



Serum miRNA-582-5p and miRNA-363 as Potential Non-Invasive Biomarkers for Glioblastoma Multiforme

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ABSTRACT

AIM: To determine expression levels of miRNA-582-5p and miRNA-363 in serum of patients with Glioblastoma Multiforme and assess their biomarker potential.

MATERIAL and METHODS: The study population consisted of 71 subjects including 35 patients and 36 healthy controls. Real-time polymerase chain reaction was used to determine serum expression levels of miRNA-582-5p and miRNA-363 in patients and control individuals. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic potential of miRNA-582-5p and miRNA-363. Serum caspase-9 level was measured using enzyme-linked immunosorbent assay.

RESULTS: Normalized expression levels of miRNA-582-5p and miRNA-363 were calculated using the 2^{-ΔΔCt} method. We found that miRNA-582-5p and miRNA-363 were significantly upregulated in patients compared with healthy controls. High levels of miRNA-582-5p (Fold change 2.86, p<0.0001) and miRNA-363 (Fold change 3.51, p<0.0001) were significantly associated with Glioblastoma Multiforme. Additionally, ROC analyses demonstrated that levels of miRNA-582-5p [area under the curve (AUC)=0.938, p=0.0001] and miRNA-363 [AUC=0.951, p=0.0001] were significantly different between the groups. In contrast, there was no correlation between levels of serum caspase-9 and those of miRNA-582-5p (p=0.144) or miRNA-363 (p=0.050).

CONCLUSION: High serum levels of miRNA-582-5p and miRNA-363 are associated with Glioblastoma Multiforme, and are potential biomarkers.

KEYWORDS: Glioblastoma multiforme, miRNA-582-5p, miRNA-363, Biomarkers, Serum

ABBREVIATIONS: AUC: Area under curve, ELISA: Enzyme-linked immunosorbent assay, cDNA: Complementary deoxyribonucleic acid, CI: Confidence interval, CNS: Central nervous system, GBM: Glioblastoma multiforme, GSC: Glioblastoma stem cell, IDH: Isocitrate dehydrogenase, miRNA: microRNA, ROC: Receiver operating characteristic, RT-PCR: Real-time polymerase chain reaction, WHO: World Health Organization, ΔCt: Delta Cycle threshold.

■ INTRODUCTION

Glioblastoma Multiforme (GBM) is the most widespread and malignant type of primary brain tumor (20). GBM is graded as grade IV glioma by the World Health Organization (WHO) (18) (Table I), and accounts for approximately 14.6% of all primary brain tumors, 48.3% of primary malignant brain tumors, 57.3% of all gliomas, and 60% of all brain tumors in adults (21). The main histological characteristics of GBM are tumor heterogeneity, necrosis, hypercellularity, microvascular proliferation, nuclear atypia, high growth rate, resistance to apoptosis, tissue invasion, angiogenesis, vascular proliferation, and necrosis (2,11). It is possible to roughly subdivide GBM into primary and secondary GBM based on their clinical features and hallmarks (14,19). Some of the molecular features of primary GBM are epidermal growth factor receptor gene mutation and amplification, overexpression of murine double minute 2, p16 deletion, and phosphatase and tensin homolog on chromosome 10 mutations. Hallmarks of secondary GBM are mutations in platelet-derived growth factor receptor alpha, retinoblastoma, isocitrate dehydrogenase 1/2 (IDH1/IDH2), and tumor protein 53 on 17p with LOH on19q (14). In addition, GBM is classified into GBM IDH wild-type and GBM IDH mutant according to the current classification of central nervous system (CNS) tumors based on molecular parameters. IDH wild-type corresponds to primary or de novo GBM, constituting 90% of cases and arising in individuals over 55 years of age; IDH mutant type corresponds to secondary GBM, constituting 10% of cases and diagnosed in those approximately 44 years of age (19).

GBM continues to be a lethal disease despite treatment strategies such as surgical resection, radiation, and chemotherapy (22). New strategies are required to understand the underlying molecular mechanisms involved in GBM, and develop therapeutics and diagnostic biomarkers.

