism. Dysregulation of miRNA expression has been implicated in various diseases, including cancer, cardiovascular disease, and neurological disorders. Besides, miRNAs can be found in a variety of biological fluids, including blood, saliva, and urine, and are increasingly being studied as potential diagnostic and therapeutic targets [74,75]. Their small size and high stability make them attractive candidates for drug development. In addition, miRNAs can exhibit tissue-specific expression patterns, which make them potentially useful as biomarkers for specific tissues or as therapeutic targets for tissue-specific diseases.

Some miRNAs are specifically expressed in specific tissues or cell types, while others are expressed more broadly. For example, in humans, two discrete genes, MicroRNA 1-1 (*MIRN1-1*) and MicroRNA 1-2 (*MIRN1-2*), residing on a genomic region on 18q11.2 produce a single mature miR-1 s [76]. While miR-122 has a liver-enriched expression and is one of the most

abundant miRNAs in the liver, accounting for about 70 % and 50 % of the whole hepatic miRNome in adult mice and humans, respectively [77]. As for the brain, miR-9 is a high brain-enriched miRNA that is involved in a negative feedback loop with nuclear receptor subfamily 2 group E member 1 (*TLX*), a nuclear receptor that controls stem cell proliferation in the developing and adult brain. Furthermore, miR-124 is one of the most abundant miRNAs in the brain as well, and targets repressor element 1 (*RE1*)-silencing transcription factor (REST), which opposes neuronal differentiation. Also, miR-92 targets eomesodermin T-box brain protein 2 (EOMES *TBR2*), a T-box transcription factor that is preferentially expressed in cortical intermediate progenitors and regulates cortical neuron production and expansion, thereby affecting the thickness of the cerebral cortex [78,79].

Many common immune-related diseases, including multiple sclerosis, systemic lupus erythematosus, type I/II diabetes, and nonalcoholic fatty liver disease, have shown established correlations with cellular miRNAs [80]. Furthermore, miRNAs are crucial posttranscriptional regulators of both non-specific (innate) and adaptive immunity. They act by regulating the expression of multiple immune genes, thus, are important elements of the complex immune regulatory network [81]. A panel of miRNAs protects cancer cells from immune clearance by decreasing the immunogenicity of cancer cells and downregulating the magnitude of an anti-cancer immune response [82]. Finally, miRNAs contribute to the progressive changes in gene expression that occur during development. The combined loss of all miRNAs results in embryonic lethality in all animals analyzed, illustrating the crucial role that miRNAs play collectively [83].

1.7. MiRNAs in glioma

Aberrant expression of miRNAs has been linked to the development and progression of this type of brain tumor. Several studies have identified specific miRNAs that are dysregulated in gliomas, and these miRNAs have been shown to regulate key pathways involved in glioma progression, such as cell proliferation, invasion, and angiogenesis [84,85]. For example, miR-21 is commonly upregulated in gliomas and promotes tumor cell proliferation and invasion by targeting multiple tumor suppressor genes [11,86–88]. Other miRNAs that have been implicated in glioma include miR-124, which is downregulated in gliomas and regulates several genes involved in glioma progression [89], and miR-128, which is also downregulated in gliomas and is involved in the regulation of glioma stem cells. MiR-128 blocks glioma self-renewal by down-regulation of Bmi-1 expression and enhance apoptosis in glioma cells via inhibition of Rnd3, which is a member of the Rho family of

small guanosine triphosphate (GTP)-binding proteins and is involved in actin cytoskeletal dynamics, apoptosis, differentiation, and other physiological processes [90]. MiRNA-338-3p inhibits glioma cell proliferation and progression by targeting Myelin transcription factor 1-like (*MYT1L*) [91]. Guessous et al. suggested miR-34a as a therapeutic target for glioblastoma since its overexpression suppresses GB cell proliferation and migration by targeting the growth arrest-specific 1 (*GAS1*) gene, a negative regulator of the sonic hedgehog signaling (SHH) pathway [92]. A set of prognostic miRNAs were also suggested in a study by Simon K. Hermansen et a. where the prognostic value of miR-107, miR-548x, miR-3125, and miR-331-3p was highlighted in GB patients' survival time estimations [93].

From the practical point of view, the most useful discoveries in the field of glioma miRNA expression, is in studies of extracellular vesicles (EV), due to the extreme risk and difficulty of performing multiple biopsies on a brain tumor tissue. EVs are cell-created delivery systems of proteins, lipids, or nucleic acids and means of extracellular communication. Generally, there are three types of EVs: exosomes (50–100 nm), microparticles (200 nm-1 um) and apoptotic bodies. The main function of these vesicles is to deliver bioactive molecules to recipient cells, affecting their biological characteristics, changing the tumor microenvironment, and producing long-distance effects [15,16]. MiRNAs can be packed in these EVs and released into the extracellular space by neurons and microglia to act locally as well as pass through the blood-brain barrier and act systemically [94]. Recent studies show that exosomal miRNA plays an important role in glioma occurrence, development, invasion, metastasis, and treatment resistance [95]. A study by Akers et al. found that exosomal are enriched with various miRNAs in cerebrospinal fluid, making it a potential diagnostic biomarker of glioblastoma [96]. For instance, miR-21-5p has been shown to be upregulated in glioma EVs and may contribute to the progression of gliomas by promoting cell proliferation and inhibiting apoptosis [18].

1.8. Review of miRNAs selected for the study

1.8.1. Housekeeping miRNAs

Housekeeping genes are genes that are constitutively expressed in all cells of an organism and are often used as reference genes for the normalization of gene expression data.

1.8.1.1. hsa-miR191-5p

miRNA191 was considered the most stable miRNA in bone marrow mesenchymal stromal cells and HS27a/HS5 cell lines [97]. It was also shown as the most stable miRNA across 5 different tumor/normal tissue adjacent to the tumor (NAT) tissue pairs (lymphoid, colon, prostate, lung, esophagus) [98]. However, it was found to be upregulated in GB as well as other cancers such as non-small cell lung carcinoma, hepatocellular carcinoma, prostate and gastric cancer [99–103].

1.8.1.2. hsa-miR-361-5p

miRNA361 was found to be stable internal control in breast cancer and the thyroid gland [104,105]. However, in another study, miRNA361 was overexpressed in breast cancer and associated with better patient outcomes [106]. The expression was lower in glioma, cervical cancer, colorectal carcinoma and gastric cancer [107–110]. It was discovered to be a tumor suppressor miRNA in glioma that inhibited cell proliferation and metastasis [111].

1.8.1.3. hsa-miR-345-5p

miRNA345 is considered one of the most stable reference miRNAs when evaluated using two techniques where stability was assessed [112]. However, pancreatic cancer tissues and cell lines have a significant downregulation of miR-345 [113,114], while it was upregulated in oral squamous cell carcinoma, gastric cancer [115,116].

1.8.1.4. hsa-miR-103a-3p

miRNA103a was considered in ovarian carcinoma tissues as a reference normalization gene. However, it was not confirmed to be stably expressed among samples [97,117]. Despite that, it was found to be the best endogenous control in Alzheimer's patients [118]. According to reports, miRNA103a-3p promotes tumor growth in various human malignancies, including colorectal cancer, gastric cancer, and oral squamous cell carcinoma. In non-small cell lung cancer patients, miR103a-3p expression was dramatically downregulated and linked with poor overall survival [119]. In contrast, in colorectal cancer, it was upregulated and associated with poor prognosis [120,121].

1.8.2. Target miRNA

1.8.2.1. hsa-miR-93-5p

miRNA93 was found to be upregulated in the GB [122–127]. Several glioma cell properties, including growth, invasion, migration, cell cycle arrest, and chemoresistance, are regulated by miRNA93 [128]. Multiple studies showed that miRNA93 regulated autophagic activity, influenced the viability of GB cells, tumor growth, and vasculogenesis, and promoted blood vessel formation. In a coculture of human glioblastoma U87 cells and endothelial cells, overexpression of miRNA93 improved vasculogenesis [122,129]. The differential regulation of miRNA93-5p in several malignancies, including hepatocellular carcinoma [130], lung adenocarcinoma [131,132], breast cancer [133], and head and neck squamous cell carcinoma, has recently been identified [134]. Patients with lung adenocarcinoma have been demonstrated to have higher levels of miRNA93-5p expression in their tissues and plasma, which encourages cancer cell growth and migration [135,136].

1.8.2.2. hsa-miR-21-5p

Numerous studies have determined miRNA21 to be an apoptosis regulator, and it has been noted that GB cells have high levels of miRNA21 expression. Cell apoptosis was increased by the miRNA21 knockdown [9]. GB cell lines have shown miRNA21 to have additional oncogenic effects, including proliferation, invasiveness, and chemoresistance [10,12,137]. Additionally, miRNA21 plays a significant role in medication resistance. By suppressing the expression of Bcl-2-associated X protein/B-cell lymphoma protein 2 (Bax/Bcl-2) and caspase-3, high levels of miRNA21 attenuated the effects of temozolomide (TMZ) in U87MG cells [12]. According to previous studies, miRNA21 has an impact on cell invasion, metastasis, and chemotherapeutic resistance [9,10,12,87,88,137–146]. It is also upregulated in many cancer types, such as gastric, colorectal, and pancreatic cancer [147].

1.8.2.3. hsa-miR-221-5p

miRNA221 is an onco-miRNA because it promotes carcinogenesis, invasion, and proliferation [148,149]. When compared to normal brain tissue, it was discovered that the expression of miRNA221 was higher in GB patient tumor tissue [150]. Data from the TCGA database show that miRNA221 expression is associated with shorter survival in glioblastoma patients, and miRNA221 was found to link with glioma grade [151]. It has been thoroughly investigated, and a lot of information is currently available. The potential relevance of miRNA221 as a new diagnostic, prognostic biomarker

and therapeutic target in numerous cancers is supported by all the existing information [152].

1.8.2.4. hsa-miR-17-5p

According to one study, miRNA17 expression is downregulated in glioma cells, and miRNA17 inhibition increases glioma cell viability and migration [153]. miRNA17 targets numerous genes involved in the cell cycle's G1/S transition and functions specifically during this phase. There are reports of its suppressive and oncogenic effects. In glioblastoma, MiRNA17 has a role in controlling the autophagy process. MiRNA17 expression has been demonstrated to be repressed, which promotes autophagy and makes GB cells more susceptible to chemotherapeutic drugs and ionizing radiation [154]. However, other studies found miRNA17 to be overexpressed in glioma tissue than in normal brains and associated with worse patient outcomes [155]. miR17-5p was overexpressed in gastric cancer, ovarian cancer, and hepatocellular cell line [156–158].

1.8.2.5. hsa-miR-143-3p

miRNA143 has been demonstrated to be important in the development of tumors, the proliferation of cancer cells, and the invasiveness, including GB cells [159,160]. MiRNA143 expression appears to be a tissue-specific [160,161]. The colon has the highest levels of miRNA143 expression in normal tissues, whereas the brain and liver have the lowest levels [162]. According to reports, high levels of miRNA143 make cells more susceptible to chemotherapeutic medicines, such as TMZ in the case of GB [163,164]. Other studies, however, have reported a link between high levels of miRNA143 and GB cells' greater ability to invade compared to their parental GB cells, pointing to an oncogenic involvement [159]. Low levels of miRNA143 have been linked to increased tumor growth in colorectal cancer patient tissues and cell lines [165]. Similar outcomes in cases of stomach cancer were found [166]. On the other hand, another study found that miRNA143 expression enhanced tumor growth [167]. One study found that miRNA143 is up-regulated in normal brain tissues compared to GB tissues [163] while others claimed the opposite [168].

1.8.2.6. hsa-miR-335-5p

Numerous malignant cancers have shown miRNA335-5p to be a tumor suppressor. For instance, miRNA335-5p affects a variety of breast cancer gene 1 (*BRCA1*) cascade targets, influencing cell proliferation and apoptosis

in breast cancer [169]. Exosomal miRNA335 was a tumor suppressor that may be used in the hepatocellular cancer treatment [170]. miRNA335 was found to be overexpressed in GB and to stimulate cell invasion and proliferation and by inhibiting miRNA335, these results were reversed [171,172]. Another study reported that miRNA335 functions as a tumor promoter by giving malignant astrocytomas tumorigenic characteristics, including proliferation and invasion in both human malignant astrocytomas and C6 astrocytoma cells [171]. MiRNA335-5p has been found to be expressed at low levels in several human tumors, including epithelial ovarian cancer, colorectal cancer, pancreatic cancer, uterine leiomyoma, gallbladder cancer, and breast cancer [173–179].

1.8.2.7. hsa-miR-193a-5p

According to studies, miRNA193a directly targets oncogenes to decrease proliferation and increase apoptosis [180]. Conversely, overexpression of miRNA193a is associated with glioma patients' survival [181]. Also, in solid tumors, such as non-small-cell lung cancer, miRNA193a controls invasion and migration; in particular, miRNA193 has been discovered to have an anti-invasion property [181]. All normal human tissues have been found to express miRNA193a recently, except specific areas of the central nervous system, such as the nucleus accumbens, hippocampus, and spinal cord, bladder, and endocervix. Interestingly, the highest levels of miRNA193a expression were seen in adipose and mammary tissues [182]. MiRNA193-5p was shown to be overexpressed in myocardial microvascular endothelial cells of diabetic rats. Its downregulation can impact the angiogenic process by promoting cell proliferation and migration, which works directly through the insulin growth factor 2 (*IGF2*) gene [183]. The suppressive role of miRNA193b in various malignancies and tumor cell lines, including glioma, pancreatic cancer, ovarian cancer, and hepatocellular carcinoma [184–187]. On the other hand, some researchers claim that miRNA193b has oncogenic properties and that colorectal and cervical malignancies are associated with its overexpression [188,189]. Also, miRNA193b overexpression has been linked to a poor prognosis for human gliomas [187].

1.8.2.8. hsa-miR-148a-3p

miRNA148a expression among glioblastoma results are inconsistent as it was found to be upregulated [190,191] and downregulated in GB [192]. It was suggested that miRNA148-3p could serve as an independent prognosis predictor as associations with overall patient survival indicated that patients survived longer with high expression of miRNA148-3p [193]. In non-small

lung cancer, miRNA148a-3p was found to inhibit the proliferation and epithelial-mesenchymal transition [194].

1.8.2.9. hsa-miR-139-5p

miRNA139-3p was found to be downregulated in glioma cells and tissues [195]. It was suggested that miRNA139-5p inhibits the development of GB. In glioblastoma, miRNA-139-5p targets specific genes that control the cell cycle to function as a tumor suppressor and inhibit cancer cell migration and invasion [196,197]. It was discovered that miRNA139-5p targets eukaryotic translation initiation factor 4 gamma 2 (*IEIF4G2*). It was previously identified as an oncogene in human malignancies and a miRNA downstream target [198]. It was found to be downregulated in GB and glioma [196,197,199–206]. It was also summarized to be downregulated in the majority of cancers except for Gastrointestinal Stromal tumors and Adrenocortical carcinoma and in some cancers such as colorectal and prostate cancer findings were controversial [207].

1.8.2.10. hsa-miR-34a-5p

miRNA34a is one of the numerous miRNAs that are down-regulated in a number of cancer types, including neuroblastoma, leukemia, pancreatic and hepatocellular carcinomas, glioblastoma, breast, lung, and colon cancer, according to expression profiling [208]. MiRNA34a may operate as a tumor suppressor gene by targeting a variety of oncogenes involved in proliferation, differentiation, growth, apoptosis, and invasion, according to some previous studies [209].

In contrast, some research indicates that it acts as an oncogene that promotes carcinogenesis in uterine malignancies, papillary thyroid carcinoma, and renal cell carcinoma [210–212]. Other types of malignancies have also shown a correlation between miRNA34a expression and tumor growth, for example, bigger prostate and hepatocellular carcinomas are linked to low miRNA34a levels [213,214]. However, other researchers did not find any relationship between miRNA34a expression and glioma size [215].

1.8.2.11. hsa-miR-181b/d-5p

MiRNA181 targets control a number of biological functions, including cell division, growth, and intercellular communication [216]. The importance of miRNA181 in oncology can be observed in several cancer types. Matrix metalloproteinase-1 and vascular endothelial growth factor expression in chondrosarcoma were shown to be downregulated by decreased miRNA181a

expression, which led to decreased tumor malignancy [217]. MiRNA181b has been demonstrated to have onco-suppressor properties in GB cell lines and naïve mouse models, and its high expression is linked to a better result [218]. A small group of primary glioblastoma tissue showed downregulation of miRNA181a, miRNA181b, and miRNA181c, which further suggests miRNA181 family participation in the GB development [138]. In addition to its ability to regulate the chemosensitivity of temozolomide, miRNA181b has been found to enhance cell proliferation, migration, and invasion when its expression is decreased [219,220]. According to other studies, miRNA181b tumoral expression is downregulated in higher-grade gliomas as opposed to lower-grade [221,222]. Another miRNA in the miRNA181 family called miRNA181d has low expression levels and is associated with poor patient survival, indicating that miRNA181d plays a significant role and has the potential to be used as a prognostic marker for GB patients [223]. MiRNA181d targets and inhibits O6-methylguanine-DNA methyltransferase (*MGMT*), which improves the response to the temozolomide treatment [224]. A tumoral miRNA181 expression may help distinguish between distinct subgroups of GB, according to recent research [218,225].

2. METHODS AND MATERIALS

2.1. Samples

The Ethics Committee for Biomedical Research of the Lithuanian University of Health Sciences (BE-2-26) approved the study protocol and consent procedures. Before sample collection, all patients gave informed consent to participate in the research study. Diagnosis of glioma was suspected by an MRI scan and confirmed by a pathologist on post-surgical tissue. Glioma tissues and blood samples were collected at the Hospital of Lithuanian University of Health Sciences Kauno Klinikos, Neurosurgery Clinic, between 2015 and 2019. Collected tissues were snap-frozen in liquid nitrogen (–196 °C) within 1–30 minutes of tumor dissection and kept frozen until RNA extraction. Blood samples were drawn on the day of tumor removal surgery. Within 1 hour of blood collection, the serum was separated from the blood cells by centrifuging the samples for 15 min. at 1300 × g.

2.1.1. Sample groups

This study consisted of 4 major patient groups: 1) non-cancerous, 2) grade II glioma, 3) grade III glioma, and 4) grade IV glioma or glioblastoma (GB) (Table 2.1.1.1). Two samples were commercially purchased as isolated RNA in the non-cancerous patient group. The remaining two samples in this group were collected during surgical treatment for epilepsy. The GB patient group was divided into groups according to the patient's age and analyzed miRNA relative expression. Glioblastoma patients' age ranged from 29.38 to 80.02 years, with an average of 58 years. 39 GB patients who were younger than the average were assigned to a "Younger" group (min. 29.38; max. 57.97; avg. 45.74), and the remaining 43 patients to the "Older" group (min. 58.72; max. 80.02; avg. 69.43). For the GB group, each miRNA value was assigned to one of the terciles: "Low", "Average", and "High" relative expression (Table 2.1.1.2). Samples for the small RNA sequencing experiment (Study I) were randomly selected from an independent glioblastoma and non-cancerous cohort: six blood samples from non-cancerous patients, seven glioblastoma tumor samples, and seven blood samples from the same glioblastoma patients.

Table 2.1.1.1. *Number of glioma patients in each sample group used in Study II and Study III*

	Grade II	**Grade III**	**Glioblastoma**
Total number of patients	15	6	82
Overlapping tissue and blood samples for the same patient	10	5	49
Tissue samples from an archive	5	1	33

Table 2.1.1.2. *Range of relative expression values for each miRNA in a glioblastoma group*

miRNA	**Low -ΔCt (n = 28)**	**Average -ΔCt (n = 27)**	**High -ΔCt (n = 27)**
hsa-miR-143-3p	(–9.536, 0.0806)	(0.0806, 1.176)	(1.176, 3.904)
hsa-miR-193a-5p	(–6.454, –4.458)	(–4.458, –3.158)	(–3.158, 0.696)
hsa-miR-139-5p	(–18.962, –5.897)	(–5.897, –4.06)	(–4.06, –0.665)
hsa-miR-7-5p	(–9.536, –3.263) n = 25	(–3.263, –1.962) n = 24	(–1.962, 7.883) n = 24
hsa-miR-34a-5p	(–18.762, 1.348)	(1.348, 2.186)	(2.186, 5.534)
hsa-miR-93-5p	(–9.536, 1.159)	(1.159, 1.886)	(1.886, 6.28)
hsa-miR-181b-5p	(–0.832, 1.698)	(1.698, 2.524)	(2.524, 4.985)
hsa-miR-181d-5p	(–9.536, 0.00134)	(0.00134, 0.828)	(0.828, 2.988)
hsa-miR-221-5p	(–8.78, –4.135)	(–4.135, –3.233)	(–3.233, 0.825)
hsa-miR-17-5p	(–0.0852, 1.276)	(1.276, 1.976)	(1.976, 6.847)
hsa-miR-335-5p	(–9.536, 1.034)	(1.034, 1.844)	(1.844, 4.932)
hsa-miR-21-5p	(0.0858, 3.667)	(3.667, 4.854)	(4.854, 8.304)
hsa-miR-148a-3p	(6.340, –1.867)	(–1.867, –0.643)	(–0.643, 2.267)
hsa-miR-10b-3p	(–10.946, –7.12) n = 21	(–7.12, –5.709) n = 20	(–5.709, 4.967) n = 21

"-ΔCt" means a relative expression of a miRNA. It is calculated as follows, -(cycle threshold value of a target miRNA – cycle threshold value of a geometric mean of all reference miRNAs). One would get the same values if a $\log_2^{FoldChange}$ formula were used.

2.2. MiRNA analysis

2.2.1. MiRNA quantification

In total, 20–40 mg of frozen, post-surgical tumor samples were mechanically grinded and homogenized with ultrasonication at 20 % amplitude for a 1-second on/off pulsation. MiRNAs were isolated from the homogenized tissue using a mirVana miRNA isolation kit (cat. #: AM1560, Invitrogen) by separating the small RNA fraction (less than 150 nt) from

the rest of the RNA. From the serum samples, small RNAs encapsulated in the EV were extracted using an exoEasy kit (cat. #: 76064, Qiagen). The universal copy-deoxyribonucleic acid (cDNA) library of the isolated small RNAs was synthesized by the TaqMan Advanced miRNA cDNA Synthesis Kit (cat. #: A28007, Applied Biosystems) from 10 ng of RNA. Pre-amplified and ten times diluted cDNA was used for the expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method using TaqMan Advanced miRNA Assays (Applied Biosystems). 4 endogenous miRNAs were used for data normalization: hsa-miR-191-5p (Assay ID: 477952_mir), hsa-miR-361-5p (Assay ID: 478056_mir), hsa-miR-345-5p (Assay ID: 478366_mir), and hsa-miR-103a-3p (Assay ID: 478253_mir). All protocols were followed according to the manufacturer's recommendations, except for the RT-qPCR reaction, which was carried out by proportionally scaling down the recommended final reaction volume of 20 µL to 12 µL. At the RNA isolation and cDNA synthesis stages, 0.0065 ng of synthetic spike-in (cel-miR-39-3p) was added to control for technical errors.

For each sample, the relative quantitation of miRNA was calculated according to the Equation.2 (1):

$$\Delta Ct_{target\ miR} = Ct_{target\ miR} - \sqrt[4]{Ct_{miR191} \times Ct_{miR361} \times Ct_{miR345} \times Ct_{miR103a}}$$

An additional normalization step was applied to serum extracellular samples normalizing to spike-in cel-miR-39-3p levels according to the Equation (2):

$$-(\Delta Ct_{target\ miR} = \Delta Ct_{target\ miR} \times \left(\frac{\overline{Ct_{miR39}}}{Ct_{miR39}} \right))$$

2.2.2. MiRNA sequencing

RNA isolation was done from frozen glioblastoma tumor tissue and extracellular vesicles of the blood as described in the Methods section "2.2.1 MiRNA quantification". Up to 100 ng of small (less than 150 nt) or extracellular RNAs were used for Small RNA sequencing. The libraries were constructed using CATS Small RNA-seq Kit (cat. #: C05010040, Diagenode) and sequenced on the MiSeq sequencer (cat. #: SY-410-1003, Illumina) applying MiSeq Reagent kit v2 (cat. #: MS-102-2001, Illumina) to obtain 50 base-pair reads. Sequencing data were analyzed on a GenomeDK computational cluster by running the nf-core/smrnaseq pipeline[1]. In short, 1) the quality of the raw sequencing reads was evaluated by FastQC software[2], 2) library construction and sequencing adapters were trimmed with the Trim Galore

[1] https://nf-co.re/smrnaseq

[2] https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

tool[3], 3) the most common contaminants were filtered out using Bowtie2[4], 4) pre-processed reads aligned to the mature reference miRnome from miRbase[5], 5) analysis of the detected human miRNA counts by PyDESeq2 software[6]. Additional analysis was carried out by applying data processing and differential expression web-based tools Oasis2[7] and DEApp[8].

2.3. Neuropsychological evaluation

Neuropsychological assessment of glioblastoma patients was performed by a medical psychologist, dr. Aistė Pranckevičienė at the Hospital of Lithuanian University of Health Sciences Kauno Klinikos, Neurosurgery Clinic, approximately three days before the tumor removal surgery. The patient exclusion criteria included severe cognitive deficits and/or neurological impairment leading to the inability to complete all study tasks. The neuropsychological evaluation was done by analyzing the health-related quality of life (HRQOL) and depression questionnaires of each patient. Both questionnaires were previously validated for HRQOL assessment in Lithuanian brain tumor patients [226]. These questionnaires comprised The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire QLQ-C30 [63] and QLQ-BN20 questionnaires [227]. The answers to the QLQ-C30 questionnaire reflect the status of global health and various abilities: social, cognitive, or emotional functioning. QLQ-BN20 questionnaire supplements the QLQ-C30 for brain cancer patients. Its task is to address the symptoms related to brain tumors, for example, motor dysfunction, visual impairments, or difficulties projecting on the future. In addition, KPS [228] was used to assess functional status. The KPS is an 11-point rating scale designed to measure a patient's ability to carry out his/her usual activities and dependence on help and nursing care.

2.4. Statistical analysis

The evaluation of patients' post-treatment survival time was done by Kaplan–Meier estimation, using a log-rank test and Cox's proportional hazard analysis. The Student's T-test was applied to evaluate the expression differences between two groups, after the F-test was performed to determine the variance between two groups. Spearman correlation was used to

[3] https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
[4] https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
[5] https://www.mirbase.org/ftp.shtml
[6] https://github.com/owkin/PyDESeq2
[7] https://oasis.ims.bio
[8] https://yanli.shinyapps.io/DEApp/

evaluate relationships between miRNA expression and neuropsychological results, while Pearson's correlation was used for the correlations of miRNA expression and other numerical variables: age, post-surgical survival time, tumor volume, tumoral/extracellular miRNA expression. Logistic and linear regression models were constructed to evaluate the diagnostic and predictive properties of analyzed miRNAs. All statistical tests were done in Python applying Lifelines[9], Statsmodels[10], and Sklearn[11] modules. Decision tree classification analysis for glioma survival prediction, evaluating miR-181 expression and other factors, was performed using the classification and regression trees (CRT) algorithm with Gini method nonlinear combinations. Classifying glioma survival from extracellular vesicle data, the classifier test size was 60 % of the dataset, the maximum tree depth was set to 5, and the Entropy evaluation method was applied. For the tumoral miRNA expression data, the test size was 20 % of the dataset, the maximum depth was 3, and the Entropy evaluation method was used. The same parameters were used for glioma grade classification using the tumoral miRNA dataset. Decision trees were visualized with the DtreeViz[12] software. The significance levels in all statistical tests were defined as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

[9] https://lifelines.readthedocs.io/en/latest/

[10] https://www.statsmodels.org/stable/index.html

[11] https://scikit-learn.org/stable/

[12] https://github.com/parrt/dtreeviz

3. RESULTS

3.1. Study I

3.1.1. Target selection by small RNA sequencing

Three small sample groups were sequenced to investigate the most promising diagnostic miRNAs. Small-RNA sequencing was done from 7 glioblastoma serums, the same patients' post-surgical GB tissue, and 6 non-cancerous serums. The highest number of unique miRNAs were detected in GB tissue, while the EVs of GB and non-cancerous patients' unique miRNA count was similar. In total, only 59 miRNAs overlapped between all three groups of samples when miRNA was detected in at least 6 out of 7 biological replicates (Fig. 3.1.1.1). Sequencing results highlighted three miRNAs as a candidate for a minimally invasive glioblastoma diagnostic test. Comparing non-cancerous blood samples with miRNA expression from GB serum EVs, miR-126-5p, miR-338-3p, and miR-338-5p were up-regulated in GB patients' blood ($\log_2^{FoldChange} = 1.500$; 2.178; 1.936, FDR adjusted $p = 0.020$; 0.002; 0.003, respectively). In addition, these miRNAs were similarly expressed in both GB patients' post-surgical tissue and the same patients' serum EVs ($\log_2^{FoldChange} = -0.102$; 0.102; 0.154, FDR adjusted $p = 0.857$; 0.861; 0.749, respectively). Significant expression differences between EVs of GB patients versus EVs from non-cancerous patients meant that the expression of miR-126-5p and miR-338-3p/5p had the potential to indicate the occurrence of glioblastoma. Furthermore, a non-significant difference in the expression of these miRNAs between GB tissue and GB EVs suggested that miR-126-5p and miR-338-3p/5p are produced mainly as signaling molecules of the cancer cells (Fig. 3.1.1.2–3.1.1.3).

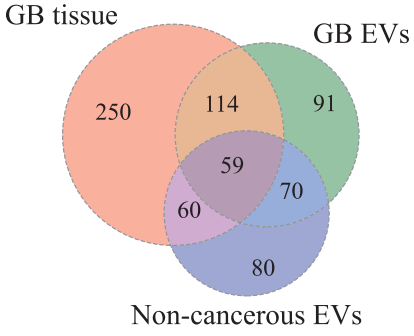

Fig. 3.1.1.1. *Venn diagram of an overlapping unique miRNAs*

Unique miRNA was considered if it was detected in at least 6 biological replicates of the group.

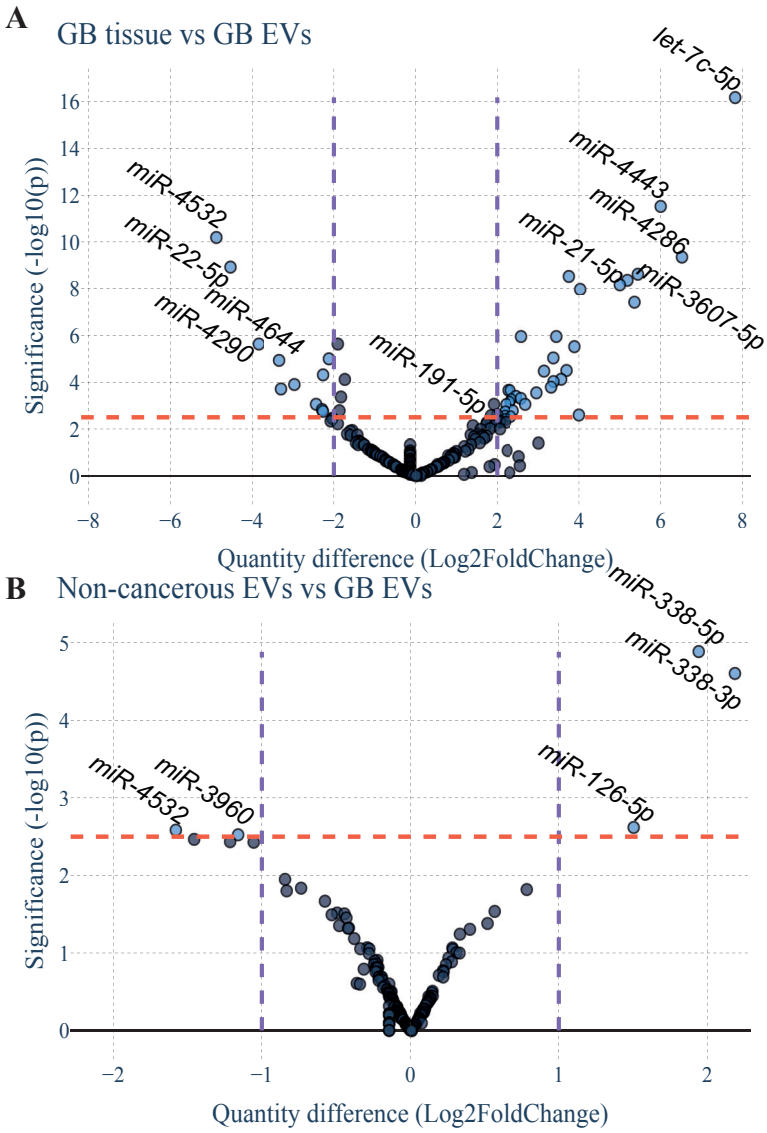

Fig. 3.1.1.2. *Volcano plot of differentially expressed miRNAs between different sample groups*

Purple vertical line represents the threshold of the expression difference. Orange horizontal line shows the significance level of alpha 0.05. Blue dots are significantly differentially expressed miRNAs between two groups. A) compares miRNAs from glioblastoma (GB) tissue and extracellular vesicles of the blood (EVs), and B) compares miRNAs in EVs of GB and non-cancerous patients.

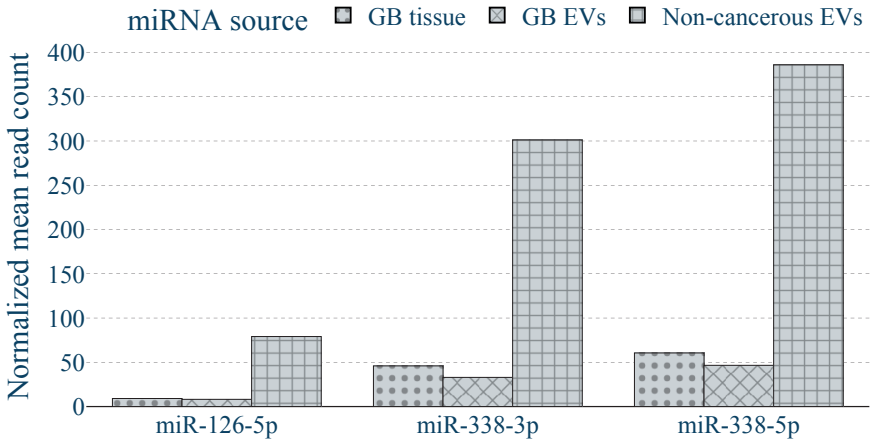

Fig. 3.1.1.3. *Selected miRNAs based on small RNA-seq results*

First column in each miRNA group represents miRNA counts from glioblastoma tissue. Second column – extracellular vesicles of glioblastoma patients. Third column – extracellular vesicles from non-cancer patients.

