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Evaluation of the Relationship Between miRNA-22-3p and Gal-9 Levels in Glioblastoma

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Abstract. *Background/Aim: Glioblastoma, the most prevalent primary malignant brain tumor, is significantly impacted by molecular mechanisms, including the function of microRNAs and galectins. The interplay between miRNA-22-3p and Galectin-9, a galactoside-binding lectin, is particularly notable. This study aimed to further investigate their roles in glioblastoma pathogenesis by analyzing the serum levels of these molecules in patients with glioblastoma. Patients and Methods: This investigation included 50 subjects, consisting of 25 patients with glioblastoma and an equal number of healthy controls. Blood serum specimens were obtained for miRNA isolation and subsequent cDNA synthesis. The expression of the miRNA-22-3p gene was assessed using polymerase chain reaction (PCR), and a sandwich enzyme-linked immunosorbent assay (ELISA) was utilized to quantify serum Gal-9 concentrations. Results: In patients diagnosed with glioblastoma, there was a significant elevation in miRNA-22-3p expression compared to healthy controls. However, despite a trend towards increased serum Gal-9 levels in the glioblastoma group, the difference did not reach statistical significance. Conclusion: Glioblastoma patients are characterized by increased Gal-9 serum levels and reduced miRNA-22-3p expression. These results indicate their potential as diagnostic and prognostic markers as well as therapeutic targets.*

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Glioblastoma multiforme (GBM), which is categorized by the World Health Organization (WHO) as a high-grade (stage IV), invasive astrocytoma, represents the most prevalent primary neoplasm of the central nervous system, characterized by aggressive malignancy and high mortality rate (1-5). Despite a comprehensive therapeutic approach for GBM, encompassing surgical resection, radiation therapy, and systemic administration of the alkylating agent Temozolomide (TMZ), the median overall survival duration remains approximately 15 months (2, 3, 6, 7). This malignancy, is predominantly observed in individuals aged 64 and above, presents global survival rates of 35%, 8%, and 4.7% at 1, 3, and 5-years post-diagnosis, respectively (5, 8, 9).

MicroRNAs (miRNAs), a class of non-coding RNA molecules typically comprising 20-25 nucleotides, critically regulate gene expression by binding to the 3'-untranslated region (3'UTR) of target messenger RNAs (mRNAs), consequently exerting post-transcriptional control *via* mechanisms such as translational repression or mRNA degradation (10-12). The aberrant production of miRNAs in cancer is frequently associated with a spectrum of genetic and epigenetic alterations, encompassing gene amplification, deletion, translocation, or epigenetic silencing (13). Notably, specific miRNAs possess dual functionality as they can act as either tumor suppressors or oncogenes in the context of human malignancies, orchestrating the coordinated regulation of multiple target genes (11, 14, 15).

miRNA-22-3p has been extensively investigated across cancer types, demonstrating a crucial regulatory role and robust tumor-suppressing properties (14-20). Importantly, its down-regulation has been strongly associated with clinicopathological characteristics and overall prognosis, indicating its potential as a metastamir. However, it is noteworthy that studies have presented contradictory findings, as miRNA-22-3p exhibits tumor-suppressive capabilities at high expression levels (6, 21-23). Moreover, functional studies have revealed that miRNA-22 hampers proliferation and migration, specifically inhibiting glioma cell proliferation and migration (24).

Galectins, a unique group within the broader family of carbohydrate-binding proteins, are differentiated by a conserved carbohydrate recognition domain (CRD) that is particularly significant due to its distinct affinity for β-galactosides (7, 25). Although they are predominantly localized within the cellular cytoplasm, galectins are particularly predisposed to be released into the extracellular environment in response to cellular trauma (25, 26).