MicroRNAs (miRNAs, miRs) have been explored as a potentially novel class of diagnostic and prognostic biomarkers in human cancers (16). miRNAs are non-coding RNA molecules 18-22 nucleotides in length, that are involved in many vital processes and regulate gene expression post-transcriptionally, including playing roles in RNA silencing (4). Several impactful studies have reported that miRNAs are non-invasive biomarkers accessible in body fluids such as saliva, serum, and urine (3,13). Many miRNAs were found to be involved in GBM progression, such as miRNA-21, miRNA-32, and miRNA-153. They have been associated with regulation of cell proliferation,

apoptosis, invasion, chemoresistance, migration, and survival in GBM (25). miRNA-582-5p and miRNA-363 have been reported to function as oncogenes in human glioma and human glioblastoma stem cells (GSCs). miRNA-582-5p inhibits apoptotic pathways via targeting the apoptotic mRNAs caspase-9 and caspase-3 (10); miRNA-363 regulates cell survival (7) by targeting the apoptotic mRNAs caspase-3 and BCL2L11 (Bim) (10).

However, the role and molecular mechanisms of miRNA-582-5p and miRNA-363 in the prognosis of GBM have not been fully understood yet. There are only a few studies that have investigated the role of miRNA-582-5p and miRNA-363 in GBM. In the present study, we investigated miRNA-582-5p and miRNA-363 expression levels in human serum samples to assess their potential as diagnostic biomarkers of GBM. In addition, miRNA-582-5p and miRNA-363 were analyzed to investigate their roles in the regulation of the intrinsic apoptotic pathway in human GBM. Combining previous studies involving gene microarray analyses, we determined that caspase-9, a key initiator of this pathway, could be the potential target mRNA in our study (10,25).

■ MATERIAL and METHODS

Study Population and Clinical Procedures

The present case-control study consisted of 35 GBM patients who underwent surgical resection and were classified per the WHO classification of tumors of the CNS (18), and 36 healthy controls who were examined and reported by physicians as normal through cranial magnetic resonance imaging. They were selected from the Department of Neurosurgery in Yeditepe University (Istanbul, Turkey) after detailed clinical examinations.

All individuals signed an informed consent form in accordance with the Helsinki Declaration. The study was approved by the Medical Ethics Committee of the Yeditepe University Medical Faculty (file no: 25.10.2018/916).

miRNA Selection and Isolation

In addition to several databases including miRBase, Target-ScanHuman, microrna.org, and Embase, literature searches were performed for miRNA selection. We selected two miRNAs (miRNA-582-5p and miRNA-363) that have been reported as oncogenic miRNAs in human glioma and human GSCs (7,10), for further examination.

Table I: Classification of Astrocytic Tumors and Their Characteristic Features According to WHO (18)

WHO Grade	CNS Tumors	Histological features	Age at diagnosis
I	Pilocytic astrocytoma	Microcysts, Rosenthal fibers	10
II	Diffuse astrocytoma	Mildly increased cell number or atypia	34
III	Anaplastic astrocytoma	Mitoses, prominent atypia	41
IV	Glioblastoma Multiforme	Necrosis, endothelial proliferation	53

CNS: Central nervous system, **WHO:** World Health Organization.

5 ml of whole blood samples from all participants were collected in covered test tubes. Serum samples were separated by centrifugation (at 4500 rpm for 10 min); then, the samples were transferred into new sterilized tubes and immediately stored at -80°C until the commencement of the experiment. miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) was used for miRNA isolation from 200 µl of the serum samples per the manufacturer's instructions. The optical density was measured using NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNAs were synthesized with the miScript II RT Kit (Qiagen) and the Applied Biosystems® 2720 Thermal Cycler. Reverse transcription was performed with incubation at 37°C for 60 min and 95°C for 5 min. The miRNA concentration was measured using the Qubit miRNA Assay Kit (Thermo Fisher Scientific) using a standard protocol on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The synthesized complementary DNA (cDNA) was stored at -20°C before the real-time polymerase chain reaction (PCR) experiments.

Real-time PCR

Real-time PCR for human (hsa)-miR-582-5p, hsa-miR-363 and U6 was performed using the miScript SYBR Green PCR Kit (Qiagen). All reactions were run on the Rotor Gene-Q real-time PCR cycler (Qiagen, Germany). 45 PCR cycles (94°C, 15 s; 55°C, 30 s; 70°C, 30 s) were run after an initial denaturation step at 95°C for 20 min. U6 small nuclear RNA (RNU6) was used as internal control and the 2^{-ΔΔCT} method (17) was used to analyze the relative quantified levels of miRNA-582-5p and miRNA-363 (23). The analyses were done using Microsoft Excel.