To strengthen the confidence in the diagnostic properties of miR-126-5p and miR-338-3p/5p, their expression was additionally measured by qPCR method in an independent cohort of GB patients (n = 39) (Fig. 3.1.1.4). Only the expression of miR-338-5p was in par with the small RNA sequencing results. Expression of miR-126-5p was significantly higher in GB's EVs (1.42 -ΔCt) compared to GB's tissue (–2.0 -ΔCt) ($p < 0.001$). Whereas miR-338-3p was up-regulated in GB's tissue rather than in EVs (–1.67 vs. –3.87 (-ΔCt); $p = 0.010$). Relative expression of miR-338-5p in the glioblastoma tissue group was –6.35 (-ΔCt), and in the EVs of the same patient's serum, the expression was not significantly lower than –7.58 (-ΔCt) ($p = 0.161$). The non-significant difference in miR-338-5p expression remained if a paired Student's T-test was performed ($p = 0.128$). The qPCR validation confirmed only that miR-338-5p expression from patients' GB tissue is reflected in the patients' bloodstream, which makes it a perspective miRNA for early diagnosis or monitoring treatment efficiency.

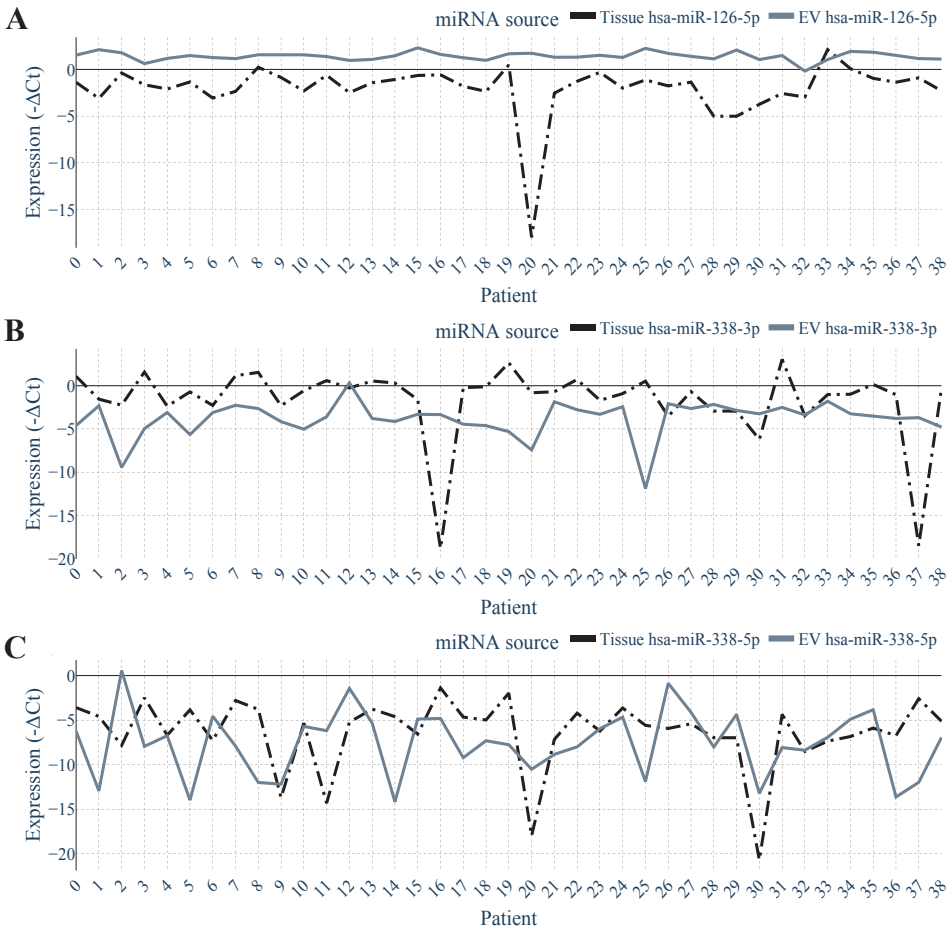

Fig. 3.1.1.4. *The reflection of tumoral miRNA expression in glioblastoma patient's extracellular vesicles of blood serum*

RT-qPCR validation results of the selected miRNAs from small RNA-seq results. A) miR-126-5p, B) miR-338-3p, C) miR-338-5p. Continues grey line shows miRNA expression in glioblastoma (GB) patient's extracellular vesicles (EVs), while the doted black line shows expression in glioblastoma tissue. Each value on the x-axis indicates individual patient whom miRNA expression was measured in blood's extracellular vesicles and tumor tissue. Similar expression in patient's EVs and GB tissue indicate that miRNA is mostly exported from the GB tissue.

However, the selected small RNA sequencing strategy of a low sequencing depth but higher replicate count resulted in a low number of detected miRNAs in the final sequencing results. The detection of the unique miRNAs ranged from 239 to 1088, with an average of 472. Therefore, further target selection was continued from the analysis of the Literature.

3.2. Study II

3.2.1. miRNA expression in brain tissue

A total of 18 miRNAs were selected for this study to analyze by the RT-qPCR method. Four reference miRNAs were selected combining the literature review and a database analysis performed by Dovydas Kičiatovas. The database analysis highlighted miR-191, miR-361, and miR-345 as mid to high-expressed miRNAs in the glioblastoma tissue without significant expression changes among different glioblastoma subtypes – a complete list of analyzed miRNAs listed in Table 3.2.1.1.

Table 3.2.1.1. *List of analyzed miRNAs*

4 reference miRNAs[13]	10 onco-miRNAs	4 onco-suppressive miRNAs
hsa-miR-191-5p	hsa-miR-34a-5p	hsa-miR-143-3p
hsa-miR-361-5p	hsa-miR-93-5p	hsa-miR-193a-5p
hsa-miR-345-5p	hsa-miR-181b-5p	hsa-miR-139-5p
hsa-miR-103a-3p	hsa-miR-181d-5p	hsa-miR-7-5p
	hsa-miR-221-5p	
	hsa-miR-17-5p	
	hsa-miR-335-5p	
	hsa-miR-21-5p	
	hsa-miR-148a-3p	
	hsa-miR-10b-3p	

Expression of miRNAs was analyzed in non-cancerous (n = 4), grade II / grade III glioma – low-grade glioma (LGG) (n = 15), and grade 4 glioma – glioblastoma (GB) (n = 82) brain tissue samples. Overall, the mean relative expression (-ΔCt) of all the analyzed miRNAs in the non-cancerous, LGG, and GB brain tissue samples were –1.12; –1.37; and –0.65 (vΔCt). The mean of the potential onco-miRNAs alone was –1.45; –1.10; and 0.25 (-ΔCt). Potential onco-suppressor-miRNAs resulted in a mean of –0.30; –2.04; and –2.90 (-ΔCt), respectively.

On the individual miRNA level, significant expression changes between all sample groups were detected in hsa-miR-21-5p and hsa-miR-10b-3p (Table 3.2.1.2). The relative expression of an oncogenic miR-21-5p and miR-10b-3p positively correlated with a higher malignancy glioma occurrence. The average expression of miR-21-5p and miR-10b-3p in non-cancerous, LGG and GB brain tissue was 0.08; 1.37; 4.06 and –12.57; –10.55; –6.31 (-ΔCt), respectively. Other miRNAs were also significantly differentially

[13] Used for the calculations of miRNA's relative expression (-ΔCt)

expressed in different tissue types but not between all of them. Arguably, the most important expression changes are between non-cancerous and LGG tissue groups since it indicates the early stages of the cancerous processes. Comparing these two groups, significant changes were detected in both onco-suppressive and oncogenic miRNAs, out of which the onco-suppressive miR-7-5p had the most noticeable expression shift in a non-cancerous group (1.79 -ΔCt), and LGG group (–1.56 -ΔCt). Comparing these two groups, less significant expression changes occurred in miR-143-3p, miR-21-5p, and miR-10b-3p. Most changes in miRNA expression were detected between LGG and GB tissue groups, where 9 out of 14 miRNAs were significantly differentially expressed (Table 3.2.1.2) Out of which, the expression of an oncogenic miR-221-5p was detected almost the same in non-cancerous and GB groups, but it was significantly lower in the LGG group (–4.87 -ΔCt), when compared to the GB group (–3.68 -ΔCt) ($p = 0.005$).

Table 3.2.1.2. *Average miRNA expression in different types of brain tissue*

		Relative expression (-ΔCt)			T-test significance		
miRNA type	**miRNA**	**Non-cancerous**	**LGG**	**GB**	**Non-cancerous *vs* LGG**	**LGG *vs* GB**	**Non-cancerous *vs* GB**
Onco-suppressive	hsa-miR-143-3p	1.92	0.43	0.39	**0.033***	0.934	0.079
	hsa-miR-193a-5p	–4.59	–5.21	–3.62	0.267	**0.000*****	0.209
	hsa-miR-139-5p	–0.33	–1.83	–5.90	0.076	**0.000*****	**0.005****
	hsa-miR-7-5p	1.79	–1.56	–2.42	**0.000*****	0.110	**0.000*****
Oncogenic	hsa-miR-34a-5p	0.13	0.21	1.02	0.909	0.114	0.655
	hsa-miR-93-5p	0.01	0.87	1.61	0.090	**0.011***	0.072
	hsa-miR-181b-5p	2.41	2.51	2.05	0.801	**0.021***	0.532
	hsa-miR-181d-5p	0.47	0.91	0.36	0.348	**0.026***	0.891
	hsa-miR-221-5p	–3.20	–4.87	–3.68	0.056	**0.005****	0.572
	hsa-miR-17-5p	0.63	1.32	1.80	0.155	0.059	0.086
	hsa-miR-335-5p	0.77	0.72	1.24	0.933	0.094	0.609
	hsa-miR-21-5p	0.08	1.37	4.06	**0.015***	**0.000*****	**0.000*****
	hsa-miR-148a-3p	–3.27	–3.54	–1.28	0.584	**0.000*****	**0.000*****
	hsa-miR-10b-3p	–12.57	–10.55	–6.31	**0.020***	**0.000*****	**0.000*****

"-ΔCt" means a relative expression of a miRNA. It is calculated as follows, -(cycle threshold value of a target miRNA – cycle threshold value of a geometric mean of all reference miRNAs). One would get the same values if a $\log_2^{FoldChange}$ formula were used. Two-tailed Student's t-Test considering group variance calculated by the F-test. * 1 % to 5 % possibility that the patterns in the data occurred by chance ($p > 0.05$). ** 0.1 % to 1 % possibility that the patterns in the data occurred by chance ($p > 0.01$). *** less than 0.1 % possibility that the patterns in the data occurred by chance ($p > 0.001$)

Enabling the logistic regression model, it was possible to accurately classify patients' tissue samples into LGG and GB glioma groups, depending on analyzed miRNA expression. When all miRNAs were taken together, the model resulted in a 90 % accuracy and an f1-score of 0.78 and 0.94 for LGG and GB, respectively. Minimizing the number of predictors to only five most promising miRNAs, which, on an individual basis, were the most significantly differentially expressed between LGG and GB groups, the logistic regression model still retained the same accuracy and f1-score values. The most increase in odds of being diagnosed with glioblastoma was assigned to an oncogenic miR-10b-3p. The model predicted that an increase of a single integer of miR-10b-3p relative expression (-ΔCt) increased the odds of a GB diagnosis by 148 %. In this model, the decrease of an onco-suppressive miR-139-5p was associated with a less malignant diagnosis. With every one integer increase in miR-139-5p expression, the odds of being diagnosed with grade IV glioma was predicted to decrease by 26 %. Other miRNAs considered in this model increased the odds of the GB outcome by 26 % (miR-148a-3p), 46 % (miR-193a-5p), and 57 % (miR-21-5p).

The decision tree classifier algorithm suggested a similar miRNA set. This classifier also selected tumoral miR-10b-3p as the most important factor for glioma grade selection. Higher miR-10b-3p expression (more than –9.11 -ΔCt) was suggested as a primary separator of a lower-grade (grade II and III) and higher-grade (grade IV) glioma. Differently from the logistic regression model, the decision tree indicated the increase of a tumoral miR-139-5p expression as a lower-grade glioma factor. In particular, miR-139-5p expression higher than –2.03 (-ΔCt) suggested a lower-grade glioma diagnosis. The accuracy of this classifier reached 94.11 % with a weighted precision, recall, and f1-score of 98 %, 94 %, and 97 %, respectively (Fig. 3.2.1.1).

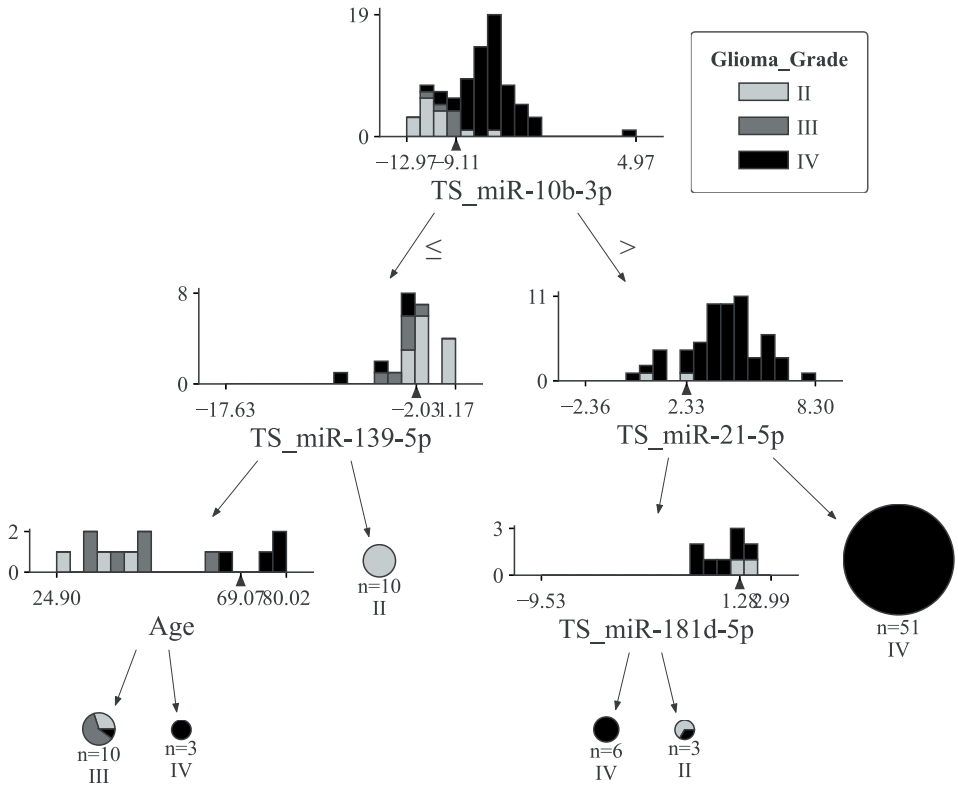

Fig. 3.2.1.1. *Decision tree classifier illustration for predicting glioma patients' survival time considering all analyzed tumoral miRNAs*

The black triangle indicates the dataset separation point of the classifier. Age presented in years, "TS" means tumoral sample.

To exclude an age group as a significant effector to the analyzed miRNA expression, both logistic regression and Support Vector Machines (SVM) methods were performed to predict the patient's age group based on a set of miRNA expression. Various combinations of miRNAs were tested: 1) all 14 analyzed miRNAs, 2) 10 oncogenic miRNAs, and 3) 4 onco-suppressive miRNAs. None of the miRNA sets were able to generate a meaningful classification model for the patient's age group since the accuracy, f1-score and the Area Under the Curve (AUC) of these models were consistently below 63 %. In addition, a linear regression model was constructed trying to predict patients' age by the expression of a miRNA set. None of the constructed linear regression models were able to explain more than 55 % of the age data (r-squared = 0.55).

Comparing miRNA expression with different stages of glioma, some miRNA's expression showed a tendency to be additionally related to the patient's age (Fig. 3.2.1.2). Expression of an onco-suppressive miR-7-5p tended to decrease less, during the increase of glioma malignancy, in older patients rather than in younger ones (Fig. 3.2.1.2. A). An opposite trend appeared in an oncogenic miR-10b-3p in which expression increased more in younger patients than in older patients (Fig. 3.2.1.2. B). Although not significant, a constantly higher levels of an oncogenic miR-17-5p were detected in younger patients within all brain tissue groups. Overall, the expression of an onco-suppressive miR-139-5p did not differ significantly between non-cancerous and LGG groups. However, when these groups were divided into age groups, it was revealed that in younger patients, the expression of miR-139-5p tended to sharply decrease in the lowest grade of glioma (Grade II) versus non-cancerous tissue group but was higher in the most malignant type of glioma (Grade IV) (Fig. 3.2.1.2. E).

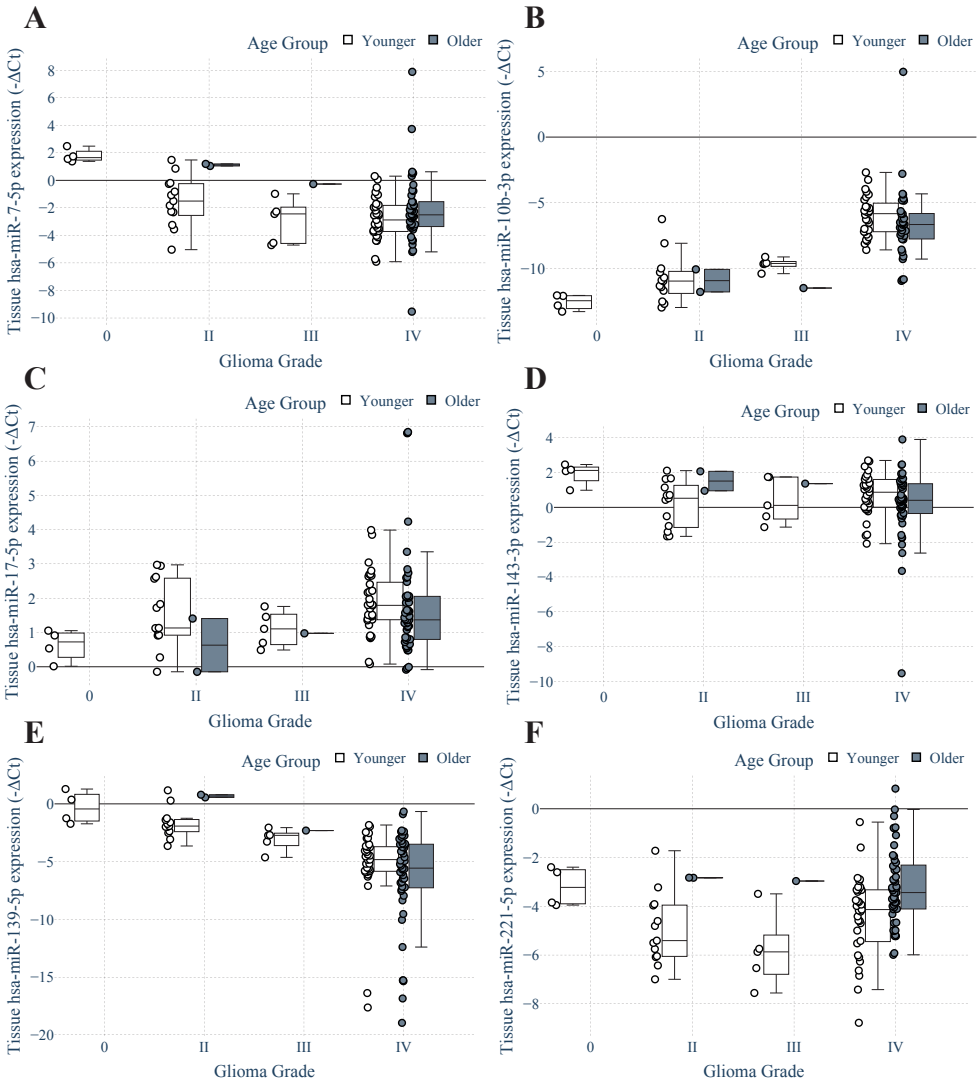

Fig. 3.2.1.2. *Tumoral miRNA expression in different grades of glioma, separated by age groups*

Relative expression of A) miR-7-5p, B) miR-10b-3p, C) miR-17-5p, D) miR-143-3p, E) miR-139-5p, F) miR-221-5p in different grades of post-surgical glioma tissue. The borders of the boxes illustrate the Q_1 and Q_3 values, The middle line in the box shows the median, and the end of the whiskers represents a maximum/minimum value before the upper/lower fence ($Q_{3/1} \pm 1.5$*Interquartile range (Q_3-Q_1)). The outliers are presented as dot outside the upper/lower fence.

Similarly, an onco-suppressive miR-143-3p expression difference among glioma grades was seen when comparing younger versus older patients.

Interestingly, the shift of an onco-suppressive miR-193a-5p expression differed depending on the patient's age. In younger patients, levels of miR-193a-5p reduced during glioma progression and showed a slight increase in the glioblastoma group. At the same time, older patients tend to have an increased expression of miR-193a-5p in every glioma grade. When comparing miRNA expression between younger and older patient groups, no noticeable expression changes were detected in miR-148a-3p, miR-181b/d-5p, miR-221-5p, and miR-335-5p.

In addition, younger GB patients with mutated *IDH1* gene had a significantly lower expression of miR-21-5p, compared to GB *IDH1* wild-type younger patients: median expression of 4.14 and 1.1 (-ΔCt), respectively ($p = 0.021$) (Fig. 3.2.1.3. A). Similarly, miR-148a-3p was less expressed in younger GB patients with mutated *IDH1* (-ΔCt median of -3.35) rather than a wild-type *IDH1* patients (-ΔCt median of -1.00) ($p = 0.009$). Although not significant, miR-34a-5p also tended to be lower expressed in younger *IDH* mutated GB patients (-ΔCt median of -0.13), compared to younger *IDH* wild-type GB patients (-ΔCt median of 2.03) (Fig. 3.2.1.3. C). No noticeable changes were detected in all other analyzed miRNAs when comparing their expression between an *IDH1* wild-type and mutated GB tissue samples.

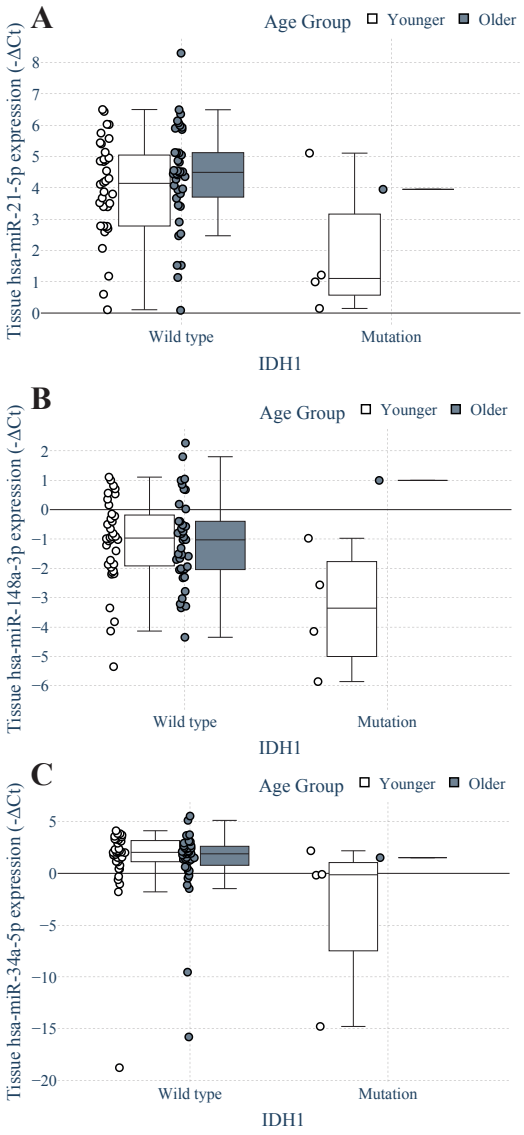

Fig. 3.2.1.3. *Tumoral miRNA expression between IDH1 gene genotypes of glioblastoma patients, separated by age groups*

Relative expression of A) miR-21-5p, B) miR-148a-3p, C) miR-34-5p in post-surgical glioblastoma tissue. The borders of the boxes illustrate the Q1 and Q3 values, The middle line in the box shows the median, and the end of the whiskers represents a maximum/minimum value before the upper/lower fence (Q3/1 ± 1.5*Interquartile range (Q3-Q1)). The outliers are presented as dot outside the upper/lower fence

Comparing miRNA expression changes in consideration of *MGMT* promoter methylation status, the expression of miR-193a-5p in younger GB

patients was significantly lower compared to older GB patients, both in *MGMT* promoter methylated (p = 0.047) and unmethylated (p = 0.021) groups (Fig. 3.2.1.4. A). Whereas miR-221-5p was upregulated only in older patients with a methylated *MGMT* promoter, compared to analogous younger GB patients (p = 0.012) (Fig. 3.2.1.4. B). The expression of other analyzed miRNAs did not differ significantly in different *MGMT* promoter methylation groups.

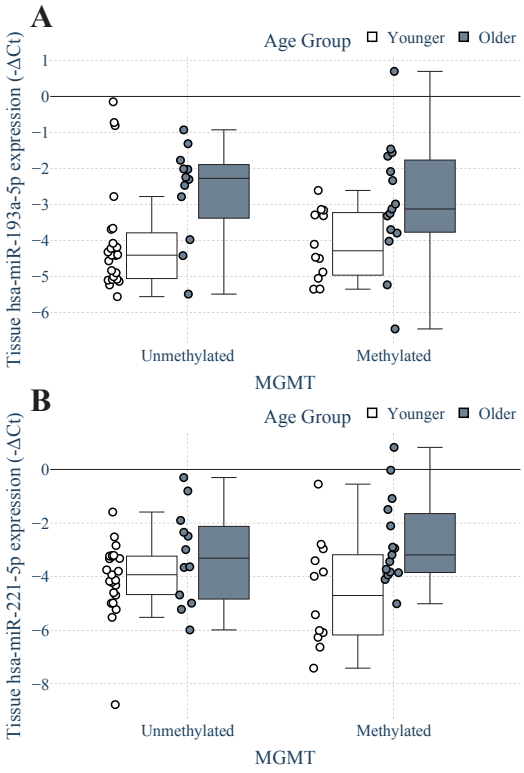

Fig 3.2.1.4. *Tumoral miRNA expression in glioblastoma patients with different MGMT promoter methylation status, separated by age groups*

Relative expression of A) miR-193a-5p, and B) miR-221-5p in a post-surgical tissue of glioblastoma patients. The borders of the boxes illustrate the Q1 and Q3 values, The middle line in the box shows the median, and the end of the whiskers represents a maximum/minimum value before the upper/lower fence (Q3/1 ± 1.5*Interquartile range (Q3-Q1)). The outliers are presented as dot outside the upper/lower fence

3.2.2. miRNA tumoral expression and glioblastoma patients' Health Related Quality of Life

Higher levels of miR-34a-5p and miR-181d-5p expression in tumor tissue were found to be associated with greater physical functioning ($p < 0.05$ for both miRNAs), and an increase of miR-181b-5p correlated with patients' better social functioning ($p < 0.05$). In contrast, the overall functioning of GB patients has only correlated with the expression of tumoral miR-34a-5p (Table 3.2.2.1). Although not significant, a tendency for improving in social and emotional functioning was observed with increasing expression of miR-34a-5p and miR-181b-5p, respectively. In addition, glioblastoma patients' performance, according to KPS, positively correlated with miR-34a-5p expression ($p < 0.05$).

Table 3.2.2.1. *Relationship between health-related quality of life indicators, clinical evaluation of patient's functioning, and miRNA expression in glioblastoma patients' tumor tissue*

Criteria	miR-34a-5p	miR-181b-5p	miR-181d-5p
	rho	rho	rho
Global evaluation of health	−0.05	−0.02	−0.03
Physical functioning	**0.30***	0.23	**0.29***
Role functioning	0.05	0.18	0.07
Emotional functioning	0.14	0.24	0.10
Cognitive functioning	0.11	0.07	−0.07
Social functioning	0.26	**0.32***	0.13
EORTC QLQ C30 Total Score	**0.31***	0.19	0.06
KPS at time of admission	**0.36***	0.09	−0.04

Higher scores represent better functioning and better functional outcomes after the surgical treatment. Spearman correlation coefficient shown under "rho" columns.

3.2.3. Glioblastoma volume and tumoral miRNA expression

A significant correlation was detected between glioblastoma volume and miR-21-5p expression (Pearson's $r = -0.52$, $p < 0.01$). Virtually equally, both for males and females, GB patients with a higher tumor volume had a lower expression of miR-21-5p (Fig. 3.2.3.1. A). As for tumoral miR-148a-3p, its expression negatively correlated with the tumor volume more significantly in the male subgroup of GB patients (Pearson's $r = –0.68$, $p < 0.05$). Despite the sex of GB patients, miR-148a-3p also was significantly correlated with tumor volume (Pearson's $r = –0.51$, $p < 0.01$) (Fig. 3.2.3.1. B). A positive correlation was observed with miR-181d-5p in all glioblastoma samples and a male subgroup (Pearson's $r = 0.43$; 0.83, $p < 0.05$; < 0.01, respectively)

(Fig. 3.2.3.1. C). The weakest association between GB volume and miRNA expression was detected with miR-93-5p (Pearson's r = 0.09, p = 0.65) (Fig. 3.2.3.1. D)

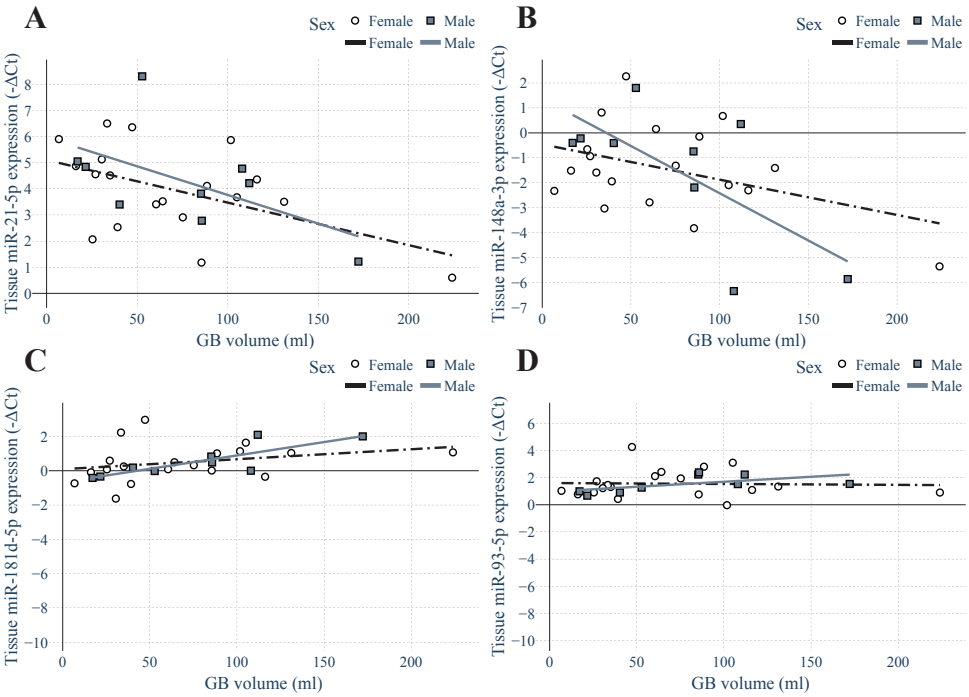

Fig. 3.2.3.1. *Correlation between tumoral miRNA expression and glioblastoma (GB) volume*

A) miR-21-5p; B) miR-148a-3p; C) miR-181d-5p; D) miR-93-5p

3.3. Study III

3.3.1. miRNA expression in extracellular vesicles

Weak to moderate correlations were observed within miRNAs from extracellular vesicles and between tumoral and extracellular miRNAs (Fig. 3.3.1.1). Strongest correlations were between tumoral miR-93-5p and tumoral miR-181d-5p; miR-17-5p; miR-335-5p (Pearson's r = 0.76, 0.73, 0.69; p < 0.001, respectively). Only weak correlations were detected between tumoral and extracellular miRNAs. The highest positive correlation was between tumoral miR-148a-3p and extracellular miR-34a-5p (r = 0.40, p < 0.01), and the highest negative correlation was observed between tumoral miR-139-5p and extracellular miR-7-5p (r = –0.40, p < 0.01).

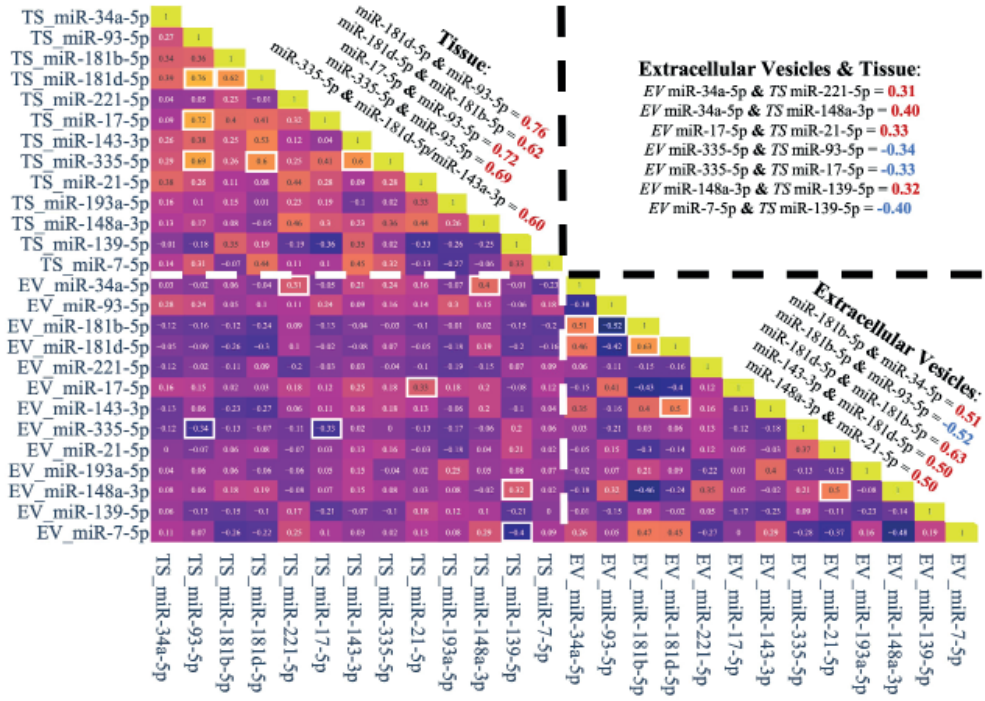

Fig. 3.3.1.1*. miRNA correlation heatmap in glioma*

Pearson's r correlations between tumoral (upper right square), extracellular (lower left square), and tumoral-extracellular (lower right square) miRNAs in glioma. Highlighted correlations are the highest correlations within the quarter.

