

Higher expression of PAR-1 may be associated with severity of Glioblastomas

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Abstract

Glioblastoma multiforme (GBM) is one of the most malignant types of central nervous system tumors. Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor that elicits several pro-tumoral responses. It has been linked to proliferation and invasiveness in glioblastomas. In the present study, prevalence of the receptor (PAR-1) and its associated downstream signaling was studied in two human glioblastoma cell lines D-54 MG and U-87 MG. The expression of PAR1 was found to be significantly higher in D-54 MG cell-line when compared to U-87 MG cell-line. The activation of PAR-1 in the presence of TFLLR (a synthetic peptide activator of PAR-1) led to an increase in ERK $\frac{1}{2}$ activity along with a significant increase in the migration and invasion of these cell-lines. Subsequent treatment with SCH-79797 (PAR-1 inhibitor) significantly decreased the migration and invasion in these glioma cell lines, respectively. The relative increase and decrease in the migration and invasion of these cell lines in presence of activator and inhibitor respectively was found to be linked with the expression of PAR-1. To conclude the study links the PAR-1 signaling with progression of Glioblastoma. PAR-1 and associated downstream signaling may be a potential therapeutic target to preclude the pathogenesis of gliomas.

Introduction

Gliomas are primary malignant brain tumors classified as astrocytomas, glioblastomas, oligodendrogliomas, ependymomas and mixed gliomas according to their origin [1–5]. They are amongst one of the highly invasive malignant brain tumors with a global burden of 2–3 new cases per 100,000 people per year worldwide [6]. Due to its aggressive behavior the median survival time for a recently diagnosed patient is approximately 1 year, with < 5% of patients surviving 5 years post-diagnosis [7, 8]. Once diagnosed, patients undergo surgical removal by adjuvant radiotherapy and chemotherapy [9, 10]. Tumor resists radiotherapy as well as its invasive ability [11] resulting in lower survival [12]. Recent data shows tumor incidence in India, to be 5 to 10 per 100,000 populations and 2% malignancy [13, 14]. Of these astrocytomas (38.7%) occur most frequently and the majority of the patients diagnosed have grade IV gliomas (60%) [15, 16]. In a study performed by Tao Sun [17], the frequency of tumor occurrence was found to be higher in males although with higher malignancy in females.

Till date many molecular targets have been screened for the early detection of gliomas and of these protease-activated receptor-1 (PAR-1) is a potential candidate. Several studies indicate that PARs play an important role in the progression of prostate cancer [18, 19], breast cancer [20] and lung cancer [21, 22]. Recently the expression of PAR-1 has been shown to play a pivotal role in migration, invasion, and angiogenesis of gliomas.

PARs are thrombin based receptors and among four types, activation of PAR-1, 3 and 4 occur by thrombin whereas PAR-2 activation takes place by trypsin-like serine proteases. Proteases bind to the extracellular domain of N-terminus at specific sites and expose the new N-terminal associated ligand thus activating the cleaved receptor [23, 24]. Expression of the thrombin based PAR-1 has a key role in the metastatic

behavior of many types of cancer. Human melanoma cells expressing PAR-1 have enhanced metastatic potential [25, 26] with upregulation $G_{\alpha q}$ and associated ERK1/2 signaling [27].

The present study examined the expression of PAR-1 in two different glioma cell lines i.e., D-54 MG (female cell line) and U-87 MG (male cell line). The PAR-1 signaling via ERK $\frac{1}{2}$ and its effect on the migration and invasion in these gliomas cell-lines was analyzed. The effect of PAR-1 activator and inhibitor confirms the role of these receptors in pathogenesis of gliomas.

Methods

Cell culture

Glioma cells D-54 MG (RRID:CVCL_5735) were purchased from Thermo Fisher Scientific and U-87 MG (RRID:CVCL_GP63) were purchased from NCCS, Pune. DMEM medium was used to culture the cells containing 10% fetal bovine serum, 2 mM glutamine, 200 penicillin/ml, and 0.2 g/ml streptomycin and incubated at 37 °C in a controlled humid atmosphere with 5% CO₂. The cells were counted using a trypan blue exclusion method by hemocytometer.

m-RNA expression of PAR-1 and RT-PCR

Total RNA was obtained from D-54 MG and U-87 MG cells lines with Purelink RNA mini kit from invitrogen (Carlsbad, CA). 1 µg of RNA was reverse-transcribed using GeneAmp RT kit (Applied biosystems). Following primers were used; forward 5' CCTATGAGACAGCCAGAATC 3' and reverse 5' GCTTCTTGACCTTCATCC 3' for PAR-1 Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control with forward 5' TTCAACGGCACAGTCAAGGC 3' and reverse 5' TCCACCACCCTGTTGCTGTAGC 3' primers respectively (Oikawa et al. 2013). PCR cycles were executed and the denaturation occurs at 95 °C for 15 min; then at 94 °C for 60 s, annealing temperature was 51 °C (GAPDH), 55 °C (PAR-1) for 90 s, and 72 °C for extension 30 s for 35 cycles; lastly the additional extension at 72 °C for 10 min.

