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NEÜ YAYINEVİ

Investigation of Toll-Like Receptor Family Expression in Glioblastoma: A Comparative Analysis of qPCR and Cell Culture

Glioblastomada Toll-Benzeri Reseptör Ailesi Ekspresyonunun Araştırılması: Karşılaştırmalı Bir qPCR ve Hücre Kültürü Analizi

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Öz

Amaç: Glioblastoma yetişkinlerde en sık görülen ölümcül beyin kanseridir. Toll-benzeri reseptörler, patojen tanıma ve doğal bağışıklığın aktivasyonu ile ilişkili 10 reseptörden (Toll-benzeri reseptör 1-10) oluşan hücre yüzey reseptörleridir. Ancak, çelişkili sonuçlar içermekle birlikte bazı çalışmalar Toll benzeri reseptör ekspresyonunun, glioblastoma dahil bazı tümörlerde, kanser hücresi proliferasyonu ve ilerlemesi ile ilişkili olabileceğini göstermiştir. Bu nedenle, bu çalışmada literatürde ilk kez glioblastomada Toll-benzeri reseptörlerin on üyesinin tamamının ekspresyon profilinin araştırılması amaçlandı.
Hastalar ve Yöntem: 2018 yılı Ocak ve Aralık ayları arasında tanı alan 25 glioblastoma hastasına ait formalinle fikse edilmiş parafine gömülmüş dokularda Toll-benzeri reseptörlerin mRNA ekspresyonu kantitatif gerçek zamanlı polimeraz zincir reaksiyonu kullanılarak değerlendirildi. Ayrıca her bir Toll-benzeri reseptörün ekspresyonu, beş farklı insan glioblastom hücre hattı (T98G, U87-MG, U373, LN18 ve A172) kullanılarak hücre kültürü analizi ile araştırıldı.
Bulgular: Glioblastoma grubunun formalinle fikse edilmiş parafine gömülmüş dokularında kantitatif gerçek zamanlı polimeraz zincir reaksiyonu analizi ile Toll-benzeri reseptör 1, Toll-benzeri reseptör 6 ve Toll-benzeri reseptör 7 mRNA düzeyleri anlamlı olarak arttığı (her biri, $p<0.001$), Toll-benzeri reseptör 4 ve Toll-benzeri reseptör 10 düzeylerinin ise kontrol grubu ile kıyaslandığında anlamlı olarak azaldığı görüldü (sırası ile, $p=0.023$, $p<0.001$). Ek olarak, Toll-benzeri reseptör mRNA ekspresyon profilleri farklı hücre hatları arasında farklılık sergiledi.
Sonuç: Çalışmamızda birçok Toll-benzeri reseptör üyesi glioblastoma mikroçevresinde farklı ekspresyon düzeyi gösteriyor ve onu farklı şekilde etkiliyor gibi görünüyordu. Glioblastomada her bir Toll-benzeri reseptörün endojen protein seviyesini doğrulamak, glioblastomanın patogenezi ve prognozu üzerindeki kesin rollerini netleştirmek ve yeni hedef tedavilere ışık tutmak için daha kapsamlı çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Glioblastoma, hücre hatları, hücre kültürü, real-time PCR, toll-benzeri reseptör

