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INSTITUTE OF HEALTH SCIENCES
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INVESTIGATION OF THE USE OF miRNA223 AS A BIOMARKER IN GLIOMA TUMORS

MASTER OF SCIENCE THESIS

GÖKHAN ÖZDEMİR, BSc

İSTANBUL – 2023

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Jury	Title, Name- Surname (Institution)	Signature
Supervisor (Chair of the Jury)		
Member		
Member		

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

02/02/2023

Gökhan ÖZDEMİR

DEDICATION

I would like to dedicate this thesis to my wonderful parents, who contributed everything to ensure that I could receive an education despite all obstacles and endeavors, and who have been my emotional support throughout my entire life.

Gökhan ÖZDEMİR

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Gökhan ÖZDEMİR

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LIST OF SYMBOLS AND ABBREVIATIONS

AGO2	: Argonaute 2
AIF	: Apoptosis Inducing Factor
Apaf-1	: Apoptotic Protease Activating Factor 1
AUC	: Area Under Curve
Bak	: Bcl-2 Antagonist/killer-1
Bax	: Bcl-2-Associated X Protein
BiCNU	: Carmustine
C.I	: Confidence Interval
CARD	: Caspase Activation Domain
CCNU	: Lomustine, CeeNU
cDNA	: Complementary DNA
CNS	: Central Nervous System
CT	: Computed Tomography
C_T	: Cycle Threshold
Cyt C	: Cytochrome C
Δ	: Delta
$\Delta\Delta C_T$	: Delta Delta C_T
ΔC_T	: Delta C_T
DGCR8	: DiGeorge Syndrome Critical Region-8
DNA	: Deoxyribonucleic Acid
DR	: Death Receptor
eg	: example
EGFR	: Epidermal Growth Factor Receptor
FADD	: Fas-Associated Death Domain
GBM	: Glioblastoma Multiforme
HGSOC	: High-grade serous ovarian cancer
IAP	: Inhibitor of Apoptosis Protein
IDH-1	: Cytosolic $NADP^+$ related isocitrate dehydrogenase
ln $(2^{-(\Delta\Delta CT)})$	: Fold change
LNA	: Locked nucleic acid

IncRNA	: Long noncoding RNA
LOH	: Loss of Heterozygosity
µl	: microliter
µg	: microgram
m^7G	: 7-methylguanosine
MDM2	: Mouse Double Mnute 2
mg	: milligram
miRNA	: Micro RNA
MRI	: Magnetic Resonance Imaging
mRNA	: Messenger RNA
NADP+	: Nicotinamid Adenin Dinucleotide Phosphate
ng	: Nanogram
ohm	: symbol Ω, unit of electrical resistance
OD	: Optic Density
OR	: Odds Ratio
PAN2	: Poli(A)-nuclease 2
PAN3	: Poli(A)-nuclease 3
PCR	: Polymerase Chain Reaction
PDGFA	: Platelet-Derived Growth Factor Receptor Alpha
pg	: picogram
piRNA	: Interacting RNA
pre-miRNA	: Precursor-miRNA
pri-miRNA	: Primer-miRNA
PTEN	: Phosphate and Tensin Homolog
RB	: Retinoblastoma
RISC	: RNA Induced Silencing Complex
RNA	: Ribonucleic Acid
RNase III	: Ribonuclease III
ROC	: Receiver Operating Characteristic
rpm	: Rounds per minute
RT-PCR	: Real-Time Polymerase Chain Reaction

SD	: Standard Deviation
shRNA	: Short hairpin RNA
siRNA	: Small Interfering RNA
SMAC	: Second Mitochondria-Derived Activator of Caspase
SNP	: Single Nucleotide Polymorphism
TMZ	: Temozolomide
TNF	: Tumor Necrosis Factor
TRAIL	: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TRBP	: Transactivating Response RNA Binding Protein
UTR	: Untranslated Region
WHO	: World Health Organization
XPO5	: Exportin 5
XRN1	: Exonuclease-1

SUMMARY

Özdemir, G. (2023). Investigation of the use of miRNA223 as a biomarker in glioma tumors. Yeditepe University, Institute of Health Sciences, Department of Molecular Medicine. Master Thesis. İstanbul.

The purpose of this study is to determine of the use of miRNA223 as a biomarker in glioma tumors. The expression of several microRNAs in glioma tumors has been studied. miRNA223 has been studied in several forms of cancer. This is the first study in literature to investigate the expression level of miRNA223 in patients having glioma at the Turkish population. This study demonstrates the role of possible biomarkers in the expression analysis of miRNA223 in glioma tumors. The blood samples used in this thesis study are previously taken samples and present at the Molecular Medicine Laboratory of Yeditepe University. Yeditepe University Non-Interventional Clinical Research Ethics Committee approval is obtained prior to the study. Blood samples are obtained from the laboratory freezer which were priorly obtained from 35 patients who have referred to Yeditepe University Hospital having glial tumors. Blood samples from 30 healthy subjects are used as a control group. Depending on the location of the tumor, 3 different serum chambers were constructed from patient sera. A serum pool was created from the control structures. Qiagen's miRNeasy Serum/Plasma Kit (Cat. No. / ID: 217184) is used to practice miRNA isolation. Subsequently the cDNA is converted by conventional PCR, using the miRCURY LNA RNA miRCURY LNA RT Kit (Cat. No./ID: 339340 - Qiagen). A fluorometer is used to measure cDNA levels. Dilutions of the samples were made. To detect miRNA expression levels, expression analysis was performed by PCR method in Rotor Gene (Rotor-Gene Q –Qiagen) using miRCURY LNA SYBR Green PCR Kit (Cat. No. / ID: 339346). Real-Time Polymerase Chain Reaction is used to detect miRNA expression levels (RT-PCR). SPSS ver. 23.0 is used for statistical analysis (SPSS Inc, Chicago, IL, USA). ROC curve analysis has been done. The p value is set to 0.05 for statistically significant results.

***Key Words*:** Glioma tumors, miRNA223

ÖZET

Özdemir, G. miRNA223'ün glioma tümörlerinde biyobelirteç olarak kullanımının araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Moleküler Tıp Anabilim Dalı. Yüksek Lisans Tezi. İstanbul, 2023.

Bu tez çalışmasının amacı, miRNA223'ün glioma tümörlerinde biyobelirteç olarak kullanımının araştırılmasıdır. Yapılan çalışmalarda Glioma tümörlerinde, farklı mikroRNA'ların genetik analizi değerlendirilmiştir. miRNA223 ise farklı kanser türlerinde incelenmiştir. Bu çalışma, Türk popülasyonundaki glioma hastalarında miRNA223'ün ekspresyon düzeyini araştıran literatürdeki ilk çalışmadır. Bu çalışma, miRNA223'ün glioma tümörlerinde ekspresyon analizinde olası biyobelirteçlerin rolünü göstermektedir. Bu tez çalışmasında kullanılan kan örnekleri, Yeditepe Üniversitesi Moleküler Tıp Laboratuvarında bulunan ve daha önceden alınmış mevcut örneklerden oluştu. Çalışma öncesinde Yeditepe Üniversitesi Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu onayı alındı. Bu çalışmada kullanılan hasta kan örnekleri laboratuvar dondurucusunda bulunan ve Yeditepe Üniversitesi Hastanesi'ne glia tümörü ile başvuran 35 hastadan alınan kan örneklerinden oluştu. 30 sağlıklı kişiden alınan kan örnekleri ise kontrol grubu olarak kullanıldı. Tümörün lokasyonuna bağlı olarak hasta serumlarından 3 farklı serum havuzu oluşturuldu. Kontrol grubundan da serum havuzu oluşturuldu. miRNA izolasyonunu sağlamak amacıyla Qiagen'in miRNeasy Serum/Plazma Kiti kullanıldı (Cat. No. / ID: 217184). Mikro RNA'lar ise miRCURY LNA RT Kit (Cat. No./ID:339340) kullanılarak cDNA ya konvansiyonel PCR yaparak dönüştürülür. cDNA seviyelerini ölçmek için florometre kullandı. Örneklerin dilüsyonları yapıldı. miRNA ekspresyon seviyelerini saptamak için miRCURY LNA SYBR Green PCR Kit (Cat. No. / ID: 339346) kullanılarak Rotor Gene' de (Rotor-Gene Q –Qiagen) PCR yöntemi ile ekspresyon analizi yapıldı. miRNA223 ekspresyon seviyelerini saptamak için Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (RT-PCR) cihazı kullanıldı. İstatistiksel analizler için SPSS paket programı (SPSS Inc, Chicago, IL, USA) sürüm 23.0 kullanıldı. ROC analizi yapıldı. İstatistiksel olarak anlamlı sonuçlar için p değeri 0,05 olarak ayarlandı.

***Anahtar Kelimeler*:** Glioma tümörleri, miRNA223,

1. INTRODUCTION AND PURPOSE

A kind of nervous system tumor called glioblastoma multiforme (GBM) develops from glial tissue of the brain. This tumor has cells that are significantly unlike from normal cells in appearance. Adults are typically affected with glioblastoma multiforme, which more frequently attacks the brain than the spinal cord.

microRNAs are one-stranded, and non-coding RNA molecules called have 21–23 nucleotides. The miRNAs control post-transcriptional gene expression regulation and RNA silencing. When miRNAs couple with complementary sequences in mRNA molecules, they are able to snuff out the genes of those mRNA molecules by, among other things, cleaving the mRNA strand into two pieces, shortening the poly(A) tail, or converting the mRNA into proteins. The use of ribosomes is necessary for this final method of gene silencing, which is also the least effective.

miRNAs, as well to extracellular circulating miRNAs, are found in a variety of mammalian cell types. Over 60% of human and other mammalian genes seem to be targets of miRNAs. Since many miRNAs have been proven to be evolutionarily conserved, this suggests that they are essential for a variety of biological processes. For instance, 85 families of miRNAs have persisted since at least the common ancestor of fish and mammals, and tests in mice in which the genes for one or more members of a family were knocked out have shown that the majority of these conserved miRNAs perform crucial functions.

MicroRNAs (miRNAs), control the stability, translation of mRNA in multicellular animals in order to control post-transcriptional gene expression. RNA polymerase II synthesizes miRNAs from beginning transcripts that have been polyadenylated and capped (pri-miRNAs), which may or may not encode for proteins.

It is postulated that many multifactorial, external, ambient, and endogenous molecular factors contribute to the development of glioma tumors. Numerous genes appear to be implicated, even though the molecular etiology of glioma tumors is not recognized.

It has been investigated how various microRNAs are expressed in glioma tumors. Numerous cancer types have been researched in relation to miRNA223. This is the first research to be published that looks at the level of miRNA223 expression in Turkish glioma patients. Consequently, the aim of the present thesis study is to determine the potential use of miRNA223 as a biomarker in glioma tumors.

2. GENERAL INFORMATION

2.1. Brain Tumors

Cancer is a global problem for people's health of all ages. Cancer in children could be mostly fatal if not diagnosed earlier and treated in a timely manner (1, 2). Therefore, it is of great importance to ensure adequate resource allocation and the functionality of the health system for increased survival (3).

WHO now emphasizes that childhood cancers are a major public health problem in developing countries (4, 5). Although the classification of brain tumors has been revised in 2021 after the 2016 classification. The WHO 2021 report renounces the century-old histogenetic classification of brain tumors and uses molecular information for the first time to diagnose brain cancers (6). The new classification is seen at Table 2.1.

Primary CNS tumors in childhood include numerous different tumor types, and approximately 50% of these arise below the tentorium (7, 8).

2.1.1. Histology of Brain Tumors

The human nervous system consists of a trillion neurons, each with a large number of interconnections. Some of these neurons have specialized receptors to receive stimuli (eg, mechanical, chemical, thermal) that can reach certain nerve centers. These stimuli are then transmitted to the other 7 neurons for processing and reach higher centers where these stimuli are recorded and/or motor responses are initiated.

