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Differential expression of microRNAs targeting genes associated with the development of high-grade gliomas

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Abstract

Background: Highly malignant high-grade gliomas are tumors of the central nervous system (CNS). They are solid tumors arising from transformed cells of the brain and/or the spinal cord. In recent years, the expression of genes and regulating miRNAs in glial brain tumors has been actively studied. The present study is devoted to assessing the expression levels of miR-215-5p, miR-22-3p, miR-122-5p, miR-107, miR-324-5p, miR-34a-5p, miR-155. -5p, miR-21-5p, miR-497-5p, miR-330-3p, miR-146a-5p, miR-92a-1-5p, miR-326 and target genes EGFR, SMAD4, SMAD7, SMO, NOTCH1, NOTCH2, HIF1A, EGLIN1/3, KDM1B, KDM1A, MSI1, MSI2, TET1 in high-grade glioma tissues.

Results: As a result of the analysis of the levels of relative expression of the studied genes, there are significant changes ($p < 0.05$) in tumor tissue for genes: *EGFR*, *SMAD4*, *SMAD7*, *SMO*, *HIF1A*, *EGLN1/3*. We obtained data on a significant change ($p < 0.05$) in the levels of relative expression for microRNA: hsa-miR-215-5p, hsa-miR-22-3p, hsa-miR-122-5p, hsa-miR-107, hsa-miR-324-5p, hsa-miR-155-5p, hsa-miR-21-5p, hsa-miR-330-3p, hsa-miR-326. Data on the association of overall survival in patients with high-grade glioma and the level of relative expression of the *EGFR* and *HIF1A* genes were obtained. The obtained data demonstrate the association of overall survival of patients with high-grade glioma and the level of relative expression of *EGFR*, *HIF1A* and hsa-miR-22-3p, hsa-miR-107 and hsa-miR-330-3p.

Conclusions: The obtained data on the expression of genes and microRNAs expand the understanding of the biology of the development of high-grade glial tumors. These data demonstrate new potential therapeutic and prognostic goals in high-grade gliomas.

Keywords: High-grade gliomas, Gene expression, Expression of miRNAs, Prognostic markers, EGFR, HIF1A

Background

Gliomas are the most common cancer of the central nervous system that develops from glial cells. These tumors are characterized by diffuse and infiltrative growth, which ensures their growth into the surrounding tissues. High-grade gliomas are characterized by nuclear atypia, cellular pleomorphism, mitotic activity, anaplasia, and rapid proliferation, alternating with aggressive invasion of brain tissue. In their microenvironment, glioma cells face many factors such as acidity, hypoxia, and low nutrient availability. To maintain rapid growth, they need to modulate metabolic activity [1]. Despite the achieved success in the complex therapy of high-quality gliomas, patients demonstrate low overall survival rates—8 months for glioblastomas and 20 months for anaplastic astrocytomas; there is an urgent need to develop new therapeutic and prognostic targets [2].

In accordance with the current WHO classifier, glioma subtypes have some molecular genetic features (Table 1).

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Table 1 Molecular classification markers of gliomas [2]

Molecular marker	Subtype WHO
IDH WT	Diffuse or anaplastic astrocytoma, primary glioblastoma
IDH mut	Diffuse or anaplastic astrocytoma, diffuse or anaplastic oligodendroglioma, secondary glioblastoma
Not co-deletion 1p–19q	Diffuse or anaplastic astrocytoma, glioblastoma
Co-deletion 1p–19q	Diffuse or anaplastic oligodendroglioma
H3-K27M mut	Diffuse midline glioma

Changes in gene expression are intensively studied in cancer, including gliomas. The identification of differentially expressed genes stimulates the development of new therapeutic and prognostic targets [3]. Gene expression is regulated by many transcriptional and posttranscriptional factors. MicroRNA is an important factor in the regulation of transcription [4]. In this study, we assessed the expression of genes involved in important signaling pathways in the development of oncological diseases in the tissues of high-grade gliomas in comparison with conventionally healthy tissue. The proteins of the studied genes are participants in a number of important signaling pathways. The epidermal growth factor receptor (EGFR) is involved in signaling to the cell leading to differentiation, proliferation, migration, and survival [5]. SMAD4 and SMAD7 are important participants in TGF-B signaling, which takes on a role in tumor development [6]. The smoothened protein (SMO) acts as a key regulator of the hedgehog signaling pathway, which regulates the formation of embryonic patterns, tissue regeneration, stem cell renewal, and is involved in cancer development [7]. Transmembrane proteins NOTCH1/2 are participants in NOTCH signaling in the regulation of many cellular processes throughout life, including cell proliferation, maintenance of stem cells, cell cycle, and differentiation [8]. Musashi proteins (MSI1/2) are also involved in NOTCH signaling by binding to mRNA of the negative regulator Numb [9]. HIF1A and EGLN1/3 are involved in the response of the tumor cell to low oxygen conditions by stabilizing and activating the hypoxia-inducible factor, a transcription factor critical for the adaptive response to reduced oxygen levels [10]. KDM1A/B plays a role in the formation of the histone H3 methylation profile, regulating the proliferation and differentiation of neural stem cells [11]. Tet methylcytosine dioxygenase 1 (TET1) is an important factor in the response of glioma therapy to ionizing radiation [12].

