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ORIGINAL ARTICLE

67kDa laminin receptor regulates the activation of MAPKs through DUSPs in glioma cell line U251

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Summary

Purpose: *All-trans-retinoic-acid (ATRA), the active derivative of vitamin A, is critical in regulating cell cycle as well as inhibiting tumor growth and angiogenesis. It has been used in the clinical treatment of leukemia. 67kDa laminin receptor (67LR), as one of the receptor of laminin, plays an important role in tumor cells invasion, proliferation and metastasis. Current research indicates that 67LR is highly expressed in glioma and is associated with tumor progression. However, the underlying molecular mechanisms, especially the signaling pathways involved, have not been reported yet. Therefore it is of great importance to clarify its downstream targets.*

Methods: *The U251 glioma cell line was used in this study. Cell Counting Kit-8 was used in cell proliferation assay. Quantitative real-time PCR (qRT-PCR) was used to determine the transcription level of dual specificity phosphatases (DUSPs). Western blot analysis was used to detect the expression of mitogen activated protein kinases (MAPKs) and phosphorylated MAPKs.*

Results: *67LR could influence the transcription of DUSPs and expression of MAPKs. ATRA could enhance the expression of 67LR in U251 cells and this enhancement was dose-dependent. ATRA was able to inhibit the growth of U251 cells.*

Conclusions: *ATRA expressed significant therapeutic effect on glioma cells, and 67LR is not the only factor that can influence the proliferation of U251 cells.*

Key words: *ATRA, 67LR, DUSPs, glioma, MAPKs, proliferation*

Introduction

Glioma, originated from astrocytes, is one of the types of cancer most difficult to treat and is often fatal [1]. In 2007, the World Health Organization histologically classified gliomas into four types: pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiforme (GBM) [2]. GBM is the most severe-grade malignant glioma and is characterized by a most aggressive growth and highest mortality. Although resection can prolong the life of patient, GBM can still relapse easily. Until recently median survival was generally less than one year fro the time of diagnosis, with less than 15% of patients being alive 2 years post diagnosis [4]. So developing effective therapeutic strategies is still a challenge in the field of neurosurgery [3]. Nowadays, some researchers have found that the extracellular matrix (ECM) around tumors is associated with the growth, differentiation, attachment, invasion and migration of tumor cells. It can be remodeled by tumors [5]. Laminin is a kind of major non-collagenous glycoprotein in ECM and is involved in many important biological activities. It performs its normal physiological function by combining with different receptors. These receptors can be divided into the integrin family and the non-integrin family. 67LR, also known as LAMR1 [6], is a non-integrin receptor which plays a key role in tumor invasion and metastasis. Studies have found that 67LR is highly expressed in gliomas, it is correlated with tumor progression [7-9], and represents a hot point in cancer research.

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Table 1. Sequences of sense and antisense primers used in quantitative real-time PCR

RNA species	*Primer pairs*	*Product size (bp)*
67LR	Sense:5'-ACGGCTTCTTGTGGTTAC-3' Antisense:5'-AGTCAGTGGTTGCTCCTA-3'	420
β-actin	Sense:5'-ACTATCGGCAATGAGCGGTTCC-3' Antisense:5'-AGCACTGTGTTGGCATAGAGGTC-3'	152
MKP-1	Sense:5'- TCTTCCTCAAAGGAGGATACGAA-3' Antisense:5'-GCTGTCAGGGACGCTAGTACTCA-3'	113
MKP-3	Sense: 5'- GAACTGTGGTGTCTTGGTACATT-3' Antisense: 5'-GTTCATCGACAGATTGAGCTTCT-3'	100
MKP-5	Sense:5'- TGGTGTTGAAAGGTGGACTTAGTA-3' Antisense: 5'- CAAGGAACAGGAAGGGCAAG-3'	159
PAC-1	Sense: 5'- CTGCGAGGAGGCTTCGAC-3' Antisense: 5'- AGGAACAGGTAGGGCAAGATC-3'	165

ATRA is the most important active derivative of vitamin A. It plays a key role in cancer development. In 1988, Chinese researchers successfully treated acute promyelocytic leukemia using ATRA [10]. At present, ATRA is regarded as a challenging therapeutic method, because of its nontoxicity and effectiveness.