Neurons and neuroglia are two categories of cells that make up the nervous system. Neurons perform the receptive, integrative and motor functions of the nervous system. Neuroglial cells support, protect, and assist neurons to function. Most neurons consist of three different units; these are the cell body, multiple dendrites, and an axon (8).

2.1.2. Clinical Symptoms of Brain Tumors

Brain tumors do not have clear pathognomonic features that describe them. This often makes early diagnosis difficult (9-14). The symptoms and signs of brain tumors are varied. These findings vary according to the affected brain part, developmental stage of the child, ability, and whether the intracranial pressure is elevated (15-18). Children often have symptoms that are reflecting increased intracranial pressure. However, these symptoms are those attributable to more common conditions (eg, migraine headache and vomiting due to gastrointestinal disease) or considered psychological in origin (18, 19). In addition to headache and vomiting, symptoms and signs listed at the time of diagnosis are macrocephaly, coordination problems, lethargy, abnormal weight loss, clinically evident growth of the head (swollen fontanel), seizure, papilledema, altered consciousness, strabismus and sight problems (20). Age is an important factor affecting the occurrence of symptoms in children with CNS tumors. The most common initial symptoms in children older than two years of age are headache, nausea/vomiting, seizures, diplopia, ataxia, and behavioral changes. The most common initial symptoms in children younger than two years of age are seizures, vomiting, and behavioral changes (21).

2.1.3. Diagnosis of Brain Tumors

Primary brain tumors' clinical symptoms can be both broad and focused (22). Increased intracranial pressure is the cause of generalized symptoms such as headaches and seizures. Damage to the tissue or compression of certain places causes focal symptoms like unilateral weakness or personality changes. Typically focused in low-grade tumors or in the early stages of the disease, widespread symptoms develop as the tumor enlarges and spreads. The traditional perception of a tumor-related headache is that it is intense, becomes worse in the morning, and comes on with nausea and vomiting. Patients with brain tumors, however, report more bifrontal headaches of this nature. Patients who experience extended nausea, protracted vomiting, seizures, changes in their headache pattern, neurological symptoms, or deterioration of their posture should all be checked for a brain tumor.

People with brain tumors frequently have cognitive impairment (such as language or concentration problems). In addition to the history and physical examination, when a tumor is detected, a fundoscopy and thorough neurological examination should be done. In addition to evaluating the patient's mental state, this examination should also check their cranial nerves, motor, sensory, and cerebellar functioning (23). After doing the necessary brain imaging, the diagnosis is confirmed using histological techniques. The diagnosis and management of brain tumors can benefit from the use of several imaging techniques. The location and biological activity of the tumor can be identified using the most recent advancements in structural and functional neuroimaging methods.

2.1.4. Glioma Tumors

Two thousand and sixteen WHO classification systems define these tumors as diffuse.

Classification of Glioma Tumors

Gliomas are classified as follows:

- Astrocytomas (astrocytoma, anaplastic astrocytoma, and glioblastoma):

Pediatric gliomas, also called astrocytomas, consist of malignant cells resembling glial cells. Over half of all primary brain and spinal cord cancers are gliomas, the most common of which is the astrocytoma. Astrocytes, star-shaped glial cells that are a component of the brain's supporting tissue, give birth to astrocytomas. Though they can occur anywhere in the brain, the cerebrum is most frequently afflicted. All ages can be affected by astrocytomas, but adults—particularly middle-aged males—are more frequently affected. The majority of childhood brain tumors are astrocytomas, which are more prevalent in young people.

- Ependymomas; anaplastic ependymoma, myxopapillary ependymoma, and subependymoma are all types of ependymomas.

- Glioblastoma multiforme (GBM)

This type of tumor is known as the aggressive type of glial tumors. These

tumors tend to spread rapidly, have a bad prognosis, and develop quickly. GBM is more common in persons aged 50 to 70, and it affects men more than women.

- Medulloblastomas

Medulloblastoma was histologically classified as classical type in the 2007 WHO classification of tumors, as large cell variant, nodular, extensive nodular medulloblastoma. In two thousand and sixteen, the WHO classification was revised and medullo-blastoma was divided into molecular subgroups: the classification system, which included both histology and molecular findings.

The occurrence of primary brain tumors in the USA was 21.4 / 100,000 person, with gliomas accounting for 6.6 per 100,000 people, over half of which are glioblastomas which was determined in a study which was investigated between years 2007 and 2011 (Figure 2.1) (12).

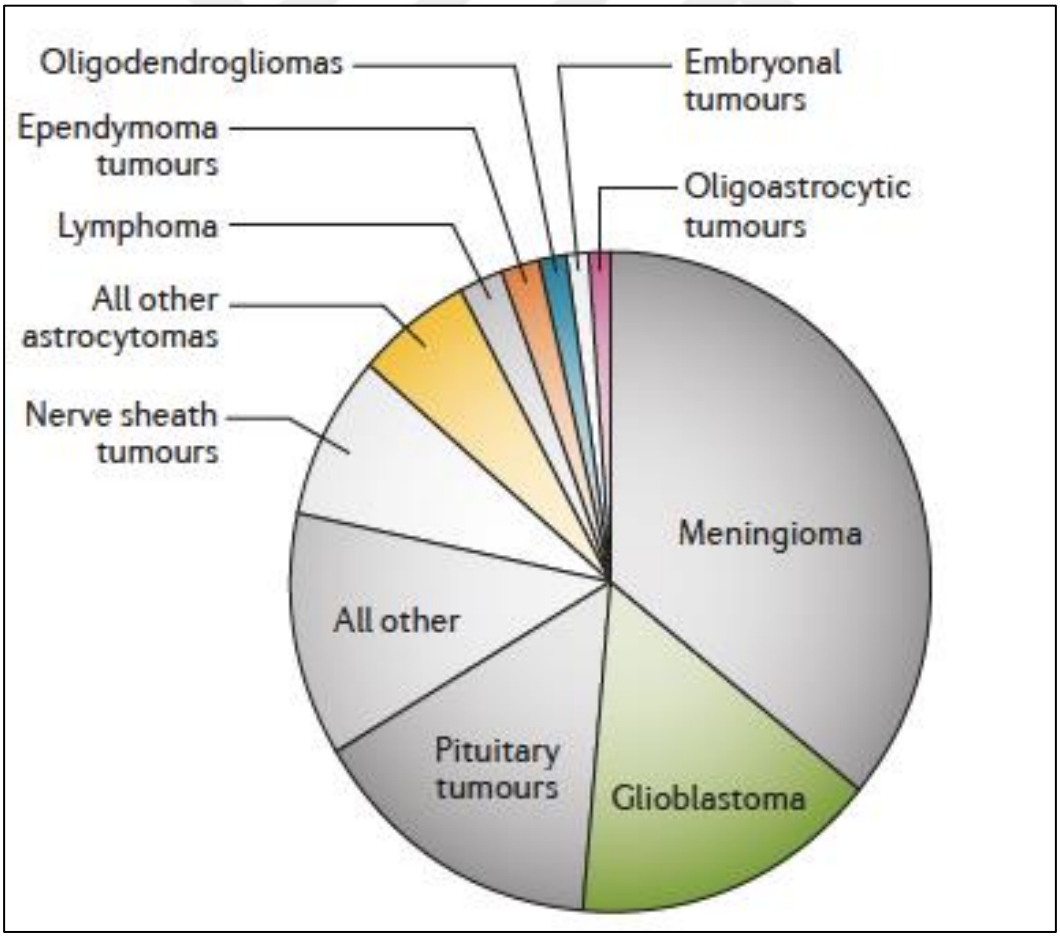

Figure 2.1: The prevalence of brain tumors (12)

Rare Types of Malignant Tumors

Hemangioblastomas are tumors that are most typically found in the brain's cerebellum. These tumors develop from blood, they could be quite enormous, and are frequently accompanied with a cyst. They are more seen in men than women around ages 40 to 60.

Rhabdoid type of tumors are extremely non common which develop withing the nervous system. They frequently occur in many locations throughout the body, particularly in the kidneys.

2.1.5. Mechanism and Pathophysiology of Gliomas

Gliomas, like most other tumors, are fundamentally genetic disorders of single cells. The clinical aspects of brain tumors are shaped by unique patterns of genetic abnormalities, which are increasingly used for categorization and diagnosis. Substantial genetic and epigenetic alterations in various forms of gliomas can be seen at Figure 2.2.

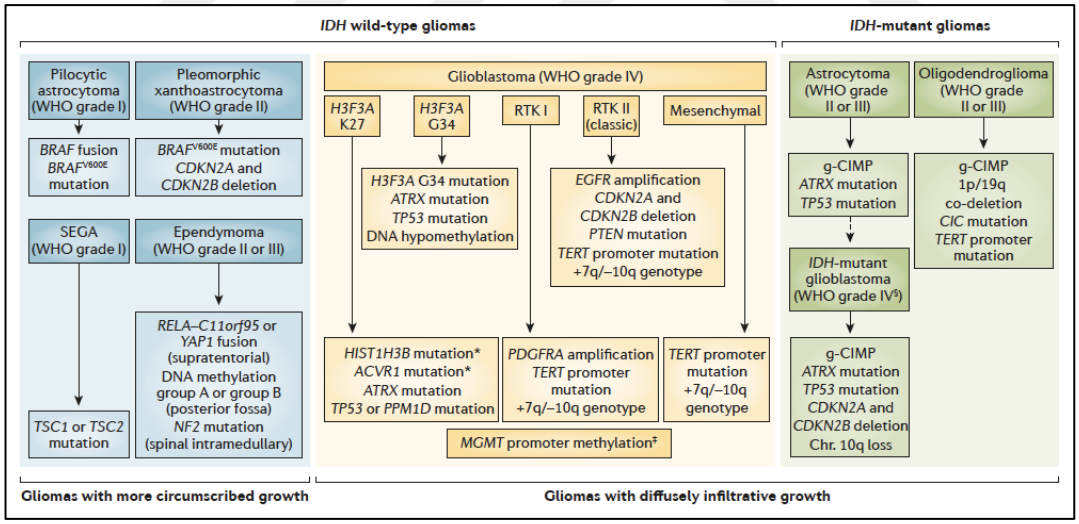

Figure 2.2: Various forms of glioma tumors (12)

2.2. Classification of Brain Tumors

World Health Organization (WHO) has devised a classification based on histological findings under a microscope to determine whether a tumor is malignant or benign (12, 13).

Table 2.1: The 2021 WHO classification of the central nervous system (CNS), tumors (5th edition; WHO CNS 5) (6)

TYPE		CNS WHO GRADE
Adult-type diffuse gliomas	Astrocytoma, IDH-mutant	2/ 3/ 4
	Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted	
	Glioblastoma, IDH-wildtype	1
Pediatric-type diffuse low-grade gliomas	Diffuse astrocytoma, MYB-or MYBL1-altered	1
	Angiocentric glioma	1
	Polymorphous low-grade neuroepithelial tumor of the young	1
	Diffuse low-grade glioma, MAPK pathway-altered	N/ A
Pediatric-type diffuse high-grade gliomas	Diffuse midline glioma, H3 K27-altered	4
	Diffuse hemispheric glioma, H3 G34-mutant	4
	Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype	4
	Infant-type hemispheric glioma	N/ A

2.3. Causes of Brain Tumors

When brain tumors form, certain genes on a cell's chromosomes are considered to break and cease to function correctly. These genes frequently regulate how frequently cells divide, as well as repair genes that fix errors in other genes and genes that should trigger the cell to self-destruct if the damage is too large to be corrected. A person may occasionally be born with some partial deficiencies in one or more of these genes.