The main goal of the present study was to expand the understanding of high-grade gliomas at the molecular level. In this study, we analyzed the differential expression of mRNA of the genes described above, which are key participants in the signaling pathways associated with carcinogenesis. Additionally, the levels and microRNAs that treat the genes under study were investigated. Potential targets for targeted therapy of gliomas and prognostic markers have been identified.

Methods

The study was approved by the ethics committee of the Federal State Budgetary Institution "National Medical Research Center of Oncology" of the Ministry of Health of Russia. In each case, the patient received voluntary informed consent for inclusion in the study. The selection included 28 patients (13 men and 15 women) with an initially diagnosed glial brain tumor. The diagnosis was confirmed by histological and molecular genetic studies in accordance with the WHO classification of CNS tumors (2016) [13]. The average age was 59.2 ± 9.1 years. Tissue samples of gliomas and conventionally healthy (control) tissue (24 cases of glioblastoma, IDH-WT and 4 cases of anaplastic astrocytoma, IDH-WT) were taken intraoperatively. Sampling points were calculated using the Medtronic S7 navigation station (Medtronic, Republic of Ireland). The control point was localized at a distance of 15 mm from the border of the tumor along the path of access to the tumor, outside the functionally significant areas of the brain. To visualize the tumor, patients took 5-aminolevulinic acid 2 h before surgery. The control biopsy specimen when evaluated with a Blue 400 module of a CarlZeiss OPMI PENTERO microscope (Zeiss AG, Germany) did not have fluorescence. Tissue samples were frozen in liquid nitrogen and stored at − 80 °C.

RNA and DNA extraction and purification

Without thawing, tissue fragments were placed in TRIzol (ThermoFisher, USA) and homogenized using MagNALyser (Roshe, Switzerland). Nucleic acids were isolated according to the manufacturer's recommendations. Additionally, DNA and RNA were purified using a DNA-sorb-B kit (AmpliSens, Russia) and a miRNA miniKit kit (Qiagen, Germany), respectively. The resulting samples of total RNA were treated with DNAase

1 (ThermoFisher, USA) to remove genomic DNA. The concentration of nucleic acids was evaluated on a Qubit fluorometer (ThermoFisher, USA) according to the manufacturer's instructions. Synthesis of cDNA on an RNA template was performed using the Poly(A) polymerase (New England Biolabs, USA) and the MMLV Reverta kit (Syntol, Russia) with universal primer as described earlier [14].

Molecular genetic diagnostics

Somatic mutations in genes IDH1 (R132), IDH2 (R172) were determined using Sanger sequencing, and 1p-19q deletions were determined by fragment analysis on an AB 3500 genetic analyzer (Applied Biosystems, USA) [15, 16].

Quantification of relative expression of mRNA 14 genes (*EGFR*, *SMAD4*, *SMAD7*, *SMO*, *NOTCH1*, *NOTCH2*, *HIF1A*, *EGLIN1*, *EGLIN3*, *KDM1B*, *KDM1A*, *MSI1*, *MSI2*, *TET1*) and 13 microRNAs (hsa-miR-215-5p, hsa-miR-22-3p, hsa-miR-122-5p, hsa-miR-107, hsa-miR-324-5p, hsa-miR-34a-5p, hsa-miR-155-5p, hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-330-3p, hsa-miR-146a-5p, hsa-miR-92a-1-5p, hsa-miR-326) were performed by RT-qPCR. MicroRNAs targeting the studied genes were selected using the TargetScan [16] and miRTarBase [17] databases. The genes *PSMC*, *TBP*, *RPLO*, miRNAs miR-191-5p, miR-103a-1-5p and small nuclear RNA RNU49 were used as reference sequences. Expression stability was evaluated using geNorm [18]. Design of specific oligonucleotide primers for genetic loci and miRNAs was performed using Primer-BLAST [19], NCBI GenBank reference sequences, and miRBase database (Additional file 1). PCR was performed on a Bio-Rad CFX96 thermocycler (Bio-Rad, USA). Quantitative PCR analysis was performed using the $2^{-\Delta\Delta Ct}$ method [20].

Data analysis

All statistical analyses were performed using R studio. Differences in expression between tumor tissue and normal brain tissue were calculated using the Wilcoxon W-test. Correlation of expression of miRNAs and their target genes was calculated using Spearman rank correlation. Kaplan–Meier survival curves were then drawn for all genes and miRNAs predicted to show a survival risk either above or below average risk, using cutoff points of gene expression levels identified by MaxStat package of R software [21]. A value of $p<0.05$ indicates statistical significance.

