Involvement of miR-31, miR-148a, & miR -221 as three risky microRNAs in the invasion and angiogenesis of glioblastoma cells via myelinated nerve fiber of white matter tracts path

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Abstract

Glioblastoma is one of the most frequently occurring and malignant brain tumors. Due to the importance of microRNAs and HIF1α gene that is involved in the angiogenesis and growth of tumor cells, this study aimed to evaluate the correlation of the expression of HIF1α gene with miR-148a, miR-31, and miR-221, which were reported by in silico analysis as risky microRNAs in GBM. The findings obtained from Real-Time PCR using TaqMan Assay indicated the significant difference in the expression of HIF1α gene and microRNAs between the patient and healthy groups and the expression changes were obtained point to point in the patients; so they can be considered as important biomarkers in glioblastoma. The obtained AUC from the ROC analysis indicated that the analysis of the expression of HIF1α and miR-148a, miR-31 & miR-221 genes can be used to distinguish healthy and patient groups nevertheless, the correlation of HIF1α gene expression with the microRNAs was reported low according to Pearson's correlation coefficient. Thus the significant increase in HIF1α gene expression is probably controlled by other molecules. By using bioinformatics analysis; Gene Ontology biological process; MSigDB Hallmark and KEGG pathway enrichment analysis of PPI network was carried out with Enrichr web-based application. The axon guidance pathway, proteoglycan in cancer and some of intracellular signaling pathways identified as the most important signaling pathways. The results of MSigDB_Hallmark pathway enrichment analysis introduced hypoxia signaling pathway with less involvement from these micoRNAs. Therefore these microRNAs can be considered in Glioblastoma cell infiltration through the myelinated nerve fibers of white matter tracts.

1. Introduction

As reported in the US, glioblastoma multiforme is the most prevalent, aggressive, and deadly type of primary brain tumor in adults (Stupp et al. 2009, Ostrom et al. 2014).

This disease is one of the most malignant cancers with a lifespan of less than one year and < 5% of people after the diagnosis of the disease have a lifespan of 5 years using new therapies (Dolecek et al. 2012, Lima et al. 2012). The annual incidence of this disease is about 5.26 per 100,000 people and the number of new cases that are diagnosed each year is 17,000 people (Brook et al. 2019). Studies have shown that the onset and progression of cancer depend not only on the cancer cells but also on the tumor microenvironment (TME) (Dachs and Chaplin 1998, Quail and Joyce 2013, Whiteside 2018). In recent years, the hypoxic conditions of solid tumors, such as glioblastoma, have been extensively investigated and it is said that the hypoxic microenvironment of such solid tumors may affect their immunotherapy. In a microenvironment with high oxygen concentration, the degree of T-cells activation is higher compared to a hypoxic microenvironment (Ohta et al. 2011). A possible explanation for the adaptation of T-cells to hypoxia is Hypoxia-Inducible Factor 1 (HIF1), which provides the conditions for the cell to switch from an aerobic to an anaerobic pathway (Majmundar et al. 2010). In addition to immunotherapy, the efficacy of conventional cancer treatments is reduced by hypoxia (Ohta et al. 2011), which is associated with a poor prognosis of the disease (Acker and Plate 2004). Tissue necrosis and hypoxia are among the main histopathological characteristics of glioblastoma. Moreover, HIF1 is one of

the major factors that regulate the response of glioblastoma to hypoxia, and its central role in reinforcing angiogenesis and invasion of tumor cells has been proven (Ohta et al. 2011, Liao et al. 2019, Xu et al. 2021). Glioblastomas are highly vascularized tumors characterized by necrotic areas that are surrounded by pseudopalisading cells, which have high expression of HIF1α and VEGF (Semenza 2003). In recent years, the expression of HIF1α gene in cancers has been studied and in about 70% of human tumors and metastatic tissues, an increase in the expression of this gene has been reported compared to the surrounding normal tissue (Zhong et al. 1999). In addition, most of the studies have reported this gene as a tumor marker in oligodendroglioma, breast cancer, lung cancer, etc.

The role of HIF1α in the malignancy phenotype of various cancers is so important that one of the ways to treat these diseases is through the HIF1α inhibitory factors (Semenza 2003, Zhang et al. 2021). A study showed that the ANKDD1A gene, as a candidate tumor suppressor interacts with FIH1 and decreases HIF1α stability to inhibit cell autophagy in the glioblastoma multiforme hypoxic microenvironment (Feng et al. 2019). Several other studies have shown that vincristine inhibits angiogenesis by inhibiting this gene in glioblastoma and can be a therapeutic target in glioblastoma (Escuin et al., Park et al. 2016). Evidence also suggests that microRNAs play an important role in the nature of TME and the function of tumor stromal cells (Chou et al. 2013, Li et al. 2013, Zhang et al. 2014) so that the deregulation of these molecules has been attributed to almost all aspects of cancer, including its onset and progression (D'Souza-Schorey and Clancy 2012, Iorio and Croce 2012, Que et al. 2019). Promisingly, they are referred to as diagnostic and prognostic biomarkers and also potential therapeutic targets in cancer (Mirzaei et al. 2016, Lelli et al. 2017). These molecules, as a large subgroup of non-coding RNAs, have a length of 18–25 nucleotides and are evolutionarily conserved in various organisms (Ruan et al. 2009, Balachandran et al. 2020). MicroRNAs regulate gene expression post-transcriptionally by mRNA degradation or inhibiting their translation (Vella et al. 2004). Depending on their target molecule, these molecules can act as oncogenes or tumor inhibitors by inhibiting the expression of cancer-related target genes (Wouters et al. 2011, Yu et al. 2018). MicroRNAs have different actions and are differentially expressed among various cancer types. For example, miR-31 expression is reduced in breast, ovarian, prostate, hepatocellular, and gastric carcinomas, but it increases in the lung, colorectal, head and neck squamous cell, and esophageal squamous cell carcinomas (Yu et al. 2018). The importance of determining the expression of microRNAs in different cancerous tissues to the extent that according to genome-wide profiling, miRNA expression signatures (miRNome) allow different cancers to be distinguished from other cancers with very high accuracy (Lu et al. 2005, Volinia et al. 2006) and the origin of cancerous tissue to be identified in tumors with little differentiation. This is in contrast to the expression profile of mRNAs, which are very inaccurate as an indicator of primary tissue or type of cancer (Lu et al. 2005, Iorio and Croce 2012). Another advantage of determining the expression of microRNAs over mRNA is that their expression remains unchanged in formalin-fixed paraffin-embedded clinical tissues, which are usually produced in clinics (Nelson et al. 2004, Iorio and Croce 2012). The present study aimed to evaluate the expression of the genes of three microRNAs, miR-31, miR-148a, & miR-221, and their correlation with the expression of HIF1α gene as one of the main genes of the HIF signaling pathway in patients with glioblastoma multiforme. However, before performing the experiment using The Cancer Genome Atlas (TCGA) analysis,

several risky microRNAs were obtained in these patients, and then, using the StarBase database, FIH1 and subsequently, HIF1α were introduced as the common target molecule of these microRNAs. After that, their expression pattern was obtained in formalin-fixed paraffin-embedded (FFPE) GBM tissues in comparison with normal brain FFPE tissues, and this expression correlation between the main gene and selected microRNAs was evaluated. Finally, bioinformatics analysis identified the main pathways in which these microRNAs have major importance.

