Original Article RKIP suppresses the proliferation and invasion ofchoriocarcinoma cells through inhibiting the MAPK signaling pathway

Meng Zhang^{1,2}, Di Zhang³, Qingxia Fan¹

¹Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China; ²Department of Oncology, The First Affiliated Hospital of Nanyang Medical College, Nanyang 473000, China; ³Office of Scientific Research and Development, Sun Yat-sen University, Guangzhou 510275, China

Received September 20, 2015; Accepted December 6, 2015; Epub December 15, 2015; Published December 30, 2015

Abstract: Raf kinase inhibitor protein (RKIP), an inhibitor of Raf-mediated activation of mitogen-activated protein/ extracellular signal-regulated kinase kinase (MEK), has been identified as a metastasis suppressor gene. However, the role of RKIP in human choriocarcinoma remains undetermined. Therefore, in the present study, we investigated the expression of RKIP in human choriocarcinoma cells and evaluated the effects of RKIP on choriocarcinoma cell proliferation and invasion. Our results indicated that RKIP was lowly expressed in human choriocarcinoma cells. Overexpression of RKIP inhibits choriocarcinoma cell proliferation, migration and invasion, promotes the apoptosis of choriocarcinoma cells. Furthermore, overexpression of RKIP significantly inhibited the expression of MEK and ERK phosphorylation in choriocarcinoma cells through inhibiting the MAPK signaling pathway, implying that RKIP may serve as a potential molecular target for the treatment of human choriocarcinoma.

Keywords: Raf kinase inhibitor protein (RKIP), choriocarcinoma, invasion

Introduction

Choriocarcinoma is a highly malignant trophoblastic tumor, and occurs in females at the origin of the chorionic epithelium of the placenta. This rare malignant tumor exhibits a high incidence of metastasis, primarily to the lungs, brain, and liver, and there is no effective treatment [1, 2]. This is largely attributed to a lack of complete understanding of the exact mechanism for this malignancy. Therefore, further understanding of the molecular mechanisms of choriocarcinoma progression and the development of novel strategies for the diagnosis, treatment and prognosis of choriocarcinoma are required.

Raf-1 kinase inhibitory protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, is a conserved, small, cytosolic protein originally purified from bovine brain. RKIP has been identified as a modulator of extracellular signal-regulated kinase (ERK) [3], nuclear factor-kappa B (NF-kB) [4], and G protein coupled receptor (GPCR) signaling cascades [5]. RKIP was found to be implicated in neural development, cardiac function, and spermatogenesis [6-8]. For example, Hellmann et al. reported that RKIP enhances neuronal differentiation in human SH-SY5Y cells [9]. In addition, RKIP has been identified as a tumor suppressor gene. Significantly decreased RKIP expression has been demonstrated in many kinds of malignant tumors, such as colorectal cancer [10], breast cancer [11] and prostate cancer [12]. RKIP was also reported to inhibit invasiveness and metastasis of malignant tumor [10]. However, the role of RKIP n human choriocarcinoma remains undetermined. Here, the expression of RKIP was evaluated in human choriocarcinoma cell lines. The in vitro effects of RKIP in relation to proliferation, invasion, apoptosisand cell signaling pathways putatively involved were also investigated.



Figure 1. RKIP is lowly expressed in human choriocarcinoma cell lines. A. RKIP mRNA expression in human choriocarcinoma cell lines (BeWo and JEG-3 cells) was significantly lower than that in normal HTR-8/SVneo cell line as determined by RT-qPCR. B. Down-regulated RKIP protein was displayed in BeWo and JEG-3 cells than that in HTR-8/SVneo cells by western blot. All the data are expressed as means \pm SD from three independent experiments. *P<0.05 as compared with the control group.

Materials and methods

Cell culture

The human choriocarcinoma cell lines (BeWo cells and JEG-3) and a normal HTR-8/SVneo cell line (American Type Culture Collection, Manassas, VA) were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin and 50 μ g/ml of streptomycin (Sigma, St. Louis, MO, USA).