Galectin-9 (Gal-9), a member of the galectin family characterized by a tandem-repeat structure and existing as a heterodimer with two CDRs connected *via* a peptide linker, has been shown to be over-expressed in glioma tissues, a phenomenon correlated with increased tumor aggressiveness (7, 25, 26). It has been shown that Gal-9 interacts with the cell surface protein T-cell immunoglobulin mucin 3 (TIM-3) present on glioblastoma cells. This interaction potentially attenuates Th1-mediated immune responses and fosters immunological tolerance through mechanisms involving apoptotic and necrotic processes (7, 27-30). Gal-9, located both extracellularly and intracellularly within the nuclei and cytoplasm, is believed to trigger intracellular calcium flux and induce Th1 cell death *via* its interaction with TIM3 (7, 29, 31). In addition, galectins, including Gal-9, can be released in the serum under stress conditions such as infection and inflammation (25). Furthermore, there is a marked discrepancy in the expression patterns of Gal-9 in neoplastic tissues compared to their healthy counterparts (7, 15, 25-27).

Multiple scientific studies suggest a negative prognostic correlation between elevated Galectin-9 (Gal-9) expression levels in glioma patients and their survival outcomes. The data indicate that glioma patients exhibiting high Gal-9 expression have significantly shorter survival times compared to those with lower expressions (7, 27, 31, 32). In a separate study conducted by Yang and colleagues investigating the interaction between Gal-9 and miRNA-22 and their potential impact on lymphocyte apoptosis and neoplastic cell proliferation, it was revealed that miRNA-22, which has been reported to be down-regulated in hepatocellular carcinoma tissues and cell lines, directly targets the 3'UTR of Gal-9 (15). Corroborating this, Chen *et al*. reported that the repression of miRNA-22 in hepatocellular carcinoma was associated with enhanced Gal-9 expression (33).

These studies highlight the intricate relationship between Gal-9 and miRNA-22, suggesting a regulatory role of this microRNA in Galectin-mediated oncogenesis. In this context, the current study was undertaken with the objective of elucidating the interplay between Gal-9 and miRNA-22-3p in a cohort of Turkish patients diagnosed with GBM.

Materials and Methods

Sample inclusion criteria. The study population comprised 25 glioblastoma patients, recruited from the Neurosurgery Clinic at Yeditepe University Hospital and a control group consisting of 25 entirely healthy volunteers. Patients who had previously undergone surgical treatment for glioblastoma were excluded from the study. Detailed information was collected for all participants, encompassing age, sex, initial symptoms, duration of symptoms, tumor location, date of surgery, post-operative follow-up duration, recurrence, and survival time (spanning from the date of surgery to the last follow-up), for patients who had passed away, the cause of death was also meticulously documented. The study was approved by the Yeditepe University Clinical Research Ethics Committee. All participants involved in the study were thoroughly informed about the study, and informed consent forms were obtained.

Control group selection. The control group comprised sex- and age-matched individuals attending routine health check-ups at Yeditepe University Hospital. Any subjects with a history of chronic illnesses were excluded from the control group.

Sample acquisition. Peripheral blood samples were collected from both patients and control subjects. These samples were then centrifuged at 4,000 rpm (1520 g-force) for a period of 15 min. The serum obtained from this process was aliquoted into three separate tubes and preserved at –80˚C until subsequent analysis. Prior to the day of analysis, serum samples were thawed and brought to room temperature to ensure optimal conditions for the planned assessments.

miRNA isolation, cDNA synthesis, and miRNA22-3p expression evaluation. MiRNA was extracted from the thewed serum samples utilizing the miReasy Kit (Cat. No./ID: 217184), as per the manufacturer's instructions. This was followed by cDNA synthesis employing the miRCURY LNA RT Kit (Cat. No./ID: 339340, Qiagen, Germantown, MD, USA). Concentrations of the samples were then measured and appropriately diluted. The expression level of miRNA-22-3p (miRCURY 22-3p, cat no: TP00204606 Qiagen) was assessed *via* polymerase chain reaction (PCR) utilizing the miRCURY LNA SYBR green PCR kit (Cat. No./ID: 339346) in a Rotor Gene system (Rotor-Gene Q; Qiagen). The expression levels of miRNA-22-3p were quantified using both the ΔCT and ΔΔCT methods, with RNU6 serving as the internal control for normalization.