Environmental variables may have negative impacts that are worse. In other

situations, environmental genetic damage could be the only contributing component. It is mysterious why some people in the same setting get brain tumors but not others (9, 10).

When a cell divides quickly and its internal systems for controlling development are damaged, the cell may eventually turn into a tumor (11, 12, 13). The immune system of the body could serve as an additional line of defense by ideally identifying and eliminating the abnormal cell. The immune system may not be able to recognize abnormal tumor cells due to substances that tumors produce. Eventually, this will allow the tumor to grow in spite of all internal and external constraints (14-17).

The local blood supply meant for normal tissue may not be able to provide the amount of oxygen and nutrients needed by a fast-growing tumor. Angiogenesis factors, which promote the development of blood vessels, can be produced by tumors. As more vessels develop, the flow of nutrients to the tumor is boosted, and the tumor finally depends on them (18, 19, 20).

2.4. Prognosis of Brain Tumors

Advances in neuroimaging, surgical technology, the application of conformal RT, and conventional chemotherapy have greatly improved outcomes for children with brain tumors. In addition to recent advances in these modalities, the molecular characterization of almost all childhood brain tumors has been determined. These developments formed the basis of stratification of therapy and revealed molecularly targeted therapeutic agents (21, 22, 23).

2.5. microRNAs (miRNAs)

Micro-RNAs are a known as endogenous which are non-coding type of the RNA molecules. They play a part in physiopathological mechanisms such as normal and cancer cell growth, proliferation, death, and differentiation (24, 25).

They are encoded by genes that are transcribed from DNA but not translated into protein. There are thousands of very conserved gene regions that produce miRNAs in the human genome. Today, over 1000 miRNAs are identified in human genome.

The pre miRNAs then interact with the dicer endonuclease in the cytoplasm to become mature miRNAs. The miRNAs are mostly used to determine the diseases like cancer, viral diseases, cardiovascular diseases and metabolic diseases (24, 25). The pool of miRNA biomarkers for cancer is not only derived from the main tumor, but it may also represent an as-yet-unidentified biological process induced by the presence of cancer (26).

2.5.1. Biogenesis of miRNAs

miRNAs are created by transcriptional procedures of RNA polymerase II / III transcripts (13-19).

miRNAs are usually transcribed as a one continuous transcript which is also known as a cluster, which is referred to as a family and may have similar seed region (20, 21, 22, 26).

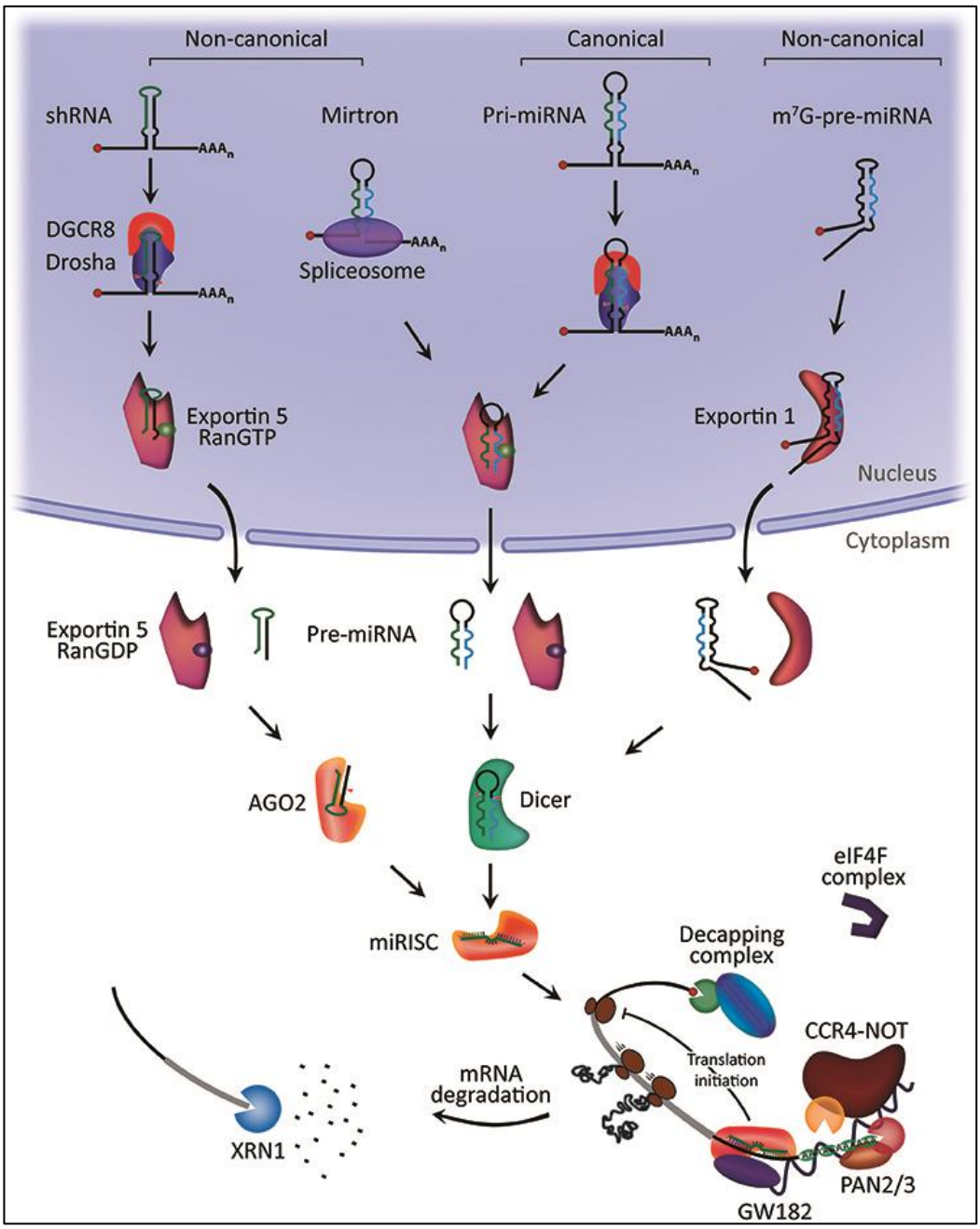

Figure 2.3: MicroRNA biogenesis and mechanism of action (23)

The microprocessor complex splits small hairpin RNA (shRNA), which is then exported non-canonically via Exportin5/RanGTP to the cytoplasm. They next go through cleavage that is dependent on AGO2 but not Dicer. Although their nucleocytoplasmic shuttling is different, mirtrons and 7-methylguanine forming maturation.

Every method eventually results in the development from a working miRISC. MiRISC often attaches to target mRNAs in order to prevent translation,

most likely through interacting. The target mRNA's m7G cap is removed by the decapping complex after PAN2/3 starts deadenylation, which is finished by the CCR4-NOT complex. The exoribonuclease XRN1 can then 5′3′ degrade decapped mRNA (23, 24).

2.5.2. miRNA223

Some miRNAs have been demonstrated to have significant impacts on lipid homeostasis (24- 30). Recently, it has been discovered that miRNAs, in particular miRNAs 33, 122, and 223, are crucial for controlling lipoprotein metabolism (24). MicroRNA 223 (miRNA 223) governs monocyte differentiation and regulates a wide range of inflammatory genes in monocytes and macrophages, according to prior research (31, 32, 33). However, analysis of the bioinformatics data showed that miRNA223 controls a number of genes related to lipid and cholesterol metabolism (34). Humans have a miRNA that is 22 nucleotides long and is situated on the q12 arm of the 223 X chromosome (Figure 2.4).

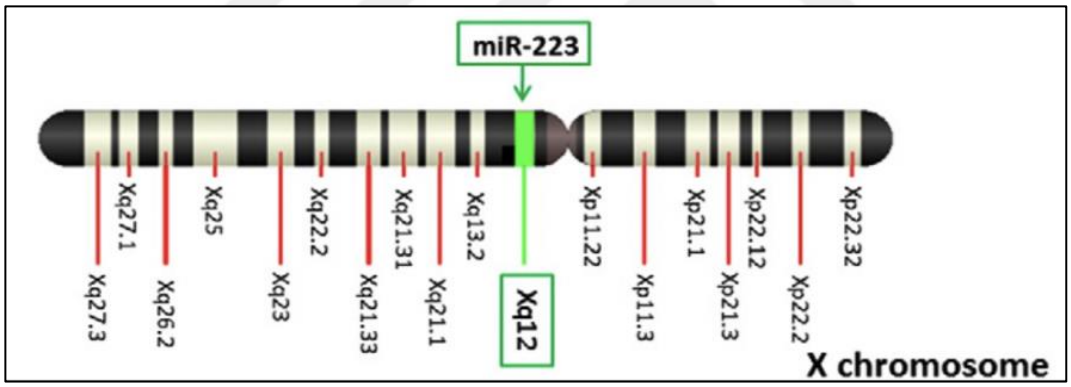

Figure 2.4: Chromosomal location of miRNA223 (33)

Although the first studies reported that miRNA223 is limited to myeloid cells (35, 36), it has been shown by multiple groups that functional miRNA223 expression also occurs in non-myeloid cell types, including hepatocytes (37). Hepatic miRNA223 levels were significantly increased in ischemic/reperfusion injury and decreased in hepatocellular carcinoma (38). With the increasing number of miRNAs involved in the regulation of cholesterol metabolism, studies have shown that miRNA223 is a versatile central regulator for complex cholesterol metabolism (39, 40).

miRNA 223 promoter activity and mature miRNA 223 expression levels correlate with cellular cholesterol status in mouse macrophages and human hepatoma cells. In humans, miRNA 223 has been shown to regulate HDL cholesterol uptake by directly suppressing the SCARB1 gene and inhibit cholesterol synthesis by targeting 3-hydroxy-3-methylthracite-CoA synthase 1 (HMGCS1) and methylsterol monooxygenase 1 (SC-4MOL). However, miRNA 223 indirectly increases cellular cholesterol efflux by increasing ABCA1 (ATP Binding Casette Transporter) gene expression via Sp3 (42).

Genetic loss of miRNA223 has been found to cause an increase in cholesterol levels, and also elevated HDL cholesterol levels. In line with these results, it has been shown that miRNA223 plays an active role in cholesterol biosynthesis, cholesterol uptake, and cholesterol regulation by direct and indirect regulation of multiple genes. Some miRNAs have been found in HDL fractions purified from human plasma. Among these miRNAs, the highest amount is miRNA223. It has been shown that HDL fragments transport miRNA223 to target recipient cells and can mediate a significant reduction in miRNA223 target gene expression via SR-B1 (43).

Additionally, it has been discovered that HDL-derived miRNA223 may be transported to endothelial cells and block intracellular adhesion molecule 1, which lessens monocyte adherence and inflammation (44). These findings could provide an explanation for how HDL cholesterol reduces inflammation (45). MiRNA223 had been recorded to have the greatest concentration of HDL-binding miRNAs (46).

2.5.3. miRNA223 and Cancer

miRNA223 is a hematopoietic system-specific miRNA detected by bioinformatics prediction tools (24). In studies, it has been associated with cancer types such as leukemia, pancreatic, colorectal, breast, stomach, ovarian and diseases such as lupus erythematosus, rheumatoid arthritis, glioblastoma, chronic asthma, type 2 diabetes (25).

miRNA223 downregulation has been linked to a high tumor burden, disease aggressiveness, and poor patient prognosis in some circumstances. As a result,

knowing the multifaceted role of miRNA223 in cancer detection and treatment is critical. The insulin-like growth factor-1 receptor (IGF-1R), monocytic enhancer factor 2C, microtubule destabilizer stathmin 1, and artemin and Forkhead box protein O1A (FOXO1A) are all targets of miRNA223 that have been linked to cancer (36)

Chronic lymphocytic leukemia and primary small cell lung cancer (SCLC) have been associated to miRNA223 downregulation, while ovarian and colorectal cancer have been linked to miRNA223 overexpression. Particularly miRNA223 is being researched as a possible biomarker in recurrent ovarian cancer (39, 41, 42).