DUSP, as an important member of protein tyrosine phosphatases (PTPs) family, could dephosphorylate threonine and tyrosine residues on MAPKs, to specifically induce their inactivation [11]. Mitogen-activated protein kinase phosphatases (MKPs) is a subclass of DUSPs. Based on the specificity of MKP to substrate, MKPs can be classified into 4 groups. The first group can dephosphorylate ERKs specifically, including DUSP-6 (MKP-3), DUSP-7 (MKP-X) and DUSP-5; the second group, including DUSP-10 (MKP-5), DUSP-16 (MKP-7) and DUSP-8, can selectively dephosphorylate JNK and p38; the third group includes DUSP-1 (MKP-1) and DUSP-4 (MKP-2), which can dephosphorylate all kinds of MAPKs; the fourth group can selectively dephosphorylate ERKs and p38, including DUSP-2 (PAC1) and DUSP-9 (MKP-4) [12]. The MKPs being used are MKP-1, MKP-3, MKP-5 and PAC1 respectively.

Our previous research has demonstrated that the expression of 67LR is high in human glioma U251 cells [9], and it has a close relationship with the physiological characteristics of U251 cells. We down-regulated the expression of 67LR using RNAi, and found that it could influence the migration, invasion, proliferation and apoptosis of U251 cells. Some authors have indicated that ATRA can improve the expression of 67LR on MCF-7 cells and melanoma B16 cells [13,14]. Our experiment showed that this improvement also exists in U251 cells. It has been reported that there exists a relationship between 67LR and MAPK pathway in melanoma cells [7], but whether this relationship exists in glioma cells is still unknown. In our research, we monitored the change of DUSPs and MAPKs in mRNA and protein levels respectively, after up- or downregulating the expression of 67LR by ATRA incubation and RNAi. We found that 67LR can not only influence the physiological characteristics of glioma cells through the MAPK pathway, but can also affect the activity of MAPKs by regulating DUSPs. Taken together, this study clarifies a possible molecular mechanism by which 67LR regulates the proliferation of glioma cells.

Methods

Reagents and cell line

ATRA was purchased from Sigma-Aldrich (St.Luis, MO, USA). U251 is a human malignant glioma cell line and was purchased from Shanghai Institutes for Biological Sciences, CAS. Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) was used for cell culture at 37°C and 5% CO_2. Antibodies being used included anti-ERK, anti-phospho-ERK, anti-p38MAPK, anti-phospho-p38MAPK, anti-SAPK/JNK and anti-phospho-SAPK/JNK (Cell Signaling Technology, Beverly, MA, USA), anti-67LR (Abcam,USA). Cell Counting Kit-8 was utilized in proliferation assays (Dojindo, Japan).

Plasmid and co-transfection

The interfering RNA expression plasmid was designed in our previous research [8]. The transfection reagent was X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland), and the transfection was conducted according to the manufacturers' protocol.