PAR-1 protein expression and Western Blotting

Serum deprived confluent cells were taken for treatment and for subsequent western blot analysis. The cells were trypsinized and centrifuged at 500g for 5 minutes. Monolayer was rinsed with the chilled phosphate-buffered saline (PBS 0.1M) and a cytoplasmic fraction was obtained (Nuclear-cytoplasmic extraction kit, G-Biosciences Cat.786-182). The lysate was mixed on a rocker at 4°C for 15 min followed by centrifugation at 14,000 g in a pre-cooled centrifuge (Eppendorf Ultracentrifuge 5810R) for 15 min. at 4°C, the supernatant containing protein was transferred to a fresh and sterile 1.5 ml centrifuge tube, and the pellet was discarded. The concentration of collected protein was determined by the Bradford Reagent using bovine serum albumin (BSA) as standard. 10% SDS-polyacrylamide gel electrophoresis was performed with 10 µg protein/lane and then transferred to Polyvinylidene fluoride (PVDF) membrane. Blocking was performed with 5% skimmed milk for 3h at 4°C followed by three washing in 0.1M PBST (PBS with 0.1% Tween-20). Membrane was incubated overnight at 4°C with specific antibodies against

PAR-1 [Anti-PAR-1] (Abcam cat., ab114346). Next day after three repeated washing, peroxidase conjugated anti-mouse or anti-rabbit IgG (1:1500, Abcam cat. (ab150157) secondary antibody was added to membranes for 2h at room temperature. After washing the membrane protein bands were imaged by enhanced chemiluminescence (ECL) substrate (Pierce™ ECL Western Blotting Substrate Cat No. 32109). The quantification of protein was performed through densitometric digital analysis of protein bands using Image J software. β -actin was used to normalize bands of PAR-1.

Expression of p-ERK by Western Blotting

Serum deprived confluent cells were taken for treatment and western blot analysis. The cells were trypsinized and centrifuged at 500g for 5 minutes. The monolayer was rinsed with the chilled phosphate-buffered saline (PBS 0.1M) and a cytoplasmic fraction was obtained (Nuclear-cytoplasmic extraction kit, G-Biosciences Cat.786-182). The lysate was mixed on a rocker at 4°C for 15 min followed by centrifugation at 14,000 g in a pre-cooled centrifuge (Eppendorf cooling centrifuge 5810R) for 15 min. at 4°C, the supernatant containing protein was transferred to a fresh and sterile 1.5 ml centrifuge tube, and the pellet was discarded. The concentration of collected protein was determined by the Bradford Reagent using bovine serum albumin (BSA) as standard. 10% SDS-polyacrylamide gel electrophoresis was performed with 25 μ g protein/lane and then transferred to Polyvinylidene fluoride (PVDF) membrane. Blocking was performed with 5% skimmed milk for 3h at 4°C followed by three washing in 0.1M PBST (PBS with 0.1% Tween-20). Membranes were allowed to incubate overnight at 4°C with specific antibodies against ERK1/2 [p44/42 MAPK (1:2,000)] (Abcam, ab17942). Next day after three repeated washing, peroxidase conjugated anti-mouse or anti-rabbit IgG (1:1000, Abcam cat. (ab150157) secondary antibody was added to the membrane for 2h at room temperature. After washing the membrane protein bands were imaged by enhanced chemiluminescence (ECL) substrate (Pierce™ ECL Western Blotting Substrate Cat No. 32109). The quantification of protein was performed through densitometric digital analysis of protein bands using Image J software. Untreated cells were taken as control to compare with the cell-line.

Transwell Migration Assay

Transwell filter chambers of 8 μ m polycarbonate with 6mm inserts (Costar Corporation, Cambridge, MA) were used for migration assay. Recently subcultured, semi confluent D-54 MG and U-87 MG glioma cells, serum starved for 12 hours were harvested with EDTA (1.5 mM) and consecutively washed with phosphate buffer saline (PBS), and resuspended in DMEM at 2×10^6 cells/ml. 200 μ l cell aliquot was placed on the upper filter surface. SCH-79797 in DMEM was mixed to the upper chamber (with cells) and the lower chamber had 600 μ L DMEM with FBS. Filters were again kept at 37°C and 95% O₂ and 5% CO₂ for 5 hr. The upper surface cells were scraped off and lower surface cells were fixed with 4% paraformaldehyde (PFA) for 5 min. The obtained lower surface cells were stained with 1% crystal violet in 0.2 M sodium borate buffer at pH 9.0.

Matrigel Invasion Assay

Subcultured, semi confluent serum starved for 12 hours cells were harvested and resuspended in DMEM at 200,000 cells/ml. 250 µl of the cell aliquot was loaded in each matrigel coated chamber and then incubated at 37°C, 5% CO₂ for 12 hr. Filters were then returned to the incubator for 5 hr. The upper surface cells were scraped off and lower surface cells were fixed with 4% paraformaldehyde (PFA) for 5 min. The obtained lower surface cells were stained with 1% crystal violet in 0.2 M sodium borate buffer at pH 9.0. Untreated cells were taken as control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software). The results were presented as mean ± standard error mean (±SEM). Differences between the means were analyzed using independent t-test and One-way ANOVA. All the statistical analysis was performed at the significance level of $p < 0.05$.

Results

m-RNA expression of PAR-1 receptor

PAR-1 expression was assessed in D-54 MG and U-87 MG cell lines using RT-PCR. As evident, the D-54 MG cell-line has relatively higher mRNA expression than U-87 MG cell line [$p < 0.001$; $F(2, 12) = 47.60$] (Figure 1). The expression was analyzed by the Densitometric analysis of the bands using Image Studio Lite Ver 5.2.