Correspondingly, miRNA223 promotes proliferation, cell cycle transition and invasion of glioblastoma cells possibly by downregulation of PAX6 expression. (39, 41, 42).

In addition, miRNA223 has been found to play a role in cell cycle, tumor spread, hematopoietic differentiation, and immune functions of cells (9). miRNA223 has been found to have the highest expression among the miRNAs contributing to the pathogenesis of T-Cell Acute Lymphoblastic Leukemia (T-ALL) and act as an Oncomir.

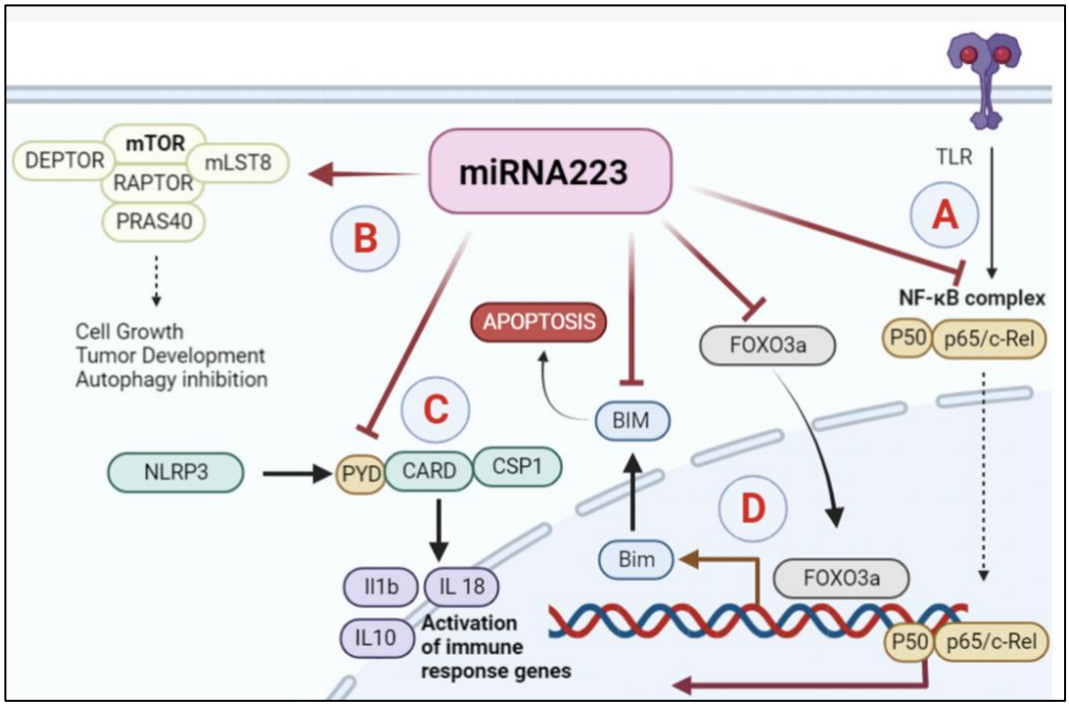

Figure 2.5: The role of miRNA223 in various cancers (33)

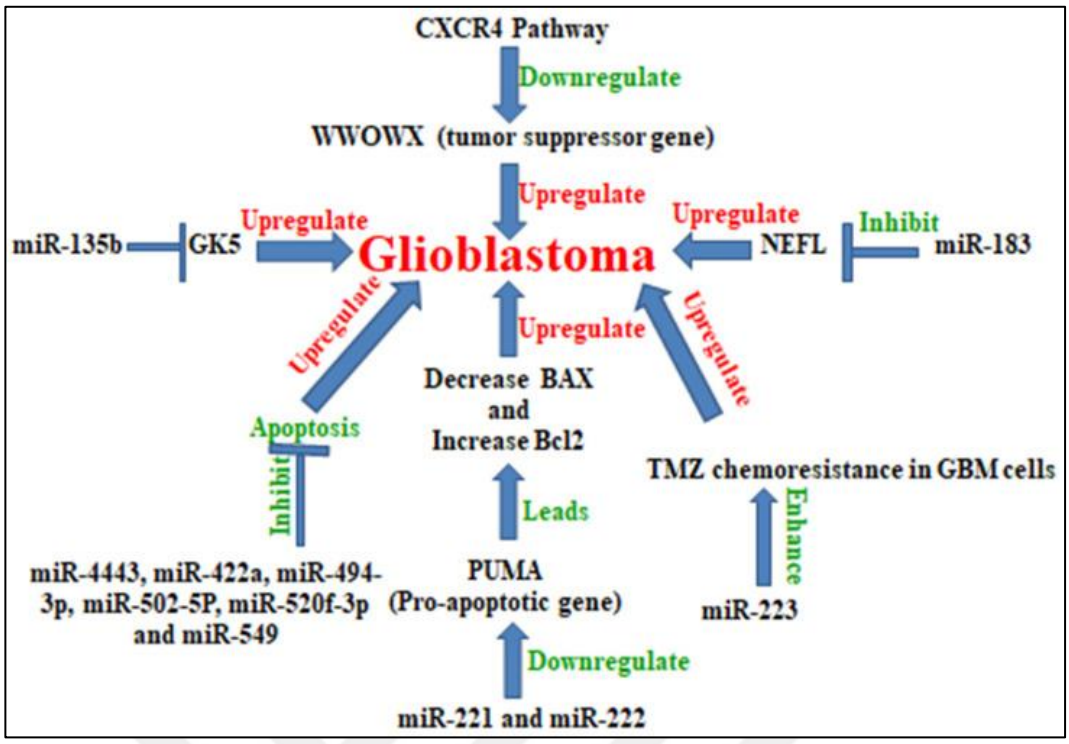

Figure 2.6: MicroRNAs up-regulate glioblastoma (34)

MicroRNAs up-regulate glioblastomas various miRNAs such as miRNA183, miRNA135b, miRNA221, miRNA222, miRNA4443, miRNA422a, miRNA494-3P, miRNA502-5P, miRNA520f-3p, miRNA549, and miRNA223 are responsible for the up-regulation of glioblastoma growth (Figure 2.6).

2.5.3.1. Gastric Cancer and miRNA223

This type of cancer is characterized by an interplay of genetics, and environment (34, 35, 36, 37). Infection with *Helicobacter pylori* is the most known chronic bacterial cause. It is a leading cause of gastritis, ulcers, and stomach cancer (32, 38, 39, 40, 41).

2.5.3.2. Hepatocellular Cancer and miRNA223

Hepatocellular carcinoma (HCC) is expected to become more common, both in terms of incidence and fatalities (42). This type of cancer has the third death rate of all cancers in the world. miRNA223 levels in HCC patients are substantially greater than in healthy people. Therefore, miRNA223 has been suggested as a

potential HCC diagnostic biomarker. However, it is thought that tissue damage, not carcinogenesis, is what leads to miRNA223 overexpression in hepatocellular diseases. Compared to HCC and healthy persons, hepatitis B patients with HCC-negative results had greater blood levels of miR-223. Chronic hepatitis caused more hepatocyte damage than HCC did, and hepatitis (tissue destruction) is more likely to lead to higher blood levels of miRNA-223 than HCC (43).

2.5.3.3. Breast Cancer and miRNA223

Both developed and developing countries rank breast cancer as one of the main causes of mortality (44, 45). Its prevalence is rising, most likely as a result of public health initiatives and early identification, among other factors (46, 47). Breast cancer cells (MDA-MB-231) express abnormally high quantities of Caprin-1 and extremely low levels of miRNA223.

Breast cancer cell invasion and proliferation are accelerated by Caprin-1 overexpression. Conversely, in MDA-MB-231 cell lines, miRNA223 expression inhibits breast cancer cell growth and invasion (48). Caprin-1 mRNA's expression is inhibited when miRNA223 attaches to its 3′-UTR. When miRNA223 biogenesis is decreased, it is expected that Caprin-1 regulation would also decrease (48).

2.5.3.4. Lung Cancer and miRNA223

Lung cancer is a serious condition, especially given its high incidence and fatality rates (49). As a result, new treatment biomarkers are required to enhance illness outcomes (50).

2.5.3.5. Ovarian Cancer and miRNA223

In ovarian cancer, miR-223-3p expression is increased. In this scenario, miRNA223 reduces the expression of the sex-determining region Y-box 11 (SOX11). Cell proliferation, migration, and invasion are all negatively regulated when the sox11 gene is inhibited. As a result, miR-223-3p can potentially govern ovarian cancer by regulating SOX11 expression (51-55).

2.5.3.6. Osteocarcoma and miRNA223

Osteosarcoma is a bone tumor cancer that affects teenagers and young adults (56). In osteosarcoma, miRNA223 is downregulated (57, 58, 59).

2.5.4. miRNA223 as a Circulating Biomarker

The possibility that miRNAs might be used as beneficial cancer indicators (43, 52, 59). Although tissue miRNA expression patterns have the potential to be exploited as cancer diagnostic biomarker (60-69). The fact that circulating miRNAs may be non-invasively acquired by blood collection is their main advantage. Detection of circulating miRNA may also be more accurate and discerning than existing blood markers like CA19-9 and CEA (70- 73).

There is a lot of potential for circulating miRNAs as diagnostic biomarker. It was determined that plasma samples from patients with stomach cancer had significantly higher levels of miRNA223. Because of this, microRNA-223 could be employed as a biomarker for stomach cancer. Significant miRNA223 expression is found in a number of different cancer types. Circulating miRNA223 may one day serve as a new, non-invasive biomarker for the early detection of cancer (70, 72). Table 2.2 shows how MiR223 regulates carcinogenesis by binding certain targets at (33).

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33)

Clinical Relevance	***Molecular Mechanism Involved or Function***	***Experimental Model***	***Specific Targets***	***References***
Breast cancer	miR-233-3p mimics inhibited the NLRP3-dependent processes in cancer cells by suppressing the NLRP3 and downstream factors, including PYD and CARD domain-containing protein, IL-1, and IL-18.	Breast cell lines: HMEC, MDA-MB231, MCF-7, and SKBR3 Xenograft mouse model	NLRP3, PYD, CARD, IL1, IL18	PMID: 30747211

Table 2.2 continued next page

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33) *(Continued)*

Clinical Relevance	***Molecular Mechanism Involved or Function***	***Experimental Model***	***Specific Targets***	***References***
Hepatocellular carcinoma	Checkpoint kinase 1, DNA methyltransferase 1, baculoviral IAP repeat-containing 5, kinesin family member 23, and collagen, type I, 1 target genes were considered the hub genes of miR-223-3p in HCC, which are significantly upregulated in HCC. miRNA223 functions as a tumor suppressor and plays a critical role in inhibiting the tumorigenesis and promoting the apoptosis of HCC through the mTOR signaling pathway by targeting Rab1.	Database: GEO database, TCGA database, Meta-analysis., Bioinformatics evaluation, Protein–protein interaction (PPI) network construction Hepatocellular cell lines: Huh7, Hep3B, Bel-7402, and the HepG2 cell lines	Checkpoint kinase 1, DNA methyltransferase 1, baculoviral IAP repeat-containing 5, kinesin family member 23, and collagen, type I, 1. Rab1, mTOR	PMID: 29207133 PMID: 27998765