Quantitative real-time PCR

Based on traditional protocol, the total RNA was

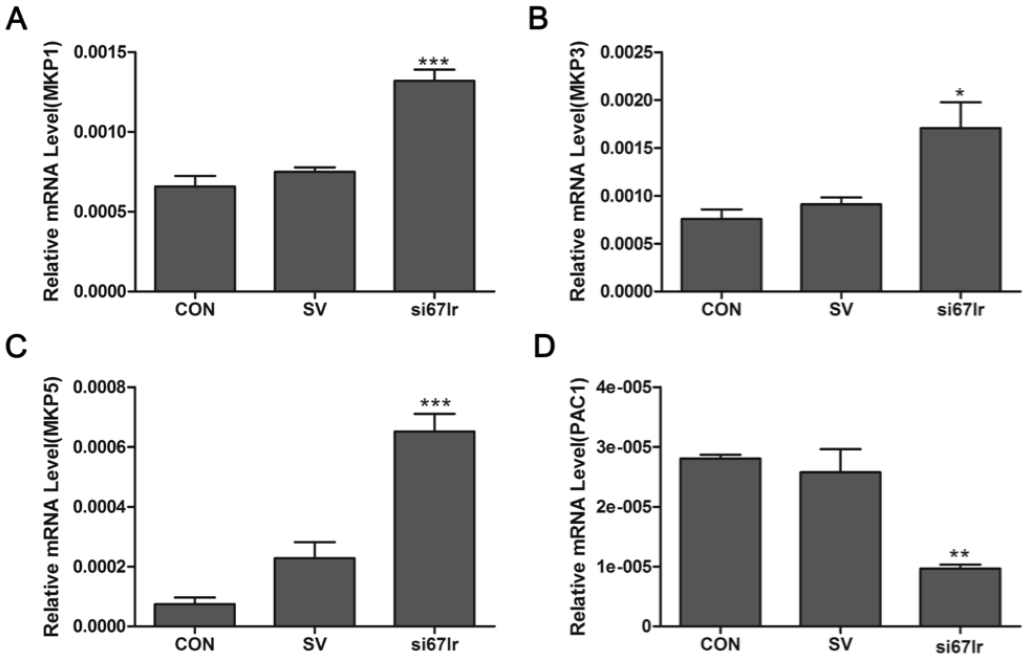

Figure 1. qRT-PCR showing the transcription levels of DUSPs. Both Con and SV are negative controls. SV was transfected with scrambled plasmid. **(A)** The transcription level of MKP-1; **(B)** The transcription level of MKP-3; **(C)** The transcription level of MKP-5; **(D)** The transcription level of PAC1 (*p<0.05, **p<0.01, ***p<0.001, compared with the control group).

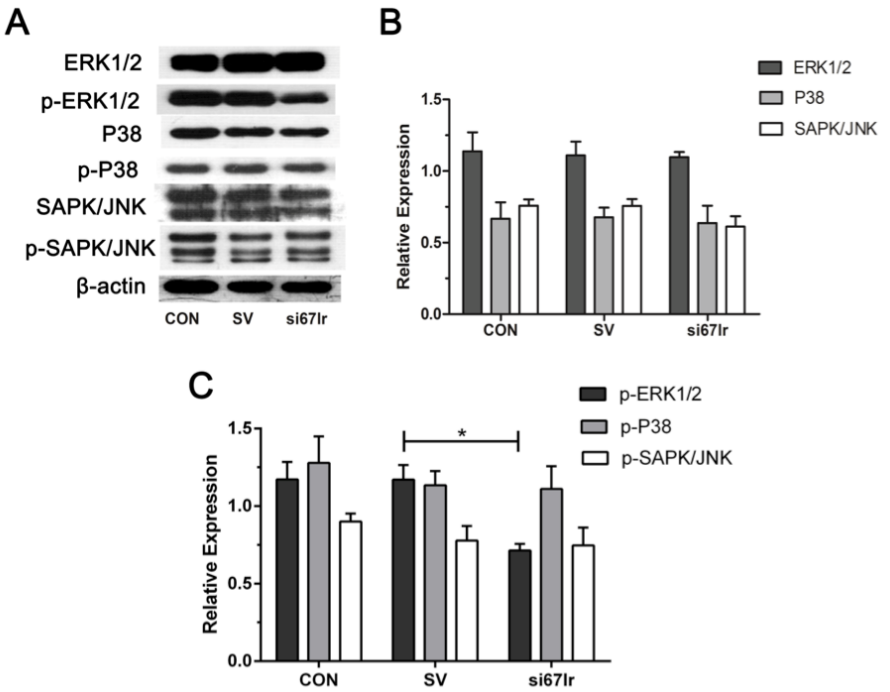

Figure 2. The expression levels of MAPKs in glioma cells transfected with 67LR-shRNA compared to scrambled shRNA transfection and control group. **(A)** Results of Western blot analysis; **(B)** Statistical analysis of the expression level of total MAPKs protein (p>0.05); **(C)** Statistical analysis of the expression level of phosphorylated MAPKs protein (*p<0.05 compared with SV group of p-ERK 1/2).