Protein expression of PAR-1 receptor

To further validate the m-RNA expression obtained for the PAR-1 receptor in cell-lines the protein expression for the same was performed. Similar results were obtained by the Western blot analysis also. The receptor was expressed more in D-54 MG cell-line than U-87 MG cell-line [$p < 0.001$; $F(2, 12) = 50.60$] (Figure 2). The intensity of the bands was analyzed by Densitometric analysis using Image Studio Lite Ver 5.2.

p-ERK a functional representative of PAR-1 activation

ERK1/2 signaling has been reportedly linked with the PAR-1 downstream signaling and has been correlated with the pathogenesis of gliomas. To analyze the effect of PAR-1 activation in D-54 MG and U-87 MG cell-lines the cells were treated with 30 µM TFLLR (PAR-1 activator) and protein was extracted at different time points for western blot. Untreated cells were taken as control. The persistent activation of p-ERK^{1/2} was found in the D-54 MG cell-line [$p < 0.001$; $F(6, 28) = 834.0$] but not in the U-87 MG cell-line [$p < 0.01$; $F(6, 28) = 2059.0$] where the activation of p-ERK^{1/2} considerably decreased (Figure 3a). p-ERK^{1/2} in D-54 cell line upregulation after 30 min also but in U-87 MG cell-line the signal intensity was persistently low after 30 min. (Figure 3b). Thus the results suggest that the ERK ½ is activated through its enhanced phosphorylation by the PAR-1 activation and it plays a role in pathogenesis of gliomas.

Migration of D-54 MG and U-87 MG cells by PAR-1 activation

Effect of PAR-1 in migration of both the cell-lines of glioblastoma was observed by transwell migration assay. TFLLR (30 μ M) activator sequence of PAR-1 was applied to the upper chamber of the transwell. TFLLR increased the migration in D-54 MG cell line ($t = 7.390$, $p < 0.001$) (Figure 4a) but no significant change in the migration of U-87 MG cell line ($t = 1.697$, $p > 0.05$) as analyzed by t-test (Figure 4b).

To confirm the role of PAR-1 in migration, we tested the effect of inhibitor SCH-79797 on TFLLR based migration. As seen in Figure 5a, PAR1 inhibitor SCH-79797 (3 μ M) inhibited TFLLR-induced migration in D-54 MG cell-line [$F(2, 12) = 60.07$, $p < 0.001$] further suggesting that PAR1 is involved in the migration of D-54 MG cell-line. Similarly, the decrease in the migration of U-87 MG cell-line was observed when equated with the TFLLR induced cells [$F(2, 12) = 23.52$, $p < 0.001$] (Figure 5b).

PAR-1 activation enhances invasion in Matrigel invasion assay

To further corroborate the PAR-1 activation and invasion by its agonist we treated the cell-lines with thrombin, an endogenous ligand of PAR-1. The effective concentration (EC_{50}) of thrombin in D-54 MG and U-87 MG cell-line was observed first. It was found that D-54 MG cells show activation at lower concentrations of thrombin (0.1U/ml) but U-87 MG shows PAR-1 activation only at higher concentrations (1U/ml) (Figure 6). Then the effect of thrombin on invasion was studied. It was found that the thrombin significantly increased the invasion of D-54 MG cell-line [$F(2, 12) = 98.72$, $p < 0.0001$] (Figure 7a) but not in U-87 MG cell-line [$F(2, 12) = 9.894$, $p > 0.05$] (Figure 7b).

We further analyzed the effect of the PAR-1 inhibitor SCH-79797 on TFLLR induced invasion of cells. SCH-79797 significantly decreased the invasion in both D-54 MG cell-line [$F(2, 12) = 98.72$, $p < 0.0001$] (Figure 8a) and U-87 MG cells [$F(2, 12) = 9.894$, $p > 0.01$] (Figure 8b). However the decrease in the invasion across the matrigel in case of D-54 MG cell-line was highly significant as compared to U-87 MG cell-line.

Discussion

Glioblastoma is one of the most malignant tumors among all other astrocytomas and pathogenicity of these cells is the chief problem associated with the cure. Many studies performed earlier have reported PAR-1 in facilitating progression and severity of gliomas [28]. The present study was performed to find an association between prevalence of PAR-1 and its impact on pathogenesis of the glioblastoma. Two cell lines of glioblastoma were gauged for expression of PAR-1 receptor followed by pharmacologic intervention to look at the involvement of this receptor in invasiveness of these cell lines and the signaling involved.

Our first aim was to find out if the cell lines being used in the study had differential expression of PAR-1 mRNA. The reverse transcription of total mRNA revealed a difference in the expression pattern of the PAR-1 in the two cell lines with significantly higher expression of PAR-1 in the D-54 MG cell line than the U-87 MG cell line. Earlier studies have shown a positive correlation between the PAR-1 receptor m-RNA

expression and expression of PAR receptors on the cell surface [29]. Further many studies have found an association between higher expression of these receptors to greater malignancy [30].

