RESEARCH ARTICLE

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Immunomodulatory Interventions Based on a Bioinformatics Study of *TLR2* in Glioblastoma Multiforme

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Abstract

Objective: One of the most malignant types of tumors with a remarkable ability of recurrence rate and aggressiveness is glioblastoma multiforme(GBM). Anyway, according to the restricted remedies accessible for the treatment of this serious tumor, there is no confident and stable therapeutic strategy. Notably, bioinformatics analysis can detect many effective genes in the diagnosis and treatment of GBM. **Materials and Methods:** Using large-scale data analysis and bioinformatics, we examined *TLR2*'s role in GBM. We analyzed gene expression datasets from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) to recognize disparately expressed diverse genes (DEGs) associated with *TLR2*. A protein-protein interaction (PPI) network was constructed to reveal potential molecular partners and functional enrichment analysis elucidated biological activities. In this account, the correlation and association between *TLR2* expression and the infiltration of immune cells within the tumor microenvironment were investigated. **Results:** Our analysis demonstrated significant differential gene expression patterns in GBM, especially *TLR2*. The PPI network highlighted interactions with key proteins in pathways related to proliferation, invasion, immune evasion, and angiogenesis. Functional enrichment analysis indicated *TLR2*'s involvement in critical signaling processes, including toll-like receptor signaling. Interestingly, *TLR2* expression was strongly associated with the infiltration of immune cells, proposing its performance and function in the tumor microenvironment. **Conclusion:** Understanding *TLR2*'s functions in glioblastoma and other cancers is vital for developing targeted therapies and immunomodulatory interventions, potentially improving clinical outcomes for patients facing these formidable diseases. Further validation and functional studies are needed to confirm *TLR2*'s role in cancer and expand the prospects for combination therapies.

Keywords: Bioinformatics study- *TLR2*- Glioblastoma- GEO- TCGA- GDC

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Introduction

In the central nervous system (CNS), grade IV gliomas, or glioblastomas multiformes, are the most prevalent brain cancer, occurring in 5.36 new cases per 100,000 people[1-3]. There has been some evidence to show that patients with a GBM treated with chemoradiotherapy and maximal surgical excision have a five-year overall survival (OS) between 0.01 and 29.1% [4, 5]. High heterogeneity in a subgroup of GBM is closely correlated with morphological features, molecular alterations, and immunotherapy [1]. Determining the molecular targets for diagnosis and reexamination is essential for both the prognosis of GBM patients and the effectiveness of treatment. Significant molecular biomarkers for GBM are prognostic or therapeutic variables, according to several investigations. Thus, the signaling pathways that are involved in conjunction with bioinformatics research are quite significant. According to this theory, the first line of defense is constituted by Toll-like receptors (TLRs), which identify nonself-molecules and initiate inflammatory reactions [6]. TLRs have thus been regarded as viable targets for cancer treatments [7, 8]. Two classes of TLRs are characterized by the location of the proteins located within the cell and the corresponding molecules corresponding to pathogen-associated molecular patterns