In the miRNA expression evaluation of the same patients' tumoral and extracellular miRNAs, the most similar expression was detected in miR-17-5p, miR-93-5p, and miR-193a-5p (Fig. 3.3.1.2). Indicating the prognostic potential of these miRNAs as an accurate biomarker for the monitoring of glioma therapy effect.

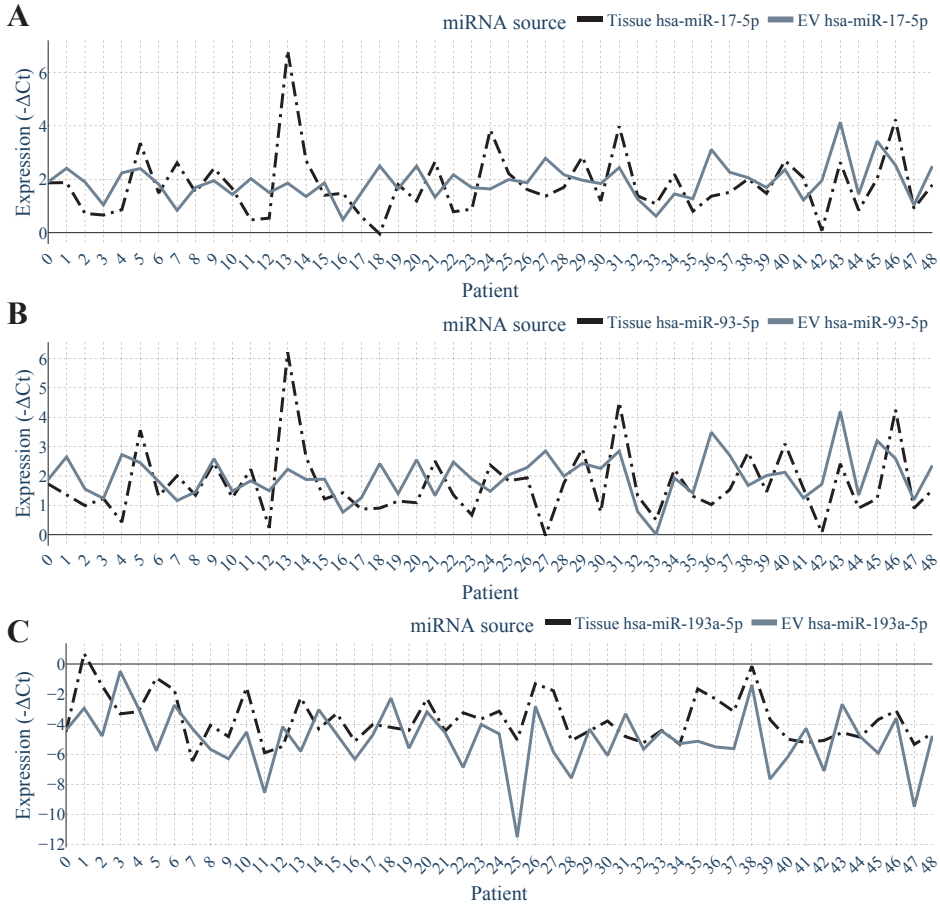

Fig. 3.3.1.2*. Reflection of tumoral and extracellular miRNA expression in glioblastoma patients*

A) miR-17-5p, B) miR-93-3p, C) miR-193-5p. Continues grey line shows miRNA expression in glioblastoma (GB) patient's extracellular vesicles (EVs), while the doted black line shows expression in glioblastoma tissue. Each value on the x-axis indicates individual patient whom miRNA expression was measured in blood's extracellular vesicles and tumor tissue. Similar expression in patient's EVs and GB tissue indicate that miRNA is mostly exported from the GB tissue.

Only two out of 14 analyzed miRNAs showed a significant differential expression in patients' EVs of blood serum when the relative expression was compared between LGG and GB patients (Table 3.3.1.1). Although, an accurate logistic regression or decision tree model for glioma grade prediction was unable to be created just from extracellular miRNA expression data. In addition, younger glioblastoma patients had a significantly higher expression of miR-21-5p in serum EVs (-ΔCt of 2.58), than older GB patients (-ΔCt

of 2.24) (p = 0.006) (Fig. 3.3.1.3. A). When *MGMT* promoter methylation status was included, the expression of miR-221-5p differed significantly between younger patients with methylated (-ΔCt of –4.07), and unmethylated (-ΔCt of –5.71) *MGMT* promoter (p = 0.039). There was no noticeable difference between this miRNA expression in older patients with different *MGMT* promoter status (Fig. 3.3.1.3. B). However, a significant differential miR-221-5p expression was observed between different age groups of GB patients with a methylated *MGMT* promoter. Younger GB patients, in their blood serum EVs, had a relative expression of miR-221-5p of –4.07 (-ΔCt), whereas older patients had an average expression of miR-221-5p of –6.77 (-ΔCt) (p = 0.037).

Table 3.3.1.1. *Average miRNA expression in extracellular vesicles of blood serum*

		Relative expression (-ΔCt)		**T-test significance**
miRNA type	**miRNA**	**LGG**	**GB**	**LGG *vs* GB**
Onco-suppressive	hsa-miR-143-3p	–0.16	–0.08	0.827
	hsa-miR-193a-5p	–4.69	–4.89	0.713
	hsa-miR-139-5p	–1.41	–0.73	0.262
	hsa-miR-7-5p	–0.87	–0.27	**0.030***
Oncogenic	hsa-miR-34a-5p	–10.24	–8.89	0.355
	hsa-miR-93-5p	1.80	1.98	0.354
	hsa-miR-181b-5p	–0.91	–1.02	0.705
	hsa-miR-181d-5p	–1.65	–1.60	0.859
	hsa-miR-221-5p	–5.32	–5.79	0.471
	hsa-miR-17-5p	1.19	1.93	0.259
	hsa-miR-335-5p	–1.78	–3.35	0.202
	hsa-miR-21-5p	2.52	2.38	0.275
	hsa-miR-148a-3p	–1.66	–2.22	0.103
	hsa-miR-10b-3p	–9.51	–7.15	**0.037***

"-ΔCt" means a relative expression of a miRNA. It is calculated as follows, -(cycle threshold value of a target miRNA - cycle threshold value of a geometric mean of all reference miRNAs). One would get the same values if a $\log_2^{FoldChange}$ formula were used. Two-tailed Student's t-Test considering group variance calculated by the F-test. * 1 % to 5 % possibility that the patterns in the data occurred by chance (p > 0.05).

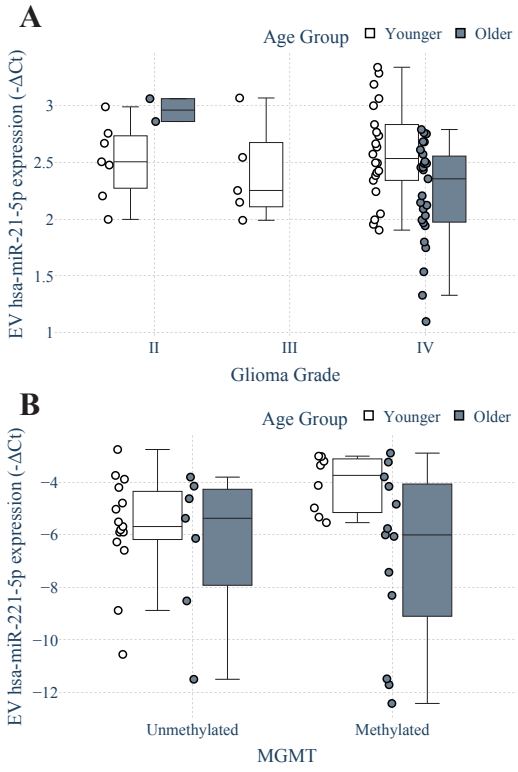

Fig. 3.3.1.3. *Extracellular miRNA expression in different grade of glioma, separated by age groups*

Relative expression of A) miR-21-5p and B) miR-221-5p in serum extracellular vesicles of patients diagnosed with different grade of glioma. The borders of the boxes illustrate the Q_1 and Q_3 values, The middle line in the box shows the median, and the end of the whiskers represents a maximum/minimum value before the upper/lower fence ($Q_{3/1} \pm 1.5$*Interquartile range (Q_3-Q_1)). The outliers are presented as dot outside the upper/lower fence.

Also, both miR-10b-3p and miR-7-5p shared a similar expression pattern in different grades of glioma: 1) a higher expression in grade II glioma, 2) a decreased expression in grade III glioma, and 3) an increase of expression in grade IV glioma to the similar level as in grade II glioma (Fig. 3.3.1.4. A and B). In contrast, relative expression of miR-139-5p, and miR-17-5p was consistently detected in all patients' blood samples at a stable level in all grades of glioma (Fig. 3.3.1.4. C and D).

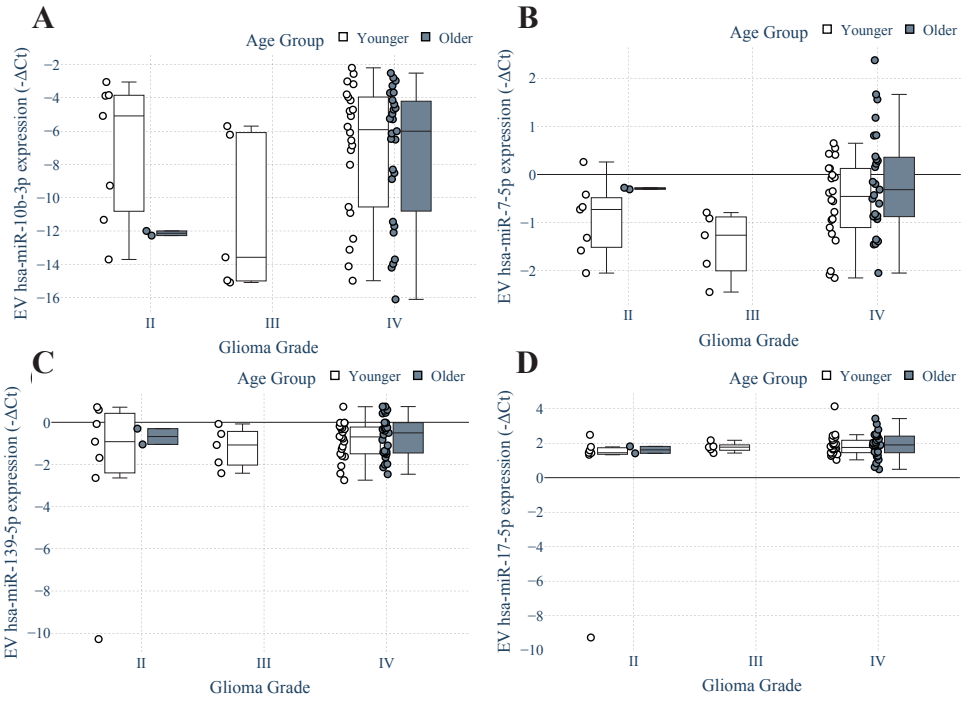

Fig. 3.3.1.4. *Extracellular miRNA expression in different grade of glioma, separated by age groups*

Relative expression of A) miR-10b-3p, B) miR-7-5p, C) miR-139-5p, and D) miR-17-5p in serum extracellular vesicles of patients diagnosed with different grade of glioma. The borders of the boxes illustrate the Q_1 and Q_3 values, The middle line in the box shows the median, and the end of the whiskers represents a maximum/minimum value before the upper/lower fence ($Q_{3/1} \pm 1.5$*Interquartile range (Q_3-Q_1)). The outliers are presented as dot outside the upper/lower fence.

3.3.2. Glioblastoma volume and extracellular miRNA expression

Interestingly, expression of an extracellular miR-7-5p was only correlated with a tumor volume of female GB patients (Pearson's $r = –0.56$, $p < 0.05$) (Fig. 3.3.2.1. A). Although not significant, but very similar pattern was observed with miR-93-5p (Pearson's $r = –0.37$, $p = 0.16$) (Fig. 3.3.2.1. B). Whereas miR-193a-5p and miR-221-5p did not show major differences between genders. Extracellular miR-193a-5p was negatively correlated with glioblastoma tumor volume (Pearson's $r = –0.40$, $p = 0.05$), and miR-221-5p was positively associated with GB volume (Pearson's $r = 0.40$, $p < 0.05$) (Fig. 3.3.2.1. C, D).

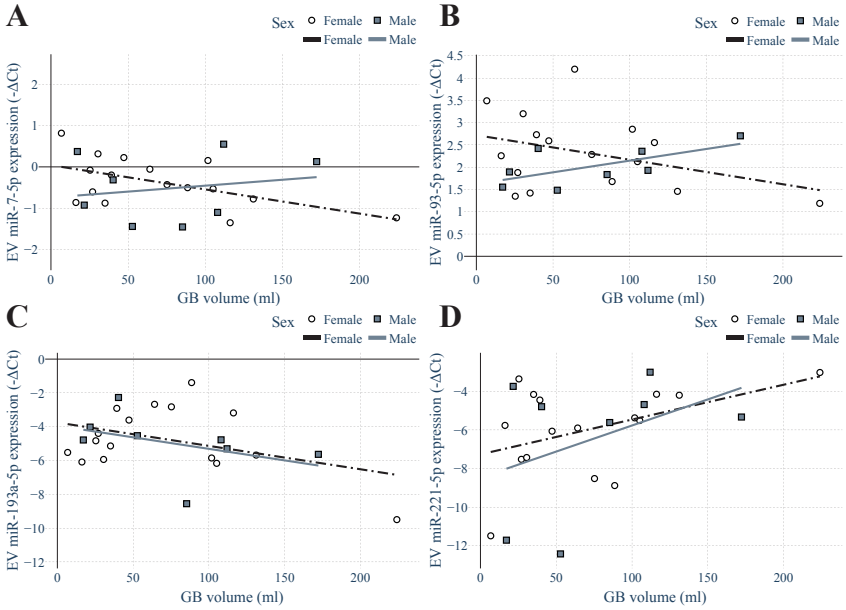

Fig. 3.3.2.1. *Correlation between extracellular miRNA expression and glioblastoma (GB) volume*

A) miR-7-5p; B) miR-93-5p; C) miR-193a-5p; D) miR-221-5p.

3.3.3. miRNA extracellular expression and glioma patients' Health Related Quality of Life

In glioblastoma patients' EVs, levels of miR-34a-5p or miR-181b-5p were not correlated with any of the European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire's (EORTC QLQ) C30 objectives. However, decreasing expression of an extracellular miR-181d-5p was found to be associated with GB patients' improved physical and emotional functioning ($p < 0.05$ and $p < 0.01$, respectively) (Table 3.3.3.1). Increasing expression of extracellular miR-181d-5p also was associated with patients' cognitive functioning and overall functioning status. However, these associations were calculated as non-significant ($p < 0.05$). No extracellular expression of analyzed miRNA tended to be related to the performance of glioblastoma patients as measured by KPS ($p < 0.05$).

Table 3.3.3.1. *Relationship between health-related quality of life indicators, clinical evaluation of patient's functioning, and miRNA expression in glioblastoma patients' extracellular vesicles of blood serum*

Criteria	miR-181b-5p	miR-181d-5p
	rho	rho
Global evaluation of health	−0.05	−0.02
Physical functioning	−0.09	**−0.27***
Role functioning	0.08	−0.03
Emotional functioning	−0.18	**−0.38****
Cognitive functioning	−0.05	−0.25
Social functioning	−0.04	−0.20
EORTC QLQ C30 Total Score	−0.08	−0.27
KPS at time of admission	0.05	−0.04

Higher scores represent better functioning and better functional outcomes after the surgical treatment. Spearman correlation coefficient shown under "rho" columns.

3.3.4. MiRNA expression and patients' overall survival time

Lower expression of miR-181b-5p in glioblastoma tissue was associated with a longer patient survival time compared to an average miR-181b-5p expression ($p = 0.003$) (Fig. 3.3.4.1. A). A similar relationship between tissue miR-181b-5p expression and GB patients' survival duration was only reflected as a tendency when predicting survival time from an extracellular expression of miR-181b-5p ($p = 0.183$) (Fig. 3.3.4.1. B). Although insignificant, a noticeable difference in GB patients' survival time was observed between patients with low and high miR-193-5p or miR-34a-5p expression ($p = 0.132$; $p = 0.151$) (Fig. 3.3.4.1. C and D). Also, a tendency for longer survival was observed in GB patients with a high expression of an oncogenic miR-21-5p in their blood serum EVs ($p = 0.068$) (Fig. 3.3.4.1. E). In contrast, GB patients with a low extracellular miR-34a-5p expression survived longer than patients with an average or high expression of miR-34a-5p ($p = 0.033$; $p = 0.03$) (Fig. 3.3.4.1. F).

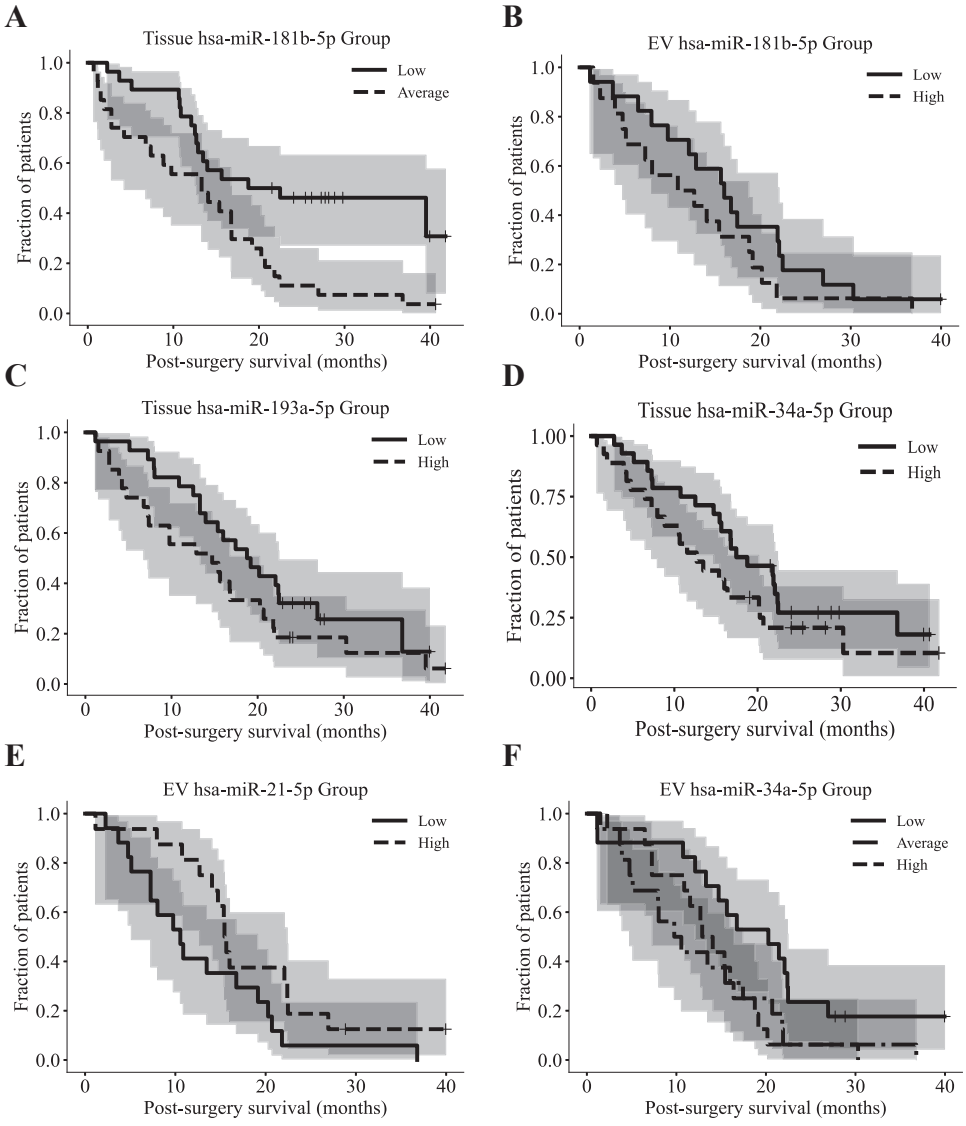

Fig. 3.3.4.1. *Kaplan-Meier survival curves by miRNA expression groups in glioblastoma patients*

Expression groups of 1) tumoral A) miR-181b-5p, B) miR-34a-5p, C) miR-193-5p, and 2) extracellular D) miR-181b-5p, E) miR-34a-5p, F) miR-21-5p in a glioblastoma patients' post-surgical tissue or extracellular vesicles of blood serum. The shadowing area of the main curve represents the 95 % confidence interval. Vertical lines on the main Kaplan-Meier curve shows the censored glioblastoma patients

To determine the significance of tumoral and extracellular miR-181 levels for all glioma patient post-surgical survival time, decision tree classifier analysis was performed (Fig. 3.3.4.2). The classifier also included known glioma biomarkers *IDH1* genotype and *MGMT* promoter methylation status. In addition, tumor-related symptoms, quality of life index, and functional patient status were also included for the survival time evaluation. The classifier's overall accuracy was 82.2 %. The accuracy of the prediction was 90.6 % for the short survival subgroup (< 16.85 months, n = 64), but it was lower (67.6 %) for the long survival group (> 16.85 months, n = 37), showing that this subgroup had more heterogeneous features. The decision tree classifier indicated that patients with gliomas with *IDH1* wild-type genotype, lower miR-181d-5p and greater miR-181b-5p tumoral expression would have the highest likelihood of short post-surgical survival times. Whereas longer survival was associated with *IDH1* mutation (R132H), severe tumoral symptoms, and higher miR-181b extracellular expression.

Fig. 3.3.4.2. *Decision tree classifier illustration for predicting grade II-IV glioma patients' survival time*

Grouped into two subgroups according to the cohort survival mean: < 16, 85 months - short survival; ≥ 16, 85 months - long survival. The earlier factor appearance (vertically going from top to bottom) shows its higher importance to the prediction model. Values on the lines indicates the factor value at which the algorithm divided the factor groups. For miR-181 expression levels, the fold change value was used. The higher tumor related symbol score reflects more pronounced symptoms, and the higher quality of life score indicates better functional and psychological well-being of the patient

Furthermore, a predictive model for glioma patient's survival time was created by evaluating all 14 analyzed tumoral miRNAs individually and as a

set, patient's age/gender, *IDH1* genotype, *MGMT* promoter methylation status, and glioma grade (Fig. 3.3.4.3). MiRNA set was constructed according to Cox's proportional hazard analysis from glioblastoma patients' data, selecting five significant miRNAs: miR-34a-5p, miR-221-5p, miR-17-5p, miR-143-3p, and miR-139-5p. The predictive model achieved a 66.6 % accuracy and a weighted average of 1) 67 % precision, 2) 67 % recall, and 3) 65 % f1-score. This classifier assigned the highest importance to patients' age, miR-143-3p, and miRNA set expression. The model mainly identified short survival time (< 11.53 months) for patients who were older than 49.93 years, and had a miRNA set score higher than 4.06. Whereas patients younger than 49.93 years with a lower than 0.93 (-ΔCt) expression of a tumoral miR-143-3p were assigned toward long survival (between 21.45 and 41.79 months). Within this set of features, neither *IDH1*, *MGMT* genes, nor glioma grade were suggested as important features for glioma survival time prediction.

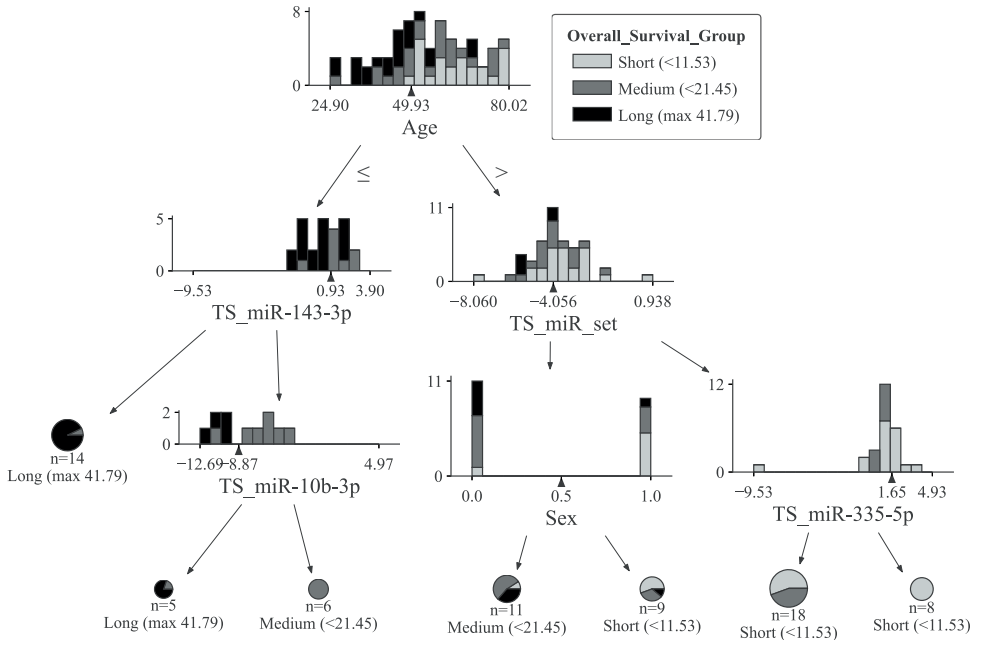

Fig. 3.3.4.3. *Decision tree classifier illustration for predicting glioma patients' survival time considering all analyzed tumoral miRNAs*

Survival time divided into terciles and shown as time in months. The higher the feature is in the "tree roots", the bigger importance it has to the survival time. The width of the "roots" represents the size of the sample group. "TS" means tumoral miRNA expression. Females are coded as 0 and males as 1. MiRNA set (TS_miR_set) consists of 5 miRNAs associated with glioblastoma patients' survival, according to Cox's proportional hazard analysis: miR-34a-5p, miR-221-5p, miR-17-5p, miR-143-3p, miR-139-5p

For a more practical glioma outcome prediction, a predictive glioma survival model was generated considering patients' age, sex, *IDH1* genotype, *MGMT* promoter methylation status, glioma grade, and extracellularly expressed 14 analyzed miRNAs and their set (Fig. 3.3.4.4). A set of extracellular miRNAs consisted of 4 miRNAs which were significantly associated with glioblastoma patient's survival, performing Cox's proportional hazard analysis: miR-181d-5p, miR-221-5p, miR-17-5p, and miR-335-5p. The model's performance was slightly worse compared to the model where tumoral miRNA expression was used. The accuracy of this classifier was 60.1 %, with a weighted average of 66 %, 61 %, and 59 % for precision, recall, and f1-score, respectively. Patients' age remained the most important feature of this classifier. Despite that, extracellular miR-7-5p, miR-10b-3p, and miR-139-5p were the top 3 miRNAs considered for predicting glioma patients' survival duration. A shorter survival time was predicated for older patients (> 49.03 years old) with a higher extracellular miR-7-5p expression (> –0.117 (-ΔCt)) and a higher extracellular miR-139-5p expression (> 0.75 (-ΔCt)). Although, other combinations of extracellular miRNA expression also resulted in a short (< 11.53 months) outcome prediction for glioma patients (Fig. 3.3.4.4).

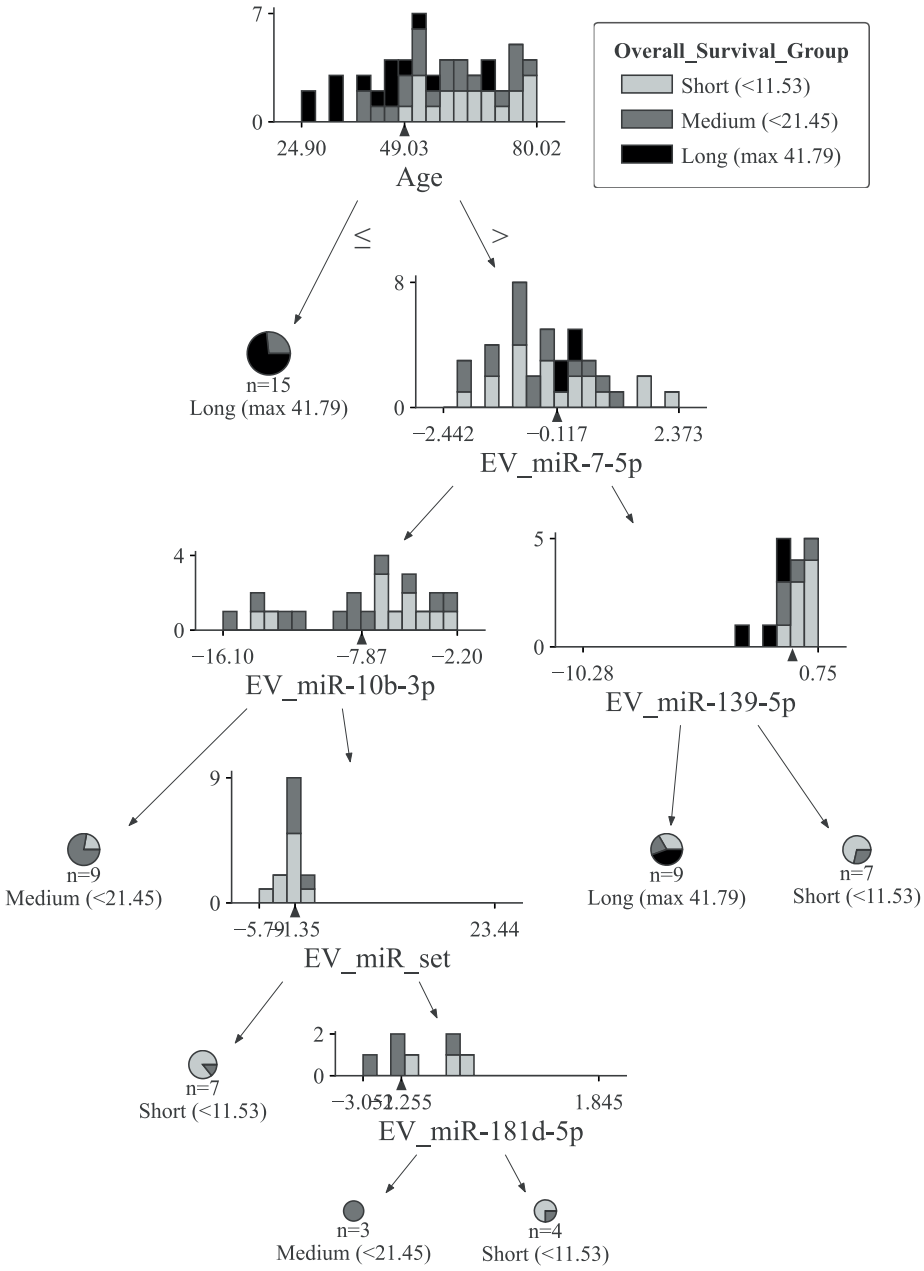

Fig. 3.3.4.4. *Decision tree classifier for glioma patients' survival time considering all analyzed extracellular miRNAs*

Survival time divided into terciles and shown as time in months. The higher the feature is in the "tree roots", the bigger importance it has to the survival time. The width of the "roots" represents the size of the sample group. EV – extracellular miRNA expression. EV miRNA set (EV_miR_set) consists of 4 miRNAs: miR-181d-5p, miR-221-5p, miR-17-5p, and miR-335-5p

Despite the insignificant effect of the tumoral miR-221-5p expression on glioblastoma patient survival time, shown by the Kaplan-Meier survival curves and log-rank test, it was detected that a survival time of younger females diagnosed with GB had a moderate negative correlation with the tumoral miR-221-5p expression (r-squared = 0.62) (Fig. 3.3.4.5). No other noticeable correlations were detected among the combination of GB patients' age group, sex, tissue miRNA expression and overall survival time after the tumor removal surgery. In addition, a weak correlation between longer survival and lower expression of extracellular miR-139-5p and miR-34a-5p was also observed in younger females (r-squared = 0.36 and 0.27). Older females tended to survive longer if their extracellular miR-21-5p expression was higher, however, the correlation was very weak (r-squared = 0.12).

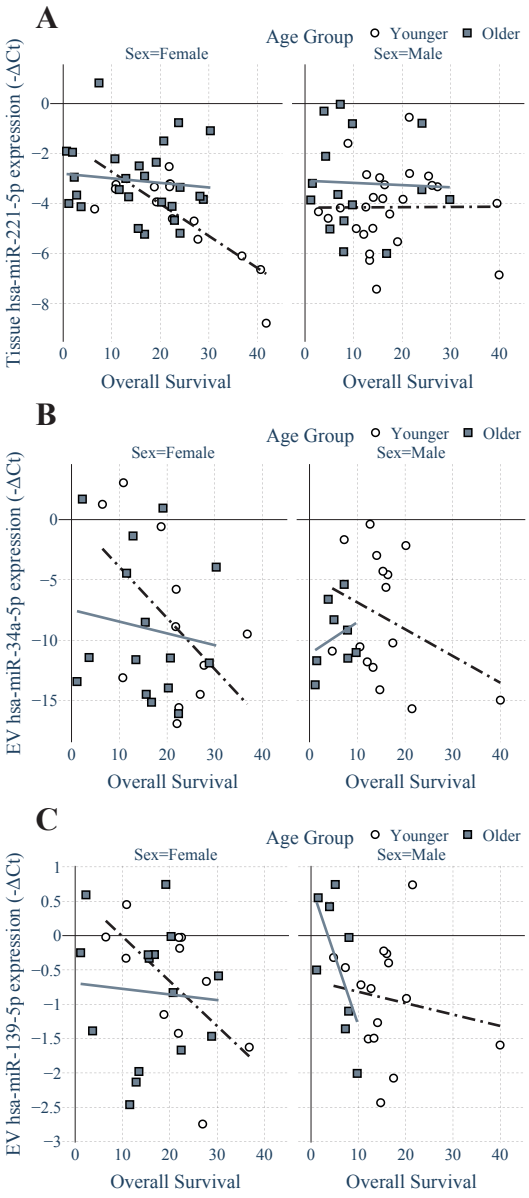

Fig. 3.3.4.5. *Correlation between glioblastoma patient post-surgical survival time (in months) and miRNA relative expression of 1) tumoral A) miR-221-5p, 2) extracellular B) miR-34a-5p, C) miR-139-5p, grouped to 2 groups by patients' age*

No significant differences were detected comparing the survival time of males and females, younger and older, or *MGMT* methylated and unmethylated

glioblastoma patients (Fig. .3.3.4.6). Although a tendency for longer survival was noticeable in younger females.