We then asked whether activation of these receptors influence the ERK $\frac{1}{2}$ dependent signaling pathway which is associated with cellular proliferation, migration and invasion in these cell-lines or not. In earlier studies the ERK $\frac{1}{2}$ signaling has been linked with PAR-1 downstream signaling and has been correlated with the pathogenesis of gliomas [31, 32]. Activation of PAR-1 has been shown to induce proliferation in astrocytes via MAPK involving distinct signaling pathways [33]. Activation of PAR-1 by a synthetic peptide PAR-1 activator TFLLR led to an increased level of phospho ERK $\frac{1}{2}$ in both D-54 MG and U-87 MG cells. However in D-54 MG cell-lines there was a persistent increased expression of p-ERK $\frac{1}{2}$ as compared to U-87 MG cell-line in which it subsided after 30 minutes and this may be because of differential signaling resulting from differential expression of PAR-1 in these cell lines. This result corroborated with the increased expression of PAR-1 in D-54 MG cell-line in contrast to U-87 MG cell-line.

A number of studies in the last decade point towards significant involvement of PAR-1 and its ligand thrombin in the progression of glioma [34–38]. Thrombin is an endogenous ligand for the PAR-1 receptor [28]. By activating the receptor it induces glioma growth and proliferation [39]. Overall the activation of PAR-1 via thrombin increases the likelihood of neuro-glial transmission, amplification of cellular signaling, inflammation, differentiation, proliferation, malignancy of glial cells, and tumor related brain edema [40, 41]. In the present study a concentration-dependent increase was observed in the migration of D-54 MG cells but not in the migration of U-87 MG cells. In U-87 MG cells the migration increased only at a higher concentration (10 U/ml). These observations further add to the role of PAR-1 in migration of glioma cells.

Next we looked for an association between the prevalence of PAR-1 receptor gene expression and migration and invasion in these cell-lines. It was found that the migration and invasion of D-54 MG cells increases in a concentration-dependent manner as opposed to that of U-87 MG cells which increased only at a higher concentration. The higher expression of PAR-1 in D-54 than U-87 cells in this study points towards an association between the PAR-1 signaling and pathogenicity. This is in line with previous studies where the prevalence of PAR-1 receptors has been shown to be associated with the pathogenesis of some glioblastoma cell lines [42]. Some later studies have also correlated angiogenesis with over-expression of PAR-1 in Glioblastoma multiforme [43].

The TFLLR induced migration and invasion in the D-54 MG cell lines was significantly higher as compared to the U-87 MG cell lines. This could be because of the over-expression of PAR-1 receptors in the D-54 MG as compared to the U-87 MG. The involvement of PAR-1 was further confirmed by use of a specific inhibitor SCH-79797. It was observed that use of specific inhibitor SCH-79797 significantly reduced the TFLLR induced increase in the migration and invasion of D-54 MG. Our results are in line with many previous studies both in rodents and human tissues that have reported active involvement of PAR-1 receptors in the progression of Glioblastoma multiforme [44]. These results along with ours suggest an association between PAR-1 expression and glioma migration. Earlier studies have found that activated

PAR-1 fails to be down-regulated in carcinomas [45] by persistently activating ERK1/2 and thus inducing cellular pathogenesis.

There are certain reports [46] which have correlated gender with incidence and survival of gliomas. The incidence of gliomas is less in women, but once it occurs it progresses more rapidly than in males. The two different human origin glioma cell lines i.e., the D-54 MG and U-87 MG used in this study are coincidentally of female origin and male origin respectively. This again provides a weightage to the findings in the present study where the rate of migration and invasion in D-54 MG cells is higher than U-87 MG cells and this may be an explanation for higher aggressiveness of D-54 MG because of the increased expression of PAR-1 as compared to U-87 MG.

Conclusion

It was found that activation of PAR-1 and its inhibition causes the increased and decreased glioma migration and invasion respectively and also had a differential effect on the associated downstream ERK signaling. These results indicate that PAR-1 and the downstream signaling have critical roles in progression of gliomas and may serve as potential drug targets for controlling and treating Glioblastoma multiforme. Thus, PARs can provide an alternative to the conventional radio chemotherapy and might help in the survival of GBM patients.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of Data and Material

The data supporting the results of the present study are provided by the corresponding author.

Author Contributions

ST and SS performed the experiments, SAS and AR helped in manuscript preparation, DRM, RCS and MB provided the consumables and space to work. The core idea was generated by AP.