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(PAMPs). *TLR1, TLR2, TLR4, TLR5*, and *TLR6* on the human cell surface form a single group that is primarily responsible for identifying lipids, lipoproteins, and proteins found in microbial membranes. The other category includes *TLR3, TLR7, TLR8*, and *TLR9*, which are only expressed in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes. They are also capable of identifying nucleic acids from microorganisms. The *TLR2* and *TLR4* receptors are also capable of detecting endogenous molecules in response to injury in tissues along with heat-shock proteins such as HSP70, HSP60 Gp96, HSP22, and HSP72, and high-mobility group box-1 proteins. Aside from this, they also recognize additional molecules found in the extracellular matrix (ECM) such as biglycan, tenascin-C, versican, and fragments of extracellular matrix molecules (oligosaccharides of hyaluronic acid and heparan sulfate). Based on the structure of the receptors, TLRs can be considered integral membrane receptors. There are two transmembrane helices in their molecule, one at the N-terminus, which has a leucine-rich repeat motif for ligand recognition, and one at the C-terminus, which is homologous to the signaling domains of members of the IL-1R family of proteins and is known as the Toll IL-1 receptor (TIR). Different signaling routes, such as the plasmatic membrane's canonical signaling pathway, may be presented by cell surface TLRs. During the response to TLRs, myeloid differentiation primary response 88 (MYD88) is recruited to the site, where it activates tumor necrosis factor receptor-associated factor 6- (TRAF6) through a protein complex with nuclear factor-kappa B (NF-B) and recruits tumor necrosis factor receptor-associated factor 6. This process results in the production of proinflammatory cytokines, including interleukin-6 (IL-6), interleukin-1β (IL-1β) and α, tumor necrosis factor (TNF), interleukin-8 (IL-8), and interleukin-18 (IL-18). A cell survival profile is the pathway's ultimate output [9]. The heterodimerization of p65 and p50 subunits constitutes NF-κB canonical activation upon TLR signaling. Following IκB kinase activation, IκB undergoes phosphorylation, ubiquitination, and proteasome degradation, allowing NF-κB translocation to the nucleus and subsequently initiating transcription [10]. A number of these activated transcripts result in the upregulation of positive cell cycle regulators, including c-Myc, which enhances the expression of genes linked to proliferation [11, 12], c-Jun [13], and serum response factor (SRF) [14]. Cyclin D1 is a key player in cellular division and DNA synthesis. route via *TLR2* dimerization with either *TLR1* or *TLR6*. When activated, *TLR2* can combine with *TLR1* and *TLR6* to create a heterodimer complex. By forming heterodimers with *TLR1* or *TLR6, TLR2* broadens its ligand spectrum, which in turn helps the innate immune system identify distinct molecular patterns linked to pathogens [15]. In general, the discovery of new biomarkers could be beneficial in enhancing the clinical results of GBM patients and offering a multimodal strategy. To identify genes unique to tumors and biomarkers that are associated with prognosis, bioinformatics analysis is a well-coordinated tool that may aid in the creation of cancer treatments [16]. Currently, gene transcript expression levels may be determined using microarray and RNA-seq data obtained from the Gene Expression Omnibus (GEO) database. Additionally, technological assistance for tracking mRNA expression and cell function prediction can be obtained [17]. By examining lin7A-silenced data samples, it is verified that high levels of RPL36A and AP1S1 were linked to a poor prognosis and the pathophysiology of GBM [18]. According to Zhou et al., the expression of CEP55 and RRM2 determines the prognosis of GBM [19]. There have been various studies that have used bioinformatics to identify differentially expressed genes during GBM carcinogenesis, however in clinical practice, the prognostic significance of these genes has not yet been recognized for the majority of cases. A more thorough investigation into the identification of many prognostic genes would improve our comprehension of potential treatment targets for GBM, prognostic assessment, and disease surveillance. GBM is a dangerous malignant tumor with a highly aggressive nature, in addition to its infiltrative growth and high recurrence rate [17].Thus, there have been notable advancements in the study and treatment of cancer; nonetheless, the prognosis for individuals with GBM is still dismal, necessitating more investigation into the molecular pathways behind this grave and lethal illness. It is crucial to have an innate immune system because it is the body's first line of defense against infections and cellular stress that can invade the body. As a result of their role in the innate immune system and signaling pathways, toll-like receptors (TLRs), among other components, are crucial for the identification of pathogen- and fecal molecular patterns (PAMPs), damage-induced apoptosis (DAMP), and subsequent inflammatory responses [20-22]. Toll-like receptor 2 (*TLR2*) is a member of the Toll-like receptor family and has drawn a lot of interest due to its versatility in ligand recognition and role in inflammatory reactions [23]. *TLR2* may have a role in oncogenesis and tumor development, as evidenced by the growing body of research linking it to several malignancies [23-25]. It is yet unknown, nevertheless, exactly what roles *TLR2* plays in glioblastoma and how important they could be for the biology of cancer. We have attempted to bridge this information gap by using publicly accessible gene expression datasets from the Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA), two databases that provide publicly accessible gene expression datasets [26, 27]. The findings of our analysis are presented in this scientific article, which also highlights the differential expression of *TLR2*-related genes in GBM. Moreover, we developed a network of protein-protein interactions (PPI) as a way to identify partners for *TLR2* and to explore its molecular potential, and a functional enrichment analysis was used to investigate the biological functions of *TLR2*, as well as how *TLR2* expression is related to the infiltration of immune cells in the tumor microenvironment [28]. Gaining more insight into the relationship between *TLR2* and glioblastoma and the possible implications for the biology of cancer may be crucial for the creation of targeted and interventional immune regulatory medicines. Comprehending *TLR2*'s function in GBM might eventually result in improved

therapeutic approaches and improved clinical results for individuals suffering from this difficult illness. By applying bioinformatics techniques, we hope to investigate *TLR2*'s function in glioblastoma and determine its significance to this malignancy.

