SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Examining the role of miRNAs in the diagnosis of glioblastoma and the determination of tumor progression

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DEBRECEN, 2025

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00 pm, on 8th December, 2025.

1. Introduction

A well-known and intensively researched representative of neoplastic lesions affecting the central nervous system is glioblastoma multiforme (GBM) of astrocytic origin, which is one of the most aggressive malignant neoplastic lesions and the most common malignant primary tumor of the brain and central nervous system, accounting for 14.5% of all tumors affecting the central nervous system and 48.6% of malignant CNS tumors [Grochans et al., 2022]. GBM can develop de novo (primary GBM) or through the malignant transformation of lower-grade diffuse astrocytomas or anaplastic astrocytomas (secondary GBM) [Oronsky et al., 2021]. However, the vast majority of cases (~90%) are de novo, which mainly occur in elderly patients and are characterised by very rapid development. In contrast, secondary variants manifest in younger individuals, are primarily localised in the frontal lobe, and are associated with a significantly better prognosis [Ohgaki et al., 2013; Kanderi et al, 2024]. Despite advances in neurosurgical techniques, therapeutic strategies, and genetic and molecular biological research, the prognosis for this condition remains extremely poor. The average survival time is estimated to be only 9-16 months, while the 1- and 5-year survival rates are 37.2% and 5.1%, respectively. Based on these data, GBM can be considered one of the most lethal forms of cancer, Therefore, it is essential to gain a deeper understanding of its pathophysiology and to identify new molecular markers that will not only provide us with broader scientific knowledge about the prognosis and diagnosis of the disease, but also bring us closer to developing a more effective

treatment strategy [Yao et al., 2018; Taylor et al., 2019; Wang et al., 2021]. One such potential marker molecule studied in relation to GBM is microRNAs (miRNAs), which have become a hot topic in molecular biology research in recent years. miRNAs are short, non-coding molecules consisting of 20-25 nucleotides that regulate a large proportion (nearly 90%) of protein-coding genes by fine-tuning gene expression at the post-transcriptional level [Jámbor et al, 2019]. In addition, thanks to their oncogenic and tumor suppressor functions, they are essential contributors to various cellular processes (e.g., cell proliferation, signal transduction, apoptosis) [Otmani et al., 2022]. They perform these tasks under physiological conditions when they are in a state of equilibrium. However, when this balance is altered due to changes in their expression levels, they can induce various diseases, including tumor formation. Taking this knowledge into account, it can be said that the characterization of miRNA expression patterns in GBM can be used as a potential diagnostic and prognostic tool and that the identified miRNAs and their verified targets may be useful in the future for selecting the appropriate therapy.

2. Aims

During my doctoral work, I set myself the goal of conducting a molecular genetic study of glioblastoma (GBM), the most common and aggressive type of brain tumor in adults. The significance of our choice of topic is justified by the disease's very high morbidity and mortality rates and the lack of early diagnostic and effective therapeutic strategies, despite ongoing efforts to date. In addition, we intended to use a 'novel' genetic and methodological approach to achieve results in order to characterize the disease and gain a deeper understanding of its molecular background. To this end, we chose to focus on miRNAs, small non-coding regulatory molecules at the post-transcriptional level, which have become the center of attention in molecular biology research in recent decades.

My research focused primarily on combining new-generation techniques (next-generation sequencing (NGS) and NanoString technology) to achieve the most efficient and reliable detection of miRNAs.

When applying these techniques, our fundamental goal was to:

- Identify new diagnostic and prognostic markers among miRNAs that would complement the molecular markers currently used in clinical practice, enabling more reliable diagnosis of the disease and more accurate determination of its progression.
- Our further goal was to perform bioinformatic analysis of the identified miRNAs, followed by a more in-depth

investigation and confirmation of the predicted miRNA targets and their actual function using mRNA sequencing.
- Finally, we aimed to compare the transcriptomic results obtained with plasma samples obtained by liquid biopsy sampling.

To achieve our goals, we had access to tissue samples from the peritumoral brain area of GBM patients and lower-grade glioma patients, defined as the control group, provided by the Brain Tumor and Tissue Bank of the Department of Neurosurgery at the University of Debrecen, as well as plasma samples taken from patients classified into the two groups.