Determination of Caspase-9 Serum Levels

Serum levels of human caspase-9 were measured using an enzyme-linked immunosorbent assay kit (eBioscience, Vienna, Austria) in accordance with the manufacturer's instructions.

Statistical Analyses

The results were expressed as mean ± standard deviation. Chi-square and Fisher's exact tests were performed to compare demographic characteristics. Student's t-test was used to examine the statistical significance of differences between the case and control groups. Pearson's correlation was used to evaluate the relationship between expression levels of miRNA-582-5p and miRNA-363, and those of caspase-9. The diagnostic value of serum miRNA-582-5p and miRNA-363 levels were determined with ROC analysis using MedCalc version 15.0 (MedCalc Software, Ostend, Belgium) at a 95%

confidence interval. These analyses were performed using the MedCalc software (NY, USA). p<0.05 was considered as statistically significant. All calculations were performed using the SPSS 20.0 software program (SPSS Inc, Chicago, IL, USA) and figures were created using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA) for Windows®. p<0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics of the study groups are shown in Table II. The study was conducted with 71 samples; 9 females, 26 males in the GBM group and 13 females, 23 males in the healthy control group. The mean age of the patients and healthy controls was 48.69 ± 18.34 and 42.75 ± 11.70 years, respectively (p=0.108). There was no statistically significant difference between the two groups in terms of gender (p=0.344). In the GBM group, 30 (85.7%) out of 35 patients had the IDH1 mutation and only 5 (14.3%) of these patients had the IDH1 wild-type sequence.

The results showed that caspase-9 levels in the serum of GBM patients (10.56 ± 5.59 mg/dL) were not significantly different compared with those of the control group (10.27 ± 1.93 mg/dL) (p=0.768).

The tumor locations were as follows; temporal lobe, 13 (37%); parietal lobe, 5 (14.3%); frontal lobe, 4 (11.4 %); thalamus, 4 (11.4%); cingulate cortex, 3 (8.6%); occipital lobe, 2 (5.7%); corpus callosum, 1 (2.9%); cerebellum, 1 (2.9%); pons, 1 (2.9%); and brainstem, 1 (2.9%). The most common tumor location was the temporal lobe (37%).

We analyzed the expression levels of miRNA-582-5p and miRNA-363 in the samples. Using RNU6 as an internal control for data normalization, we found that the miRNAs levels were significantly different between the two study groups. The results showed that levels of miRNA-582-5p and miRNA-363 in GBM samples were significantly upregulated (Fold change 2.86 and 3.51, respectively, p<0.0001; Figures 1 and 2, and Table III) compared to those in control samples.

To determine the diagnostic potential of miRNA-582-5p and miRNA-363 serum expressions in GBM, we used ROC analysis (MedCalc software). ROC results showed that the area under the ROC curve (AUC) of miRNA-582-5p was 0.938 (sensitivity, 84.4%; specificity, 97.0%; 95%CI 0.849–0.982; p<0.0001) and the AUC of miRNA-363 was 0.951 (sensitivity, 89.3%; specificity, 86.1%; 95%CI 0.866–0.989; p<0.0001). Our results demonstrate that miRNA-582-5p and miRNA-363 could be

Table II: Demographic and Clinical Values for the Patients with GBM and Healthy Control Groups

Characteristic	Healthy Controls (n=36)	GBM Patient (n=35)	p
Gender	Male / Female 63.9% / 36.1% (n=23) / (n=13)	Male / Female 74.3% / 35.7% (n=26) / (n=9)	0.344
Age (Year)	42.75 ± 11.70	48.69 ± 18.34	0.108
Caspase-9 (mg/dl)	10.27 ± 1.93	10.56 ± 5.59	0.768