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Figures

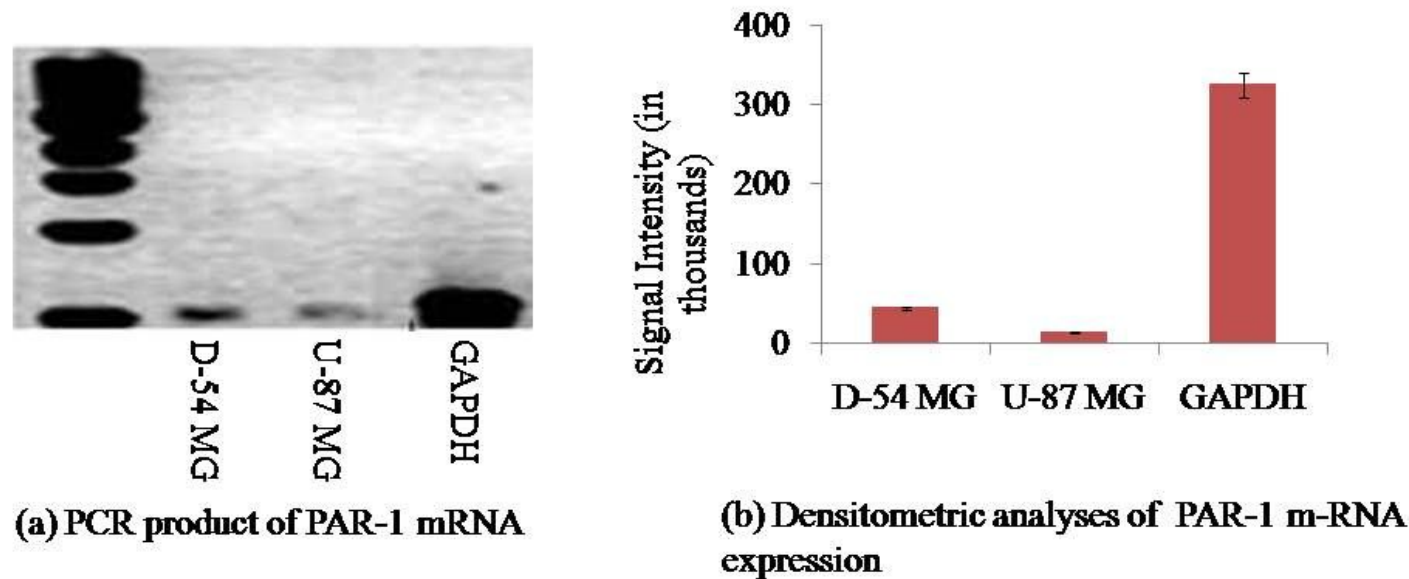


Figure 1

(a) Represents the RT-PCR analyses of mRNA expression encoding PAR1 receptor. PCR was performed using specific sets of primers for PAR-1 and GAPDH. (b) Densitometric analysis of PAR-1 m-RNA expression in D-54 MG and U-87 MG cells-lines. m-RNA was normalized to the density of GAPDH (control), and expressed as mean (\pm SEM).

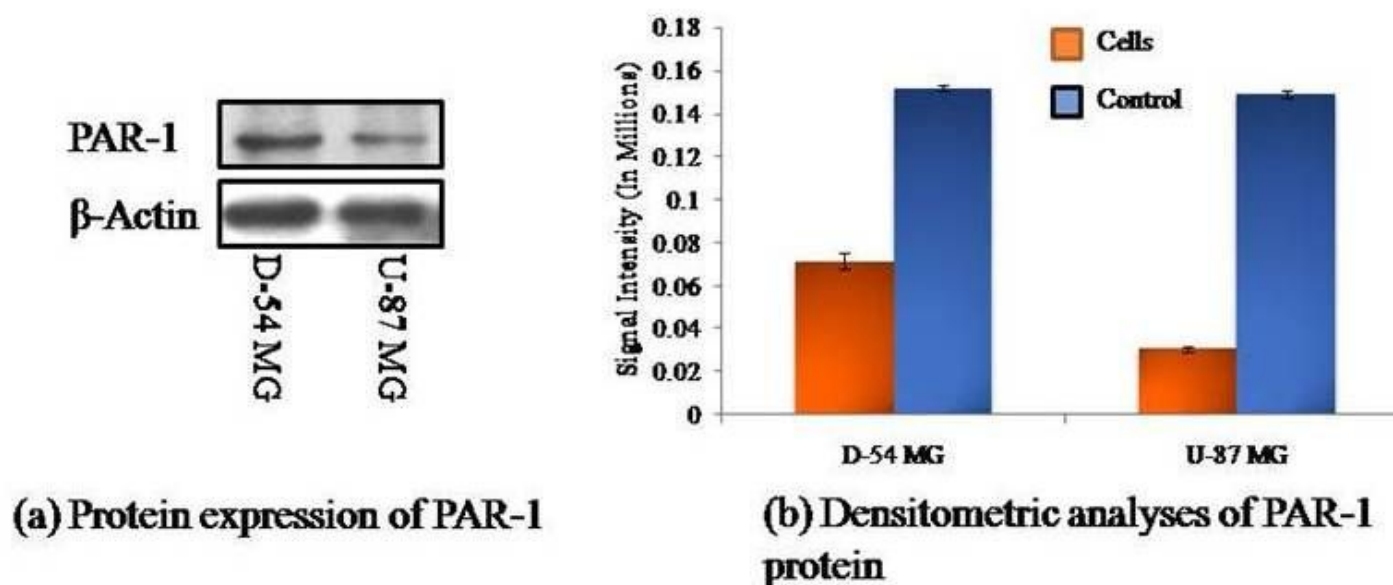


Figure 2

(a) Represents the protein expression of PAR-1 receptor. (b) Represents the protein expression of PAR-1 receptor in D-54 MG and U-87 MG cell-lines. Expression of PAR-1 was normalized to the density of β -actin and expressed as mean (\pm SEM).