3. Patients and methods

3.1. Brain tissue samples used

In the first phase of our research, we used 5 tissue samples (GBM and control) from the above-mentioned tissue pool for next-generation RNA (miRNA and mRNA) sequencing. In the case of each of the tissues used as controls, histopathological examination confirmed that they were clearly peritumoral, i.e., brain tissue free of tumor cells. In addition to the controls, the samples belonging to the patient group were also used after histological confirmation. When selecting individuals for the GBM group, we ensured that none of them had received chemotherapy and/or radiotherapy prior to their inclusion in the study. The average age of GBM patients was 63 years, while that

of the control group was 64 years. The demographic and clinical data of the patients were collected from a review of the relevant medical records.

Our research was approved by the Scientific and Research Ethics Committee of the Medical Research Council of the Ministry of Health, Budapest, Hungary, with the following license: ETT TUKEB; project identification code: IV/1753-/2021/EKU.

3.1.2. Total RNA isolation from brain tissue samples

Total RNA containing the miRNA fraction required for next-generation miRNA and mRNA sequencing was purified from the five tissue samples mentioned above using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). During isolation, 30 mg was used from each of the ten brain tissue samples in order to ensure the most accurate comparison of the results. After isolation, information on the concentration and quality of our RNA samples was obtained using a Nanodrop (Thermo Scientific, Waltham, MA, USA) spectrophotometer based on the A260/A280 and A260/A230 absorbance ratios.

3.1.3. Bioinformatic analysis of data obtained by next-generation sequencing

The analysis of changes in miRNA expression patterns was performed using next-generation small RNA sequencing with an Illumina NextSeq 500 device. We used the normalized

results of differentially expressed miRNAs and mRNAs obtained from the sequencing to determine the extent of changes in the transcriptome in comparison with the GBM control group. Bioinformatic evaluation of these data was performed using the iDEP.96 integrated web program.

3.1.4. In silico miRNA target gene prediction, construction of protein-protein interaction networks, gene ontology, functional annotation, and pathway analysis

We identified the potential target genes of miRNAs showing different levels of expression using the web-based miRNet and miRNA Enrichment Analysis and Annotation (miEAA) programs, which predicted targets based on the miRTargetLink 2.0 database. Only experimentally validated target genes were considered in the analysis. Based on these, we created an miRNA-target network using miRNet, in which the top miRNAs were determined based on their degree centrality value. In addition, using the NetworkAnalyst 3.0 visual analytics platform, we created comprehensive protein-protein interaction (PPI) networks using the top 50 up- and down-regulated mRNAs and miRNAs, where the hubs, i.e., central proteins, were also ranked according to their degree centrality values. In addition, using the NetworkAnalyst 3.0 platform, we performed network-based gene ontology (GO) analysis describing biological processes, as well as functional enrichment and pathway analysis using the pathway enrichment option of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. During the analysis, we considered a p-value of <0.05 to be statistically significant.

3.1.5. Validation of miRNA sequencing results using RT-qPCR

The results obtained by sequencing were confirmed using a larger sample size, for which 28 control and 30 GBM tissue samples were used. The total RNA required for the measurement was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol described in section 3.1.2, also using 30 mg of tissue sample. The concentration and quality of the purified RNA were measured using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Gene expression levels were determined using real-time quantitative polymerase chain reaction (RT-qPCR) with a LightCycler® 96 (Roche, Pleasanton, California, USA) instrument. The cDNA samples required for RT-qPCR measurements were transcribed from our total RNA samples, which also contained the miRNA fraction, using the miRCURY LNA RT Kit (Qiagen, Hilden, Germany). For validation, based on normalized data from iDEP analysis and sequencing, the following five miRNAs were selected based on their log2FC values: three upregulated (hsa-miR-196a-5p (log2FC = 5.6); hsa-miR-21-3p (log2FC = 4.39); hsa-miR-10b-3p (log2FC = 3.66)) and two downregulated miRNAs (hsa-miR-383-5p (log2FC = -6.33); hsa-miR-490-3p (log2FC = -5.61)). To detect and quantify the mature miRNAs to be validated, we used commercially available primers (miRCURY LNA miRNA PCR Assay (Qiagen, Hilden, Germany)), using hsa-miR-103a-3p as a reference gene, which was selected based on information available in the literature.

