Exploration of CD47 Expression in Glioblastoma and Oligodendroglioma: Implications for Tumor Microenvironment and Treatment Strategies

Irem Yenidogan Aydın

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Koç University

Graduate School of Health Sciences

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Irem Yenidoğan Aydın

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and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Doc. Dr. Yildiz Tutuncu

Prof. Dr. Savas Guzel

Doc. Dr. Ibrahim Kulac

Date: 27/08/2024

ABSTRACT

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Glioblastoma (GBM) is the most common and aggressive type of primary malignant brain tumor with a median survival time of 16 to 19 months

The diagnosis of GBM is typically made by imaging techniques such as magnetic resonance imaging (MRI) and confirmed by biopsy. Treatment is mainly surgery, but also includes radiation therapy and supportive chemotherapy. Surgical resection is the most decisive factor in patient survival, but the use of temozolomide chemotherapy in combination with radiotherapy has been shown to improve the 2-year survival rate.

GBM is characterized by its rapid growth and infiltrative nature, making it difficult to completely remove by surgery. Despite advances in treatment modalities, GBM remains largely incurable and has a high rate of recurrence even after treatment, which contributes to its poor prognosis.

Alternative treatment modalities for GBM are needed because current treatment options such as surgery, adjuvant chemotherapy and radiation therapy have limited effectiveness in improving long-term survival. While these treatments can help manage symptoms and prolong survival to some extent, they are not curative.

CD47 is a membrane protein belonging to the immunoglobulin superfamily and is known as the “don't eat me” signal. It plays a role in immune evasion by inhibiting phagocytosis, the process by which immune cells engulf and destroy cancer cells. In cancer, high levels of CD47 expression have been observed in several tumor types, including prostate carcinoma, ovarian cancer, clear cell renal cell carcinoma, non-small cell lung cancer ,endometrial carcinoma, breast cancer, bladder cancer and more. In these cancers, CD47 expression has been associated with adverse clinicopathological features such as higher tumor grade, lymphovascular invasion and advanced disease stages, revealing a link between expression level and poor prognosis and reduced survival rates.

Between 2004 and 2013, only a limited number of studies have examined the level and role of CD47 in GBM. In these studies, in summary, high CD47 levels were associated with radiotherapy and temozolomide resistance, targeted inhibition of CD47 was shown to suppress tumor growth in vitro, CD47 was shown to work together with SIRPalpha, calreticulin, Tenascin C molecules in GBM and to be associated with PI3K/Akt pathway, and PDL-1 and VEGF inhibition in addition to CD47 inhibition was shown to be synergistic in suppressing tumor growth.

Our aim in this study is to add to the literature on the role of CD47 in GBM in different aspects by revealing the concentration and prevalence of CD47 levels in pathology specimens and its relationship with patient survival, treatment resistance and tumor grade.

ÖZETÇE

Glioblastoma ve Oligodendrogliomada CD47 Ekspresyonunun Araştırılması: Tümör Mikroçevresi ve Tedavi Stratejileri için Çıkarımlar

Immunoloji Yüksek Lisans Programı

Ağustos,2024

Glioblastoma (GBM) en yaygın ve agresif primer malign beyin tümörü türüdür ve medyan sağkalım süresi 16 ila 19 aydır .GBM tanısı tipik olarak manyetik rezonans görüntüleme (MRI) gibi görüntüleme teknikleriyle konur ve biyopsi ile doğrulanır. Tedavi esas olarak cerrahidir, ancak radyasyon tedavisi ve destekleyici kemoterapiyi de içerir. Cerrahi rezeksiyon hasta sağkalımında en belirleyici faktördür, ancak radyoterapi ile birlikte temozolomid kemoterapisinin kullanılmasının 2 yıllık sağkalım oranını iyileştirdiği gösterilmiştir.

GBM, hızlı büyümesi ve infiltratif doğası ile karakterize olup, cerrahi ile tamamen çıkarılmasını zorlaştırmaktadır. Tedavi yöntemlerindeki ilerlemelere rağmen, GBM büyük ölçüde tedavi edilemez olmaya devam etmektedir ve tedaviden sonra bile yüksek oranda nükse sahiptir, bu da kötü prognozuna katkıda bulunur. GBM için alternatif tedavi yöntemlerine ihtiyaç duyulmaktadır çünkü cerrahi, adjuvan kemoterapi ve radyasyon tedavisi gibi mevcut tedavi seçenekleri uzun süreli sağkalımı iyileştirmede sınırlı etkinliğe sahiptir. Bu tedaviler semptomları yönetmeye ve sağkalımı bir dereceye kadar uzatmaya yardımcı olabilirken, küratif değildir.

CD47, immünoglobulin süper ailesine ait bir membran proteinidir ve “beni yeme” sinyali olarak bilinir. Bağışıklık hücrelerinin kanser hücrelerini yutup yok ettiği süreç olan fagositozu inhibe ederek bağışıklıktan kaçışta rol oynar. Kanserde, prostat karsinomu, yumurtalık kanseri, berrak hücreli renal hücreli karsinom, küçük hücreli olmayan akciğer kanseri, endometriyal karsinom, meme kanseri, mesane kanseri ve daha fazlası dahil olmak üzere çeşitli tümör tiplerinde yüksek düzeyde CD47 ekspresyonu gözlenmiştir. Bu kanserlerde CD47 ekspresyonu, daha yüksek tümör derecesi, lenfovasküler invazyon ve ileri hastalık evreleri gibi olumsuz klinikopatolojik özelliklerle ilişkilendirilmiş ve ekspresyon düzeyi ile kötü prognoz ve düşük sağkalım oranları arasında bir bağlantı olduğunu ortaya koymuştur.

2004 ve 2013 yılları arasında, CD47'nin GBM'deki seviyesi ve rolünü inceleyen sınırlı sayıda çalışma yapılmıştır. Bu çalışmalarda özetle, yüksek CD47 düzeylerinin radyoterapi ve temozolomid direnci ile ilişkili olduğu, CD47'nin hedefe yönelik inhibisyonunun in vitro tümör büyümesini baskıladığı, CD47'nin GBM'de SIRPalpha, calreticulin, Tenascin C molekülleri ile birlikte çalıştığı ve PI3K/Akt yolağı ile ilişkili olduğu, CD47 inhibisyonuna ek olarak PDL-1 ve VEGF inhibisyonunun tümör büyümesini baskılamada sinerjistik olduğu gösterilmiştir.

Bu çalışmadaki amacımız, CD47 düzeylerinin patoloji örneklerindeki konsantrasyonunu ve yaygınlığını ve hasta sağkalımı, tedavi direnci ve tümör derecesi ile ilişkisini ortaya

koyarak CD47'nin GBM'deki rolü hakkındaki literatüre farklı açılardan katkıda bulunmaktır

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Abbreviations

- **IHC**: Immunohistochemistry
- **TMA**: Tissue Microarray
- **GBM**: Glioblastoma
- **TME**: Tumor Microenvironment
- **TAMs**: Tumor-Associated Microglia/Macrophages
- **BBB**: Blood-Brain Barrier
- **MDSCs**: Myeloid-Derived Suppressor Cells
- **NK**: Natural Killer
- **TLRs**: Toll-Like Receptors
- **GAMs**: Glioma-Associated Microglia/Macrophages
- **ADCC**: Antibody-Dependent Cellular Cytotoxicity
- **ADCP**: Antibody-Dependent Cellular Phagocytosis

CHAPTER 1: INTRODUCTION

Brain tumors are very important problems requiring new treatment approaches by basic science due to prevalence, undefined background, and subsequent consequences in patients and their relative's lives. According to the World Health Organization, 6.511 new brain tumor cases are being diagnosed in Turkey per year while the number of deaths is 6.016 per year. Among these tumors, it is estimated that around 30 percent are malignant with high morbidity and mortality risk, while the remaining 70 percent are classified as nonmalignant, although they can still cause a significant decrease in quality of life. Meningiomas and glial tumors including glioblastoma, astrocytoma, and oligodendroglioma are about two-thirds of all primary brain tumors diagnosed in adults[1].Glioblastoma (GBM) is an aggressive and highly malignant primary brain tumor, categorized as a grade IV astrocytoma.

CHAPTER 2: BACKGROUND INFORMATION AND OBJECTIVES

GBM is one of the most concerning diagnoses in oncology considering the prevalence and bad prognosis resisting to all conventional treatment modalities. The median age of onset is about 61 years, with a slight predominance in males (male-female ratio of 1.2:1). Known risk factors include exposure to radiation and certain genetic syndromes[2]. For instance, specific Aurora Kinase polymorphisms, such as AURKB rs2289590 and AURKC rs11084490, have been linked to a reduced risk of developing GBM[3]. Conversely, certain HLA variants, including the genotypes B13 and B07-Cw07, are positively associated with an increased risk of GBM, while the Cw01 variant is negatively associated with the disease[4]. The role of DNA repair genes is also complex; for example, the BRCA1 rs799917 polymorphism has been shown to either increase or decrease GBM risk depending on the genetic model used[5]. Additionally, polymorphisms in MMP genes reveal that the 2G/2G genotype of MMP-1 correlates with a heightened GBM risk, whereas MMP-2 polymorphisms do not exhibit the same association[6]. Polymorphisms in asthma-related genes like IL-4RA and IL-13 display an inverse relationship with GBM risk, suggesting a potential protective

effect[7]. In the Han Chinese population, polymorphisms in CCDC26 are associated with increased susceptibility to GBM[8].

GBM's clinical presentation varies based on the tumor's location in the brain. Generalized symptoms are those that affect the brain as a whole and are not confined to a specific area. For example, many patients experience headaches that are persistent and worsen over time, often being more intense in the morning or when lying down. Another common generalized symptom is seizures, which may occur suddenly and involve the entire body, leading to loss of consciousness and convulsions. Additionally, patients might suffer from nausea and vomiting, which are often linked to increased intracranial pressure caused by the tumor. As the condition progresses, some individuals may exhibit a depressed level of consciousness, characterized by increased drowsiness, confusion, or difficulty in maintaining alertness. Neurocognitive dysfunction is another generalized symptom, manifesting as memory problems, difficulty with concentration, or changes in personality, all of which can significantly impact daily life[9].

Focal symptoms, on the other hand, are related to the specific area of the brain that is affected by the tumor. For instance, if the tumor affects the motor cortex, the patient might experience weakness or paralysis in one part of the body, such as a hand, arm, or leg, which is typically on one side. Sensory loss is another focal symptom, where patients may lose sensation or experience abnormal sensations like tingling or numbness in certain parts of the body. When the tumor impacts the language centers of the brain, it can lead to aphasia, which is the loss of ability to understand or express speech, making communication difficult. In cases where the tumor affects the brain regions responsible for vision and spatial awareness, visual-spatial dysfunction may occur, leading to difficulties in perceiving and navigating space, such as bumping into objects or misjudging distances. These symptoms, whether generalized or focal, provide crucial clues to healthcare providers in diagnosing the location and nature of the brain tumor, guiding further investigation and treatment[10].

Diagnosis usually involves imaging techniques like MRI and CT scans. Magnetic resonance imaging (MRI) with contrast is the best method for evaluating brain tumors due to its superior detail of soft tissues. Computed tomography (CT), which has lower soft-tissue resolution, is mainly used in urgent cases, such as bone structures, bleeding, or when MRI is not available for the patient. High-grade gliomas characteristically show hypointense masses on T1-weighted MRI images and heterogeneous enhancement

following contrast infusion. For example, in the figure-1, the enhancing tumor can be distinguished from the surrounding hypointense edema. Axial T1-weighted MRI reveals edema in the left temporal lobe with loss of sulci and blurred gray-white matter distinction, and after gadolinium infusion, a 2.5 cm × 2.5 cm circular area of irregular contrast enhancement is observed (arrow).

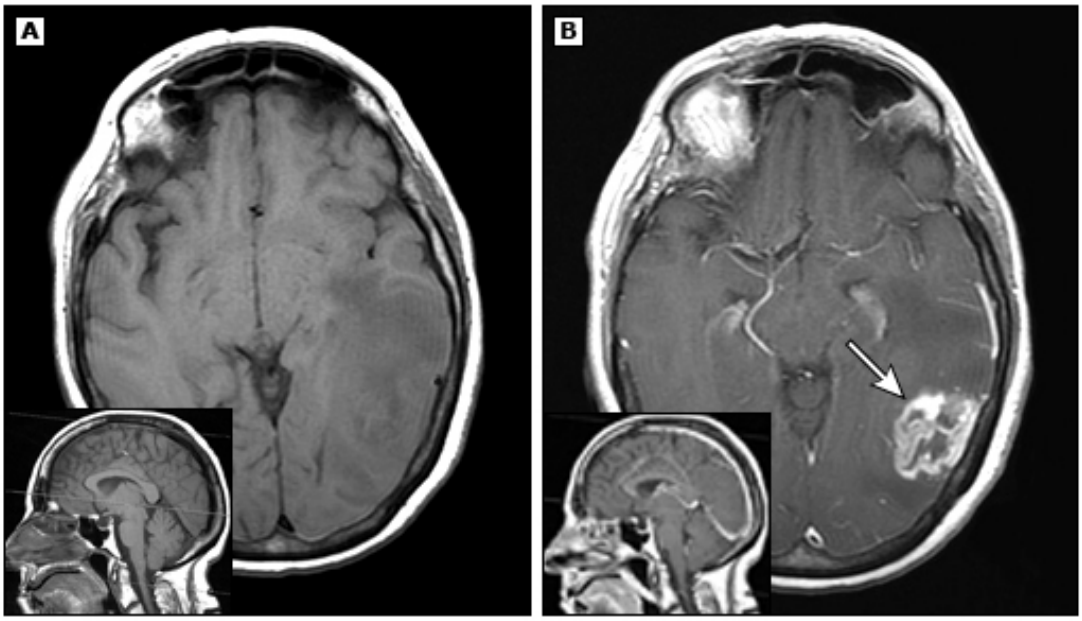


Figure 1: An overview of GBM Extracted from Dietrich, Jorg. "Clinical presentation, diagnosis, and initial surgical management of high-grade gliomas." *UpToDate; Uptodate: Waltham, MA, USA* (2022).