The visualization of the interaction network was implemented using Cytoscape 3.7.2 [22].

Results

Samples were taken from 28 patients with high-grade glioma IDH-WT and no 1p-19q co-deletion. Data on clinical and morphological parameters and relative expression of 13 microRNAs and 14 target genes in high-grade glioma samples are given in Additional file 1.

A statistically significant increase ($n<0.05$) in mRNA levels was demonstrated by the genes *EGFR* (60% of patients, median FC—2.4), *SMO* (63% of patients, median FC—1.3), and *HIF1A* (73% of patients, median FC—1.8). A significant decrease in mRNA expression was observed for genes *SMAD4* (50% of patients, median FC—0.9), *SMAD7* (83% of patients, median FC—0.3), *EGLN1* (90% of patients, median FC—0.66) and *EGLN3* (67% of patients, median FC—0.56). There were no significant changes in the relative expression of the *NOTCH1*, *NOTCH2*, *KDM1B*, *KDM1A*, *MSI1*, *MSI2*, and *TET1* genes in tumors relative to healthy tissue (Fig. 1, Table 2).

Analysis of the TargetScan and miRTarBase databases made it possible to isolate a pool of 104 microRNAs targeting 3'UTR mRNA of the *EGFR*, *SMAD4*, *SMAD7*, *SMO*, *NOCTH1*, *NOTCH2*, *HIF1A*, *EGLN1*, *EGLN3*, *KDM1B*, *KDM1A*, *MSI1*, *MSI1*, *MSI1*. Based on a literature search at PubMed, 13 microRNAs found in publications regarding glial tumors were selected from the list (Fig. 2).

In the study sample, a statistically significant increase ($p<0.05$) in the relative expression was found for hsa-miR-155-5p (for 73% of cases, median FC—3) and for hsa-miR-21-5p (67% of patients, median FC—2.9). There was a statistically significant ($p<0.05$) decrease in the relative expression for hsa-miR-215-5p (73% of patients, median FC—0.55), hsa-miR-22-3p (63% of patients, median FC—0.81), hsa-miR-107 (80% of patients, median FC—0.55), hsa-miR-324-5p (100% of patients, median FC—0.81), hsa-miR-330-3p (100% of patients, median FC—0.09), hsa-miR-326 (70% of patients, median FC—0.71) and hsa-miR-122-5p (83% of patients, median FC—0.66), respectively (Fig. 3, Table 2). For the rest of microRNAs (hsa-miR-34a-5p, hsa-miR-497-5p, hsa-miR-146a-5p, hsa-miR-92a-1-5p), no significant changes were revealed in the relatively healthy tumor tissue.

Additionally, according to the analysis of associations of changes in the transcriptional activity of miRNA and genes, significant negative correlations of *HIF1A* expression with miR-215-5p ($r=-0.55$, $p=0.0026$) and miR-122-5p ($r=-0.52$, $p=0.0046$), EGLN1 with miR-155-5p ($r=-0.39$, $p=0.0407$).

Analysis of clinical data and transcriptional activity of genetic loci revealed that increased expression of *HIF1A* or *EGFR* is associated with a decrease in patient survival. A low level of *HIF1A* expression leads to an increase in

