

Original Article

Raf kinase inhibitor protein (RKIP) inhibits the cell migration and invasion in human glioma cell lines in vitro

Xuhui Lei¹, Liang Chang², Wei Ye¹, Chuanlu Jiang¹, Zhiren Zhang³

¹Department of Neurosurgery, The Second Affiliated Hospital of Harbin Medical University, Harbin, China;

²Department of Neurosurgery, The Tumor Hospital of Harbin Medical University, Harbin, China; ³Departments of Clinical Pharmacy and Cardiology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China

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Abstract: Objective: To investigate the effects and the potential mechanisms of RKIP on cell migration, invasion and proliferation in human glioma cell lines in vitro. Methods: The RKIP over-expressing and RKIP knockdown human U87 glioma cells were used to reveal the effects of RKIP on human glioma cells migration, invasion and proliferation. After the recombinant plasmid pcDNA3.0-RKIP or RKIP-shRNA was transfected into the cell lines U87 by the means of liposome assay, the cells migration, invasion and proliferation were detected by wound healing, Transwell and MTT assay. Then, the levels of RKIP, MMP-3, MMP-9 and HMGA2 mRNA transcription were measured by means of RT-qPCR and levels of proteins expressions were determined using Western blot. Results: The results of MTT assay suggested that the PKIP have little inhibitive effects on glioma cells proliferation ($P>0.05$). The present paper showed that the migration distances in the group of RKIP-shRNA were markedly increased compared to the pcDNA3.0-RKIP and control. Similarly, the results showed that the numbers of invasion cells in RKIP-shRNA were remarkably increased than the pcDNA3.0-RKIP group and control group. Western blot and RT-qPCR suggested that over-expressions of RKIP lessened the MMP-2, MMP-9 and HMGA2 expression, however, turning down the RKIP expression showed the inverse effects. Conclusion: RKIP inhibits the cells migrations and invasions. Meanwhile, RKIP might inhibit the glioma cells through inhibiting MMPs and HMAG2 expression. Therefore, we demonstrated that RKIP is an underlying target for the treatment of glioma.

Keywords: Raf kinase inhibitor protein (RKIP), glioma, HMGA2, migration, invasion

Introduction

Glioma is a common kind of tumors in nervous system, which generates from the glial cell [1]. Glioma has a high migration and invasion character which frequently metastasize into surrounding brain tissues [2]. Metastasis of glioma into surrounding nervous tissues makes it hard to get an efficient treatment [3]. To get full appreciation of all the treatment strategies, more than 97% of glioma patients died less than five years after being diagnosed [4]. The capable abilities of migration and invasion in glioma cell may be responsible for the rate of mortality. Although there were mass of achievements for glioma, the potential fundamental mechanism and characteristic biomarker in glioma migration and invasion are much or a lot unclear. As a result, a volume of efforts should

be done to explore and reveal the potential fundamental mechanism and characteristic biomarker targets in glioma cell migration and invasion for novel treatment strategy.

Raf Kinase Inhibitory Protein (RKIP) play important roles in inhibition of key signal transduction including MAPK and NF- κ B pathway [5]. Meanwhile, RKIP also adjusts cell proliferations and differentiations [6]. RKIP play vital roles in a large amount of processes of cell, for examples membranes synthesis, neurodevelopment and tumors metastases [7-9]. A lot of researches reported that RKIP inhibited the cells metastases of cancers. Zheng Fu et al. performed immunohistochemistry to detect RKIP expression in primary prostate tumor cells and metastases of prostate carcinoma. Over-expression of RKIP cells was established and ability of cells

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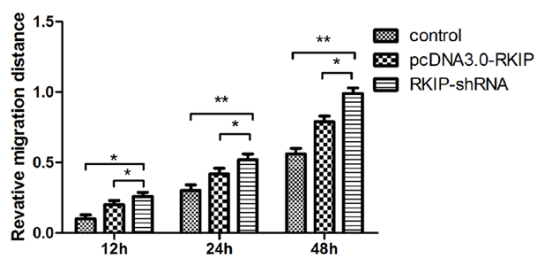


Figure 1. Effects of RKIP on cells migration in human U87 glioma cell by means of wound healing assay. The relative distance was ratio of experimental group distance vs. the distance of the cell-blank scratch at 12 h, 14 h and 48 h after transfection. * $P < 0.05$, ** $P < 0.01$.

