ANTICANCER RESEARCH *40*: 3265-3270 (2020)
doi:10.21873/anticanres.14308

Investigation of the Effects of MicroRNA-221 Expression Levels in Glioblastoma Multiforme Tumors

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Abstract. *Background/Aim: The aim of our study was to examine miRNA-221 as a candidate biomarker to define prognosis and/or classification for glial tumors. Materials and Methods: This study included 39 patients who underwent glial tumor surgery and 40 healthy individuals as the control group. miRNA expression levels were determined by real-time polymerase chain reaction (RT-PCR). Receiver operating characteristic curve analysis was used for analyzing the predictive ability of miRNA-221. Results: The levels of miRNA-221 expression were determined by comparing the ΔCT values of miRNAs and the internal control. When the expression levels of miRNA-221 were compared according to the ΔCT method, miRNA-221 was found to be significantly increased in the patient group compared to the control group (p<0.0001). Conclusion: Increased expression levels of miRNA-221 could be a biomarker for glial tumors.*

RNA interference (RNAi) is an epigenetic post-transcriptional regulatory mechanism, which inhibits (silences) gene expression. MicroRNAs (miRNAs) consist of 18-25 base pairs of ribonucleic acid (RNA) molecules, which control gene expression *via* RNAi. miRNAs bind and cause degradation of messenger RNAs (mRNA) through the formation of an RNAi complex. miRNAs, as noncoding RNAs, have enhanced our understanding of the epigenetic mechanisms controlling gene expression at the post-transcriptional level.

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Key Words: Glial tumors, glioblastoma, microRNA, miRNA-221, biomarker.

According to the literature, miRNA-221 may have oncogenic properties and is correlated with cell proliferation and migration (1-3). miRNAs have been found to correlate with pathological processes and have begun to be used as disease biomarkers (4, 5). Glial tumors comprise about 80% of primary brain tumors (6). Low grade glial tumors have higher survival ratios compared to high grade tumors. Biomarkers are needed to define the progress of glial tumors. There are only a few studies that have investigated the role of miRNA-221 in glial tumors.

The purpose of our study was to examine miRNA-221 as a candidate biomarker to define prognosis and/or to classify glial tumors. If miRNA-221 is defined as a biomarker, it would allow the prognosis of glial tumors and the application of treatments earlier, resulting in an increase of survival rates.

Materials and Methods

This hospital-based prospective case-control study consisted of 39 patients who were operated for glial tumors and 40 healthy subjects as the control group. All participants were recruited from the Neurosurgery Department of Yeditepe University, Istanbul, Turkey. Demographic characteristics of patients and controls were recorded and followed-up prospectively. The control group was examined by cranial magnetic resonance imaging and was reported as normal. Tumor classification was determined according to their grades, location and sides. Pathological examinations of brain tumors were performed according to the World Health Organization Classification of Tumors.

The study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki and the study protocol has been approved by the Yeditepe University Medical Faculty Ethics Committee (file no: 23.06.2016/634). Furthermore, all individuals gave informed consent for participating in the study. The peripheral blood samples were withdrawn from the patients and the control group and collected into EDTA-tubes. miRNA-221 expression was assessed in all blood samples.

miRNA isolation. To obtain serum samples, peripheral blood samples were taken into plain 5 ml vacuum gel blood collection tubes and centrifuged for 10 min at 1500 × *g* at room temperature. The collected serum samples were then transferred in a sterilized tube and frozen at –80˚C until miRNA experiments. miRNA isolation was performed using Trizol and its purity and concentration were measured using NanoDrop2000 (Thermoscientific, Waltham, MA, USA).

miRNA isolation from 200 μl serum was performed using the miRCURY™ RNA Isolation Kit (Exiqon, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolation of miRNA is based on spin column chromatography using a proprietary resine as a separation matrix. Serum samples were lysed with the provided lysis solution and proteins were precipitated by the protein precipitation solution. After the precipitation process, isopropanol was added to the collected supernatant and the solution was loaded to the spin column. Subsequently, the column was washed and the miRNAs were eluted in RNase free water.

cDNA synthesis. The isolated miRNA samples were exposed to reverse transcriptase for first-strand cDNA synthesis using the miRCURYLNA RT Kit (Exiqon, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Measurement of miRNA purity. The purity of the miRNA samples was assayed using NanoDrop 2000 (Thermoscientific, Waltham, MA, USA). The purity of the miRNA samples was determined by the OD260/OD280 ratio. Samples with optical density ratios higher than 2 were accepted as pure.