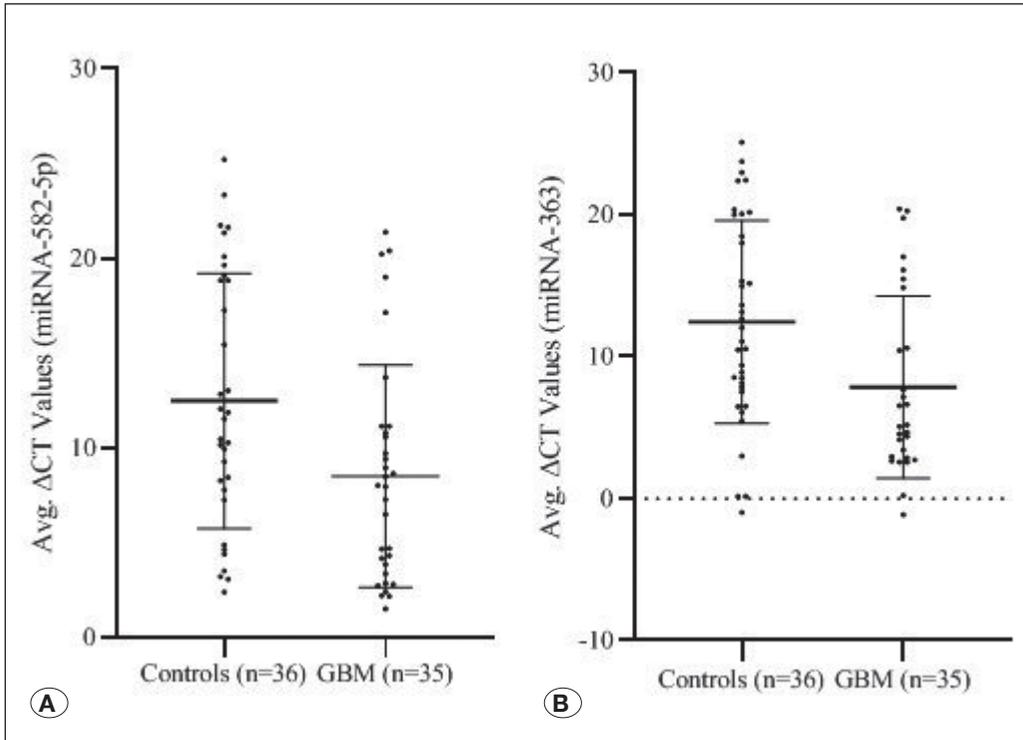


Figure 1: A, B) Real-time polymerase chain reaction (RT-PCR) to assess human serum expression levels (Δ CT values) of miRNA-582-5p and miRNA-363 in the study subjects. **(A)** Serum expression level (Δ CT values) of miRNA-582-5p in GBM patients (n=35) vs. control subjects (n=36) (p=0.014). **(B)** Serum expression level (Δ CT values) of miRNA-363 in GBM patients (n=35) vs. control subjects (n=36) (p=0.010). **GBM:** Glioblastoma multiforme, **Δ CT:** Delta Cycle threshold, **miRNA:** microRNA.

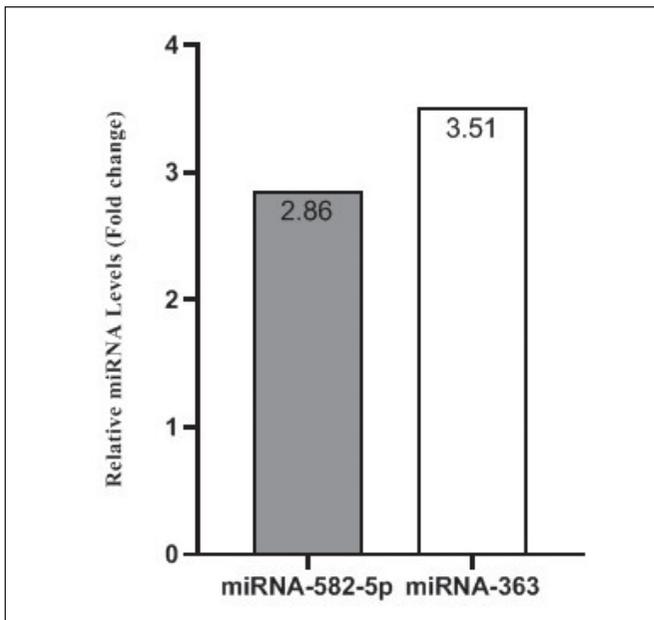


Figure 2: Relative fold changes in miRNA-582-5p and miRNA-363 levels in GBM patients compared to those in healthy subjects. The expression levels of miRNA-582-5p and miRNA-363 were significantly increased in serum of GBM patients compared to the control group (p<0.0001). **GBM:** Glioblastoma multiforme, **miRNA:** microRNA.