2. Materials And Methods

2.1. Study design and population

This case-control study was performed on paraffin-embedded brain tissues of patients and healthy individuals. The sample size was estimated to be 100 people of both genders, who were divided into two groups each with 50 people.

The case group included paraffin-embedded glioblastoma tissues of patients aged > 48 years old collected from the pathology ward of Shohadaye Tajrish Hospital in Tehran from March 2015 to March 2018 and the control group included brain tissue samples of healthy individuals aged > 48 years old who were autopsied in Tehran Forensic Medicine due to ambiguous death from March 2015 to March 2018. Demographic information of subjects was collected from their medical records. Consent forms were completed by patients or their relatives and they were assured that their information would remain confidential.

2.2 In-silico analysis

To select candidate miRNAs, microRNA expression data was downloaded from The Cancer Genom Atlas (TCGA) portal (http://cancergenome.nih.gov/). After finding risky miRNAs accompany with the survival of patients with glioblastoma, three microRNAs, miR-31, miR-148a, and miR-221 were selected.

Using the StarBase database (http://starbase.sysu.edu.cn/), hypoxia-inducing factor1α inhibitor (HIF1AN) was proposed as a common target of these microRNAs. Despite the difference in the expression of the three microRNAs between the two groups, after obtaining a low expression coefficient between the three microRNAs with the target gene, bioinformatics analysis was used to find an appropriate signaling pathway.

2.2.1 miRNAs-targets prediction

The hsa-miR-31-5p, hsa-miR-148a-5p, and hsa-miR-221-5p miRNA targets prediction was performed using four computational target predicting databases, including

Targetscan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), and DIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index). Then, the overlapping predicted genes

between different databases were screened by the Venn diagram (https://bioinfogp.cnb.csic.es/tools/venny/). Subsequently, to increase the accuracy of the hsa-miRNAs-targets prediction, only genes predicted by all four databases were considered as identified targets

2.2.2. Network construction and Enrichment analysis

The STRING database (http://string.embl.de/) was performed in Cytoscape version 3.9 to construct a protein-protein interaction network (PPI) for predicted genes. Finally, Gene Ontology (GO) biological process terms, MSigDB Hallmark, and KEGG pathway enrichment analysis of PPI network was carried out with Enrichr web-based application (https://maayanlab.cloud/Enrichr/), by p-value < 0.05 as the statistically significant level

2.3. Real-Time PCR (RT-PCR)

Examination of gene expression consists of 3 steps. The first step includes total cellular RNA extraction. The RNA is then converted into cDNA by reverse transcription PCR. In the next step, the expression of RNAs is examined by real-time PCR. To do this, first, before examining the expression of genes, to ensure that tumor samples do not have any normal tissue, the necessary histopathological examination and tissue evidence were performed by a pathologist and due to the sensitivity of hypoxia conditions and the effects of heterogeneity in this study, the needed sections of each sample were prepared only from specific tumor areas with a thickness of 20 µm under the supervision of a pathologist. After the removal of paraffin from the sections with xylene, RNA was extracted from the paraffin-embedded tissue using the MagMAX FFPE DNA/RNA Ultra Kit (Applied Biosystems™; Thermo Fisher, USA). After the removal of proteins, RNA was isolated. Then, NanoDrop (Roch) was used to measure the amount of the extracted RNA and its degree of purity. The ratios of the first and second optical densities were 1.8–2.2 and 1.1–7.9, respectively, which were acceptable for cDNA synthesis. In the current study, the expression of both microRNA and mRNA genes had to be examined, so, two different kits were used. For reverse transcription of microRNAs, miR-U54 (as internal control) and TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific Company) with characteristics of: Taqman microRNA - ID: 002279, ID: 000470, ID: 001210, ID: 000524 with PN: 4427975 were used. Due to having specific primers for the selected microRNAs, this kit produces specific cDNA for each microRNA, and thus the expression of microRNAs is evaluated with very high specificity and sensitivity.

Also, the primeScriptTM RT reagent kit (Takara) was used for the reverse transcription of the mRNAs of HIF1α and TBP (internal control) genes.

Sequences of TBP and HIF1α genes were obtained from www.rtprimerdb.org and confirmed with BLAST in NCBI. The probes and forward and reverse primers were designed by www.rtprimerdb.org. The concentration of RNA obtained from different samples was different, so in order to study the expression, in addition to gene normalization using the reference gene, RNA samples were also normalized in order to be used in the cDNA synthesis stage with almost the same concentrations.

Table 1
Sequence of the primers and probes applied at this study

Sequence	TBP	HIF1α
Forward	GCATATTTTCTTGCTGCCAGTCT	GCTCCCTATATCCCAATGGA
Reverse	ACCACGGCACTGATTTTCAGTT	GCTTGCGGAACTGCTTTC
Probe	ACTGTTCTTCACTCTCTTGGCTCCGTGGCA	CCAGTTACGTTCCTTCGATCAGTTGTCA

2.4. Statistical analysis of data

In this study, $P < 0.05$ was considered statistically significant. SPSS v20 (https://www.ibm.com/analytics/spss-statistics-software) was used for data analysis. Also, in order to select the appropriate test for data analysis, first, the quantitative variables were evaluated in terms of normal distribution of data and equality of variance using One-Sample Kolmogorov-Smirnov test and Levene's test, respectively. Independent Samples T-test was used to evaluate the significance of the mean difference Δct between healthy and patient groups. The protocol of Thomas D Schmittgen & Kenneth J Livak (Livak and Schmittgen 2001) showed the expression ratio between the case and control groups, which was reassessed with REST Analysis (https://www.gene-quantification.de/rest.html). Cycle threshold factor was used in all protocols to study the pattern of changes in gene expression, and gene expression was reported based on fold change. Receiver operating characteristic (ROC) curve analysis was then performed to the diagnostic value evaluation of HIF1α and microRNAs by means of Graphpad-Prism-9 (https://www.graphpad.com/scientific-software/prism)

3. Results

3.1. Results of examining the normality of data

According to the One-Sample Kolmogorov-Smirnov test, the significance level in both healthy and patient samples was obtained, which demonstrated the normality of data (Table 1).