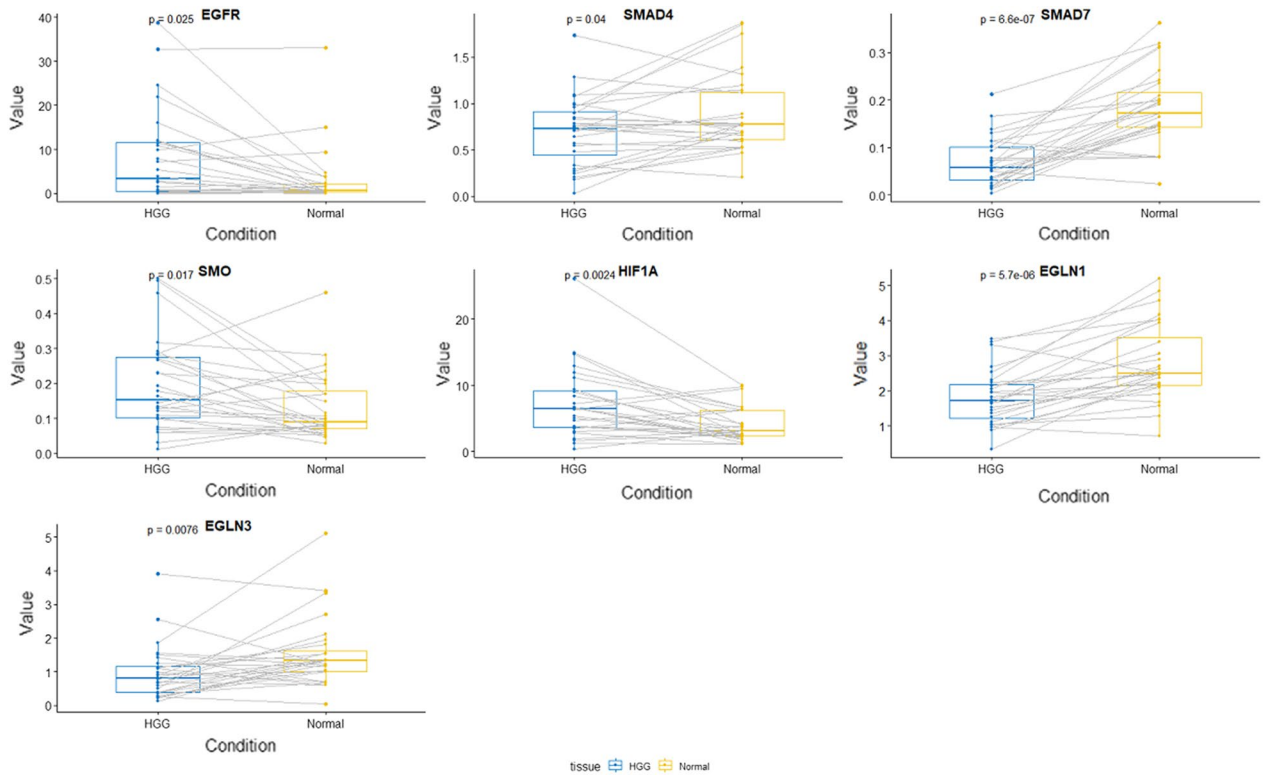

Fig. 1 Comparison of the relative expression of genes showing a level of statistical significance $p < 0.05$ Wilcoxon test in paired samples from patients with high-grade glioma. HGG—tissue high-grade glioma, normal—healthy tissue, value—$2^{-\Delta Ct}$

Table 2 Statistically significant ($p < 0.05$) changes in gene and microRNA expression in high-grade tissue relatively healthy

Gene	Median FC	Regulatory microRNA	Median FC	Correlation Spearmen
EGFR	2.42	hsa-miR-21-5p	2.9	r = 0.38, p = 0.044*
		hsa-miR-155-5p	3	r = 0.34, p = 0.08*
		hsa-miR-122-5p	0.6	r = 0.15, p = 0.44
SMAD4	0.9	hsa-miR-155-5p	3	r = −0.2, p = 0.3
SMAD7	0.31	hsa-miR-330-3p	0.09	r = 0.62, p = 4e−04*
		hsa-miR-21-5p	2.9	r = −0.35, p = 0.067
SMO	1.3	hsa-miR-324-5p	0.48	r = −0.4, p = 0.033*
		hsa-miR-330-3p	0.09	r = −0.05, p = 0.8
HIF1A	1.79	hsa-miR-330-3p	0.09	r = −0.25, p = 0.2
		hsa-miR-21-5p	2.9	r = 0.29, p = 0.12
		hsa-miR-22-3p	0.81	r = −0.01, p = 0.96
		hsa-miR-107	0.55	r = −0.29, p = 0.13
		hsa-miR-155-5p	3	r = 0.48, p = 0.01*
EGLN1	0.66	hsa-miR-21-5p	2.9	r = −0.26, p = 0.18
EGLN3	0.56	hsa-miR-122-5p	0.6	r = −0.27, p = 0.16

* *p* values correlation Spearman < 0.05

median of overall survival of patients with HGG from 40.5 to 220 days ($p = 00.063$), and *EGFR* from 43 to 306 days ($p < 0.0001$) (Fig. 4).

A similar relationship was found between the survival of patients with HGG and miR-22-3p, miR-107 and miR-330-3p expression (Fig. 5). Reduced expression of