Table 2.2 continued next page

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33) *(Continued)*

Clinical Relevance	*Molecular Mechanism Involved or Function*	*Experimental Model*	*Specific Targets*	*References*
Prostate Cancer	miRNA223 inhibited the malignant behavior of prostate cancer cells while EYA3/c-Myc had the opposite effect. Moreover, circGNG4 enhanced the expression of EYA3/c-Myc by sponging miRNA223 to promote the growth of prostate cancer tumors in vivo.	Prostate clinical samples: Prostate Cancer and adjacent normal tissues. Prostate cell lines: PC-3, LNCaP, VCaP, and DUL145, and the human normal prostatic epithelial cell line, RWPE-1 Xenograft mouse model	circGNG4, EYA transcriptional coactivator, phosphatase 3 (EYA3)/c-Myc	PMID: 34395419
Esophageal squamous cell carcinoma (ESCC)	High expression level of miR-223 had a significant adverse impact on the survival of ESCC patients through repression of the function of FBXW7.	ESCC clinical samples: ESCC Primary ESCC tissue samples and human ESCC cell lines: TE1, TE4, TE6, TE8, TE9, TE10, TE14, and TE15.	FBXW7	PMID: 22108521

Table 2.2 continued next page

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33) *(Continued)*

Clinical Relevance	*Molecular Mechanism Involved or Function*	*Experimental Model*	*Specific Targets*	*References*
Gastric cancer	miRNA223 induced by the transcription factor Twist, post-transcriptionally downregulates EPB41L3 expression by directly targeting its 30-untranslated regions.	Gastric clinical samples: Gastric cancer and normal gastric tissues, Gastric cell lines: HEK293 cell, GPG29 cell, immortalized normal gastric mucosa GES cell, normal stomach fibroblastic cell NSFC, non-metastatic gastric cancer SUN-1, KATO-III, NUGC-3 cells, and metastatic gastric cancer XGC-9811L, AGS, and N87.	EPB41L3	PMID: 21628394
Lung cancer	miRNA223 targets IGF-1R, a transmembrane receptor tyrosine kinase related to lung cancer oncogenesis, tumor growth, cancer cell survival. miRNA223 overexpression leads to tumor suppression		IGF-1R	PMID: 10857553

Table 2.2 continued next page

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33) *(Continued)*

Clinical Relevance	*Molecular Mechanism Involved or Function*	*Experimental Model*	*Specific Targets*	*References*
Liver cancer	miR-223-3p down-regulated the expression of FAT1, and inhibited the proliferation, migration, invasion, and EMT of liver cancer cells by targeting FAT1. FAT1 was highly expressed in liver cancer tissues and cells while miR-223-3p was lowly expressed.	Liver clinical samples: Liver Tumor tissues and corresponding adjacent tissues. Liver cell lines: Normal human hepatocyte, L-02 and human liver cancer cell lines, Hep G2, SMMC-7721, Hep 3b, and Li-7,	FAT1	PMID: 32233593
Brain cancer	miR-223-3p could be decreased by NLRP3 overexpression , which was considered as one of target genes of miR-223-3p. miR-223-3p might act as a suppressor and a potential therapy target of glioblastoma.		NLRP3	PMID: 30033329

Table 2.2 continued next page

Table 2.2: Roles of miRNA223 in the regulation of carcinogenesis by binding certain targets (33) *(Continued)*

Clinical Relevance	***Molecular Mechanism Involved or Function***	***Experimental Model***	***Specific Targets***	***References***
Colon cancer	miRNA223 regulate the FoxO3a/BIM signaling pathway and colorectal cancer cell proliferation and apoptosis. Downregulating the expression of miRNA223 increased the expression of FoxO3a and BIM and weakened cell proliferation and induced apoptosis.	Colon cell lines: NCM460 and SW620 cancer cells	FoxO3	PMID: 29949152

Cancer therapy is challenging due to a variety of reasons (73-77). Some microRNAs have tumor suppressing properties, making them promising targets for cancer treatment (78). Despite the significant advancements in miRNA-based treatment, a number of challenges still need to be overcome before it can be used in clinical settings (79). Nevertheless, several studies found that miRNA223 expression may be a useful diagnostic and treatment target (43, 79, 80, 81).

2.5.4.1. miRNA223 as a Diagnostic Biomarker for Hepatocellular Carcinoma

The likelihood of recurrence in hepatocellular carcinoma (HCC) is high. Additional research for the treatment of HCC is required because to this recurrence (82, 83). Both chronic hepatitis B and HCC patients have been shown to

overexpress miRNA223. The use of miR-223 is constrained by the common miRNA223 profile. High levels of miRNA223 are also seen in chronic hepatitis B, although this overexpression is caused by inflammation and liver damage rather than hepatocellular carcinoma (43, 84, 85).

miRNA223 downregulation was determined as reducing the HCC cell proliferation, implying that miRNA223 which plays a crucial role in HCC progression and metastasis (85). Furthermore, circulating miR-223-3p was found to be a promising predictive biomarker. (86)

2.5.4.2. miRNA223 as a Diagnostic Biomarker for Breast Cancer

The impacts of macrophages and miRNA on breast cancer cells were highlighted by Escobar et al. Overexpression of miRNA223 in combination which resulted in an aggressive cancer phenotype (81). It was also determined that elevated miRNA223 expression works as a tumor suppressor and is associated with longer life in triple-negative breast cancer patients. As a result, miRNA223 may represent a novel predictive biomarker in breast cancer (87, 88). miRNA223 has been found to be a possible diagnostic biomarker for breast cancer (81, 87).

2.5.4.3. miRNA223 as a Diagnostic Biomarker for Esophageal Cancer

The type of tumors as esophageal cancer is the second largest reason of death rate for cancer in the world. When esophageal carcinomas are in their early stages, most doctors favor surgical resection (89). Because therapy options are limited, more study is essential to specify the molecular pathways implicated (80, 89, 90).

Cell migration, invasion, and metastasis are also aided by ARTN (90, 91, 92). Artemin expression after treatment is substantially associated with relapse and death in cancer patients (93, 94).

miRNA223 directly binds the ARTN, which is a growth factor, 3′-UTR and regulates this protein production, according to Xu et al. (93). Overexpression of miRNA223 inhibits ARTN activity, reducing esophageal cancer cell movement and invasion. ARTN overexpression via miRNA223 silencing, on the other hand,

enhances cell migration and invasion (93, 94, 95). These findings support the use of microRNA-223 as therapeutic targets for esophageal cancer treatment.

2.5.4.4. miRNA223 as a Diagnostic Biomarker for Lung Cancer

Early detection of lung cancer, like all malignancies, improves patient outcomes. Discovering new early diagnostic biomarkers associated with carcinogenesis is critical for future breakthroughs in cancer detection and therapy.

miRNA223 specifically aims IGF-1R, which is a receptor tyrosine kinase implicated in lung cancer, as well as cancer cell survival. The initial dysregulation that results in the change of initially healthy cells into tumors and malignancy is thought to be caused by IGF-1R overexpression. Tumor suppression in lung cancer has been connected to miRNA223 overexpression (95, 96, 97).

miRNA223 may serve as a blood biomarker for non-small cell lung cancer in its early stages (97, 98). Overall, miRNA223 inhibits cell growth and proliferation by targeting IGF-1R and its downstream signaling pathway (97, 99-103). Overexpression of miRNA223 could be a promising technique for lung cancer treatment (103-106).

3. MATERIALS AND METHODS

3.1. Materials

The serum samples of a patient group consisted of 35 people having a diagnosis of Glioblastoma multiforme and a control group consisted of 30 healthy people were obtained priorly from Yeditepe University Hospital for another study. The samples were already stored at -80°C at the Molecular Medicine Laboratory of Yeditepe University freezer.

3.2. Selection of the Samples

The inclusion criteria of patient group samples;

- Patients whose age are between 3-71 years
- Patients with Glioblastoma multiforme diagnosis

The inclusion criteria of control group samples;

- Patients with Glioblastoma multiforme diagnosis
- Patients with no any systemic diseases

In this study, the samples used are priorly obtained from patients who have visited the neurology polyclinic with some clinical complaints of headache, vomiting, and fainting.

3.3. Collection of the Samples

The blood samples used in this thesis study are previously taken samples and present at the Molecular Medicine Laboratory of Yeditepe University. Blood samples are obtained from the laboratory freezer which were priorly obtained. Symptoms are examined using physical, neurological, optical, and radiological tools.

3.4. Ethical Approval

Yeditepe University Non-Interventional Clinical Research Ethics Committee approval is obtained prior to the study. The ethical approval is present at Appendix 7.1.

3.5. Chemicals and Consumables

Chemicals used in this study are PCR grade water, Trizol (Quiazol, Qiagen), Chloroform (Sigma, Aldrich), 100% ethanol (Sigma Aldrich), miReasy Kiti ile (Cat. No. / ID: 217184 Qiagen) miRCURY LNA RT Kit. Consumables used in this study are NanoDrop2000 (Thermoscientific, Waltham, Massachusetts, USA) (Invitrogen, Thermo-Fisher Scientific Incorporated). Conventional PCR - Rotor Gene (Rotor-Gene Q - Qiagen).

3.6. Preparation of the Samples

3.6.1. Serum Pool

The serum pools are prepared according to the criteria defined by Feliciano et al (108). In accordance with the location of glioma tumors, patient and control samples were categorized by pools. Randomly selected patients were divided into three pool groups, by the location of the tumors, according to tumor incidence occurrence. First pool group has 9 patients, tumors located at the temporomandibular. Second pool group has 13 patients, tumors located at the frontal, thalamus, and parietal. Third pool group has 13 patients, tumors located at the cingulate, occipital, brainstem, pons and corpus. The control group is consisted of 30 healthy people. Each pool was prepared from the samples belonging to its own group, with a final volume of 600 microliters. Pool mixtures has been exercised at least 5 times in this study.

Table 3.1: Patient groups and control group pool classification

NUMBER	PATIENT GROUP	DIAGNOSIS
9	POOL 1	Temporomandibular
13	POOL 2	4 Frontal, 4 Thalamus, 5 Parietal
13	POOL 3	5 Cingulate, 3 Occipital, 2 Brainstem, 1 Cerebellum, 1 Pons, 1 Corpus
30	POOL	CONTROL

3.6.1.1. miRNA Isolation

Qiagen's miRNeasy Serum/Plasma Kit (Kit no: 217184) is used to isolate the miRNA. The composition consisted of lysis, homogenization, 70% Ethanol addition, washing and elution steps.

RWT Buffer is established by immediately adding 44 microliters (µl) of ethanol before use. RPE Buffer is used to remove salts and deposits from colons. The RPE Buffer is performed with 30 liters of ethanol.

600 µl of serum samples are inserted in sterile Eppendorf tubes, and 3000 µl of lysis solution (Qiazol Lysis Solution) is added and combined. Samples are then incubated at room temperature for five minutes before being mixed with). 3,5 µl 39 spike. Each sample receives 600 µl of chloroform, vortexed and incubated for 3 minutes.

For 15 minutes, the mixture is centrifuged at four degrees at 12.000 g. The mixture has separated into two distinct phases after centrifugation. The mixture's top phase is clean, and 1200 µl of this phase is transferred to clean Eppendorf tubes. 900 microliters of 100% ethanol is added to each 600 microliter of the collected clear fraction and vortexed. 700 microliters of this solution are transferred to the columns. It is centrifuged at 8000g for 30 seconds. After centrifugation, the liquid part in the tube under the column is poured. The same process is repeated until the remaining liquid is used up. 700µl of RWT solution is added to the columns and centrifuged at 8000g for 30 seconds. After centrifugation, the liquid part in the tube under the column is poured. 500µl of RPE solution is added to the columns and centrifuged at 8000g for 2 minutes. After centrifugation, the liquid part in the tube under the column is poured. 500µl of 80% ethyl alcohol is added to the columns and centrifuged at 8000g for 2 minutes. After centrifugation, the liquid part in the tube under the column is poured, and the tubes at the bottom are discarded. The columns are inserted in new 2 ml collection tubes and centrifuged for 5 minutes at maximum speed. The bottom tube is discarded. Columns are placed in 1.5 ml collection tubes. After that, 14 µl of RNA-free water is added. The microRNA isolate is obtained after 1 minute of highest rate centrifugation.