Table 2
Results of One-Sample Kolmogorov-Smirnov

Null Hypothesis Sample	Sig.	Null Hypothesis Control	Sig.
MTBPs	0.607	MTBPc	0.405
MHIF1s	0.909	MHIF1c	0.542
MU54s	0.932	MU54c	0.858
M31s	0.075	M31c	0.152
M148s	0.181	M148c	0.599
M221s	0.143	M221c	0.690

3.2. Independent Samples T-test

The results of Levene's test for HIF1α, miR-31, miR-148A, and miR-221 genes at significance levels of < 0.05 showed p-values of < 0.0001, 0.043, 0.731, and < 0.0001, respectively that proved the inequality of variances. Also, the T-test showed a significance level of < 0.05, indicating a significant relationship between the mean difference Δct of the case and control groups. On the other hand, |t|>2 (7.526, 101.924, 14.193, and 22.712, for HIF1α, miR-31,miR-148A, and miR-221 genes, respectively) showed a significant difference in the expression of the mentioned genes between the case and control groups.

3.3 Analysis of gene expression using Thomas D Schmittgen & Kenneth J Livak formula

According to the protocol of Thomas D Schmittgen & Kenneth J Livak, considering 100% efficiency for all Real-Time PCRs, the gene expression showed a significant increase in both case and control groups. Log fold change (case group/control group) was 3.64, -1.77, 2.74, and 2.64 for HIF-1α, miR-31, miR-148A, and miR-221 genes, respectively.

3.4. Results of Gene Expression Analysis by REST Analysis

The efficiency of probe and primers were determined using the CTs obtained from their different concentrations, which were 0.973, 0.942, 0.942, 0.981, 0.962, and 0.931 for HIF-1α, TBP, U54, miR-31, miR-148A, and miR-221, respectively.

Considering 4000 replications to create random pairs, as well as considering different performance qualities of primers and probes, the results of REST analysis were as follows. Changes in the expression of HIF1α, miR-148A, and miR-221 genes in the case group compared to the control samples have shown an increase to 5.903, 6.225, and 5.114, respectively, with a p-value of < 0.0001, which was significant. But, in case of miR-31, the expression change was in reduced form (0.253).

3.5. Pearson Correlation Test

After determining the relative frequency of gene expression, Pearson Correlation test was used to evaluate the expression correlation of miRNA with mRNA. The correlation coefficients obtained for miR-31, miR-148A, and miR-221 were − 0.096, 0.236, and − 0.097, respectively (Fig. 2).

3.6. ROC curve analysis of HIF1α and microRNAs

The ROC curve was used to explain whether the expression of HIF1α and microRNAs genes could be used as a tool to distinguish between the case and control groups (Table 3).

Table 3
sensitivity and specificity of ROC curve analysis

Gene	AUC	Sensitivity	Specificity
HIF1α	0.85(CI:0.76 to 0.94, P < 0.0001)	82.61%, (CI:69.28–90.91%)	94%, (CI:83.78–98.36%)
miR-31	0.94(CI:0.89 to 0.99, P < 0.0001)	86.96%, (CI:74.33–93.88%)	96%, (CI:86.54–99.29%)
miR-148a	0.98(CI:0.95 to1.00, P < 0.0001)	97.83%, (CI:88.66–99.89%)	94%, (CI:83.78–98.36%)
miR-221	0.98(CI:0.94 to1.00, P < 0.0001)	95.65%, (CI:85.47–99.23%)	96%, (CI:86.54–99.29%)

3.7. miRNA target prediction and functional analysis

To reveal the functional role of hsa-miR-31, hsa-miR-148a, and hsa-miR-221 miRNAs, we first determined its target genes and then studied those genes on the network level. To identify these miRNAs targets, we employed four computational target predicting databases, including Targetscan, miRDB, miRPathDB, and DIANA-microT. Then, using the Venn diagram, 106, 153, and 85 predicted targets were identified for hsa-miR-31, hsa-miR-148a and hsa-miR-221, respectively (Fig. 4).

Next, we used the STRING database to illustrate the PPI network of these predicted targets. After removing isolated nodes, the PPI network analysis predicted the significant hub genes and the specific connections among them, that contained 278 nodes and 588 edges (Fig. 5A). Finally, utilizing Enrichr, we uncovered functional annotation of this network. The KEGG pathways enrichment results indicated that the "Axon guidance" was the most significant pathway of this network. Also, we observed that some critical pathways were significantly enriched in this network, such as "Proteoglycans in cancer," "Insulin resistance," or "MAPK signaling pathway". Furthermore, MSigDB_Hallmark pathways enrichment results revealed these miRNAs targets associated with several hallmark pathways (Fig. 5B).

4. Discussion

In recent years, many efforts have been made to fully determine the biology of specific tumors so that with accurate tumor diagnosis, patients can appropriately and beneficially be treated. Researchers try to find biomarkers with high specificity and sensitivity. Examination of biomarkers, such as microRNAs, is an effective way to detect diseases early and track their progression or response to treatment. Therefore, microRNAs have been proposed as new target molecules for the diagnosis and treatment of glioblastoma (Shea et al. 2016). TCGA analysis using Cox regression and Kaplan-Meier analyses has identified that miR-31, miR-148a, and mir-221 as risky microRNAs that accompany with the survival of patients with glioblastoma (Srinivasan et al. 2011, Wong et al. 2015). Wong et al. (2014) used two Antagomirs to inhibit miR-31 and miR-148a in orthotopic xenograft mouse models and reported an inhibited tumor

growth and increased mouse lifespan. This result is inconsistent with the result obtained from cells in culture medium (Wong et al. 2015). Using StarBase V2.0, FIH1 molecule or hypoxia-inducing factor1α inhibitor was obtained as the common target of these microRNAs. The results obtained in our study showed a significant difference in the expression of the three microRNAs between the case and control groups, and is in line with the results of some previous studies and contradicts some other studies. For example, Michela Visani et al. (2013) compared the expression of 19 microRNAs in 60 paraffin-embedded glioblastoma samples with other types of grade I-III brain neoplasias. Examination by Real-Time PCR showed that the expression of miR-31 was inhibited or decreased in glioblastoma samples (Visani et al. 2014). Moreover, Zhou et al. (2015) reported that miR-31 inhibits glioblastoma tumors and acts as an apoptosis enhancer. Also, it increases the sensitivity of glioblastoma cells to temozolomide)TMZ(and can be a predictor of the tumor by inhibiting STAT3 activation (Zhou et al. 2015). Growing evidence shows that the miR-148/152 family has the potential to act as both tumor suppressor and oncogene in various cancers (Ilango et al. 2020). Using deep sequencing microarray technique, Monika Pwecka et al. (2015) compared the expression level of microRNA in tumor tissue and tumor margin tissue and observed that the expression of miR-148a was increased in tumor tissue (Piwecka et al. 2015). In the study of Daming Cui et al., it was found that miR-148a promotes human glioma cell with IDH1R132H mutation invasion and tumorigenesis by downregulating GADD45A (Cui et al. 2017). Many previous studies of miR-221 have shown increased expression of this gene (Le Sage et al. 2007, Conti et al. 2009, Silber et al. 2009). Le Sage et al. (2007) showed that the levels of miR-221 and miR-222 increase in primary glioblastoma samples, and this increase is associated with low levels of p27Kip1 (Le Sage et al. 2007). In some tumors (e.g. GBM), the oncogenic properties of miR-221 and miR-222 increase through the inactivation of suppressors, P27 and P57 (Gillies and Lorimer 2007). Chen et al. (2011) found that downregulation of miR-221/222 increases the sensitivity of glioma cells to temozolomide, and this occurs by regulating apoptosis, which is independent of the TP53 pathway (Chen et al. 2012). Chun-Hua Xu et al. (2019) reported that by silencing the microRNA-221/222 cluster, the glioblastoma angiogenesis process can be inhibited, which is performed by the suppressor of cytokine signaling-3 dependent JAK/STAT pathway (Xu et al. 2019).