Total RNA extraction, reverse transcription and quantitative real-time quantitative PCRs (RT-qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 2 μ g of total RNA was reversely transcribed for cDNA

using the reverse transcription (RT) kit (Takara Biotechnology, Dalian, China) and Oligo dT primer according to the manufacturer's instruction. RT-qPCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) with Fast Start Universal SYBR Green Master (Roche, USA). The specific primers were as follows: RKIP, forward: 5'-AGCAGTGGCA-CAGTCCTC-3', reverse 5'-TGGTCTCCAGATCGG-TTG-3'; and β-actin, forward: 5'-AGAAAATCTG-GCACCACACC-3', reverse: 5'-TAGCACAGCCTG-GATAGCAA-3'. The PCR cycling program was 95°C for 3 min, then 35 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 15 s, and a final extension at 72°C for 5 min.Relative quantification of RKIP mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

Total proteins were extracted from cells using RIPA lysis buffer (Beyotime, Nantong, China). The protein concentration was measured using Bradford protein dye reagent (Bio-Rad). Proteins (30 µg/lane) were separated by 10% SDSpolyacrylamide gel electrophoresis and transferred onto transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline. After blocking, the target proteins were probed with primary antibodies (anti-RKIP, anti-phospho-MEK, anti-MEK, antiphospho-ERK, anti-ERK or GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then, the blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody. The signal was visualized with an enhanced chemiluminescence detection reagent (Millipore, Boston, MA, USA). GADPH was used as the loading control.

Plasmid construction and transfection

FLAG-tagged RKIP expression vector was constructed by inserting PCR amplified RKIP fragment into a pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) linked with FLAG tag at the amino terminus. The RKIP expression vector and empty pcDNA3 were transfected into human choriocarcinoma cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were selected with G418 at the concentration 800 µg/ml, and the resistant clones were further confirmed by Western blotting.



Figure 2. RKIP inhibits the proliferation of choriocarcinoma cells. A, B. BeWo and JEG-3 cells were transfected with empty vector or Flag-RKIP for 48 h. Expression of RKIP protein was assessed by Western blotting and normalized to GAPDH. C, D. Overexpression of RKIP significantly the proliferation of BeWo and JEG-3 cells, respectively. All the data are expressed as means \pm SD from three independent experiments. *P<0.05 as compared with the empty vectorgroup.Vector, transfected with an empty vector; OE, overexpression.

Proliferation assay

Cell proliferation was evaluated by 3-(4. 5-methylthiozol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay. In brief, BeWo cells and JEG-3 cells were seeded in a 96-well culture plate at a density of 1×10^4 cells/well, respectively. After 24, 48 or 72 h of incubation, the medium was discarded and replaced with an equal volume (100 µL) of fresh medium containing 0.456 mg/mL MTT and incubated for 4 h at 37°C in the dark. The culture media was removed and 200 µl DMSO was added to each well. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Migration and invasion assays

In vitro Transwell migration assays were performed in modified Boyden chambers with 8-mm pore filter inserts in 24-well plates (BD Biosciences, Eugene, OR, USA). Briefly, the lower chamber was filled with DMEM containing 10% FBS. Cells transfected with empty vector or Flag-RKIP (1×10^5 cells per well) suspended in 0.1% FBS medium were seeded in the upper compartment. After 24 h of incubation, cells were fixed in methanol for 15 minutes and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells on the under-



Figure 3. RKIP inhibits the migration and invasion of choriocarcinoma cells. BeWo and JEG-3 cells were transfected with empty vector or Flag-RKIP for 24 h. A. Effect of RKIP overexpression on BeWo cell migration using Transwell assay. B. Effect of RKIP overexpression on BeWo cell invasion using Matrigel assay. C. Effect of RKIP overexpression on JEG-3 cells invasion using Matrigel assay. All the data are expressed as means \pm SD from three independent experiments. *P<0.05 as compared with the empty vector group.Vector, transfected with an empty vector; OE, overexpression.

side of the filters were viewed and counted under a microscope (Olympus, Tokyo, Japan).