candidate biomarkers for the diagnosis of GBM (Figure 3). Further, expression levels of miRNA-582-5p and miRNA-363 were correlated with the IDH1 wild-type or mutant status in GBM patients. As shown in Table IV, there was no statistically

significant correlation between expression levels of the target miRNAs (miRNA-582-5p or miRNA-363) and the IDH1 variants (p=0.805 and p=0.541, respectively). Additionally, our results showed that serum miRNA-582-5p and miRNA-363 levels were not correlated with serum caspase-9 levels (p=0.144 and p=0.050, respectively) (Table V).

DISCUSSION

GBM is a glial cell-derived malignant type of primary brain tumor, characterized by poor survival and cellular tumor heterogeneity (18). miRNAs are considered to be an important factor in GBM because of their impact on the regulation of gene expression via post-transcriptional regulation of target mRNA in a sequence-specific manner. They have emerged as potential biomarker candidates, and are involved in many vital processes such as cell proliferation, apoptosis, and differentiation and may function as oncogenes or tumor suppressors (29). Several studies have reported that miRNAs can be detected in body fluids such as serum, plasma (1,5), cerebrospinal fluid (6), breast milk (30), urine, tears, colostrum (27), ovarian follicular fluid (8), and saliva (12). Extracellular miRNAs circulate via exosomes, microvesicles, apoptotic bodies, and proteins such as AGO2 (1,12,15). In addition, they are highly stable in blood (9). Therefore, miRNAs have become potential non-invasive biomarker candidates (24). A number of studies have demonstrated that miRNAs are involved in the development of GBM and might be of diagnostic and prognostic value (25). Among these miRNAs, miRNA-582-5p and miRNA-363 have been reported to contribute to proliferation, apoptosis, and survival of GBM cells (10,25). However, the association of miRNA-582-5p and miRNA-363 with development of GBM has not been fully understood.

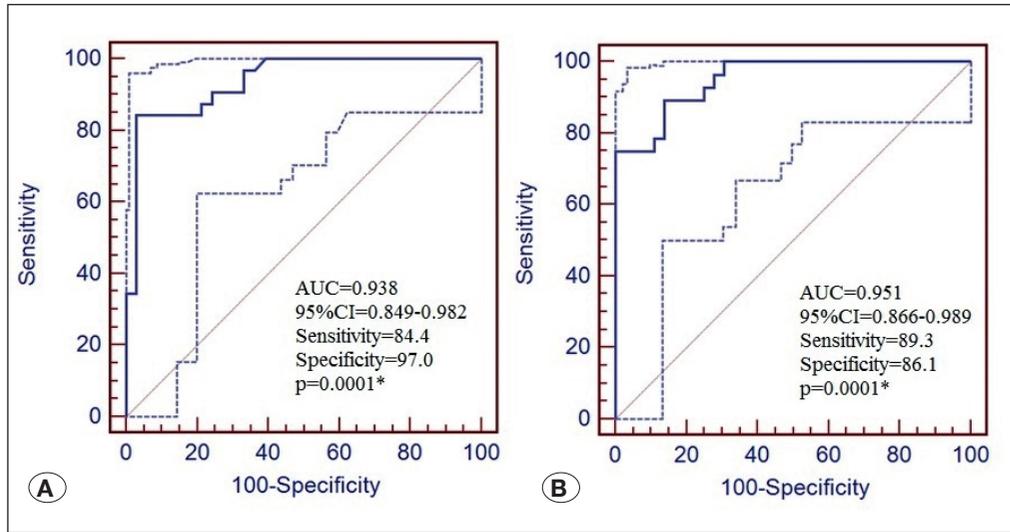


Figure 3: A, B: Diagnostic potential of serum miRNA-582-5p and miRNA-363 assessed using ROC curve analysis. **(A)** Diagnostic potential of miRNA-582-5p, AUC=0.938 (0.849 to 0.982 at 95% CI) with 84.4% sensitivity and 97% specificity. **(B)** Diagnostic potential of miRNA-363, AUC = 0.951 (0.866–0.989 at 95% CI) with 89.3% sensitivity and 86.1% specificity. *p<0.05; **ROC:** Receiver operating characteristic, **miRNA:** microRNA, **AUC:** Area Under the Curve, **95% CI:** 95% confidence interval.