Materials and Methods

This research was accomplished according to the ethical number: IR.SEMUMS.REC.1402.035, Semnan University of Medical Sciences and Guilan University of Medical Sciences.

Patient Samples

To obtain information on mRNA expression in gliomas, we have used the Gene Expression Omnibus (GEO) database [17] and the TCGA-GDC portal [20] (as part of the Cancer Genome Atlas project). A total of 100 samples were collected for GSE4290 (platform = GPL570), including 77 glioblastoma and 23 control samples. GSE50161 (platform = GPL570) included 34 glioblastoma samples and 13 samples from healthy individuals who did not develop glioblastoma. The TCGA database (https: //portal.gdc.cancer.gov/) contains 156 samples from glioblastoma and 5 samples from normal tissues. The total number of glioblastoma samples was reduced to 154 after removing duplicate samples.

Identification of differentially expressed genes

We normalized and log2transformed all raw expression data from the GEO database. A hidden batch effect was also removed and two datasets were combined using the sva package [21] (V 3.45.0) in R software. By using the R limma package [22] (V 3.53.3), differentially expressed genes (DEGs) were screened using the cutoff criteria of $|\log2FoldChange| \geq 1.5$ and adj. p-value < 0.01.

Data from the GDC portal RNA-Seq was normalized using TMM [26] and VOOM [29] algorithms in the R package GDCRNATools [23]. DESeq2 [25] (V 1.37.0), was used to analyze expression data for RNA between primary tumors and normal solid tissues. Statistically significant DEGs were selected by the following thresholds: |log2FoldChange| >1.5 and FDR 0.01, respectively. In R, a volcano plot was generated based on the analysis of DESeq2 data using EnhancedVolcano. The volcano plot showed the relationship between fold change and the negative log of the FDR.

Construction of protein-protein interaction (PPI) network

We created the PPI (Protein-Protein Interaction) network, which illustrates the connections between proteins, using GeneMANIA [30], a Cytoscape [31] plugin. We used GeneMANIA, a platform that predicts genetic interactions, protein-DNA interactions, and interactions between genes; additionally, it analyzes pathways, inspects gene and protein expression, analyzes protein domains, and provides phenotypic profiles to help us better understand gene-gene interactions. As a result of the network structure analysis, the linkages between proteins and their interacting partners may be determined with network structure visualization.

Functional enrichment analysis

To determine the extent to which functional and pathway information was enriched for mRNA targets in PPI, the clusterProfiler package [28] in R was used to analyze gene ontology information (GO) [26]. In the PPI network, ClusterProfiler detects enriched genes using hypergeometric distribution tests such as groupGo, enrichGO, and enrichKEGG, which are based on the hypergeometric distribution. Functional annotations had a p-value less than 0.05, and a p-value less than 0.05 was considered statistically significant.

Immune Infiltration Analysis

The connection between the expression levels of our final target genes and the quantity of six immune cell types—B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells—was evaluated using TIMER [30] (version 2.0; https://cistrome.shinyapps.io/timer). The TIMER method was used to assess GBM tumors for connections between immune infiltration and target gene expression. The Spearman's rank correlation test was used to determine P-values < 0.05 and $-1 < R < 1$ as statistically significant thresholds.

Results

Investigation of TLR2

In this study, we used two microarray datasets from GEO on the same platform from GBM patients for analysis. It was first necessary to log2 transform the raw expression data using the R software. We could remove the hidden batch consequences after merging the two datasets using the ComBat function included in the R SVA package (Figure 1). Importantly, based on the microarray data that we collected, we employed differential expression investigation and analysis using the R Limma package, which returned 1966 variables (upregulated genes = 832, downregulated genes = 1134) that had statistical significance based on the results (Figure 2, A).

The second step in the analysis of the expression of genes was the analysis of RNAseq data collected from the TCGA. With the help of the DESeq2 package in R, it was possible to identify DEGs between primary tumors and normal solid tissues from TCGA_GBM data. There were 3240 DEGs (upregulated genes were 1608 and downregulated genes were 1632). Based on the criteria $|\log2FoldChange| > 1.5$ and FDR < 0.01 we determined the DEGs (Figure 2, B). Following this, the results of the expression analysis performed in GEO and TCGA were combined (Figure 3). Our next step was to investigate *TLR2* further based on our results of the expression analysis of the GEO and TCGA_GBM data to learn more about this protein (Figure 4).