Vasogenic edema can be observed frequently in patients and demonstrates a hyperintense signal in the white matter on T2 or FLAIR-weighted imaging. The thick rim enhancement around the tumor margins, with a central area lacking enhancement, related to the central necrosis or cystic changes (Figure 2) is also very pathognomonic in diagnostic process. FLAIR (A) and postcontrast T1-weighted images (B) reveal a large, T2-hyperintense mass in the left temporal lobe with heterogeneous enhancement and central necrosis.

High-grade gliomas show increased blood flow and volume, resulting in increased permeability on perfusion imaging. Magnetic resonance spectroscopy (MRS) often reveals a high choline signal, decreased N-acetyl aspartate (NAA), and increased lactate levels, which are characteristic features of these tumors. In image-3 the color chart displays choline levels, ranging from low (blue) to high (red), so high choline levels (red) are observed in the medial portion of the glioblastoma.

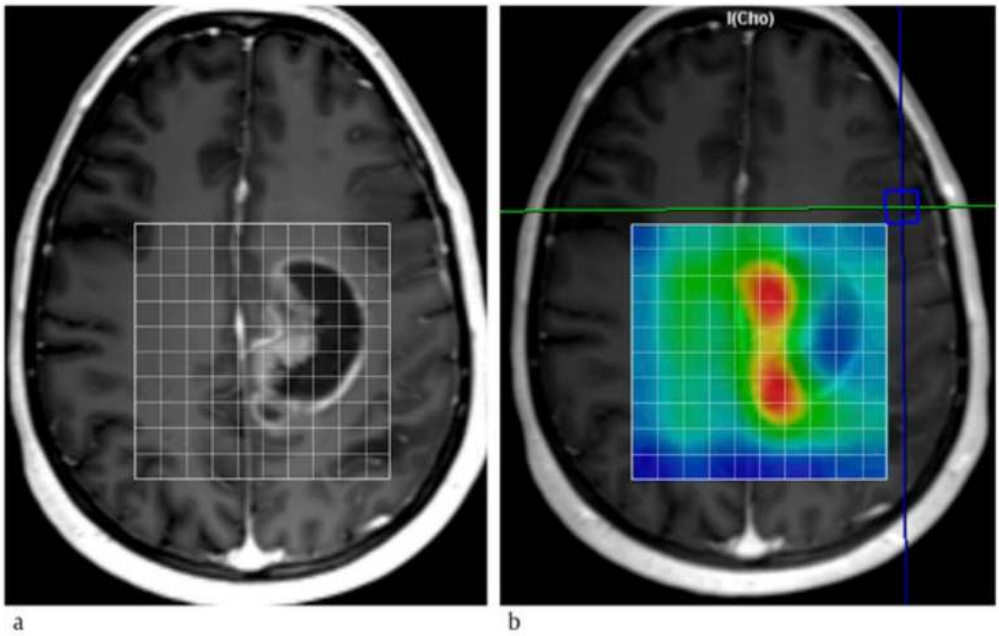


Figure 2.: An overview of Increased Choline in GBM Extracted from Barker, Peter B., et al. *Clinical MR spectroscopy: techniques and applications*. Cambridge University Press, 2009].

The radiology evaluation is followed by histopathological confirmation through biopsy or surgical removal. The histopathologic evaluation of glioblastoma (GBM) is a comprehensive process involving histological examination, immunohistochemistry (IHC), and molecular analysis, as well as genetic and cytogenetic analysis.

The first step in the evaluation of GBM determines the classification and grade of the tumor due to its histological features. GBM is characterized by high cellularity, pleomorphism, mitotic activity, necrosis, and microvascular proliferation, all of which are assessed using hematoxylin and eosin (H&E) staining. These features help in identifying the aggressiveness of the tumor. Reevaluation of histopathological samples is also crucial, in one study, 25% of long-term survivors initially diagnosed with GBM were reclassified to other types of malignant gliomas upon reevaluation[11].

Necrosis is a fundamental feature of GBM, and its extent is closely examined with assessing both coagulation and ischemic necrosis. While high levels of VEGF are being associated with ischemic necrosis, low levels of IL-8 are linked to coagulation necrosis[12]. Additionally, evaluating the extent of residual viable tumor versus therapy-induced necrosis is important since the extent of recurrent tumor, for both high-grade and non-high-grade components, is associated with the survival of the patients.

Immunohistochemistry and molecular analysis provide information about specific markers that influence the classification and prognosis of GBM. Testing for IDH1/2 mutations is made for classification and prognosis, as IDH-wildtype status in lower-grade astrocytomas can predict behavior similar to that of IDH-wildtype GBM[13]. EGFR amplification is another commonly assessed marker, particularly in both primary and recurrent GBMs, associated with tumor growth[14]. MIB-1 labeling is used to evaluate proliferative activity, even though no prognostic value has been identified[15].

Additional molecular markers are also tested to provide further insights. For example, H3-K27M mutations are significant in midline gliomas, as these tumors exhibit WHO grade IV behavior[16]. The methylation status of the MGMT promoter is also evaluated, as it can influence the tumor's response to chemotherapy, making it an important factor in treatment planning[17].

The genetic and cytogenetic analysis of GBM identifies common and characteristic genetic aberrations of the tumor such as losses of chromosomes 10 and 9p, and gains of chromosome 7[18]. Additionally, specific gene polymorphisms, such as the 2G/2G genotype and 2G allele of the -1607 MMP-1 polymorphism, are supportive findings for the diagnosis of GBM[19].

The pathogenesis of GBM is complex and involves numerous genetic mutations and altered gene expressions, affecting various signaling pathways (Figure 3).

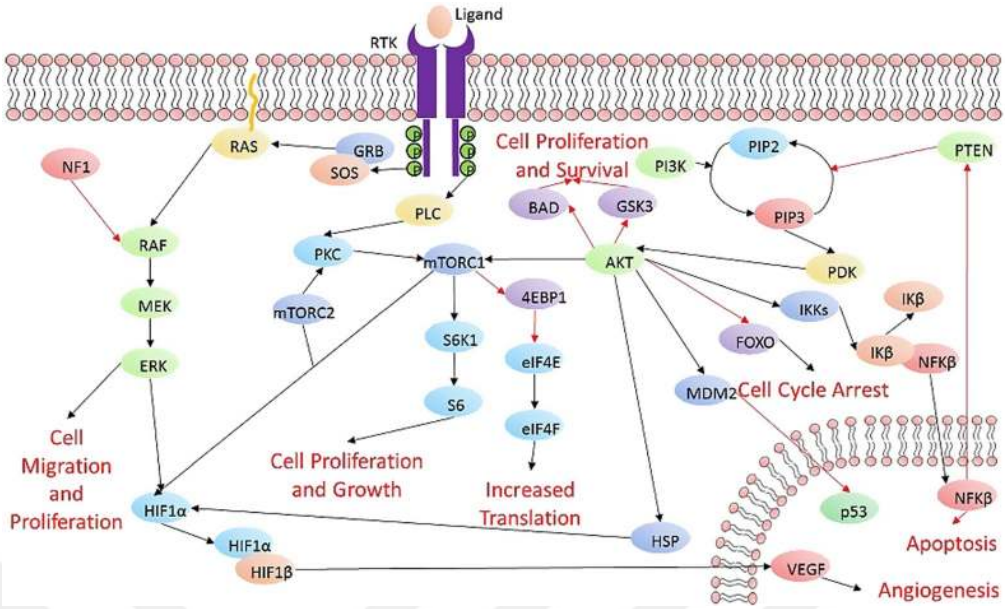


Figure 3 : An overview of pathways in GBM Extracted from Pearson, Joshua RD, and Tarik Regad. "Targeting cellular pathways in glioblastoma multiforme." *Signal transduction and targeted therapy* 2.1 (2017): 1-11.].

Key molecular markers include hypoxia-inducible factor-1 alpha (HIF-1α), telomerase reverse transcriptase (TERT), isocitrate dehydrogenase 1 (IDH1), and tumor protein p53. These genetic aberrations lead to the tumor's development, progression and treatment resistance so the life quality and survival of the patients. The tumor microenvironment, particularly the blood-brain barrier (BBB) (Image-4) and the immunosuppressive microenvironment (Image-5), significantly contributes to the invasion of the tumor and resistance to treatment. The BBB acts as a formidable barrier, preventing many therapeutic agents from reaching the brain and effectively targeting the tumor cells.

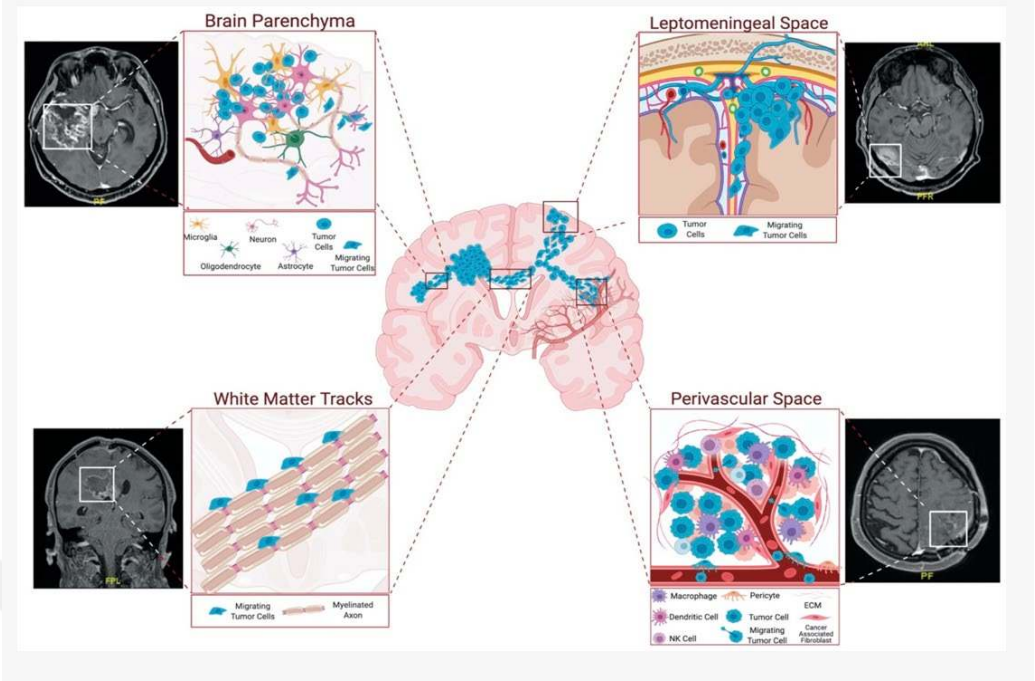


Figure 4 : An overview of BBB Extracted from Seker-Polat, Fidan, et al. "Tumor cell infiltration into the brain in glioblastoma: from mechanisms to clinical perspectives." *Cancers* 14.2 (2022): 443.]

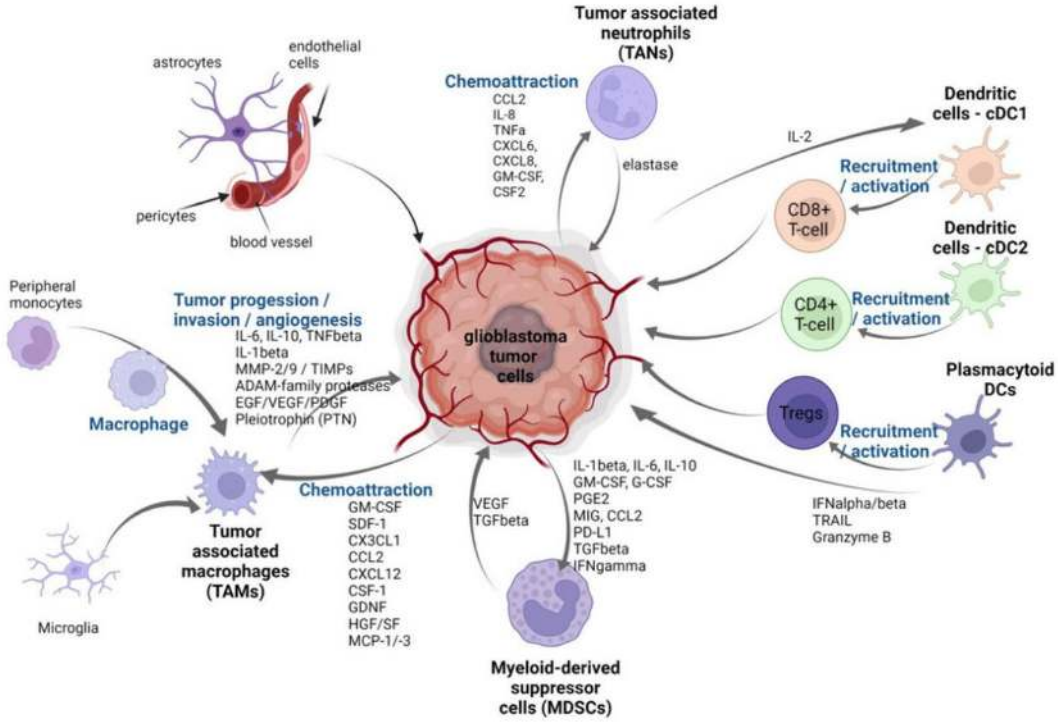


Figure 5: An overview of tumor microenvironment Extracted from Codrici, Elena, et al. "Friends with benefits: chemokines, glioblastoma-associated microglia/macrophages, and tumor microenvironment." *International journal of molecular sciences*23.5 (2022): 2509.