Table III: miRNA-582-5p and miRNA-363 Expression Levels in Patients with GBM (n=35) and/vs Healthy Control (n=36) Groups. Diagnostic Potential of the miRNAs and Their Regulation

miRNA	Median ΔCT GBM	Median ΔCT Control	p	Fold change	p	AUC	ss (%)	sp (%)	p	Regulation
miRNA-582-5p	8.31	12.44	0.014*	2.86	<0.0001*	0.938	84.4	97.0	0.0001*	Up
miRNA-363	7.36	12.43	0.010*	3.51	<0.0001*	0.951	89.3	86.1	0.0001*	Up

AUC: Area under curve, **GBM:** Glioblastoma multiforme, **ΔCT:** Delta Cycle threshold. **sp:** specificity, **ss:** sensitivity. *p statistically significant, at least p<0.05.

Table IV: Association Between IDH1 Status and Serum miRNA-582-5p, miRNA-363 Expression Levels in GBM Patients

miRNA	IDH1 Type	n	X ± SD	p
miRNA-582-5p	IDH1 Mutant	30	2.10 ± 4.32	0.805
	IDH1 Wild Type	5	1.60 ± 2.48	
miRNA-363	IDH1 Mutant	30	3.00 ± 4.59	0.541
	IDH1 Wild Type	5	4.70 ± 2.96	

GBM: Glioblastoma multiforme.

*p<0.05, X± SD (Mean ± Standard Deviation), n: number of sample

Table V: Correlation Between Serum Caspase-9 Levels and miRNA Expressions of Patients with GBM

Parameter miRNA	Caspase-9 (mg/dl) p
miRNA-582-5p	0.144
miRNA-363	0.050

GBM: Glioblastoma multiforme.

* p statistically significant, at least p<0.05

The main aim of the present study was to investigate the expression levels of miRNA-582-5p and miRNA-363 in the serum of GBM patients and their diagnostic potential. To our knowledge, this is the first study investigating the diagnostic

potential of miRNA-582-5p and miRNA-363 in serum of Turkish patients with GBM. We analyzed the expression levels of miRNA-582-5p and miRNA-363 in serum samples from patients and healthy controls; our results showed that both target miRNAs (miRNA-582-5p and miRNA-363) were significantly upregulated in GBM patients. This suggests that increased expression levels of circulating miRNA-582-5p and miRNA-363 might be a potential biomarker in the diagnosis of GBM patients. Floyd et al. (10) previously reported that miRNA-582-5p and miRNA-363 are oncogenic in human GSCs. Similar to our results, they reported that miRNA-582-5p and miRNA-363 were overexpressed in their case group. In addition, Conti et al. reported that inhibition of miRNA-363 reduces the viability of glioma cell lines. Consistent with our results regarding miRNA-363 expression levels, they suggested that miRNA-363 can be a marker of glioma (7).

Finally, similar to our findings, another study reported that miRNA-363p is an onco-miRNA (28).

IDH status, a molecular signpost, has a specific clinical value in GBM prognosis (21). In the present study, 85.7% of the patients had the IDH1 mutation with only 14.3% of patients harboring the IDH1 wild-type sequence. To explore whether serum level of miRNA-582-5p or miRNA-363 is more specific to the IDH1 mutant than the wild type, correlation between IDH1 mutation status and target miRNA level was analyzed. Such data has not yet been reported, to our knowledge. We found that IDH1 mutation status is not significantly associated with expression of miRNA-582-5p or miRNA-363 in GBM patients. Based on this data, high serum levels of these miRNAs cannot be considered discriminative biomarkers for GBM subtypes.