Contradiction in the results obtained from gene expression studies is due to different reasons, especially sample selection or preparation, which is presented as heterogeneity and this has been covered a lot in recent years. This is one of the issues that has caused glioblastoma to be considered as a treatment-refractory brain tumor (Brennan et al. 2013, Patel et al. 2014, Meyer et al. 2015, Neftel et al. 2019).

According to the study of Anoop P. Patel et al. in 2018, the correlation of individual cells within a tumor showed a wide range (correlation coefficient ~ 0.2–0.7), which confirmed the intratumoral heterogeneity within the tumor and these heterogeneous mixtures have also been observed in different subtypes of glioblastoma. Each tumor has a dominant subtype in terms of gene expression but contains cells with alternate gene expression (Sottoriva et al. 2013, Patel et al. 2014). Comparing the expression profiles of microRNAs that are spatially located at different sites of a glioblastoma tumor has shown intratumoral heterogeneity (Soeda et al. 2015) and these expression changes occur in three different areas of core, rim, and invasive margin (Alfardus et al. 2021). The study conducted by Puchalski et al. that was published in

2018 as the Ivy GBM Atlas reported that the heterogeneity at different regions of a single tumor is greater than expected and even much higher between areas anatomically similar to different patients' tumors (Puchalski et al. 2018). Intratumoral exchange of microRNAs by extracellular vesicles (EVs) increases the heterogeneity of glioblastoma stem cells (GSCs) and alters subpopulation-specific microRNA signature (Bronisz et al. 2016). Both miR-148a and miR-31 in these vesicles increase heterogeneity in these cells (Godlewski et al. 2017). Importantly, findings from various studies of tumor heterogeneity indicate that a single biopsy is insufficient to determine tumor signature (Khalafallah et al. 2021).Unlike other genes, hypoxia gene signatures, such as HIF1α signatures, correspond to multiple biopsies from multiple areas throughout a tumor, and intratumoral heterogeneity is highly true about them. When only one biopsy of a tumor sample is available, hypoxia gene signature may give us a more reliable estimate of the total hypoxia of the tumor than other genes, but in these cases, multiple biopsies provide complete assurance of tumor classification. However, unfortunately, they are not always available (Lukovic et al. 2019). Tumor environmental stresses, such as hypoxia, are factors that increase epigenetic heterogeneity in tumor cells (Singh et al. 2012, Eriksen et al. 2016, Ramón y Cajal et al. 2020) and thus make a difference in the results of expression analysis. The experimental design and data analysis are also very important (Xu and Wong 2010, Iorio and Croce 2012). Another important issue is the use of different controls for data normalization, which explains some of the differences observed in different studies (Peltier and Latham 2008). In this study, the TBP gene was used as the HIF1α reference gene. Kreth, S., et al. investigated the expression of 19 typical reference genes in order to evaluate the expression of HIF1α, and found that among different types of glioma, glioblastoma has the highest level and diversity of reference genes and this is unlike normal brain cells. Due to the difference in the expression levels between the glioblastoma and normal brain cells, the selection of the reference gene in brain tumors has more sensitivity than other tumors and is very important in expression analysis. In their study, Normfinder analysis showed the most stable expression of IPO8 and TBP genes (Kreth et al. 2010). In the study of Susanne Grube et al. (2014), geNorm expression stability analysis, BestKeeper expression stability analysis, and NormFinder expression stability analysis showed that TBP is an appropriate reference gene for studying the expression in glioblastoma and normal brain cells (Grube et al. 2015). The same results were obtained from the study of Kang IN et al. in 2015 (Kang et al. 2015). Another important issue that complicates the situation is that in response to cellular stress conditions such as hypoxia, there is an immediate and dynamic regulation in the miRNA expression rate, and the expression can be different at the time of sample collection and examination (Marsit et al. 2006, Kulshreshtha et al. 2007). Hypoxia occurs in solid tumors (e.g. glioblastoma) by increasing the tumor diameter to about 1 mm (Vaupel et al. 1989) and stimulates the self-repair of glioblastoma cancer stem cells. This phenomenon is common in glioblastoma (Heddleston et al. 2009, Colwell et al. 2017). There is ample evidence that hypoxia induces cancer stem cells to cause tumor resistance and recurrence after conventional therapy (Tang et al. 2021, Zhang et al. 2021). Contrary to this, there is other evidence that hypoxia makes tumor cells more sensitive to chemotherapy (Emami Nejad et al. 2021). Therefore, studying this pathway as one of the major pathways in this disease, is very important. During hypoxic conditions and therapeutic stress, CSCs use special signaling pathways to regulate their stemness, and HIF signaling plays a key role in regulating these pathways (Qiang et al. 2012, Mimeault and Batra 2013, Vadde et al. 2017, Yu et al. 2018, Yang et al.

2020). Based on the results of MsigDB Hallmark and KEGG pathways enrichment, it was found that in glioblastoma, the three mentioned microRNAs have the highest importance in signaling pathways, which are involved in specific patterns of glioma cell infiltration. Pathways that make these three microRNAs important for the migration and invasion of glioblastoma are shown schematically in Fig. 6.

This movement and invation is through the myelinated nerve fibers of white matter tracts Which is different from the perivascular space around blood vessels and subarachnoid space (Lefranc et al. 2005, Cuddapah et al. 2014). Cell motility along white matter tracts, a second route of glioma cell invasion, is mediated by a group of proteins called axonal guidance molecules (Armento et al. 2017). In addition to, these molecules being involved in the development of the CNS, they play an important role in cancer-associated processes (Mancino et al. 2011). The most distinguished axonal guidance molecules are: ephrins; netrins; Slits; semaphorins; plexin; neuropilin and Robo, which show different expression in different cancers (Ronca et al. 2017) In addition, they are not narrowing to nervous system and these are prominently expressed in many developing and mature organs (Hinck 2004). Their normal function in the adult CNS and other adult tissues is essentially unknown and There is a need for more research (Chedotal et al. 2005). Altered expression of axonal guidance proteins contributes to tumor cell migration and invasion, this special infiltrative route has been observed in glioblastoma cells (Chedotal et al. 2005)

It has been determined from previous studies extracellular matrix molecules in addition to constructing a structural scaffold can significantly influence axon guidance cue function, so proteoglycans can be considered as Modulators of Axon Guidance Function (Wit and Verhaagen 2007).