Abstract

Aim: Glioblastoma is the most frequent, and fatal brain cancer in adults. Toll-like receptors are cell surface receptors comprised of 10 receptors (Toll-like receptor 1−10) related to triggering innate immunity by recognizing pathogens. However, some studies suggested that the expression of Toll-like receptors might be related to cancer cell proliferation and progression in some tumors including glioblastoma with some contradictory results. Thus, we aimed to investigate all ten members of the Toll-like receptor expression profile in glioblastoma for the first time in the literature to contribute additional data to the literature.
Patients and Methods: Quantitative real-time polymerase chain reaction was applied to formalin-fixed paraffin-embedded tissues of 25 glioblastoma patients, diagnosed between January and December 2018, to evaluate the mRNA expression of Toll-like receptors. Also, the expression of each Toll-like receptor was investigated by cell culture analysis using five different cell lines of human glioblastoma (T98G, U87-MG, U373, LN18, and A172). The results were compared statistically.
Results: Toll-like receptor 1, Toll-like receptor 6, and Toll-like receptor 7 mRNA levels were significantly increased in the formalin-fixed paraffin-embedded tissues of the glioblastoma group ($p<0.001$, each) whereas the expression of Toll-like receptor 4 and Toll-like receptor 10 was downregulated compared to the control group ($p=0.023$, $p<0.001$, respectively), by quantitative real-time polymerase chain reaction analysis. Additionally, Toll-like receptor mRNA expression profiles differed among the cell lines.
Conclusion: In our study, many Toll-like receptor members seemed to display different expression level in the glioblastoma microenvironment and affect it diversely. Further comprehensive studies are required to confirm the endogenous protein level of each Toll-like receptor in glioblastoma, to clarify their precise role in the pathogenesis and prognosis of glioblastoma, and to shed light on new target therapies.

Keywords: Glioblastoma, cell lines, cell culture, real-time PCR, toll-like receptor

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INTRODUCTION

Glioblastoma (GBM) is a grade 4 glioma according to World Health Organization (WHO) classification that is the most frequent and lethal primary central nervous system cancer in adults (1-3). It usually shows recurrence and resistance to the current therapies of surgery, radiotherapy, and chemotherapy (4-5). Therefore, in terms of discovering more effective therapies to prolong survival, targeting some molecules mainly in the tumor microenvironment that may act in the pathogenesis and the prognosis of GBM are being investigated intensively in the literature. Immunotherapy, targeting Toll-like receptors (TLRs) and using their receptor agonists is one of the most attractive treatments for GBM.

TLRs are cell surface receptors that are fundamentally related to pathogen recognition and activation of innate immunity. The human TLR family comprises 10 receptors (from TLR1 to TLR10, numerically) (6). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are present on the cell membrane and bind to particles of microorganisms. TLR3, TLR7, TLR8, and TLR9 are located in endosomes and activated by nucleic acids of dying cells and microorganisms (6).

In addition to the main functions of immunity, some studies have claimed that expression of various members of the TLRs family might be associated with tumor cell proliferation and progression in many neoplasms (7-9). There are only a few studies in the literature regarding the expression of TLRs, mostly focused on TLR2, TLR4, and TLR9, that have reported GBM initiates the innate immune system by activating TLRs (6, 10). Through binding its ligand, TLRs have been displayed to activate intracellular cascades to stimulate downstream signaling that plays contradictory roles as either tumor progression or suppression (6). Thus, we aimed to investigate the expression profile of each member of TLRs in GBM in a single study for the first time in the literature and contribute additional data about TLRs in GBM to the literature, to assist the development of novel targeted immunotherapies.

PATIENTS AND METHODS

FFPE GBM tumor samples

After obtaining ethics committee approval from the Institutional Review Board of Bozok University (2019/04), 25 formalin-fixed and paraffin-embedded (FFPE) tumor samples of GBM diagnosed in the Pathology Department of Istanbul Yeni Yuzyil University Gaziosmanpasa Hospital and Pathology Department of Bozok University between January and December 2018 were examined. Clinicopathologic parameters were obtained from patients' records for each sample. Non-tumoral tissue samples of 8 patients diagnosed with hematoma were used as a control group. Confirmation of histopathological diagnosis and grading was performed according to the WHO classification.

Initially, FFPE tissue block samples were stained with Hematoxylin-Eosin (H&E) and evaluated under a light microscope to select samples. Tissue containing more than 90% tumor was selected. FFPE tissue samples for each patient and control were cut into 10-µm sections. Necrotic and bleeding areas were not included in the study.

Cell Culture

Human GBM cell lines of T98G, U87-MG, U373, LN18, and A172 (ATCC, USA) and Wi38 human fibroblast cell line (ATCC, USA) were used for this study. All cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 2mM L-glutamine (Gibco, USA), penicillin (20 units/ml) and streptomycin (2 µg/ml) (Gibco, USA) at 37^{O}C in a humidified CO_2 incubator (NuAire, USA) containing 95% air and 5% CO_2. Cells were monitored daily and passaged when they reached 80% confluency.