Numerous signaling pathways are deregulated in GBM including apoptotic pathways. Considering the role of apoptotic mechanisms in GBM progression, it is important to examine factors involved in regulation of cell homeostasis such as caspases-9 (26). Caspase-9, a pivotal player in intrinsic apoptosis leading to activation of executioner caspase-3, is known to be targeted by miRNAs under GBM-like pathological conditions. In relation to GBM, there is inadequate information on the association of miRNA-582-5p and miRNA-363 with caspase-9. A human GSC-based study investigated miRNA-582-5p and miRNA-363 as anti-apoptotic miRNAs promoting human GSC survival. They suggested that miRNA-582-5p inhibits apoptotic pathways via targeting of caspase-9 and caspase-3 mRNAs (10); and that miRNA-363 regulates cell survival by targeting caspase-3 and BCL2L11 (Bim) mRNAs (10). Their studies have shown that caspase activation is blocked in GSCs due to overexpression of miRNA-582-5p and miRNA-363. Another study investigated the association of miRNA-363-3p, which acts as an onco-miRNA, with modulation of cell growth and invasion in glioma. Their results suggested that miRNA-363-3p directly targets pyruvate dehydrogenase B and promotes cell proliferation, protects against apoptosis, and enhances invasion (28). In the present study, we compared the expression levels of miRNA-582-5p and miRNA-363 with serum caspase-9 levels in GBM patients and control individuals; the results showed that up-regulation of miRNA-582-5p and miRNA-363 expression is not remarkably associated with serum caspase-9 levels. Thus, in contrast to some reported data, our results do not indicate upregulated miRNA-582-5p and miRNA-363 serum levels as mediators of apoptosis inhibition via caspase-9 targeting. This may be the result of several factors. First, miRNA-582-5p and miRNA-363 may not target caspase-9 mRNA in the progression of GBM. Second, the small sample size of the study population (n=71) could have limited identification of an association between them. Moreover, our study was performed with a sample of the Turkish population; studies with a larger sample size and different ethnic populations would be justifiable to validate this finding.

In summary, our results demonstrated an association between miRNAs (miRNA-582-5p, miRNA-363) and GBM. The results also show that miRNA-582-5p and miRNA-363

are upregulated while its potential target caspase-9 is not altered in GBM serums. Importantly, these findings suggest a potential relationship between the altered expression of miRNA-582-5p and miRNA-363 and the neurobiological changes underlying GBM. Taken together, our data suggest that miRNA-582-5p and miRNA-363 may play a role in GBM development. Therefore, determining the serum expression levels of miRNA-582-5p and miRNA-363 may be useful as a non-invasive diagnostic strategy in individuals with GBM. On the other hand, this study indicates that miRNA-582-5p and miRNA-363 probably do not influence apoptotic pathways via caspase-9 mRNA targeting in GBM. Despite animal experiments and *in vitro* studies regarding miRNAs as biomarkers in GBM, limited human data is available on the oncogenic effects of miRNA-582-5p and miRNA-363. Overexpression of miRNA-582-5p and miRNA-363 in GBM is intriguing, and further studies should be performed to clarify the non-invasive biomarker potential of miRNA-582-5p and miRNA-363 and their possible association with caspase-9 in GBM patients. Collectively, our results clearly indicate that miRNA-582-5p and miRNA-363 play a significant role in GBM progression, with no effect on direct regulation of caspase-9 expression. However, more experimental investigations along with computational analyses are needed to validate our findings.

■ CONCLUSION

Our findings reveal oncogenic roles of miRNA-582-5p and miRNA-363 in GBM. Importantly, caspase-9 expression in GBM was not regulated by miRNA-582-5p or miRNA-363. Altogether, our study not only identifies oncogenic roles of miRNA-582-5p and miRNA-363 in GBM, but also provides insights into targetable apoptosis pathways in GBM, and miRNA candidates as serum biomarkers of GBM.

In conclusion, overexpression of miRNA-582-5p and miRNA-363 could be a potential non-invasive biomarker in patients with GBM.

■ AUTHORSHIP CONTRIBUTION

Study conception and design: TI, DB

Data collection: UT, CKY, SO

Analysis and interpretation of results: SGY, DB

Draft manuscript preparation: TI, DB

Critical revision of the article: TI

Other (study supervision, fundings, materials, etc...): TI

All authors (DB, SGY, CKY, SO, UT, TI) reviewed the results and approved the final version of the manuscript.

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