CHAPTER 3: CHALLENGES IN GBM TREATMENT

The current standard of care for GBM involves maximal surgical resection followed by radiotherapy and concurrent chemotherapy with temozolomide. This multidisciplinary approach has been shown to improve survival rates, but the prognosis remains poor due to the tumor's poor tissue accessibility, invasiveness, and rapid growth, patients typically relapse, with a median survival of about two years following initial diagnosis. The complex biology of GBM presents big challenges. The tumor's ability to invade surrounding brain tissue makes complete surgical resection difficult, sometimes impossible, and often leaves residual cancer cells causing high recurrence rates. The genetic heterogeneity of GBM means that different cells within the same tumor can have distinct genetic profiles, complicating the development of targeted therapies. This heterogeneity is responsible for the tumor's ability to develop resistance to the different treatment options, as different subpopulations of cancer cells may respond differently to therapeutic agents[20].

Recent advancements in treatment include immunotherapy, such as immune checkpoint inhibitors, adoptive cell transfer therapy, therapeutic vaccines, and oncolytic virus therapy aim to modify the patient’s immune system to target and destroy tumor cells. Additionally, targeted therapies, such as tyrosine kinase inhibitors, are being explored for their potential to inhibit specific pathways involved in tumor growth and survival. Nanotechnology-based drug delivery systems are also under investigation to overcome the challenges posed by the BBB, enhancing drug solubility, targeted activity, and minimizing side effects[21], [22], [23].

Research into the tumor microenvironment has revealed that the immunosuppressive environment created by GBM not only protects the tumor from the immune system but also promotes its growth and survival. The BBB's role in limiting the delivery of therapeutic agents to the brain further complicates treatment, making it difficult for drugs to reach and effectively target tumor cells. the delivery of therapeutic agents to brain tumors, posing a major obstacle in GBM treatment. The BBB's selective permeability, due to tightly joined endothelial cells, astrocyte end-feet, and pericytes,

limits the passage of large and hydrophilic molecules, thereby reducing the efficacy of many chemotherapeutic agents. This limitation is particularly problematic for high molecular weight drugs, which struggle to cross the BBB and reach the tumor site effectively. Additionally, the BBB expresses efflux transporters, such as P-glycoprotein, which actively pump chemotherapeutic agents out of the brain, further hindering drug accumulation at the tumor site and complicating treatment efforts. Efforts to develop novel drug delivery systems, such as nanoparticles and other nanotechnology-based approaches, aim to overcome these barriers and improve the delivery and efficacy of treatments[24], [25].

Emerging therapies, including personalized medicine approaches that tailor treatment based on the genetic profile of an individual's tumor, offer hope for more effective interventions. Advances in immunotherapy are being investigated for their potential to enhance the body's immune response against GBM. Additionally, research into the molecular mechanisms underlying GBM's resistance to treatment is ongoing, intending to identify new targets for therapy and developing drugs that can overcome this resistance.

CHAPTER 4: TUMOR MICROENVIRONMENT

The immune system with both innate and adaptive components evokes the body to constantly monitor malignant cells. This mechanism helps to detect and eliminate the malignant cells before they can form tumors. This interaction, defined as immune surveillance, is one of the fundamental steps for suppressing the development of cancers like glioblastoma multiforme (GBM). The tumor microenvironment (TME) of glioblastoma multiforme (GBM) has been investigated frequently in recent studies and revealed key components are microbiomes, vasculature, extracellular matrix (ECM), infiltrating parenchymal and peripheral immune cells and molecules, chemical gradients, neural stem cell phenotypic variations, fibroblasts, immune and inflammatory cells, blood and lymphatic vascular networks, as well as signaling molecules and inflammatory mediators like chemokines, cytokines, and growth factors.

In the environment surrounding the tumor, the most common immune cells are resident microglia (a type of brain cell) and circulating macrophages (immune cells that travel through the blood). Together, these cells are referred to as tumor-associated macrophages/microglia (TAMs), and they establish a big portion—about 30% to 50%—

of the immune cells in the tumor mass. The proportion of these immune cells varies depending on the type of GBM, for example mesenchymal subtype has the highest percentage of TAMs. Gene expression analyses of TAMs in high-grade gliomas show that these cells cannot be defined as a single phenotype, highlighting their dynamic and adaptable nature, influenced by the tumor microenvironment. Current research is focused on understanding how TAMs promote tumor growth, invasion, survival, immune suppression, and angiogenesis in GBM[26].

Tumor cells interact with TAMs and trigger the formation of tumor-promoting M2-like phenotype. M2-like macrophages secrete anti-inflammatory and immune-suppressive factors, which help facilitate tumor progression and immune evasion. In GBM, TAMs with various phenotypes coexist, including an antitumor, proinflammatory M1-like phenotype[27], [28].

The M2 phenotype of tumor-associated macrophages/microglia (TAMs) is characterized by increased activity of the STAT3 signaling pathway (Figure 6). The combined effects of STAT3 activation through TAMs decide the direction of the tumor immune response. Activated STAT3 interacts with immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs), M2-like tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), into the GBM microenvironment by cytokine signals like IL-6, IL-10, and TGF-β. Additionally, STAT3 plays a role in drawing immune cells, such as T cells, dendritic cells (DCs), and natural killer (NK) cells, to the tumor center[29].

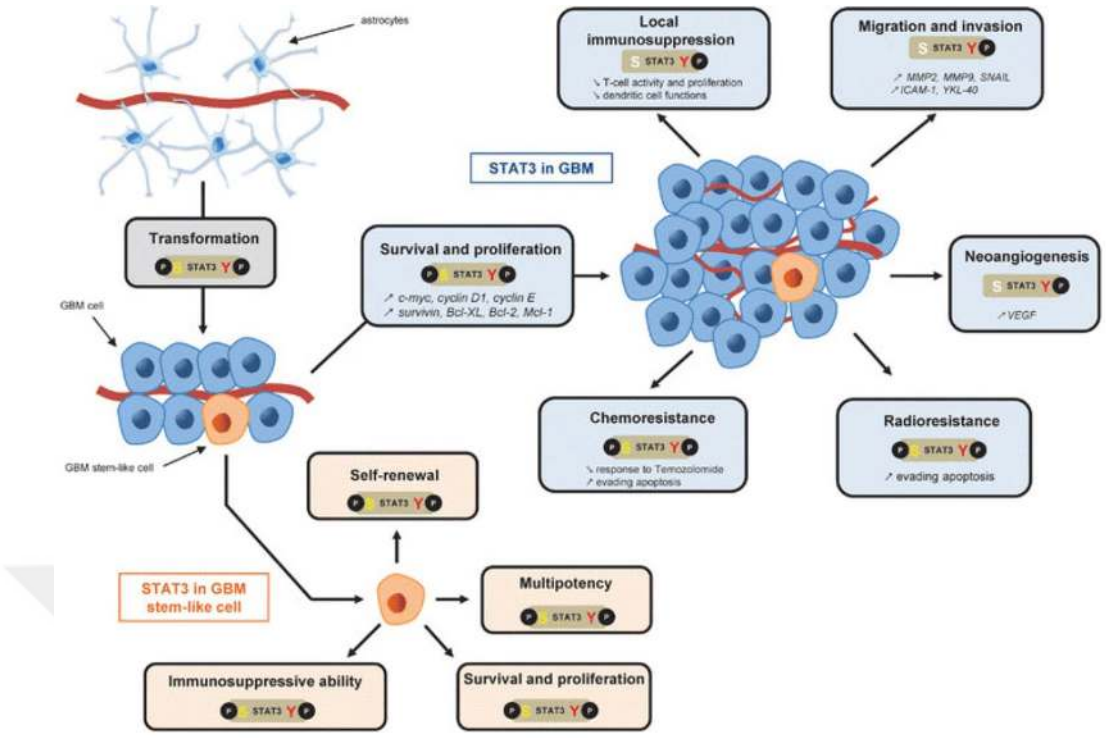


Figure 6: An overview of STAT pathway in GBM Extracted Ouedraogo, Zangbewende Guy, et al. "Role of STAT3 in genesis and progression of human malignant gliomas." *Molecular neurobiology* 54 (2017): 5780-5797.].

The higher production of anti-inflammatory cytokines such as interleukin-10 and transforming growth factor-beta reduces the polarization of microglia. This polarization induces the invasion of tumor cells through extracellular matrix degradation into healthy brain cells. The recruitment of TAMs to the tumor microenvironment is stimulated by the cytokine colony-stimulating factor (CSF)-1, released by glioma cells. CSF-1 binds to its receptor (CSF-1R) on TAMs, maintaining the M2 phenotype favoring tumor growth[30].

TAMs, a major component of the TME, exhibit remarkable plasticity, allowing them to adapt to various signals and cues within the tumor. This plasticity is a double-edged sword; while it enables TAMs to support tissue repair and homeostasis, it also allows them to promote tumor progression when influenced by the tumor microenvironment. Research has shown that TAMs can be reprogrammed to adopt an antitumor phenotype, offering a potential therapeutic strategy to enhance immune responses against GBM[31].

TAMs can influence the activity of T cells, natural killer (NK) cells, and dendritic cells, either promoting or suppressing their function depending on the signals they receive. TAMs can secrete TGF-β, which inhibits the cytotoxic activity of NK cells and T cells, thereby promoting immune evasion. Conversely, reprogramming TAMs to produce pro-inflammatory cytokines can enhance the activation and proliferation of these immune cells, boosting antitumor immunity. Strategies targeting TAMs fall into three main categories: preventing the recruitment of bone marrow-derived infiltrating macrophages/monocytes, promoting TAM-mediated phagocytosis of tumor cells to restore their innate antitumor activity, and reprogramming TAMs to antitumor macrophages/microglia[32].

Toll-like receptors (TLRs) are membrane proteins responsible for innate immune response. TLRs are the gates of the innate immune system that recognize and stop pathogens from entering. Communication between immune cells and glioma through Toll-like receptors (TLRs) in the tumor microenvironment (TME) plays a critical role in glioma progression (Figure-8). When TLR3, 7, 8, and 9 on microglia are activated, they suppress glioma growth by releasing pro-inflammatory cytokines like interleukins (ILs), tumor necrosis factors (TNFs), and interferons (IFNs). Agonists that stimulate TLR7/9 on plasmacytoid dendritic cells (pDCs) enhance the activity of NK cells and microglia via IFN secretion while reducing the presence of infiltrating regulatory T cells (Tregs). Conversely, blocking TLR7/9 leads to an accumulation of Tregs and accelerates glioma growth. The activation and proliferation of cytotoxic T-cells are driven by pDCs and TLR2/7 on myeloid dendritic cells (mDCs), which contribute to antitumor effects[33]. However, tumor-infiltrating microglia can be reprogrammed by glioma cells into a protumor phenotype, known as glioma-associated microglia/macrophages (GAMs), which support glioma invasion and progression by releasing matrix metalloproteinases (MMPs), ILs, and inducible nitric oxide synthase (iNOS). TLR2, 3, and 4 are crucial for glioma stem cell (GSC) differentiation and development[34]. GSCs activate GAMs that express TLR4, leading to the release of IL-6, which further enhances GSC development, creating a positive feedback loop that supports glioma progression. The green and red arrows indicate processes that promote or inhibit glioma development, respectively.