3.1.6. Validation of mRNA sequencing results using RT-qPCR

The results obtained by mRNA sequencing were confirmed using the same total RNA samples as in the validation of miRNA sequencing. However, in this case, the cDNA molecules required for the RT-qPCR reaction were transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For validation, the following 14 mRNA genes were selected based on their log2FC values from normalized iDEP analysis and sequencing data: E2F2 (log2FC = 3.59), HOXD13 (log2FC = 3.69), VEGFA (log2FC = 4.3), CDC45 (log2FC = 4.31), AURKB (log2FC = 4.6), HOXC10 (log2FC = 4.9), and MYBL2 (log2FC = 5.73), as well as the downregulated FABP6 (log2FC = -2.3), PRLHR (log2FC = -4.37), NEUROD6 (log2FC = -5.72), CBLN1 (log2FC = -6.16), HRH3 (log2FC = -6.39), HCN1 (log2FC = -7.36), and RELN (log2FC = -8.5), whose relative expression was determined using the Maxima™ SYBR Green qPCR Master Mix and the Lightcycler® 96 (Roche, Pleasanton, California, USA) using GAPDH as a reference gene [Kreth et al., 2010]. In contrast to validation performed on miRNAs, the expression of mRNAs was examined using primers designed by us, which were created using the primer design interface available on the NCBI (National Center for Biotechnology Information) website.

3.1.7. Statistical analysis

The distribution of data obtained by validating miRNSs and mRNAs was analyzed uniformly using the Kolmogorov-Smirnov test, which provides information on whether the data examined show a normal distribution by analyzing their mean and standard deviation values. Since our samples did not show a normal distribution, the statistical significance of the normalized expression data from the RT-qPCR measurements was calculated using the non-parametric Mann-Whitney U test, in which the expression difference observed between the control and patient groups was considered statistically significant at a value of $p < 0.05$. The diagnostic efficiency of the validated miRNAs was determined based on ROC-AUC curves generated using EasyROC (ver. 1.3.1.) analysis, based on sensitivity and specificity values. The significance level in this case was also $p<0.05$.

3.1.8. Determination of the correlation between miRNS and mRNA expression determined by next-generation sequencing

To determine the correlation between our deregulated miRNA and mRNA datasets, we examined miRNA-mRNA interactions using the miRTarBase and miRTargetLink 2.0 databases, considering only experimentally validated target gene interactions.

3.2. Blood plasma samples used

Another important objective of our research was to compare the results obtained from tissue examination with those obtained from plasma samples obtained by liquid biopsy. All individuals involved in the research were also undergoing treatment at the Department of Neurosurgery at the University of Debrecen. The blood samples used in our work came from six patients with GBM confirmed by histological examination and six individuals undergoing treatment for herniated discs, the latter forming the control group in our study. The average age of the individuals in the latter group was 58.6 years, while that of the patients in the GBM group was 61.3 years. As with the tissue samples, GBM patients who had not received chemotherapy or radiation therapy prior to participating in the study were selected for the sample.

Our research was approved by the Scientific and Research Ethics Committee of the Health Sciences Council, Budapest, Hungary, with the following license: ETT TUKEB; project identification code: 51450/2015/EKU (0411/15).

3.2.1. Isolation of total RNA from blood plasma

Total RNA purification from plasma samples, including miRNAs, was performed using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After isolation, information on the concentration and quality of our isolated RNA samples was obtained using a Nanodrop (Thermo Scientific, Waltham, MA, USA)

spectrophotometer based on the A260/A280 and A260/A230 absorbance ratios. Reverse transcription of the mature miRNAs obtained by isolation was performed using the miScript II RT Kit according to the manufacturer's instructions. The concentration of cDNAs was determined using a Qubit® 2.0 Fluorometer (Thermo Fischer Scientific, USA).