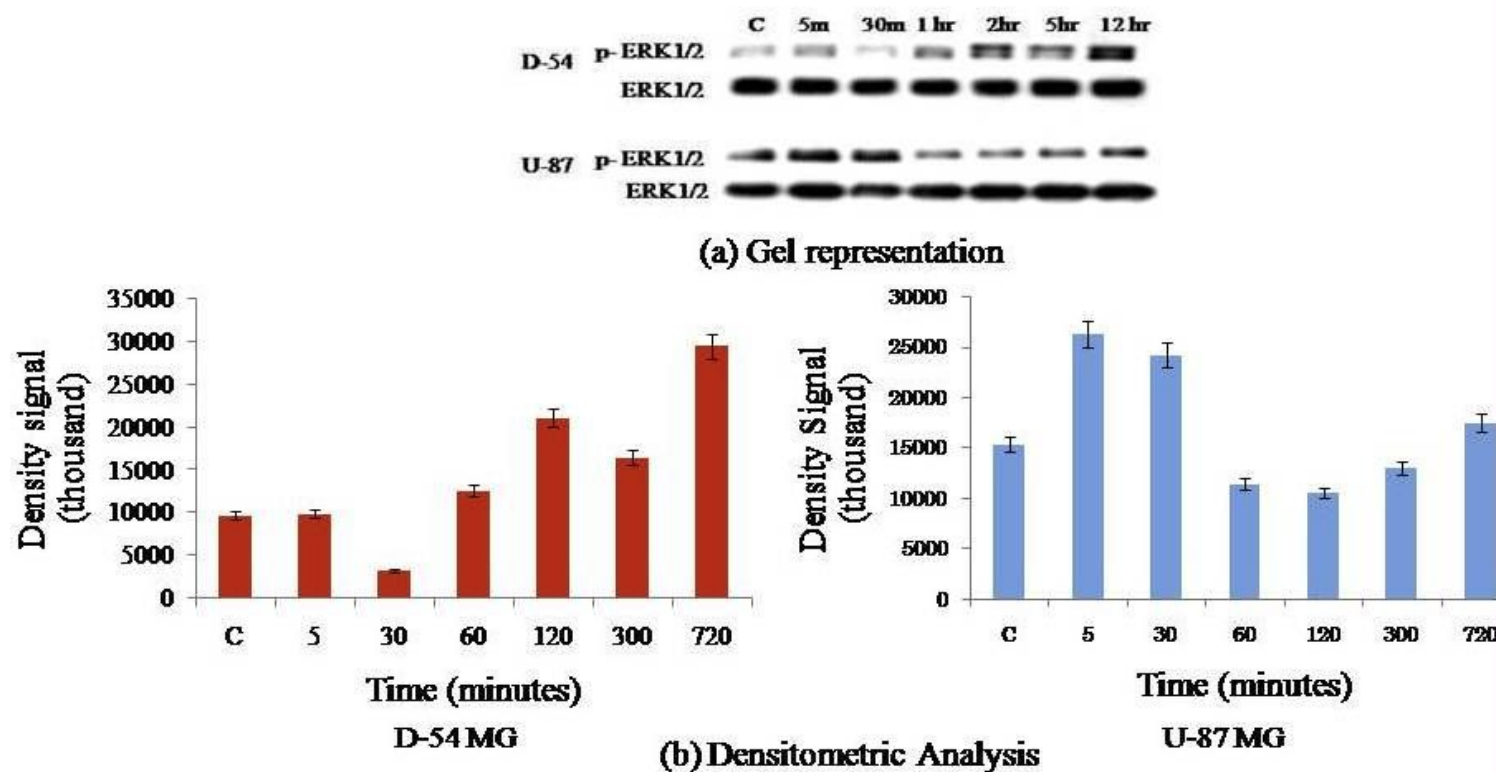


Figure 3

(a) Cells were stimulated by TFLLR and ERK ½ phosphorylation was assessed by Western blotting. (b) Time course of ERK ½ activation by TFLLR (30 µM). Densitometric analysis results are expressed as means ±S.E.M. D54- MG cells showed a persistent ERK ½ activation beyond 12 h whereas in U87-MG cells ERK ½ activation was evident only up to 30 min.