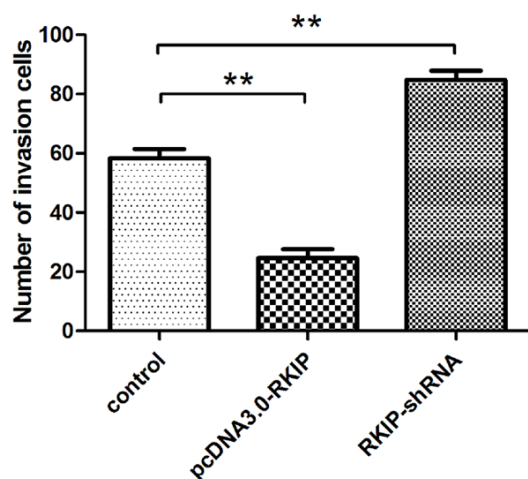


Figure 2. Effects of RKIP on cells invasion in human U87 glioma cell by means of Transwell assay. The number of glioma cells cross through the membrane was recorded. * $P < 0.05$, ** $P < 0.01$.

proliferation, and in vitro cell invasion were detected. The results showed that over-expression of RKIP cells were associated with decreased in vitro cells invasion and RKIP over-expressing in prostate tumor cell reduced metastasis of tumor in vivo [10].

The extracellular matrix (ECM) is a crucial role in glioma cells metastasis because that the tumors cell lives in it [11, 12]. As we know that MMPs is a positive factor in degradation of ECM. As a result, matrix metalloproteases (MMPs) have been known as vital factors in cancer cells invasion and migration. The mechanisms that RKIP inhibit tumors invasion and metastasis may negative regulation for MMPs expressions by means of suppression of NF- κ B signaling [13-15].

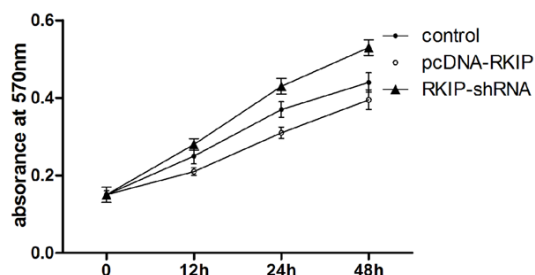


Figure 3. The effects of RKIP on the proliferation of U87 glioma cells. To determine the effects of RKIP on the ability of proliferation in U87 cells, the MTT assays were carried out to detected the proliferations of cells that over-expressing or down regulating of RKIP. The results showed that RKIP had little effects on the proliferation of U87 glioma cells.

Although RKIP had been reported to be an inhibitor in cell migration and invasion, there is little paper research the effects of RKIP on cell migration and invasion of glioma cells. In present research, we detected the effects of RKIP on cell migration and invasion of human U87 glioma cells and explore to reveal the potential mechanism.

Materials and methods

Cell cultures and transfections

The human glioma cell line U87 were pursued from The Second Affiliated Hospital of Harbin Medical University and were cultured in DEME (Boster Biology Co., Wuhan, China) supplemented with 10% FBS. All cells were incubated in a 37°C atmosphere of 5% CO₂ and cell cultures were broken down with 0.1% trypsin. RKIP open reading frame clones were cloned into pcDNA3.0 vector for building the RKIP-over-expressing cell. According to the RNA interference theory, the RKIP knockdown cell was constructed as a negative control of the RKIP over-expressing one which was built using the scrambled shRNA. Lipofectamine™ 2000 (Life Technologies) was used to transient transfections.