3.2.2. Identification of differentially expressed miRNAs using NanoString analysis

The miRNS expression pattern of control and GBM patient plasma samples was determined using the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) nCounter Human v3 miRNS panel at the RT-Europe Research Center in Mosonmagyaróvár. Background correction of the obtained data was performed by subtracting the average ± 2 standard deviations of the negative controls. Technical variation per lane was corrected by the geometric mean of the positive controls. Normalization of the entire dataset was performed using the geometric mean of 10 housekeeping miRNAs. Subsequently, miRNAs showing significantly different expression between the GBM and control groups were identified using the non-parametric Mann-Whitney U test, and the differential miRNA expression data were analyzed using the DESeq2 package of the iDEP.95 web program (using $FC \geq 1$ and $FDR \leq 0.1$ settings).

3.2.3. In silico prediction of differentially expressed miRNA targets

During in silico target prediction, we first created an miRNA target gene network using the web-based miRNet visual network analysis platform, which ranked the top miRNAs in the network based on their degree and betweenness centrality values. We predicted the experimentally validated target genes of these miRNAs using miRNet, miRTarBase, and TargetScan software. The target interactions obtained were then further validated using the miRWalk2 database, and the general and GBM-specific protein-protein interaction (PPI) networks of the target genes were created using the NetworkAnalyst 3.0 platform. Using differentially expressed miRNA target genes, we performed network-based gene ontology (GO) analysis describing biological processes and functional enrichment and pathway analysis using the pathway enrichment option of the KEGG database of the DAVID software. During the analyses, we considered a p-value of <0.05 to be statistically significant.

3.2.4. Validation of results obtained with NanoString analysis using RT-qPCR

Cell-free total RNAs used to confirm the results obtained with NanoString analysis were extracted from plasma samples from 28 healthy controls and 26 GBM patients using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany).The concentration of the miRNA fraction in the isolated total RNA samples was measured using the Qubit® 2.0 (Thermo Fischer Scientific, Waltham, MA, USA) miRNA-specific fluorometric

device. The amount of mature miRNAs was measured by RT-qPCR using a LightCycler® 96 instrument (Roche, Pleasanton, CA, USA). The miScript II RT Kit (Qiagen, Hilden, Germany) was used for reverse transcription of the miRNAs to be quantified during the reaction. During RT-qPCR measurements, the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used to validate randomly selected hsa-miR-433-3p, hsa-miR-29-3p, hsa-miR-195-5p, hsa-miR-362-3p, hsa-miR-133a-3p, and hsa-miR-1286-3p, which were detected in both the GBM and control groups using specific primers (miScript Primer Assays, Qiagen, Hilden, Germany) provided by the manufacturer. We used hsa-miR-16-5p as a reference miRNA [Ma et al., 2018]. Three parallel measurements were performed during the experiments.

3.2.5. Statistical analysis

The significance of miRNS expression levels obtained by RT-qPCR measurements was calculated using the non-parametric Mann-Whitney U test, in which a value of $p<0.05$ was considered statistically significant. Using the normalized expression data of miRNAs showing significant differences from RT-qPCR measurements, we created ROC-AUC curves with the help of the easyROC program to assess the diagnostic potential of our miRNAs. The significance level in this case was also $p<0.05$.

4. Results

4.1. miRNA and mRNA expression profiles identified by next-generation sequencing from tissue samples of glioblastoma and control groups

Using normalized data from sequencing, hierarchical cluster analysis and principal component analysis (PCA) were performed using the iDEP.96 program to visualize changes in the expression patterns of miRNAs in the GBM-control group comparison. Based on the clusters formed by the program for each sample, ranked according to the 200 most significant differences in the global expression patterns of miRNS and mRNA genes, it can be said that the expression of the miRNS and mRNAs examined shows a clear difference between the control and GBM groups. Our findings were confirmed by PCA analyses performed to better interpret and visualize the distribution of miRNA and mRNA expression values, based on which we determined that the GBM samples formed a single cluster based on their expression patterns and were clearly separated from the control samples. This data distribution suggests that GBM biogenesis induced drastic changes in the expression of numerous miRNAs and mRNAs.