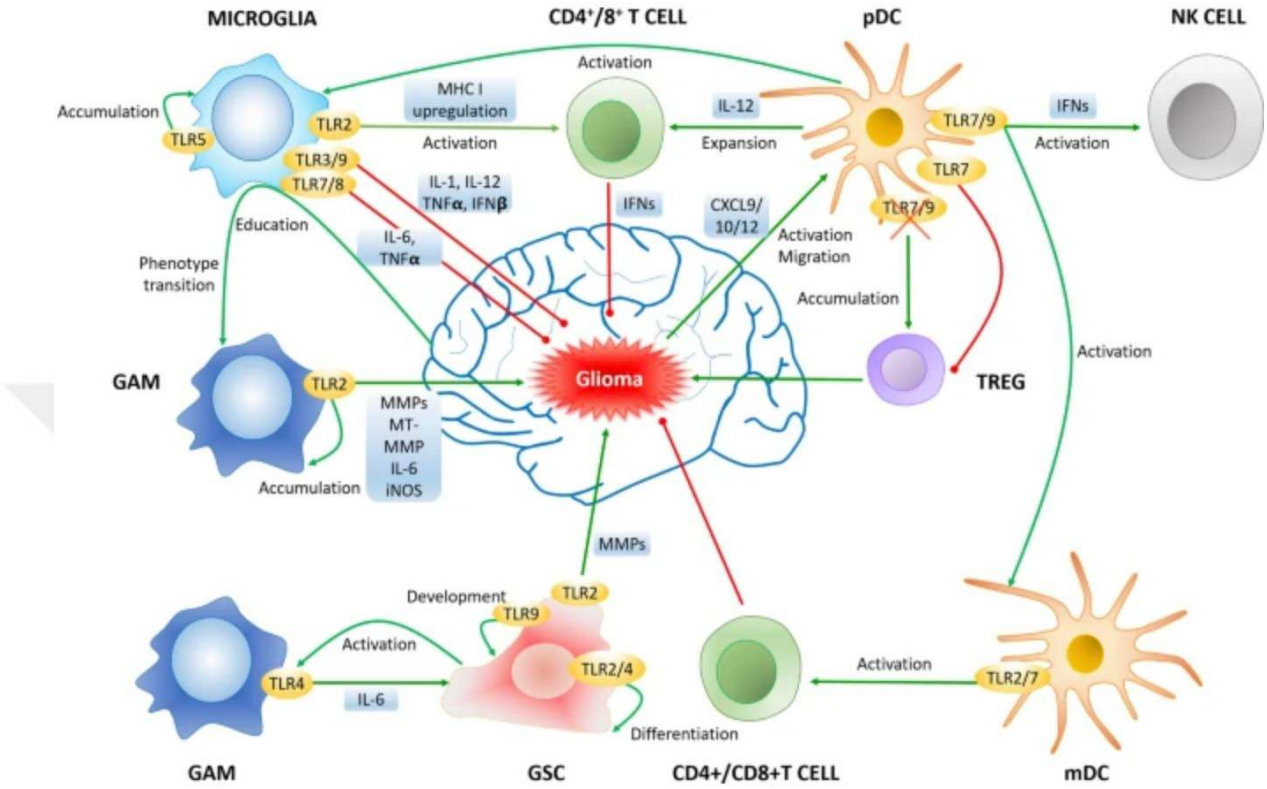


Figure 8: An overview of TLRs in GBM xtracted from Xun, Yang, et al. "Toll-like receptors and toll-like receptor-targeted immunotherapy against glioma." *Journal of hematology & oncology* 14 (2021): 1-32.

In TAMs, TLR2 expression is increased compared to others, favoring glioma invasion through extracellular matrix degradation[35]. The degradation precedes the activation of matrix metalloproteinases 2 and 9. There are preclinical studies that have shown that using a monoclonal antibody against TLR2 on microglia can inhibit tumor growth[36]. Although phase I trials using OPN-305, a TLR2-targeting monoclonal antibody, have shown promising safety results in healthy subjects and patients with myelodysplastic syndromes, there are currently no clinical trials for glioma treatment using TLR2 antibodies[37]. TLR4, another Toll-like receptor, triggers the release of interleukin-6 by TAMs which results in upregulation of PD-L1[38]. This cascade of TLR4 and PD-L1 promotes glioma growth through the immune checkpoint inhibition resulting in T-cell inactivation. A phase II clinical trial is currently underway to evaluate

the effectiveness of durvalumab, a PD-L1-targeting monoclonal antibody, alone or in combination with tremelimumab, which targets the checkpoint protein CTLA-4, in treating high-grade glioma[39]. Additionally, a phase I/II trial is examining the effects of combining hypofractionated stereotactic radiation therapy with durvalumab[40].

The immune checkpoint inhibitors (ICIs) work by targeting cancer-immune cell interactions via immune checkpoints, which suppress anti-cancer immune responses and create an immunosuppressive, pro-tumoral environment. Currently, seven ICIs that specifically activate cytotoxic T cells have been clinically approved[41]. While effective in "hot" tumors like melanoma and non-small cell lung carcinoma, which have a high proportion of cytotoxic T cells, only a minority of patients respond to these treatments. Moreover, they are less effective in non-T-cell inflamed "cold" tumors like GBM, which show only modest responses to current ICIs. Thus, focusing on innate immune cells to boost anti-tumoral activity may offer complementary and synergistic potential for treating GBM.

The CD47-SIRPα axis in TAMs is currently one of the most extensively studied innate immune checkpoints[42]. Targeting this pathway changes innate and adaptive antitumor immunity responses and a promising approach for GBM treatment emerges (Figure-9).

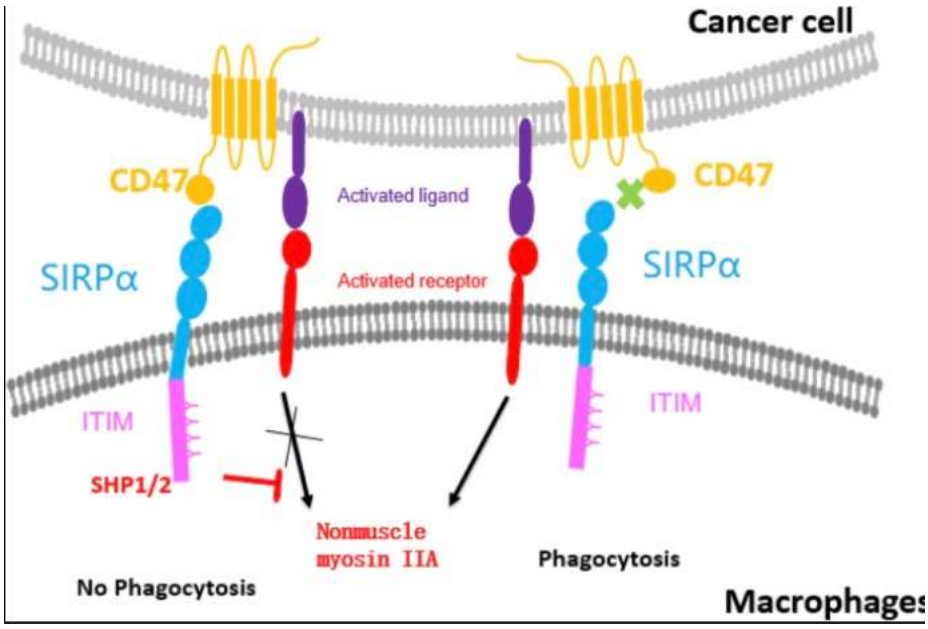


Figure 9: An overview of CD47 SIRPalpha interaction Extracted from Qu, Tailong, Baiyong Li, and Yifei Wang. "Targeting CD47/SIRPα as a therapeutic strategy, where we are and where we are headed." *Biomarker research* 10.1 (2022)].

TAMs can enhance the antigen presentation through cancer cell phagocytosis, leading to adaptive T cell immunity associated with an M1-like phenotype. Tumor-associated macrophages (TAMs) have dual, yin and yang effects on immune responses, with the yin representing M2-like TAMs and the yang representing M1-like TAMs. M1-like TAMs are associated with pro-inflammatory activities, while M2-like TAMs are linked to anti-inflammatory functions. The shift in TAM polarization from an M1 to an M2 phenotype is often linked to tumor progression[43]. TAMs are thought to contribute to glioblastoma (GBM) progression through several pro-tumorigenic mechanisms, including: 1) promoting the proliferation of GBM cells; 2) enhancing GBM cell migration and invasion; 3) stimulating angiogenesis within the tumor; 4) aiding in the degradation of the extracellular matrix (ECM); and 5) supporting an immunosuppressive tumor microenvironment (TME) (Figure-10).

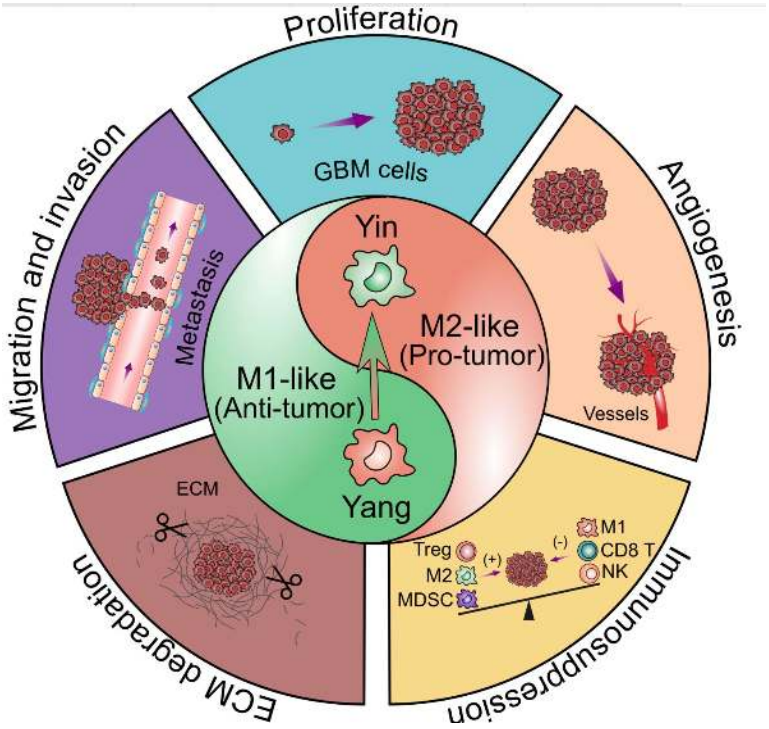


Figure 20: An overview of M1-M2 balance in tumor microenvironment Extracted from Papadakos, Stavros P., et al. "Unveiling the Yin-Yang Balance of M1 and M2 Macrophages in Hepatocellular Carcinoma: Role of Exosomes in Tumor Microenvironment and Immune Modulation." *Cells* 12.16 (2023): 2036.

The GBM microenvironment is very dynamic, consisting of a variety of cell types, extracellular matrix components, and signaling molecules. These components create a complex network of interactions that cause tumor growth through the inhibition of immune responses and leading to treatment resistance. Understanding the intricate biology of the TME is crucial for developing effective therapeutic strategies.

CHAPTER 5: Understanding the CD47-SIRPα Axis in Immune Evasion

The CD47-SIRPα axis is one of the immune evasion mechanisms in various cancers. CD47 is a transmembrane protein that serves as a key player in the immune system by delivering an antiphagocytic signal, known as the "don't eat me" signal[44]. It binds to SIRPα on macrophages which induces the phosphorylation of SIRPα's cytoplasmic immunoreceptor tyrosine-based inhibition motifs.

The CD47 gene, located on chromosome 3q13, encodes an integrin-associated protein crucial for immune evasion. It contains an immunoglobulin variable-like amino-terminal domain, five transmembrane domains, and a carboxy-terminal intracellular tail. SIRPs, inhibitory immune receptors encoded by a cluster of genes on chromosome 20p13, include SIRPα, SIRPβ1, SIRPγ, SIRPβ2, and SIRPδ. SIRPα binds to CD47 with high affinity. Structurally, the extracellular domain of SIRPα consists of three immunoglobulin-like domains (the NH2-terminal V-like domain and two C1 domains), a single transmembrane segment, and an intracellular segment containing four tyrosine residues forming two ITIMs. When CD47 on tumor cells binds to the NH2-terminal V-like domain of SIRPα on myeloid cells, it results in the recruitment and activation of tyrosine phosphatases SHP1/SHP2, inhibiting the phagocytosis of tumor cells by macrophages. This process recruits Src homology 2 domain-containing tyrosine phosphatases, which inhibit myosin-IIA accumulation at the phagocytic synapse, resulting in blocking phagocytosis[45].

This interaction referred to as the "don't eat me" signal serves as a negative immune checkpoint for innate immunity[46]. The mechanism is exploited by various cancer cells to avoid being destroyed by the immune system (Table-1).

Table-1 CD47 Related Mechanisms Utilized by Various Cancer Cells to Evade Immune System

Epithelial Ovarian Cancer	CD47 is associated with worse prognosis, FIGO stage, CA125, HE4 levels, lymph-node metastasis, platinum resistance, and BRCA/TP53 mutations. CD47 shows strong predictive capacity.
Endometrial Carcinoma	High CD47 expression correlates with high tumor grade, particularly in high-grade tumors, indicating its potential as a therapeutic target.
Non-Small Cell Lung Cancer	CD47 is overexpressed in patients with the tumor. High levels are associated with shorter progression-free survival and overall survival in patients with EGFR mutations.
Bladder Cancer	The decrease in CD47 expression was observed in patients receiving neoadjuvant chemotherapy.
Gastric Cancer	Co-expression of CD47 and B7-H3 is associated with poor prognosis, advanced tumor stage, poor differentiation, and increased macrophage infiltration.
Diffuse Large B-Cell Lymphoma	CD47 is highly expressed in intestinal non-germinal center B-cell type DLBCL, particularly with 18q21 gain/amplification which lower cytotoxic T-cell infiltration.
Soft Tissue Sarcomas	Higher expression of CD47 in high-grade tumors. Cd47 is associated with increased

	progression-free survival.
Prostate Cancer	CD47 is expressed in all prostate carcinoma cases. High CD47 expression is linked to increased expression of CD68 and CD163, markers of macrophage polarization, promoting immune evasion.