RNA isolation and complementary DNA (cDNA) synthesis

All cells were passaged with trypsin, stained with Trypan Blue Dye, and counted with a Neubauer cell counting chamber. Cells were seeded at 3x105 cells/well in a 6-well tissue culture plate and incubated for 24 hours in a 2 ml medium. At the end of the incubation, the medium was removed, and cells were washed with cold PBS for 1 minute. After PBS removal, 500 µl Trizol reagent was added to cells in each well, and suspension cells were transferred to a microcentrifuge tube. Total RNA isolation from cells was performed via Trizol reagent (Roche Diagnostic, GmBH, Mannheim, Germany) according to the manufacturer's instructions.

Total RNA isolation from tumor and control tissues was carried out by using a High Pure FFPET RNA isolation kit (Roche Diagnostic, GmBH, Mannheim, Germany) by the manufacturer's protocol. The quality and concentration of isolated RNAs from both cells and tissues were determined with Nanodrop (Thermo, USA). RNAs were kept at -80^{O}C until cDNA synthesis. cDNA synthesis from tissue, control, and cells was

performed by using the "OneScript Plus cDNA synthesis kit" (Abmgood, Canada) according to the manufacturer's instructions. cDNAs were stored at -80^{O}C until a quantitative real-time polymerase chain reaction (qPCR) experiment.

Determination of TLR gene expression with quantitative real-time polymerase chain reaction (qPCR)

The gene expression profiling of TLR 1-10 was performed by using specific primers and Universal Probe Library probes on Light Cycler 480 real-time PCR system (Roche, Germany). Relative gene expression profiles of TLR 1-10 were calculated using the β-actin housekeeping gene as a reference. All primer and probe set for TLR 1-10, and β-actin were summarized in Table 1. Totally, 20 µl reaction mixture was prepared for TLRs and β-actin as follows: 1 µl from primer-probe set, 4 µl from TaqMan Master Mix, 2 µl cDNA, and 13 µl molecular grade water. PCR reaction conditions were set as 1 cycle for 10 minutes at 95^{O}C, 45 cycles for 10 seconds at 95^{O}C, for 30 seconds at 60^{O}C, and for 1 second at 72^{O}C. For each run, DNase- and RNase-free water included mixture was loaded for no template control (NTC). Each reaction was performed in triplicate. TLR1-10 expressions were calculated via Light Cycler 480 software after normalized to β-actin.

Statistical Analysis

The ΔΔCT method was applied to detect the expression profiles of TLRs. The statistical analysis was examined by "Statistical Package for Social Sciences" (SPSS) version 22 (SPSS Inc., Chicago, IL). The Shapiro–Wilk test was used to check the normal distribution. For comparing the two groups

Table 1. Primers and UPL probes used for real-time gene expression analysis (5'→3')

Primer sequences	UPL number
TLR1	
CCTAGCAGTTATCACAAGCTCAAA (Forward)	
CCTTGGGCCATTCCAAATA (Reverse)	#79 (04689020001)
TLR2	
GGCCAGCAAATTACCTGTGTG (Forward)	
AGGATCAGCAGGAACAGAGC (Reverse)	#56 (04688538001)
TLR3	
GTGGCCCTTAAAAATGTGGA (Forward)	
GTGTTTCCAGAGCCGTGCTAA (Reverse)	#151 (04694376001)
TLR4	
TCATTGTCCTGCAGAAGGTG (Forward)	
TCC CAC TCC AGG TAA GTG TT (Reverse)	#62 (04688619001)
TLR5	
TGAGGGACTTTCTCATCTTCAAGT (Forward)	
CCTTAATGCAGTCAGATGGCTA (Reverse)	#31 (04687647001)
TLR6	
TTTGGATTTATCTCATAATCAGTTGC (Forward)	
GATCTAAATGCCTGAAACTCACAA (Reverse)	#121 (04693558001)
TLR7	
GTCTAAAGAACCTGGAAACTTTGG (Forward)	
TCTCAGGGACAGTGGTCAGTT (Reverse)	#102 (04692209001)
TLR8	
CAGAATAGCAGGCGTAACACATCA (Forward)	
TGTTGTCATCATCATTCCACAA (Reverse)	#59 (04688562001)
TLR9	
CTGGGACCTCTGGTACTGCT (Forward)	
CTGCGTTTTGTCGAAGACCA (Reverse)	#98 (04692152001)
TLR10	
TGTCACCATTGTGGTTATTATGC (Forward)	
GCAGATCAAAGTGGAGACAGC (Reverse)	#76 (04688996001)
β-actin	
ATTGGCAATGAGCGGTTC (Forward)	
CGTGGATGCCACAGGACT (Reverse)	#11 (04685105001)