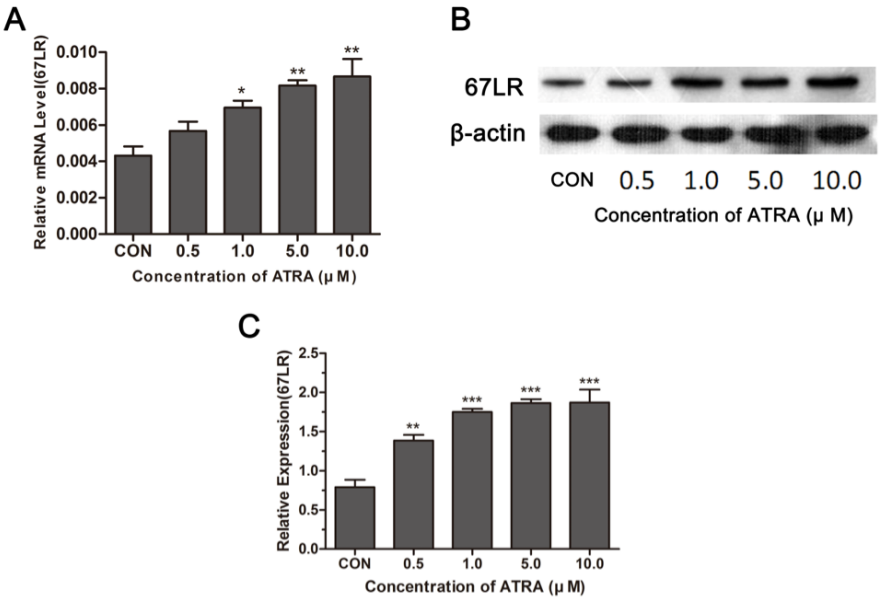

Figure 3. The transcription and expression levels of 67LR were dramatically increased in glioma cells after ATRA incubation for 48 hrs at different concentrations. **(A)** Results from qRT-PCR; **(B)**, **(C)** The expression level of 67LR in U251 cells incubated with ATRA at different concentrations was analyzed by Western blot analysis. 67LR and β-actin expressions were detected with anti-67LR and anti-actin antibodies. Band intensities were quantified by Quantity One software. (*p<0.05, **p<0.01, ***p<0.001, compared with the control group).

extracted from U251 cells and reverse transcription system was used to synthesize the corresponding cDNA. The primers listed in Table 1 were designed to detect 67LR [8], MKP-1, MKP-3, MKP-5, PAC-1 and β-actin (positive control) respectively. Cycle parameters were: 67LR—denature at 95°C for 10 sec, anneal and extend at 55°C for 30 sec, for 30 cycles; β-actin, MKP-1, MKP3—denature at 95°C for 10 sec, anneal and extend at 58°C for 30 sec, for 30 cycles; MKP-5, PAC-1—denature at 95°C for 10 sec, anneal and extend at 58°C for 40 sec, for 35 cycles.

Western blotting

After abstracting the total proteins from U251 cells, electrophoresis with 10% SDS-PAGE gel was carried out to disperse the proteins in different scales. Then, PVDF membranes were utilized for the purpose of proteins transfer. Incubation with no-fat milk was conducted overnight in 4°C. Primary and secondary antibodies were incubated with the target proteins to determine their expression level. An enhanced chemiluminescence detection system was used for proteins detecting purpose. In this experiment, the antibodies used included anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-SAPK/JNK, anti-phospho-SAPK/JNK and anti-67LR.

Proliferation assays in vitro

Cell Counting Kit-8 was used for accurate analysis of cell proliferation; the absorbance was read at 400nm. The containing reagent WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-etrazolium, monosodium salt) can react with the dehydrogenase in living cells to generate a kind of yellow formazan dye. Cells were seeded onto 96-well plates at a concentration of 1×10^4 cells per well and the ultimate volume of cell culture medium was 0.2 ml per well. Cells were then treated with ATRA at different concentrations or transfected by 67LR RNA interfering plasmid both for 48 hrs. For time course experiments, cell numbers were counted at fixed periods.

Statistics

The experimental data were processed and compared using one-way analysis of variance (ANOVA) by Graph Prism 5. All data are presented as mean ± SEM (standard error of the mean). The statistical significance of all tests was set at $p<0.05$.