Invasion assays were done using modified Boyden chambers with filter inserts for 24-well dishes containing 8-µm pores (Millipore, Boston, MA, USA). The same procedures described above were used, except that the filters were precoated with 100 ml Matrigel at a 1:4 dilution in DMEM to form a genuine reconstituted basement membrane.

Cell apoptosis assay

JEG-3 cells (1×10⁵ cells/ml) were seeded into each well of a 24-well plate for 48 h. The staining was carried out according to the instructions provided by the manufacturer of the Pl Annexin-V Apoptosis Detection Kit I, BD Pharmingen (BD Biosciences, USA). Briefly, cells were washed with PBS, suspended in 1× binding buffer and then treated with annexin-V APC and propidium iodide (PI) for 15 min. The percentage of cells undergoing apoptosis and necrosis was quantified using a flow cytometer (Abcam, Cambridge, UK) equipped with Cell Quest software within 1 h.

Statistical analysis

All experiments were repeated three times, and data were expressed as the mean \pm SD from a representative experiment. The statistical significance of the difference was analyzed by ANOVA and post hoc Dunnett's test. Statistical significance was defined as P<0.05.

Results

RKIP is lowly expressed in human choriocarcinoma cell lines

The expression profile of RKIP at mRNA in two human choriocarcinoma cell lines (BeWo and



Figure 4. RKIP promotes the apoptosis of choriocarcinoma cells. A. JEG-3 cells were transfected with empty vector or Flag-RKIP for 24 h. Apoptosis was determined by AnnexinV-FITC/PI staining detected by flow cytometry. B. Quantification of A. C. The expression of apoptotic proteins Bax and BcI-2 in JEG-3 cells was assessed by Western blot. GAPDH was used as internal control to ensure that equal amounts of proteins were loaded in each lane. All the data are expressed as means \pm SD from three independent experiments. *P<0.05 as compared with the empty vector group.Vector, transfected with an empty vector; OE, overexpression.

JEG cells), and a normal HTR-8/SVneo cell line was examined by RT-qPCR analysis. As indicated in **Figure 1A**, RKIP mRNA expression was down-regulated in BeWo and JEG-3 cells compared with that of HTR-8/Svneo cells. Similarly, Western blot analysis demonstrated that the expression of RKIP protein was also decreased in BeWo and JEG cells (**Figure 1B**).

Effect of RKIP on the proliferation of choriocarcinoma cells

To determine the cellular function of RKIP, BeWo and JEG-3 cell lines were transiently transfected with RKIP. We observed that overexpression of RKIP significantly increased endogenous RKIP protein expression in BeWo and JEG-3 cells, respectively (**Figure 2A** and **2B**). Next, the effect of RKIP on the proliferation of choriocarcinoma cells was determined using MTT assay. We found that overexpression of RKIP significantly inhibited the proliferation of BeWo and JEG-3 cells, respectively (**Figure 2C** and **2D**).

Effect of RKIP on themigration and invasion of choriocarcinoma cells

Next, the effect of RKIP on the migration and invasion of choriocarcinoma cells was exam-

Int J Clin Exp Med 2015;8(12):22183-22190

RKIP suppresses choriocarcinoma cell proliferation and invasion



Figure 5. RKIP suppresses choriocarcinoma cell migration and invasion via MAPK signaling. JEG-3 cells were transfected with empty vector or Flag-RKIP for 24 h. (A) The protein level of p-MEK/MEK and p-ERK/ ERK were determined by western blot analysis, GAP-DH was used as a loading control. Quantification of (B) p-MEK/MEK and (C) p-ERK/ERK. All the data are expressed as means \pm SD from three independent experiments. *P<0.05 as compared with the empty vector group.Vector, transfected with an empty vector; OE, overexpression.

ined. We found that overexpression of RKIP significantly reduced the migratory ability of BeWo and JEG-3 cells, respectively (**Figure 3A** and **3C**). In addition, Boyden chamber invasion assays demonstrated that the invasiveness of BeWo and JEG-3 cells was also significantly decreased after overexpression of RKIP (**Figure 3B** and **3D**).