UPL: Universal Probe Library

Mann-Whitney U was used. Continuous variables were given as mean and standard deviation (mean ± SD). p<0.05 was considered statistically significant.

RESULTS

Subjects

The mean age of the GBM patients was 53±13 (38-83) and the control group was 52.75±9.98 (42-63). While 11 (44%) of GBM patients were female and 14 (56%) were male, 3 (37.5%) of the individuals constituting the control group were female and 5 (62.5%) were male.

TLR1–10 mRNA expression patterns

TLR1, TLR6, and TLR7 mRNA levels were significantly increased in the GBM group (p<0.001, each) whereas TLR4 and TLR10 mRNA levels were significantly decreased in the patients' group (p=0.023, p<0.001, respectively) (Figure 1). In this study, human

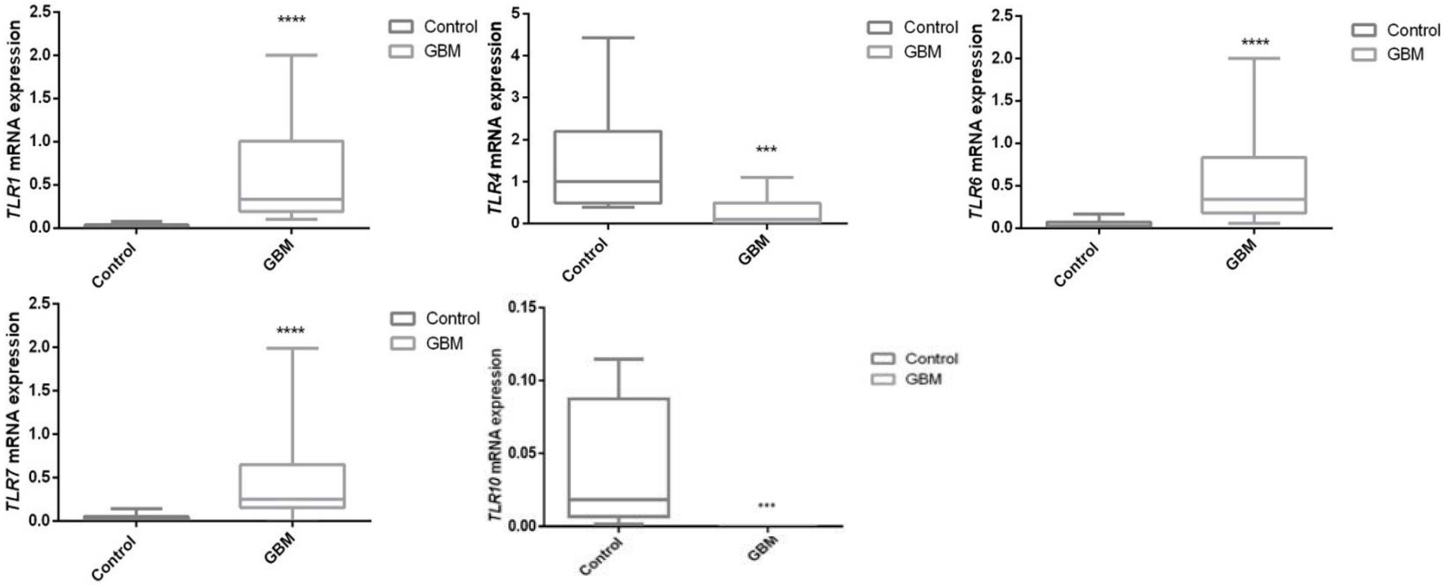

Figure 1. Comparison of TLR1, TLR4, TLR6, TLR7, and TLR10 mRNA expression levels between GBM and the control groups.