Results

Role of 67LR in the activation of MAPKs in U251 cell line

To elucidate the molecular mechanism by

which 67LR acts on the proliferation of glioma U251 cells, we tested the transcription levels of MKP-1, MKP-3, MKP-5 and PAC1, and the expression levels of p38, ERK1/2 and SAPK/JNK and their phosphorylated form after 67LR-RNAi for 48 hrs. The result of qRT-PCR showed that the mRNA levels of MKP-1,MKP-3 and MKP5 were all significantly increased (Figure 1), resulting in a change of MAPKs phosphorylation, which is shown in the following Western blot analysis (Figure 2).

The results above indicated that p-ERK1/2 were downregulated by 67LR-RNAi after 48 hrs of treatment. This might be related to DUSPs, due to their ability to dephosphorylate MAPKs. However, no significant changes were found in p38 and p-SAPK/JNK protein levels.

Upregulating the expression of 67LR by incubating with ATRA for 48 hrs decreases U251 cells proliferation

ATRA at different concentrations were incubated with U251 cells for 48 hrs. The transcription and expression levels of 67LR were determined by qRT-PCR and Western blot analysis, respectively. ATRA could also increase the transcription and expression of 67LR in a dose-dependent manner (Figure 3).

Since we already showed that U251 cells can be interfered by 67LR RNAi and had a significant decrease in their proliferation rate, we next examined the effect of ATRA treatment on cell growth by Cell Counting Kit-8 (CCK-8) (Figure 4). The status of U251 cells is depicted in Figure 5. When the concentration of ATRA changed from 0.1μM to 50μM, the cell morphology was transformed and this transformation was dose-dependent. Although the expression level of 67LR was enhanced, the cell growth was still significantly

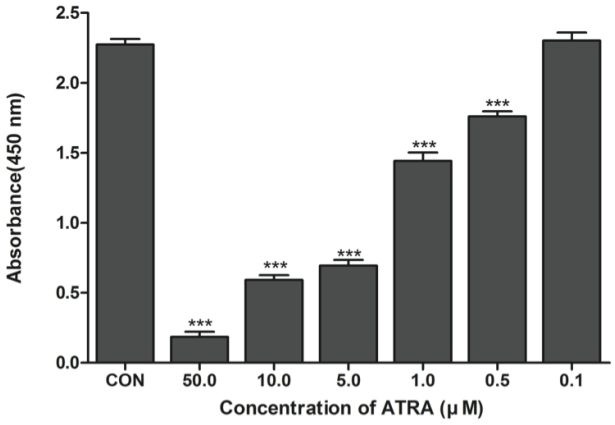

Figure 4. The results of CCK-8 test. U251 cells were incubated with ATRA at different concentrations for 48 hrs. The results show that the cell growth was inhibited with the increase of ATRA concentration (***p<0.001, compared with the control group).