In this study Proteoglycan in cancer, the next important signaling pathway, that contains molecules that provide the same extracellular matrix (ECM) needed for glioblastoma cell motility. Many compounds of this pathway are involved in glioma cell infiltration. These molecules construct structural support and work as a leader scaffold or barrier (Friedl and Alexander 2011, McGranahan and Swanton 2017). The brain parenchyma unlike the ECM of the other cells, lacks tight and stiff components such as collagens; fibrinogen and laminin (Vollmann-Zwerenz et al. 2020) and instead have proteoglycan; hyaluronic acid; tenascin-C; Aggrecan; Nidogen; agrin and ... (Dityatev et al. 2010, Friedl and Alexander 2011, McGranahan and Swanton 2017, Ferrer et al. 2018). Proteoglycans (PGs) are complex macromolecules composed of a central core protein that linked to sulfated glycosaminoglycan (GAG) chains covalently (Nikitovic et al. 2018, Schwartz and Domowicz 2018). Those which are common component of the extracellular matrix and include, aggrecan (ACAN), versican (VCAN), neurocan (NCAN) and brevican (BCAN) (Wade et al. 2013); those which attach to the cell surface via a glycosylphosphatidylinositol (GPI) anchor and include glypican (GPC) family; and those are the transmembrane family, including the syndecans, neuroglycan, appicans and NG-2 (Schwartz and Domowicz 2018). It has been shown that the ECM, cell–cell and cell–ECM connecting molecules and proteases, have an important role on glioma cell migration (Baumann et al. 2009, Onken et al. 2014). The composition of the ECM highlights specific glioblastoma stem cell niches and causes migration and invasion in specific form (Nguyen-Ngoc et al. 2012). One of the most important niches within the diseased brain is the hypoxic niche. This is A hypoxic environment stimulates the expression of the hypoxia-inducible factors (HIFs) HIF1 and HIF2 that stimulate invasiveness (Li et al.

2009). An acidic environment also stimulates HIF function (Filatova et al. 2016) and then MMPs are activates (Cong et al. 2014) of course, the results of MSigDB_Hallmark pathways enrichment also introduced hypoxia signaling pathway as one of the target signaling pathways that There is less involvement from these micoRNAs and the importance of these microRNAs is highlighted in other signaling pathways. Due to the function of the obtained signaling pathways, these three microRNAs can be considered in Glioblastoma cell infiltration through the myelinated nerve fibers of white matter tracts.

5. Conclusion

MicroRNA-based therapies have becoming more attractive for researchers because of their multi-gene targeting functions. These molecules are involved in different signaling pathways of diseases. In recent years, the issue of glioblastoma cell invasion and migration is considered as one of the main causes for the refractory-treatment property of this disease. In this study, the involvement of these three microRNAs in the migration of glioblastoma cells from the myelinated nerve fiber of white matter tracts path was confirmed. Due to the difference in the expression of these microRNAs between the two studied groups, they can be used to distinguish between healthy and patient groups. Thus, this microRNAs can be considered as biomarkers with diagnostic significance in glioblastoma. This way of migration, which is specific to nerve cells, is very important in the malignancy of nerve tissue and more regulatory molecules need to be investigated in this way, of course, tumor heterogeneity issue, which is common in many glioblastoma tumors, must be considered.

Declarations

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Figures

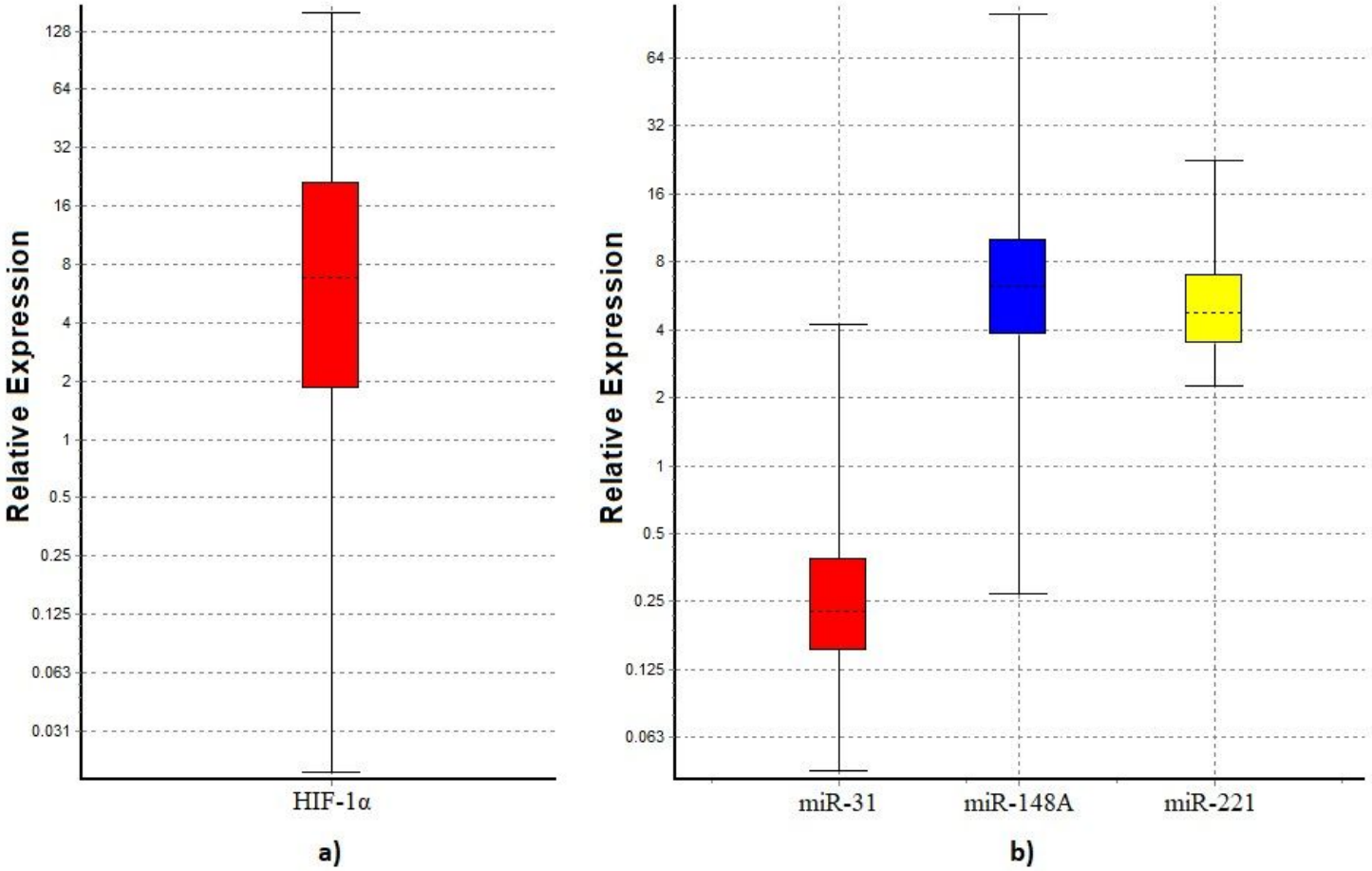
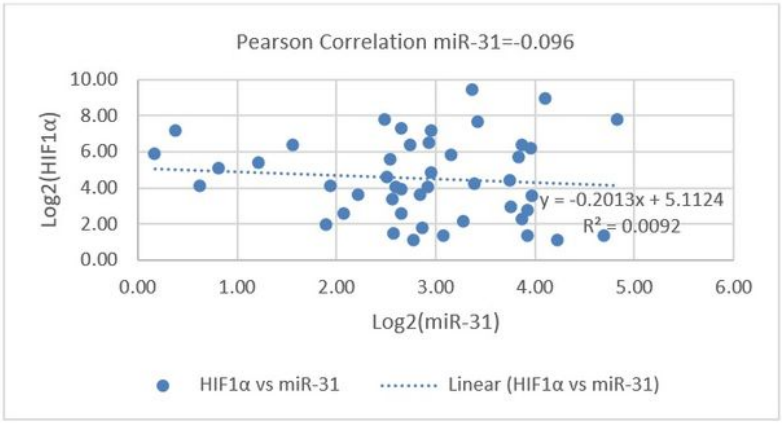
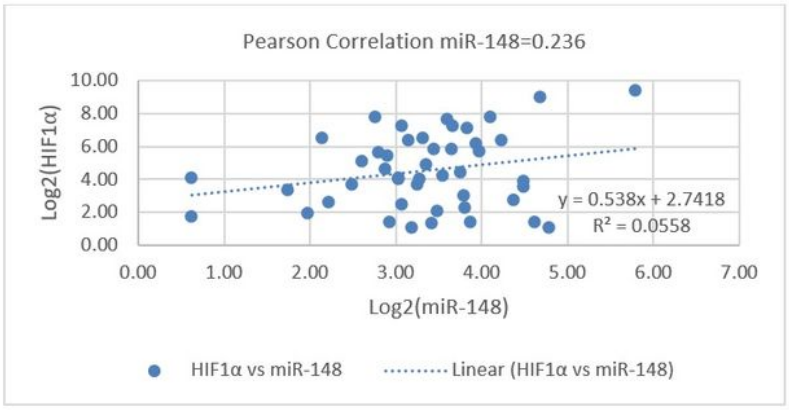