Enzyme-linked immunosorbent assay (ELISA). Thawed serum samples underwent centrifugation at 3,000 rpm (850 g-force) for 20 min. Gal-9 levels in the serum were quantified using a sandwich ELISA technique with a commercially available kit (ab273161 Human Galectin-9 ELISA kit, Abcam, Cambridge, UK) adhering strictly to the manufacturer's instructions. The optical density values were measured at a wavelength of 450 nm using an ELISA plate reader (WHYM201, Poweam Medical Co., Ltd., Nanjing, PR China). The concentrations of Gal-9 were then determined based on a standard curve generated from known concentrations.

Statistical analysis. Statistical evaluations were conducted using the IBM SPSS Statistics software, version 22 (IBM Corp., Armonk, NY, USA). A *p*-value of less than 0.05 was considered indicative of statistical significance. The Mann-Whitney *U*-test was employed to compare the expression levels of miRNA-22-3p between the two groups, given the non-normal distribution of the data. To assess the

Table I. *Patients' survival time. According to a five-year patient follow-up, the annual and overall mean (± SD) survival time of patients diagnosed with glioblastoma. The mean survival time of GBM patients was 26.08 months.*

Mean (SD) survival time (months)	95% CI (lower - upper bound)	1-Year mean survival time	2-Years mean survival time	3-Years mean survival time	4-Years mean survival time	5-Years mean survival time
26.08±12.56	21.15-31.01	88±44	40±12	24±8	8±4	0

CI: Confidence interval; SD: standard deviation

Table II. *miRNA expression levels. Comparison of serum miRNA 22-3p expression levels between glioblastoma patients and healthy subjects. Calculated using the Mann-Whitney U-test.*

		N	Mean	S.D.	Mean rank	Sum of ranks	*p*-Value
miRNA 22-3p	Patient	24	2.32	1.04	28.69	688.50	0.038*
	Control	24	2.62	4.11	20.31	487.50	

SD: Standard deviation. *$p<0.05$ indicates statistical significance. N: Number of samples.

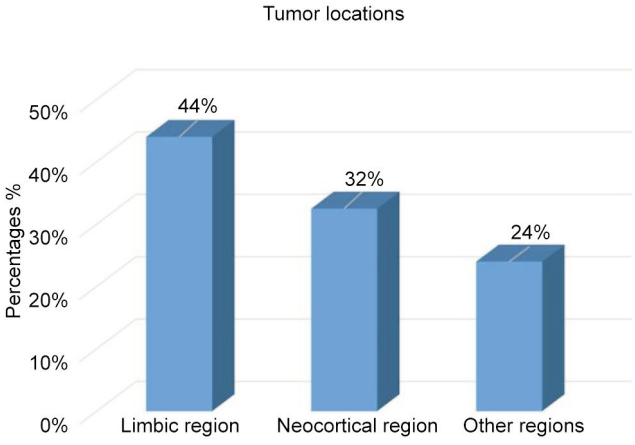

Figure 1. *Distribution percentages (%) upon assessment of tumor location among GBM (Glioblastoma multiforme) patients.*

diagnostic value of miRNA-22-3p serum levels in distinguishing glioblastoma patients from control subjects, a receiver operating characteristic (ROC) curve analysis was performed using the MedCalc software. In addition, the Mann-Whitney *U*-test was utilized to compare serum Gal-9 levels between the patient and control group.

Results

Demographic data: In relation to demographic characteristics, the study comprised a total of 50 participants, including 27 females and 23 males. The average age of the glioblastoma multiforme (GBM) patients was found to be comparable to that of the control group, with mean ages of 42.9 and 40.9, respectively. Upon assessment of tumor location, a significant prevalence was observed in the limbic (n=11) and neocortical regions (n=8) (Figure 1). Additionally, 14 tumors were situated on the left side, 11 on the right side and one presented bilaterally. According to our review from patient follow-ups, the mean survival time of GBM patients was 26.08 months (Table I).

miRNA-22-3p expression: A statistically significant elevation in the expression levels of miRNA-22-3p was observed in the patient group when compared to the control group (Mann-Whitney *U*, $p=0.038$) (Table II). The diagnostic potential of miRNA-22-3p as a putative biomarker for glioma was evaluated through the application of receiver operating characteristics (ROC) curve analysis. Utilizing ΔΔCT values, miRNA 22-3p demonstrated a specificity of 100% and sensitivity of 41.7% [95% confidence interval (CI)=0.524-0.802, AUC=0.674, $p=0.025$] (Figure 2), affirming its diagnostic efficacy.