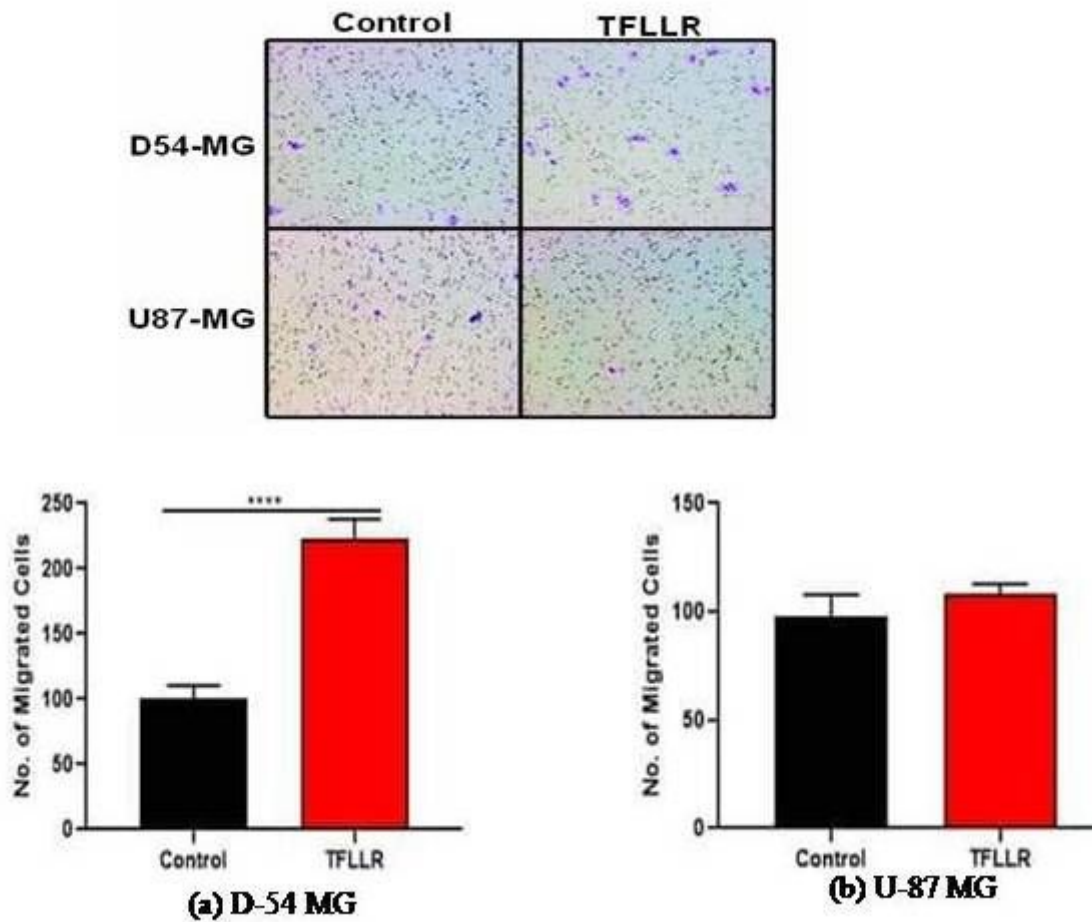


Figure 4

PAR-1 activation and migration by TFLLR in D-54 MG and U-87 MG cells was analyzed by Transwell migration assay (n=3). (a) Migration of cells is significantly higher in D-54 MG cell-line when compared to untreated control. (b) The number of migrated cells was not significant in U-87 MG cell-lines.

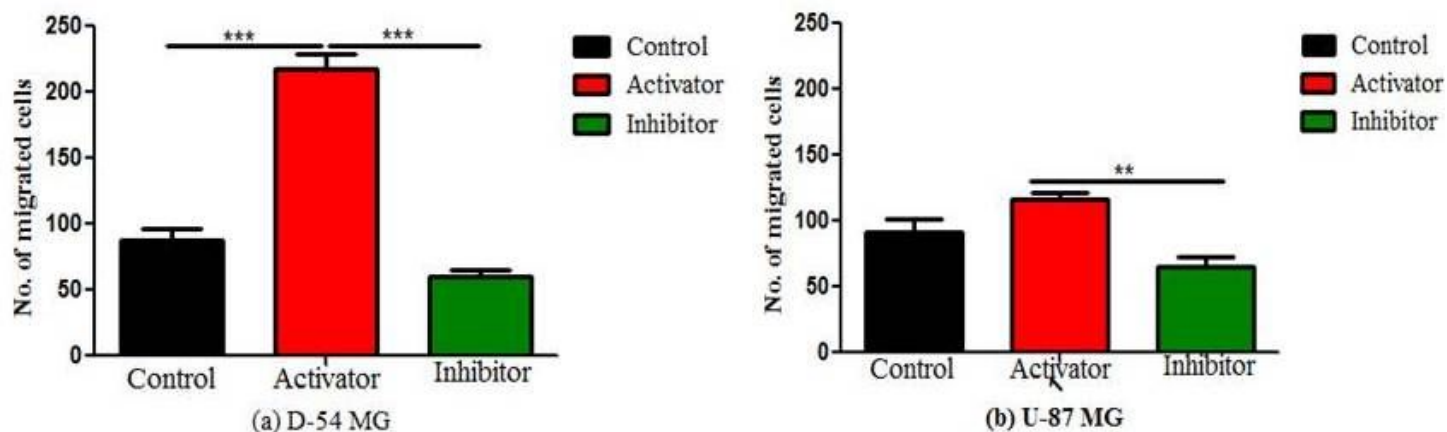


Figure 5

To confirm the role of PAR-1 in migration thrombin (an endogenous ligand) based stimulation of PAR-1 was performed. The figure represents the effective concentration (EC₅₀) of thrombin in D-54 MG and U-87 MG cell-line. D-54 MG cells show activation at lower concentrations of thrombin (0.1U/ml) but U-87 MG shows PAR-1 activation only at higher concentrations (1U/ml).