Figure 1

Diagram of gene expression and scattering of gene expression in glioblastoma samples compared to control samples. a) HIF1α; b) miR-31, miR-148A, & miR-221.



(a)



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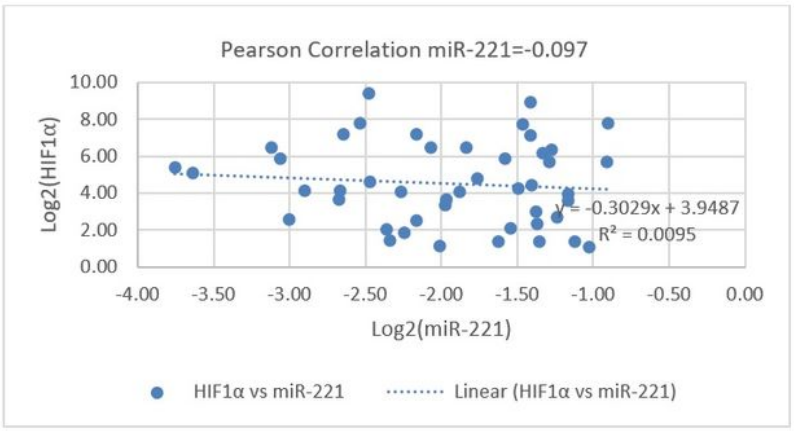

(c)

Figure 2

Diagram of the expression correlation of HIF1α with a) miR-31, b) miR-148A, & c) miR-221.