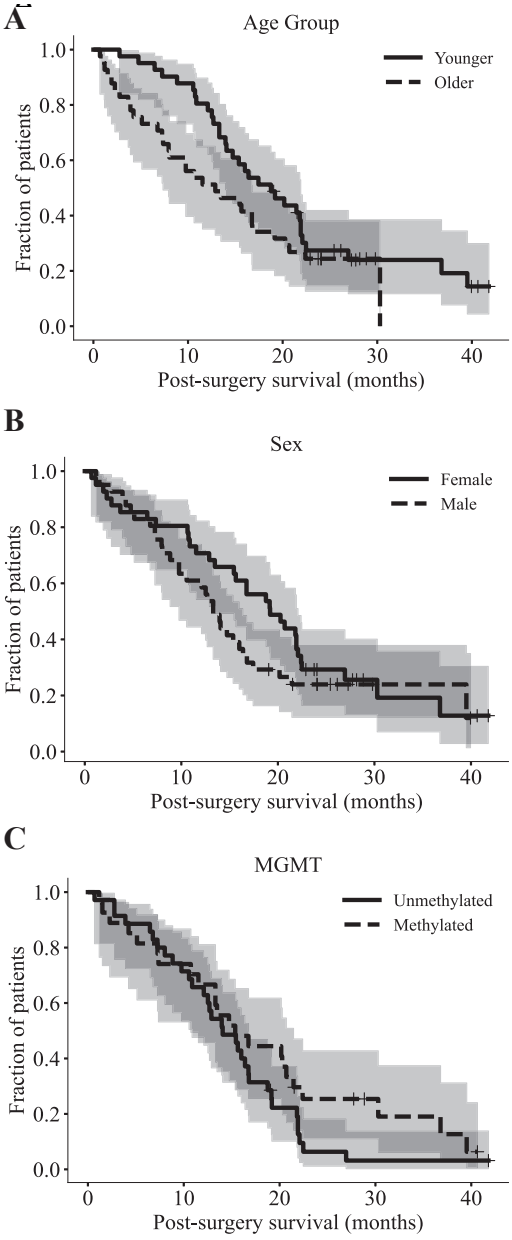

Fig. 3.3.4.6. *Kaplan-Meier survival curves in glioblastoma patients*

Expression groups of A) Age, B) Sex, C) MGMT promoter status in a glioblastoma patients' post-surgical. The shadowing area of the main curve represents the 95 % confidence interval. Vertical lines on the main Kaplan-Meier curve shows the censored glioblastoma patients.

Finally, Cox's proportional hazard analysis was performed with a set of all analyzed miRNAs to evaluate how numerical changes in miRNA expression relate to glioblastoma patients' survival. When analyzing miRNAs, the significant impact on GB patients' survival time had an expression of tumoral: miR-34a-5p, miR-221-5p, miR-17-5p, miR-143-3p, and miR-139-5p (Table 3.3.4.1). Furthermore, the expression of miRNAs from EVs was investigated to determine the most significant miRNAs for the least invasive monitoring of patients' health. Significant extracellular miRNAs associated with GB patients' survival time were: miR-181d-5p, miR-221-5p, miR-17-5p, and miR-335-5p (Table 3.3.4.2). Not significant due to the wide confidence interval, but the most effective miRNA for the shift of patients' baseline hazard of survival was miR-93-5p, measured both in glioblastoma tissue and in serum EVs. For every increase in the relative expression (-ΔCt) of tumoral miR-93-5p, an increase of 69 % for baseline hazard followed. Extracellularly expressed miR-93-5p had an even stronger effect of increasing the baseline hazard of 194 % with every integer increase in its relative expression (Fig. 3.3.4.7).

Table 3.3.4.1. *Cox's proportional hazard importance of miRNA expression, in glioblastoma tissue, for post-surgical survival*

Covariate	Coefficient	Effect for baseline hazard	Significance
hsa-miR-34a-5p	0.13	Increase of 14 %	**p = 0.04**
hsa-miR-93-5p	0.53	Increase of 69 %	p = 0.12
hsa-miR-181b-5p	0.23	Increase of 25 %	p = 0.43
hsa-miR-181d-5p	–0.31	Decrease of 27 %	p = 0.34
hsa-miR-221-5p	0.48	Increase of 62 %	**p < 0.005**
hsa-miR-17-5p	–0.71	Decrease of 51 %	**p = 0.02**
hsa-miR-143-3p	–0.47	Decrease of 38 %	**p < 0.005**
hsa-miR-335-5p	0.26	Increase of 30 %	p = 0.11
hsa-miR-21-5p	–0.31	Decrease of 26 %	p = 0.05
hsa-miR-193a-5p	–0.05	Decrease of 5 %	p = 0.64
hsa-miR-148a-3p	0.24	Increase of 28 %	p = 0.05
hsa-miR-139-5p	0.18	Increase of 19 %	**p = 0.01**
hsa-miR-7-5p	–0.16	Decrease of 15 %	p = 0.13
hsa-miR-10b-3p	0.09	Increase of 10 %	p = 0.35

Table 3.3.4.2. *Cox's proportional hazard importance of miRNA expression, in glioblastoma extracellular vesicles, for post-surgical survival*

Covariate	Coefficient	Effect for baseline hazard	Significance
hsa-miR-34a-5p	0.06	Increase of 6 %	p = 0.12
hsa-miR-93-5p	1.08	Increase of 194 %	p = 0.05
hsa-miR-181b-5p	–0.13	Decrease of 12 %	p = 0.61
hsa-miR-181d-5p	–0.83	Decrease of 56 %	**p = 0.03**
hsa-miR-221-5p	–0.26	Decrease of 23 %	**p = 0.03**
hsa-miR-17-5p	–2.29	Decrease of 90 %	**p = 0.01**
hsa-miR-143-3p	0.25	Increase of 28 %	p = 0.23
hsa-miR-335-5p	0.12	Increase of 13 %	**p = 0.02**
hsa-miR-21-5p	–0.15	Decrease of 14 %	p = 0.78
hsa-miR-193a-5p	0.10	Increase of 10 %	p = 0.40
hsa-miR-148a-3p	0.10	Increase of 10 %	p = 0.68
hsa-miR-139-5p	–0.07	Decrease of 7 %	p = 0.81
hsa-miR-7-5p	0.44	Increase of 56 %	p = 0.20
hsa-miR-10b-3p	0.02	Increase of 2 %	p = 0.70

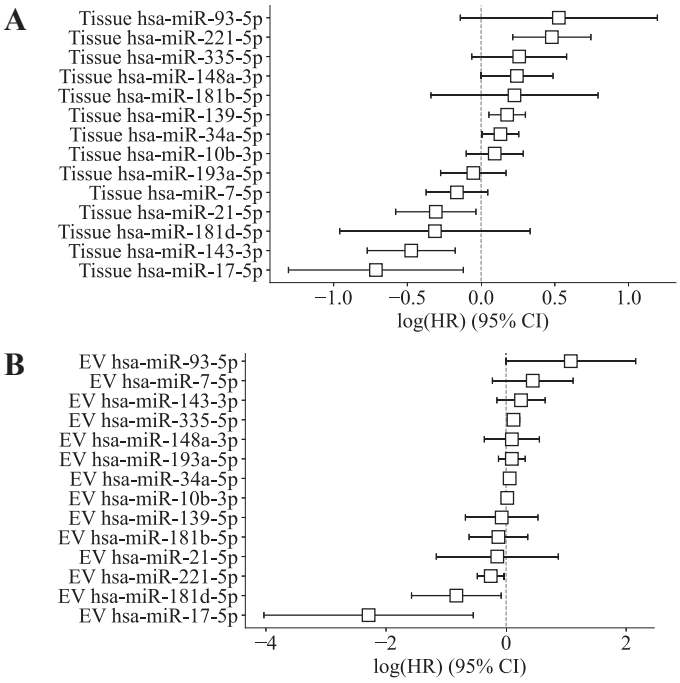

Fig. 3.3.4.7. *Weight of tumoral and extracellular miRNA expression on glioblastoma patients' post-surgical survival*

Weight of A) tumoral and B) extracellular miRNA expression on glioblastoma patients' post-surgical survival. The end of whiskers shows the 95 % confidence

4. DISCUSSION

Glioma is one of the worst prognoses-bearing cancer type in humans, mostly due to its low early detection rates and difficult treatment of its most malignant form – glioblastoma. In theory, it is possible to detect early stages of glioma by an MRI scan and monitor their progression, however, in practice, this type of strategy, both economically and technically, is hard to maintain since glioma is a relatively rare disease with no known strong genetic predisposition to it [3,4]. Ideally, like for all other cancers, it would be to have a minimally invasive, low-cost diagnostic and prognostic test for glioma patients, which would let the physicians detect gliomas at their early stages and effectively treat them. MiRNA properties are ideal for this purpose. They are relatively stable both in tissue and extracellularly, loaded to extracellular vesicles, which can pass the brain-blood barrier, and are reported to be differentially expressed in various types and stages of cancer. This study explored the diagnostic and prognostic potential of selected miRNAs for the diagnosis and prognosis of different grades of glioma.

Overall, dysregulation of selected miRNA expression was more noticeable in GB, rather than in LGG samples, when compared to non-cancerous tissue. The gradual increase of onco-miRNAs was observed in LGG and GB, compared to non-cancerous brain tissue. Similarly, the inverse tendency was observed for the onco-suppressor-miRNAs.

Looking at the diagnosis of glioma, a list of promising miRNAs was identified to be differentially expressed in different stages of glioma. The increasing expression of miR-21-5p was detected in a tissue of the higher grades of glioma. Even though miR-21-5p has been previously reported to have oncogenic properties in glioma and negatively impact patients' survival time [86,229], this study found no significant result for GB patients' survival association with tumoral or extracellular miR-21-5p expression. This inconsistency in clinical associations with miR-21 expression was also noticed by Guli Jiang et al., which motivated their group to perform a meta-analysis on miR-21 expression and patients' overall survival [230]. Due to this, in all future studies exploring miR-21 expression changes in glioma, it would be beneficial also to analyze the protein levels of its main targets: Phosphatase and Tensin Homolog (*PTEN*), Tropomyosin 1 (*TPM1*) and Programmed Cell Death 4 (*PDCD4*) gene. By doing that, the molecular effect of miR-21-5p would be determined as well, giving more confidence in miR-21-5p expression results [231–233]. However, even if the results of expression studies of miR-21-5p are obtained in high confidence, miR-21-5p remains an extremely abundant miRNA in the whole human body. At first glance, it

doesn't seem like an issue, but as Ana E. Jenike & Marc K. Halushka pointed out, miR-21-5p lacks specificity to be a useful biomarker since its expression is reported to be differentially expressed in numerous diseases [234]. They also indicate that miR-21-5p is mainly expressed in macrophages and other cells responsible for inflammatory response. This might explain why in this study, there were no significant associations found between an extracellular miR-21-5p expression and glioma grade or patients' survival time – probably because most of the miR-21-5p expression came from the inflammatory process (which is both present in patients with LGG and GB) and not from the glioma cells.

Other analyzed promising miRNAs were miR-143-3p, miR-7-5p, and miR-10b-3p – all these miRNAs had a significantly different expression between non-cancerous and LGG tissue. The expression results of these miRNAs are on par with other scientific articles, where miR-143-3p and miR-7-5p are described as onco-suppressive miRNAs, and miR-10b-3p is considered to be an oncomiRNA [235–237]. Differential expression combination of these miRNAs in non-cancerous and low-grade glioma tissue returns hope that an early, minimally invasive diagnostic or prognostic test can be created for glioma patients.

Although miRNA expression changes between non-cancerous and LGG groups are the most beneficial for early brain cancer diagnosis, it is also important to notice miRNA expression differences among LGG and GB. These expression changes between different grades of glioma can expand the knowledge of glioma progression and give directions to future treatment strategies. In some rare cases, when it is pathologically hard to distinguish an advanced grade III glioma from the early development of a glioblastoma, expression measurements of the analyzed miRNAs: miR-193a-5p, miR-139-5p, miR-21-5p, miR-148a-3p, and miR-10b-3p would help to make an accurate diagnostic decision.

The logistic regression model for predicting the diagnosis of glioma assigned the increase of a miR-193a-5p expression to the increasing odds of being diagnosed with glioblastoma. Interestingly, based on miR-193a-5p expression in other types of cancer, it is considered as an onco-suppressive miRNA [238,239]. Usually, its expression decreases in cancer tissue when compared to non-cancerous tissue. In the cohort of this study, the expression of miR-193a-5p was practically the same in non-cancerous and LGG brain tissue. However, a significant difference in its expression was measured between LGG and GB tissue. Unlike other types of cancer, miR-193a-5p had a higher expression in a more malignant grade of glioma (glioblastoma) samples instead of a lower grade glioma. This unusual pattern could mean that the body more efficiently releases a miR-193a-5p as a response to the

increased malignancy of glioma, but GB cells effectively eliminate its onco-suppressive abilities. An example of this silencing of miR-193a-5p could be with the mechanism of sponging with long non-coding RNAs (lncRNAs), where an increase of a specific lncRNA transcription is stimulated in order to capture miRNAs, which would otherwise silence survival-import mRNA transcripts of the cancer cell. In this manner, onco-suppressive miR-193a-5p was shown to be silenced in triple-negative breast cancer cell lines by the long non-coding RNA LINC01224 [240]. Similarly, Jun Li et al. suggested that in breast cancer, lncRNA SNHG1 sponges miR-193a-5p, to increase homeobox A1 (*HOXA1*) oncogenic activity [241]. Luo JF et al. also reported that miR-193a-5p is downregulated in prostate cancer by a lncRNA Titin-antisense RNA1 (*TTN-AS1*) [242].

This study detected significant expression differences in extracellular miR-7-5p and miR-10b-3p between LGG and GB patients. Both miRNAs were expressed lower in higher-grade glioma, even though miR-7-5p is considered as onco-suppressive miRNA, and miR-10b-3p, in most cancers, acts as an onco-miRNA. Higher expression of onco-miRNA in the EVs of glioblastoma patients could be explained by a hypothesis that tumor cells are packaging oncogenic molecules to EVs for faster growth and spread through the body. Similarly to this study, miR-10b-3p was found to be higher expressed in EVs of lung adenocarcinoma patients compared to non-cancerous patients [243]. An opposite hypothesis is also valid, that non-cancerous cells are packaging onco-suppressive molecules to EVs to prevent the growth and spread of a tumor. The findings in this study of extracellular miR-7-5p would fall under this hypothesis. A study by Huan Wang et al. showed a similar cellular response when a medication treatment of Verbascoside was applied to GB [244]. Another possible explanation for the higher expression of an onco-suppressive miRNA in a patient's EVs of a higher-grade glioma could be that cancer cells are deliberately packaging onco-suppressive miR-7-5p to exclude it from its environment.

The sequencing results of this study suggested miR-338-5p as a promising miRNA for monitoring glioblastoma progression in patients' serum EVs. MiR-338-5p had a similar relative expression in post-surgical tissue and in the patient's blood serum. These results were also confirmed on an independent cohort with an RT-qPCR method. However, this doesn't necessarily mean that miR-338-5p is the only suitable miRNA for minimally invasive monitoring of glioblastoma patients. The selected sequencing approach in this study had some flaws. First, too little spike-in was added to each sample during smallRNA-seq library preparation, which resulted in non-detected or minimally detectable spike-in sequences in the sequencing results. Second, the sequencing depth was selected too low, thinking that including a higher

amount of biological replicates would be more beneficial, which is generally true, but after a minimum sequencing depth is reached [245]. Also, there is still a debate on miRNA annotation. Some studies reveal that the current miRNA nomenclature could be inaccurate, and in practice, only certain miRNAs from the miRBase can be detected in humans [246].

In addition, the expression of some miRNAs in this study had an association with patients' age. At a younger age, the human immune system, naturally, has a very limited experience in recognizing various types of cancerous alterations in the body but is more resilient, undergoing destructive changes during higher stages of cancer, compared to an older patient. The faster decrease of an onco-suppressive miR-139-5p and miR-143-3p expression in the early stages of glioma (relative to non-cancerous tissue) in younger patients compared to older patients and their higher expression in grade IV glioma in younger patients compared to older ones, could suggest that miR-139-5p and miR-143-3p are involved in the immune response to glioma. Recent studies have shown that miRNAs are involved in the immune response. For example, miR-155 is associated with interferon response, activity of natural killer cells, and macrophage polarization [247]. In contrast, the knock-out of miR-146a in mice resulted in immunoproliferative and autoimmune disease development [248].

Another angle of miRNA studies, which was not considered in this PhD thesis, was estimating miRNA biological activity. MiRNA quantities do not always reflect its activity. In fact, miR-34 was shown to be activated after damaging deoxyribonucleic acid (DNA)in the cells. One way of activating miRNAs is through their 5-prime (5') phosphorylation. The phosphorylation of miRNA enables it to be loaded to Argonaute RISC Catalytic Component 2 (AGO2), which is one of the RISC proteins and is responsible for the cleavage of targeted mRNA [249]. This concept was suggested by Nina Mørup et al. when analyzing circulating levels of miR-30-5p in different stages of puberty in boys [250].

Overall, this study has flaws and weak points, such as the debatable choice of housekeeping miRNAs for relative expression analysis in EVs. However, there is still no clear consensus on which miRNAs are best to use in different types of cancer. Although, there is probably, truth in one of the thoughts expressed by prof. Arvydas Skeberdis, in one of his lectures: "*If a choice of a housekeeping gene radically changes your results, there is a big chance that your findings have little biological meaning*". Besides selecting housekeeping genes, this study also lacks quality control for the isolation of EVs. For the isolation of the vesicles, a commercially available kit was used. However, only later in the study it was realized that additional measurements had to be done to evaluate the size and surface markers of extracted vesicles. Because

of this, the results from this study cannot confirm whether the expression of extracellular miRNAs came from exosomes, microparticles or apoptotic bodies.

Despite these weak points, this study expands the knowledge of miRNA quantity alterations in different grades of glioma. Of course, further experiments are needed to confirm that the loss or increase of a specific miRNA leads to any effect on its target mRNAs. Furthermore, the detected changes of miR-7-5p, miR-10b-3p and other miRNAs expression in LGG and GB tissues and EVs can improve the diagnosis and prognosis of glioma patients. Finally, the results from this PhD thesis can serve as a foundation for a more complex project of a combined miRNA knock-out in glioma cell cultures or glioblastoma mouse models.

CONCLUSIONS

1. The tumoral expression of miR-21-5p, miR-10b-3p significantly and consistently were differentially expressed in non-cancerous brain, low-grade glioma, and glioblastoma tissues making these miRNAs most promising candidates for a creation of a novel molecular diagnostic test for glioma grade. Extracellular expression of miR-10b-3p and miR-7-5p were significantly differentially expressed between low-grade glioma and glioblastoma samples, which enables these miRNAs to be further studied for a minimally invasive glioma progression screening.
2. MiR-338-5p, miR-17-5p, miR-93-5p, and miR-193a-5p had the most similar expression in glioblastoma tissue and extracellular vesicles of the same patient. Meaning that these miRNAs are promising candidates for studies of monitoring the smallest glioblastoma treatment efficiencies, since their tumoral expression is reflected in the extracellular vesicles of the blood.
3. MiR-181b-5p expression was significantly associated with glioblastoma patients' survival time after the tumor-removal surgery. In contrast, *IDH1* genotype or *MGMT* promoter methylation status was not significantly associated with glioblastoma patients' survival time. Because of this, tumoral miR-181b-5p has a better prognostic value for glioblastoma patients than *IDH1* or *MGMT* promoter methylation status.
4. The strongest effect on the hazard ratio of glioblastoma post-surgical survival had a combination of tumoral miR-221-5p, miR-17-5p, and extracellular miR-17-5p expression. These miRNAs are promising candidates for future studies on treatment development in glioblastoma xenografts.
5. Several glioma survival models have been developed by identifying combinations of the most important molecular features. Prognostic indicators of short survival: IDH1 wild-type genotype, lower miR-181d-5p and higher miR-181b-5p expression in tumor tissue, while better survival prognosis was associated with IDH1 mutation (R132H) and higher extracellular expression of miR-181b
6. When including all analyzed miRNAs in the survival model, better survival predictions were found in patients who were younger, had lower tumor miR-143-3p expression, or higher miR-143-3p expression but in combination with lower miR-10b-3p expression. Shorter glioma survival associated with older patient age, higher expression of five tumoral miRNA set and higher expression of tumoral miR-335-5p.

SANTRAUKA

ĮVADAS

Glioma yra labiausiai paplitęs pirminių piktybinių smegenų auglių tipas, kuris atsiranda centrinės nervų sistemoje įvykus vėžiniams glijos ląstelių pokyčiams. Gliomos piktybiškiausia forma – glioblastoma, kuri sudaro beveik 50 proc. visų piktybinių smegenų auglių ir yra labiausiai paplitęs piktybinis centrinės nervų sistemos (CNS) navikas. Tik 6.8 proc. glioblastomos pacientų išgyvena ilgiau nei penkerius metus, o tik 42.5 proc. pacientų pasiekia vienerius metus [1,2]. Taip yra daugiausia dėl to, kad smegenų vėžys aptinkamas vėlai. Techniškai įmanoma identifikuoti gliomos pacientus, kuriems dar nėra išreikštų simptomų, naudojant rutininį magnetinio rezonanso tomografijos (MRT) skenavimą. Tačiau ši strategija yra labai imli darbui ir nėra ekonomiška [3,4].

Norint anksti nustatyti gliomas, reikalinga tyrimo strategija panaši į bendro kraujo tyrimą - minimaliai invazyvi, nereikalaujanti didelių žmogiškųjų resursų bei greitai atliekama. Šiuo tikslu gali būti naudojamas mikro RNR (miRNR) ekspresijos nustatymas. MiRNR yra mažos nekoduojančios RNR molekulės, kurios atlieka svarbų reguliavimo vaidmenį genų ekspresijoje [5]. Vėžio atveju miRNR gali veikti kaip onkogenai arba naviko slopikliai, priklausomai nuo specifinės miRNR ir ląstelių sąveikos. Kai kurios miRNR gali skatinti vėžio progresavimą nukreipdamos ir slopindamos naviko slopinimo genus, o kitos gali veikti priešingai – slopindamos onkogenus [6] . Be to, miRNR gali reguliuoti kitus vėžio biologijos procesus, tokius kaip angiogenezė, invazyvumas ar metastazavimas. Tam tikrų miRNR raiškos išbalansavimas jau yra nustatytas gliomų atveju bei įrodyta, kad šios miRNR kontroliuoja svarbius gliomų progresavimo kelius [7] . Pavyzdžiui, miR-124 yra mažiau ekspresuojama aukštesnio laipsnio gliomose ir yra reikšmingai nuslopinta gliomos audiniuose, lyginant su sveiku smegenų audiniu. Funkciniai ląstelių tyrimai parodė, kad miR-124 veikimas nukreiptas į genus, dalyvaujančius ląstelių ciklo reguliavime ir proliferacijoje [8] . Kitoje spektro pusėje yra miR-21 - viena iš dažniausiai išbalansuotų miRNR gliomose. Tyrimai parodė, kad miR-21 ekspresijos lygis didėja esant aukštesnio laipsnio gliomoms ir yra susijęs su prastesne pacientų išgyvenimo trukme [9–12].

MiRNR yra stabilios ir gali būti supakuojamos į pūsleles ir taip patekti į kraujotaką [13,14]. Ekstraląstelinės pūslelės (EP) yra mažos, su membrana surištos dalelės, kurias išskiria įvairūs ląstelių tipai, įskaitant vėžines ląsteles. EP skirstomos į tris pagrindinius tipus: egzosomas, mikropūsleles ir apoptotinius kūnelius. Šiose pūslelėse yra įvairių bioaktyvių molekulių, įskaitant baltymus, lipidus ir nukleorūgštis, tokias kaip miRNR. EP atlieka reikšmingą

vaidmenį tarpląstelinėje komunikacijoje, nes gali perduoti savo turinį į kaimynines ar tolimas ląsteles, moduliuodamos įvairius ląstelių procesus, tokius kaip diferenciacija, proliferacija ar imuninis atsakas. Vėžio atveju, EP gali skatinti naviko augimą ir progresavimą pristatydamos onkogenines molekules, tokias kaip miRNR, taip keisdamos naviko mikroaplinką [15,16]. Norint stebėti gliomų vystymąsi, galima išskirti tarpląstelines pūsleles ir kiekybiškai įvertinti jų miRNR.

Naujausi tyrimai ištyrė su miRNR esančių EV potencialą būti naudojamomis, kaip biomarkeriais gliomos diagnozei ir prognozei nustatyti. Pavyzdžiui, įrodyta, kad su EV susijusi miR-10b yra galimas gliomos diagnozės biomarkeris, o miR-21 ir miR-221, aptiktos EV, buvo susijusios su prasta glioma sergančių pacientų prognoze [17–21]. Tačiau vis dar trūksta nuoseklių duomenų ekstraląstelinių pūslelių miRNR srityje, kad būtų galima sukurti mažai invazinį diagnostinį ar prognostinį tyrimą gliomos pacientams, kurio specifiškumas ir jautrumas būtų minimaliai pageidaujamą 95 proc. ribą.

Darbo tikslas: Nustatyti diagnostinių ir prognostinių mikroRNR rinkinį gliomos progresavimui ir baigčiai.

Darbo uždaviniai:

1. Įvertinti ryšį tarp gliomos laipsnio ir miRNR ekspresijos profilio a) naviko audinyje ir b) ekstraląstelinėse kraujo pūslelėse.
2. Nustatyti ryšį tarp miRNR ekspresijos naviko audinyje ir tarpląstelinėse kraujo pūslelėse
3. Įvertinti tirtų miRNR prognostinį potencialą pagal standartinius gliomų molekulinius žymenis – *MGMT* geno promotoriaus metilinimą ir *IDH* geno mutacijas.
4. Nustatyti miRNR ekspresijos profilio ryšį su glioma sergančių pacientų klinikine baigtimi
5. Remiantis miRNR ekspresija, *MGMT* geno promotoriaus metilinimo būsena, *IDH* genų mutacijų duomenimis ir pacientų klinikinėmis charakteristikomis – sukurti algoritmą, leidžiantį pacientus skirstyti į išgyvenamumo prognozines grupes.

Darbo naujumas ir reikšmingumas. Šis tyrimas praplečia žinias apie mikroRNR ekspresiją gliomas pooperaciniuose audiniuose ir kraujo ekstraląstelinėse pūslelėse. Be to, šiame tyrime pirmą kartą buvo ištirta Lietuvos glioblastomų kohortos miRNR ekspresija navikiniame audinyje ir pacientų ekstraląstelinėse kraujo pūslelėse. Taip pat, pirmą kartą glioma sergančių pacientų funkcionavimo kokybė buvo įvertinta atsižvelgiant į naviko ir tarpląstelinę miRNR raišką. Šis tyrimas suteikia įžvalgų apie specifinių miRNR rinkinį, tinkantį tolesniems tyrimams, siekiant sukurti minimaliai invazinius

diagnostinius ir prognostinius gliomos ir glioblastomos tyrimus. Šiame tyrime pateikti duomenys gali būti atspirties taškas būsimiems miRNR bioaktyvumo gliomose tyrimams.

Disertacijos planas. Disertaciją sudaro 3 dalys: I) potencialių mikroRNR paieška gliomos prognozei ir diagnozei; II) MikroRNR analizė gliomos audinyje po operacijos; III) MikroRNR analizė glioma sergančių pacientų kraujo serumo ekstraląstelinėse pūslelėse.

Šių tyrimų derinys padeda geriau suprasti gliomos molekulinę biologiją ir miRNR ryšį su gliomos laipsniu, išgyvenamumą po operacijos ir glioma sergančių pacientų gyvenimo kokybę. II ir III tyrimo dalies rezultatai buvo paskelbti recenzuojamuose žurnaluose "International Journal of Molecular Sciences" ir "Cancers".

MEDŽIAGOS IR METODAI

Mėginiai. Tyrimo protokolą ir sutikimo procedūras patvirtino Lietuvos sveikatos mokslų universiteto Biomedicininių tyrimų etikos komitetas (BE-2-26). Prieš mėginių paėmimą, visi pacientai pasirašė informuotą sutikimą dalyvauti tyrime. Gliomos diagnozė buvo įtariama atlikus magnetinio rezonanso tomografijos (MRT) tyrimą, o patologas patvirtino diagnozę ištirdamas pooperacinį audinį. Gliomos audiniai ir kraujo mėginiai buvo paimti Lietuvos Sveikatos Mokslų Universiteto Ligoninės Kauno klinikose, Neurochirurgijos klinikoje 2015–2019 m. Paimti audiniai buvo užšaldyti skystame azote (–196 °C) per 15–30 minučių po naviko pašalinimo operacijos ir laikomi užšaldyti iki RNR išskyrimo. Kraujo mėginiai buvo paimti likus 7 dienoms iki naviko pašalinimo operacijos. Per 1 valandą nuo kraujo paėmimo serumas buvo atskirtas nuo kraujo ląstelių, centrifuguojant mėginius 15 min. 1300 ×g. Atskirtas serumas buvo laikomas užšaldytas (–80 °C), kol buvo išskirtos tarpląstelinės pūslelės ir jų RNR.

Mėginių grupės. Į šį tyrimą buvo įtrauktos 4 pagrindinės pacientų grupės: 1) sveiki (neturintys vėžinių susirgimų) 2) II laipsnio glioma, 3) III laipsnio glioma ir 4) IV laipsnio glioma arba glioblastoma. Ne vėžiu sergančių pacientų grupėje 2 mėginiai buvo įsigyti išskirtos RNR pavidalu. Likę 2 šios grupės mėginiai buvo paimti chirurginio epilepsijos gydymo metu. Be to, glioblastomos pacientų grupė buvo suskirstyta į grupes pagal pacientų amžių ir analizuota miRNR santykinė ekspresija. Glioblastoma sergančių pacientų amžius svyravo nuo 29,38 iki 80,02 metų, vidutiniškai 58 metų. 39 glioblastoma sergantys pacientai, kurie buvo jaunesni už vidurkį, buvo priskirti „Jaunesniųjų" (min. 29,38; maks. 57,97; vid. 45,74), o likę 43 pacientai – „Vyresnio amžiaus" grupei (min. 58,72; maks. 80,02; vid. 69,43). Glioblastomos grupei kiekviena miRNR reikšmė buvo priskirta vienai iš tercilių: „Žema",

„Vidutinė“ ir „Aukšta“ santykinė išraiška. Mėginiai mažųjų RNR sekoskaitos eksperimentui (Studija I) buvo atsitiktinai atrinkti iš nepriklausomos glioblastomos ir ne vėžinės imčių: 6 kraujo mėginiai iš nesergančiųjų vėžiu, 7 glioblastomos naviko mėginiai ir 7 kraujo mėginiai iš tų pačių glioblastoma sergančių pacientų.

Mikro RNR raiškos nustatymas. Iš viso 20–40 mg šaldytų, pooperacinių naviko mėginių buvo mechaniškai sumalti ir homogenizuoti ultragarsu. MikroRNR buvo išskirtos iš homogenizuoto audinio naudojant mirVana miRNR izoliavimo rinkinį (kat. Nr.: AM1560, Invitrogen), atskiriant mažą RNR frakciją (mažiau nei 150 nt) nuo likusios RNR. Iš serumo mėginių, naudojant exoEasy rinkinį (kat. Nr.: 76064, Qiagen), buvo išskirtos mažosios RNR, patalpintos ekstraląstelinėse pūslelėse. Universali išskirtų mažų RNR kDNR biblioteka buvo susintetinta TaqMan Advanced miRNA cDNA sintezės rinkiniu (kat. Nr.: A28007, Applied Biosystems) iš 10 ng RNR. Iš anksto amplifikuota ir 10 kartų praskiesta kDNR buvo naudojama raiškos analizei RT-kPGR metodu, naudojant TaqMan Advanced miRNA Assays (Applied Biosystems). Duomenims normalizuoti buvo panaudotos 4 referentinės mikro RNR: hsa-miR-191-5p (zondo nr: 477952_mir), hsa-miR-361-5p (zondo nr: 478056_mir), hsa-miR-345-5p (zondo nr: 478366_mir), ir hsa-miR-103a-3p (zondo nr: 478253_mir). Visų protokolų buvo laikomasi pagal gamintojo rekomendacijas, išskyrus RT-kPGR reakciją, kuri buvo atlikta proporcingai sumažinant rekomenduojamą galutinį reakcijos tūrį nuo 20 µl iki 12 µl. RNR išskyrimo ir kDNR sintezės etapuose buvo pridėtas 0,0065 ng sintetinė mikro RNR (cel-miR-39-3p), kad būtų galima normalizuoti technines klaidas.

Mikro RNR sekoskaita. Mažųjų RNR sekoskaitai buvo panaudota iki 100 ng mažų (mažiau nei 150 nt) arba ekstraląstelinių RNR. Bibliotekos buvo sukurtos naudojant CATS Small RNA-seq rinkinį (kat. Nr.: C05010040, Diagenode) ir sekvenuotos naudojant MiSeq sekvenatorių (kat. Nr.: SY-410-1003, Illumina), taikant MiSeq Reagent kit v2 (kat. Nr.: MS-102-2001, Illumina), kad būtų gauta 50 bazinių porų nuskaitytos sekos. Sekvenavimo duomenys, analizuoti GenomeDK skaičiavimo klasteryje paleidžiant nf-core/smrnaseq analizės kodų seką. Trumpai, 1) neapdorotų sekos nuskaitymų kokybė buvo įvertinta FastQC programine įranga, 2) bibliotekos konstravimo ir sekos nustatymo adapteriai, pašalinti Trim Galore įrankiu, 3) dažniausiai pasitaikančios RNR sekos iš aplinkos, išfiltruotos naudojant Bowtie2, 4) apdorotos sekos, prilygintos prie referentinio miRnomo iš miRbase, 5) aptiktų žmogaus miRNR kiekio analizė naudojant DESeq2 programinę įrangą. Papildoma analizė atlikta taikant duomenų apdorojimą ir miRNR kiekių skirtumo palyginimą internetiniais įrankiais Oasis2 ir DEApp.