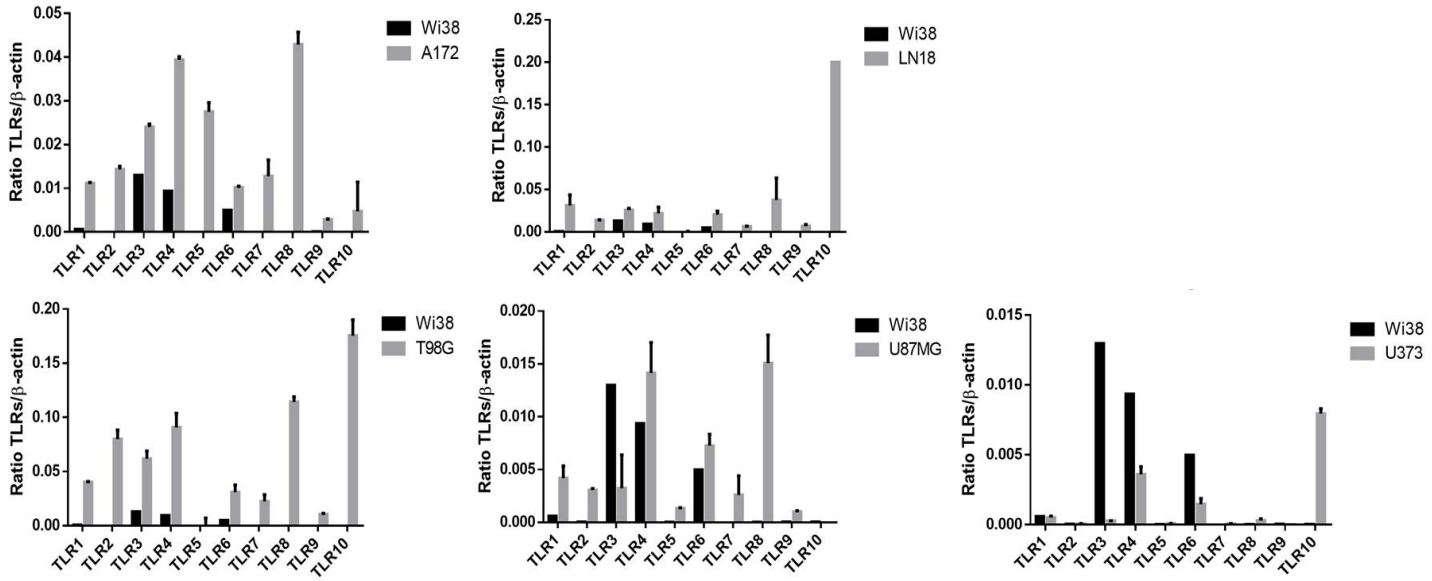

Figure 2. Comparison of TLR 1-10 expression levels between glioblastoma cell lines A172, LN18, T98G, U87-MG, U373, and the control group (Wi38).

GBM cell lines of T98G, U87-MG, U373, LN18, and A172, and for the control group Wi38 fibroblast cells were used. TLR mRNA expression profiles differed according to the cell lines. mRNA expression of all TLRs, from TLR1 to TLR10, were significantly increased in human GBM cell lines of A172, LN18, and T98G ($p<0.001$, each). TLR3 and TLR10 showed downregulation in the U87-MG cell line, while the others were upregulated ($p<0.001$, each) (Figure 2). TLR3, TLR 4, and TLR6 downregulation and TLR10 upregulation were demonstrated in the U373 cell line ($p<0.001$, each) (Figure 2).