Construction of PPI network related to TLR2

TLR2 protein-protein interaction (PPI) networks were generated using the GeneMANIA plugin in Cytoscape. Each edge of the composite network is weighted according to its individual sources of data. The PPI network consisted of 21 nodes and 251 edges in total. According to the results of the protein-protein interaction analysis, the

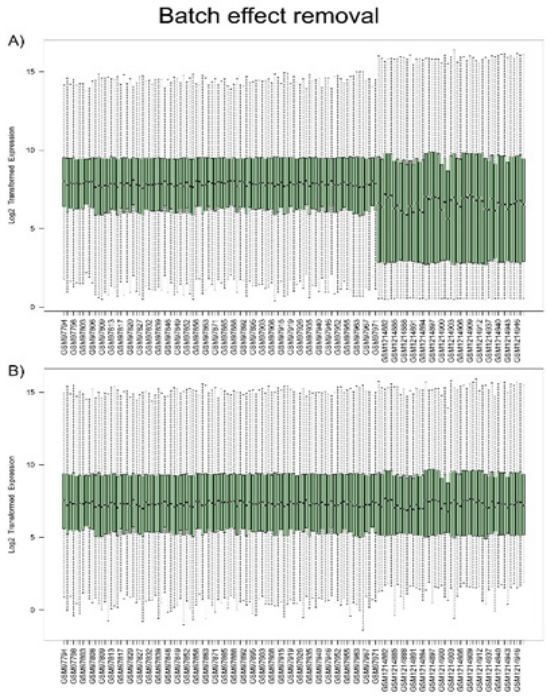

Figure 1. It is the Box Plots that Indicate the Main Overall Expression Profiles of Two Affymetrix Gene Chips (A) after they have been normalized by the normalized quantiles method, and (B) after batch effects have been deleted by the ComBat method using the SVA package in R.

top 20 proteins that are related to *TLR2* include *CD14, TLR1, CXCR4, TLR6, EGFR, LY96, SAA1, TIRAP, TLR8, SIGIRR, TLR10, TLR5, CLEC7A, TLR7, IRAK3, IRAK4, MYD88, TOLLIP, SFTPA1* and *TLR4* (Figure 5).

Functional enrichment analysis of TLR2 PPI network

To analyze and investigate the main biological performance and activity and also the signaling mechanism related to the *TLR2* PPI network, a Gene Ontology (GO) enrichment analysis has been performed. There is also a cnetplot associated with each analysis separately showing the role that each gene plays in the different pathways.

To analyze *TLR2* PPI network proteins, GO analysis was accomplished and applied. According to Figure 6, the top three GO findings in terms of biological process (BP) have a direct and impressive correlation with the "MyD88-dependent TLR signaling pathway", "pattern identification receptor signaling pathway", and importantly, the "TLR signaling pathway".

Based on molecular performance (Molecular function(MF), the top three GO findings illustrate that these particular genes were enriched in the activities of "NAD+ nucleosidase", "hydrolase activity, hydrolyzing N-glycosyl compounds", and "Toll-like receptor binding" (Figure 7).

According to the top three GO findings for cellular

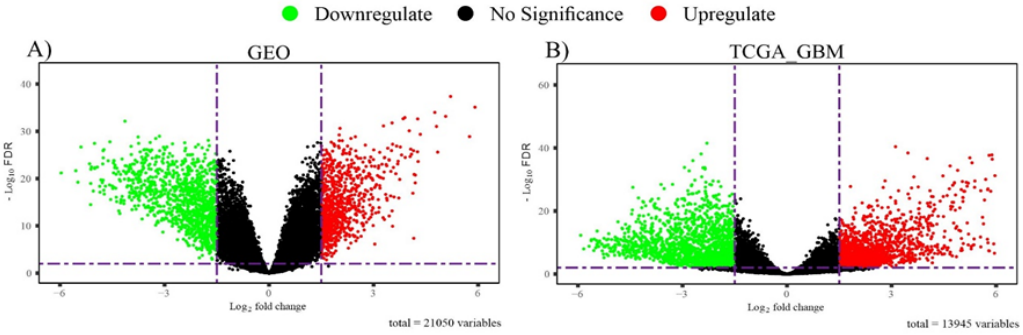

Figure 2. (A) Volcano plots for differentially expressed genes in GEO, (B) differentially expressed genes in TCGA_GBM. The graph shows down expression and up expression as green and red points, respectively. "EnhancedVolcano" was used to generate volcano plots.