Neuropsichologinis įvertinimas. Glioblastoma sergančių pacientų neuropsichologinį vertinimą atliko medicinos psichologė dr. Aistė Pranckevičienė

Lietuvos Sveikatos Mokslų Universiteto Ligoninės Kauno klinikų Neurochirurgijos klinikoje, likus maždaug 3 dienoms iki naviko pašalinimo operacijos. Pacientai, kurie turėjo sunkių kognityvinių ar neurologinių sutrikimų, dėl kurių nepavyko atlikti visų tyrimo užduočių, buvo nevertinami. Neuropsichologinis vertinimas buvo atliktas analizuojant kiekvieno paciento su sveikata susijusios gyvenimo kokybės ir depresijos klausimynus. Abu klausimynai patvirtinti HRQOL vertinimui Lietuvos smegenų auglių pacientams [226]. Šiuos klausimynus sudarė Europos vėžio tyrimų ir gydymo organizacijos gyvenimo kokybės klausimynai QLQ-C30 [63] ir QLQ-BN20 [227]. QLQ-C30 klausimyno atsakymai atspindi pasaulinės sveikatos būklę, įvairius gebėjimus: socialinį, pažintinį ar emocinį funkcionavimą. QLQ-BN20 klausimynas papildo QLQ-C30 smegenų vėžiu sergantiems pacientams. Jos užduotis yra pašalinti simptomus, susijusius su smegenų augliais, pavyzdžiui, motorinių sutrikimų, regėjimo sutrikimų ar sunkumų, susijusių su ateities prognozėmis. Be to, funkcinei būklei įvertinti buvo naudojama Karnofsky skalė (KS) [228]. KS yra 11 balų vertinimo skalė, skirta įvertinti paciento gebėjimą atlikti įprastą veiklą ir priklausomybę nuo pagalbos ir slaugos.

Statistinė analizė. Pacientų išgyvenamumo laikas po gydymo buvo įvertintas Kaplan-Meier metodu, naudojant log-rank testą ir Cox proporcingą pavojaus analizę. Student T testas buvo pritaikytas dviejų grupių ekspresijos skirtumams įvertinti, po to, kai buvo atliktas F testas, siekiant nustatyti dviejų grupių dispersiją. Spearman koreliacija buvo naudojama vertinant ryšį tarp miRNR ekspresijos ir pacientų neuropsichologinių bei KS rodiklių, o Pearson koreliacija naudota vertinant miRNR raišką su amžiumi, išgyvenamumo po operacijos laiku, auglio tūriu bei audinio ir EV miRNR raiškos tendencijas. Buvo sukurti logistinės ir linijinės regresijos modeliai, skirti įvertinti analizuojamų miRNR diagnostines ir ligos išeities savybes. Visi statistiniai testai buvo atlikti Python naudojant Lifelines, Statsmodels ir Sklearn modulius. Sprendimų medžio klasifikacijos analizė gliomos išgyvenamumo prognozei, įvertinant miR-181 raišką ir kitus veiksnius, atlikta naudojant klasifikavimo ir regresijos medžių (CRT) algoritmą su Gini metodo netiesiniais deriniais. Klasifikuojant gliomos išgyvenamumą pagal ekstraląstelinių pūslelių duomenis, klasifikatorius testuotas su 60 proc. duomenų, nustačius maksimalus 5 sluoksnių medžio gylį ir pritaikant entropijos vertinimo metodą. Naudojant naviko miRNR raiškos duomenis, testavimo imtis sudarė 20 proc. duomenų rinkinio, didžiausias gylis buvo 3 ir buvo naudojamas entropijos vertinimo metodas. Tie patys parametrai buvo naudojami gliomos laipsnio klasifikavimui naudojant naviko miRNR duomenų rinkinį. Sprendimų medžiai buvo vizualizuoti naudojant DtreeViz programinę įrangą. Visų statistinių testų reikšmingumo lygiai buvo apibrėžti kaip $p < 0,05$ (*), $p < 0,01$ (**) ir $p < 0,001$ (***).

REZULTATAI

Pirmoji dalis

MiRNR paieška pritaikant mažųjų RNR sekoskaitą. Mažųjų RNR sekoskaita buvo atlikta iš 7 glioblastomos (GB) sergančių pacientų serumų, tų pačių pacientų pooperacinio GB audinio ir 6 vėžiu nesergančių pacientų serumų. Sekvenavimo rezultatai parodė, kad 3 miRNR turi potencialą būti naudojamos minimaliai invaziniam GB diagnostiniui testui. Lyginant ne vėžinius kraujo mėginius su miRNR ekspresija iš GB serumo ekstraląstelinių pūslelių, miR-126-5p, miR-338-3p ir miR-338-5p raiška buvo padidėjusi GB pacientų kraujyje ($\log_2^{\text{Kartų pokytis}} = 1,985$; 2,109; 1,748; FDR pakoreguotasis $p = 0,041$; 0,032; 0,049; atitinkamai kiekvienai aukščiau išvardintai miRNR). Be to, šios miRNR buvo panašiai ekspresuojamos tiek GB sergančių pacientų pooperaciniame audinyje, tiek tų pačių pacientų serumo ekstraląstelinėse pūslelėse ($\log_2^{\text{Kartų pokytis}} = –1,058$; –0,638; –0,806; FDR pakoreguotasis $p = 0,480$; 0,331; 0,302; atitinkamai). Reikšmingi raiškos skirtumai tarp GB pacientų EV ir ne vėžiu sergančių pacientų EV reiškė, kad miR-126-5p ir miR-338-3p/5p ekspresija gali indikuoti apie glioblastomos atsiradimą. Be to, nereikšmingas šių miRNR ekspresijos skirtumas tarp GB audinio ir GB tarpląstelinių pūslelių rodo, kad miR-126-5p ir miR-338-3p/5p gaminamos daugiausia kaip vėžio ląstelių signalinės molekulės.

Šios miRNR buvo patikrintos atskiroje GB imtyje ($n = 39$), atvirkštinės transkripcijos kiekybinio polimerazinės grandininės reakcijos (AT-kPGR) metodu. Tik miR-338-5p rezultatai sutapo tarp miRNR sekoskaitos ir AT-kPGR rezultatų. AT-kPGR metodu, miR-126-5p raiška GB EV buvo reikšmingai didesnė negu GB audinyje –2.0 santykinė raiška ($-\Delta Ct$) ($p < 0.001$). O miR-338-3p raiška buvo didesnė GB audinyje negu tų pačių pacientų EV (–1.67 prieš –3.87 ($-\Delta Ct$); $p = 0.010$). Tuo tarpu, miR-338-5p raiška GB audinyje buvo –6.35 ($-\Delta Ct$), o tų pačių pacientų EV buvo –7.58 ($-\Delta Ct$). Šis nustatytas raiškos skirtumas nebuvo reikšmingas lyginant miR-338-5p raišką tarp dviejų grupių ($p = 0.161$) ar lyginant individualų raiškos pokytį kiekviename paciente ($p = 0.128$). Rezultatai iš AT-kPGR patvirtino, kad miR-338-5p ekspresija iš pacientų GB audinių atsispindi pacientų kraujyje, todėl tai yra perspektyvi miRNR ankstyvai diagnostikai arba gydymo efektyvumo stebėjimui.

Tačiau pasirinkta maža RNR sekoskaitos strategija, turinti mažą sekos nustatymo gylį, bet didesnį pakartojimų skaičių, lėmė mažą aptiktų miRNR skaičių galutiniuose sekoskaitos rezultatuose. Unikalių miRNR aptikimas svyravo nuo 239 iki 1088, vidutiniškai 472. Todėl tolesnė taikinių atranka buvo tęsiama iš literatūros analizės.

Antroji dalis

MiRNR raiška smegenų audinyje. MiRNR raiška buvo analizuojama 1) ne vėžinėse (n = 4), 2) II/III laipsnio gliomos – žemo laipsnio gliomos (LGG) (n = 15) ir 3) 4 laipsnio gliomos – glioblastomos (GB) (n = 82) smegenų audiniuose. Visų analizuotų mikro RNR vidutinė santykinė raiška nevėžinių, LGG ir GB smegenų audinių mėginiuose buvo –1,12; –1,37; ir –0,65 (-ΔCt). Vien potencialių onko-miRNR vidurkis buvo –1,45; –1,10; ir 0,25 (-ΔCt), o onkosupresorinių-miRNR vidurkis –0,30; –2,04; ir –2,90 (-ΔCt) atitinkamai.

Individualiu miRNR lygiu reikšmingi raiškos pokyčiai tarp visų mėginių grupių buvo aptikti tiriant hsa-miR-21-5p ir hsa-miR-10b-3p. Santykinė onkogeninio miR-21-5p ir miR-10b-3p raiška teigiamai koreliavo su piktybiškesniu gliomos laipsniu. Vidutinė miR-21-5p ir miR-10b-3p raiška ne vėžiniame, LGG ir GB smegenų audinyje buvo 0,08; 1,37; 4,06 ir –12,57; –10.55; –6,31 (-ΔCt) atitinkamai. Kitos miRNR taip pat buvo reikšmingai skirtingai ekspresuojamos skirtinguose audinių tipuose. Galima teigti, kad svarbiausi išraiškos pokyčiai yra tarp nevėžinių ir LGG audinių grupių, nes tai rodo ankstyvas vėžinių procesų stadijas. Lyginant šias dvi grupes, reikšmingi pokyčiai buvo aptikti tiek onkosupresinėse, tiek onkogeninėse miRNR, iš kurių onkosupresinė miR-7-5p turėjo ženkliausią raiškos pokytį tarp ne vėžinės grupės (1,79 -ΔCt) ir LGG grupės (–1,56 -ΔCt). Lyginant šias dvi grupes, miR-143-3p, miR-21-5p ir miR-10b-3p raiškos pokyčiai buvo taip pat reikšmingi. Dauguma miRNR ekspresijos pokyčių buvo aptikti tarp LGG ir GB audinių grupių, kur 9 iš 14 miRNR buvo ekspresuojamos reikšmingai skirtingai. Iš kurių onkogeninio miR-221-5p raiška buvo aptikta beveik tokia pati ne vėžinių ir GB grupių, tačiau LGG grupėje ji buvo reikšmingai mažesnis (–4,87 -ΔCt), lyginant su GB grupe (–3,68 -ΔCt) (p = 0,005).

Logistinės regresijos modelis naudotas klasifikuoti pacientų audinių mėginius į LGG ir GB gliomų grupes, atsižvelgiant į analizuojamą miRNR raišką. Kai visos miRNR buvo paimtos kartu, modelio tikslumas buvo 90 proc.. Sumažinus miRNR skaičių iki 5 perspektyviausių miRNR, kurios individualiai buvo reikšmingiausiai išreikštos tarp LGG ir GB grupių, logistinės regresijos modelis vis tiek išlaikė tą patį tikslumą. Labiausiai tikėtina, kad bus diagnozuota glioblastoma, buvo priskirtas onkogeniniam miR-10b-3p. Modelis numatė, kad miR-10b-3p santykinės išraiškos padidėjimas vienu sveikuoju skaičiumi padidino GB diagnozės tikimybę 148 proc.. Šiame modelyje onkosupresinio miR-139-5p sumažėjimas buvo susijęs su mažiau piktybine diagnoze. Kaskart padidėjus miR-139-5p ekspresijai, tikimybė, kad bus diagnozuota GB, sumažėjo 26 proc.. Kitos šiame modelyje tiriamos miRNR pa-

didino GB diagnozės tikimybę 26 proc. (miR-148a-3p), 46 proc. (miR-193a-5p) ir 57 proc. (miR-21-5p).

Panašų miRNR rinkinį pasiūlė sprendimų medžio klasifikatoriaus algoritmas. Šis klasifikatorius taip pat pasirinko navikinę miR-10b-3p kaip svarbiausią veiksnį gliomos laipsnio pasirinkimui. Didesnė miR-10b-3p raiška (daugiau nei –9,11 -ΔCt) buvo pasiūlyta kaip pagrindinis žemesnio laipsnio (II ir III laipsnio) ir aukštesnio laipsnio (IV laipsnio) gliomos atskyriklis. Skirtingai nuo logistinės regresijos modelio, sprendimų medis parodė auglio miR-139-5p ekspresijos padidėjimą kaip žemesnio laipsnio gliomos faktorių. Visų pirma, miR-139-5p ekspresija, didesnė nei –2,03 (-ΔCt), parodė žemesnio laipsnio gliomos diagnozę. Šio klasifikatoriaus tikslumas siekė 94,11 proc., o svertinis tikslumas, atšaukimas ir f1-įvertis atitinkamai buvo 98 proc., 94 proc. ir 97 proc..

Lyginant miRNR raišką su skirtingomis gliomos stadijomis, kai kurių miRNR raiškos tendencija buvo papildomai susijusi su paciento amžiumi. Vyresnio amžiaus pacientams, o ne jaunesniems, onkosupresinio miR-7-5p raiška mažėjo, kai didėjo gliomos piktybiškumo laipsnis. Priešinga tendencija nustatyta su onkogeniniu miR-10b-3p, kurio raiška jaunesniems pacientams padidėjo labiau nei vyresnio amžiaus pacientams piktybėjant gliomai. Nors ir nereikšmingas, bet nuolat didesnis onkogeninio miR-17-5p lygis buvo aptiktas jaunesniems pacientams visose smegenų audinių grupėse. Onkosupresinės miR-139-5p raiška reikšmingai nesiskyrė tarp nesergančių vėžiu ir LGG grupių, tačiau suskirsčius šias grupes į amžiaus grupes, paaiškėjo, kad jaunesniems pacientams miR-139-5p raiška buvo linkusi mažėti esant žemiausio laipsnio gliomai (II laipsnis), palyginti su ne vėžinių audinių grupe, bet buvo didesnis sergant piktybiškiausiu gliomos tipu (IV laipsnis/GB). Panašūs raiškos skirtumai buvo pastebėti su onkosupresine miR-143-3p tarp gliomos laipsnių, lyginant jaunesnius ir vyresnius pacientus. Įdomu tai, kad onkosupresinės miR-193a-5p raiškos pokytis skyrėsi priklausomai nuo paciento amžiaus. Jaunesniems pacientams miR-193a-5p raiška buvo mažesnė gliomos piktybėjimo metu ir šiek tiek padidėjo GB grupėje. Tuo tarpu vyresnio amžiaus pacientams miR-193a-5p raiška progresyviai didėjo kiekvienoje gliomos stadijoje. Palyginus miRNR raišką tarp jaunesnių ir vyresnių pacientų grupių, pastebimų miR-148a-3p, miR-181b/d-5p, miR-221-5p ir miR-335-5p raiškos pokyčių neaptikta.

Be to, jaunesni GB pacientai su mutavusiu *IDH1* genu turėjo reikšmingai mažesnę miR-21-5p raišką, palyginus su GB *IDH1* laukinio tipo jaunesniais pacientais: raiškos mediana buvo atitinkamai 4,14 ir 1,1 (-ΔCt) ($p = 0,021$). MiR-148a-3p buvo mažiau išreikšta jaunesniems GB pacientams, sergantiems *IDH1* mutacija (-ΔCt mediana –3,35), lyginant su laukinio tipo *IDH1* pacientais (-ΔCt mediana –1,00) ($p = 0,009$). Nors ir nereikšmingas, bet miR-34a-

5p taip pat buvo mažiau ekspresuojama jaunesniuose, su *IDH1* mutacija, GB pacientams (-ΔCt mediana –0,13), lyginant su jaunesniais *IDH1* laukinio tipo GB pacientais (-ΔCt mediana 2,03). Nebuvo aptikta jokių pastebimų pokyčių visose kitose analizuotose miRNR, lyginant jų raišką tarp *IDH1* laukinio tipo ir mutavusių GB audinių mėginių. Lyginant miRNR raiškos pokyčius, atsižvelgiant į *MGMT* promotoriaus metilinimo būseną, miR-193a-5p raiška jaunesniems GB pacientams buvo reikšmingai mažesnė, palyginti su vyresniais GB pacientais, tiek metilinto *MGMT* promotoriaus ($p = 0,047$), tiek nemetilinto ($p = 0,021$). grupėse. Tuo tarpu miR-221-5p raiška buvo didesnė tik vyresnio amžiaus pacientuose su metilintu *MGMT* promotoriumi, palyginus su analogiškais jaunesniais GB pacientais ($p = 0,012$). Kitų analizuotų miRNR raiška skirtingose *MGMT* promotoriaus metilinimo grupėse reikšmingai nesiskyrė.

MiRNR glioblastomos audinio raiška ir pacientų gyvenimo kokybės rodikliai.

Nustatyta, kad didesnis miR-34a-5p ir miR-181d-5p raiškos lygis naviko audinyje yra susijęs su geresniu pacientų fiziniu funkcionavimu ($p < 0,05$ abiem miRNR), o miR-181b-5p padidėjimas koreliuoja su geresniu pacientų socialiniu funkcionavimu ($p < 0,05$). Tuo tarpu bendras glioblastoma sergančių pacientų funkcionavimas koreliavo tik su naviko miR-34a-5p raiška. Nors ir nereikšminga, tačiau socialinio ir emocinio funkcionavimo gerėjimo tendencija buvo pastebėta didėjant atitinkamai miR-34a-5p ir miR-181b-5p raiškai. Be to, glioblastoma sergančių pacientų būklė, remiantis Karnofskio skale, teigiamai koreliavo su miR-34a-5p raiška ($p < 0,05$).

Glioblastomos tūris ir miRNR audinio raiška.

Nustatyta reikšminga koreliacija tarp glioblastomos tūrio ir miR-21-5p raiškos (Pearsono $r = –0,52$, $p < 0,01$). Beveik vienodai, tiek vyrams, tiek moterims, GB pacientams, kurių naviko tūris buvo didesnis, miR-21-5p raiška buvo mažesnė. Kalbant apie navikinį miR-148a-3p, jos raiška neigiamai koreliavo su naviko tūriu glioblastoma sergančių pacientų vyrų pogrupyje (Pearsono $r = –0,68$, $p < 0,05$). Nepaisant GB pacientų lyties, miR-148a-3p taip pat reikšmingai koreliavo su naviko tūriu (Pearsono $r = –0,51$, $p < 0,01$). Visuose glioblastomos mėginiuose ir vyrų pogrupyje buvo pastebėta teigiama koreliacija su miR-181d-5p (Pearsono $r = 0,43$; $0,83$, $p < 0,05$; $< 0,01$). Silpniausias ryšys tarp GB tūrio ir miRNR raiškos buvo aptiktas matuojant miR-93-5p raišką (Pearsono $r = 0,09$, $p = 0,65$).

MiRNR raiška gliomos ekstraląstelinėse pūslelėse.

Tik dvi iš 14 analizuotų miRNR turėjo reikšmingai skirtingą raišką pacientų ekstraląstelinėse kraujo serumo pūslelėse, lyginant LGG ir GB pacientų mėginius. Jaunesniems glioblastoma sergantiems pacientams miR-21-5p raiška serumo ekstraląstelinėse pūslelėse buvo reikšmingai didesnė (-ΔCt 2,58), nei vyresnio amžiaus GB pacientams (-ΔCt 2,24) ($p = 0,006$). Kai buvo įtraukta *MGMT* promotoriaus metilinimo būsena, miR-221-5p raiška reikšmingai skyrėsi tarp jaunesnių pacientų, turinčių metilintą (-ΔCt –4,07) ir nemetilintą (-ΔCt –5,71) *MGMT* promotorių ($p = 0,039$). Vyresnio amžiaus pacientams, turintiems skirtingą *MGMT* promotoriaus statusą, nebuvo pastebimo skirtumo tarp šios miRNR raiškos. Tačiau reikšmingai skirtinga miR-221-5p raiška buvo nustatyta tarp skirtingų GB pacientų amžiaus grupių, turinčių metilintą *MGMT* promotorių. Jaunesnių GB pacientų kraujo serumo ekstraląstelinėse pūslelėse santykinė miR-221-5p raiška buvo –4,07 (-ΔCt), o vyresnio amžiaus pacientų vidutinė miR-221-5p raiška buvo –6,77 (-ΔCt) ($p = 0,037$). Tiek miR-10b-3p, tiek miR-7-5p pasižymėjo panašiu raiškos lygiu skirtingose gliomos stadijose: 1) didesnė raiška sergant II laipsnio glioma, 2) sumažėjusi raiška sergant III laipsnio glioma ir 3) GB raiškos lygis panašus į II laipsnio glioma. Be to, santykinė miR-139-5p, miR-17-5p raiška buvo nuosekliai aptikta panašiu lygiu visų gliomos laipsnių pacientų kraujo mėginiuose.

Glioblastomos tūris ir ekstraląstelinė miRNR raiška.

Įdomu tai, kad ekstraląstelinės miR-7-5p raiška koreliavo tik su GB sergančių moterų naviko tūriu (Pearson $r = –0,56$, $p < 0,05$). Nors ir nereikšmingas, bet labai panašus raiškos lygis buvo pastebėtas tiriant miR-93-5p (Pearson $r = –0,37$, $p = 0,16$). Tuo tarpu miR-193a-5p ir miR-221-5p didelių skirtumų tarp lyčių neparodė. Ekstraląstelinės miR-193a-5p raiška neigiamai koreliavo su GB naviko tūriu (Pearson $r = –0,40$, $p = 0,05$), o miR-221-5p buvo teigiamai susieta su GB tūriu (Pearson $r = 0,40$, $p < 0,05$).

MiRNR ekstraląstelinių pūslelių raiška ir gliomos pacientų gyvenimo kokybė.

GB sergančių pacientų ekstraląstelinėse pūslelėse miR-34a-5p bei miR-181b-5p raiška nekoreliavo su jokiu Europos vėžio tyrimų ir gydymo organizacijos gyvenimo kokybės klausimyno C30 kriterijumi. Tačiau nustatyta, kad sumažėjusi ekstraląstelinio miR-181d-5p raiška buvo susijusi su GB sergančių pacientų fizinės ir emocinės būsenos pagerėjimu (atitinkamai $p < 0,05$ ir $p < 0,01$). Didėjanti ekstraląstelinio miR-181d-5p raiška taip pat buvo susijusi su pacientų kognityvinėmis funkcijomis ir bendra funkcionavimo būkle, tačiau šios sąsajos nebuvo statistiškai reikšmingos ($p < 0,05$). Jokia analizuotos

miRNR ekstraląstelinė raiška neparodė tendencijos būti susijusi su GB sergančių pacientų produktyvumu, vertinant pagal Karnofskio skalę ($p < 0,05$).

MiRNR raiška ir glioblastomos pacientų gyvenimo trukmė po operacijos.

Mažesnė miR-181b-5p raiška GB audinyje buvo susijusi su ilgesniu pacientų išgyvenimo laiku, lyginant su vidutine miR-181b-5p raiška ($p = 0,003$). Panašus ryšys tarp audinio miR-181b-5p raiškos ir GB pacientų išgyvenimo trukmės atsispindėjo tik kaip tendencija prognozuojant išgyvenimo trukmę pagal ekstraląstelinę miR-181b-5p raišką ($p = 0,183$).

Siekiant nustatyti navikinių ir tarpląstelinių miR-181 raiškos pokyčio reikšmę gliomos pacientų išgyvenimo trukmei, atlikta sprendimų medžio klasifikatoriaus analizė. Klasifikatorius taip pat apėmė žinomus gliomos biožymenis kaip *IDH1* genotipą ir *MGMT* promotoriaus metilinimo statusą. Be to, vertinant išgyvenamumo trukmę taip pat buvo įtraukti su naviku susiję simptomai, gyvenimo kokybės indeksas ir funkcinė paciento būklė. Bendras klasifikatoriaus tikslumas buvo 82,2 proc.. Prognozės tikslumas buvo 90,6 proc. trumpo išgyvenamumo pogrupyje (< 16,85 mėn., n = 64), tačiau buvo mažesnis (67,6 proc.) ilgo išgyvenamumo grupėje (> 16,85 mėn., n = 37), o tai rodo, kad šis pogrupis turėjo heterogeniškesnių bruožų. Sprendimų medžio klasifikatorius parodė, kad pacientams, sergantiems gliomomis, kuriems buvo *IDH1* laukinio tipo genotipas, mažesnė miR-181d-5p ir didesnė miR-181b-5p naviko raiška, greičiausiai bus trumpas išgyvenimas po operacijos. Tuo tarpu ilgesnis išgyvenamumas buvo susijęs su *IDH1* mutacija, sunkiais naviko simptomais ir didesne miR-181b ekstraląsteline raiška.

Be to, buvo sukurtas prognozuojamasis gliomos paciento išgyvenamumo modelis, įvertinus visas 14 analizuotų navikų miRNR atskirai ir kaip rinkinį, paciento amžių/lytį, IDH1 genotipą, MGMT promotoriaus metilinimo būseną ir gliomos laipsnį. MiRNR rinkinys buvo sudarytas pagal Cox proporcingą pavojaus analizę iš glioblastoma sergančių pacientų duomenų, atrinkus 5 reikšmingas miRNR: miR-34a-5p, miR-221-5p, miR-17-5p, miR-143-3p ir miR-139- 5p. Nuspėjamasis modelis pasiekė 66,6 proc. tikslumą ir svertinį vidurkį: 1) 67 proc. tikslumo, 2) 67 proc. atšaukimo ir 3) 65 proc. f1-įvertis. Šis klasifikatorius didžiausią reikšmę skyrė pacientų amžiui, miR-143-3p ir miRNR rinkinio išraiškai. Modelis nustatė daugiausia trumpą išgyvenamumo laiką (< 11,53 mėnesio) pacientams, kurie buvo vyresni nei 49,93 metų ir kurių miRNR rinkinio balas buvo didesnis nei 4,06. Tuo tarpu jaunesni nei 49,93 metų pacientai, kurių naviko miR-143-3p ekspresija buvo mažesnė nei 0,93 ($-\Delta Ct$), buvo priskirti ilgam išgyvenamumui (nuo 21,45 iki 41,79 mėnesio). Atsižvelgiant į šį savybių rinkinį, nei IDH1, nei MGMT genai, nei gliomos laipsnis nebuvo pasiūlyti kaip svarbios gliomos išgyvenimo laiko prognozavimo savybės.

Praktiškesniam gliomos baigties prognozavimui buvo sukurtas nuspėjamasis gliomos išgyvenimo modelis, atsižvelgiant į pacientų amžių, lytį, IDH1 genotipą, MGMT promotoriaus metilinimo būseną, gliomos laipsnį ir ekstraląsteliniu būdu ekspresuotą 14 analizuotų miRNR ir jų rinkinį. Ekstraląstelinių miRNR rinkinį sudarė 4 miRNR, kurios buvo reikšmingai susijusios su glioblastomos paciento išgyvenimu, atliekant Cox proporcingą pavojaus analizę: miR-181d-5p, miR-221-5p, miR-17-5p ir miR-335-5p. Modelio veikimas buvo šiek tiek prastesnis, palyginti su modeliu, kuriame buvo naudojama naviko miRNR ekspresija. Šio klasifikatoriaus tikslumas buvo 60,1 proc., o tikslumo, atšaukimo ir f1-įverčio svertinis vidurkis atitinkamai buvo 66 proc., 61 proc. ir 59 proc.. Pacientų amžius vis dar išliko svarbiausias šio klasifikatoriaus požymis. Nepaisant to, tarpląstelinis miR-7-5p, miR-10b-3p ir miR-139-5p buvo 3 geriausios miRNR, kurios buvo laikomos prognozuojant glioma sergančių pacientų išgyvenamumą. Vyresnio amžiaus pacientams (> 49,03 m.), kurių ekstraląstelinė miR-7-5p ekspresija buvo didesnė (> –0,117 (-ΔCt)) ir didesnė ekstraląstelinė miR-139-5p ekspresija (> 0,75 (-)), buvo numatytas trumpesnis išgyvenimo laikas. dCt)). Nors kiti ekstraląstelinės miRNR ekspresijos deriniai taip pat lėmė trumpą (< 11,53 mėnesio) gliomos pacientų baigties prognozę.

Nors ir nežymiai, tačiau pastebimas GB pacientų išgyvenamumo skirtumas tarp pacientų, kurių miR-193-5p arba miR-34a-5p raiška buvo maža ir didelė ($p = 0,132$; $p = 0,151$). Taip pat buvo pastebėta ilgesnio išgyvenamumo tendencija GB pacientams, kurių kraujo serumo ekstraląstelinėse pūslelėse buvo didelė onkogeninio miR-21-5p raiška ($p = 0,068$). Tuo tarpu GB pacientai, kurių ekstraląstelinė miR-34a-5p raiška buvo žema, išgyveno ilgiau nei pacientai, kurių miR-34a-5p ekspresija buvo vidutinė arba aukšta ($p = 0,033$; $p = 0,03$).

Nepaisant nereikšmingo naviko miR-221-5p raiškos poveikio GB pacientų išgyvenamumo laikui, parodyto Kaplan-Meier išgyvenamumo kreivėmis ir log-rank testu, buvo nustatyta, kad jaunesnių moterų, kurioms diagnozuota GB, išgyvenamumo laikas vidutiniškai neigiamai koreliavo su naviko miR-221-5p raiška ($r^2 = 0,62$). Nebuvo aptikta jokių kitų pastebimų koreliacijų tarp glioblastoma sergančių pacientų amžiaus grupės, lyties, audinių miRNR raiškos ir bendro išgyvenimo laiko po naviko pašalinimo operacijos. Be to, silpna koreliacija tarp ilgesnio išgyvenamumo ir mažesnės ekstraląstelinės miR-139-5p ir miR-34a-5p raiškos taip pat buvo pastebėta jaunesnėms moterims ($r^2 = 0,36$ ir $0,27$). Vyresnės moterys išgyveno ilgiau, jei jų ekstraląstelinė miR-21-5p raiška buvo didesnė, tačiau koreliacija buvo labai silpna ($r^2 = 0,12$). Lyginant vyrų ir moterų, jaunesnių ir vyresnių ar skirtingą *MGMT* promotoriaus metilinimą turinčius GB pacientus, reikšmingų skirtumų tarp išgyvenamumo trukmės nenustatyta išgyvenamumą reikšmingų skirtumų ne-

nustatyta. Nors jaunesnių moterų tendenciją į ilgesnį išgyvenimą buvo pastebėta.

Galiausiai, norint įvertinti, kaip skaitiniai miRNR raiškos pokyčiai yra susiję su GB sergančių pacientų išgyvenimu, Cox proporcinga pavojaus analizė buvo atlikta su visų analizuotų miRNR rinkiniu. Analizuojant miRNR, reikšmingą įtaką GB pacientų išgyvenamumui turėjo naviko raiška: miR-34a-5p, miR-221-5p, miR-17-5p, miR-143-3p ir miR-139-5p. Be to, buvo tiriama miRNR raiška iš ekstraląstelinių pūslelių, siekiant nustatyti reikšmingiausias miRNR, kad būtų galima mažiausiai invaziniu būdu stebėti pacientų būklę. Reikšmingos tarpląstelinės miRNR, susijusios su GB pacientų išgyvenimo laiku, buvo: miR-181d-5p, miR-221-5p, miR-17-5p ir miR-335-5p. Nereikšminga dėl plataus pasikliautinojo intervalo, tačiau veiksmingiausia miRNR pacientų išgyvenimo pavojaus poslinkiui buvo miR-93-5p, išmatuota tiek glioblastomos audinyje, tiek serumo ekstraląstelinėse pūslelėse. Kiekvieną kartą padidinus santykinę naviko miR-93-5p raišką, pradinis pavojus padidėjo 69 proc.. Ekstraląsteliniu būdu išreikšta miR-93-5p turėjo dar stipresnį poveikį, padidindama pradinį pavojų 194 proc., kiekvienu sveiku skaičiumi padidėjus jos santykinei raiškai.

IŠVADOS

1. MiR-21-5p, miR-10b-3p navikinė raiška nuosekliai buvo skirtingai išreikšta ne vėžiniuose smegenų, žemo laipsnio gliomos ir glioblastomos audiniuose, todėl šios miRNR yra perspektyviausi kandidatai kuriant naują molekulinės diagnostikos testą gliomos piktybiškumo laipsniui nustatyti. Tarpląstelinė miR-10b-3p ir miR-7-5p raiška buvo reikšmingai skirtingai išreikšta tarp žemo laipsnio gliomos ir glioblastomos mėginių, o tai leidžia toliau tirti šias miRNR minimaliai invaziniam gliomos progresavimo nustatymui.
2. MiR-338-5p, miR-17-5p, miR-93-5p ir miR-193a-5p raiška buvo panašiausia to paties paciento glioblastomos audinyje ir ekstraląstelinėse pūslelėse, o tai reiškia, kad šios miRNR yra perspektyvios kandidatės tyrimams stebėti mažiausius glioblastomos gydymo efektyvumus, nes jų navikų raiška atsispindi tarpląstelinėse kraujo pūslelėse.
3. MiR-181b-5p raiška buvo reikšmingai susijusi su glioblastoma sergančių pacientų išgyvenamumo laiku po naviko pašalinimo operacijos. Priešingai, *IDH1* genotipas arba *MGMT* promotoriaus metilinimo būsena nebuvo reikšmingai susijusi su glioblastoma sergančių pacientų išgyvenimo laiku. Dėl šios priežasties navikinė miR-181b-5p raiška yra pranašesnė prognozuojant glioblastoma sergantiems pacientams išgy-

venimo trukmę negu *IDH1* arba *MGMT* promotoriaus metilinimo nustatymas.