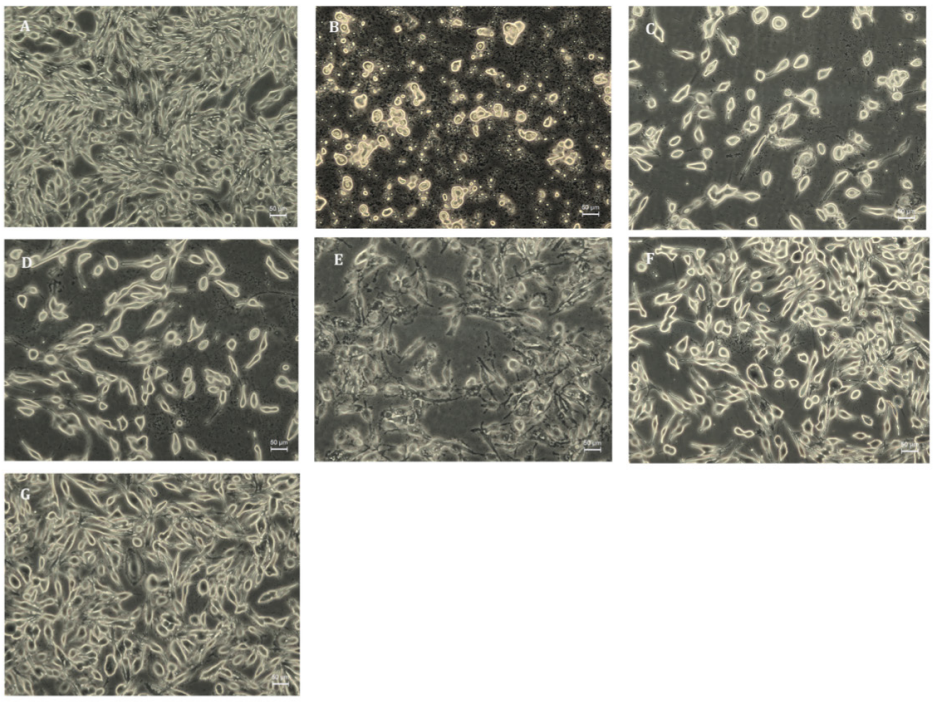

Figure 5. Cell morphology after incubation with ATRA at different concentrations for 48 hrs. **(A)** Blank control; **(B)** Incubation with 50μM ATRA for 48 hrs; **(C)** Incubating with 10μM ATRA for 48 hrs; **(D)** Incubation with 5μM ATRA for 48 hrs; **(E)** Incubation with 1μM ATRA for 48 hrs; **(F)** Incubation with 0.5μM ATRA for 48 hrs; **(G)** Incubation with 0.1μM ATRA for 48 hrs.

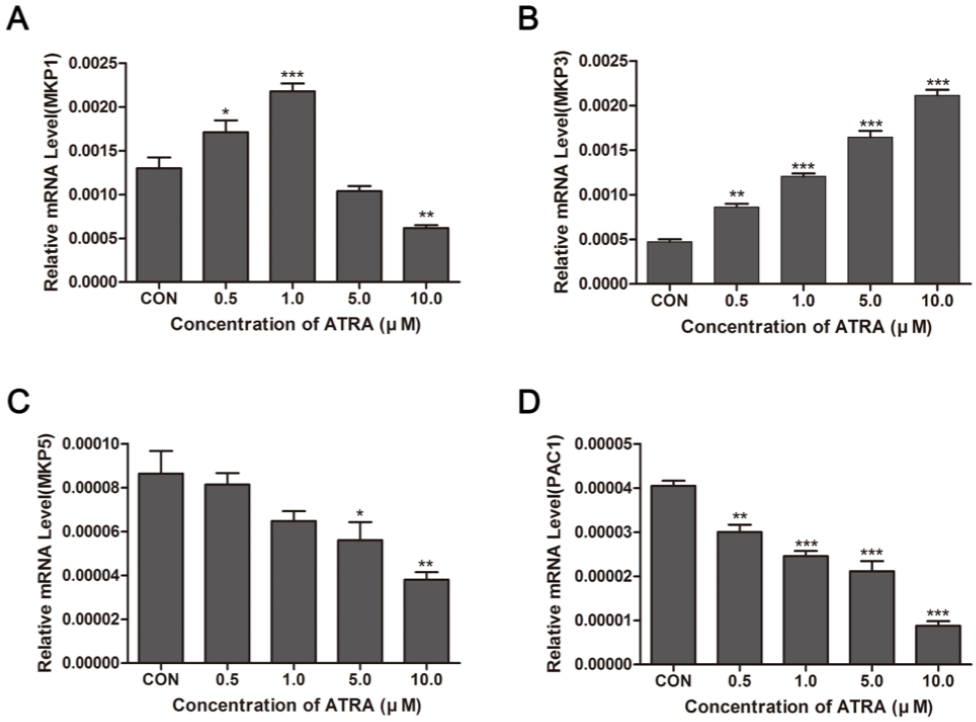

Figure 6. qRT-PCR showing the transcription levels of DUSPs with U251 cells incubated with ATRA at different concentrations. Con is negative control. **(A)** The transcription level of MKP-1; **(B)** The transcription level of MKP-3; **(C)** The transcription level of MKP-5; **(D)** The transcription level of PAC1 (*p<0.05, **p<0.01, ***p<0.001, compared with the control group).