Wound healing assay

The wound healing assay was used to detect the ability of glioma cell migration and performed as previous paper described [16]. Forty-eight hours after transfection, the plastic micropipette tip was used to make a cell-blank scratch. Then, the cells were washed using PBS

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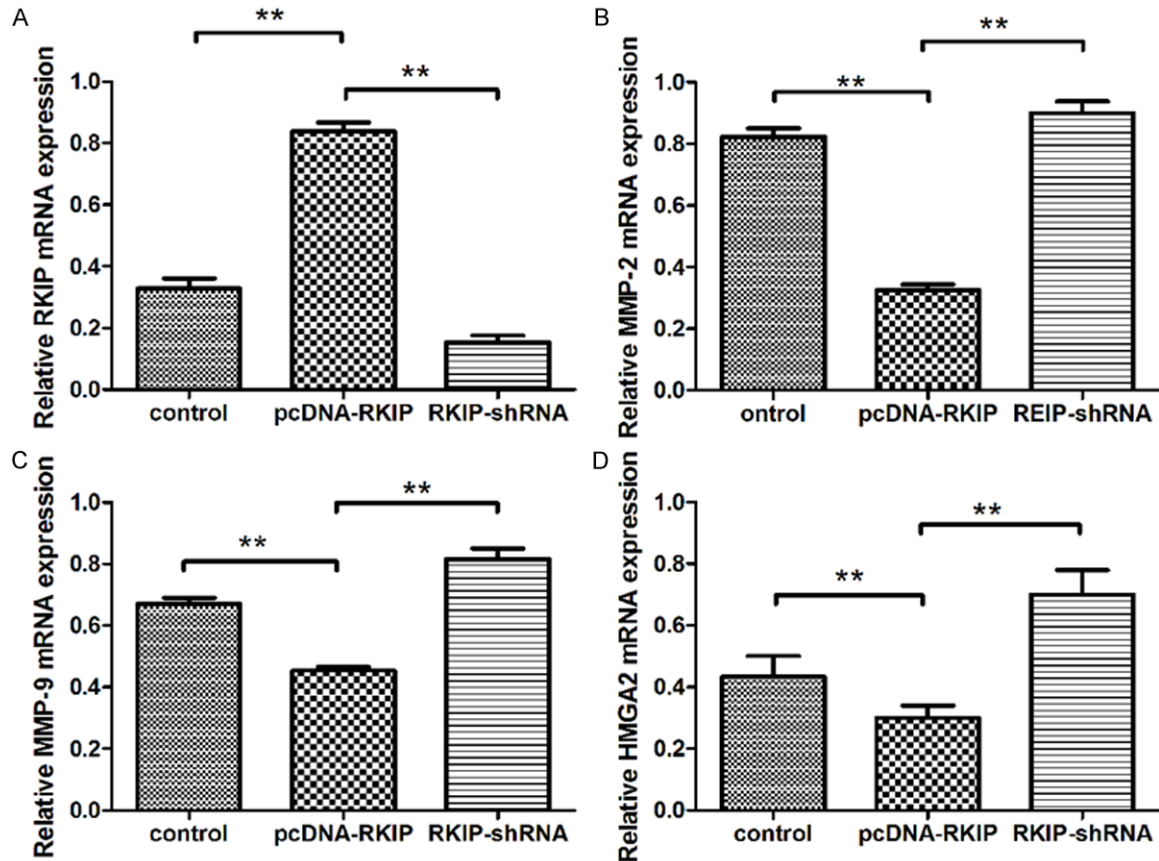


Figure 4. Effects of over-expressing and knockdown RKIP on mRNA expressions in human U87 glioma cells. A. The RKIP mRNA relative expressions (vs. GAPDH), B. The MMP-2 mRNA relative expression (vs. GAPDH), C. The MMP-9 mRNA relative expressions (vs. GAPDH), D. The HMGA2 mRNA relative expression (vs. GAPDH), * $P < 0.05$, ** $P < 0.01$.

in order to wipe off the trashy cells. The remnant glioma cells were maintained in DEME serum for another 24 h. The distances that the cells moved were observed and recorded.