Determination of miRNA levels by fluorometer. MicroRNA levels were determined fluorometrically. miRNA concentration of the transcripted samples was determined and equalized by the Qubit miRNA Assay Kit's standard protocol on the Qubit 3.0 Fluorometer (Thermoscientific). The microRNA accurate concentration levels were determined by fluorometric analysis (7).

The miRNA expression levels analyzed by real -time polymerase chain reaction. The target miRNA selected for this study was determined by using the "mirbase" and "targetscan" databases (8, 9). miRNA and its targets were analyzed using these data bases regarding molecular mechanisms in glioblastoma. miRNA-221 was chosen as the target miRNA for this study.

The miRNA expression levels were determined by using the cycle threshold (Ct) and delta CT (ΔCT) methods. The small nuclear RNA (RNU6) is the most frequently and widely used reference gene as normalization factor for cancer studies (10). RNU6 (internal control or housekeeping assay) was used for the normalization of the miRNA expression levels. The relative expression of miRNA-221 (ΔCT) was calculated based on the arithmetic mean Ct value of internal control (11).

Statistical analyses. All results were evaluated with SPSS 23 statistical analysis program. Pearson and Spearman correlation test were used for correlations. Differences between the patient and control groups were evaluated with the student's *t*-test or the Mann-Whitney *U*-test. Relationships between variables were determined with the Chi-square and Fischer Exact tests and one-way analysis of variance (ANOVA). We performed ROC analysis using the MedCalc software (NY, USA). Sensitivity values are located on the vertical axis of the ROC curve and specificity values are located on the horizontal axis. ROC curves (Receiver operating characteristic curves) were generated depending on the sensitivity-specificity features. A confidence interval (CI) of 95% and a *p*-Value <0.05 were considered statistically significant.

Table I. *Tumor types and grades*.

Tumor type	n	%
Glioblastoma Grade-4	21	53.9
Oligodendroglioma Grade-2	12	30.8
Oligodendroglioma Grade-3	2	5.1
Astrocytoma Grade-2	2	5.1
Astrocytoma Grade-3	2	5.1
Total	39	100

Table II. *Comparison of ΔCm levels miRNA expression levels between patients and control groups*.

MiRNA221-3p	ΔCт	*p*-Value	95%CI
Patient (n=39)	2.98±2.11	0.000*	1.177-3.145
Control (n=40)	0.82±2.27		

n, Number of samples; *$p<0.05$; X±SD (Mean±Standard Deviation). The difference between the groups was analyzed by the independent sample student's *t*-test.

Results

We evaluated a total of 39 patients of whom 23 were male (59%) and 16 were female (41%). The control group comprised 40 individuals of whom 25 were male (62.5%) and 15 were female (37.5%). The mean age of the patient group was 43.10±20.21, and that of the control group was 42.58±20.21. Demographic characteristics of the patients and the control group were statistically insignificant regarding age ($p=0.883$) and gender ($p=0.748$).

Tumors were classified according to the grade, location and side. Pathological examinations of brain tumors were performed according to the WHO Classification of Tumors. Tumors were classified and evaluated according to their grades as shown at Table I. Regarding tumor location, 16 (41%) were in the temporal lobe, 5 (12.8%) in the frontal lobe, 5 (12.8%) in the parietal lobe, 3 (7.7%) in the occipital lobe, 1 (2.6%) in the cerebellum, 4 (10.3%) in the thalamus, 4 (10.3%) in the cingulate cortex and 1 (2.6%) in the bulbus. Cytosolic NADP+ related isocitrate dehydrogenase (IDH-1) status in the GBM patient group was determined by using