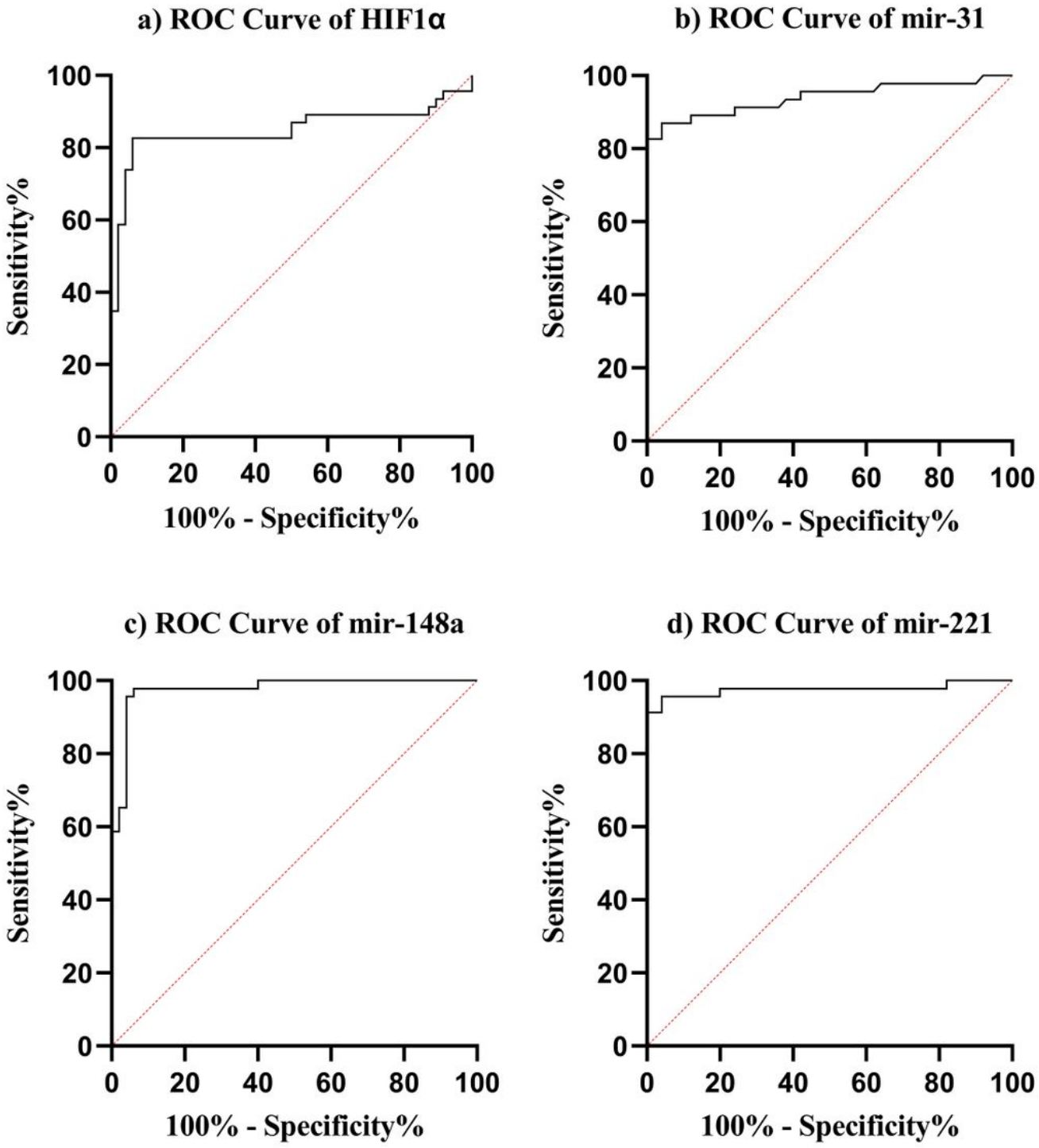

Figure 3

ROC was performed on HIF1α expression level (a), mir-31 (b), mir-148a (c) and mir-221 (d) in GBM FFPE tissues (n = 50) compared to normal brain FFPE tissues (n = 50) to determine the optimal cutoff values.