4.1.1. Identification and validation of differentially expressed miRNA and mRNA genes using RT-qPCR

Using the DESeq2 algorithm of the iDEP.96 web program, with FDR < 0.05 and FC > 2 thresholds, we identified a total of 117 miRNAs and 1590 mRNAs that showed significantly different expression in GBM patient samples compared to control samples. Of these, 35 miRNAs and 365 mRNAs showed upregulation (log2FC ≥ 1), while 82 miRNAs and 1225 mRNAs showed downregulation (log2FC ≤ −1). Based on the p-values calculated using the Mann-Whitney U test following RT-qPCR measurements performed on the miRNAs and mRNAs selected for validation described in sections 3.1.5 and 3.1.6, all three were upregulated (hsa-miR-196a-5p: $p<0.00001$; hsa-miR-21-3p: $p<0.00056$; hsa-miR-10b-3p: $p<0.00001$) and both downregulated (hsa-miR-383-5p: $p<0.00028$; hsa-miR-490-3p: $p<0.00056$), and all seven were upregulated (E2F2: $p=0.00056$; HOXD13: $p<0.00001$; VEGFA: $p<0.00001$; CDC45: $p=0.00024$; AURKB: $p=0.0001$; HOXC10: $p<0.00001$; and MYBL2: $p<0.00001$) and seven downregulated mRNAs (FABP6: $p=0.02088$; PRLHR: $p=0.00028$; HCN1: $p=0.00194$ and RELN: $p<0.00001$). Thus, it can be said that for both miRNAs and mRNAs, we successfully confirmed the different expression observed during sequencing in the GBM group compared to the control group.

4.1.2. Characterization of the diagnostic efficacy of validated miRNAs using the EasyROC program

Based on ROC-AUC curves generated using normalized expression data of validated miRNAs, we determined that the miRNAs examined have outstanding diagnostic potential based on their AUC, sensitivity, and specificity values, which were as follows for hsa-miR-196a-5p, hsa-miR-21-3p, hsa-miR-10b-3p, hsa-miR-383-5p, and hsa-miR-490-3p. AUC: 0.96032, 0.97768, 0.99206, 0.9375, and 0.9648. The highest sensitivity values were observed for our two downregulated miRNAs, hsa-miR-383-5p (95%) and hsa-miR-490-3p (95%). Slightly lower, but still remarkable values were obtained for the upregulated hsa-miR-10b-3p (94%), hsa-miR-21-3p (93.8%), and hsa-miR-196a-5p (88%). The 1-specificity values followed a different order. In this case, the highest values were associated with the upregulated miRNAs. The highest value was observed for hsa-miR-10b-3p (100%), followed by hsa-miR-21-3p (92.9%) and hsa-miR-196a-5p (92%), followed by the two downregulated hsa-miR-383-5p (95%) and hsa-miR-490-3p (85%).

4.1.3. Correlation between the new generation sequencing-derived miRNA and mRNA datasets

Based on the correlation data obtained from the analysis, we hypothesized that significantly up- or downregulated miRNAs in GBM patients may be involved in the regulation of gene expression in cell cycle (AURKB, CDC45, CDK6), cell proliferation (EGFR, VEGFA), and angiogenesis (VEGFA), thereby promoting tumor growth. Furthermore, in the same context, they interact with upregulated transcription factors such as E2F2 and MYBL2, which regulate the transcription of genes

involved in the cell cycle, cell differentiation, and cell proliferation. In addition, other target genes (AJAP1, MMP9, POSTN, STC2) promote metastasis by regulating adhesion or migration, while the upregulated HOXC10 participates in the transcription of genes that enhance migratory capacity. In addition, we have identified genes (LTBP-1, POSTN) through which the regulatory function of tumor-associated macrophages can influence the tumor microenvironment.