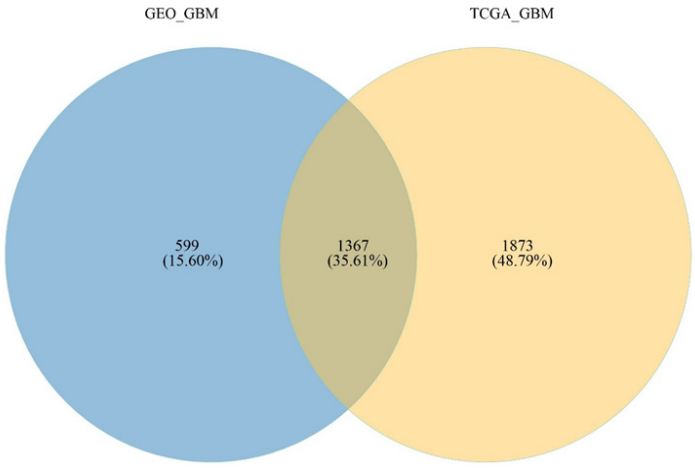

Figure 3. The Venn Diagram Shows the Result of Combining the Results from GOE and TCGA Expression Analyses in One Diagram.

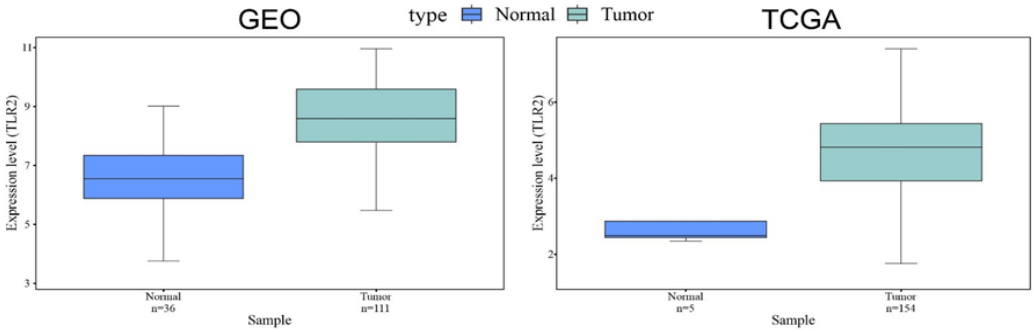

Figure 4. Variations in TLR2 Transcription Levels between Normal and Tumor Samples in the GEO and TCGA Databases.

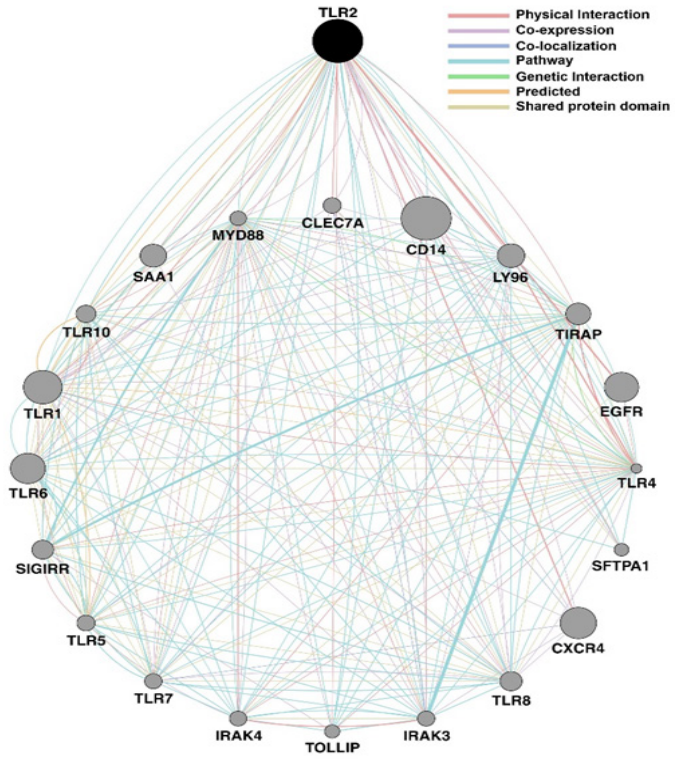

Figure 5. This Picture Indicates the Protein-Protein Interaction(PPI) network of TLR2 in GBM.