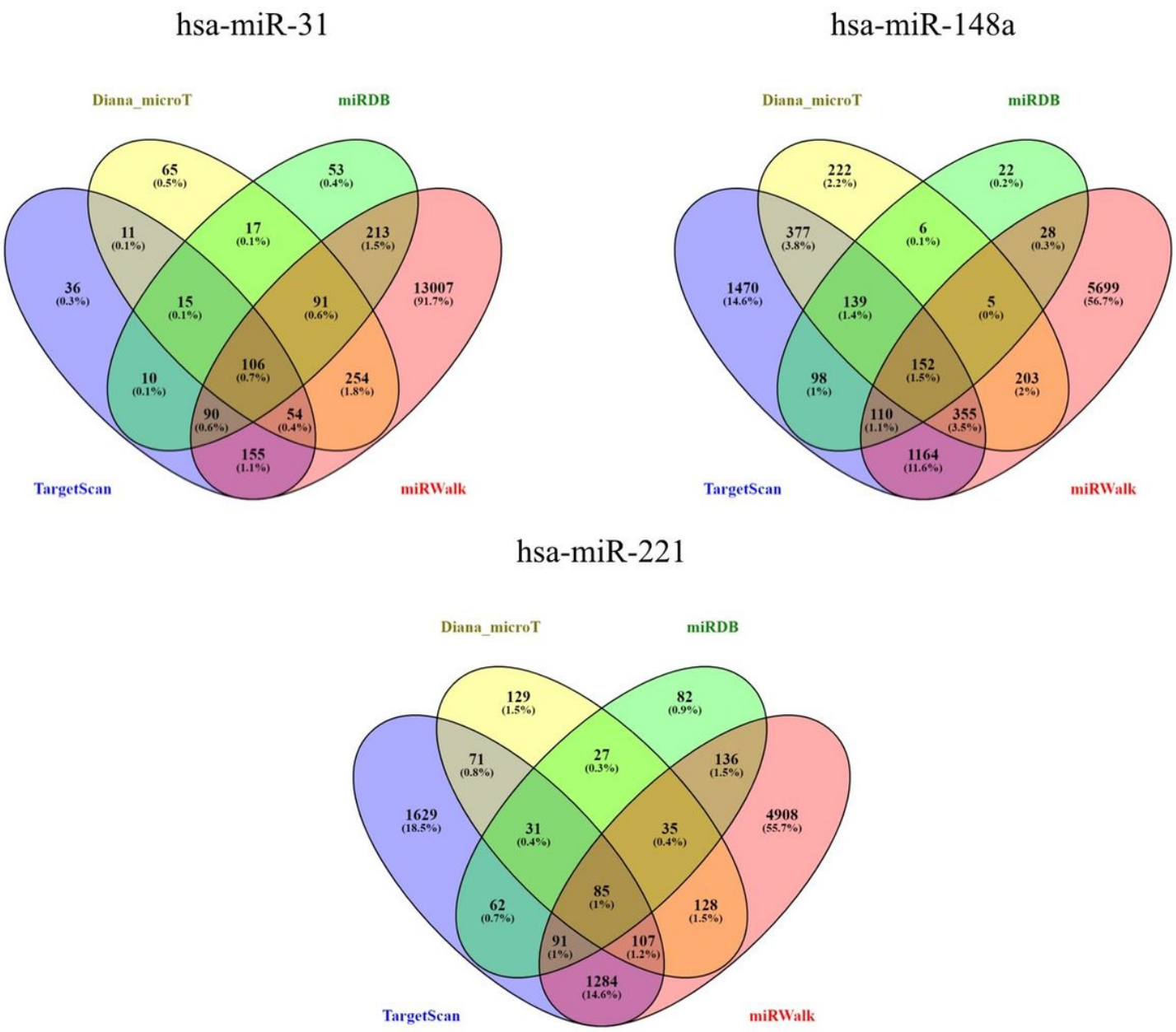

Figure 4

Venn diagram for hsa-miR-31, hsa-miR-148a and hsa-miR-221

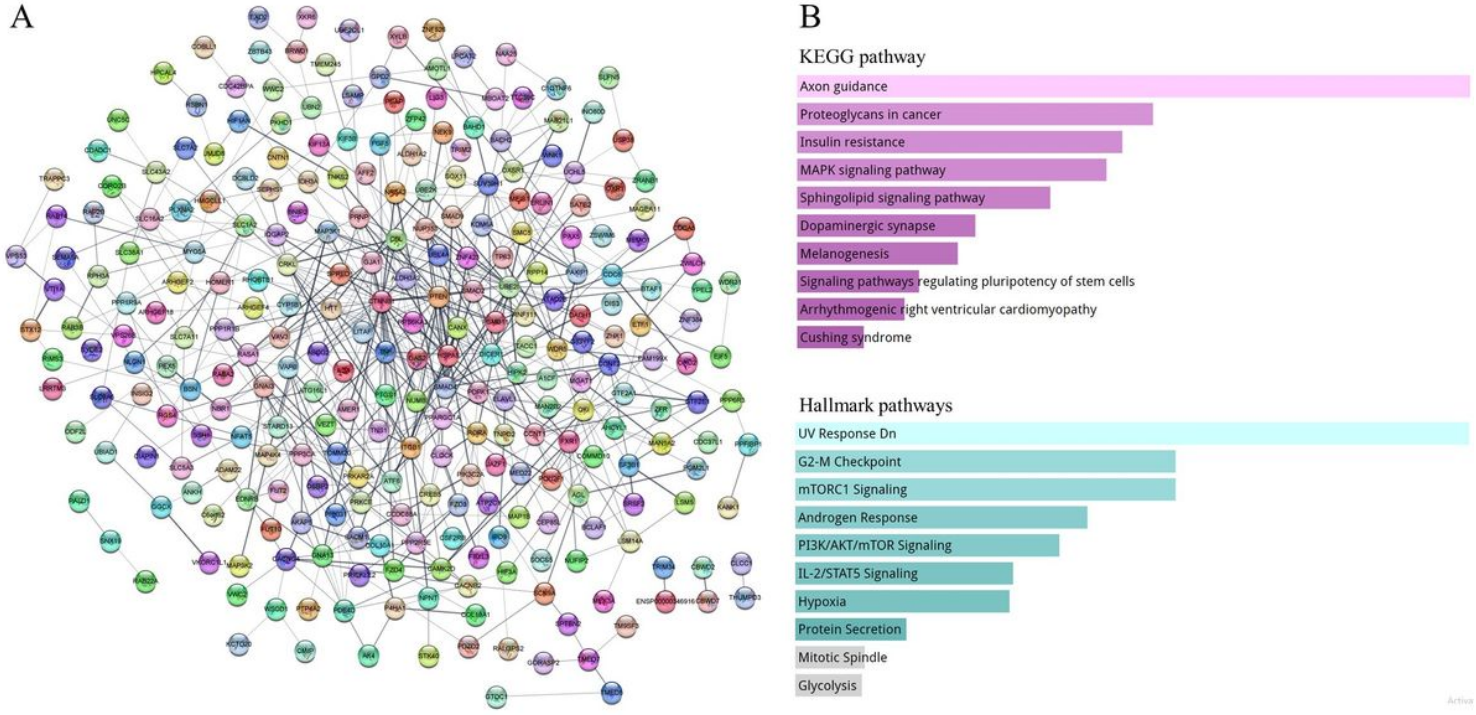

Figure 5

PPI network of the predicted targets (A) and the KEGG pathways enrichment and MSigDB_Hallmark pathways enrichment results (B)

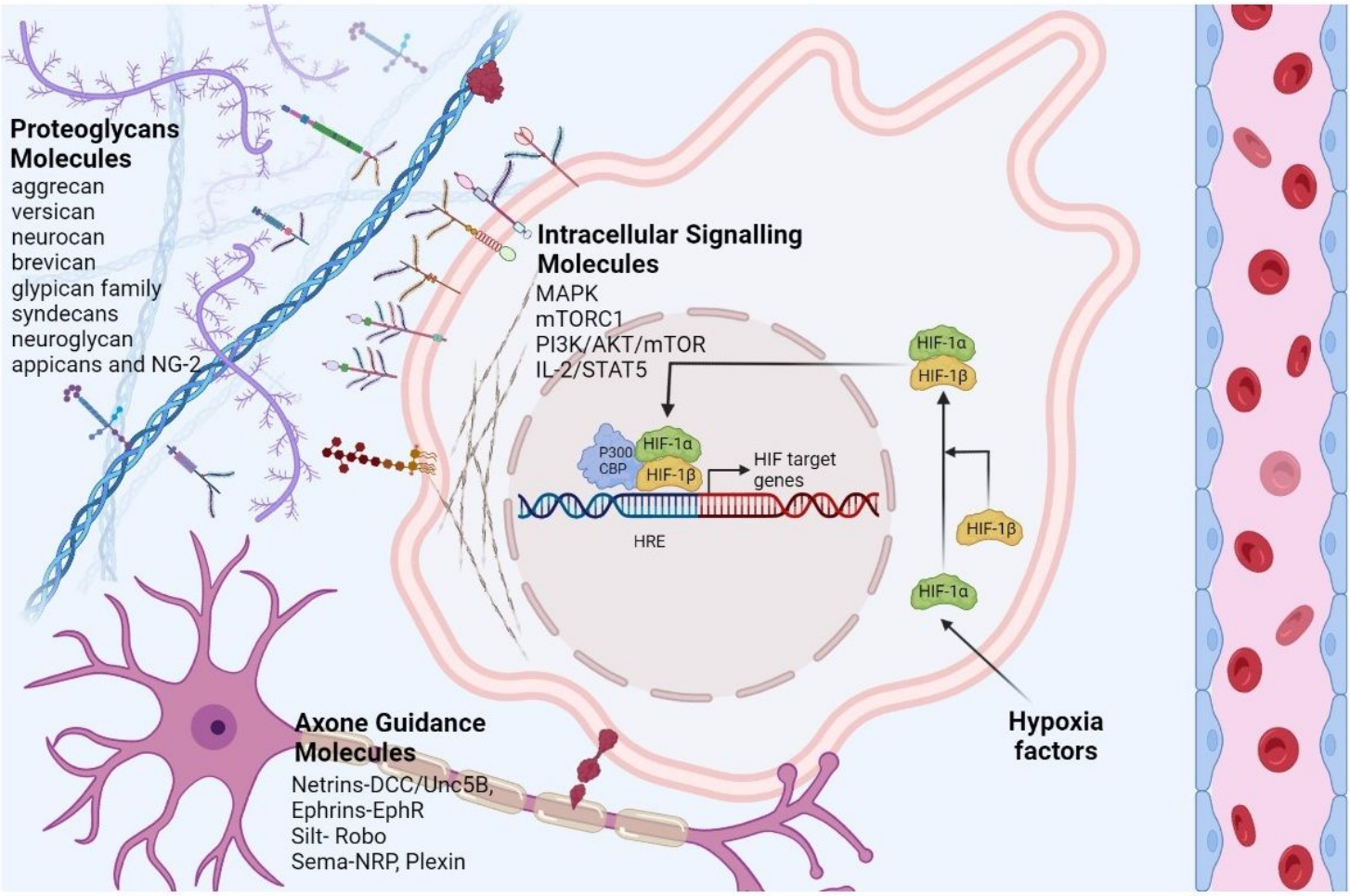

Figure 6

The main signaling pathway in glioblastoma cell migration and invasion in which three microRNAs, mir-31, mir-148a and mir-221 are involved. Intracellular signaling pathways have been shown also which include the MAPK signaling pathway, mTORC1 signaling pathway, PI3K/AKT/mTOR signaling pathway, IL2/STAT5 signaling pathway Figure created with BioRender (https://biorender.com).