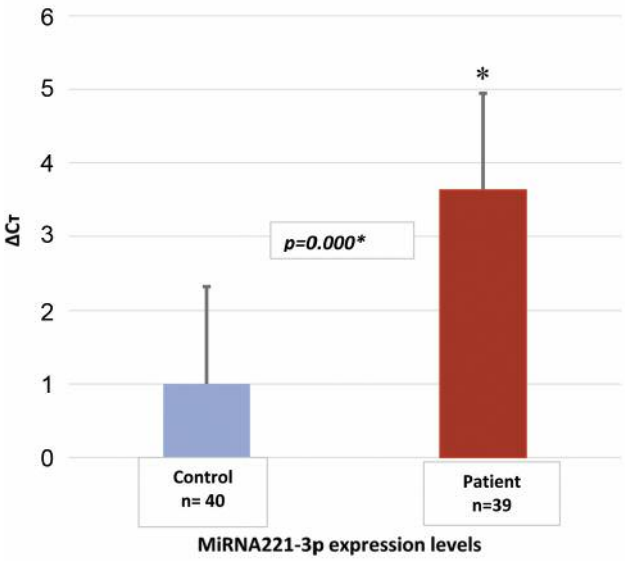

Figure 1. *Comparison of miRNA221 expression levels between patient and control groups according to mean ΔCT value. The difference between the groups was analyzed by the independent sample student's t-test. *p-Value<0.05.*

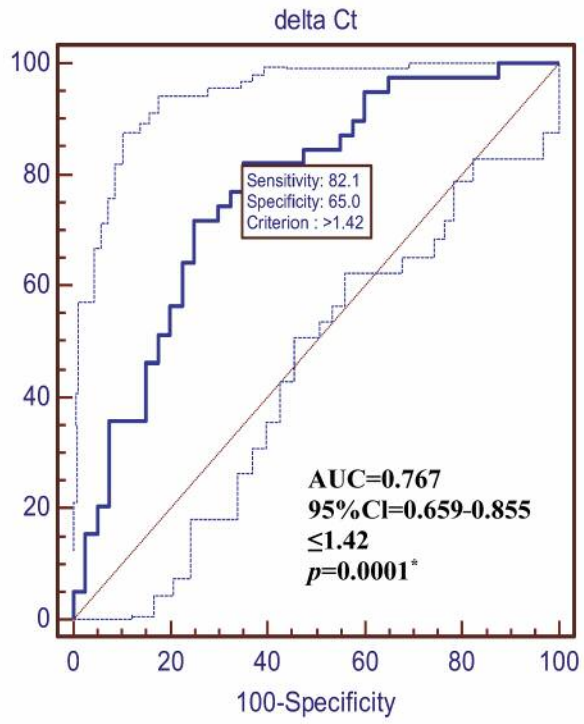

Figure 2. *ROC analysis graph of plasma mir221 expression levels in the control and GBM groups. *p-Value<0.05.*

Table III. *Comparison of plasma miRNA221 expression levels as ΔCm according to glial tumor type.*

MiRNA221-3p	n	ΔCT	*p*-Value
Oligo-3	2	2.15±2.18	0.693
GBM	21	3.09±2.31	
Oligo-2	12	2.94±2.01	
Astro-3	2	4.46±1.01	
Astro-2	2	1.48±0.43	

n, Number of samples; *$p<0.05$; X±SD (Mean±Standard Deviation). The difference between the groups was analyzed by one-way analysis of variance (ANOVA).

Table IV. *Comparison of plasma miRNA221 expression levels as ΔCm according to glial tumor location.*

MiRNA221-3p	n	ΔCT	*p*-Value
Thalamus	4	2.57±0.98	0.840
Cingulate	4	2.35±1.63	
Parietal	5	1.93±1.25	
Cerebellum	1	4.64±0.00	
Bulbus	1	2.30±0.00	
Occipital	3	2.99±1.99	
Frontal	5	3.97±2.53	
Temporal	16	3.20±2.61	

n, Number of samples; *$p<0.05$; X±SD (Mean±Standard Deviation). The difference between the groups was analyzed by one-way analysis of variance (ANOVA).

immunohistochemical methods. Thirty-four patients with glioblastoma had an *IDH-1* mutant variant (87.2%) whereas 5 patients had the wild-type (12.8%). *IDH-1* mutations, which present in early stage of gliomagenesis, alter the function of the enzymes, causing them to produce 2-hydroxyglutarate that does not produce NADPH. Regarding the side of the brain, 18 (46.2%) tumors were found in the right hemisphere and 21 (53.8%) in the left hemisphere.