Targeting the CD47-SIRPα axis can significantly enhance the phagocytosis of tumor cells by both M1 and M2 macrophage subtypes. This approach shifts macrophages toward an M1 phenotype, known for its antitumor activity. CD47 monoclonal antibody Hu5F9-G4 and SIRPα monoclonal antibody FSI-189 block the anti-phagocytosis signal and activate macrophages to attack tumor cells in leukemia, non-Hodgkin lymphoma, and solid tumors like breast and ovarian cancer[47]. This model has revealed better survival time in animal models without toxic effects on normal tissues. CD47 antibody-induced apoptosis of tumor cells can occur independently or through complement-dependent cytotoxicity. This antitumor activity is linked to cell death caused by CD47 ligation, potentially involving cAMP regulation by heterotrimeric Gi and subsequent PKA-mediated effects. This mechanism has been observed in cancers like leukemia, where CD47 blockade induces apoptosis in malignant cells.

TTI-621 and TTI-622, composed of the N-terminal V domain of human SIRPα fused to the human IgG Fc region, bind to CD47 on tumor cells, preventing inhibitory signals to macrophages and tumor antigen presentation. These fusion proteins are undergoing clinical evaluation for their potential to treat hematologic malignancies and solid tumors[48].

NI-1701 (anti-CD19/anti-CD47) and NI-1801 (anti-CD47/mesothelin) target tumor-specific antigens and CD47 to promote phagocytosis and immune response. These bispecific antibodies are in early-phase clinical trials and show promise in targeting both hematologic and solid tumors[49].

CD47 is highly expressed in 63.7% of patients with epithelial ovarian cancer. The expression level is associated with a worse prognosis. The worse prognosis has been

identified through clinicopathological factors, including FIGO stage, CA125 and HE4 levels, lymph-node metastasis, and platinum resistance. It also correlates with genetic mutations such as BRCA and TP53. A risk score model incorporating CD47, CA125, and BRCA mutations demonstrated a strong predictive capacity, with a concordance index of 0.777, for managing high-risk populations[50], [51]. CD47 expression is significantly correlated with the tumor grade of patients with endometrial carcinoma. This correlation is identified particularly in high-grade tumors, suggesting its potential as a therapeutic target[52]. CD47 is overexpressed in 84% of Non-Small Cell Lung Cancer patients. High CD47 levels are related to shorter progression-free survival and overall survival in patients with EGFR mutations[53]. In muscle-invasive bladder cancer, CD47 expression is detectable in 44% of samples. Although there is no significant association between CD47 levels and clinicopathological parameters or survival outcomes, a slight decrease in CD47 expression was observed in patients receiving neoadjuvant chemotherapy[54].

Co-expression of CD47 and B7-H3 in gastric cancer is associated with poor prognosis of patients with gastric cancer. High CD47 expression correlates with advanced tumor stage, poor differentiation, and increased macrophage infiltration[55]. CD47 is highly expressed in intestinal non-germinal center B-cell type diffuse large B-Cell lymphoma, particularly in cases with 18q21 gain/amplification. This subtype is characterized by lower cytotoxic T-cell infiltration and a distinct biological profile compared to non-intestinal DLBCL[56].

CD47 is prevalent across various soft tissue sarcoma subtypes, with higher expression levels in high-grade tumors. Interestingly, high CD47 expression is associated with increased progression-free survival, suggesting its potential as an immunotherapeutic target. CD47 is expressed in all prostate carcinoma cases, with 52.5% exhibiting high levels. High CD47 expression is strongly associated with increased expression of CD68 and CD163, markers of macrophage polarization, indicating its role in promoting immune evasion.

Tumor cells can be targeted through antibody Fc-dependent mechanisms, including neutrophil cell-mediated antibody-dependent cellular cytotoxicity (ADCC) and macrophage-mediated antibody-dependent cellular phagocytosis (ADCP). Bispecific

antibodies that target membrane-proximal epitopes can enhance ADCC activity and improve ADCP by more efficiently blocking the CD47-SIRPα axis. This dual-targeting strategy has shown promising results in ovarian and lung cancers.

The CD47-SIRPα axis has been shown as a critical target in various cancer types. Targeting this pathway can modify the tumor microenvironment, restore innate and adaptive immune functions, and favor patients with lower morbidity and mortality. While ongoing studies are promising, these theories require further investigation and evaluation of potential toxicities to become standard clinical treatments for various cancers. Ongoing researchs combining basic science and clinical impacts will be vital in translating these promising preclinical findings into effective therapies for patients, finally improving their prognosis and quality of life.

The primary concern with CD47 inhibitors is hematological toxicity, such as anemia, thrombocytopenia, and leukopenia, due to high CD47 expression on normal red blood cells and platelets. Optimizing initiation and maintenance doses is very important to balance efficacy and avoid toxicity.

Personalized medicine approaches, including the use of biomarkers to identify patients who are most likely to respond to CD47-SIRPα blockade, are essential. Understanding the genetic and molecular landscape of tumors in individual patients can guide the selection of appropriate therapies and improve outcomes. Biomarkers that predict response to CD47-targeting therapies are being identified, which can help tailor treatments to individual patients' needs.

CHAPTER 6: Role of CD47 in Glioblastoma

The published studies and updated conclusions related to the role of CD47 in GBM have been evaluated and summarized before the study (Table-2). One research investigates the critical function of microglia, the brain's resident immune cells, in demonstrating an immune response against glioblastoma when the CD47-SIRPα pathway is disrupted. The study utilized both in vitro cultured glioblastoma cells and primary microglia, as well as in vivo animal models treated with CD47-blocking antibodies to show how microglia contribute to the anti-tumor response post-CD47-SIRPα blockade. The results demonstrated that blocking the CD47-SIRPα interaction

significantly activates microglia. This interaction normally inhibits phagocytosis by sending a "don't eat me" signal from CD47 on glioblastoma cells to SIRPα on microglia. Disrupting this cascade triggers microglia activation and enhances their phagocytic activity against tumor cells, resulting in significant tumor cell clearance in both in vitro and in vivo models. The findings demonstrated that blocking CD47 not only eliminates the inhibitory signal but also promotes the release of pro-phagocytic signals, thereby enhancing the overall immune response against glioblastoma cells. This highlights the key role of microglia in the immune response against glioblastoma following the disruption of the CD47-SIRPα antiphagocytic pathway. The therapeutic potential of targeting this axis to activate microglia is very promising in terms of possible improvement in treatment outcomes for glioblastoma patients[57].

Another study investigates the prognostic significance of MGMT methylation status with a combination of CD47 and TIGIT expression in adult diffuse gliomas. Analyzing through molecular and immunohistochemical techniques of tissue samples from adult diffuse glioma patients indicated that patients with unmethylated MGMT promoter status responded poorly to treatment and had worse overall survival compared to those with methylated MGMT. Elevated levels of CD47, which provides a "don't eat me" signal to immune cells, were associated with poorer prognosis, as were high levels of TIGIT, an immune checkpoint receptor that inhibits T cell activity. The combination of MGMT unmethylation, high CD47, and high TIGIT expression indicated a particularly poor prognosis, suggesting that these factors together create a more immunosuppressive tumor environment and contribute to more aggressive disease progression. Thus, MGMT unmethylation and elevated CD47 and TIGIT levels serve as significant indicators of poor prognosis in adult diffuse gliomas, potentially guiding more tailored and effective treatment strategies. Targeting CD47 and TIGIT, alongside considering MGMT methylation status, could open new therapeutic avenues to improve outcomes for these patients[58].

In their article, Chen et al. explores an innovative approach to glioblastoma treatment using genetically engineered macrophages to target glioma stem cells. Researchers established chimeric antigen receptor macrophages designed to specifically target glioma stem cells and gave them directly into the surgical cavity post-tumor resection in animal models of glioblastoma. The immune response and tumor recurrence were then monitored in vivo. The engineered CAR macrophages successfully targeted

and recognized antigens on glioma stem cells. Post-surgery initiation of engineered macrophages into the tumor cavity modified the local immune environment. The increased immune system activity was able to identify and attack residual glioma stem cells. These CAR macrophages increased infiltration and activation of other immune cells, like T cells, within the tumor microenvironment, amplifying the overall anti-tumor immune response. Animal models treated with CAR macrophages revealed significant reductions in tumor recurrence and improved survival rates compared to control groups[59].

The research of Gholamin et al. investigates how conventional treatment modalities such as irradiation and temozolomide chemotherapy can amplify the effectiveness of anti-CD47 immunotherapy. Researchers evaluated whether irradiation or temozolomide (TMZ) chemotherapy could boost the efficacy of anti-CD47 therapy in glioblastoma treatment, using glioblastoma cell lines to assess the impact of combining these treatments. The study showed the increased expression of calreticulin with irradiation and TMZ chemotherapy. Calreticulin protein takes part on the surface of glioblastoma cells and makes them more vulnerable to phagocytosis when CD47 is blocked. The combination treatments are associated with significantly higher infiltration of macrophages and other anti-tumor immune cells into the microenvironment. This infiltration created anti-tumor immune response leading to significant tumor size reduction. Anti-tumor Immune response-related better survival rates were observed in glioblastoma models treated with either irradiation or TMZ in combination with anti-CD47 therapy compared to either treatment alone[60].

The study of Liu et al. creates a new lipid nanoparticle (LNP) system designed to target delivery to glioblastoma cells by siRNA mechanism. Researchers developed an ionizable cationic lipid-based nanoparticle for efficient siRNA delivery to glioblastoma cells. They designed and synthesized a new ionizable cationic lipid used to formulate lipid nanoparticles (LNPs) capable of encapsulating siRNA. The main approach was reducing off-target effects and silencing target genes in glioblastoma cells. The targeted gene and subsequent immune suppression enhanced apoptosis and reduced tumor cell proliferation. The combination of siRNA delivery and immunotherapy resulted in significant tumor reduction and increased survival rates in glioblastoma models. This demonstrates that optimized ionizable cationic lipid-based nanoparticles provide an

effective and targeted siRNA delivery system, enhancing the efficacy of immunotherapy for improved therapeutic outcomes in glioblastoma patients[61].

Zhou et al. in their study targets glioma cells by co-delivering a phagocytosis checkpoint inhibitor and a STING agonist directly to glioma cells through a nanocapsule named as The Trojan horse. This model has been created by delivering therapeutic agents particularly to glioma cells, ensuring the drugs are released in the tumor microenvironment. The anti-CD47 provides "don't eat me" signal, allowing macrophages to recognize and phagocytose glioma cells, while the STING agonist activates the STING pathway in immune cells, enhancing the production of type I interferons and other cytokines that promote a robust anti-tumor immune response. Anti-tumor immunologic microenvironment enhances phagocytosis of glioma cells and creates positive feedback loop on immune response. In orthotopic glioma models, the combination therapy delivered by the nanocapsule resulted in significant tumor reduction and extended survival compared to single-agent treatments or controls. This study demonstrates that co-delivery of a phagocytosis checkpoint inhibitor and a STING agonist via a Trojan horse nano capsule is a very promising treatment strategy for glioma immunotherapy[62].

Table-2

Study Title	Summary
Microglia as Effector Cells of CD47-SIRPα Axis Disruption Against Glioblastoma	In vitro and in vivo models demonstrated that blocking CD47-SIRPα activates microglia, enhancing their phagocytic activity and significantly clearing tumor cells.
MGMT Unmethylation and High Levels of CD47 and TIGIT in Adult Diffuse Gliomas	The patients with unmethylated MGMT promoter status, combined with high CD47 and TIGIT expression, have a poor prognosis in adult diffuse gliomas.
Intracavity Generation of Glioma Stem Cell–Specific CAR Macrophages	The genetically engineered CAR macrophages target glioma stem cells. in

for Postoperative Glioblastoma Therapy	the tumor cavity post-surgery modified the immune environment, reducing tumor recurrence and improving survival rates in animal models.
Irradiation or Temozolomide Chemotherapy Enhances Anti-CD47 Treatment of Glioblastoma	The irradiation and temozolomide chemotherapy can amplify the effectiveness of anti-CD47 therapy. The combination treatments increased macrophage infiltration and enhanced anti-tumor immune responses, leading to significant tumor reduction and improved survival rates.
An Optimized Ionizable Cationic Lipid for Brain Tumor-Targeted siRNA Delivery and Glioblastoma Immunotherapy	A new lipid nanoparticle system for efficient siRNA delivery to glioblastoma cells. The targeted delivery reduced off-target effects, enhanced apoptosis, and reduced tumor cell proliferation, improving therapeutic outcomes.
Co-delivery of Phagocytosis Checkpoint and STING Agonist by a Trojan Horse Nanocapsule for Orthotopic Glioma Immunotherapy	The delivering of both a phagocytosis checkpoint inhibitor and a STING agonist, the study demonstrated significant tumor reduction and extended survival through enhanced immune responses.

Chapter 7: The Aim of the Study

Glioblastoma multiforme (GBM) is the most common and aggressive type of primary malignant brain tumor[63]. It has a poor prognosis, with an average survival time of 16 to 19 months. GBM diagnosis is typically made using imaging techniques such as magnetic resonance imaging (MRI) and confirmed by biopsy. Treatment usually involves surgery, supported by radiation therapy and chemotherapy. The most decisive

factor for patient survival is surgical resection, but the use of temozolomide chemotherapy along with radiotherapy has been shown to improve the two-year survival rate[64].

GBM is characterized by its rapid growth and infiltrative nature, making it difficult to completely remove surgically. Despite advancements in treatment methods, GBM remains largely incurable and has a high recurrence rate even after treatment, contributing to its poor prognosis[65]. There is a need for alternative treatment methods for GBM because the effectiveness of current treatment options, such as surgery, adjuvant chemotherapy, and radiation therapy, in improving long-term survival is limited. While these treatments can help manage symptoms and extend survival to some extent, they are not curative.