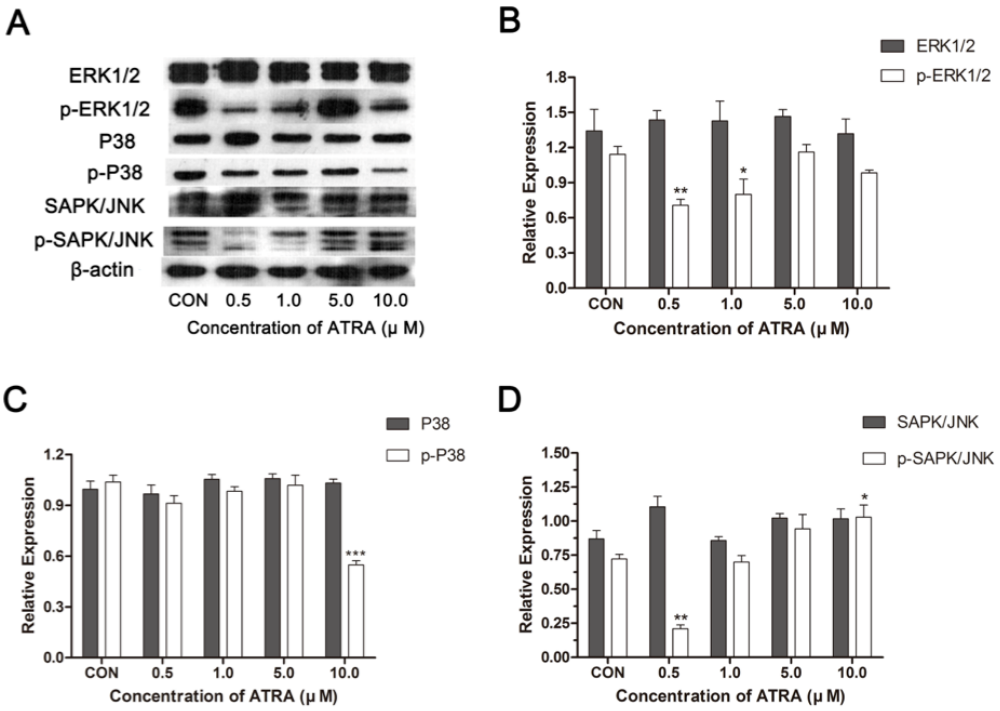

Figure 7. The expression of MAPKs in U251 cells incubated with ATRA at different concentrations. **(A)** Results from Western-blot analysis; **(B)** Statistical analysis of the expression of ERK1/2 and p-ERK1/2 proteins; **(C)** Statistical analysis of the expression of p38 and p-p38 proteins; **(D)** Statistical analysis of the expression of SAPK/JNK and p-SAPK/JNK proteins (*p<0.05, **p<0.01, ***p<0.001, compared with the control group of phosphorylated MAPKs).

inhibited. These results suggest that ATRA exerts obvious inhibitory effect on the proliferation of U251 cells.

Upregulating 67LR reverses the transcription of DUSPs and expression of MAPKs

To further investigate the role of 67LR on DUSPs and MAPK cascades, we studied the transcription of DUSPs (Figure 6) and the expression of MAPKs (Figure 7) and compared them with the results before (Figures 1 and 2). We chose the same DUSPs members: MKP-1, MKP-3, MKP-5 and PAC1. Similar to the situation of down-regulating 67LR (Figure 6 B and D), the mRNA level of MKP-3 was increased and that of PAC1 was decreased upon treatment of ATRA in a dose-dependent manner. However, the mRNA level of MKP-5 was reversed completely (Figure 6 C) compared to Figure 1 C. Interestingly, different from any other DUSPs, the level of MKP-1 was improved at 0.5 and 1.0 μM, but was significantly decreased at 5.0 and 10.0μM, which was also reversed. These results indicated that the transcription of DUSPs was related to the expression of 67LR.

Since 67LR is associated with DUSPs and DUSPs can regulate the activity of MAPKs, we next used Western blot to test the expression of MAPKs (Figure 7). Although the expression of ERK1/2, p38 and SAPK/JNK did not change obviously, the phosphorylation status of MAPKs was altered significantly. Figure 7 A shows that the phosphorylation of MAPKs changed irregularly after treatment with ATRA for 48 hrs in different concentrations. The p38 and ERK signaling was inactivated by ATRA incubation in a dose-dependent manner, but SAPK/JNK signaling was activated. Moreover, the expression level of phosphorylated ERK1/2 was downregulated after incubation with 0.5 and 1.0μM ATRA, while upregulated obviously with 5.0 μM ATRA, and then decreased again when the ATRA's concentration was 10.0 μM. These results suggest that the expression of 67LR not only relates with DUSPs, but also affects the expression of phosphorylated MAPKs.