Serum GAL-9 concentrations: While the serum levels of Gal-9 were not found to be statistically different between the groups (Mann-Whitney *U*, $p=0.063$), a trend towards higher levels was noted in the patient group when compared to the control group (Figure 3, Table III).

Relationship between Gal-9 and miRNA-22-3p levels: A statistically negative correlation was found between serum Gal-9 levels and miRNA-22-3p expression levels in the patient group (r=–0.154). Conversely a positive correlation was observed in the control group (r=0.255) (Table IV, Figure 4).

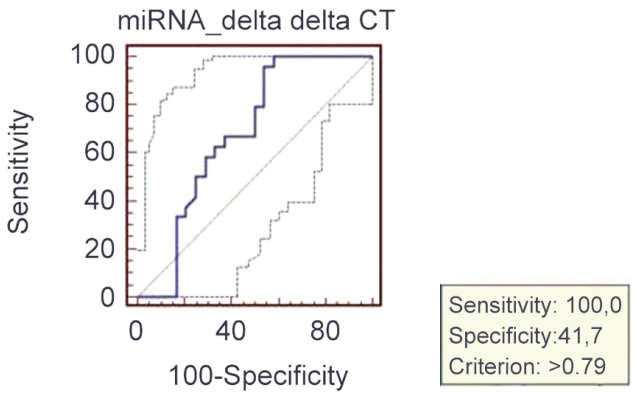

Figure 2. *Receiver operating characteristic (ROC) analysis of mRNA-22-3p expression in patients with glioblastoma comparing with healthy subjects.*

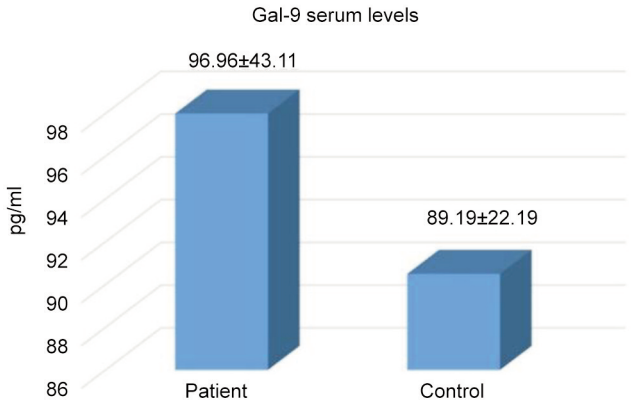

Figure 3. *Comparison of galectin-9 (Gal-9) levels in serum between patients diagnosed with glioblastoma (GBM) and healthy subjects.*

Table III. *Galectin-9 levels. Comparison of galectin-9 (Gal-9) levels as pg/ml in serum between patients diagnosed with glioblastoma and healthy subjects. Calculated using the Mann-Whitney U-test.*

		N	Mean	S.D.	Mean rank	Sum of ranks	*p*-Value
Gal-9 pg/ml	Patient	23	96.96	43.11	22.48	517.00	0.481
	Control	21	80.19	22.19	22.52	473.00	

SD: Standard deviation. *$p<0.05$ indicates statistical significance. N: Number of samples.

Table IV. *Association between Gal-9 and miRNA 22-3p. Correlation analysis between serum levels of galectin-9 (Gal-9) and miRNA 22-3p expression in patients diagnosed with glioblastoma (GBM) and healthy subjects, individually.*

	Patient		Control	
	Gal-9 pg./ml	miRNA-22-3p	Gal-9 pg./ml	miRNA-22-3p
Gal-9 pg./mg	1	–0.154*	1	0.255*
miRNA-22-3p		1		1

*Correlation is significant at the 0.05 level (2-tailed).