Micro RNA was isolated from serum pools in accordance with the kit

procedure (miRCURY LNA RT Kit Cat. No./ID: 339340) and converted to cDNA without waiting.

3.6.1.2. cDNA Synthesis

RNase-free water, Rection Buffer, Reverse transcriptase enzyme are included in the complementary DNA (cDNA) reverse transcription kit (miRCURY LNA RT Kit, Cat No/ID:339340-Qiagen). cDNA translation of miRNA samples was performed following the kit instructions. For this, the following volume of mix solution was prepared.

Table 3.2: Reagens and amounts in the mixture

REAGENTS	AMOUNT
RNase-free water	5µl
Rection Buffer	2 µl
Reverse transcriptase enzyme	1 µl
Template RNA	2 µl
Total	**10 µl**

Table 3.3: PCR procedure conditions

STEP	TIME	TEMPERATURE
Reverse transcription step	60 min	42°C
Inactivation of reaction	5 min	95°C
Storage	∞	4°C

3.6.1.3. miRNA Levels Calculation Employing Fluorometer

Utilizing miRNA buffer and miRNA-specific reagent, the experimental solution is created. Using standards 1 and 2, which contain 10 ng/ µl and 250 pg/µl

rRNA, respectively, the fluorometer is calibrated. The 500 µl Qubit tubes are filled with the working solution and RNA before the measurement (Qubit 3.0 microRNA Assay Kit, Invitrogen, Thermo Fisher Scientific Inc.). The samples were diluted by RNase free water (1/10ng/µl nanogram/microliter).

3.6.1.4. miRNA223 Expression Analysis

PCR analysis was performed using rotor gene device for miRNA223 expression analysis of cDNA samples obtained from pool 1, pool 2, pool 3 patient serum and control pool serum as internal control (housekeeping assay, RNU6). Mixes were prepared as defined at instructions of the miRCURY LNA RNA miRCURY LNA RT Kit (Cat. No./ID: 339340 - Qiagen) for PCR which is indicated at Table 3.4.

Table 3.4: Components and amounts added in the mixture

COMPONENT	**AMOUNT**
2x miRCURY SYBR Green Master Mix	5µl
RNase-free water	2µl
cDNA Template	2µl
miRNA 223	1µl
Total volume	**10µl**

Table 3.5: Ingredients of the mixture for miRNA expression analysis for RNU6

COMPONENT	AMOUNT
2x miRCURY SYBR Green Master Mix	5µl
RNase-free water	2µl
cDNA Template	2µl
(Housekeeping GENE) RNU6	1µl
Total volume	**10µl**

3.7. miRNA223 Calculation

ΔCT and ΔΔCT values were calculated to determine the miRNA223 expression level in the patient and control groups.

Comparing microRNA expression levels

Comparing microRNA expression levels delta Ct (ΔΔCT) and fold change 3 internal controls (housekeeping assay = mirRU6) were used to show the differences. miRNA level determination calculations Livak formula (2 delta CT = 2ΔΔCT). When calculating (ΔCT target-ΔCT internal control) - (ΔCT all control-ΔCT internal control) value was determined and delta delta CT was calculated in logarithm base 2 (2^(-ΔΔCT)) (107).

The resulting value

Taking the logarithm to the base LN gives the fold change value.

Livak formula

DNA, mRNA or miRNA expression of samples in structural tissue and cell cells used to identify constraints (107).

microRNA Expression Analyzes with Simultaneous PCR

microRNAs planned to be investigated within the framework of the study are “mirbase” and “targetscan” databases (http://www.mirbase.org/, http://www.targetscan.org/) was determined using on the molecular mechanism of glioma, different microRNAs and their target proteins with effects were analyzed with these databases.

3.8. Statistical Analysis

The receiver operating characteristic (ROC) analysis was performed using the MedCalc statistical program Version 20.215 to reveal the diagnostic value of microRNA223. One-way Anova test was performed for the results. The p values which are less than 0.05 was considered as statistically significant.

4. RESULTS

4.1. Demographic Features of the Groups

The demographic features of the groups are indicated at Table 4.1. The table shows the pool distributions by age and gender.

Table 4.1: Pool distributions by age range and gender

GROUPS	n (NUMBER)	AGE RANGE	GENDER	
			MALE	FEMALE
POOL 1	9	36 - 70	4	5
POOL 2	13	28 - 60	6	7
POOL 3	13	3 - 71	5	8
POOL CONTROL	30	8 - 70	16	14

4.2. miRNA Results

miRNA 223 expression levels (ΔΔCT values) of pool 1 which included the patients who have tumor at the temporomandibular, pool 2 which has patients who has tumor at the frontal, thalamus, and parietal, and pool 3 that has patients who has tumor at the cingulate, occipital, brainstem, cerebellum, pons, corpus parts of the brain, were higher than the control pool values that contained with health patients. Pool 1 that is consisted of tumor group at the Temporomandibular part of the brain, has the highest miRNA 223 expression levels (ΔΔCT values).

Table 4.2: ΔΔCT values of patient groups and control group pool classification

NUMBER	PATIENT GROUP	DIAGNOSIS	ΔΔCT values
9	POOL 1	Temporomandibular	5,12
13	POOL 2	4 Frontal, 4 Thalamus, 5 Parietal	2,88
13	POOL 3	5 Cingulate, 3 Occipital, 2 Brainstem, 1 Cerebellum, 1 Pons, 1 Corpus	2,68
30	POOL	Control	0,23

Table 4.3: Pool distributions by age range, complaint, P53n, OLIG2, IDH, and average survival time (month)

GROUPS	AGE RANGE	COMPLAINT	P53n	OLIG2	IDH	AVERAGE SURVIVAL TIME (MONTH)
POOL1	36-70	5 Seizure 4 Headache	60.00	12 Positive 1 Negative	12 Positive 1 Negative	22
POOL 2	28-60	5 Seizure 5 Headache 3 Other	50.00	Positive	Positive	26
POOL 3	3-71	5 Seizure 4 Headache 4 Other	50.00	Positive	Positive	24

Delta ΔCT (ΔΔCT) has made progress in understanding miRNA expression levels, hence miRNA223 was selected as the thesis's target miRNA. ΔCT and delta (Δ) CT techniques have been used to understand miRNA expression levels. The ΔΔCT equations were used to calculate the internal control (housekeeping assay, RNU6) and to determine the miRNA expression level.

ROC curve analysis for the analysis of the effect of miRNA223 on prognosis in glial tumors has been performed. When ΔΔCT values are used, the specificity of miRNA223 is 100.0, sensitivity 75% (95% CI=0.593–0.983, AUC=0.875, p=0.0007).

There is a significant difference between patient pool ΔΔCT values and control group ΔΔCT values. There are no significant differences between pool 1 ΔΔCT values and control group pool ΔΔCT values, pool 2 ΔΔCT values and control group pool ΔΔCT values, pool 3 ΔΔCT values and control group ΔΔCT values.

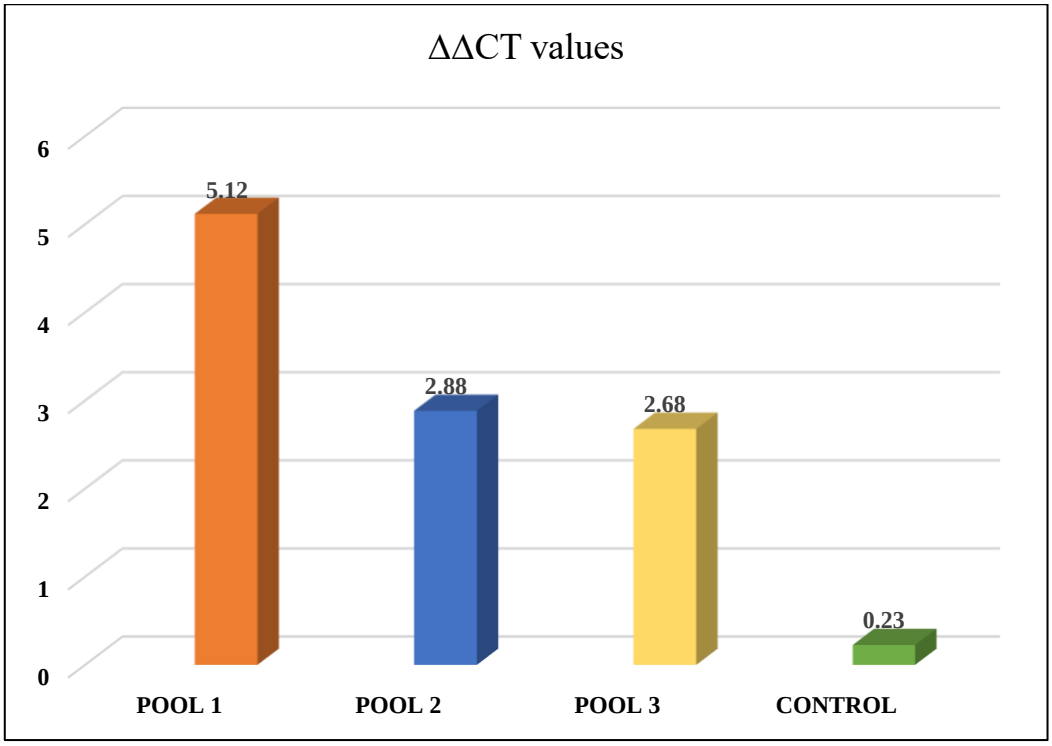

Figure 4.1: ΔΔCT values of pool group classification

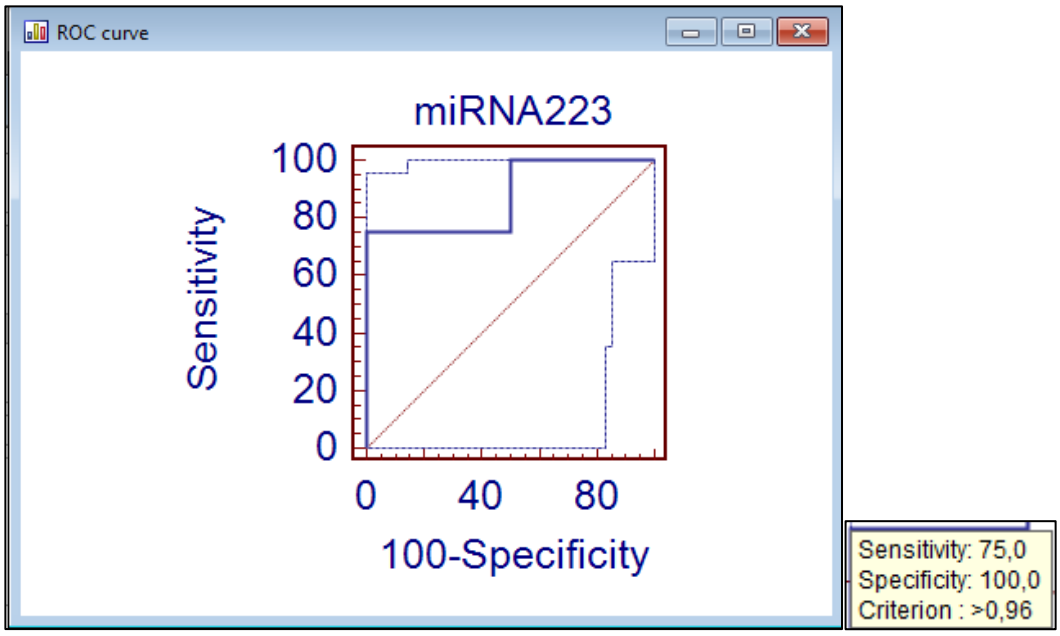

Figure 4.2: POOL1, POOL2, POOL3, and control pool ROC curve

Table 4.4: POOL1, POOL2, POOL3, and control pool ROC curve diagnosis results

ROC curve

Variable	miRNA223
Classification variable	diagnosis

Positive group	
diagnosis	= 1
Sample size	12
Negative group	
diagnosis	= 0
Sample size	2