CD47 is a membrane protein belonging to the immunoglobulin superfamily and is known as the "don't eat me" signal. It plays a role in immune evasion by inhibiting phagocytosis, the process by which immune cells engulf and destroy cancer cells. High levels of CD47 expression have been observed in various tumor types, including prostate carcinoma, ovarian cancer, clear cell renal cell carcinoma, non-small cell lung cancer, endometrial carcinoma, breast cancer, bladder cancer, and more. In these cancers, CD47 expression has been associated with adverse clinicopathological features, such as higher tumor grade, lymphovascular invasion, and advanced disease stages. A correlation between the level of CD47 expression and poor prognosis and reduced survival rates has also been established[66], [67], [68], [69], [70], [71], [72].

Between 2004 and 2013, a limited number of studies examined the levels and role of CD47 in GBM. These studies summarized that high levels of CD47 are associated with resistance to radiotherapy and temozolomide, and inhibiting CD47 can suppress tumor growth in vitro. It has been shown that CD47 interacts with SIRPα, calreticulin, and Tenascin C molecules in GBM, and is associated with the PI3K/Akt pathway. Additionally, the combined inhibition of CD47, PDL-1, and VEGF has demonstrated synergistic effects in suppressing tumor growth[73], [74], [75], [76], [77], [78], [79], [80], [81], [82], [83], [84].

Our study aims to contribute to the literature on the role of CD47 in glioblastoma (GBM) by investigating several aspects. Specifically, we intend to compare CD47 levels between high- and low-grade gliomas and, within the high-grade group, correlate these levels with the tumor's invasive characteristics. Additionally, we will investigate the relationship between CD47 expression, patient survival, treatment resistance, and prognosis. While previous studies have identified significant changes in CD47 levels in GBM and outlined the molecular pathways it interacts with at the cellular level, our study seeks to further emphasize the importance of CD47 in tumor infiltration and its impact on clinical outcomes for patients.

CHAPTER 8: METHODOLOGY

8.1 Sample Collection and Preparation

This study involved samples from initial surgeries of patients diagnosed with IDH wild type glioblastoma (GBM) at our institution between 2017 and 2022, before the commencement of their treatment. Stereotactic biopsy samples were excluded. The medical pathology clinic reviewed and confirmed all included samples. Clinical follow-up data were sourced from the neurosurgery and medical oncology departments.

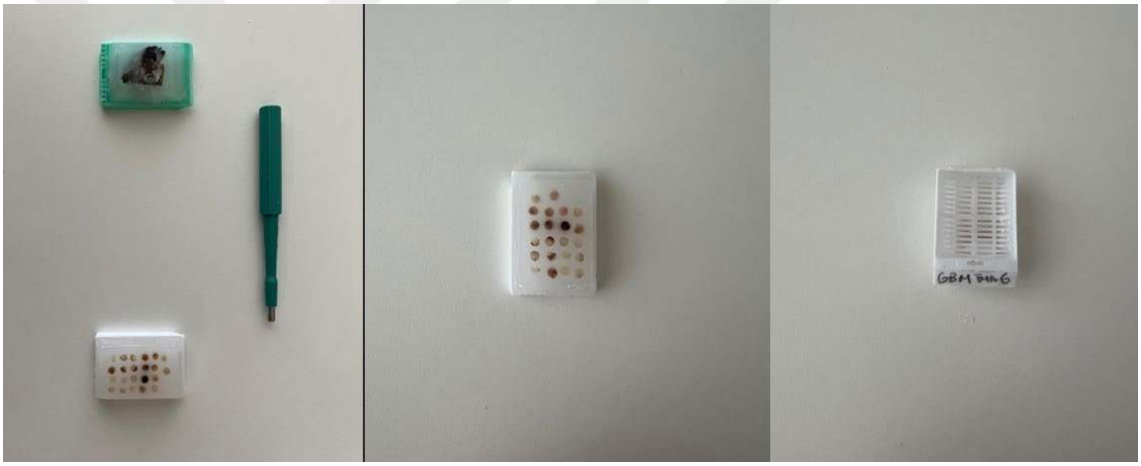
8.2. Tissue Microarray (TMA) Construction

Tissue microarrays are paraffin blocks made by extracting cylindrical tissue core “biopsies” from different paraffin donor blocks and re-embedding these into a single recipient (microarray) block at defined array coordinates. Tissue microarray (TMA) technology is a high-throughput method that enables the simultaneous analysis of multiple tissue samples. This technique involves extracting small cores of tissue from various donor blocks and arranging them into a single recipient block, which can then be examined on a single slide. TMA allows for the analysis of multiple samples simultaneously, conserving valuable tissue specimens. All samples within the array are treated under identical conditions, reducing variability in the results. The method requires smaller volumes of reagents, making the process more resource efficient. The

original tissue blocks remain largely intact, allowing for future analyses. MAs are compatible with various methods, including IHC. Steps are explained below;

1. Each paraffin block has standard microscopic slides that are stained with hematoxylin and eosin. Dr. Ibrahım Kulac with Irem Yenidogan, examined the slides to mark the core of the tumor after which the samples can be arrayed.
2. A tissue microarray instrument is used to acquire a tissue core from the block. This core is then placed in an empty paraffin blocK, the recipient block. The core is placed at a specifically assigned coordinate (X-Y guide), which is accurately recorded, typically on a spreadsheet, such as Microsoft Excel. Every step has been repeated two times for each case (Figure 11-12-13).

GBM_TMA1						
	X					Y
CONTROL-1	X1Y1: B18-01100-B5	X2Y1: B18-01788-D2	X3Y1:B18-02659-A2	X4Y1: B19-02921-A9	X5Y1: B19-04270-A5	
CONTROL-2	X1Y2: B18-01100-B5	X2Y2: B18-01788-D2	X3Y2: B18-02659-A2	X4Y2: B19-02921-A9	X5Y2: B19-04270-A5	
	X1Y3: B18-01282-A1	X2Y3: B18-02281-A7	X3Y3: B18-02910-B2	X4Y3: B19-03725-A4	X5Y3:B19-04311-A3	
	X1Y4: B18-01282-A1	X3Y4: B18-02281-A7	X3Y4: B18-02910-B2	X4Y4: B19-03725-A4	X5Y4: B19-04311-A3	



8.3 Immunohistochemistry (IHC) Protocol

Antibody CD47 Clone: MC0027

Manufacturer: Medaysis

Dilution: 1/120

Automated Protocol (Dako Omnis system):

1. Deparaffinization: The tissue sections are first deparaffinized to remove the paraffin wax, preparing the samples for staining.

2. Antigen Retrieval: The sections undergo antigen retrieval using EnVision FLEX Target Retrieval Solution (TRS) at a high pH, heated to 97°C for 30 minutes. This step is essential to unmask antigens and enhance antibody binding.
3. Antibody Incubation: The primary antibody is applied to the tissue sections and incubated for 30 minutes, allowing for specific binding to the target antigen.
4. Blocking: A blocking step is performed to prevent non-specific binding of the antibody to the tissue, reducing background staining.
5. EnVision FLEX HRP Polymer Application: The sections are then treated with EnVision FLEX HRP (Horseradish Peroxidase) polymer for 20 minutes. This polymer binds to the primary antibody and amplifies the signal.
6. Chromogen Development: The chromogen, EnVision FLEX Substrate Working Solution, is applied for 5 minutes to visualize the antibody-antigen complexes. This reaction produces a visible color indicating the presence of the target antigen.
7. Washing: The tissue sections are washed to remove any excess reagents, ensuring clear and specific staining.
8. Hematoxylin Counterstaining: The sections are counterstained with hematoxylin for 6 minutes. This stains the cell nuclei, providing contrast to the chromogen-stained target antigens.
9. Final Washing: The sections undergo a final wash to remove any residual stain or reagents.
10. Cover Slip Mounting: Finally, the sections are mounted with a cover slip to preserve the staining and allow for microscopic examination.

Control Tissue: Tonsil, placenta, and bladder mucosa were used as controls on each slide. After confirming appropriate staining in positive controls, the assessment was performed. Although the bladder mucosa showed variable results, the squamous epithelium in the tonsil consistently showed strong staining.

IHC Evaluation

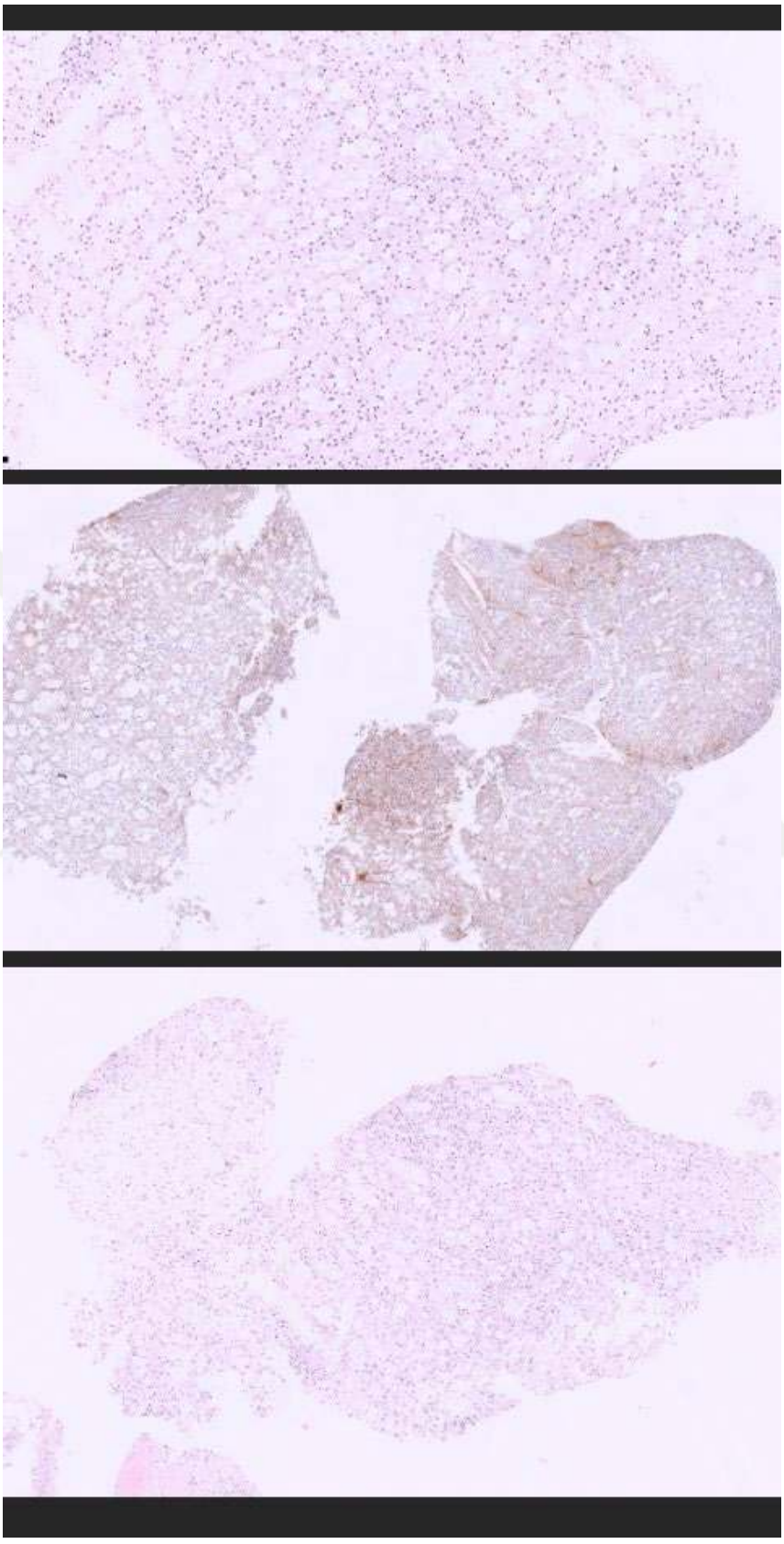
CD47 staining was assessed only in tumor areas, excluding non-tumor regions. Two TMA cores were evaluated for each case, and separate evaluations were conducted for each core and the average of two calculation has been taken into consideration (Figure 14-22.