Effect of RKIP on the apoptosis of choriocarcinoma cells

Then, we investigated whether RKIP can regulate apoptosis of choriocarcinoma cells. As indi-

cated in Figure 4A and 4B, the percentage of apoptotic cells was higher in the RKIPoverexpressing JEG-3 cells than that in the empty vector-containing JEG-3 cells (from 22.3% to 5.6%). Consistent with the results of RKIP modulation of apoptosis, RKIP overexpression increased the expression of Bax, and decreased the expression of Bcl-2 in JEG cells (Figure 4C).

RKIP suppresses choriocarcinoma cell migration and invasion via MAPK signaling

RKIP has been identified as a suppressor of the mitogen-activated protein kinase (MAPK) pathway [13]. So, we investigated the effect of RKIP on MAPK signal-related molecules expression. As indicated in **Figure 5**, we found that the expression of MEK and ERK phosphorylation was dramatically decreased in transfected JEG-3 cells, whereas no change in total MEK and ERK was observed. These results imply that RKIP may directly influence activation of the ERK/MAPK pathway.

Discussion

RKIP can inhibit cell proliferation and migration/invasion of gastric cancer [14], colorectal cancer [10] and glioma [15]. However, the role of RKIP in human choriocarcinoma is unknown. This study suggests critical function of RKIP in human choriocarcinoma. First, RKIP was lowly expressed in human choriocarcinoma cells. Second, overexpression of RKIP inhibits choriocarcinoma cell proliferation, migration and invasion. Third, overexpression of RKIP promotes choriocarcinoma cell apoptosis. Mechanistically, overexpression of RKIP significantly inhibited the expression of MEK and ERK phosphorylation in choriocarcinoma cells.

RKIP is a metastasis suppressor whose expression is reduced in nasopharyngeal carcinomatissues and is absent in nasopharyngeal carcinoma metastases [16]. The expression of RKIP in gastric cancer cells was also decreased significantly in comparison to that of normal gastric mucosal epithelial cells, and up-regulation of RKIP significantly decreased the cell viability and the S-phase fraction in SGC-7901 cells [14]. Consistent with the previous studies, in this study, it was found that the expression of RKIP was down-regulated in BeWo and JEG cells compared with that of HTR-8/Svneo cells, and overexpression of RKIP inhibits choriocarcinoma cell proliferation. These findings indi-

Int J Clin Exp Med 2015;8(12):22183-22190

cate that RKIP may play an important role in the development and progression of choriocarcinoma.

Cell motility is determined by dynamic cytoskeleton and contributes significantly to the migration, invasion and metastasis of malignant tumor cells [17]. Tumor invasion is an early marker of metastasis. Therefore, understanding of tumor invasion is of great importance. Wang et al. reported that overexpression of RKIP suppressed colorectal cancer cell metastasis in vitro and in vivo, whereas knockdown of RKIP expression in colorectal cancer cells and its murine model increased metastatic ability [10]. Overexpression of RKIP in prostate cancer cells was also associated with decreased in vitro cell invasion, decreased development of lung metastases in vivo [12]. In line with these results, our study confirms the critical roles of RKIP implicated in motility of choriocarcinoma cells. Overexpressed RKIP largely inhibited cell migration and invasion. These results suggest that the expression level of RKIP is a negative regulator of migration and invasiveness in choriocarcinoma cells.

Apoptosis, which is known as programmed cell death, is a physiological process which is mediated by a series of gene regulation and cellsignaling pathways. The Bcl-2 family of proteins represents key regulators of apoptosis and can be classified into two functionally distinct groups: anti- and pro-apoptoticproteins. Bcl-2 protein is a critical cell death inhibitor, whereas Baxacts as pro-apoptoticprotein. Alterations in the levels of anti- and pro-apoptotic proteins influence apoptosis [18]. In the present study, we found that overexpression of RKIP promotes apoptotic processes along with decreased ratio of Bcl-2/Bax in choriocarcinoma cells.