4. Stipriausias poveikis mirties rizikai po glioblastomos šalinimo operacijos buvo nustatytas navikinės miR-221-5p, miR-17-5p ir ekstraląstelinės miR-17-5p raiškos derinį. Šios miRNR yra perspektyvios tyrimų kandidatės glioblastomos gydymo tobulinimui ksenografтų modeliuose.
5. Sukurta keletas gliomos pacientų išgyvenamumo modelių, nustatant svarbiausių molekulinių savybių derinius. Trumpo išgyvenamumo prognoziniai rodikliai: *IDH1* laukinio tipo genotipas, mažesnė miR-181d-5p ir didesnė miR-181b-5p raiška navikiniame audinyje, tuo tarpu geresnė išgyvenamumo prognozė susijusi su *IDH1* mutacija (R132H) ir didesne miR-181b ekstraląsteline raiška.
6. Į išgyvenamumo trukmės modelį įtraukiant visas analizuotas miRNR, nustatytos geresnės išgyvenimo prognozės pacientams, kurie buvo jaunesnio amžiaus, turėjo mažesnę naviko miR-143-3p raišką arba didesę miR-143-3p raišką, bet derinyje su mažesne miR-10b-3p raiška. Trumpesnis gliomos išgyvenamumas, susijęs su vyresniu pacientų amžiumi, esant didesnei 5 naviko miRNR rinkinio raiškai ir didesnei naviko miR-335-5p raiškai.

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PUBLICATIONS

Thesis publications:

1. Skiriutė, Daina; **Stakaitis, Rytis**; Steponaitis, Giedrius; Tamašauskas, Arimantas; Vaitkienė, Paulina. *The Role of CASC2 and miR-21 Interplay in Glioma Malignancy and Patient Outcome* // International journal of molecular sciences. Basel : MDPI. ISSN 1422-0067, 2020, vol. 21, no. 21, p. 1-9. doi:10.3390/ijms21217962. Link: . Science Citation Index Expanded (Web of Science); MEDLINE. [S1] [Field of science: N010, N004] [Impact factor: 5.923, aggregate impact factor: 6.387, quartile: Q1 (2020. InCites JCR SCIE)]
2. **Stakaitis, Rytis**; Pranckevičienė, Aistė; Steponaitis, Giedrius; Tamašauskas, Arimantas; Bunevičius, Adomas; Vaitkienė, Paulina. *Unique Interplay Between Molecular miR-181b/d Biomarkers and Health Related Quality of Life Score in the Predictive Glioma Models* // International journal of molecular sciences. Basel : MDPI. ISSN 1422-0067, 2020, vol. 21, iss. 20, p. 1-15. doi:10.3390/ijms21207450. Link: . Science Citation Index Expanded (Web of Science); MEDLINE. [S1] [Field of science: N010, N004, M001] [Impact factor: 5.923, aggregate impact factor: 6.387, quartile: Q1 (2020. InCites JCR SCIE)]
3. Vaitkienė, Paulina; Pranckevičienė, Aistė; **Stakaitis, Rytis**; Steponaitis, Giedrius; Tamašauskas, Arimantas; Bunevičius, Adomas. *Association of miR-34a expression with quality of life of glioblastoma patients: a prospective study* // Cancers. Basel : MDPI. ISSN 2072-6694, 2019, vol. 11, no. 3, p. 1-11. doi:10.3390/cancers11030300. Link: . Science Citation Index Expanded (Web of Science); MEDLINE; Scopus. [S1] [Field of science: M001] [Impact factor: 6.126, aggregate impact factor: 4.837, quartile: Q1 (2019. InCites JCR SCIE)]

Non-thesis publications:

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2. **Stakaitis, Rytis**; Pranckevičienė, Aistė; Steponaitis, Giedrius; Tamašauskas, Arimantas; Bunevičius, Adomas; Vaitkienė, Paulina. Prognostic potential of microRNAs expression levels in different grade glioma // COINS 2019 - 14th international conference of life sciences : abstracts book : [February 26-28, 2019, Vilnius, Lithuania] / Vilnius University Students Representation. Vilnius : Vilnius University Students Representation, 2019, p. 52-53. [T2] [Field of science: N010, M001]
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Association of miR-34a Expression with Quality of Life of Glioblastoma Patients: A Prospective Study

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Received: 4 February 2019; Accepted: 26 February 2019; Published: 4 March 2019

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Abstract: MiR-34a acts as tumor-suppressor by targeting many oncogenes related to proliferation, apoptosis, and invasion of gliomas. We studied the relationships between health-related quality of life (HRQOL), depression, and miR-34a expression status in patients with newly diagnosed glioblastoma (GBM). A comprehensive HRQOL assessment was completed by 38 patients with glioblastoma prior to surgical resection and included the European Organization for Research and Treatment of Cancer (EORTC) questionnaire for cancer patients (QLQ-C30) and the Brain Cancer-Specific Quality of Life Questionnaire (QLQ-BN20), the Patient Health Questionnaire-9 (PHQ-9), the Karnofsky performance index (KPS), and The Glasgow Outcome Scale (GOS). The miR-34a expression in glioblastoma tissue was measured using quantitative reverse transcription PCR. Our findings show that lower miR-34a expression is significantly associated with higher tumor volume, worse physical functioning, lower KPS, and greater depressive symptom severity of GBM patients. Moreover, analysis reveals that miR-34a effects might be gender specific, as stronger relationships between miR-34a and patient functioning measures were observed in males when compared to females. Despite the fact that, due to small sample size, our results should be considered as preliminary, our study suggests that miR-34a is associated with tumor burden and can be important for health-related quality of life, functional status, and mood symptoms of glioblastoma patients.

Keywords: glioblastoma; health-related quality of life; miR-34a expression; depression; survival; prognosis

1. Introduction

Glioma is a rare and often devastating disease associated with significant functional impairment and short survival time [1,2]. Prediction of outcomes after brain tumor surgery is critical for treatment guidance and optimized use of healthcare resources. Currently, besides traditional outcome measures such as overall survival, progression-free survival, and radiological response to treatment, the value of patient-centered outcome measures is widely acknowledged [3]. Health-related quality of life (HRQOL), as an outcome measure, reflects the patient's perspective on his or her disease, covering physical, psychological, and social aspects of patient's functioning, as well as symptoms induced by the disease and/or its treatment [4]. Decreased HRQOL and depression in patients are sensitive

predictors of shorter survival in glioma patients [3]. However, patient-centered outcome measures are rarely investigated in relation to biological biomarkers.

The need for glioma biomarkers with improved sensitivity and specificity has inspired research of small non-coding micro-RNAs (miRNAs). Previous studies report that MIR34A (miR-34a) can directly down-regulate several target mRNAs which encode proteins required for cell cycle transition (E2F3, MYCN, CCND1, c-MET, Notch1/Notch2), invasion and metastasis (Fra-1), mitogen-activated protein kinase pathways (MAP3K9), and anti-apoptotic function (Bcl-2) [5]. Expression profiling identifies miR-34a as one of the several microRNAs that are down-regulated in various types of cancer including neuroblastoma, leukemia, pancreatic and hepatocellular carcinomas, glioblastoma, breast, lung, and colon cancers [6]. On the contrary, other studies find that it functions as an oncogene promoting tumorigenesis in renal cell carcinoma, papillary thyroid carcinoma (PTC), and uterine cancers [7–9]. These studies across different types of cancers have contradictory results regarding miR-34a role in tumor progression.

Gender is an important factor that affects the risk of cancer occurrence and development, incidence, prognosis, and treatment response and sex-specific therapeutic strategies should be quite urgent in cancer treatment [10]. There is increasing evidence that miR-34a expression may be related to gender [10,11]. Sex and treatment-dependent regulation of miRNAs may explain the different treatment response of males and females. Therefore, it is important to examine the impact of miRNA expression in both sexes under different disease conditions. Although extensive studies explore the role of miR-34a in the glioblastoma cell lines [12], little is known about the relationship between the expression level of miR-34a in glioblastoma tissues and the quality of patient functioning. Therefore, in this study, we examine the associations between expression level of miR-34a in glioblastoma tissue and the spectrum of a patients' presenting symptoms.

2. Results

2.1. Demographic and Clinical Characteristics Relationship with miR-34a Expression Levels

Social, demographic, and clinical characteristics of the sample are presented by miR-34a expression status in Table 1. Expression of miR-34a in tumor tissue was not related to any of demographic variables. The tendency that patients with lower miR-34a expression more frequently had frontal tumors can be observed and less of them were diagnosed with tumors located in more than one lobe of the brain. Patients with higher miR-34a expression were more frequently diagnosed with multifocal tumors, however none of these differences were statistically significant, most likely due to a small number of patients in the subgroups.

Patients with lower miR-34 expression had significantly greater tumor volume in contrast-enhanced T1-weighted sequences, when compared with patients with higher miR-34a expression ($U = 34.0$, $p = 0.03$), however no volume differences were found in T2 fluid-attenuated inversion recovery (FLAIR) image sequences (Table 1).

Additional analysis of tumor volume and miR-34a expression relationships in gender subgroups revealed stronger relationship between miR-34a expression and tumor volume in males when compared with females.

In males, miR-34a expression correlated negatively with T1-weighted contrast-enhanced tumor volume (Spearman rho = -0.53, $p = 0.05$) (Figure 1). The correlation between miR-34a expression and tumor volume on FLAIR sequences was insignificant (Spearman rho = -0.25, $p = 0.31$). In females, there was no correlation between miR-34a expression and T1 contrast volume (Spearman rho = -0.09, $p = 0.78$), as well as no correlation between miR-34a expression and FLAIR tumor volume (Spearman rho = 0.18, $p = 0.54$).

Table 1. Social, demographic and clinical characteristics in total study sample and miR-34 subgroups.

Characteristics	Total Sample	Lower than Median miR34 Expression	Equal or Higher than Median miR34 Expression
N (%)			
Gender			
Females	23 (56.1%)	13 (56.5%)	10 (43.5%)
Males	18 (43.9%)	7 (38.9%)	11 (61.1%)
Marital status			
Living alone	6 (14.6%)	3 (50.0%)	3 (50.0%)
With partner	35 (85.4%)	17 (48.6%)	18 (51.4%)
Education			
Lower than university	18 (43.9%)	8 (44.4%)	10 (55.6%)
University degree	23 (56.1%)	12 (52.2%)	11 (47.8%)
Tumor location			
Frontal	14 (34.1%)	10 (71.4%)	4 (28.6%)
Temporal	8 (19.5%)	3 (37.5%)	5 (62.5%)
Parietal	6 (14.6%)	3 (50.0%)	3 (50.0%)
Occipital	-	-	-
Two or three lobes	13 (31.7%)	4 (30.8%)	9 (69.2%)
Tumor side			
Right	19 (46.3%)	10 (52.6%)	9 (47.4%)
Left	19 (46.3%)	9 (47.4%)	10 (52.6%)
Bilateral	3 (7.3%)	1 (33.3%)	2 (66.7%)
Lesion			
Solitary	34 (82.9%)	19 (55.9%)	15 (44.1%)
Multifocal	7 (17.1%)	1 (14.3%)	6 (85.7%)
Median			
Volume			
T1 Contrast enhanced	31.0	58.3	24.4*
T2 FLAIR weighted	116.7	144.3	114.2

* U = 34.0, $p = 0.03$.

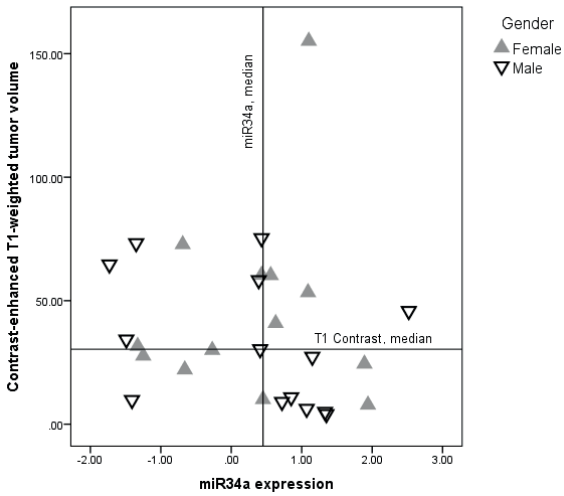

Figure 1. Relationship between miR-34a expression and contrast-enhanced T1-weighted glioblastoma tumor volume. Solid lines represent median values of either miR-34a expression (vertical) or T1 contrast median (horizontal). Gender dependent correlation between T1-weighted contrast-enhanced tumor volume and miR-34a expression was found in the male ▽ (Spearman rho = −0.53, $p = 0.05$) but not in the female ▲ (Spearman rho = −0.09, $p = 0.78$) subgroup.

2.2. Health Related Quality of Life and miR-34a Expression

Relationships between miR-34a expression levels and HRQOL indicators are shown in Table 2. Correlation analysis revealed a statistical tendency for higher miR-34a expression in tumor tissue to be positively related with physical functioning and total HRQOL reported by glioblastoma patients (Table 2). Correlation between miR-34a expression and physical functioning was stronger in males. Tendency for positive correlation between miR-34a expression and cognitive and social functioning was also observed in males, but not in females. Higher miR-34a expression was significantly negatively related to subjectively reported complaints in drowsiness. Similarly, the relationship between drowsiness and miR-34a expression was stronger in the male subsample.

Table 2. Relationship between health-related quality of life indicators, clinical evaluation of patient's functioning, and miR-34 expression in glioblastoma patients. Spearman rho.

Scales and Domains	miR-34 Expression					
	Total Sample		Females		Males	
	rho	Sig.	rho	Sig.	rho	Sig.
Health-related Quality of life						
EORTC QLQ-C30 [A]						
Global evaluation of health	−0.05	0.76	−0.11	0.62	0.05	0.86
Physical functioning	**0.30**	**0.06**	0.18	0.40	**0.66**	**0.01**
Role functioning	0.05	0.78	−0.13	0.56	0.27	0.32
Emotional functioning	0.14	0.39	0.15	0.49	0.27	0.32
Cognitive functioning	0.11	0.52	−0.09	0.68	**0.44**	**0.09**
Social functioning	0.26	0.12	0.09	0.67	**0.44**	**0.09**
QLQ C30 Total Score	**0.31**	**0.06**	0.26	0.25	0.24	0.36
EORTC QLQ-BN20 [B]						
Future uncertainty	−0.11	0.50	−0.14	0.52	−0.07	0.81
Visual difficulties	0.06	0.74	0.25	0.25	−0.22	0.43
Communication	0.15	0.38	0.18	0.42	−0.14	0.62
Motor difficulties	0.13	0.42	0.23	0.30	−0.17	0.52
Headaches	−0.08	0.64	0.07	0.70	−0.19	0.49
Seizures	0.15	0.36	−0.16	0.46	0.42	0.12
Drowsiness	**−0.34**	**0.03**	−0.20	0.37	**−0.49**	**0.05**
Hair loss	−0.01	0.97	0.33	0.13	−0.42	0.11
Itchy skin	−0.06	0.71	0.13	0.54	−0.27	0.32
Leg weakness	−0.26	0.11	−0.17	0.45	−0.42	−0.11
Bladder control	0.19	0.24	**0.37**	**0.08**	−0.01	0.98
Depression						
PHQ-9 [C]	**−0.36**	**0.03**	**−0.36**	**0.09**	**−0.37**	0.16
Level of functioning						
KPS at time of admission [D]	**0.36**	**0.03**	0.19	0.41	0.34	0.22
GOS at time of discharge [E]	0.17	0.30	0.11	0.62	0.09	0.74

[A] The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire QLQ-30. Higher scores represent better functioning; [B] The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, Brain tumor module QLQ-BN20. Higher scores represent higher symptom burden. [C] Patient Health Questionnaire-9. Higher scores indicate higher levels of depression. [D] Karnofsky Performance Scale. Higher scores represent better functioning.[E] Glasgow Outcome Scale. Bolded values indicate significant associations. Higher scores represent better functional outcomes after surgical treatment.

2.3. Depression Correlation with miR-34a Expression

In addition to making criteria-based diagnoses of depressive disorders, the PHQ-9 is a reliable and valid measure of depression severity. The examination was conducted before the operation. Higher miR-34a expression was statistically significantly negatively correlated with depressive symptom

severity, preoperatively (Table 2), both in the total sample of GBM patients and in males and females separately.

2.4. Functional Status

The Karnofsky Performance Scale (KPS) allows patients to be classified as to their functional impairment. This can be used to compare effectiveness of different therapies and to assess the prognosis in individual patients. Higher miR-34a expression in tumor tissue is significantly positively correlated with the KPS score on admission, indicating that patients with higher miR-34a expression have a better functional status before surgery. This association was slightly stronger in males when compared to females (Table 2). The miR-34a expression was not related to functional outcomes at discharge, assessed with the GOS.

2.5. Correlation of miR-34a Expression and Patient Survival

The observed association of miR-34a expression with health-related quality of life and functional status indicated for us to check the association between patient survival and miR-34a expression. For this purpose, the miR-34a expression level values obtained from the complete set of 41 glioblastoma samples were divided into two categories as follows: Values that were lower than or equal to the median expression were ranked as "low" miR-34a expression levels and values that were higher than the median were ranked as "high" miR-34a expression levels. The Kaplan–Meier analysis using the log-rank test showed no association between overall patient survival and miR-34a expression (Log-rank test, $\chi 2 = 0{,}471$, df = 1, $p = 0.493$) (see Figure 2).

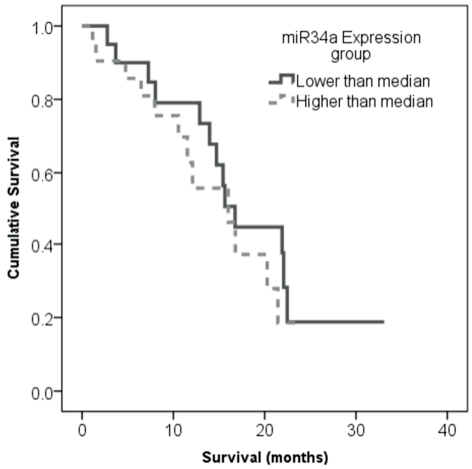

Figure 2. Kaplan–Meier survival curves in high and low miR-34a expression groups. No association between overall patient survival and miR-34a expression was found (Log-rank test, $\chi 2 = 0.471$, df = 1, $p = 0.493$).

3. Discussion

This study, for the first time, reveals the relationships between HRQOL and miR-34a expression in patients with newly diagnosed glioblastomas. Previous studies indicate that miR-34a expression might be decreased in glioblastomas, as compared to lower grade gliomas and non-tumor brain tissue. Moreover, low levels of miR-34a were associated with a poor survival prognosis. However, more comprehensive studies are needed to confirm the significance of miR-34a expression levels for the glioblastoma patients [6,13–15].

Firstly, we analyzed the relationship between social, demographic, and clinical characteristics of glioblastoma patients and miR-34a expression levels in their tumor tissue and found that patients with lower miR-34a expression had significantly higher tumor volumes. These results are expected as previous studies show that miR-34a may act as tumor suppressor gene by targeting many oncogenes related to proliferation, differentiation, growth, apoptosis, and invasion [16]. The association of miR-34a expression with tumor size is also reported in other types of tumors, e.g. low miR-34a levels are associated with larger sizes of hepatocellular carcinoma and prostate cancer [17,18]. Meanwhile, Gao and colleagues did not notice any association between miR-34a expression and glioma tumor sizes [13]. Different results in the Gao et al. study may be attributed to a different study design, as gliomas of different grades are analyzed together. We analyze only glioblastoma patients, thus making our sample more homogeneous. Behaviour of low-grade gliomas is different than that of high-grade gliomas, thus different relationship between tumor volume and miR-34a expression might be expected as a function of tumor grade. Gao and colleagues also evaluate tumor size by using tumor diameter while we employ volumetric analysis [13]. More detailed research in larger patient samples is needed to confirm these results.

Some gender differences in GBM risk and the course of the illness were recently reported. GBM incidence rates in males are slightly higher when compared to females, however males have some survival advantages over females during the first year after diagnosis but with no difference thereafter [19,20]. Gender might be important for treatment-dependent regulation of miRNA expression and may explain the differential treatment response of males and females [10]. Thus, it is important to examine the impact of miRNA-expression in both sexes individually and under different disease conditions. There is increasing evidence that the expression of miR-34a may be related to gender and in response to therapy. For example, miR-34a was up-regulated in prostate adenocarcinoma, male group, and was not abnormally expressed in the other related cancer groups [10]. There are sex differences in response to miRNA-34a therapy in mouse models of cardiac disease [11]. Thus, it is important to examine the impact of miRNA expression in both sexes individually. In our study, additional analysis of tumor volume and miR-34a expression relationships in gender subgroups reveals stronger relationship between miR-34a expression and tumor volume in males, when compared to females. Although the exact mechanism of miR-34a regulation across genders still needs to be discovered, preliminary findings indicate that various hormone factors might participate in miR-34a expression regulation. For example, the thyroid hormone 3,3,5-triiodo-L-thyronine (T3) is shown to induce the expression of miR-34a [21] and reduced T3 levels are linked to worse HRQOL and shorter survival of brain tumor patients, including those with gliomas [22,23]. Additional hormones to be considered in miR-34a regulation are estradiol (E2), as shown in human breast cancer [24]. However, in our sample of glioblastomas, the difference in expression between miR-34a and gender has not been established. Nevertheless, with increasing evidence that miR-34a expression can be controlled by the hormone, we have decided to explore the differences of HRQOL, functional status, or depression linking to miR-34a expression in more detail, not only across all the glioblastoma samples but also between genders.

Worse perceived HRQOL is shown to predict shorter survival of glioma patients [25]. Identification of molecular markers, which could act as predictors of patients' health status, is important in order to develop novel therapeutic strategies aiming to improve prognosis and to optimize the health status of glioblastoma patients. There is a tendency for an association between higher miR-34a expression and better physical functioning and overall HRQOL. The correlation between higher miR-34a expression and better physical functioning was stronger in males. The tendency for a positive correlation between miR-34a expression and cognitive and social functioning is also observed in males, but not in females. Patients with a higher miR-34a expression also scored higher on the KPS before surgery. However, miR34a expression was not significantly related to short term patients' functional outcomes at the time of discharge.

Given the poor prognosis of glioblastoma, depression stands to worsen outcomes when it develops concomitantly [26]. Despite this common interaction, relatively little research has been performed on the development of depression associated with glioblastoma. One reason for this is that the pathophysiological development of depression and glioblastoma share several pathways, including altered regulation of the 5-HT receptor, norepinephrine, and 3′:5′-cyclic monophosphate [26]. We find that patients with a lower expression of miR-34a reported more severe depressive symptoms. These results are in line with Azavedo et al. [27], who report an association between miR-34a expression in postmortem brain tissue of patients with Major Depressive Disorder and Bipolar Disorder. In animal models, miR-34 family is related to stress and anxiety response [28]. Current evidence suggests that the miR-34 family might have a critical function in regulating the behavioral and neurochemical response to acute stress and in inducing stress-related amygdala neuroplasticity [29]. However, studies with many more cases will be needed to carefully elucidate the better awareness of depression when it occurs in conjunction with miR-34a expression and to encourage optimal patient care and future research to identify potential molecular pathways between them.

Previous studies provide contradictory results on the importance of miR-34a expression for the survival of glioma patients. Gao and colleagues find that grade III glioma and glioblastomas with lower miR-34a expressions correlates with worse progression-free patient survival and overall survival [10]. Meanwhile, Toraih and colleagues do not find any significant associations between miR-34a expression levels and overall survival of glioblastoma patients [6]. In contrast to these previous studies, Genovese and colleagues, in two independent cohorts of glioblastoma, show that glioblastomas with low-expressing miR-34a have better outcomes, with longer survival overall [15]. In our sample of glioblastoma patients, no statistically significant associations between miR-34a expression and overall survival are found. Further studies are needed to confirm miR-34a expression significance in glioblastomas.

Several limitations of the current study should be acknowledged. A relatively small sample size limited the statistical power of our analysis and prevented us from employing more sophisticated and multivariate statistics. Preoperative MRI images were collected retrospectively and they were available for only 61% of total sample. It might be expected that the size of the tumor is a significant covariate linking miR-34a expression with various aspects of patients functioning, thus, further studies investigating miR-34a in the context of clinical factors are needed. However, this study presents one of the first attempts to link molecular tumor data with patients functioning, assessed by patients themselves as well as their doctors. Patient assessments were performed prospectively and provide us with interesting relationships between micro and macro levels of patient functioning.

4. Materials and Methods

4.1. Procedures

The study protocol and consent procedures were approved by the Ethics Committee for Biomedical Research of the Lithuanian University of Health Sciences (LUHS) (P2-9/2003 and BE-2-3). Written informed consent was obtained from each study patient before inclusion in the study.

Consecutive adult patients admitted for surgery for suspected glioblastoma based on brain MRI, at the Department of Neurosurgery, Hospital of LUHS, Kaunas, Lithuania in a period from October 2015 until May 2017, were invited to participate in this prospective observational cohort study. The study exclusion criteria included severe cognitive deficits and/or neurological impairment leading to inability to complete all study tasks. Neuropsychological assessment was performed, from two to three days before brain tumor surgery, by a certified medical psychologist. The medical history, clinical characteristics, and functional status of the study patients were recorded by the study neurosurgeon. Histological brain tumor diagnoses were verified from postoperative pathology reports. Pre-operative MRI images were obtained from medical documentation.

4.2. Samples

Forty-six patients with histologically confirmed glioblastoma participated in the study. Data of five patients was excluded due to failed miRNA analysis. Thirty-eight (92.7%) patients completed health related quality of life (HRQOL) and depression questionnaires. Functional status was assessed in 37 (90.2%) patients. Preoperative MRI data was available for 25 (61.0%) patients.

4.3. Questionnaires

The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire QLQ-30 [30] and QLQ-BN20 questionnaires [31,32] were used to evaluate preoperative health related quality of life (HRQOL) and brain tumor related symptoms. Both questionnaires were previously validated for HRQOL assessment in Lithuanian brain tumor patients [33].

The QLQ-C30 contains 30 items that were designed to assess global health status, functional status, role functioning, emotional functioning, cognitive functioning, social functioning, and various cancer related symptoms. Raw scores were linearly transformed to 0–100 scales with higher scores indicating better global health, functional status, and greater general HRQOL.

The QLQ-BN20 is a 20-item self-rating instrument that was designed as the QLQ-C30 supplement for evaluation of HRQOL specifically in brain tumor patients. It addresses future uncertainty, visual disorder, motor dysfunction, communication deficits, and other common BT-related symptoms. The QLQ-BN20 scores were linearly transformed to a 0–100 scale with higher score indicating greater BT-related symptom severity.

The Patient Health Questionnaire-9 (PHQ-9) [34] is a brief self-report tool for screening, diagnosing, monitoring, and measuring the severity of depression. The PHQ-9 is based on the Diagnostic Statistical Manual-IV depression diagnostic criteria and it is recommended for depression screening in glioma patients [35,36].

The Karnofsky performance scale (KPS) [37], was used for assessment of functional status. The KPS is an 11-point rating scale that is designed to measure a patient's ability to carry his/her normal activities and dependence on help and nursing care. The total KPS score ranges from 100 (normal functioning) to 0 (death).

Functional outcomes at hospital discharge were evaluated by a neurosurgeon using The Glasgow Outcome Scale (GOS) [38]. The GOS ranges from 1 (death) to 5 (good recovery) and it is widely used for research purposes in neurosurgical patients.

4.4. Tumour Volume Measurements

Tumor segmentation and volume measurements were performed using the 3D Slicer medical image computing platform, version 4.3.1 (www.slicer.org) [39]. We used the most recent pre-operative structural MRI imaging data, which was performed on 1.5T or 3T MRI scanners. For the purpose of this study, we used T1-weighted contrast enhanced and T2-FLAIR weighted sequences. T1-contrast enhancing tumor volume (in cm^3), representing tumor necrotic core, and T2-FLAIR hyper-intense tumor volume, representing tumor infiltrations/edema, were calculated. All volumetric analyses were performed by a trained neurosurgeon. The rater was blinded to microRNA and psychological assessment data.

4.5. Small RNA Extraction, Micro RNA cDNA Synthesis and qPCR Performance

Small RNA (<200 nt) was extracted from snap-frozen (−196 °C) post-surgical tumor samples applying cryogenic mechanical grinding, ultrasonic homogenization, and using a "mirVana™ miRNA Isolation Kit" (Catalog nr: AM1560). Quality and quantity of extracted small RNAs were evaluated with Agilent "2100 Bioanalyzer" (Part nr: G2939BA) and "Small RNA analysis kit" (Part nr: 5067-1548). A measure of 10 ng of purified micro RNA was synthesized to cDNA using a "TaqMan™ Advanced miRNA cDNA Synthesis Kit" (Catalog nr: A28007) and the expression profile of mature micro RNA 34a

(hsa-miR-34a-5p) was detected by performing quantitative RT-PCR (qPCR) on "Applied Biosystems 7500 Fast Real-Time PCR System" in 3 replicates using "TaqMan™ Fast Advanced Master Mix" (Catalog nr: 4444557) in addition to hsa-miR-191-5p, hsa-miR-361-5p (as referenced), and hsa-miR-34a-5p probes from "TaqMan™ miRNA Advanced Assay" product line (Assays ID: 477952_mir, 478056_mir, and 478048_mir, respectively). Fluorescent data were converted to cycle threshold (Ct) measurements and relative quantitation of hsa-miR-34a-5p was calculated according to the following formulas:

1) $\Delta Ct_{miRNA} = MeanCt_{miRNA} - \sqrt{MeanCt_{miR-191} \times MeanCt_{miR-361}}$
2) $2^{-\Delta Ct_{miRNA}}$

In order to quantify samples in 95% of the cases, samples with a standard deviation of more than 0.25 were eliminated from the analysis.

4.6. Statistical Analysis

The SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) software package was used for statistical analysis. Chi-square and Mann–Whitney tests were used to evaluate associations among miR-34a expression levels and clinical parameters. The relationship between patients' functioning and miR-34a expression was evaluated using Spearman correlation analysis. A Kruskal–Wallis test was used to reveal the difference across medians of miR-34a expression. The significance level was defined as a p value less than 0.05. The Kaplan–Meier method was used to estimate survival functions. For comparing survival time distribution between groups, the log-rank test was used.

5. Conclusions

Taken together, the findings of our study suggest that some molecular markers might be important for health-related quality of life, functional status, and depressive symptoms of glioblastoma patients. That is, the slower proliferation rate of tumors with higher miR-34a expression may allow for greater neuroplasticity by offering the brain more time for reorganization in response to invading tumors. Due to the small sample size, our results should be considered as preliminary. Thus, further studies in miR-34a expression in glioblastoma patients, addressing possible gender differences, are strongly encouraged.

Author Contributions: P.V., A.B., A.P., and G.S. generated the idea; P.V. and A.P. drafted the manuscript; R.S. and G.S. performed molecular analysis; A.P. and A.B. collected patient clinicopathological data, performed neuropsychological tests, and survey according to questionnaires; A.P. and P.V. performed all computational and statistical analysis of the data; A.T. and A.B. coordinated postoperative patient sample acquisition. All authors have read and approved the final version submitted.

Funding: This work was funded by the Research Council of Lithuania (grant no. S-MIP-17-108).

Acknowledgments: We kindly thank Jūratė Žeglienė for assistance in tissue sampling and patient clinical data gathering.

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of
Molecular Sciences

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Unique Interplay between Molecular miR-181b/d Biomarkers and Health Related Quality of Life Score in the Predictive Glioma Models

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Received: 26 August 2020; Accepted: 7 October 2020; Published: 9 October 2020

Abstract: In the last decade, an increasing amount of research has been conducted analyzing microRNA expression changes in glioma tissue and its expressed exosomes, but there is still sparse information on microRNAs or other biomarkers and their association with patients' functional/psychological outcomes. In this study, we performed a combinational analysis measuring *miR-181b* and *miR-181d* expression levels by quantitative polymerase chain reaction (qPCR), evaluating isocitrate dehydrogenase 1 (*IDH1*) single nucleotide polymorphism (SNP), and O-6-methylguanine methyltransferase (*MGMT*) promoter methylation status in 92 post-surgical glioma samples and 64 serum exosomes, including patients' quality of life evaluation applying European Organization for Research and Treatment of Cancer (EORTC) questionnaire for cancer patients (QLQ-30), EORTC the Brain Cancer-Specific Quality of Life Questionnaire (QLQ-BN20), and the Karnofsky performance status (KPS). The tumoral expression of miR-181b was lower in grade III and glioblastoma, compared to grade II glioma patients ($p < 0.05$). Additionally, for the first time, we demonstrated the association between miR-181 expression levels and patients' quality of life. A positive correlation was observed between tumoral miR-181d levels and glioma patients' functional parameters ($p < 0.05$), whereas increased exosomal miR-181b levels indicated a worse functional outcome ($p < 0.05$). Moreover, elevated miR-181b exosomal expression can indicate a significantly shorter post-surgical survival time for glioblastoma multiforme (GBM) patients. In addition, both tumoral and exosomal miR-181 expression levels were related to patients' functioning and tumor-related symptoms. Our study adds to previous findings by demonstrating the unique interplay between molecular miR-181b/d biomarkers and health related quality of life (HRQOL) score as both variables remained significant in the predictive glioma models.

Keywords: glioblastoma; miR-181; prognosis; exosomes; quality of life

1. Introduction

In the early stages of glioblastoma formation, usually no specific symptoms are present leading to its late detection, usually only when the tumor is already grown significantly and/or spread to other parts of the brain [1]. Glioblastoma multiforme (GBM) symptoms are not well defined and depend on

the location of the tumor, but most commonly include headache, nausea, visual impairment, motor disorders, seizures, personality changes, or even disorientation, and very severe memory impairments in severe cases [2].

Standardized diagnosis of glioma consists of patient's evaluation by computed tomography or magnetic resonance imaging, followed by histological analysis of the suspected tumor tissue [3]. However, even after histological examination, the characterized and grouped tumors often differ in their transcriptomal profile within the same malignancy group, which leads to complicated and limited-efficiency standardized treatment [4]. Therefore, it is necessary to determine the transcriptomal markers of gliomas in high sensitivity. Although vast amounts of genetic and epigenetic data from tumor tissue have been already collected and are publicly available from The Cancer Genome Atlas (TCGA) and other consortia, there is still a lack of epigenetic data from glioma patients' serum exosomes, which could lead to improved glioma characterization and non-invasive diagnosis.

Intensive research over the last years proven the importance of miRNAs in the molecular biology of glioma [5]. Mature miRNAs are small non-coding RNA molecules which act as gene silencers in post-transcriptional manner [6]. Some of these small RNAs are associated with major depression, suicide behavior, and anxiety [7,8]. The small footprint of miRNAs and their ability to stimulate behavior changes makes miRNAs an attractive target analyzing varying psychological state of glioma patients.