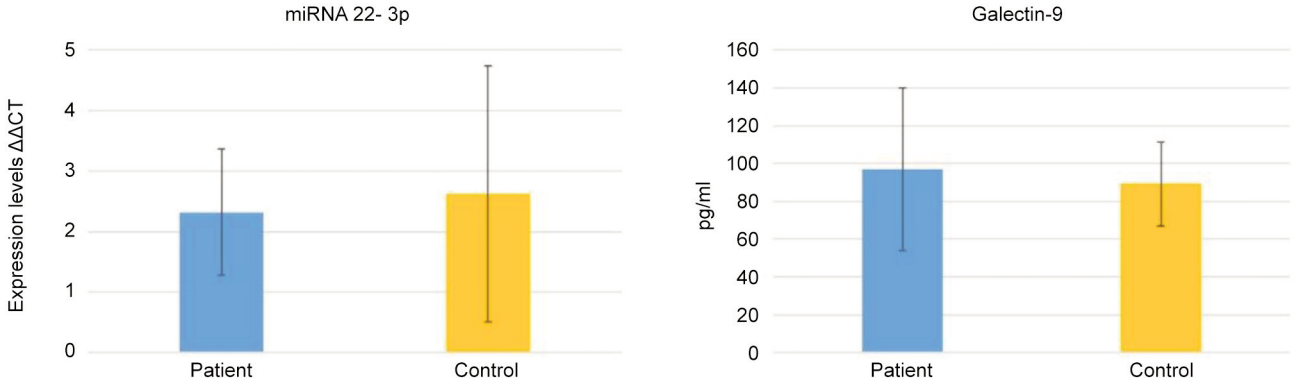

Figure 4. *Correlation between serum levels of galectin-9 (Gal-9) and microRNA-22-3p (miRNA-22-3p) expression in patients diagnosed with glioblastoma (GBM) and healthy subjects, individually.*

Discussion

Glioblastoma multiforme (GBM), characterized by histopathological hallmarks including necrosis and endothelial proliferation, accounts for an estimated 3-4% of mortality associated with neoplastic diseases (1-4, 6, 9). Recent advancements in the fields of genomics and proteomics have facilitated a more precise classification of GBM, elucidating the potential role of miRNAs in delineating the molecular phenotype of various GMB subtypes (8, 34, 35). Research has shown that miRNAs, possessing the capability to interact with the microRNA recognition element (MRE) located within the 3'UTR of mRNAs (15, 35-37), are implicated in facilitating diverse malignant transformations and metastatic processes, due to their aberrant expression in oncological patients (20, 22, 37). There is evidence suggesting that miRNAs can function as either oncogenes or tumor suppressors depending on the cellular context, the nature of various tumor systems, and specific roles of target genes (38). This extends to miRNA-22-3p, which has been documented to exhibit notable tumor suppressive attributes in specific cancer types (6, 14, 16-18, 21, 37).

The literature has documented a systematic decrement in miRNA-22-3p expression in tissue samples, which chronologically represents the progression from a non-metastatic primary neoplasm to a metastatic primary tumor, and ultimately to a metastatic lesion (22). A discernible down-regulation of miRNA-22-3p has been identified in an array of malignancies, including human hepatocellular carcinoma (14), liver cancer (15), renal cell carcinoma (18), colorectal cancer (39), breast cancer (40), non-small cell lung cancer (41), cholangiocarcinoma (42) and glioma cells (6). Conversely, miRNA-22-3p over-expression has been observed in prostate cancer (15, 43). This dichotomous expression pattern underscores the concept that a single miRNA can exhibit dual functionality, depending on its influence on cancer cell proliferation dynamics (44, 45). These observations show the complex and context-dependent roles of miRNA-22-3p in the tumorigenic process and underscore its potential as a diagnostic and therapeutic target with varying implications across different cancer types.