ΔCT values were calculated for analyzing miRNA-221 expression levels that are shown in Table II. The levels of miRNA-221 expression were determined by comparing the ΔCT values of miRNAs and the internal control (RNU6). The mean expression value of the patient group was 2.98±2.11 while that of control group was 0.82±2.27, the difference between the groups being statistically signifiant ($p<0.0001$). The expression levels of miRNA-221 was found to be significantly increased in the patient group compared to the control group (Figure 1).

There were no statistically significant difference regarding the ΔCT values of plasma miRNA-221 exǧression levels neither according to glial tumor type ($p=0.693$) (Table III) nor according to glial tumor location ($p=0.840$ (Table IV). In addition, the ΔCT values of miRNA-221 were calculated and compared with the IDH-1 variants of GBM patients. As it is shown in Table V there was no statistically significant

Table V. *Comparison of plasma miRNA221 expression levels as ΔCm according to for IDH-1 types.*

MiRNA221-3p	n	ΔCT	*p*-Value	95%CI
IDH-1 Mutant	34	3.13±2.17	0.273	–0.921-3.170
IDH-1 Wild Type	5	3.09±2.31		

n, Number of samples; X±SD (Mean±Standard Deviation). The difference between the groups was analyzed by the independent sample student's *t*-test.

Table VI. *Comparison of plasma miRNA221 expression levels as ΔCm according to tumor location.*

MiRNA221-3p	n	ΔCT	*p*-Value	95%CI
Right sided	18	3.21±2.62	0.480	–0.897-1.873
Left sided	21	2.72±1.32		

n, Number of sample; X±SD (Mean±Standard Deviation). The difference between the groups was analyzed by the independent sample student's *t*-test.

correlation between miRNA221 expression levels and IDH-1 variant. The ΔCT values of miRNA-221 were also compared with the location of the tumors. There was no statistically significant correlation between miRNA221 expression and location (Table VI).

The receiver operating characteristic (ROC) analysis was performed using the MedCalc Program to determine all serum miRNA levels, and its diagnostic value Figure 2 indicates that miRNA-221 expression levels could be used to determine the threshold values in patient groups. The area under curve (AUC) was 0.767 and the threshold value >1.42 (p=0.0001).

Discussion

MicroRNAs are defined as RNA sequences that do not encode proteins and are approximately 20-25 nucleotides in length; act as negative regulators of gene expression at the transcriptional and post-transcriptional level (12, 13). miRNAs regulate gene expression by base matching and specific binding to sites located at the 3 'end non-transcribed region of the target mRNAs (13). miRNAs are recognizes the target mRNA by a matching region called seed sequence that contains 2-9 bases. The specificity of the miRNA is assigned by this short seed sequence, although miRNAs could bind long mRNA sequences. Owing to the low specificity binding capacity of miRNAs, several mRNAs can be regulated by more than one miRNA. Additionally, a single miRNA may target more than one mRNA. Consequently, miRNAs play a crucial role in the regulation of gene expression (14). miRNAs are involved in various diseases by affecting protein synthesis. In addition, various miRNA have been identified as biomarkers and are used in the early diagnosis of different diseases (15).

As miRNAs are very stable in the blood, they are considered important candidate biomarkers for the diagnosis and treatment of diseases. miRNAs are highly resistant to RNAases, and are not affected by pH changes. Furthemore, they can be easily detected in serum and plasma samples without the need for invasive procedures. Because of these advantages, the expression levels of miRNAs can provide significant information regarding the tumor and its treatment (16).