Transwell assay

In order to detect the invasion ability of glioma cells, the Transwell assay was carried out as previous reported [17]. Forty-eight hours after transfection, cell suspension (1×10^5 cell/well) at a volume of 100 μ L was added to every upper chambers and DEME of 600 μ L was added to the bottom cell well. After incubation at 37°C in a 5% CO₂ incubator for 24 h, the cells that grow on the polycarbonate membranes were wiped off using tampons. Then, the polycarbonate membranes were handled with 4% paraformaldehyde for 30 mins. After that, the polycarbonate membranes were washed using PBS for twice and placed on the cell plate well which filled with trypan blue for cell staining. The cells number that had cross through the

polycarbonate membranes was recorded and analyzed.

RT-qPCR

Cellular total RNA was extracted using the TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instruction. 2 μ g of RNA were annealed to oligo(dT) at 65°C for 10 min. The samples were cooled at 24°C. Reverse transcriptase and dNTPs were added to the RNA oligo(dT) mixture and the reactions were carried out at 43°C for 1 h. Primers used are listed as below. RKIP: forward: 5'-GTAAAGCTT-TTAACTGCACTCGGGACTCG-3', reverse: 5'-GTA-CTCGAGTACGACTTGACTGTACTGTG-3'; MMP-3: forward: 5'-AGTGGAGGAAAACCCACCTT-3', reverse: 5'-CCAGGTCCATCAAAGGGTA-3'; MMP-9: forward: 5'-CATCGTCATCCAGTTTGGTG-3', reverse: 5'-TCGAAGATGAAGGGGAAGTG-3'; HMGA2: forward: 5'-CGAAAGGTGCTGGGCAGCTCCGG-3', reverse: 5'-CCATTCCTAGGTCTGCCTCTTG-3'; GAPDH: forward: 5'-CGGAGTCAACGGATTTGGT-

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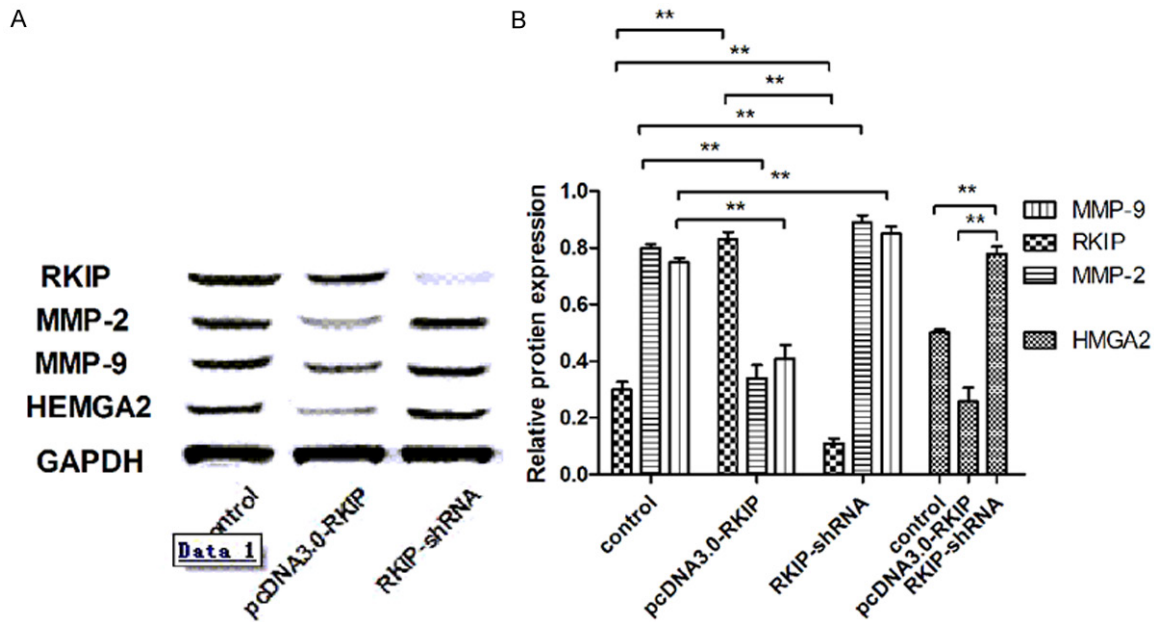