DISCUSSION

TLR1, TLR2, and TLR6 are similar receptors that are present in cells of immunity as well as neoplastic cells (6). TLR2 heterodimerizes with TLR1 or 6. They have a significant function to identify bacterial and viral ligands and signals from nonviable cells (6). It has been demonstrated that the upregulation of TLR2 in tissues and cell lines of glioma displayed a negative correlation with the survival time in GBM by Li et al. (6, 11). In addition, overexpression of TLR2 mRNA and higher protein levels are claimed to enhance tumor cell activity and progression that is directly correlated with higher histological grades and shorter survival (11-12). We have found overexpression of TLR1 and TLR6 in the FFPE tissues of GBM patients. Whereas TLR2 expression did not exhibit a difference between the groups.

Overexpression of TLR1 and TLR6 in U87-MG and A172 cell lines, and overexpression of TLR2 in U87-MG, A172, and GL261 cell lines have been reported in the literature, (6). While TLR1, TLR2, and TLR6 mRNA expression was significantly increased in the present study in GBM of human A172, LN18, T98G, and U87-MG cell lines, TLR6 expression has been decreased in the U373 cell line.

TLR3 has a crucial role in the recognition of viral RNA to activate adaptive immunity that is targeted for antiviral and antitumor therapies. Compared with the nonneoplastic tissues, TLR3 expression has been significantly elevated in GBM in a recent study reported by Zou et al. (13). However, TLR3 expression did not display a significant difference between the tumoral and nontumoral groups in our study. In the literature, TLR3 overexpression has been established in human glioma tissues, as well as human GBM cell lines of U87-MG, U251, A172, and LN229 (6). Similarly, TLR3 mRNA expression was significantly increased in human GBM cell lines of A172, LN18, and T98G in the present study. On the contrary, TLR3 downregulation has been exhibited in U87-MG and U373 cell lines.

TLR4 expression has been revealed to be notably more upregulated in GBM than in anaplastic astrocytoma, associated with higher grades and poorer prognosis in the literature (14-16). TLR4 overexpression has been thought to downregulate Wnt/Claudin signaling to prevent apoptosis and assist the progression of GBM (17). Similarly, TLR4-mediated signals have been claimed to induce immune evasion, migration, proliferation, and survival (15). Reducing TLR4 expression has been suggested to abolish GBM invasiveness, and induce apoptosis (17). Nevertheless, contradictory effects have been reported by Alvarado et al and Cruz et al. (18-19). They have presented parallelly that glioma cells cause TLR4 downregulation, leading them to survive due to immune evasion (18-19). Similar to those reports, we have shown downregulation of TLR4 in GBM. In the literature, TLR4 upregulation has been demonstrated in human GBM cell lines of U87-MG, LN229, A172, SF126, U118, U251, U87, and GI261, and glial stem cells (6). Similarly, we have found increased TLR4 mRNA expression in U87-MG, A172, LN18, and T98G cell lines. In addition, we showed decreased TLR4 mRNA expression in the U373 cell lisimilararly to our GBM tissue samples. These conflicting results might be attributed to the self-renewal cycle arrest and receptor-restricted tumor growth. Evaluation of those controversial results attentively is crucial to clarify the role of TLR4 in GBM.

TLR5 has been claimed to be related to various neurological disorders such as neurodegenerative diseases. However, its expression in GBM is not widely explored in the literature. Ifuku et al. (20) have recently declared that TLR5 has no impact on the growth of an ex vivo GL261 glioma mouse model. Moretti et al. (14). reported higher expression of TLR5 along with TLR1, TLR2, TLR4, and TLR6 in astrocytomas and GBM than in non-neoplastic brain tissue. However, we have not demonstrated any statistically significant difference between the expression of TLR5 in GBM cells and the control group in FFPE tissues. In the literature, upregulation of TLR5 has been reported in human GBM cell lines of U87-MG, and A172 (6). Similarly, our results of cell lines T98G, U87-MG, LN18, and A172 about TLR5 have confirmed the literature.