Disease prevalence (%)	85,7

Area under the ROC curve (AUC)	0,875
Standard Error	0,111
95% Confidence Interval	0,593 to 0,983
z statistic	3,388
Significance level P (Area=0.5)	0,0007

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI	+PV	95% CI	-PV	95% CI
>=-11,16	100,00	73,4 - 100,0	0,00	0,0 - 80,7	1,00				85,7	57,2 - 97,8		
>-11,16	100,00	73,4 - 100,0	50,00	8,2 - 91,8	2,00	0,5 - 8,0	0,00		92,3	63,9 - 98,7	100,0	16,5 - 100,0
>-0,45	91,67	61,5 - 98,6	50,00	8,2 - 91,8	1,83	0,5 - 7,4	0,17	0,02 - 1,7	91,7	61,5 - 98,6	50,0	8,2 - 91,8
>-0,09	83,33	51,6 - 97,4	50,00	8,2 - 91,8	1,67	0,4 - 6,8	0,33	0,05 - 2,2	90,9	58,7 - 98,5	33,3	5,5 - 88,4
>0,76	75,00	42,8 - 94,2	50,00	8,2 - 91,8	1,50	0,4 - 6,2	0,50	0,09 - 2,7	90,0	55,5 - 98,3	25,0	4,1 - 79,7
>0,96 *	75,00	42,8 - 94,2	100,00	19,3 - 100,0			0,25		100,0	66,2 - 100,0	40,0	6,5 - 84,6
>1,59	66,67	34,9 - 89,9	100,00	19,3 - 100,0			0,33		100,0	62,9 - 100,0	33,3	5,3 - 77,3
>3,36	58,33	27,8 - 84,7	100,00	19,3 - 100,0			0,42		100,0	58,9 - 100,0	28,6	4,5 - 70,7
>4,04	50,00	21,2 - 78,8	100,00	19,3 - 100,0			0,50		100,0	54,1 - 100,0	25,0	3,9 - 65,0
>4,12	41,67	15,3 - 72,2	100,00	19,3 - 100,0			0,58		100,0	48,0 - 100,0	22,2	3,5 - 59,9
>4,16	33,33	10,1 - 65,1	100,00	19,3 - 100,0			0,67		100,0	40,2 - 100,0	20,0	3,1 - 55,6
>4,51	25,00	5,8 - 57,2	100,00	19,3 - 100,0			0,75		100,0	30,5 - 100,0	18,2	2,8 - 51,8
>4,89	8,33	1,4 - 38,5	100,00	19,3 - 100,0			0,92		100,0	16,5 - 100,0	15,4	2,4 - 45,5
>6,08	0,00	0,0 - 26,6	100,00	19,3 - 100,0			1,00				14,3	2,2 - 42,8

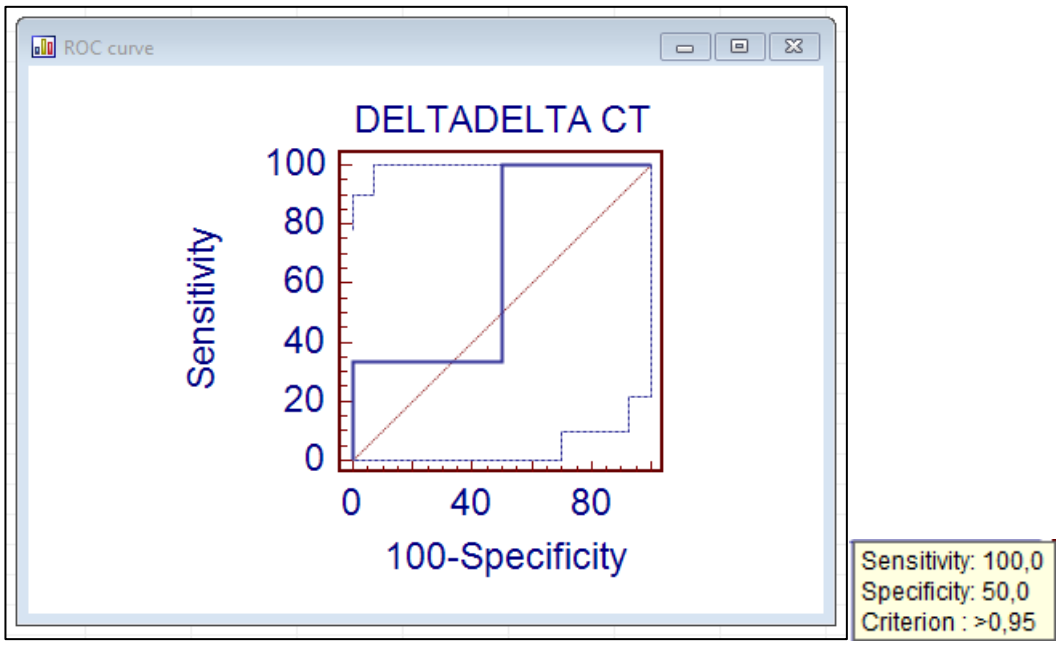

Figure 4.3: POOL1 / Control ROC curve analysis curve

Table 4.5: POOL1 / Control ROC curve diagnosis results

ROC curve

Variable	DELTADELTA_CT DELTADELTA CT
Classification variable	DATA

Positive group	
DATA	= 1
Sample size	3
Negative group	
DATA	= 0
Sample size	4

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)	0,667
Standard Error	0,224
95% Confidence Interval	0,255 to 0,938
z statistic	0,745
Significance level P (Area=0.5)	0,4561

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI
>=-11,16	100,00	30,5 - 100,0	0,00	0,0 - 59,8	1,00			
>0,95 *	100,00	30,5 - 100,0	50,00	8,3 - 91,7	2,00	0,8 - 5,3	0,00	
>4,15	33,33	5,5 - 88,4	50,00	8,3 - 91,7	0,67	0,1 - 4,4	1,33	0,4 - 4,7
>5,7	33,33	5,5 - 88,4	100,00	40,2 - 100,0			0,67	
>6,07	0,00	0,0 - 69,5	100,00	40,2 - 100,0			1,00	

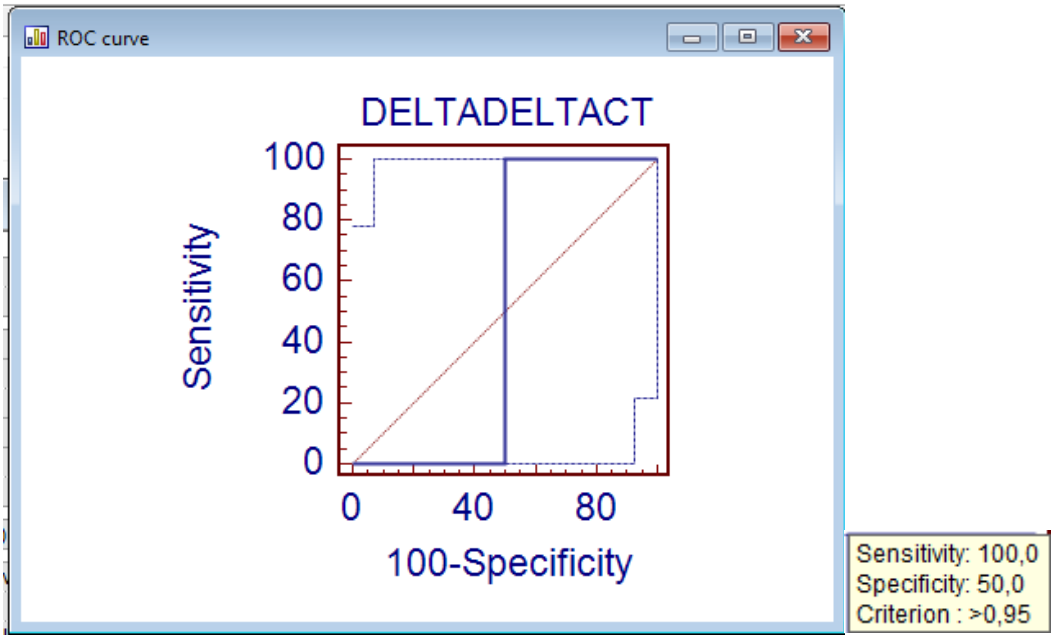

Figure 4.4: POOL2 / Control ROC curve analysis curve

Table 4.6: POOL2 / Control ROC curve diagnosis results

ROC curve

Variable	DELTADELTACT
Classification variable	DATA

Positive group	
DATA	= 1
Sample size	3
Negative group	
DATA	= 0
Sample size	4

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)	0,500
Standard Error	0,236
95% Confidence Interval	0,143 to 0,857
z statistic	0,000
Significance level P (Area=0.5)	1,0000

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI
>=-11,16	100,00	30,5 - 100,0	0,00	0,0 - 59,8	1,00			
>0,95 *	100,00	30,5 - 100,0	50,00	8,3 - 91,7	2,00	0,8 - 5,3	0,00	
>4,45	0,00	0,0 - 69,5	50,00	8,3 - 91,7	0,00		2,00	0,8 - 5,3
>5,7	0,00	0,0 - 69,5	100,00	40,2 - 100,0			1,00	

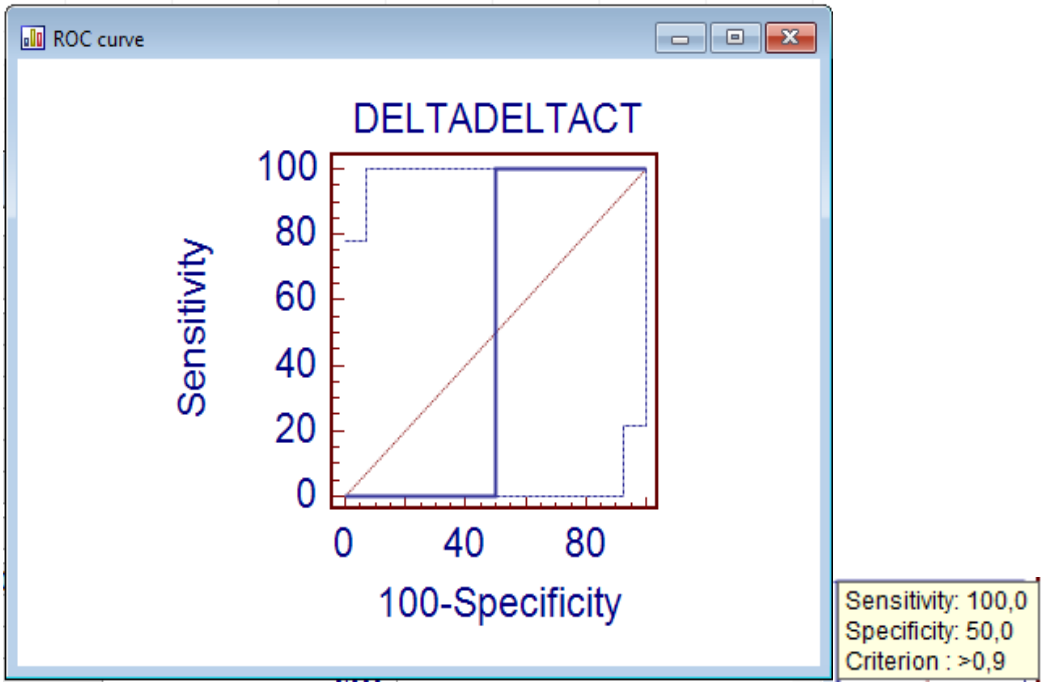

Figure 4.5: POOL3 / Control ROC curve analysis

Table 4.7: POOL3 / Control ROC curve diagnosis results

ROC curve

Variable	DELTADELTACT
Classification variable	DATA

Positive group	
DATA	= 1
Sample size	3
Negative group	
DATA	= 0
Sample size	4

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)	0,500
Standard Error	0,236
95% Confidence Interval	0,143 to 0,857
z statistic	0,000
Significance level P (Area=0.5)	1,0000