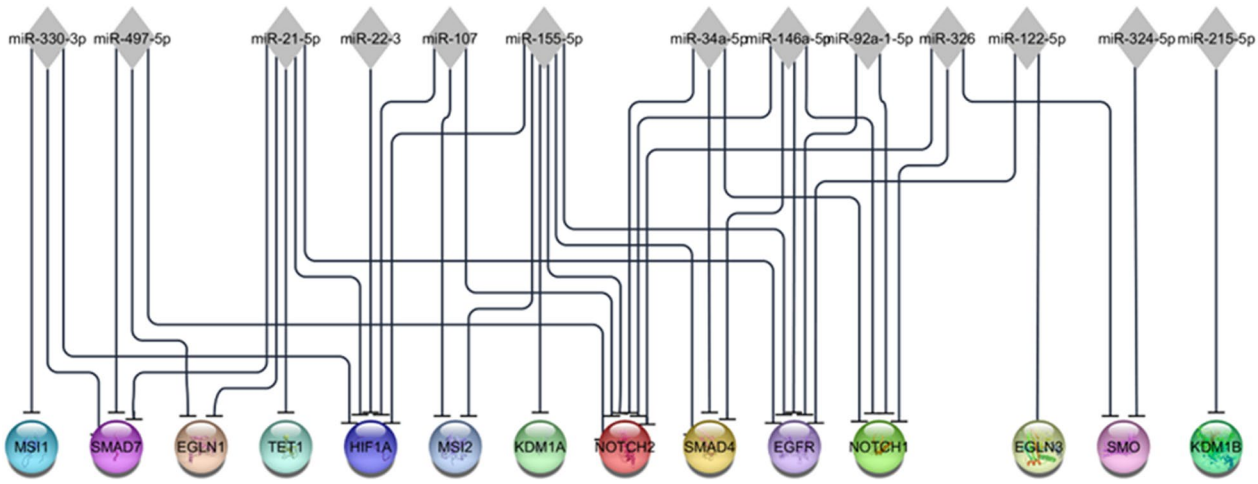

Fig. 2 MicroRNAs targeting the studied genes according to information in the TargetScan, miRTarBase databases and published data (PubMed)

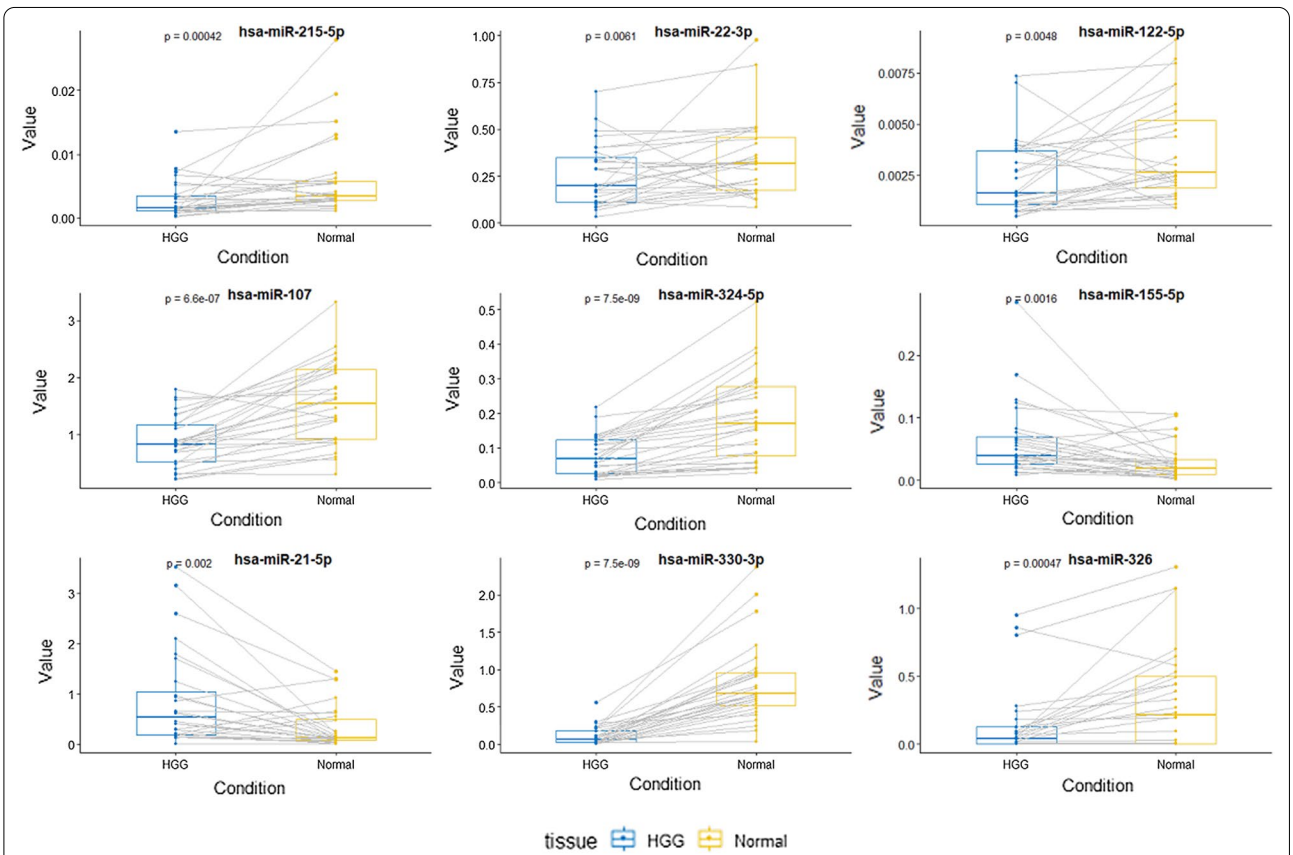

Fig. 3 Comparison of the relative expression of miRNAs showing a level of statistical significance $p < 0.05$ Wilcoxon test in paired samples from patients with high-grade glioma. HGG—tissue high-grade glioma, normal—healthy tissue, value—$2^{-\Delta Ct}$

miR-22-3p, miR-107, or miR-330-3p led to an increase in median overall survival by 6 ($p = 0.038$), 4 ($p = 0.048$), and 4 ($p = 0.0088$) times, respectively.