4.2. Differentially expressed miRNAs identified by NanoString nCounter analysis from plasma samples of glioblastoma and control individuals

As a result of the NanoString analysis, 107 of the 798 unique miRNAs examined showed significant differences between tumor and normal plasma samples after screening and differential expression analysis. Adjusting the expression data obtained to the fold-change (FC) and false discovery rate (FDR) cut-off values (log2FC 1 and FDR 0.1), only one miRNA, hsa-miR-181a-3p, showed downregulation, and a total of 52 miRNAs showed upregulation. Based on the literature and our search results in the miRCancer and miR2Disease databases, it can be said that the majority of the miRNAs we identified with different expression levels (~94%) have previously been found to be associated with various malignant tumors, and many of them (~57%) can also be associated with GBM. However, we also identified two new associations related to GBM that have not yet been described by other research groups. These are hsa-miR-1252-5p and hsa-miR-591.

4.2.1. Validation of miRNAs expressed at different levels during NanoString nCounter analysis using the RT-qPCR method

To validate the results obtained with NanoString analysis, we successfully confirmed the significantly increased expression levels of hsa-miR-433-3p, hsa-miR-195-5p, and hsa-miR-29a-3p in the GBM group from randomly selected miRNAs. The Kruskal-Wallis p-values were p= 0.00714 for hsa-miR-433-3p, p= 0.0466 for hsa-miR-195-5p, and p= 0.0041 for hsa-miR-29a-3p, while we were unable to detect expression of hsa-miR-362-3p and hsa-miR-133a-3p in either GBM or healthy control samples. Similar to our results, Wang et al. also demonstrated increased expression of hsa-miR-195-5p in blood samples from GBM patients. They hypothesize that this miRNA may be involved in the regulation of fatty acid metabolism in GBM [Wang & Lu, 2020]. According to some observations, cancerous diseases characterized by relapse are closely related to certain metabolic disorders, such as decreased fatty acid biosynthesis, which has been found to play a role in the pathogenesis of several cancerous diseases [Kannan et al., 1980]. After examining the expression of hsa-miR-195, Jia and colleagues concluded that increased expression of this miRNA is associated with longer median survival (56.53 months), compared to patients with decreased expression, for whom this value was 15 months. Furthermore, their multivariate Cox regression analysis of survival showed that high hsa-miR-195 levels were associated with reduced disease mortality [Jia et al., 2020]. For our next validated miRNA, hsa-miR-29a, Zhao et al. found that this miRNA activates a complex post-transcriptional program of GBM growth and invasion through the downregulation of PTEN,

EphB3, and SOX4, thereby supporting the aggressive nature of GBM. They also observed that increased expression of hsa-miR-29a correlates with reduced survival in patients [Zhao et al., 2019]. The last circulating miRNA we identified, hsa-miR-433-3p, has been described in previous studies as being deregulated in various cancers, and a significant association has been found between the altered expression of this miRNA and the clinical outcome of these diseases. However, contrary to our results, downregulation of hsa-miR433-3p has been observed in gastric carcinoma and hepatitis B virus-associated hepatocellular carcinoma, among others [Luo et al., 2009; Wang et al., 2012]. There may be several reasons for this conflicting result, including differences in sample sources, detection methods, tumor subtypes, and individual differences. Therefore, further studies are needed to further evaluate the role of hsa-miR-433-3p in GBM.

4.2.2. Characterization of the diagnostic efficacy of validated miRNAs using the EasyROC program

Using the normalized expression data obtained from validation, we generated ROC-AUC curves and determined that, based on the AUC, sensitivity, and specificity values, hsa-miR-433-3p, hsa-miR-195-5p, and hsa-miR-29a-3p, which showed significantly different expression between the two groups, were 0.98214, 0.9704, and 0.98214, respectively, all of which give the examined miRNAs outstanding diagnostic potential, thus predicting their potential applicability as biomarkers in clinical practice. Our hypothesis was supported by the sensitivity and

specificity values of the studied miRNAs. Namely, hsa-miR-433-3p and hsa-miR-29a-3p had the same sensitivity (92%) and specificity (96%) values, while hsa-miR-195-5p had a slightly lower sensitivity (88%) with an unchanged specificity value (96%).