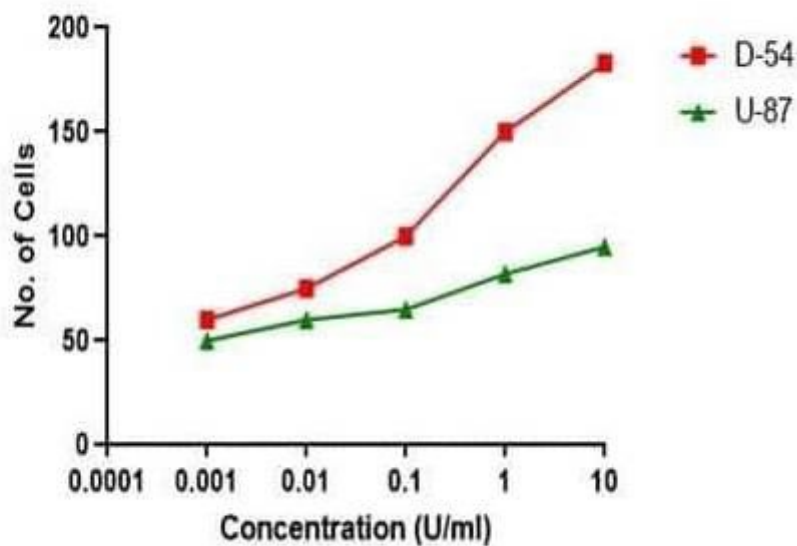
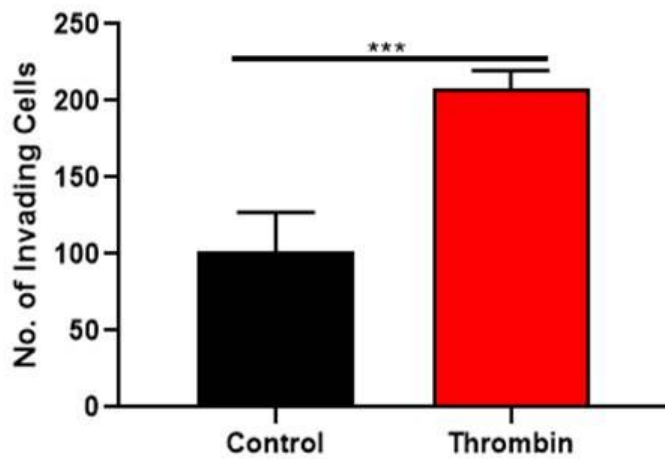
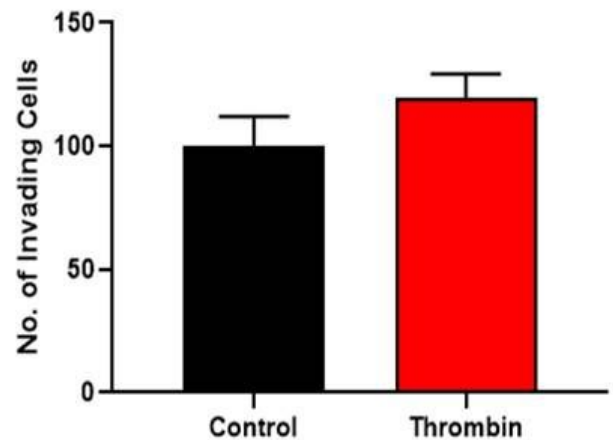


Figure 6

Represents the effective concentration of thrombin an endogenous ligand on activation and invasion of glioma cell-line. It was observed that D-54 MG cells show activation at lower concentrations of thrombin (0.1U/ml) but U-87 MG shows PAR-1 activation only at higher concentrations (1U/ml).



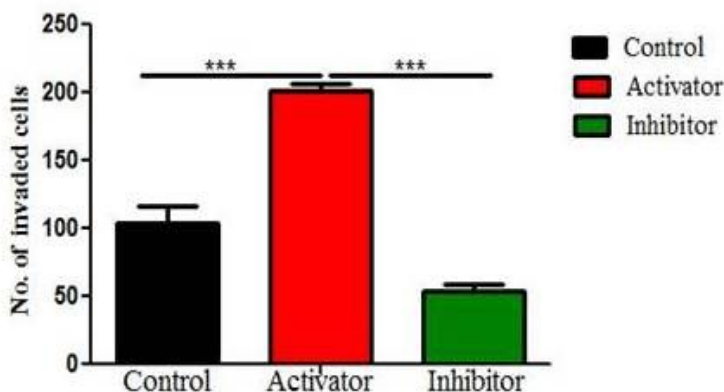
(a) D-54 MG



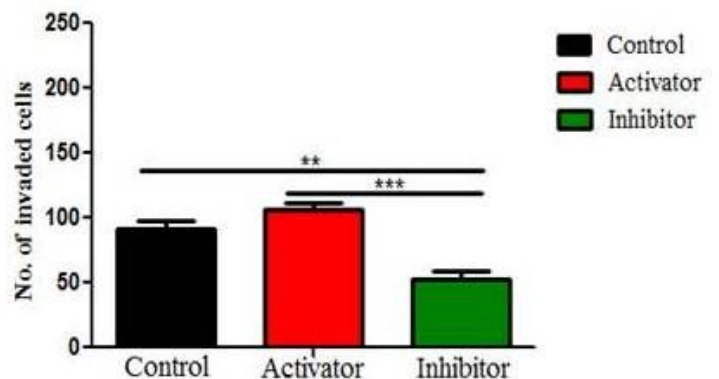
(b) U-87 MG

Figure 7

Represents the effect of activator and inhibitor of PAR-1 in migration of cells. The increase in migration of D-54 MG cells by TFLLR was blocked by PAR1 inhibitor SCH-79797 (3 μ M). SCH-79797 decreased the number of migrated cells in a concentration-dependent manner. (a) Effect of inhibitor SCH-79797 in the presence of TFLLR on the migration of cells D-54 MG (b) Effect of inhibitor SCH-79797 in the presence of TFLLR on the migration of cells U-87 MG



(a) D-54 MG



(b) U-87 MG

Figure 8

Represents the effect of activator and inhibitor of PAR-1 in invasion of cells. The increase in invasion of D-54 MG cells by TFLLR was blocked by PAR1 inhibitor SCH79797 (3 μ M). SCH-79797 decreased the number of invaded cells in a concentration-dependent manner. (a) Effect of inhibitor SCH79797 in the presence of TFLLR on the invasion of D-54 MG (b) Effect of inhibitor SCH79797 in the presence of TFLLR on the invasion of U-87 MG.

Supplementary Files

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- [Graphical.jpg](#)