Discussion

High-grade gliomas have a complex pathogenesis, which includes mutations of many key factors of

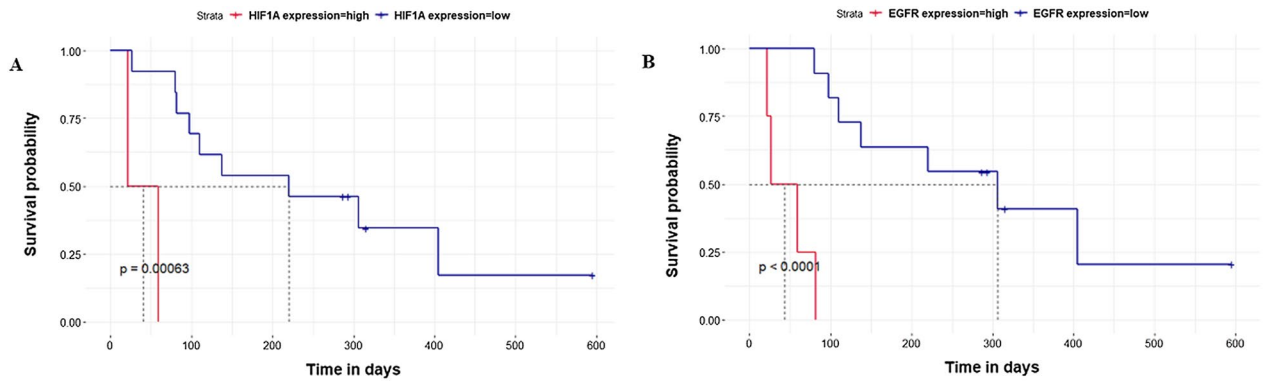

Fig. 4 The overall survival of patients with HGG depending on the expression of the HIF1A (**A**) and EGFR (**B**) genes