4.2.3. Gene ontology (GO) and pathway enrichment analysis of miRNA targets

Our GO_BP and KEGG pathway-based functional annotation analysis using our miRNA-specific target list revealed that genes regulated at the post-transcriptional level by deregulated miRNAs are enriched in signaling pathways that are key to tumorigenesis. Related to this, biological processes include DNA methylation, O-linked glycosylation of proteins, positive and negative regulation of RNA polymerase II promoter transcription, gene expression regulation, negative regulation of the G1/S transition in the mitotic cell cycle, and their role in apoptotic processes. Based on the results of the KEGG pathway analysis, a correlation was found between several types of cancer, such as glioma, prostate cancer, bladder cancer, small cell lung cancer, non-small cell lung cancer, melanoma, endometrial cancer, pancreatic cancer, and pathways associated with viral infections (hepatitis B, HTLV-I infection).

4.2.4. Protein-protein interaction (PPI) analysis of miRNA targets

We mapped the potential interactions between target proteins and their functionally important interaction partners using general and cortex-specific PPI networks. The main biologically relevant proteins forming the nodes were BCL2, RB1, PTEN, ERBB2, CCND1, ZEB1, FSCN1, WNT1, XIAP, FOXO1, UBA2, DNMT3B, ANXA2, and WEE1. Based on our results, we can say that we observed essentially the same proteins in both networks, from which we can conclude that the differentially expressed miRNAs regulate target genes that are involved in the fundamental processes that trigger tumor formation. Using the NetworkAnalyst network analysis platform, we observed enrichment in processes such as the cell cycle, viral carcinogenesis, ubiquitin-mediated proteolysis, apoptosis, transcriptional dysregulation in cancer, glioma genesis, EGFR tyrosine kinase inhibitor resistance, and FoxO, p53, ErbB, PI3K-Akt, and neurotrophin signaling pathways. Key biological processes included cell cycle, apoptosis, protein modification processes, and positive and negative regulation of cellular metabolism. In terms of molecular functions, the most significant enrichments were enzyme binding, negative and positive regulation of transcription, transcription factor binding, kinase binding, and chromatin binding.

Based on the results of enrichment analyses, it can be stated that most miRNA targets are involved in signaling pathways and biological processes critical for tumor formation, suggesting that circulating miRNAs may be potential regulatory molecules in the process of tumorigenesis. However, the data also show that these observations are not specific to a particular tumor type. Nevertheless, the network-based approach may provide a

tool for discovering new proteins and interactions that physically and functionally interact with so-called core proteins, thereby representing new tumor-associated genes or biomarkers.

5. New scientific results

- Hsa-miR-196a-5p, hsa-miR-10b-3p, hsa-miR-21-3p, hsa-miR-383-5p, and hsa-miR-490-3p showed significantly different expression in tissue samples from patients with glioblastoma. In addition, they are characterized by outstanding diagnostic indicators, making them promising marker molecules for the disease.

- We observed significant deregulation of each of the MYBL2, AURKB, VEGFA, CDC45, E2F2, HOXC10, HOXD13, HRH3, CBLN1, RELN, HCN1, NEUROD6, PRLHR, and FABP6 genes in tissue samples from glioblastoma patients.

- As a result of our research, we have compiled a panel containing 5 miRNAs and 14 mRNAs that may help to better understand glioblastoma tumorigenesis and facilitate tissue-based diagnosis in the Hungarian population.

- In addition to tissue samples, we also successfully identified miRNAs with different expression levels in plasma samples from glioblastoma patients. We detected 53 miRNAs with significant deregulation.

- During validation, the expression of hsa-miR-29a-3p, hsa-miR-195-5p, and hsa-miR-433-3p was found to be significantly higher in plasma samples from glioblastoma patients. Similar to the miRNAs identified in tissue samples, these miRNAs have excellent diagnostic potential and may therefore be valuable non-invasive biomarkers for the disease.

- We were the first in this geographical region to perform miRNA and mRNA profiling of tissue samples and plasma samples from glioblastoma patients.

I believe that our results could form a good basis for further validation in a large number of prospective cohort studies in the future.