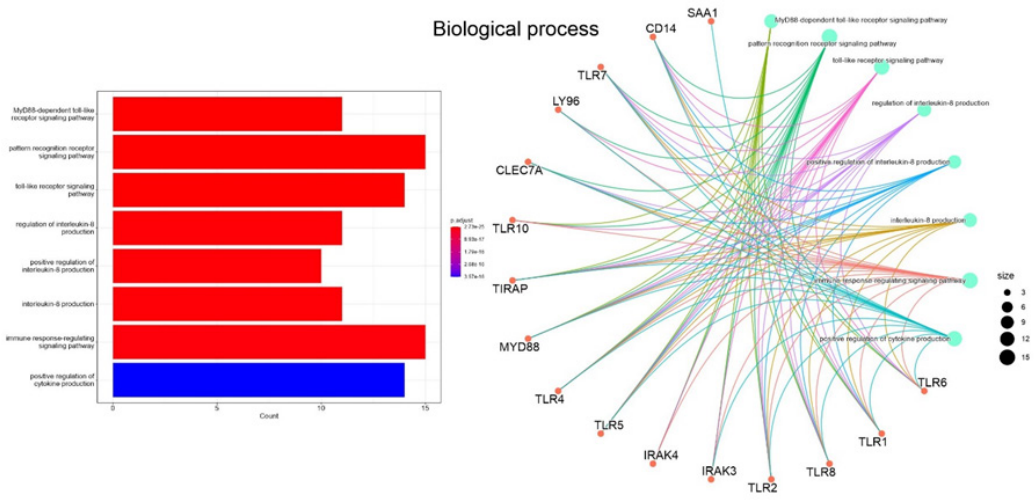

Figure 6. The Results of Biological Process Related to Gene Ontology Analysis.

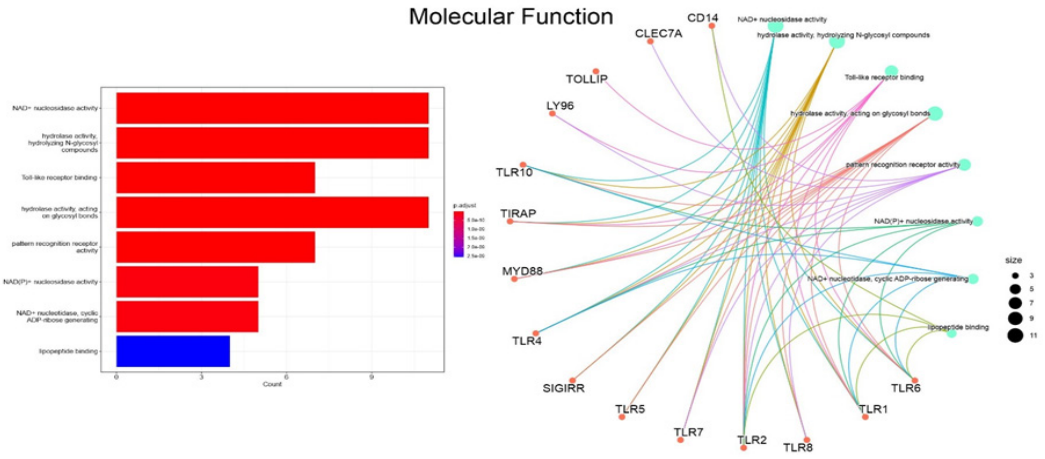

Figure 7. The Results of Molecular Function Related to Gene Ontology Analysis.

component (CC), most genes belong to "endocytic vesicle", "phagocytic vesicle", and "membrane raft" (Figure 8).

TLR2 Expression is strongly associated with and related to the Immune Infiltration

Immune reactions are one of the hallmarks of cancer. A solid tumor usually contains lymphocytes, macrophages,