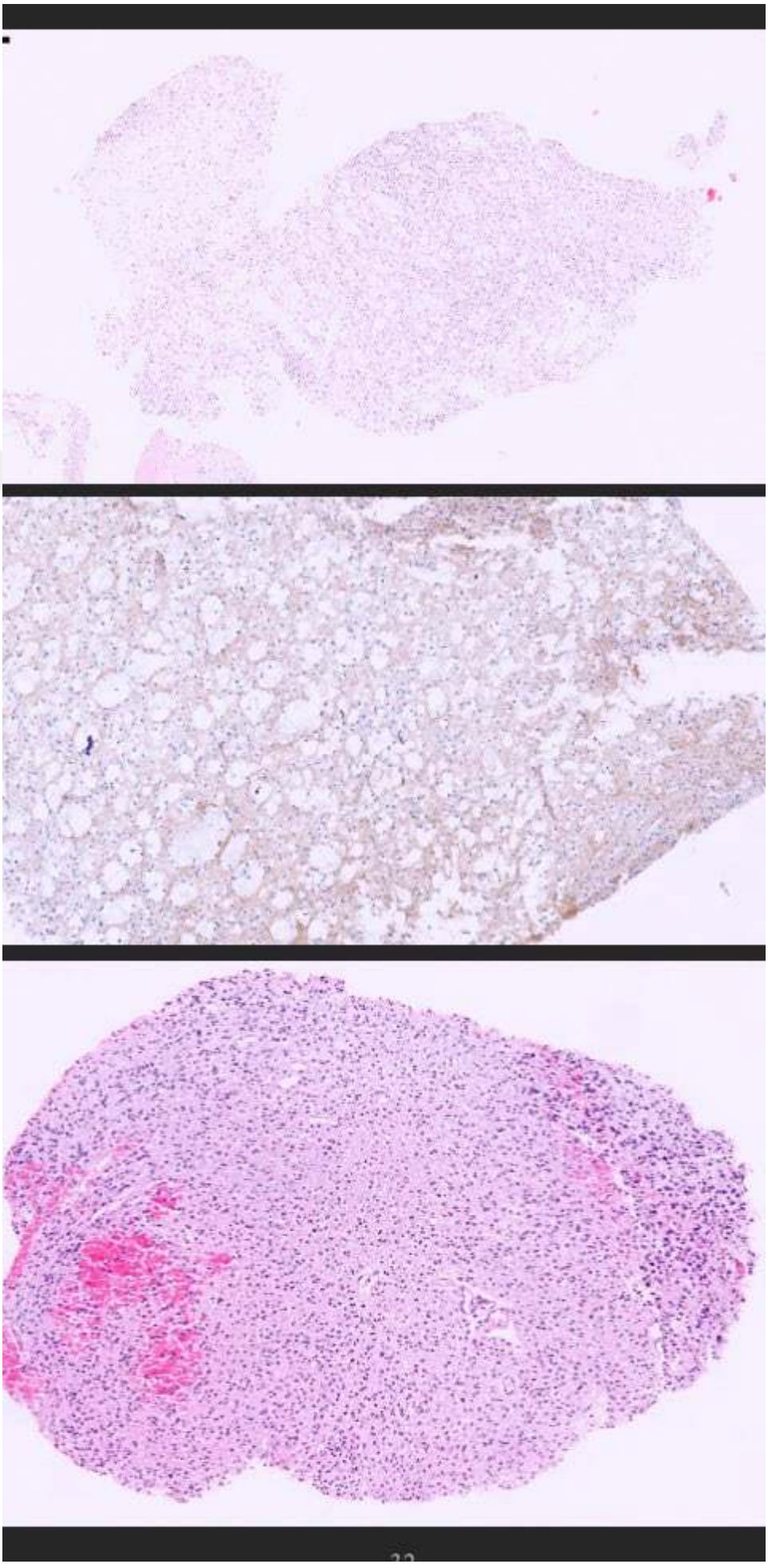
Staining Extent: Scored from 0 to 3

- 0: No staining
- 1: 0-25%
- 2: 25-50%
- 3: >60%

Staining Intensity: Scored from 0 to 3

- 0: No staining
- 1: Weak positivity
- 2: Moderate positivity
- 3: Strong positivity





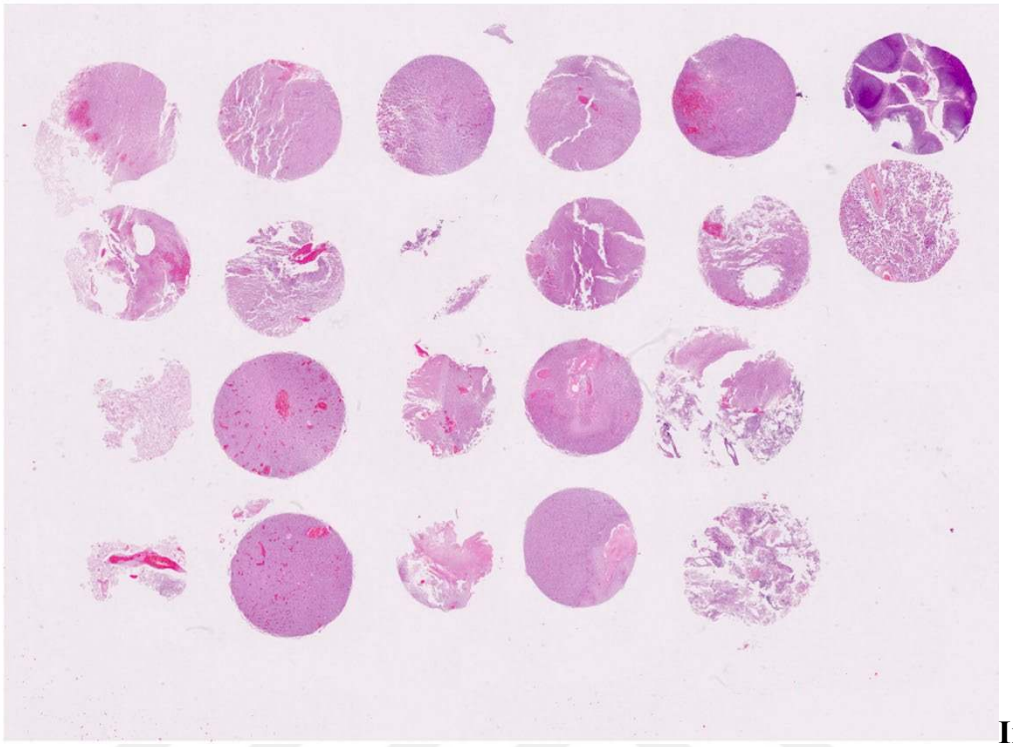


Image Analysis

The stained TMA slides were scanned using a high-resolution slide scanner (Aperio AT2, Leica Biosystems) to produce digital images. Digital images were analyzed using ImageJ software (NIH). The analysis steps included:

- Identifying and selecting individual tissue cores as regions of interest (ROIs)
- Separating DAB chromogen and hematoxylin counterstain using the color deconvolution plugin
- Applying a consistent threshold to the DAB channel to identify positive staining
- Measuring the area of positive staining and expressing it as a percentage of the total ROI area.

8.4 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism (version 9.0). Differences in CD47 expression between the peripheral and core regions of the tumors were evaluated using paired t-tests. Correlations between CD47 expression levels and

clinical outcomes, including patient survival, treatment resistance, and prognosis, were assessed using Pearson correlation coefficients. A p-value of <0.05 was considered statistically significant.

8.4 Ethical Considerations

The Institutional Review Board (IRB) of our institution approved this study. All procedures adhered to the Declaration of Helsinki. Informed consent was obtained from all patients or their legal guardians.

CHAPTER 9: Conclusions

9.1. CD47 Expression and its Impact on Patient Status at Last Control

The comprehensive analysis of the CD47 expression data reveals significant insights into its role in patient outcomes. CD47, a protein involved in various cellular processes, exhibits varied intensity and extent of positivity across the patient samples in this dataset. The average CD47 intensity of positivity is 1.26 on a scale from 0 to 3, while the extent of positivity averages at 1.79, suggesting a moderate level of expression in most cases (Table-3).

One of the most telling indicators of CD47's impact is the status of patients at their last control date. This status provides a clear picture of the patient's condition over time, with categories such as "good, stable" or "ex" (deceased) indicating significant clinical endpoints. A detailed examination shows that patients with higher CD47 positivity often have worse outcomes. Specifically, those with higher intensity and extent of CD47 positivity are more frequently found to be deceased at their last control date. For instance, among the patients who had a CD47 intensity of 2 or higher, a significant proportion were recorded as deceased at their last control date. (Table-3).

Patients categorized as "good, stable" at their last control date generally exhibit lower CD47 positivity, suggesting that lower CD47 levels may be associated with more

favorable outcomes. Conversely, those recorded as deceased ("ex") typically show higher CD47 intensity and extent of positivity.

Protocol Number	CD47 Intensity of Positivity (Scale: 0-3)	CD47 the extent of positivity (Scale: 0-3)	Clinic at Last Visit
1	1	2	ex
2	1	1	ex
3	0	0	good,stabile
4	2	2	good,stabile
5	1	1	good,stabile
6	1	2	ex
7	1	2	ex
8	2	2	unknown
9	2	3	good,stabile
10	1	2	unknown
11	1	1	ex
12	unknown	unknown	ex
13	1	1	unknown
14	1	1	good, stabile

15	0	0	ex
16	1	1	unknown
17	2	2	good,stabile
18	0	0	good,stabile
19	1	1	ex
20	1	3	good, stabile
21	1	2	good, stabile
22	1	3	good, stabile
23	3	2	good, stabile
24	1	1	unknown
25	1	2	unknown
26	1	2	good, stabile
27	1	2	bad,stabile
28	1	1	unknown
29	0	0	unknown
30	2	3	bad,stabile
31	unknown	unknown	good, stabile
32	unkno	unkno	good,

	wn	wn	stabile
33	2	3	unknown
34	2	3	good, stabile
35	1	2	good, stabile
36	2	2	good, stabile
37	1	2	bad,stabile
38	1	3	unknown
39	0	0	unknown
40	0	0	unknown
41	1	1	unknown
42	1	2	unknown
43	1	3	unknown
44	1	3	unknown
45	1	2	unknown
46	1	1	unknown
47	1	3	ex
48	1	2	ex
49	3	3	ex
50	1	1	unkno

			wn
51	2	3	unknown
52	2	2	unknown
53	2	3	unknown
54	1	1	unknown
55	2	2	unknown
56	1	3	unknown
57	2	3	unknown
58	1	1	unknown
59	0	0	ex
60	2	3	ex

9.2. Gender, Age, and Their Relationship with Clinical Outcomes

The demographic analysis of the dataset reveals a higher prevalence of male patients (78 out of 120) compared to female patients. The ages of these patients range from 34 to 82 years, with a mean age of 64.03 years, indicating that older adults are predominantly represented in the study.

When examining the status of patients at their last control date, it becomes apparent that gender and age significantly influence clinical outcomes. Older patients and male patients tend to have a higher frequency of being recorded as deceased at their last control date. Specifically, male patients and those aged 60 and above exhibit a higher

incidence of adverse outcomes, as indicated by the "ex" status (Table-4). These results indicate that there is no statistically significant correlation between age and CD47 intensity or extent.

Protocol Number	**CD47 Intensity of Positivity (Scale: 0-3)**	**CD47 the extent of positivity (Scale: 0-3)**	**Gender**	**Age**	**Clinic at Last Visit**
1	1	2	Male	62	ex
2	1	1	Female	60	ex
3	0	0	Female	72	good,stabile
4	2	2	Male	55	good,stabile
5	1	1	Male	72	good,stabile
6	1	2	Male	68	ex
7	1	2	Male	71	ex
8	2	2	Male	54	unknown
9	2	3	Male	61	good,stabile
10	1	2	Male	56	unknown
11	1	1	Fe	5	ex

			male	8	
12	unknown	unknown	Male	54	ex
13	1	1	Male	62	unknown
14	1	1	Female	70	good, stabile
15	0	0	Male	65	ex
16	1	1	Male	77	unknown
17	2	2	Male	54	good,stabile
18	0	0	Male	66	good,stabile
19	1	1	Female	38	ex
20	1	3	Male	69	good, stabile
21	1	2	Male	81	good, stabile
22	1	3	Female	64	good, stabile
23	3	2	Female	76	good, stabile
24	1	1	Female	54	unknown
25	1	2	Male	69	unknown
26	1	2	Female	72	good, stabile
27	1	2	Fe	7	bad,stabile

			male	1	
28	1	1	Female	69	unknown
29	0	0	Male	75	unknown
30	2	3	Male	64	bad,stabile
31	unknown	unknown	Male	59	good, stabile
32	unknown	unknown	Male	65	good, stabile
33	2	3	Male	73	unknown
34	2	3	Female	71	good, stabile
35	1	2	Male	56	good, stabile
36	2	2	Female	56	good, stabile
37	1	2	Male	72	bad,stabile
38	1	3	Male	67	unknown
39	0	0	Male	34	unknown
40	0	0	Male	74	unknown
41	1	1	Male	72	unknown
42	1	2	Male	41	unknown
43	1	3	Fe	7	unknown

			male	5	
44	1	3	Female	53	unknown
45	1	2	Male	66	unknown
46	1	1	Male	63	unknown
47	1	3	Female	61	ex
48	1	2	Female	61	ex
49	3	3	Female	67	ex
50	1	1	Male	65	unknown
51	2	3	Male	66	unknown
52	2	2	Female	53	unknown
53	2	3	Female	46	unknown
54	1	1	Male	69	unknown
55	2	2	Male	69	unknown
56	1	3	Male	82	unknown
57	2	3	Male	82	unknown
58	1	1	Female	68	unknown
59	0	0	Ma	6	ex

			le	3	
60	2	3	Male	54	ex

9.3. Treatment Efficacy and Long-term Patient Status

The dataset includes detailed information on various treatment modalities, including the use of Temodal, other chemotherapies, and radiotherapy. These treatments show significant variability in both duration and combination, reflecting the diverse therapeutic strategies employed in managing patients with high CD47 expression.

Despite these varied treatment approaches, a substantial number of patients still reached an "ex" status at their last control date. For example, among the 60 patients who received Temodal, the outcomes were mixed, with many still recorded as deceased at their last control. This indicates that current treatment protocols may have limited efficacy in preventing disease recurrence and improving long-term survival for patients with elevated CD47 levels (Table-5).