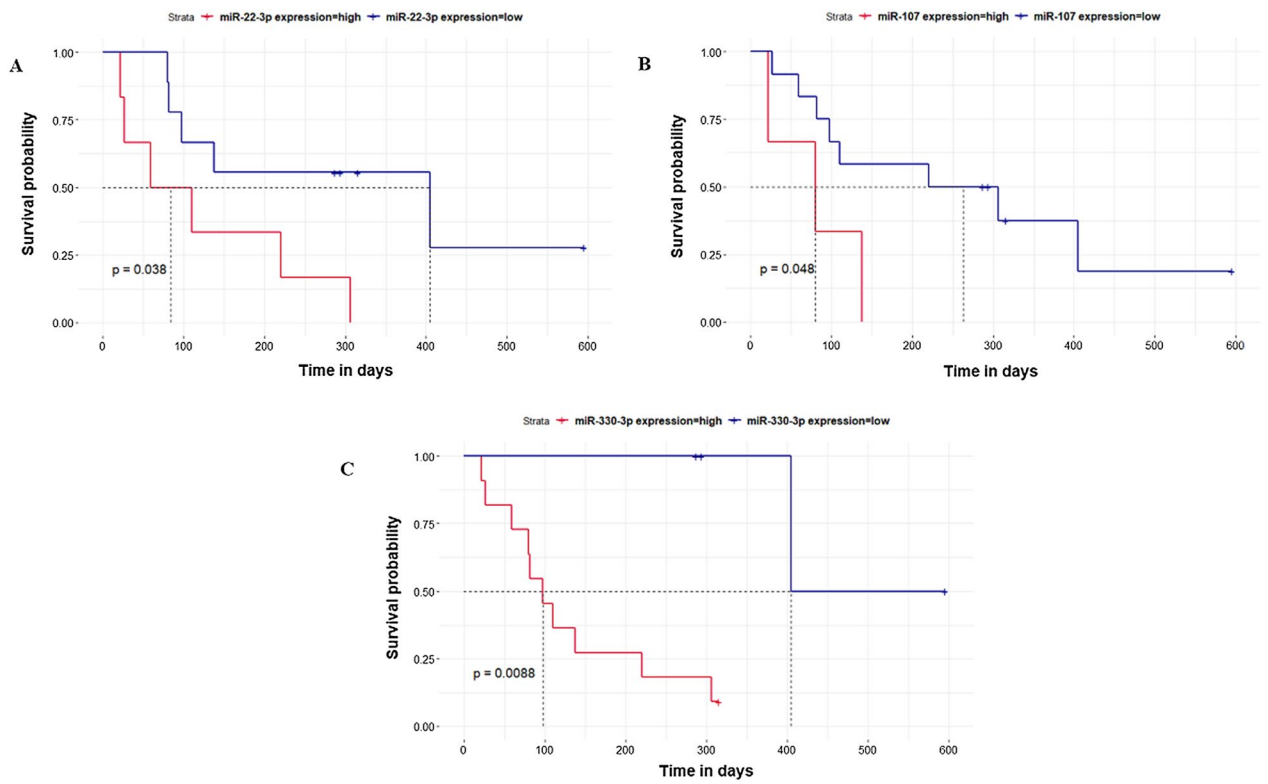

Fig. 5 Overall survival of patients with HGG depending on the expression of miR-22-3p (**A**) miRNA, miR-107 (**B**), miR-330-3p (**C**)

cellular signaling pathways involved in proliferation, cell migration, angiogenesis, and survival [6, 7, 10]. The study of mRNA levels of genes and their microRNAs opens up an understanding of tumor biology and new therapeutic or prognostic goals. HGGs are characterized by abnormal metabolic activity, especially in the late stages of oncogenesis [10]. The data obtained in this study indicate significant shifts in gene and miRNA expression associated with the regulation of the effector of the hypoxic response induced by hypoxia of transcription factor 1-alpha (HIF1-alpha) (Fig. 6). The level of HIF1A expression in the tumor in the study group of patients was higher than in normal brain tissue (Fig. 1, Table 2). Expression of the gene, activated by HIF1, promotes metabolic adaptation of the cell to a reduced partial pressure of oxygen and in the tumor also mediates angiogenesis and cell invasion [23].

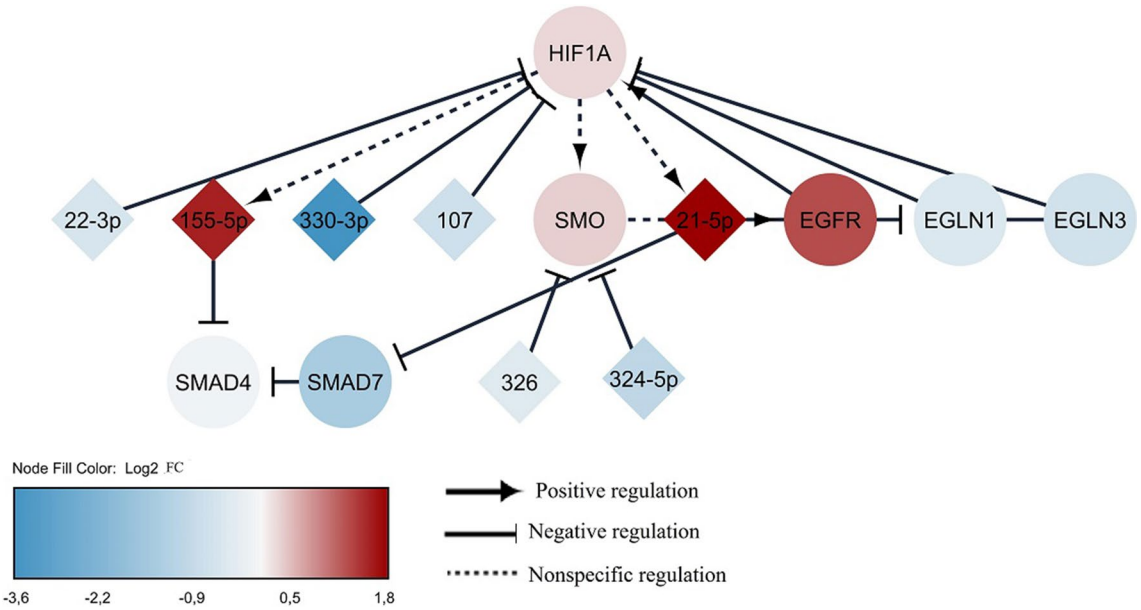

Fig. 6 Cooperative signaling pathway interactions in high-grade gliomas

The increase in HIF1A expression was accompanied by a decrease in the transcriptional activity of its main modulators—EGLN1 and EGLN3, respectively (Fig. 1, Table 2). The role of the products of these genes in the cell is to inactivate HIF1-alpha by hydroxylation of proline, which requires the presence of molecular oxygen [24]. In glial tumor samples, we found a decrease in the level of three miRNAs (Fig. 3) targeting HIF1A: miRNA-330-3p, miRNA-22-3p, and miRNA-107. In addition, there is a negative correlation between the expression of *HIF1A* and miR-122-5p, as well as *EGLN1* with miR-155-5p. It is known that miR-155-5p reduces the expression of the tumor suppressor pVHL, which is involved in the degradation of HIF1-alpha and, therefore, in the cellular response to hypoxia [25].