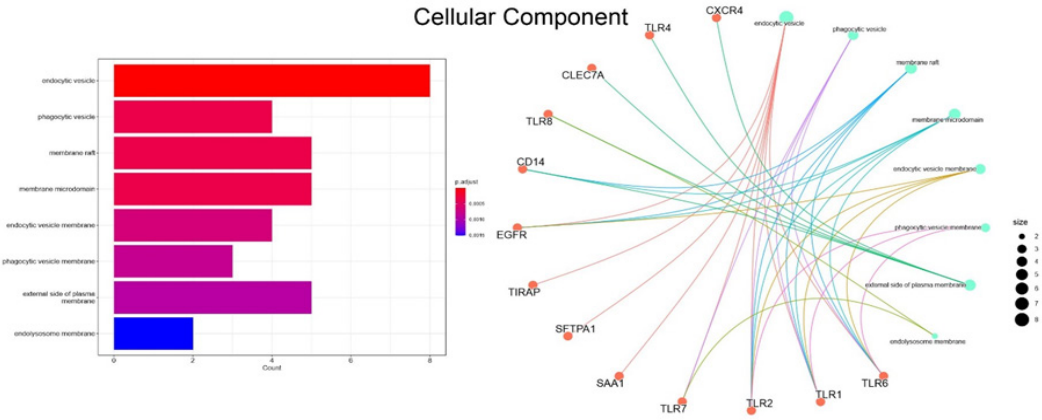

Figure 8. The Results of Cellular Components Related to Gene Ontology Analysis.

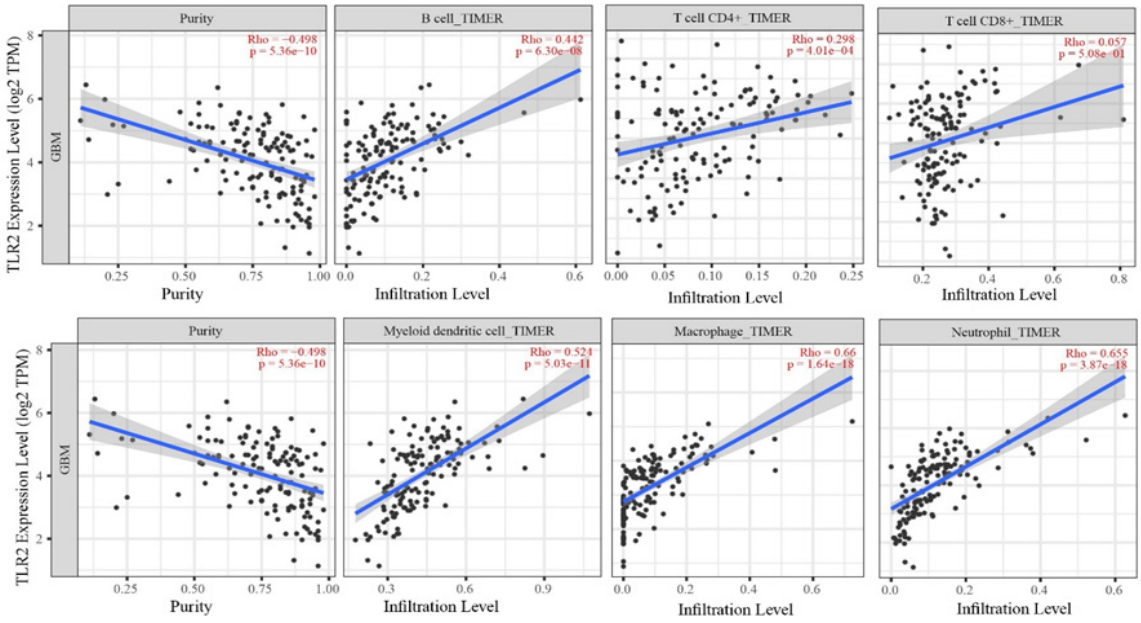

Figure 9. The Immune Infiltration Analysis.

neutrophils, dendritic cells (DCs), and other immune cells. Chronic inflammation is caused by cell infiltration. The accumulation of data has demonstrated that local inflammation plays a powerful role in the development of cancer. Medical researchers are currently exploring ways to use this knowledge to develop therapies that target the immune reaction in cancer patients. By targeting the immune reaction, scientists hope to reduce inflammation, improve immune response, and ultimately reduce cancer progression.

According to TIMER, *TLR2* is associated with and correlated to immune cell infiltration levels. In Figure 9, the final results and data illustrate and describe that *TLR2* expression was strongly associated with T cell CD4+ (Rho = 0.298, p = 4.01e−04), Macrophage (Rho = 0.66, p = 1.64e−18), B cell (Rho = −0.442, p = 6.30e−08), Myeloid dendritic cell (Rho = −0.524, p = 5.03e−11) and Neutrophil (Rho = 0.655, p = 3.87e−18) infiltration. Conversely, no association and significance were observed between RCAN1 expression and CD8+ T cells (Rho = 0.057, p = 5.08e−01).

Discussion

Among the most aggressive and deadly brain cancers, glioblastoma (GBM) has limited treatment options. There is increasing evidence that Toll-like receptor 2 (*TLR2*), an important component of the innate immune system, may play a role in the development and progression of cancer.