Figure 5. Effects of over-expressing and knockdown RKIP on protein expression in human U87 glioma cells. A. The RKIP protein relative expressions (vs. GAPDH), B. The protein relative expression of MMP-2, MMP-9 and HEMGA2, * $P < 0.05$, ** $P < 0.01$.

CGTAT-3', reverse: 5'-AGCCTTCTCCATGGTGGTG-AAGAC-3'. GAPDH was used as the loading control. PCR reaction was stopped after 25 cycles.

Protein extraction and Western blotting

Glioma cells was lysed and homogenized in lysis buffer (2% deoxycholic acid, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.2). After gel electrophoresis, proteins were blotted onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in phosphate-buffered saline and incubated at 4°C for a night with primary antibodies against RKIP (1:500 dilution), MMP-2 (1:500 dilution), MMP-9 (1:500 dilution), HEMGA2 (1:500 dilution) or GAPDH (1:1000 dilution). This was followed by incubation with secondary antibody (1 hour at room temperature). Relative density of the bands of protein of interest to GAPDH band was analyzed using the Image Pro Plus image analysis system.

Statistical analysis

Data are shown as mean \pm standard deviation. The GraphPad Prism 5 (GraphPad Software Inc., USA) was used for statistical analysis. The one-way ANOVA was used for multiple compari-

sons, while the Student's t test was used to test the differences between two groups. $P < 0.05$ was considered as a statistically significant result.

Results

Effects of RKIP on human glioma cells migration

Forty-eight hours after transfection, U87 glioma cell migration was detected by wound healing assay. Our results showed that the migration distances in RKIP-shRNA group were significantly increased than the pcDNA3.0-RKIP group and control group after transfection of 12 h, 24 h and 48 h (Figure 1). The results suggested that the over-expression of RKIP could inhibit the glioma cell migration.

Effects of RKIP on human glioma cells invasion

Then, the Transwell assay was carried out to determine the effects of RKIP on glioma cell invasion. Similarly, the results showed that the numbers of invasion cells in RKIP-shRNA group were significantly increased than the pcDNA3.0-RKIP and control (Figure 2). The RKIP over-expressing cells showed an inhibiting invasive

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ability. These results revealed that RKIP can suppress the ability of glioma cell invasion.

Effects of RKIP on human glioma cells proliferation

To determine the effects of RKIP on the ability of proliferation in U87 cells, the MTT assay was carried out to measure the proliferation of cells that over-expressing or down regulating of RKIP. Our data suggested that RKIP had not have the ability of inhibitory ability of proliferation (**Figure 3**).