TLR7 and TLR8 represent similar biological functions and architectures, that respond to

cytokines. Substantially, Buonfiglioli et al (21) have reported recently that binding TLR7 to its ligand let-7 miRNA triggers the release of cytokines and antigen presentation that inhibits migration and growth of glioma cells. In addition, two main agonists of TLR7/8 exhibited antitumoral response by diminishing cell proliferation and prolonging survival (6). To the best of our knowledge, overexpression of TLR7 and TLR8 were not demonstrated in glioma cell lines up to date (22). Remarkably, we found TLR7-8 mRNA overexpression in T98G, U87-MG, LN18, and A172 cell lines in the present study. Also, herein, we have revealed overexpression of TLR7 in GBM tissues of FFPE, for the first time in the literature, however, TLR8 expression was not different from the control group.

TLR9 upregulation has been reported to be relevant to a higher grade, disease progression, and poorer survival, due to the interaction with CCL2/CCL5, STAT3, MMP-2, and MMP-9, especially in supratentorial GBM (26). TLR9 expression has been documented in human glioma biopsies, glioma stem cells, and cell lines of U251, and U87, in the literature (6). We have demonstrated TLR9 mRNA overexpression in T98G, U87-MG, LN18, and A172 cell lines, in parallel to the literature. However, the mRNA level of TLR9 in GBM patients' tissues did not show a significant difference from the control group.

TLR10 is one of the least known TLRs in the literature. Even its ligand is still uncertain. TLR10 shares the same locus as TLR1, and TLR6, which are structurally similar to each other (27-28). Unlike TLR1 and TLR6, TLR10 does not lead to a classic downstream signaling pathway (29-30). However, TLR10's biological utility is unclear which is attributed to the complicated modulatory processes, activation of various signaling pathways, competition for ligands, and heterodimerization with other TLRs (27). Furthermore, there is extremely little information about TLR10 expression in GBM in the literature. According to the database of The Cancer Genome Atlas (CGA), TLR10 overexpression in GBM might be relevant to higher tumor grade, poorer overall, and progression-free survival (31). Based on the CGA database, Ge et al. (31) have exposed by gene correlation analysis that GBM shows a high expression level of TLR10 and also confirmed their analysis immunohistochemically. In contrast, we have detected downregulation of TLR10 mRNA in FFPE tissues. On the other hand, we have shown TLR10 overexpression in cell lines of T98G, U373, LN18, and A172, while there is downregulation in U87-MG. To our knowledge, this is the only study in the literature investigating TLR10 expression in cell lines.

Our results comparing the literature of each TLRs from TLR1 to TLR10, consequently, in GBM are stated above. In summary, we have analyzed mRNA levels of the whole TLRs family in both FFPE tissues of GBM patients and compared them with five different GBM cell lines (T98G, U87-MG, U373, LN18, and A172) for the first time in the literature and overview those issues. Additionally, we have used cell lines of U373 and LN18 in our study firstly that were not used in the literature before to investigate TLR expression. We have demonstrated overexpression of TLR1, TLR6, and TL7; and lower expression of TLR4 and TLR10 in FFPE tissues. mRNA expression of all TLRs, from TLR1 to TLR10, were significantly increased in human GBM cell lines of A172, LN18, T98G. TLR3 and TLR10 showed downregulation in the U87-MG cell line, while the others were upregulated. TLR3, TLR4, and TLR6 downregulation and TLR10 upregulation were demonstrated in the U373 cell line.

To conclude, while high expression was detected in some TLR members, low expression was detected in others, in the present study. Thus, all members of TLRs do not seem to have a similar level of expression or affect the GBM microenvironment in the same way. Indeed, we are willing to confirm the present findings by evaluating the endogenous protein level with a larger cohort using fresh tissue samples, to rule out the possible errors due to the FFPE tissue processing steps. Additionally, we claim that conducting more comprehensive studies including a higher number of patients with survival data is crucial to clarify the exact role of each TLRs on the pathogenesis and prognosis of GBM and shed light on observing more effective novel targeted therapies.

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