Discussion

Glioma, as the most prevalent intracranial tumor of the central nervous system, has a poor cure rate regardless of chemotherapy or traditional therapies [15]. The aim of our study was to generally understand the role of 67LR in glioma cells proliferation and DUSPs-MAPK signaling. Recently, a large number of studies have shown that overexpression of 67LR in tumor cells is correlated with the proliferation, invasion, metastasis, and disease poor prognosis of tumor [9,16-19].

In a variety of tumors, ATRA has been used as chemopreventive and therapeutic agent, or combined with other medicines for oncotherapy [13]. When U251 cells were incubated with 50μM ATRA for 48 hrs, the cell morphology changed significantly (Figure 5) from normal shape to almost round shape, indicating that ATRA could also lead to the death of U251 cells. However, no obvious changes were observed with ATRA at the concentration of 0.1μM (Figure 5). Therefore, ATRA can be an efficient therapeutic agent. When used in combination with other agents, it may improve treatment results significantly.

Although 67LR overexpression is correlated with increased cell proliferation, aggressiveness and malignancy in many tumors, our results (Figure 3 and Figure 4) show that enhancement of the expression of 67LR is not completely associated with the proliferation of U251 cells.

Research on signal transduction related to integrin has implicated the ability of laminin-binding integrins to activate MAPKs [7]. In our study, the phosphorylation level of ERK1/2 was significantly reduced in U251 cells transfected with 67LR RNAi, compared to non-transfected cells (Figure 2 C). As shown in Figure 1, MKP-1, which can specifically dephosphorylate MAPKs, has an obvious enhancement in the transcription level. These two results strongly suggest that the downregulation of 67LR would induce dephosphorylation of ERK1/2. Although the transcription levels of MKP-3, MKP-5 and PAC1 were also changed significantly, the dephosphorylation of p38 and SAPK/JNK did not alter. Since there are 11 kinds of MKPs, the reason of the above phenomenon maybe that MKPs except MKP-1, change differently, and synthetically induce non-obvious changes to the expression of p-P38 and p-SAPK/JNK.

DUSP family plays a key role in regulating the activity of MAPKs through adjusting protein phosphorylation [20]. To further investigate 67LR-induced dephosphorylation of MAPK, we upregulated 67LR by ATRA incubation at different concentrations for 48 hrs. The transcription levels of DUSPs, including MKP-1, MKP-3, MKP-5 and PAC1, were then detected by qRT-PCR (Figure 6). The results of MKP-3 and PAC1 (Figure 6 B,D) were similar to those of downregulating 67LR (Figure 1 B,D), while the results of MKP-1 and MKP-5 (Figure 6,A,B) were different from those of downregulating 67LR (Figure 1 A,C). The ex-

pression of phosphorylated MAPKs also had significant changes at some specific concentrations of ATRA (Figure 7). The interesting phenomenon that the mRNA level of MKP-1 first rises and then falls (Figure 6 A) along with the changes of p-ERK1/2 and p-SAPK/JNK suggests that there may be a feedback mechanism between ATRA and MKP-1. Besides, because of the 11 members of MKPs that have been identified only 4 of them were analyzed, permits us to speculate that others may also work on 67LR related signaling pathway. Our results indicate that the mechanism of the proliferation regulation system in U251 cells is complicated, and although we upregulated the expression of 67LR, the proliferation was still inhibited.

We also used Western blot analysis to detect the expression of MMP-2 in U251 cells when 67LR was down regulated by iRNA (data not shown). What we observed was a significant decrease in the expression level of MMP-2. This result further proves that 67LR not only is involved in the MAPKs pathway, but it can also affect many other aspects in U251 cells. In summary, 67LR has the ability to affect basic signal transduction pathways in cancer, and these mechanisms may enrich our knowledge about tumor proliferation regulation.

Acknowledgement

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