RKIP regulate the mRNA and protein expression of related migration and invasion related proteins

In order to explore the mechanisms that the RKIP regulate the migrations and invasions of glioma cell, we performed RT-qPCR and Western blot analysis to measure the mRNA and protein expressions of MMP-2, MMP-9 and HMGA2. As we can see in **Figures 4A, 5A and 5B**, the mRNA and protein expressions of RKIP in the pcDNA3.0-RKIP group was remarkably increased comparing to the control ones ($P<0.01$). On the contrary, in the PKIP-shRNA group, the mRNA and protein expression of RKIP had a significant reduction ($P<0.01$). We can also see that the mRNA and protein expression of MMP-2 and MMP-9 in group of pcDNA3.0-RKIP was remarkably higher than the control group ($P<0.01$) (**Figures 4B, 4C and 5B**). However, those in group of PKIP-shRNA conversely had a significant reduction. Our results also showed that the mRNA and protein expression of HMGA2 was remarkably lower in the pcDNA3.0-RKIP and significantly higher comparing to the control ($P<0.01$, respectively) (**Figures 4D and 5B**). As we know that MMPs protein and HMGA2 play vital roles in migration and invasion of many kinds of cancers, our results suggests that RKIP inhibit glioma cell invasion through down regulating the MMPs and HMGA2 expression.

Discussion

Glioma is one of the common malignant tumors in human which has a very low level of survival rate. The primary reason on the low efficiency of existing treatments is the high ability of glioma cell invasion [18-20]. As a result, it is important and necessary to explore the mechanism

of the glioma cell migrations and invasions. There are many previous researches reveal the molecular mechanisms of glioma cell migration and invasion. Zhou et al. reported that knock-down of GOLPH3 expressions led to decreases of glioma cells migrations and invasions [21]. Gao et al. revealed that over-expression of SERPINB1 inhibited the migration and invasion of glioma cells, however, knockdown expression of SERPINB1 promoted those effects. In addition, SERPINB1 inhibited glioma migration and invasion probably by inhibiting MMP-2 expression [22]. Tian et al. demonstrated that let-7b/i inhibit the glioma cell invasion through inhibiting the expression of IKBKE and promoting the expression of E-cadherin [16].

RKIP was known as a member of phosphatidyl ethanolamine binding protein. Then, it was regarded as a blocker of Raf kinase activation in MEK pathway. Lots of papers had proved that RKIP regulates NF- κ B pathway [23-25]. A volume of researches had reported that over-expression of RKIP could inhibit the cell migration and invasion in many kinds of cancers. H. Xinzhou et al. revealed the effects of RKIP on cells proliferations, migration and invasion in human prostate cancer PC-3M cells model. Their results suggested that RKIP did not have an inhibitive effect on proliferations of PC-3M cells, however, RKIP could inhibit the cell migration and invasion in PC-3M cell system. In their research, the molecular mechanisms that PKIP inhibited the cell invasion by downregulating the expression of MMPs was also reported [26]. The evidences suggest that RKIP may be a novel targets for the treatments of cancers. But the effects of RKIP on glioma cell migration and invasion were little known.

Migration and invasion of cells are tanglesome courses including cytoskeletal-reorganizations and degradations of ECM [27]. Normally, degradations of ECM are primary steps of cancer cell invasions. MMP-2 and MMP-9 may be the mediations of these courses. MMP-2 and MMP-9 degraded native collagen type IV, promoting degradations of the ECM, which helps the cancer cells to run away from the primary sites and motivate cell invasion [28-31]. The high mobility group AT-hook 2 (HMGA2) are involved in many biological events including developments of several cancers [32]. HMGA2 is highly expressed in lots of cancers including rectal carcinoma, breast cancer, and gastric carcinoma.

ma. Therefore, HMGA2 play vital roles in the developments of cancers [33, 34]. Liu et al. found that the over-expression HMGA2 could promote proliferations and invasions in gastric cancer cells [33].

In our present paper, we revealed roles of RKIP in U87 cell migrations and invasions. Our results showed that over-expression of RKIP lessened cell migration and invasion, however, turning down the RKIP expression showed the inverse effect. Therefore, we demonstrated that RKIP is an underlying target for the treatment of glioma.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhiren Zhang, Departments of Clinical Pharmacy and Cardiology, The Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Road, Nangang District, Harbin 150086, China. E-mail: zhngzhiren@163.com

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