miRNAs could have oncogenic or tumor suppressor properties according to the molecular pathways of the targeted mRNA. In normal tissues, some miRNAs have been reported to inhibit the translation of protooncogenes. These suppressor miRNAs have an important role in controlling oncogene expression. Therefore, decreased expression of tumor suppressor miRNAs can cause increased oncogene expression and accelerate tumor formation. On the other hand, increased expression of oncogenic mirRNAs can enhance tumor formation. Thus, microRNAs both for oncogenic and tumor suppressor mRNAs can be used as a potential target (17).

miR-221/222 has been reported to play a role in carcinogenesis and is considered to be among the most disregulated miRNAs in cancer, including thyroid cancer, hepatocarcinoma, estrogen receptor negative breast cells and melanoma cells. The cell cycle regulator $p27^{Kip1}$ is one of the target genes of miR-221/222. Galardi *et al*. have studied $p27^{Kip1}$ and miR-221/222 expression levels in pancreatic cells. They found an inverse correlation between miR-221/222 overexpression and regulation of cell cycle progression (18). This result was supported by studies on lung cancer, glioblastoma, breast cancer, thyroid papillary carcinoma and hepatocellular carcinoma in later years (19).

Zhang *et al*. have found that miR-221/222 regulates apoptosis by targeting PUMA in GBM and that there was a reverse correlation between PUMA and miR-221/222 expression. MiR-221/222 is overexpressed and regulates tyrosine phosphatase PTPμ expression in patients with advanced glioma. miR-221/222-induced PTPμ overexpression effects tumorogenesis of glioma cells (20).

Primary malignant brain tumors cause death and the median survival time is 2 years. GBM, is the most malignant glial tumor of central nervous system. GBM is composed of malignant astrocytomas in 80% of cases and has the shortest survival ratio. The median survival time is 14.2-16 months following diagnosis. It has been shown that only 2-3% of patients with these tumors survive for more than 3 years (21).

Ciafre *et al*. have studied miRNA expression by microarray analysis in GBM to elucidate the role of miRNAs in the development of brain tumors. They found an upregulation of miR-221, and also confirmed that the

expression levels of miR-221 were increased in a subset of glioblastoma samples. The results of our study also indicate overexpression of miR-221 in gliomas (22).

Gillies and Lorimer have shown that miR-221 inhibited the expression of the cell cycle regulatory protein $p27^{Kip1}$ in glioblastoma cells. $p27^{Kip1}$ gene, the Cip/Kip family member of cyclin-dependent kinase (CDK) inhibitors, negatively controls cell cycle progression. $p27^{Kip1}$ protein blocks cell cycle progression from G_1 to S phase and also acts as a tumor suppressor in human cancers (23).

Le Sage *et al*. have performed an *in vitro* study on GBM cell lines. They determined the relation between the high expression levels of miR-221 and miRNA-222 and the low levels of $p27^{Kip1}$ gene in GBM cell lines They showed that high levels of miR-221 and 222 inhibit $p27^{Kip1}$ gene expression thus cancer cells continuously proliferate (24).

Slingerland *et al*. claimed that because of the oncogenic effect of increased miRNA-221 expression levels, the up-regulation of miRNA-221 plays very important roles in the deregulation of the cell cycle in high-grade gliomas. Additionally, up regulation of miRNA-221 is related with poor prognosis not only in GBM but also in pancreatic adenocarcinoma and thyroid papillary carcinomas (25).

Zhang *et al*. have performed a meta-analysis of thirty-two publications that include fifteen types of tumor and 2,693 patients. The results of univariate and multivariate analyses showed that miR-221/222 cluster overexpression in many tumors was significantly associated with poor overall survival (26).

In conclusion, we found that miRNA-221 expression could be a biomarker for glial tumors. But further studies should be performed to determine its sensitivity and specifity for the prognosis of glial tumors.

Conflicts of Interest

The Authors declare no potential conflicts of interest associated with this manuscript.

Authors' Contributions

Clinical data and sample collection was performed by UT, SÖ, CKY and AHD. Analysis of the clinical data and samples was performed by SGY and KS. MicroRNA analysis was performed by Tİ, SGY and FTA. Statistical analysis was performed by SGY and KS. Analysis of the results and supervision was performed by Tİ. All Authors read, revised, and approved the final article.

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Received April 14, 2020
Revised April 29, 2020
Accepted May 8, 2020