6. Summary

When it comes to cancer affecting the central nervous system, glioblastoma (GBM) is by far the biggest challenge for both healthcare providers and basic researchers. Due to the therapeutic difficulties arising from the high degree of heterogeneity and infiltrative capacity of the tumor, as well as its increased tendency to recur, it is essential to gain a deeper understanding of its development. In this context, it is necessary to identify additional key molecules that could serve as new therapeutic and/or prognostic targets, enabling more effective treatment of the disease, better outcomes, and increased survival time. In the hope of making progress in this area, we have chosen to focus on miRNAs, which are currently the subject of considerable attention in the field of molecular biology. As post-transcriptional regulators, these molecules play an essential role in the regulation of biological processes. During my doctoral work, I determined the miRNA profile of brain tissue samples obtained by tissue biopsy, which is still considered the gold standard procedure today, using next-generation small RNA sequencing. The primary goal of this was to understand the role these molecules play in GBM tumorigenesis. Our research identified a set of five miRNAs (hsa-miR-196a-5p, hsa-miR-10b-3p, hsa-miR-21-3p, hsa-miR-383-5p, and hsa-miR-490-3p) that can function as diagnostic markers in addition to their proven role in tumor formation. In addition, we also wanted to use next-generation RNA sequencing to verify the actual regulatory function of miRNAs showing deregulation characteristic of GBM. In the process, we successfully identified 14 mRNA

molecules (MYBL2, AURKB, VEGFA, CDC45, E2F2, HOXC10, HOXD13, HRH3, CBLN1, RELN, HCN1, NEUROD6, PRLHR, FABP6) that are regulated by deregulated miRNAs and showed significantly different expression in GBM. Subsequently, our goal was to detect the five validated miRNAs in plasma samples using the RT-qPCR method, but we were unable to detect measurable amplification for any of the miRNAs.

Thus, in the second half of our research, we aimed to map the miRNA expression profile of plasma samples obtained by liquid biopsy using a different method. During the validation of the NanoString method results, we successfully identified three circulating miRNAs (hsa-miR-29a-3p, hsa-miR-195-5p, and hsa-miR-433-3p) that may be involved in the development of GBM. The significance of our research lies not only in the high mortality and morbidity rates of the disease but also in the fact that our analyses were performed using real patient samples, which are able to represent the changes that occur during the course of the disease to the greatest extent possible.

7. References

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Registry number: DEENK/423/2025.PL
Subject: PhD Publication List

Candidate: Dóra Géczi
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Géczi, D.**, Klekner, Á., Balogh, I., Penyige, A., Szilágyi, M., Virga, J., Bakó, A., Nagy, B., Torner, B., Hádáné Birkó, Z.: Identification of Deregulated miRNAs and mRNAs Involved in Tumorigenesis and Detection of Glioblastoma Patients Applying Next-Generation RNA Sequencing.
   Pharmaceuticals. 18 (3), 1-29, 2025.
   DOI: http://dx.doi.org/10.3390/ph18030431
   IF: 4.8 (2024)
2. **Géczi, D.**, Nagy, B., Szilágyi, M., Penyige, A., Klekner, Á., Jenei, A., Virga, J., Hádáné Birkó, Z.: Analysis of Circulating miRNA Profile in Plasma Samples of Glioblastoma Patients.
   Int. J. Mol. Sci. 22 (10), 1-20, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22105058
   IF: 6.208

List of other publications

3. Torner, B., **Géczi, D.**, Klekner, Á., Balogh, I., Penyige, A., Hádáné Birkó, Z.: Construction of a miRNA Panel for Differentiating Lung Adenocarcinoma Brain Metastases and Glioblastoma.
   Cancers. 17 (4), 1-23, 2025.
   DOI: http://dx.doi.org/10.3390/cancers17040581
   IF: 4.4 (2024)

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4. Jakab, Á., Emri, T., Csillag, K., Szabó, A., Nagy, F., Baranyai, E., Sajtos, Z., **Géczi, D.**, Antal, K., Kovács, R. L., Szabó, K., Dombrádi, V., Pócsi, I.: The negative effect of protein phosphatase Z1 deletion on the oxidative stress tolerance of Candida albicans is synergistic with betamethasone exposure.
J. Fungi. 7 (540), 1-24, 2021.
DOI: http://dx.doi.org/10.3390/jof7070540
IF: 5.724

Total IF of journals (all publications): 21,132
Total IF of journals (publications related to the dissertation): 11,008

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

24 June, 2025

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