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI
>=-11,16	100,00	30,5 - 100,0	0,00	0,0 - 59,8	1,00			
>0,9 *	100,00	30,5 - 100,0	50,00	8,3 - 91,7	2,00	0,8 - 5,3	0,00	
>4,88	0,00	0,0 - 69,5	50,00	8,3 - 91,7	0,00		2,00	0,8 - 5,3
>5,7	0,00	0,0 - 69,5	100,00	40,2 - 100,0			1,00	

Table 4.8: POOL1, POOL2, POOL3, and control pool ROC curve statistics analysis results (One-way Anova test)

One-way analysis of variance

Data	DELTADELTACT
Factor codes	DATA

Sample size	4

Levene's Test for Equality of Variances

Levene statistic	3,812
DF 1	1
DF 2	2
Significance level	P = 0,190

ANOVA

Source of variation	Sum of squares	DF	Mean square
Between groups (influence factor)	8,3167	1	8,3167
Within groups (other fluctuations)	3,6704	2	1,8352
Total	11,9871	3	

F-ratio	4,532
Significance level	P = 0,167

Factor	n	Mean
(1) 0	1	0,2300
(2) 1	3	3,5600

Save residuals
Multiple comparison graph

5. DISCUSSION and CONCLUSION

The patients were grouped from the most common to the least common GBM tumors in the randomly selected patient group by pooling the serum, and we saw that the expression of miRNA223 was different between the groups. Pool 1 which included the patients who have tumor at the temporomandibular, has the most aggressive cancer type and has the minimum average survival rate, when compared with other pool groups, according to the demographic tables.

Recent research has linked miRNAs to the growth and death of human cancer cells. When compared to normal tissues, osteosarcoma tissues were shown to have lower levels of the miRNA223 (109).

All body fluids contain miRNAs that circulate and may one day be used as clinically useful indicators of human cancers. It was discovered that serum levels of miRNAs found in veins of the portal system that drain tumors did not exactly match the abundance of these molecules in primary cancer tumors. Further evidence that these miRNAs are not released from the tumor comes from the dramatically increased levels of various possible biomarkers in peripheral blood (110).

Numerous studies were conducted to validate serum and plasma profiling in search of potential cancer biomarkers as a result of the extraordinary stability of miRNAs under a variety of environmental circumstances. Such efforts were made for gastric cancer as well, indicating that blood miRNA assays may perform noticeably better than the present options. The results of earlier research, however, have raised doubts about any physiological function of miRNAs for the majority of cancers. The possibility that putative biomarker candidates actually come from sources other than the primary tumor, such as normal tissues of other organs or blood cells, is one of the possible causes for this disparity (94, 96, 97, 110).

The present research based on a well-defined cohort of Western patients with gastric cancer offer a rare opportunity to investigate the sources of possible blood biomarkers by comprehensive expression profiling of miRNAs. At first, patients with cancer had significantly higher levels of expression of 20 of the 434 miRNA sequences found in peripheral blood serum. Only seven of these, nevertheless, demonstrated primary tumor levels that were higher than the nearby normal mucosa. Two possible indicators also showed more expression in peripheral

blood than in the vein that drains the tumor. Overall, the findings clearly imply that the bulk of circulating miRNA biomarkers have non-tumor origins.

Comprehensive miRNA profiling of primary malignancy and surrounding healthy tissues is done in conjunction with serum samples from portal and peripheral blood to look into potential diagnostic indicators. Poor correlations between miRNAs that are variably expressed in tissues and blood imply that a significant part of putative biomarker molecules come from tissues other than the primary tumor, reflecting a biological phenomenon that is yet not fully understood. This should be confirmed by other research teams because it has significant ramifications for future study on the function and origins of miRNAs that are related with cancer (110, 111).

The findings demonstrated that miRNA expression is significantly altered in human cancer. Furthermore, miRNA223 overexpression is linked to poor metastasis-free survival in individuals with gastric cancer. For the treatment of cancer patients moving forward, this might have therapeutic or prognostic implications.

Aberrant gene or miRNA expression is linked to the development and spread of cancer. To understand the molecular mechanisms underlying cancer pathogenesis and identify new diagnostic and therapeutic targets, it is critical to identify dysregulated miRNAs and the genes that they target. miRNA223 may act as a tumor suppressor or an oncogene in cancers of the same type, according to the recent studies.

Although it is becoming increasingly obvious that miRNAs play important roles in the control of gene expression during development, little is known about the expression levels or function of miRNAs in normal and gastric cancer cells. Recent gene expression profiling studies have discovered miRNAs linked to diseases of the stomach. miRNA expression was found to differ noticeably between a healthy stomach, chronic gastritis, and gastric cancer in a study of the onset and evolution of the disease. A miRNA cluster was found to influence variations in tumor growth and apoptosis (108).

Another recent investigation on the relationship between miRNA and gastric clinical outcome revealed that while miRNA may be a useful diagnostic

marker for gastric cancer, it has no impact on patients' clinical prognoses. This study found that primary gastric malignancies and the comparable noncancerous gastric tissues have dramatically distinct miRNA expression profiles across the whole genome. By using miRNA expression signature analysis in the testing and validation cohorts, it was discovered that 22 miRNAs were differently expressed in tumor tissues. The ability of 22 miRNAs to distinguish between tumor and nontumorous tissue raises the possibility that carcinogenesis involves predictable and systematic changes in miRNA expression patterns, which may be indicative of sporadic stomach malignancies. Only 11 miRNAs amongst the 140 tumors versus nontumor miRNAs reported in our investigation and the 19 miRNAs discovered by Petrocca and colleagues overlap. It is confirming that numerous miRNAs are present in both signatures, despite the variation in miRNAs that may have resulted from the different platforms and specific samples used in each investigation (77).

The results point to the existence of a previously unidentified regulatory mechanism whereby a pleiotropic transcription factor stimulates the production of a particular miRNA, which in turn suppresses its direct targets and controls the invasion and metastasis of gastric cancer cells. Future research should be done to thoroughly understand the miRNA complement and their mRNA targets in order to better understand how these miRNAs contribute to high-grade malignancy. Our research also demonstrated that anti-miRNA223 oligonucleotides may be used as a foundation for the creation of novel treatments for the metastatic spread of human cancer.

The findings demonstrated that miRNA expression has been significantly altered in human stomach cancer. Furthermore, patients with gastric cancer who have miRNA223 overexpression had poor metastasis-free survival. For the treatment of cancer patients moving forward, this might have therapeutic or prognostic implications (99, 108, 102, 112).

miRNAs are very stable in a variety of environmental settings, which sparked substantial study to support serum and plasma profiling in the hunt for potential cancer biomarkers.

Despite the increased interest in analyzing microRNA-based cancer prediction models, most of the reported research still require further analysis in larger cohorts of breast cancer patients.

Overall, the results of this investigation point to miRNA223 as a potential new therapeutic target for glioblastoma. This shows that glioblastoma is a direct target of miRNA223. Even the findings in this study demonstrates that human glioblastoma cells' expression of miRNA223 inhibits the growth of GBM tumors, this conclusion requires additional research.

The fact that the expression level of miRNA223 was higher in the patient group compared to the healthy group reveals the importance of miRNA223 in the prognosis of glioma. This study may reveal that GBM tumor locations would have different miRNA expression levels.

This study may indeed provide us with illuminating information about glioblastoma localities in studies with larger serum pools and different miRNAs to be created.

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7. APPENDICES

7.1. Ethical Approval

YEDITEPE UNIVERSITY

No : E.83321821-805.02.03-87
Subject : Non Interventional Clinical Research Ethics Board Decision

To Prof. Dr. Turgay İsbir

Yeditepe University Non-Interventional Clinical Research Ethics Board has reviewed the research proposal placed upon ethical approval for the project as the research title, the researchers, the application number, the ethics board meeting details, and the provided documents have been outlined below. This research proposal has been evaluated by the members of the ethics board and full ethical **APPROVAL** has been granted.

Research Title:	Investigation of the use of miRNA223 as a biomarker in glioma tumors
Researchers:	Gokhan Ozdemir, Prof. Dr. Turgay Isbir
Application Number:	202211Y0310

ETHICS BOARD MEETING DETAILS			
Meeting Date:	14 November 2022	**Meeting Place:**	Online (Google Meet)

PROVIDED DOCUMENTS
Wet signed application file, file uploaded to a CD or USB media, and electronic submission
Title of research and the names of the researchers
Letter of application
Application Form
Nature of the Research;
• Type
• Significance and distinctive value
• Aims and objectives
• Method
• Management of the research
• Widespread impact
• Pertinence of the budget and reason (if applicable)
• Timeframe and convenience
• References
Informed consent form (uniquely prepared for the research)
Letter of Undertaking-1 A commitment to undertake the responsibility of obtaining institutions permission from where the data collection will be made
Letter of Undertaking-2 A commitment to read the latest version of the World Medical Association Declaration of Helsinki and all relevant guidelines of the Ministry of Health
Letter of Undertaking-3

True copy by e-signature.
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Profession: Başkan
Phone Number:

A commitment on whether there are previous ethical board applications
Letter of Undertaking-4 A commitment that no action will be taken during the research that is not included in the research budget and that will bring additional burden to the volunteer or the Social Security Institution
Letter of Undertaking-5 A commitment to reading the circular on treatment approaches and scientific research in COVID-19 patients
Letter of Undertaking-6 A commitment to reading the document of Research Application Permissions that is published by Ministry of Education
'Resume Forms' filled separately by all the researchers and signed
Additional documents (Scale permissions used, if any, etc.)

Prof. Dr. Didem ÖZDEMİR ÖZENEN Chair	Assoc. Prof. Dr. Gökhan ERTAŞ Deputy Chair	Assoc. Prof. Dr. Elif SUNGURTEKİN EKÇİ Reporter
Prof. Dr. Feryal SUBAŞI Member	Assoc. Prof. Dr. Binnur OKAN BAKIR Member	Assist. Prof. Dr. Elif Çiğdem KELEŞ Member
Assist. Prof. Dr. Emine Nur ÖZDAMAR Member	Assist. Prof. Dr. Sevim ŞEN Member	

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7.2. Raw Data of the Pools' CT values

	Pool 1 CT	**Pool 1 CT (U6)**	**Pool 2 CT**	**Pool 2 CT (U6)**	**Pool 3 CT**	**Pool 3 CT (U6)**	**Pool control CT**	**Pool control CT (U6)**
	Patient	Patient U6	Patient	Patient U6	Patient	Patient U6	Control	Control U6
	37,67	33,99	36,42	35,46	38,61	36,12	25.5	39,06
	36,94	35,18	39,84	37,73	34,32	35.96	37.07	31,76
			35,08	33,36	35.68	36.49	38	35
			32,25	35,1	35.96	34.32	32.93	34,37
AVERAGE	**37,30**	**34,58**	**35,89**	**35,41**	**36,55**	**36,27**	**32.79**	**39,16**
AVERAGE							**32,858**	**35,87**