The ability to detect circulating small RNAs in human blood has opened the vast potential for use of miRNAs as complication-free biomarkers for the diagnosis of various cancers [9,10]. However, subsequent studies have shown that most circulating miRNA are also highly expressed in different blood cells [11]. In order to avoid misleading results, the research strategy should only include miRNA analysis of circulating exosomes that are likely to be produced by highly invasive tumors such as glioblastoma and only those with very low leukocyte expression [12].

According to the bioinformatic analysis of Z. Yeng et al., the *miR-181* family is calculated to have more than 500 reliable targets. These targets are responsible for various biological processes such as cell proliferation, division, growth, and intercellular communication [13]. *miR-181* importance in oncology is observed in different types of cancer. A decrease in *miR-181a* expression was reported to cause downregulation of matrix metalloproteinase-1 and vascular endothelial growth factor expression in chondrosarcoma leading to a decrease in the tumor malignancy [14]. In glioblastoma cell lines and nude mice models, *miR-181b* has been shown to have onco-suppressor abilities, and its high expression is associated with a better outcome [15]. Downregulation of *miR-181a*, *miR-181b*, and *miR-181c* was observed in a small cohort of primary glioblastoma tissue, also suggesting miR-181 family involvement in glioblastoma development [16]. To further investigate the importance of different miR-181 family members in glioma, we decided to analyze *miR-181b* and *miR-181d* expression levels in a bigger patient cohort of glioma tissue and exosome samples.

Since glioblastoma still remains an uncurable disease, it is crucial to improve the quality of life of GBM patients and predict their quality of functioning after the surgery [17]. Therefore, this study included known and potential biomarkers of glioma, trying to better understand the molecular profile of gliomas and assess other microRNA connections with patients' quality of life measurements. Furthermore, in this study, we tried to find a relationship between patients' quality of life scores and exosomal *miR-181* levels. The assumption was that the radiologically non-detectable damage occurs in the healthy brain tissue surrounding the tumor. We hypothesized that these small, early damages to the surrounding tissue might evoke the variety of functional/psychological symptoms and could be caused by tumoral microRNAs transported in exosomes. To date, there was no information on *miR-181* tumoral or exosomal expression levels' association with patients' functional/psychological parameters.

2. Results

2.1. miR-181 Expression within Different Grades of Glioma

We evaluated both tumor and exosomal *miR-181b* and *miR-181d* (*miR-181b/d*) expression as a diagnostic biomarker for the identification of different grade gliomas. *MiR-181b* and *miR-181d* showed a tendency to be downregulated in grade 3 glioma in both tumor and exosome samples. However, a significant difference between grade 2 and grade 3 glioma was only detected when measuring *miR-181b* expression in tumor samples ($p < 0.05$) (Figure 1A). A vast distribution of *miR-181b/d* was detected in both GBM tumor and exosome samples, as was expected due to the heterogenic nature of grade 4 glioma.

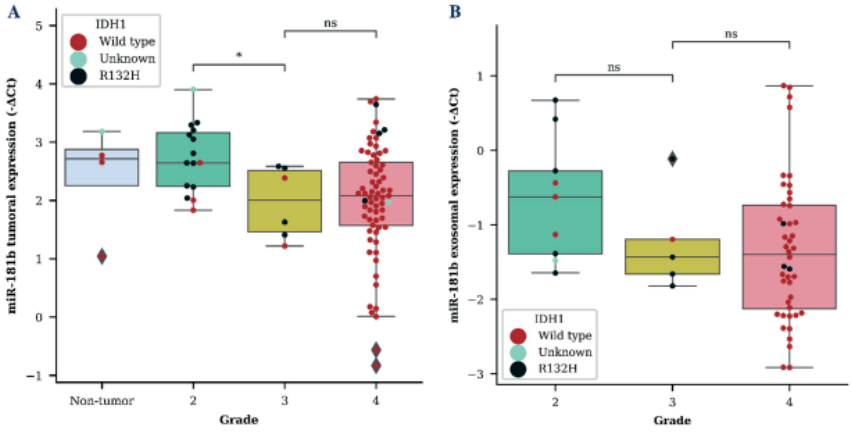

Figure 1. *MiR-181b* tumoral (**A**) and exosomal (**B**) expression levels within different grade gliomas. Colored dots represent different isocitrate dehydrogenase 1 (*IDH1*) C. 395G>A (R132H) variant status in the cells of the brain tissue. The box squares represent the data within 25 and 75 percentiles; the line in the middle shows the median.

Next, we wanted to compare *miR-181b/d* levels together with other known glioma biomarkers. Higher tumoral expression of both *miR-181b* ($p < 0.05$) and *miR-181d* ($p < 0.01$) was associated with isocitrate dehydrogenase 1 (*IDH1*) mutation (Figure 2A,B). *MiR-181d* level was significantly higher in GBM tumor tissues of patients with a *IDH1* R132H variant, which is primarily found in secondary GBMs, compared to *miR-181d* levels of GBM patients with a *IDH1* wild type ($p < 0.001$) (Figure 2C). Additionally, GBM patients that survived longer than an expected 12-month period and had an unmethylated O-6-methylguanine methyltransferase (*MGMT*) promoter also had a significantly higher *miR-181d* expression in their tumor tissue ($p < 0.05$) (Figure 2D). No significant results were observed while comparing exosomal *miR-181b/d* levels with different *IDH1* or *MGMT* patient groups.

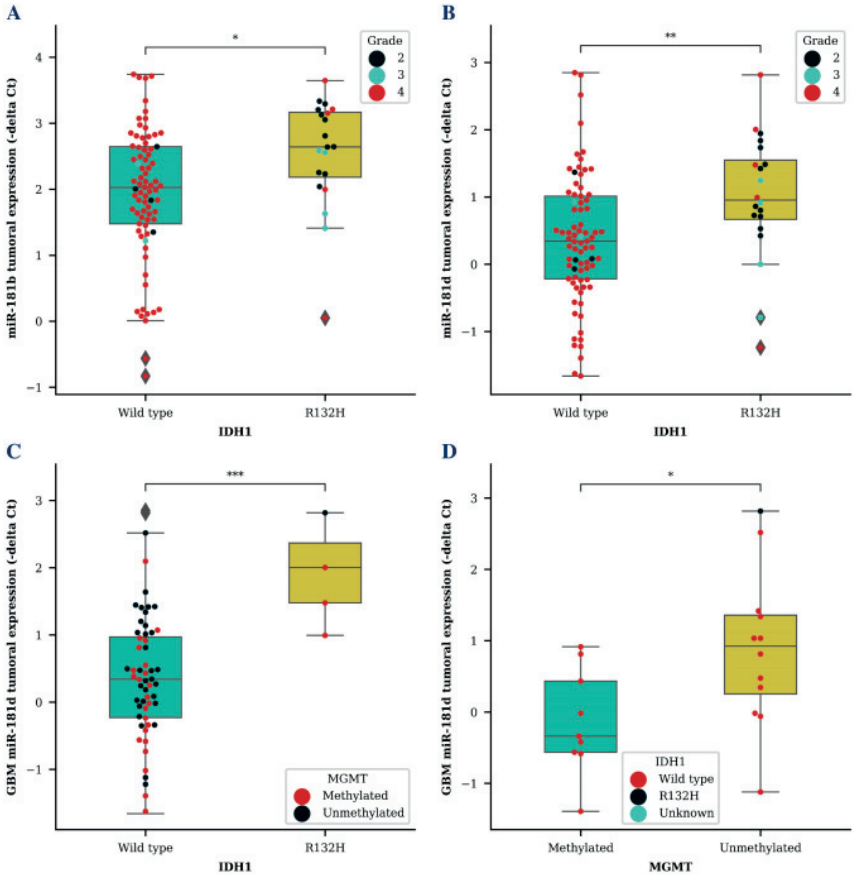

Figure 2. *MiR-181b* and *miR-181d* tumoral expression level association with known glioma biomarkers. Tumoral *miR-181b* (**A**) and *miR-181d* (**B**) expression differences between glioma patients (**A**,**B**) or GBM patients (**C**) with *IDH1* wildtype and *IDH1* R132H variant. Tumoral miR-181d levels in GBM patients with methylated *MGMT* promoter and unmethylated *MGMT* promoter status (**D**). Colored dots represent different *IDH1* R132H variant (**D**) or *MGMT* promoter methylation status (**C**) and glioma grade (**A**,**B**). The box squares represent the data within 25 and 75 percentiles; the line in the middle shows the median.

2.2. *miR-181 Expression and Functional Status of Patients*

To investigate relationships between *miR-181b/d* levels, the functional status and symptom profile of patients' correlation analysis was performed.

As can be seen in Tables 1 and 2, *miR-181b/d* was significantly related to subjectively evaluated patients' functioning; however, different trends were observed for tumoral and exosomal *miR-181* expressions. Tumoral *miR-181b* expression was positively correlated with better physical role and social functioning, as well as better general quality of life. Similarly, though non-significant trends were observed in the subsample of glioblastoma only, tumoral *miR-181d* expression was also positively related

to physical functioning in total and glioblastoma samples. Exosomal *miR-181b* was not significantly related to any of the functioning indicators. Exosomal *miR-181d* showed a significant inverse correlation with physical and emotional functioning in the total sample; a similar but non-significant trend was observed in the glioblastomas subsample.

Table 1. Correlations between *miR181b* expression and subjectively reported quality of functioning.

Subjectively Reported Quality of Functioning Groups	*miR-181b*			
	Tumoral		Exosomal	
	GBM only	Total sample	GBM only	Total sample
Global health	−0.02	0.05	−0.09	−0.05
Physical functioning	0.23	0.27 *	−0.08	−0.09
Role functioning	0.18	0.23 *	0.09	0.08
Emotional functioning	0.24	0.15	−0.21	−0.18
Cognitive functioning	0.07	0.12	0.03	−0.05
Social functioning	0.32 *	0.33 **	−0.02	−0.04
Summary Quality of Life Score	0.19	0.28 *	−0.12	−0.08
Karnofsky Performance Scale	0.09	0.08	0.10	0.05

* $p < 0.05$; ** $p < 0.01$.

Table 2. Correlations between *miR181d* expression and subjectively reported quality of functioning.

Subjectively Reported Quality of Functioning Groups	*miR-181d*			
	Tumoral		Exosomal	
	GBM only	Total sample	GBM only	Total sample
Global health	−0.03	0.02	0.04	−0.02
Physical functioning	0.29 *	0.32 **	−0.20	−0.27 *
Role functioning	0.07	0.13	−0.03	−0.03
Emotional functioning	0.10	0.07	−0.27	−0.38 **
Cognitive functioning	−0.07	0.01	−0.16	−0.25
Social functioning	0.13	0.18	−0.05	−0.20
Summary Quality of Life Score	0.06	0.17	−0.15	−0.27
Karnofsky Performance Scale	−0.04	0.00	0.08	−0.04

* $p < 0.05$; ** $p < 0.01$.

Several significant correlations were observed when analyzing relationships between *miR-181b/d* and patients' reported tumor related symptoms. Higher tumoral *miR-181b* level was related to less expressed drowsiness in glioblastoma patients (Spearman rho = −0.30, $p < 0.05$). Tumoral *miR-181d* was related to greater seizure probability both in the total sample and in glioblastomas only (Spearman rho 0.26 and 0.29, respectively, $p < 0.05$). Exosomal *miR-181b* correlated positively with greater tumor related visual difficulties both in the total sample and glioblastoma patients only (Spearman rho 0.32, and 0.27, respectively, $p < 0.05$). Exosomal *miR-181d* also was positively related to vision impairment in the total and glioblastoma patient samples (Spearman rho 0.34 and 0.32, $p < 0.04$), with more expressed drowsiness in the total sample, and with a similar non-significant trend in glioblastoma patients (Spearman rho = 0.35, $p < 0.05$, and 0.28). Exosomal *miR-181* was negatively correlated with seizure probability, both in the total sample and glioblastomas only (Spearman rho −0.36 and −0.32, respectively, $p < 0.05$).

2.3. miR-181 Expression and Patients' Survival outcome

2.3.1. miR-181 Levels in Post-Surgical Glioma Tissue

We also analyzed *miR-181b* and *miR-181d* expression in glioma tissue and evaluated its effect on patients' survival time after the tumor dissection. The survival analysis revealed no significant

survival time differences between high and low *miR-181b* or *miR-181d* levels in all stages of glioma. Furthermore, the analysis was supplemented with patients' *IDH1* and *MGMT* status; however, tumoral *miR-181* levels, within the same *IDH1* status patient group, also did not indicate any changes in patients' survival. However, a strong tendency was observed comparing *miR-181d* tumor expression between GBM patients with methylated *MGMT* promoter ($p = 0.065$). Patients within this group had a 6.22 month longer median survival when tumoral *miR-181d* expression was higher than the cohorts median tumoral expression.

2.3.2. miR-181 Levels in Glioblastoma Patients' Serum Exosomes

Despite the fact that there was no significant difference between *miR-181* tumoral expression and glioma patients' survival time, we wanted to see if the same result was reflected measuring *miR-181* in glioma patients' serum exosomes. Interestingly, survival analysis revealed a difference between different *miR-181* expression groups. A noticeable difference was only detected in GBM patients when the cohort was grouped into low (lower than median) and high (higher than median) exosomal *miR-181* expression groups. GBM patients who had low *miR-181b* serum exosomal expression survived significantly longer compared to patients with a high exosomal expression ($p = 0.017$; $df = 1$; $\chi 2 = 5.629$) (Figure 3A). Patient groups with different *miR-181d* or *miR-181b/d* expression showed only a tendency, indicating better prognosis for patients with low *miR-181d* ($p = 0.239$) or *miR-181b/d* ($p = 0.08$) expression in serum exosomes.

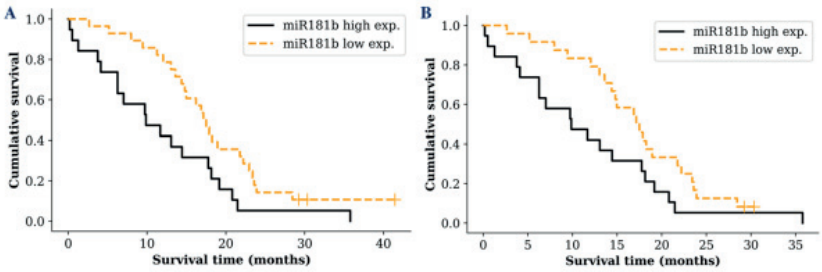

Figure 3. Kaplan-Meier survival curves comparing higher and lower *miR-181b* exosomal expression levels in: **A**—all GBM patients ($p = 0.017$); **B**—GBM patients with *IDH1* wild type ($p = 0.049$). Censored cases indicated by a vertical line.

In addition, well known glioma biomarker *IDH1* mutation status was included in the survival analysis. However, analysis of *miR-181* expression in GBM patients with *IDH1* wild type did not reveal a more sensitive survival prediction ($p = 0.049$; $df = 1$; $\chi 2 = 3.871$) (Figure 3B). No significant increase in the survival prediction was observed including *IDH1* or *MGMT* status comparing exosomal *miR-181d* expression levels.

Furthermore, we investigated the effect of *miR-181* exosomal expression differences among GBM patient ages. Samples of GBM patients were divided into two groups: younger than median cohort age (younger) and older than median cohort age (older) GBM patients. Older patients who had lower *miR-181b/d* exosomal expression showed a strong tendency ($p = 0.086$) surviving longer, compared to older patients with higher *miR-181b/d* exosomal expression—median survival of 15.6 and 7.65 months, respectively. The younger GBM patient group with higher *miR-181b/d* exosomal expression had a 2.3 month shorter median survival than the older GBM patient group with lower *miR-181b/d* exosomal expression.

In order to evaluate *miR-181b/d* exosomal expression as a prognostic biomarker, GBM patients were grouped into two specific groups: group A—GBM patients who were older than 55.3 years, had *IDH1* wild-type genotype, a hypermethylated *MGMT* promoter, and higher than median *miR-181b/d*

exosomal expression; group B—the same previous criteria but with a lower than median *miR-181b/d* exosomal expression. A significant difference in patient survival time was observed within these two small GBM patient groups. Patients from group A ($n = 7$), on average, survived 2.36 times shorter than patients from group B ($n = 4$) ($p = 0.025$; $df = 1$; $\chi 2 = 4.989$) (Figure 4).

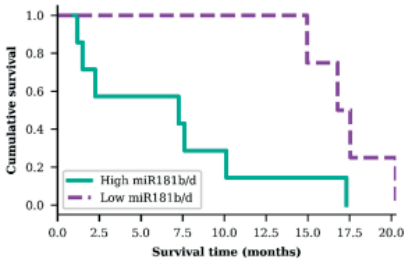

Figure 4. Survival analysis of both exosomal *miR-181b* and *miR-181d* high and low levels in older (>55.3 years) glioblastoma patients, diagnosed with *IDH1* wild type and methylated *MGMT* promoter ($p = 0.025$)

Finally, a combinational analysis was performed to estimate the importance of tumoral and exosomal *miR-181* levels, *IDH1* and *MGMT* status, tumor related symptoms, quality of life index, and functional patients' status for the survival outcome prediction. Decision tree classification was applied to evaluate the impact of measured features as a complex for patient survival as well as to estimate its importance. The overall accuracy of the tree classifier was 82.2%. The short survival subgroup (< 16.85 months) prediction accuracy was higher (90.6%), while the long survival group (≥16.85 months) showed slightly poorer accuracy (67.6%). Although the long survivor subgroup sample size was smaller (37 vs. 64), the prediction accuracy was lower indicating that the subgroup exhibits greater heterogeneity, in terms of analyzed features, as compared to short survivors. The decision tree classifier predicted the highest possibility rate of short post-surgical survival time for the glioma patients with the combination of *IDH1* wild-type genotype, lower *miR-181d* tumoral expression, higher *miR-181b* tumoral expression, and weaker tumor related symptoms. The highest probability of longer survival was associated with the combination of *IDH1* mutation (R132H), severe tumoral symptoms, and higher *miR-181b* exosomal expression (Figure 5).

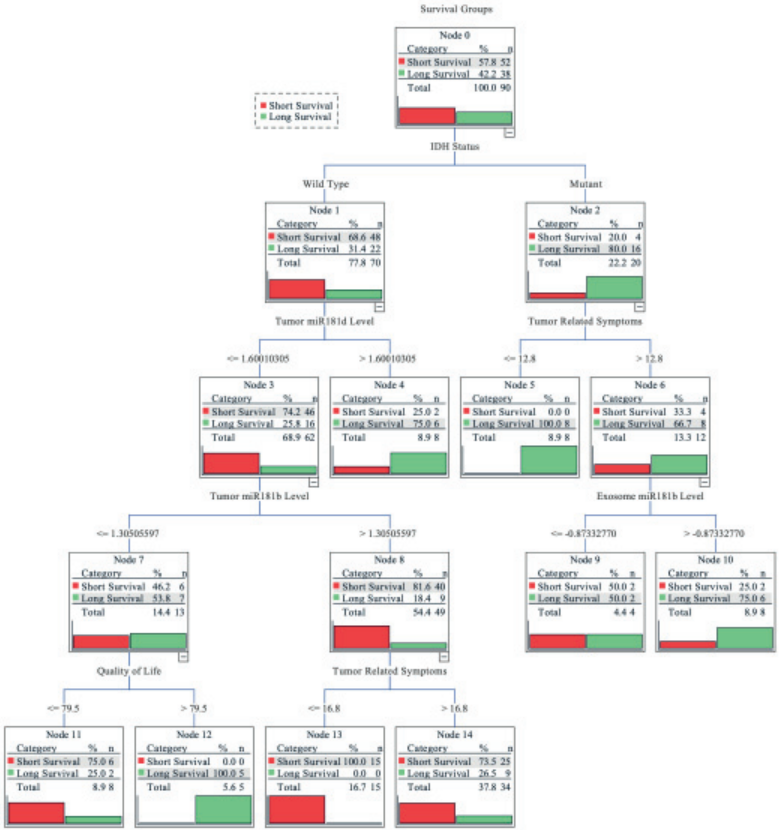

Figure 5. Decision tree for predicting grade II-IV glioma patients' survival. Grouped into two subgroups according to the cohort survival mean: <16.85 months—short survival; ≥16.85 months—long survival. The earlier factor appearance (vertically going from top to bottom) shows its higher importance to the prediction model. Values on the lines indicate the factor value at which the algorithm divided the factor groups. For *miR-181* expression levels, the fold change value was used; the higher tumor related symbol score reflects more pronounced symptoms and the higher quality of life score indicates better functional and psychological well-being of the patient.

3. Discussion

The *MiR-181* family is strongly associated with glioma and glioblastoma development, according to other in vivo and in vitro studies [15,18]. Multiple interactions of various mRNA and lncRNAs with *miR-181* family members indicate the importance of this microRNA in glioma [19]. Additionally, *miR-181b* could be involved in the regulation of tumorigenesis and epithelial to mesenchymal transition of glioma [15]. Decreased expression of *miR-181b* has been shown to stimulate cell proliferation, migration, and invasion, in addition to its ability to regulate chemosensitivity of temozolomide [20,21]. In our study, we found that the expression of *miR-181b* differs among malignancies of glioma, thus indicating that *miR-181b* expression could be associated with the grade of glioma. According to our

data, *miR-181b* tumoral expression is downregulated in higher grade gliomas, compared to lower grade, which is consistent with other studies [22,23].

MiR-181d is another miRNA that belongs to the *miR-181* family, and its low expression levels are related to poor patient survival, suggesting the important role of *miR-181d* and its potential as a prognostic factor for glioblastoma patients [24]. In particular, W. Zhang and colleagues showed that *miR-181d* targets *MGMT* and downregulates it, leading to better response to temozolomide treatment [18]. In addition to the findings of W. Zhang et al., our study showed a noticeable tendency of prolonged survival time in GBM patients with methylated *MGMT* promoter and higher *miR-181d* tumoral expression levels. These results strengthen the suggestion that *miR-181d* is activated during *MGMT* promoter methylation processes, in order to suppress *MGMT* oncogenic activity in GBM patients [25].

A wide spectrum of both *miR-181b* and *miR-181d* expression levels was observed in serum exosomes and tumor tissue samples of GBM patients. The main reason for this could be the extreme heterogeneity of the glioblastoma. Recent studies have suggested that tumoral *miR-181* expression could help indicate different subgroups of GBM [15,26]. Our results clearly show that *miR-181b* and *miR-181d* expression are not consistent in GBM tissue, but further analysis and standardized GBM subgroup evaluation guidelines have to be performed in order to apply these data for GBM subgrouping.

This study reveals, for the first time, the prognostic potential of measuring *miR-181b* and *miR-181d* expression in GBM patients' serum exosomes. Interestingly, in our study, exosomal *miR-181b* expression showed completely different predictive association to tumoral *miR-181d* levels. Longer survival time was observed in GBM patients with a lower exosomal *miR-181b* expression. Usually, a similar expression pattern is detected in tumor tissue and serum exosomes [27,28]. A different prognostic association of *miR-181b* expression in GBM post-surgical tissue and serum exosomes could be explained by a prevention of *miR-181b* packaging to exosomes in the tumor cells and exporting them out of the tumor's environment. One could consider that *miR-181b* expression is promoted in GBM cells as a defense mechanism against tumor development. At the same time, tumor cells would try to export this onco-suppressive *miR-181b* out of the GBM cells in order to survive. In that case, an indication of a good prognosis would be high tumoral and low exosomal expression of *miR-181b*, which we observe in our single and combinational analyses. However, this theory should be tested thoroughly, including functional analysis of *miR-181b*.

Molecular research traditionally relies on very formal outcome measures such as overall survival or progression-free survival. However, none of these variables reflect the current health status of a patient, the symptom burden, or the quality of his or her functioning. It is known that decreased health related quality of life (HRQOL) in glioma patients is a sensitive predictor of shorter survival [17,29]. Recent meta-analysis by Coomans et al. [30] demonstrated that some HRQOL variables were independent predictors of overall patient survival and progression-free survival in glioma patients. Significant correlation was reported between deterioration of HRQOL scores and tumor progression in glioblastoma patients in longitudinal studies [31]. Thus, HRQOL is a very informative outcome measure as it reflects the subjectively perceived burden of tumor-related symptoms at the moment of assessment, and also, it has predictive value for long-term overall survival prognosis [32].

However, patient-centered outcome measures are rarely investigated in relation to biomarkers. Only a few studies have tried to associate molecular biomarkers with patients' quality of life measurements. The work of S. V. Chatzikyriakou et al. suggests that the levels of circulating collagen metabolites could be used as a quality of life indicator for chronic heart failure patients [33]. Similar studies were carried out by S. Kay et al., who revealed changes of matrix metalloproteinase levels in idiopathic pulmonary fibrosis patients with different HRQOL scores [34], and by J. Hu et al., whose work linked serum *miR-206* levels to the quality of life of Duchenne muscular dystrophy patients [35]. Besides our previous work on *miR-34a* [36], brain cancer patients' quality of life and its association with circulating biomarkers have only been investigated by A. Bunevicius et al., whose study suggests the importance of free triiodothyronine and thyroid stimulating hormone levels [37].

All of this research indicates the possibility of patients' quality of life prediction in various diseases and invites us to look at biomarkers from a patients' psychological and functional point of view.

To the best of our knowledge, this study investigated relationships between *miR-181b* and *miR-181d* and glioma patients' functioning for the first time. Functioning was assessed using HRQOL measures as well as by clinical evaluation performed by the treating neurosurgeon. Both tumoral and exosomal *miR-181b/d* expressions were weakly, but significantly related to patients' reported functioning and symptoms. Tumoral *miR-181b/d* expression showed the tendency towards correlation with better functioning, while exosomal *miR-181d* were related to lower physical functioning and a slightly more negative tumor-related symptoms profile. Exosomal *miR-181d* was statistically significantly correlated with a smaller probability of epileptic seizure; still, this finding could be interpreted as a negative indicator since seizures are reported to be related to longer survival in glioma patients due to earlier tumor diagnosis and initiation of treatment [38]. In line with previous findings, HRQOL and subjectively reported tumor-related symptoms were significant independent predictors in the combinational analysis of survival outcome prediction. However, our study adds to previous findings by demonstrating the unique interplay between molecular *miR-181b/d* biomarkers and HRQOL, as both variables remained significant in the predictive models. These findings encourage further research on molecular markers and HRQOL connections.

Finally, the combinational analysis revealed the importance of both tumoral and serum *miR-181* transcript levels in predicting glioma patients' post-surgical outcomes. The decision tree classifier revealed that *miR-181* played an important part in different predictive subgroups. In the scenario of an *IDH1* wild-type patient, both exosomal *miR-181* had no significant influence on patients' outcome prediction; instead, the tumoral *miR-181* played an important part, especially the lower tumoral *miR-181d* level, which was the second most important factor for patients' short survival prediction. Interestingly, only higher exosomal *miR-181b* levels, but neither levels of tumoral *miR-181*, were selected as a major factor predicting longer survival in the context of patients with *IDH1* mutation and more expressed tumoral symptoms. These findings indicate the possible interplay between *IDH1* and the regulation of tumoral/exosomal *miR-181* transcript levels and could serve as an additional factor for other radiological- and clinical-data-based prediction models [39,40].

It is important to mention that the study cohort was slightly younger and did not have the usual 1.57 to 1 (male to female) gender ratio. Additionally, due to difficult microRNA detection in low amounts of serum exosomes, some of the patients' exosomal samples were unsuited for quantitative polymerase chain reaction (qPCR) analysis leading to a smaller data set. However, this study shows the importance of the *miR-181* family in GBM patients' outcome, and it is one of the first studies evaluating the influence of exosomal *miR-181b* and *miR-181d* expression levels on GBM patients' outcome and their quality of life prediction.

In conclusion, the findings of our study suggest that elevated *miR-181b* exosomal expression can indicate significantly shorter post-surgical survival time for GBM patients. Like other researchers, we demonstrate *miR-181b* and *miR-181d* expression decrease during glioma progression. More importantly, both tumoral and exosomal *miR-181* expression levels were related to patients' functioning and tumor-related symptoms. Furthermore, glioma patients' quality of life index, their tumor-related symptoms, *IDH1* status, and tumoral *miR-181b* levels are important factors predicting patients' survival time. Furthermore, adding GBM patients' *MGMT* promoter methylation, age, and exosomal *miR-181b* expression information improves predictive significance and should be considered in all future research regarding predictive exosomal biomarkers for glioblastoma patients.

4. Materials and Methods

4.1. Study Cohort

The patients' age on the day of the surgery varied from 24.6 to 80.0 years with a median of 55.3 and an average of 54.9 years. The cohort consisted of 55.5% males and 45.5% females.

The study cohort reflected common glioma patients' molecular and survival characteristic. Patients who had *IDH1* mutation had a 11.1-month longer median survival compared to patients with *IDH1* wild-type ($p = 0.008$; $df = 1$; $\chi 2 = 6.855$). Patients who were younger than 55.3 years showed a 10.1-month increase in median survival compared to older patients ($p = 0.002$, $df = 1$, $\chi 2 = 10.077$). The median survival of GBM patients was 12.3 months in contrast to 22.4 months for lower grade glioma patients ($p < 0.001$; $df = 1$; $\chi 2 = 19.9$).

4.2. Samples

The research was performed in accordance with the Lithuanian regulations, principles of the Helsinki and Taipei Declarations. Written informed consent was obtained from every patient and protocols used in this work were evaluated and approved by the Ethics Committee of Kaunas region, Lithuania (protocol: L6.1-07/09, permission code: P2-9/2003, date: 10 October 2010; and protocol: BE-10-6, permission code: BE-2-3, date: 18 April 2016).

In total, 92 different grade glioma samples were surgically removed at Lithuanian University of Health Sciences Hospital Kaunas Clinics (LUHS KC) Neurosurgery department during the period of 2016–2019. The grade of glioma was histologically confirmed at LUHS KC Department of Pathological Anatomy: 15-stage II; 7-stage III; and 70-stage IV (glioblastoma/GBM). In addition, 64 matched blood serum samples were collected: 10–stage II; 7–stage III; and 46–GBM. Due to the rare occurrence of the disease, the maximum number of samples was included into the study.

4.3. Patients Functional Status Assessment

The functional status of patients was assessed prospectively before neurosurgery by a certified medical psychologist and neurosurgeon. Patients' functional status was assessed using two different paradigms—asking patients subjectively to evaluate their health, symptoms, level of functioning, and general quality of life using standardized questionnaires; asking a neurosurgeon to evaluate the level of patient independent functioning by using a clinical scale. Three measures were used in the current study:

The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, EORTC QLQ-30 [41] is an internationally validated cancer-specific health related quality of life measure. The EORTC QLQ-C30 contains 30 items that were designed to assess global health status, functional status, role functioning, emotional functioning, cognitive functioning, social functioning, and various cancer related symptoms. In the current study, we used functional scales and the total score as indicators of subjective patients' functioning [42,43].

The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, Brain cancer module, EORTC QLQ-BN20 [44] was used to evaluate subjectively reported brain tumor related symptoms. The QLQ-BN20 is a 20-item self-rating instrument. It addresses future uncertainty, visual disorder, motor dysfunction, communication deficits, and other common brain tumor-related symptoms.

The Karnofsky performance status scale (KPS) [45] was used for assessment of functional status. The KPS is an 11-point rating scale that is designed to measure a patient's ability to carry out his/her normal activities and dependence on help and nursing care.

Data on patients' functional status evaluated by the neurosurgeon were available for 77 patients (83.7%); psychological assessment was performed for 75 (81.5%) patients from a 92 brain tumor samples cohort, and for 52 (81.3%) patients from a 64 matched blood serum samples cohort.

4.4. DNA Isolation

DNA was extracted from ~40 mg frozen tumor tissue using the desalting method with chloroform, and Proteinase K. DNA concentration was measured with a NanoDrop 2000 system (Thermo Fisher Scientific, Cat. #: ND-2000, Wilmington, DE, USA).

4.5. IDH1 Mutation and MGMT Promoter Methylation Analysis

IDH1 gene mutation in gliomas-R132H was analyzed in all the specimens applying custom TaqMan SNP genotyping assays. PCR was carried out consisting of TaqMan Universal Master Mix II (Thermo Fisher Scientific, Cat. #: 4440047, Carlsbad, CA, USA), TaqMan probes, and 20 ng purified tumor DNA. All the procedures were accomplished according to the TaqMan chemistry manufacturer recommendations. Fluorescence was measured with a 7500 Fast Real-Time PCR system (Applied Biosystems, Cat. #: 4351107, Foster City, CA, USA).

MGMT promoter methylation status was determined using methylation-specific PCR (MSP). The reaction consisted of 7.5 µL Hot Start PCR Master Mix with Hot start Taq DNA polymerase (Thermo Fisher Scientific, Cat. #: EP0701); 4.5 µL nuclease-free water; 1 µL (10 pmol/µL) of each primer, specific to methylated/unmethylated promoter; and ~20 ng of bisulfite-treated DNA as a template. Primer sequences for the methylated MGMT sequence were 5′-GGACGTTAAGGGTTTAGAGC-3′ (sense) and 5′-CAATACACGACCTCGTCAC-3′ (antisense), and for unmethylated—5′-GGATGTTAAGGGTTTAGAGT-3′ (sense) and 5′-CAATACACAACC TCATCAC-3′ (antisense). Additionally, three controls were performed: positive—"Bisulfite converted Universal Methylated Human DNA Standard & Control primer" (Zymo Research, Cat. #: D5015, Irvine, CA, USA); negative—bisulphite treated human blood lymphocytes DNA; and water control (no template control). MSP was performed in 38 cycles with the following conditions: Taq Polymerase activation at 95 °C for 5 min, denaturation at 95 °C for 15 sec, annealing at 59 °C for 30 sec, extension at 72 °C for 15 sec, and final extension at 72 °C for 5 min. Products after amplification were visualized using agarose gel electrophoresis. Each sample methylation status was evaluated according to visible signals and documented using a 0 (unmethylated) and 1 (methylated) system.

4.6. RNA Isolation and cDNA Synthesis

In total, 20–40 mg of frozen, post-surgical tumor samples was mechanically grinded and homogenized with ultrasonication at 20% amplitude for 1 second on/off pulsation prior to enriched small RNA extraction using a mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Cat. #: AM1560). An amount of 250–1250 µl of frozen serum samples was used for exosome extraction with exoEasy Maxi Kit (Qiagen, Cat. #: 76064, Valencia, CA, USA) including cel-miR-39-3p spike-in (0.0065 ng) in each sample after exosome collection step. The quality and quantity of extracted microRNAs were evaluated with a Small RNA analysis kit (Agilent, Cat. #: 5067-1548, Santa Clara, CA, USA) and NanoDrop 2000. In order to be able to analyze the broad range of micro RNAs, 10 ng extracted RNA was synthesized to cDNA with a TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. #: A28007, Pleasanton, CA, USA). Pre-amplified and 10 times diluted cDNA was used for micro RNA expression analysis afterwards.