Patients undergoing multiple regimens, including those combining chemotherapy and radiotherapy, did not uniformly achieve better survival rates. This underscores a critical gap in treatment effectiveness, suggesting that new therapeutic interventions are needed (Table-5).

Table-5

Protocol Number	Temozolomide	Duration of Temodal	Other CT	Clinic at Last Visit
1	unknown	unknown	unknown	ex
2	1	5 regimen	0	ex
3	1	4 regimen	0	good,stabile
4	1	6 months	bevasizumab	good,stabile

5	1	44 regimen	bevasizumab	good,stabile
6	1	4 regimen	0	ex
7	unknown	unknown	unknown	ex
8	unknown	unknown	unknown	unknown
9	1	6 regimen	0	good,stabile
10	unknown	unknown	unknown	unknown
11	1	2 regimen	0	ex
12	unknown	unknown	unknown	ex
13	unknown	unknown	unknown	unknown
14	1	6 regimen	bevasizumab	good, stabile
15	unknown	unknown	unknown	ex
16	unknown	unknown	unknown	unknown
17	1	unknown	bevasizumab	good,stabile
18	1	11 regimen	bevasizumab	good,stabile
19	unknown	unknown	unknown	ex
20	1	unknown	0	good,stabile
21	1	4 regimen	bevasizumab, irinotkean	good,stabile
22	1	unknown	bevasizumab, irinotkean	good,stabile
23	1	4 regimen	bevasizumab, irinotkean	good, stabile
24	unknown	unknown	unknown	unknown
25	unknown	unknown	unknown	unknown
26	1	unknown	unknown	good, stabile
27	unknown	3 regimen	bevasizumab, irinotekan	bad,stabile
28	1	6 regimen	bevasizumab	unknown
29	0	0	0	unknown
30	1	10 regimen	bevasizumab, irinotekan	bad,stabile
31	1	5 regimen	bevasizumab	good, stabile
32	1	1 year	bevasizumab	good, stabile
33	unknown	unknown	unknown	unknown
34	1	5 months	0	good, stabile
35	1	unknown	0	good, stabile

36	1	6 regimen	unknown	good, stabile
37	1	1 regimen	0	bad,stabile
38	unknown	unknown	unknown	unknown
39	unknown	unknown	unknown	unknown
40	unknown	unknown	unknown	unknown
41	unknown	unknown	unknown	unknown
42	unknown	unknown	unknown	unknown
43	unknown	unknown	unknown	unknown
44	unknown	unknown	unknown	unknown
45	unknown	unknown	unknown	unknown
46	unknown	unknown	unknown	unknown
47	1	6 regimen	0	ex
48	1	3 regimen	bevasizumab	ex
49	1	unknown	bevasizumab, irinotekan	ex
50	unknown	unknown	unknown	unknown
51	unknown	unknown	unknown	unknown
52	unknown	unknown	unknown	unknown
53	unknown	unknown	unknown	unknown
54	unknown	unknown	unknown	unknown
55	unknown	unknown	unknown	unknown
56	unknown	unknown	unknown	unknown
57	unknown	unknown	unknown	unknown
58	unknown	unknown	unknown	unknown
59	unknown	4 regimen	bevasizumab, irinotekan	ex
60	unknown	12 regimen	bevasizumab	ex

9.4. CD47 Expression and Chemotherapy / Radiotherapy

The results showed no significant relationship between CD47 expression and treatment modalities.

Table-6

Protocol Number	CD47 Intensity of Positivity (Scale: 0-3)	CD47 the extent of positivity (Scale: 0-3)	Duration of Temodal	Other CT	Duration of other CT	Radiotherapy	Duration of RT
1	1	2	unknown	unknown	unknown	unknown	unknown
2	1	1	5 regimen	0	0	1	2 weeks
3	0	0	4 regimen	0	0	1	2 weeks
4	2	2	6 regimen	bevasizumab	unknown	unknown	unknown
5	1	1	44 regimen	bevasizumab	44 regimen	1	2 weeks
6	1	2	4 regimen	0	0	1	3 weeks
7	1	2	unknown	unknown	unknown	unknown	unknown
8	2	2	unknown	unknown	unknown	unknown	unknown
9	2	3	6 regimen	0	0	1	6 weeks
10	1	2	unknown	unknown	unknown	unknown	unknown
11	1	1	2 regimen	0	0	1	5 weeks
12	unknown	unknown	unknown	unknown	unknown	unknown	unknown
13	1	1	unknown	unknown	unknown	unknown	unknown
14	1	1	6 regimen	bevasizumab	6 regimen	1	6 weeks
15	0	0	unknown	unknown	unknown	unknown	unknown
16	1	1	unknown	unknown	unknown	unknown	unknown
17	2	2	unknown	bevasizumab	unknown	1	unknown
18	0	0	11 regimen	bevasizumab	11 regimen	1	5 weeks
19	1	1	unknown	unknown	unknown	unknown	unknown
20	1	3	unknown	0	0	1	unknown
21	1	2	4 regimen	bevasizumab, irinotkean	1 regimen	1	5 weeks
22	1	3	unknown	bevasizumab, irinotkean	12 regimen	1	unknown
23	3	2	4 regimen	bevasizumab, irinotkean	14 regimen	1	5 weeks
24	1	1	unknown	unknown	unknown	unknown	unknown
25	1	2	unknown	unknown	unknown	unknown	unknown
26	1	2	unknown	unknown	unknown	1	4 weeks
27	1	2	3 regimen	bevasizumab, irinotekan	unknown	1-	3 weeks
28	1	1	6 regimen	bevasizumab	4 regimen	1	3 weeksx2
29	0	0	0	0	0	1	3 weeks
30	2	3	10 regimen	bevasizumab, irinotekan	11 regimen	1	5 weeks
31	unknown	unknown	5 regimen	bevasizumab	unknown	1	5 weeks
32	unknown	unknown	7 regimen	bevasizumab	unknown	1	unknown
33	2	3	unknown	unknown	unknown	unknown	unknown
34	2	3	5 regimen	0	0	1	5 weeks
35	1	2	unknown	0	0	1	unknown
36	2	2	6 regimen	unknown	unknown	1	3 weeks
37	1	2	1 regimen	0	0	1	3 weeks
38	1	3	unknown	unknown	unknown	unknown	unknown
39	0	0	unknown	unknown	unknown	unknown	unknown
40	0	0	unknown	unknown	unknown	unknown	unknown
41	1	1	unknown	unknown	unknown	unknown	unknown
42	1	2	unknown	unknown	unknown	unknown	unknown

43	1	3	unknown	unknown	unknown	unknown	unknown
44	1	3	unknown	unknown	unknown	unknown	unknown
45	1	2	unknown	unknown	unknown	unknown	unknown
46	1	1	unknown	unknown	unknown	unknown	unknown
47	1	3	6 regimen	0	0	1	unknown
48	1	2	3 regimen	bevasizumab	3 regimen	1	unknown
49	3	3	unknown	bevasizumab, irinotekan	unknown	1	6 weeks
50	1	1	unknown	unknown	unknown	unknown	unknown
51	2	3	unknown	unknown	unknown	unknown	unknown
52	2	2	unknown	unknown	unknown	unknown	unknown
53	2	3	unknown	unknown	unknown	unknown	unknown
54	1	1	unknown	unknown	unknown	unknown	unknown
55	2	2	unknown	unknown	unknown	unknown	unknown
56	1	3	unknown	unknown	unknown	unknown	unknown
57	2	3	unknown	unknown	unknown	unknown	unknown
58	1	1	unknown	unknown	unknown	unknown	unknown
59	0	0	4 regimen	bevasizumab, irinotekan	10 regimen	1	0
60	2	3	6 regimen	bevasizumab	12 months	1	unknown

9.5. CD47 Expression and Recurrence

The analysis indicates that there is no statistically significant relationship between CD47 positivity and recurrence or the need for additional surgeries based on the data provide.

Table-7

Protocol Number	**CD47 Intensity of Positivity (Scale: 0-3)**	**CD47 the extent of positivity (Scale: 0-3)**	**Recurrence**	**Second Surgery**	**Third Surgery**
1	1	2	unknown	unknown	unknown
2	1	1	0	0	0
3	0	0	0	0	0
4	2	2	0	0	0

5	1	1	1	0	0
6	1	2	0	0	0
7	1	2	unknown	unknown	unknown
8	2	2	unknown	unknown	unknown
9	2	3	0	0	0
10	1	2	unknown	unknown	unknown
11	1	1	0	0	0
12	unknown	unknown	unknown	unknown	unknown
13	1	1	unknown	unknown	unknown
14	1	1	0	0	0
15	0	0	unknown	unknown	unknown
16	1	1	unknown	unknown	unknown
17	2	2	0	0	0
18	0	0	0	0	0
19	1	1	unknown	unknown	unknown
20	1	3	1	unknown	0
21	1	2	1	0	0
22	1	3	0	0	0
23	3	2	0	0	0
24	1	1	unknown	unknown	unknown
25	1	2	unknown	unknown	unknown
26	1	2	1	1	0

27	1	2	0	0	0
28	1	1	1	unknown	unknown
29	0	0	0	unknown	unknown
30	2	3	1	1	0
31	unknown	unknown	0	0	0
32	unknown	unknown	1	0	0
33	2	3	unknown	unknown	unknown
34	2	3	1	1	0
35	1	2	unknown	unknown	unknown
36	2	2	1	1	0
37	1	2	0	0	0
38	1	3	unknown	unknown	unknown
39	0	0	unknown	unknown	unknown
40	0	0	unknown	unknown	unknown
41	1	1	unknown	unknown	unknown
42	1	2	unknown	unknown	unknown
43	1	3	unknown	unknown	unknown
44	1	3	unknown	unknown	unknown
45	1	2	unknown	unknown	unknown
46	1	1	unknown	unknown	unknown
47	1	3	1	1	0

48	1	2	0	0	0
49	3	3	0	0	0
50	1	1	unknown	unknown	unknown
51	2	3	unknown	unknown	unknown
52	2	2	unknown	unknown	unknown
53	2	3	unknown	unknown	unknown
54	1	1	unknown	unknown	unknown
55	2	2	unknown	unknown	unknown
56	1	3	unknown	unknown	unknown
57	2	3	unknown	unknown	unknown
58	1	1	unknown	unknown	unknown
59	0	0	0	0	0
60	2	3	0	0	0

9.6. Comparison of CD47 between Low- and High-Grade Gliomas

- Low-Grade Glioma (Table-8):
    - Average CD47 Intensity: 0.6
    - Average CD47 Abundance: 1.2
- High-Grade Glioma :
    - Average CD47 Intensity: 1.35
    - Average CD47 Abundance: 1.725

CD47 Intensity is higher on average in high-grade gliomas compared to low-grade gliomas. CD47 Abundance is also higher on average in high-grade gliomas compared to

low-grade gliomas. This suggests that there is an increase in CD47 expression as the glioma grade increases.

Table-8

Case_No	Intensity	Abundancy	Diagnosis
1	1	3	Oligodendroglioma, IDH mutant, 1p19q codeleted, grade 2
2	1	3	Astrocytoma, IDH mutant, grade 2
3	0	0	Astrocytoma, IDH mutant, grade 2
4	0	0	Astrocytoma, IDH mutant, grade 2
5	0	0	Oligodendroglioma, IDH mutant, 1p19q codeleted, grade 2
6	0	0	Astrocytoma, IDH mutant, grade 2
7	0	0	Oligodendroglioma, IDH mutant, 1p19q codeleted, grade 2
8	1	2	Astrocytoma, IDH mutant, grade 2
9	1	1	Astrocytoma, IDH mutant, grade 2
10	2	3	Astrocytoma, IDH mutant, grade 2

CHAPTER 10: Discussion

The relationship of myeloid immune cells with tumor cells is variable, with varying effects of supporting or suppressing cancer cells depending on subtype and microenvironment interactions. One way to inhibit the positive contribution of myeloid cells is by signaling through the cluster of differentiation 47 (CD47)/signal regulatory protein alpha (SIRPα) axis. CD47 is overexpressed in GBM as in many other cancer cells, and therapies targeting CD47/SIRPα are very promising. Interestingly, although

the CD47/SIRPα axis primarily acts through the innate immune system, it also affects the adaptive pathway due to its association with T cells, creating a boost environment. This review suggests that the optimal use of CD47/SIRPα immune checkpoint therapy is an exciting theory in a field like GBM where our hands are tied in terms of treatment. There are many studies on the relationship between CD47 and tumor at the 'micro' level, there are many invitro studies in the literature that support each other, and the common opinion is that the high level of the molecule is directly proportional to the advanced degree of the disease and treatment resistance, and if it is suppressed, it will lead to results in favor of treatment and increase the chance of survival of patients. However, these are all indirect results obtained in the laboratory. Although there are a few animal studies and these show promising results, there are very few of them in the literature. Additional studies are needed to contribute to the acceleration of the initiation of in vivo studies that directly reveal clinical links in a subject where the results are all similar, even though they are performed through many different mechanisms. With our study, we tried to explain the molecular information provided by the laboratory environment by providing a direct link with patient data. Despite the limit in the number of cases, which is the biggest limitation of the study, the direct correlation between the health status of the patients at the last clinical visit and the grade of the tumor was shown. However, a correlation could not be established for treatment resistance and recurrence due to insufficient information obtained during patient follow-up. We need further information and more studies to improve outcomes of GBM.