TLR2 in Glioblastoma: We found that *TLR2* genes were significantly differentially expressed in glioblastoma. There is an upregulation of 832 genes and a downregulation of 1134 genes in a complex network of molecular events that contribute to the aggressive nature of GBM. In the PPI network, CD14, TLR1, CXCR4, EGFR, MYD88, and TLR4 are the main genes that interact with *TLR2*. Various cellular processes can be affected by these interactions, including proliferation, invasion, immune evasion, and angiogenesis. Furthermore, functional enrichment analysis revealed that the *TLR2*-related network is involved in important biological processes, including MyD88-dependent toll-like receptor signaling pathways, protein recognition receptors, and signaling pathways of toll-like receptors. These pathways are known to regulate innate immune responses and are implicated in cancer development and progression. There is an enrichment of these pathways in the *TLR2* network, consistent with its potential immunomodulatory role in GBM, where the tumor microenvironment plays an important role in disease progression.

TLR2 in Other Cancers: Although this study focuses on GBM, *TLR2* plays an important role in many types of cancer. Increasing evidence suggests that *TLR2* is also involved in the pathogenesis of other cancers. Activation of *TLR2* has been shown to promote proliferation, migration, and invasion of breast cancer cells by activating the NF-κB and MAPK signaling pathways. There is an association between *TLR2* expression and tumor growth and metastasis in colorectal cancer. Additionally, *TLR2*-mediated signaling is implicated in tumor progression in cancers such as lung cancer and melanoma. As a result of these findings, it can be concluded that *TLR2* plays multifaceted roles in cancer biology and has the potential to be a therapeutic target for different types of cancer.

Immunomodulatory Role of *TLR2*: Tumor growth and progression are strongly influenced by the immune microenvironment. *TLR2* expression and immune cell infiltration were significantly correlated in our analysis of immune infiltrates in GBM. A positive correlation was found between *TLR2* expression and immune cell subtypes such as CD4+ T cells, macrophages, and neutrophils, suggesting that *TLR2* may regulate the recruitment and activity of these receptors. Immune cells in the tumor microenvironment. However, a negative correlation was observed between B cells and myeloid dendritic cells, suggesting that *TLR2* may regulate the immune response in GBM in a complex manner.

Therapeutic Implications: It is confirmed that *TLR2* has a significant association in the pathology of many different malignancies, comprising glioblastoma, proposing that it could be used as a therapeutic target. A strategy to inhibit tumor growth and enhance anti-tumor immune responses could be developed through the manipulation of *TLR2* signaling. With limited treatment options available for GBM, targeting *TLR2* in combination with existing therapies may provide a novel approach to improve patient clinical outcomes.

Limitations and Future Directions: Although our bioinformatics analysis provides valuable insights into the role of *TLR2* in glioblastoma, we must acknowledge several limitations. Although this study is based on publicly available information, there may be some differences in the methods used to collect, process, and conduct experiments. It is important to note that the in silico nature of the analysis limits the ability to confirm a causal relationship between *TLR2* and other genes or pathways identified in the study. Further validation of these bioinformatics analysis results using in vitro and in vivo glioblastoma models is required in the future. To gain a more complete understanding of how *TLR2* functions in cancer biology, functional studies can be conducted to study the effects of modulating *TLR2* expression or activity in tumor cells and tumor microenvironment. Additionally, further research into the interactions between *TLR2* and other receptors and immune pathways may uncover new targets for the development of combination cancer therapies.

In conclusion, the results of our bioinformatic analysis indicate that *TLR2* may play an important role in the pathogenesis of glioblastoma, influencing many biological processes and interacting with key proteins in the tumor microenvironment. The findings of our study also suggest that *TLR2* may be important in other forms of cancer, thus expanding its potential use as a therapeutic target. It may be possible to develop novel strategies for improving clinical outcomes in GBM and other malignancies by understanding the multifaceted roles of *TLR2*.

Author Contribution Statement

AAS and AR conceived and designed the study and

also wrote and edited the article, SM, and SEN performed the bioinformatics experiments and wrote the manuscript. FN, SHYCH, BY, and ME accompanied many other parts of the manuscript, including data validity, writing, and scientific investigation. All authors read the manuscript comprehensively and confirmed the paper's final version.

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It is part of an approved student thesis for a PhD.

The project has been approved by Semnan University and Guilan University of Medical Sciences. The ethical code for this project is IR.SEMUMS.REC.1402.035.

Conflict of interest

No conflict of interest is observed for the authors.

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