As a transcription factor, HIF-1α is involved in the activation of the Hedgehog signaling pathway (HH/GLI) [26, 27]. HH/GLI can influence the success of GBM therapy in the case of temozolomide treatment, since the *MGMT* gene is one of its targets [26, 27]. Our study found an increase in the relative expression of the *SMO* gene (Fig. 1, Table 2), a key regulator of HH/GLI, which was accompanied by a decrease in the level of targeting for *SMO* and miR-324-5p (Fig. 3, Table 2).

It is known that hypoxia leads to overexpression of EGFR and promotes long-term activation of the EGFR signaling pathway [28]. In non-hypoxic conditions, EGFR activation, on the contrary, promotes the stabilization and accumulation of HIF-1α in the cell [29]. In the studied HGG samples, the average FC level of the EGFR gene was more than two times higher (Fig. 1, Table 2) compared to normal brain tissue, which was inversely proportional to the overall survival of patients (Fig. 4). The increase in the transcriptional activity of EGFR in the tumor was accompanied by a significant decrease in the expression of miRNA-122-5p, for which it is a potential target. On the other hand, there was an increase in miR-155-5p and miR-21-5p, which may also affect the expression of EGFR (Fig. 3, Table 2). Previously, Zhou X and colleagues showed that inhibition of miR-21-5p by siRNA leads to a decrease in EGFR activity [30].

According to our data (Fig. 1), the transcriptional activity of genes of the SMAD family decreased in HGG. By preventing the formation of the SMAD2/SMAD4 complex, SMAD7 is an antagonist of the TGF-β signaling pathway, which ensures the invasion and malignancy of tumor cells [31]. Recent studies strongly suggest that miRNAs act as effectors of the hypoxic response mediated by HIF1-alpha. Among the small noncoding RNAs we studied, miR-21-5p, miR-107, and miR-155-5p belong to controlled hypoxia [32]. MicroRNA-21-5p is the most typical example of overexpression in glioma tissue associated with a poor prognosis of the course of the disease [28]. It should be noted that SMAD7 is a direct target for miRNA-21-5p, which may explain the significant changes in its expression in our study.

Thus, the revealed statistically significant patterns indicate the critical role of *EGFR*, *HIF1A*, *SMADs* in HGG oncogenesis (Fig. 6). In the HGG tumor sample, there are both the activation of the transcription of HIF1A

itself and the key participants in the associated signaling pathways EGFR, TGF-β, HIF1-alpha, HH/GLI, as well as changes in the level of microRNA (miR-215-5p, miR-122-5p, miR-21-5p, miR-326, miR-324-5p, miR-155-5p, miR-330-3p, miR-22-3p, miR-107). In addition, the altered expression pattern of *HIF1A*, *EGFR* and miRNA miR-22-3p, miR-107, and miR-330-3p is associated with decreased overall survival in HGG patients. The involvement of miRNAs in the regulation of the signaling pathways EGFR, TGF-β, HIF1-A, HH/GLI makes them attractive tools for gene therapy for oncological diseases, which currently do not have effective therapies.

Conclusion

In conclusion, it should be noted that our results demonstrate significant changes in the mRNA levels of the *EGFR*, *SMAD4*, *SMAD7*, *SMO*, *HIF1A*, *EGLN1/3* genes. And microRNA hsa-miR-215-5p, hsa-miR-22-3p, hsa-miR-122-5p, hsa-miR-107, hsa-miR-324-5p, hsa-miR-155-5p, hsa-miR-21-5p, hsa-miR-330-3p, hsa-miR-326. Expression levels of *HIF1A* and *EGFR*, as well as hsa-miR-22-3p, hsa-miR-107, and hsa-miR-330-3p, act as markers of life expectancy in patients with high-grade glioma.

Abbreviations

miRNA: MicroRNA; HIF1A: Hypoxia-inducible factor 1-alpha; EGFR: Epidermal growth factor receptor; TGF-β: Transforming growth factor beta; HH/GLI: Hedgehog/glioma-associated oncogene; WHO: World Health Organization; IDH: Isocitrate dehydrogenase; WT: Wild-type; NOS: Not otherwise specified; GB: Glioblastoma; HGG: High-grade gliomas; MGMT: O[6]-methylguanine-DNA methyltransferase; FC: Fold change.

Supplementary Information

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Additional file 1. Clinical data, fold change gene and microRNA data, DNA primer sequences.

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Authors' contributions

OIK was responsible for conceptualization and project administration. IAA was responsible for writing original manuscript. AAP was responsible for investigation, methodology, formal analysis, and writing original manuscript. NNT was responsible for writing original manuscript and data visualization. DYG was responsible for data curation and methodology. EER and NSK were responsible for methodology, review and editing manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The work was approved by the Ethics Committee of the Medical Research Center of Oncology, Protocol №8 on April 7, 2015. Written participation in the study was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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