Recently, the critical role of Gal-9, endowed with two CRDs, that functions as a key modulator of T-cell activity, has been increasingly recognized in both physiological and pathological states (46). In addition, the role of Gal-9, associated with tumor cell adhesion and metastasis in solid tumors (47), in orchestrating a plethora of biological processes integral to tumorigenesis such as cellular aggregation and adhesion, induction of tumor cell apoptosis, facilitation of immune escape mechanisms, and angiogenesis, underscores its potential as a crucial therapeutic target in cancer intervention (25-27, 30, 31). Research evidence indicates that Gal-9, which is significantly up-regulated in GBM compared to normal brain tissues and low-grade gliomas, can down-regulate TIM-3, and negatively correlated with overall patient survival (7, 27, 31, 32).

Intriguingly, the notable disparity in Gal-9 expression across different cancerous tissues (15, 28, 48) anticipates its critical role in the intricate network of cellular interactions directing cancer progression.

Previous studies have shown that elevated levels of Gal-9 expression in acute myeloid leukemia can hinder the anti-cancer activities of natural killer cells in an *ex vivo* setting (49). However, in the context of pancreatic cancer, Gal-9 has demonstrated a tumor-suppressive role, thereby presenting potential therapeutic implications (50). In primary breast tumor cells, a notable over-expression of TIM-3 and especially Gal-9, has been observed when compared to healthy tissue samples (28). Conversely, the expression of miRNA-22-3p was found to be significantly reduced in breast cancer tissue as compared to normal breast tissue (40), further highlighting the complex interplay of these molecules in cancer progression. These observations present the intricate role of Gal- 9 in the pathogenesis of neoplastic diseases, underscoring the necessity for additional in-depth research in this domain.

Given these findings, the pivotal role of Gal-9 in brain cancer pathogenesis is increasingly recognized, positioning it not only as a promising therapeutic target but also an emerging oncological biomarker in GBM (7, 15, 25-27). These observations suggest that different galectins may orchestrate distinct biological functions across diverse cancer types and that Gal-9 expression could be subject to modulation by a spectrum of miRNAs. As physiological entities with the capacity to concurrently regulate a multitude of genes (51), it is plausible that miRNA-22-3p could impact an array of pathways and function as a modulator of Gal-9 expression (17, 20). Given the established linkage between the TIM-3/Gal-9 pathway and immune responses, as well as the progression of GBM, the primary objective of this research was to explore the relationship between Gal-9 and miRNA-22-3p in the context of this specific disease.

In the present study, our findings with patients diagnosed with GMB indicate a statistically significant reduction in miRNA-22-3p expression in the patient cohort compared to the control group. Although Gal-9 serum concentrations were elevated in the patient cohort relative to the control, the variation did not surpass the threshold for statistical significance. It's important to note that the patient population in our study was limited yet, the data generated have promising implications for future research. Significantly, our data collected from the GBM patient cohort revealed a strong inverse correlation between Gal-9 and miRNA-22-3p, in stark contrast to the control cohort where we observed a

pronounced positive association between Gal-9 and miRNA-22-3p. Given that the Gal-9 gene is a known target of miRNA-22-3p (15), this association may be tied to the role of miRNA-22-3p in immune response (10), potentially enhancing our comprehension of the regulatory role of miRNA within the Gal- 9 pathway.

To the best of our knowledge, this study is the first to document the simultaneous up-regulation of Gal-9 and down-regulation of miRNA-22-3p in GBM, adding a unique dimension to our findings. Further exploratory work is required to decipher the molecular interplay and association between miRNA-22-3p and Gal-9. We anticipate that these findings will serve as a foundation for further investigations into the miRNA-22-3p/Gal-9 axis within the molecular mechanisms underpinning GBM and could potentially aid in the identification of novel therapeutic targets.

Conflict of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

All Authors contributed significantly to this study. Z.B., T.A. devised the research plan, provided materials, and participated in the analysis. The first draft of the manuscript was written by Z.B. and all authors commented on previous versions of the manuscript. Finally, all the Authors read, approved, and agreed to the publication of the manuscript.

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