4.7. Micro RNA Expression Analysis

QPCR reaction consisted of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Cat. #: 4444557, Austin, TX, USA), hsa-miR-181b-5p (Assay ID: 478583_mir) or hsa-miR-181d-5p (Assay ID: 479517_mir) probes, and 3 µl diluted cDNA. Gene expression was measured on a 7500 Fast Real-Time PCR system using a fast cycling program. In addition, 4 endogenous micro RNAs were used for data normalization: hsa-miR-191-5p (Assay ID: 477952_mir), hsa-miR-361-5p (Assay ID: 478056_mir), hsa-miR-345-5p (Assay ID: 478366_mir), and hsa-miR-103a-3p (Assay ID: 478253_mir). Additional levels of spike-in were measured in serum exosome samples (Assay ID: 478293_mir) (Thermo Fisher Scientific, Cat. #: A25576, Pleasanton, CA, USA).

For each sample, relative quantitation of hsa-miR-181b-5p and hsa-miR-181d-5p was calculated according to the Equation (1):

$$\Delta Ct_{target\ miR} = Ct_{target\ miR} - \sqrt[4]{Ct_{miR191} \times Ct_{miR361} \times Ct_{miR345} \times Ct_{miR103a}} \quad (1)$$

An additional normalization step was applied to serum exosome samples normalizing to spike-in cel-miR-39-3p levels according to the Equation (2):

$$\Delta Ct_{target\ miR} = \Delta Ct_{target\ miR} \times \left(\frac{\overline{Ct_{miR39}}}{Ct_{miR39}} \right) \quad (2)$$

4.8. Statistical Analysis

Kaplan–Meier estimation, using a log-rank test was performed to evaluate patient groups during survival analysis. A Student's independent t-test was applied evaluating the difference between two groups, and One-Way ANOVA with Bonferoni correction was applied for the comparison of three or more groups. Spearman correlation was used to evaluate relationships between miRNA expressions and the functional status of the patients. Decision tree classification analysis was performed using the CRT algorithm with Gini method nonlinear combinations. The significance level was defined as $p < 0.05$ (*).

Author Contributions: Conceptualization, P.V.; Data curation, P.V.; Formal analysis, R.S., A.P. and G.S.; Funding acquisition, P.V.; Investigation, A.P. and A.B.; Methodology, A.P., G.S. and P.V.; Project administration, P.V.; Resources, A.T. and P.V.; Supervision, A.T. and P.V.; Validation, R.S. and A.B.; Visualization, R.S. and G.S.; Writing—original draft, R.S.; Writing—review and editing, A.P., G.S., A.T., A.B. and P.V. All authors have read and approved the final version submitted. All authors have read and agreed to the published version of the manuscript

Funding: This work was funded by the Research Council of Lithuania (grant #: S-MIP-17-108).

Acknowledgments: We kindly thank Jūratė Zėglienė and Raminta Bagdanavičienė for assistance in tissue sampling and patient clinical data gathering.

Conflicts of Interest: The authors declare no conflict of interest.

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International Journal of Molecular Sciences

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Article

The role of *CASC2* and *miR-21* interplay in glioma malignancy and patient outcome

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Received: date; Accepted: date; Published: date

Abstract: Recently lncRNAs were highlighted for its regulatory role in tumour biology. The novel human lncRNA *CASC2* (cancer susceptibility candidate 2) has been characterised as a potential tumour suppressor in several tumour types. However, the roles of *CASC2* and its interplay with *miR-21* in different malignancy grade patient gliomas remain unexplored. Here we screened 99 different malignancy grade astrocytomas for *CASC2*, and *miR-21* gene expression by RT-qPCR in *IDH1* and *MGMT* assessed gliomas. *CASC2* expression was significantly downregulated in glioblastomas (p=0.0003). Gliomas with low *CASC2* expression exhibited a high level of *miR-21*, which was highly associated with the higher glioma grade (p=0.0001), *IDH1 wild type* gliomas (p<0.0001) and poor patient survival (p<0.001). Taken together, these observations suggest that *CASC2* acting as a tumour suppressor and potentially as ceRNA for *mir-21* plays an important role in *IDH1 wild type* glioma pathogenesis and patient outcome.

Keywords: *CASC2*; *miR-21*; glioma; *IDH1* status; patient survival

1. Introduction

Malignant gliomas, especially glioblastomas, are highly infiltrative, rapidly growing, aggressive, heterogeneous, chemo-resistant, and lethal neoplasms [1]. The accurate distinction between the different malignancy types has significant prognostic and therapeutic implications [2]. A thorough study of the molecular mechanisms of the formation and progression of glioma is essential for the screening of valuable diagnostic and prognostic molecular markers. Long-non coding RNAs (lncRNAs) were first recognised as being crucial regulators of gene expression in a wide range of biological context, including cancer [3]. Various lncRNAs, including *HOTAIR, MALAT, CRNDE*, have been identified as novel players in glioma pathogenesis demonstrating associations with tumour subtype, histological stage, tumour *IDH* mutational status, chemosensitivity and patient survival [4–8].

Steadily growing evidence on the ability of lncRNAs to interact with DNA, RNA and proteins acting as tethers, guides, decoys, and scaffolds, includes them in the posttranscriptional regulatory network in cancer biology [9]. Moreover, the increasing evidence suggests an interplay between microRNAs and lncRNAs [10,11]. A large number of lncRNAs acts as a competing endogenous RNAs (ceRNA) or sponges for microRNAs, for example, *PTENpg1*, *HOTAIR* [11,12]. The novel lncRNA gene *CASC2* (cancer susceptibility gene 2) has been characterised as tumour suppressor in various human malignancies [13–19]. Although the deregulated expression of *CASC2* in cancer enhances its tumorigenic properties, however, the literature evidence limits current knowledge on the pathophysiological implications and the roles of *CASC2*, and it's interplay with *miR-21* in the pathology of gliomas [20].

In this study, we assessed levels of *CASC2* and *miR-21* and their interplay in different malignancies of glioma. Our findings indicate that *CASC2* was proportionally downregulated in progressed gliomas, while *miR-21* expression was inversely associated with *CASC2* expression, malignancy grade, and patient survival. Here we demonstrate *CASC2* acting as tumour suppressor and likely interacting with *miR-21* in *IDH1* wild type gliomas.

2. Results

2.1. CASC2 and miR-21 associations with patient clinical parameters

To evaluate whether *CASC2* and *miR-21* were associated with glioma patient clinical parameters, we divided the sample into "low" and "high" (below and above the gene's mean expression of all samples, respectively) gene expression groups. As shown in Table 1, lower *CASC2* and higher *miR-21* expression were observed more frequently in patients with advanced tumour stage (IV grade gliomas/glioblastomas) ($p<0.0001$). Furthermore, *IDH1* wild-type gliomas more frequently had lower *CASC2* and higher *miR-21* expression ($p=0.037$ and $p<0.0001$, respectively).

Table 1. The relationship between *CASC2* and *miR-21* gene expression in glioma tissue and patient clinical characteristics. Pearson's χ^2-test was used for comparison of categorical variables.

Variable	Total No	*CASC2* expression			Total No	*miR-21* expression		
		Low (%)	High (%)	p-value		Low (%)	High (%)	p-value
Gender								
Male	45	25 (55.6)	20 (44.4)	0.422	37	13 (35.1)	24 (64.9)	0.182
Female	54	25 (46.3)	29 (53.7)		46	24 (52.2)	22 (47.8)	
Age, yr								
<56	47	23 (48.9)	24 (51.1)	0.841	42	24 (57.1)	18 (42.9)	0.027
≥56	52	27 (51.9)	25 (48.1)		41	13 (31.7)	28 (68.3)	
Grade								
II-III	17	2 (11.8)	15 (88.2)	<0.0001	17	16 (94.1)	1 (5.9)	<0.0001
IV	82	48 (58.5)	34 (41.5)		66	21 (31.8)	45 (68.2)	
IDH1								
Wt	78	44 (56.4)	34 (43.6)	0.037	64	21 (32.8)	43 (67.2)	<0.0001
Mut	18	5 (27.8)	13 (72.2)		16	14 (87.5)	2 (12.5)	
MGMT								
Unmeth	48	25 (52.1)	23 (47.9)	1.000	40	16 (40)	24 (60)	0.812
Meth	41	22 (53.7)	19 (46.3)		33	15 (45.5)	18 (54.5)	

miR-21				
low	37	11 (29.7)	26 (70.3)	0.002
high	46	30 (62.2)	16 (34.8)	

2.2. CASC2 and miR-21 expression in high grade and IDH1wt gliomas

Whether the activity of *CASC2* and *miR-21* was linked to the clinical progression of gliomas, we examined gene expression in grade II-III and IV gliomas. Here we show significant *CASC2* expression loss and drastic rise of *miR-21* expression in glioblastomas as compared to the average expression levels in lower grade (II-III) gliomas (p=0.0003 and p<0.0001 respectively) and control non-cancerous brain tissues (p=0.005 and p<0.0001, respectively) (Figure 1A, B). When all samples were divided into *IDH1* gene mutated (*IDH1mut*, n=18) and *IDH1* wild-type (*IDH1wt*, n=78) gliomas, we observed a tendency of lower expression of *CASC2* in *IDH1wt* (p=0.053) and highly significant relationship between higher *miR-21* expression and *IDH1wt* gliomas (p<0.0001) (Figure 2A, B).

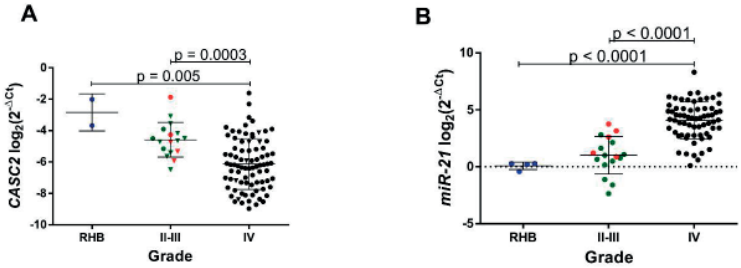

Figure 1. *CASC2* and *miR-21* gene expression are associated with glioma malignancy grade. (a) *CASC2* expression measured by RT-qPCR in RHB (reference human brain, n=2), II-III malignancy grade gliomas (n=17) and IV grade gliomas, n=82 (glioblastomas). (b) *miR-21* expression measured in the same patient postoperative tumour tissue by RT-qPCR in RHB (n=4), II-III (n=17) and IV grade (n=66) gliomas. The line in the graph is the mean with the SD. Colour corresponds to different glioma malignancy grade: green – grade II, red – grade III, black – grade IV gliomas. Triangle shape corresponds to *IDH1mut* glioma, circle shape – *IDH1wt*.

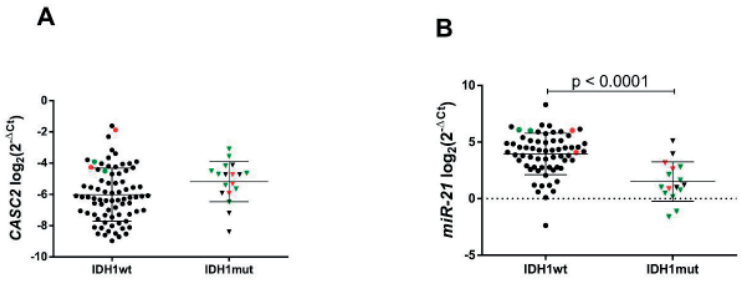

Figure 2. *CASC2* and *miR-21* gene expression in *IDH1wt* and *IDH1mut* gliomas. The line indicates mean with the SD. Colour corresponds to different glioma malignancy grade: green reflect grade II, red – grade III, black – grade IV gliomas.

2.3. CASC2 and miR-21 interplay in gliomas

Several reports have suggested that lncRNAs may function as a molecular sponge or competing endogenous RNA in modulating miRNAs, suggesting that it could be an inverse correlation between lncRNA and miRNAs [21]. It was shown that *miR-21* can bind to *CASC2* directly by the putative miRNA response element (MRE) [20]. Here at the clinical level, we further confirm the recently reported interaction between *CASC2* and *miR-21* in glioma cell lines. We show that significantly higher *miR-21* gene expression was observed in the "low" *CASC2* group as compared to "high" and vice versa (p=0.0004, Figure 3A-B). Correlation analysis revealed moderate negative association between *CASC2* and *miR-21* expression in gliomas (r^2=-0.42, n=83, p<0.0001, Figure 3C).

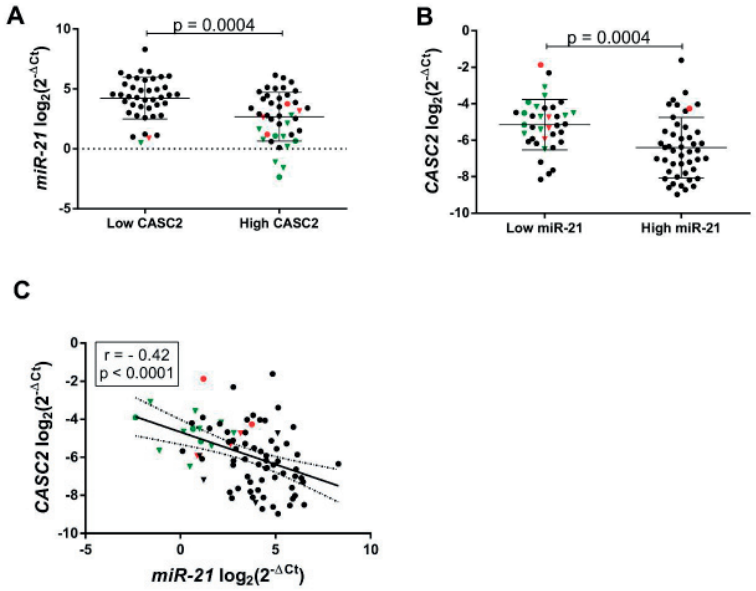

Figure 3. *CASC2* and *miR-21* interplay in gliomas. (a) *miR-21* gene expression in low (n=41) and high (n=42) *CASC2* expression groups in all patient gliomas. (b) *CASC2* gene expression in low (n=37) and high (n=46) *miR-21* gene expression groups in all patient gliomas. (c) Expression correlation between *CASC2* and *miR-21* in gliomas (r=-0.42, p<0.0001, n=83) visualized as a scatter plot. The lines in the graphs indicate mean with the SD. Colour corresponds to different glioma malignancy grade: green reflect grade II, red – grade III, black – grade IV gliomas. Triangle shape corresponds to *IDH1mut* glioma, circle shape – *IDH1wt*.

2.4. Survival analysis

Kaplan-Meier survival analysis showed highly significant association between low *CASC2* expression levels (n=50) and worse patients' outcome (Log-rank test, $\chi2$=7.777, df=1, p=0.0053; Figure 4A), while patients with low *miR-21* expression (n=37) showed significantly increased overall survival, compared to patients with high *miR-21* expression (n=46) (Log-rank test, $\chi2$=8.518, df=1, p=0.0035; Figure 4B). The combined effect of low *CASC2* and high *miR-21* expression (n=41) in glioma was shown to be associated with significantly decreased overall survival compared to patients with the combination of high *CASC2* and low *miR-21* expression (n=26) in tumour tissue (Log-rank test, $\chi2$=12.91, df=1, p=0.0003; Figure 4C). Univariate Cox regression model revealed that patients' clinical characteristics such as age and tumour stage, *IDH1* status was associated with their survival as well as *CASC2*, *miR-21*, and combined *CASC2*/*miR-21* expression. However, multivariate analysis showed that only patient age and tumour stage were covariates associated with the overall survival of glioma patients (Table 2).

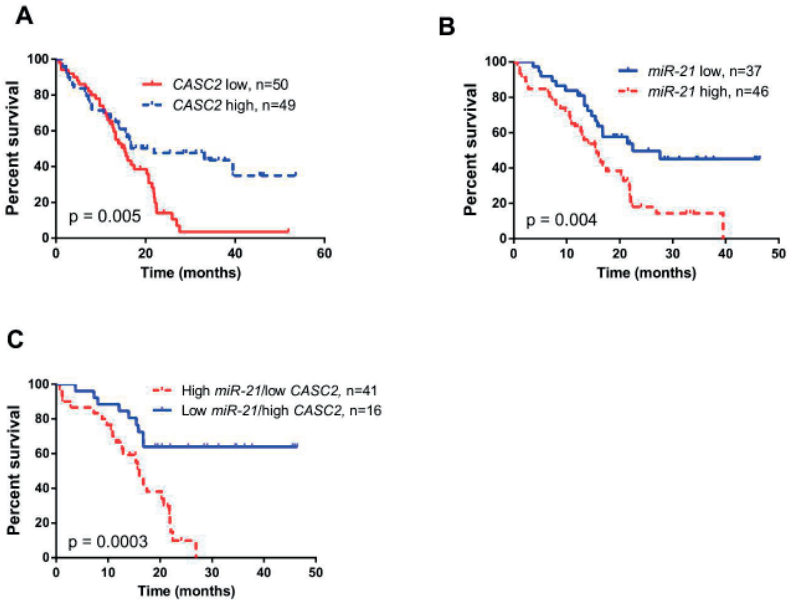

Figure 4. Kaplan-Meier curves for glioma patient survival correlation with (a) *CASC2* expression (Log-rank test, $\chi2=7.777$, df=1, p=0.005) (b) *miR-21* expression (Log-rank test, $\chi2=8.518$, df=1, p=0.004) and c) combined *miR-21* and *CASC2* expression (Log-rank test, $\chi2=12.91$, df=1, p=0.0003) in glioma tumour tissue.

Table 2. Cox regression analysis of different clinicopathological variables, *CASC2* and *miR-21* expression.

Characteristics	Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95 % CI)	p-value
Age (<56 vs. ≥56)	0.216 (0.123-0.381)	<0.0001	0.408 (0.215-0.775)	0.006
Gender (female vs. male)	0.868 (0.537-1.404)	0.564	NA	
Tumor grade (II-III vs. IV)	0.069 (0.017-0.284)	<0.0001	0.100 (0.019-0.526)	0.007
IDH1^{R132H} (wild-type vs. mutated)	0.160 (0.064-0.404)	<0.0001	0.809 (0.244-2.682)	0.729
MGMT (methylated vs. non methylated)	0.722 (0.435-1.199)	0.208	NA	
CASC2 low vs. high	0.497 (0.301-0.821)	0.006	0.751 (0.389-1.450)	0.393
miR-21 low vs. high	0.438 (0.247-0.775)	0.005	0.852 (0.424-1.712)	0.653
CASC2 low/*miR-21* high vs. *CASC2* high/*miR-21* low	0.259 (0.118-0.570)	0.001	NA	

3. Discussion

The key finding of the current study is that down-regulation of lncRNA *CASC2* and up-regulation of *miR-21* expression is associated with glioma progression. Our results show that *CASC2* downregulation is associated with highly expressed *miR-21* and poor patient outcome.

Moreover, here we show that highly active *CASC2* significantly might have suppressed *miR-21* levels in *IDH1* wild-type gliomas.

Current knowledge on the involvement and function of lncRNA *CASC2* in glioma evidences the availability of small amount of data from clinical samples. Downregulation of *CASC2* in glioma tissue was showed by Wang et al [20] in a limited sample of 24 patients, while Liao et al revealed *CASC2* playing a role in modulating glioma temozolomide (TMZ) chemoresistance in 57 pateint samples [22]. In agreement with our data, *CASC2* expression in both studies was shown to correlate with glioma malignancy grade inversely. Recently, several studies including patient samples have been carried out on *CASC2* expression in other malignancies. In particular, *CASC2* acts as a tumour suppressor in endometrial, colorectal, lung, stomach, renal, gastric cancers, and osteosarcomas [13–19]. In NSCLC (Non-small-cell lung carcinoma) patients (n=76), *CASC2* expression was downregulated proportionally to the pathological stage and associated with tumour size, and this gene was an independent predictor for overall survival [13]. In 76% CRC (colorectal cancer) patients (n=68) *CASC2* low expression was associated with tumour stage [14]. In RCC (renal cell carcinoma) (n=32), *CASC2* was significantly downregulated compared with the matched normal tissue [15]. In gastric cancer tissue (n=67) and cell lines, *CASC2* expression was downregulated [17] and low *CASC2* level in tissue correlated with the vessel invasion, tumour stage, metastasis, and poor patient survival [19]. In osteosarcoma, *CASC2* expression downregulation was observed in patient tissue samples and cell lines, and low expression in tissue was associated with poor tumour differentiation, higher malignancy grade, and shortened patient survival [18]. To sum up, recent scientific work and our research in astrocytic gliomas support evidence that *CASC2* gene expression is downregulated proportionally to tumour stage, indicating the suppressive role of *CASC2* in malignancy progression. Functional studies *in vitro* in various cancer cell lines confirmed *CASC2* acting as a tumour suppressor as when overexpressed *CASC2* was able to inhibit cell proliferation, cell growth, migration and invasion, and to induce apoptosis [20].

Emerging evidence revealed a new mechanistic role of lncRNAs as part of a posttranscriptional regulatory network in cancer biology. Recent data suggest that coding and non-coding RNAs can regulate one another through their ability to compete for miRNA binding through a typical MREs (miRNA response elements). LncRNAs can act as competing endogenous RNAs (ceRNA) or miRNA "sponges", which can sequester miRNAs, therefore preventing a single or multiple miRNA from binding to their proper target RNAs and protecting them from suppression [23]. Importantly, micro RNAs also regulate lncRNAs [10]. Recently, it was demonstrated that *CASC2* in colorectal cancer is functioning as ceRNA for *miR-18a*, thereby modulating the expression of target gene *PIAS3*, and subsequently inhibiting CRC cell proliferation and tumour growth [14]. Studies in hepatocellular carcinoma revealed that *CASC2* prohibited mesenchymal-epithelial transition progression and exerted anti-metastatic effect via *CASC2*/*mirR-396*/*FBXW7* axis [9]. Wang and colleagues demonstrated *CASC2* and *mir-21* reciprocal interaction in glioma cell lines U251 and U87 [20]. Similarly, Liao et al [22] study showed *CASC2* interaction with *miR-181a* and *PTEN* gene in regulating chemosensitivity in temozolomide resistant glioma cells. However, as to our knowledge, no studies are demonstrating *CASC2* and *miR-21* interaction in patient glioma samples and evaluating its clinical relevance.

Consistent with published reports on *CASC2*/*mir-21* interaction in glioma and non-small cell lung cancer cells *in vitro* [20,24], we provide evidence in patient gliomas that *CASC2* and *miR-21* play antagonistic roles and potentially interact in glioma progression. In support of this, the RT-qPCR analysis showed that *miR-21* expression is moderately upregulated in low-grade

astrocytoma and even highly upregulated in malignant glioblastoma, while high expression of *CASC2* in tumours might be responsible for the decrease of *miR-21* expression. Agreeing with our findings, *miR-21* has been well studied in gliomas with particularly high expression. *miR-21* is consistently upregulated in astrocytic tumours (grade II-IV) and downmodulates an entire set of oncosuppressor genes, for example, *PTEN* [25]. High *miR-21* expression in tumour tissue was highly associated with aggressive clinicopathological features and poor overall patient survival (n=152) [26]. In the current study, we found a correlation between high *mir-21* expression and *IDH1wt* gliomas. It is known that mutation in isocitrate dehydrogenase 1 (*IDH1mut)* is associated with distinct glioma cell metabolic profile, hypermethylated phenotype, and significantly longer overall survival as compared to patients with *IDH1wt* [27,28]. Our results further indicate *miR-21* predictive value in *IDH1wt* associated gliomagenesis.

4. Material and Methods

4.1. Ethics

The research was reviewed and approved by the Kaunas Regional Bioethics Committee (protocol No: 9/2003) and performed following the Lithuanian regulations alongside with the principles of the Helsinki and Taipei Declarations [29,30].

4.2. Patient sample

Due to rare occurrence of the disease, the maximum possible number of samples were included into the study. Total 99 samples of different malignancy grade astrocytomas were analysed for *CASC2* and *miR-21* expression: 17 grade II-III astrocytic gliomas and 82 grade IV astrocytic gliomas/glioblastomas. *CASC2* expression was analysed in 99 samples, while *miR-21* in 83 samples. *IDH1* status was obtained for 96 patients, *MGMT* promoter methylation status was determined for 89 samples. Tissue samples were prospectively collected at the Department of Neurosurgery of Lithuanian University of Health Sciences, during the period of 2015 – 2018. The pathological review was performed on each sample to confirm the diagnosis of astrocytic glioma. All tissue samples were stored in liquid. None of the patients had received preoperative chemotherapy or radiotherapy. All patients signed written consent forms. Overall survival was calculated from the day of surgery to the death or last follow-up.

4.3. RNA and DNA extraction

Total and small RNAs (<200 nt) were extracted from 30-40 mg snap-frozen (-196°C) post-surgical tumour samples applying cryogenic mechanical grinding, ultrasonic homogenization at 20% amplitude, 1 second on/off pulsation and using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, USA). Procedures were done according to the manufacturer's instructions. The RNA concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific, USA). Quality of extracted small RNAs was evaluated with Small RNA analysis kit (Agilent, USA) on 2100 Bioanalyzer (Agilent, USA).

DNA was extracted from ~40 mg frozen tumour tissue using the desalting method with chloroform, and Proteinase K. DNA concentration was measured with NanoDrop 2000 system.

4.4. CASC2 gene expression analysis

cDNA synthesis was performed using 2 μg of RNA, hexamer primers, "Multiscribe™ Reverse Transcriptase" reverse transcriptase, according to the manufacturer's recommendations, using, "High-Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, USA). RT-qPCR was conducted using "AB 7500 Fast Real-time PCR system" (Applied Biosystems, USA). *CASC2* gene primers sequences were as follows: forward 5′-GCACATTGGACGGTGTTTCC-3′; reverse 5′-CCCAGTCCTTCACAGGTCAC-3′ [31]. All amplification reactions were performed in 96-well plates and each sample was tested in 3 replicates. For normalization, the geometric expression average of five housekeeping genes (*GAPDH, YWHAZ, β-actin, 18s rRNA, HPRT1*) was used. As endogenous control "FirstChoice Human Brain Reference Total RNA" (RHB) (Ambion, USA) was used. In order to quantify samples in 95% of the cases, samples with a standard deviation of more than 0.25 were eliminated from the analysis. Gene expression was calculated as $2^{-\Delta Ct}$ values and in figures presented as log-transformed values.

4.5. miR-21 gene expression analysis

10 ng of purified micro RNAs were synthesised to cDNA using "TaqMan Advanced miRNA cDNA Synthesis Kit" (Thermo Fisher Scientific, USA). Expression profile of mature micro RNA 21 was detected performing RT-qPCR on "7500 Fast Real-Time PCR system" (Applied Biosystems, USA) in 3 replicates using "TaqMan Fast Advanced Master Mix" (Thermo Fisher Scientific, USA) and *hsa-miR-21-5p* probes (Assay ID: 477975_mir). In addition, *hsa-miR-191-5p* (Assay ID: 477952_mir) and *hsa-miR-361-5p* (Assay ID: 478056_mir) were measured in order to normalize the data. Relative quantitation of *hsa-miR-21-5p* expression for each sample was calculated according to the formulas:

$$\Delta Ct_{miR21} = Ct_{miR21} - \sqrt[2]{Ct_{miR191} \times Ct_{miR361}} \text{ and } 2^{-\Delta Ct_{\text{miR-21}}}. \quad (1)$$

4.6. IDH1 mutation detection

The most common *IDH1* gene mutation R132H in gliomas was analysed in all the specimens applying custom TaqMan SNP genotyping assays. PCR was carried out in a total volume of 12 μl consisting of "TaqMan™ Universal Master Mix II" (Thermofisher Scientific, USA), TaqMan probes and 20 ng of purified tumour DNA. All the procedures were accomplished according to the manufacturer's recommendations. Fluorescence was measured with "AB7500 Fast Real-Time PCR System". Amplification of DNA with Wild or Mutant allele labelled with VIC or FAM dyes indicated different gene variants, respectively.

MGMT methylation detection

MGMT promoter methylation status determined using methylation-specific PCR (MSP). Reaction performed in 15 μL total volume, consisting of 7.5μL "Hot Start PCR Master Mix" (Thermofisher Scientific, USA), 4.5 μL nuclease-free water (Thermofisher Scientific, USA), 1 μL (10pmol/ μL) of each primer, specific to methylated/unmethylated promoter (Metabion International) and ~20 ng of bisulfite-treated DNA as a template. Primers sequences for methylated *MGMT* sequence were 5′- GGACGTTAAGGGTTTAGAGC - 3′ (sense), 5′-CAATACACGACCTCGTCAC - 3′ (antisense), for unmethylated - 5′-GGATGTTAAGGGTTTAGAGT - 3′ (sense), 5′-CAATACACAACCTCATCAC - 3′ (antisense). Three controls were performed: positive - "Bisulfite converted Universal Methylated Human DNA Standart & Control primer" (ZymoResearch, USA), negative – bisulphite treated human blood lymphocytes DNA and water control. PCR products visualized using agarose gel

electrophoresis. Each sample methylation status evaluated according to visible signals and documented using 0 (unmethylated) and 1 (methylated) system.

4.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (San Diego, CA, USA). Continuous variables were checked for normal distribution using Shapiro-Wilk statistics and compared by Student's t-test when normally distributed or by Mann-Whitney U test when data distributed non-normally. Pearson's correlation coefficient was calculated to test the association between two gene expression. Pearson's chi-squared test was used for comparison of categorical variables. Kaplan-Meier curves were compared using Log-rank analysis in different gene expression groups. For regression analysis gene expression values were categorised as "low" or "high" according to log-transformed gene expression values were above or below all sample expression mean, respectively. Statistically significant was considered when p-value < 0.05.

5. Conclusions

In summary, lncRNA *CASC2* was found as a tumour suppressor and downregulated in low-grade astrocytomas and highly malignant glioblastomas as compared to healthy brain tissue. *miR-21* was inversely expressed with *CASC2* in gliomas and correlated with *IDH1wt* glioma and poor patient prognosis.

Author contributions. Conceptualization, Daina Skiriute and Paulina Vaitkiene; Data curation, Paulina Vaitkiene; Formal analysis, Daina Skiriute, Rytis Stakaitis and Giedrius Steponaitis; Funding acquisition, Paulina Vaitkiene; Investigation, Daina Skiriute; Methodology, Rytis Stakaitis and Paulina Vaitkiene; Project administration, Paulina Vaitkiene; Resources, Arimantas Tamasauskas; Supervision, Daina Skiriute and Arimantas Tamasauskas; Validation, Daina Skiriute and Giedrius Steponaitis; Visualization, Giedrius Steponaitis; Writing – original draft, Daina Skiriute; Writing – review & editing, Rytis Stakaitis, Giedrius Steponaitis, Arimantas Tamasauskas and Paulina Vaitkiene

Funding: This research was funded by The Research Council of Lithuania, grant number S-MIP-17-108.

Acknowledgments. We kindly thank Jūratė Žeglienė for help in tissue sampling and acquiring patient data, also we thank Vilnius University master's degree student Aistė Stefanskytė for technical support in *CASC2* gene expression experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. *List of assay IDs from Thermo Fisher Scientific of the analyzed miRNAs*

miRNA	Assay ID
hsa-miR-143-3p	477912_mir
hsa-miR-193a-5p	477954_mir
hsa-miR-139-5p	478312_mir
hsa-miR-7-5p	478341_mir
hsa-miR-34a-5p	478048_mir
hsa-miR-93-5p	478210_mir
hsa-miR-181b-5p	478583_mir
hsa-miR-181d-5p	479517_mir
hsa-miR-221-5p	478778_mir
hsa-miR-17-5p	478447_mir
hsa-miR-335-5p	478324_mir
hsa-miR-21-5p	477975_mir
hsa-miR-148a-3p	477814_mir
hsa-miR-10b-3p	477868_mir
hsa-miR-126-5p	477888_mir
hsa-miR-338-5p	478038_mir
hsa-miR-338-3p	478037_mir
hsa-miR-191-5p	477952_mir
hsa-miR-361-5p	478056_mir
hsa-miR-345-5p	478366_mir
hsa-miR-103a-3p	478253_mir
cel-miR-39-3p	478293_mir

CURRICULUM VITAE

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Education:

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2012–2016	LSMU, bachelor's degree in medical and veterinary genetics

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2018–present	Junior researcher, LSMU, Neuroscience Institute, Laboratory of Molecular Neurooncology.
2018–2019	Medical biologist, UAB Invitro diagnostika

Scientific Projects:

2021–2025	Co-initiator of a COST action – *Harmonizing clinical care and research on adrenal tumors in European countries* (HARMONISATION)
2020–2022	Junior researcher at a LRC project – *Epitranscriptome changes of non-coding RNAs involved in brain cell differentiation and pathogenesis* (EPIC)
2020–2021	Junior researcher at a LRC project – *Long non-coding RNA epitranscriptome profiling in glioma stem cells and tumors for identification of novel biomarkers* (LEADING)
2017–2020	Junior researcher at a LRC project – *miRNA profiling in gliomas for diagnosis and prognosis* (GREAT)

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2018–2022	LSMU PhD students council

Traineeships:

2021.08–2022.07	OHSU, Center for Embryonic Cell and Gene Therapy, Conrad lab (Portland, Oregon, USA)
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2020	The Lithuanian Research Council Scholarships for the academic achievements
2019	The Lithuanian Research Council Scholarships for the academic achievements

ACKNOWLEDGEMENTS

The outstanding scientific and administrative staff of the Neuroscience institute of the Lithuanian University of Health Sciences supported this work and the whole process of the PhD studies. I'm grateful for their understanding and encouragement to expand my scientific knowledge.

Dr. Paulina Vaitkienė guided me throughout this process, providing practical advice along the way and, at the same time, letting me grow as a scientist.

Also, I am grateful for all the opportunities associate professors Kristian Almstrup and Don Conrad provided. Their invaluable lessons and insights on science will always keep me motivated.

Most of all, I am thankful to my fantastic wife Ieva Stakaitienė! She never stopped believing in me during this scientific journey. Her discipline and diligence taught me to focus and notice small things that make a difference.