Accumulating evidence suggests that the MAPK pathway plays an important role in several tumors [19-21]. The Ras-dependent Raf/ MEK1/2-ERK1/2 signaling cascade is one of most extensively studied MAPK pathways, hyperactivation of which has been demonstrated by previous studies to play major roles in choriocarcinoma cell proliferation and metastasis [22, 23]. In addition, it has been documented that RKIP, which negatively regulates the mitogen-activated protein kinase (MAPK) pathway.In hepatocellular carcinoma, reduced RKIP expression has been demonstrated, which contributes to dysregulated MAPK signaling pathway and enhanced proliferation and migration of Hepatocellular carcinoma cells *in vitro* [24]. In the present study, we found that the expression of MEK and ERK phosphorylation was dramatically decreased in transfected JEG cells, whereas no change in total MEK and ERK was observed. These results imply that RKIP may suppress the proliferation and invasion of choriocarcinoma cells through inhibiting the MAPK signaling pathway.

In conclusion, RKIP suppresses the proliferation and invasion of choriocarcinoma cells through inhibiting the MAPK signaling pathway. These data suggest that RKIP plays an important role in the pathogenesis of choriocarcinoma and is a potential therapeutic target for human choriocarcinoma treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qingxia Fan, Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China. Tel: +86-0371-66913114; Fax: +86-0371-669131-14; E-mail: fqxoncol@163.com

References

- [1] Zheng J, Lu J, Cheng M and Xu C. Lung metastasis of human choriocarcinoma in mice: establishment of experimental metastatic model and its biological characteristics. Zhonghua Fu Chan Ke Za Zhi 2010; 45: 519-524.
- [2] Mamelak AN, Withers GJ and Wang X. Choriocarcinoma brain metastasis in a patient with viable intrauterine pregnancy: Case report. J Neurosurg 2002; 97: 477-481.
- [3] Schuierer MM, Bataille F, Hagan S, Kolch W and Bosserhoff AK. Reduction in Raf kinase inhibitor protein expression is associated with increased Ras-extracellular signal-regulated kinase signaling in melanoma cell lines. Cancer Res 2004; 64: 5186-5192.
- [4] Yeung KC, Rose DW, Dhillon AS, Yaros D, Gustafsson M, Chatterjee D, McFerran B, Wyche J, Kolch W and Sedivy JM. Raf kinase inhibitor protein interacts with NF-κB-inducing kinase and TAK1 and inhibits NF-κB activation. Mol Cell Biol 2001; 21: 7207-7217.
- [5] Lorenz K, Lohse MJ and Quitterer U. Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. Nature 2003; 426: 574-579.
- [6] Wen Z, Shu Y, Gao C, Wang X, Qi G, Zhang P, Li M, Shi J and Tian B. CDK5-mediated phosphor-

ylation and autophagy of RKIP regulate neuronal death in Parkinson's disease. Neurobiol Aging 2014; 35: 2870-2880.

- [7] Faramawy YE, Fu X, Agwa SH, Langer A, Elzahwy SS and Quitterer U. RKIP enhances angiotensin II-stimulated signaling. Forum on Immunopathological Diseases and Therapeutics 2011; 2: 71-78.
- [8] Stanton PG, Sluka P, Foo C, Stephens AN, Smith Al, McLachlan RI and O'Donnell L. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation; impact on meiotic cells. PLoS One 2012; 7: e41718.
- [9] Hellmann J, Rommelspacher H, Mühlbauer E and Wernicke C. Raf kinase inhibitor protein enhances neuronal differentiation in human SH-SY5Y cells. Dev Neurosci 2010; 32: 33-46.
- [10] Wang Y, Wang LY, Feng F, Zhao Y, Huang MY, Shao Q, Chen C, Sheng H, Chen DL and Zeng ZL. Effect of Raf kinase inhibitor protein expression on malignant biological behavior and progression of colorectal cancer. Oncol Rep 2015; 34: 2106-2114.
- [11] Li HZ, Gao Y, Zhao XL, Liu YX, Sun BC, Yang J and Yao Z. Effects of raf kinase inhibitor protein expression on metastasis and progression of human breast cancer. Mol Cancer Res 2009; 7: 832-840.
- [12] Fu Z, Smith PC, Zhang L, Rubin MA, Dunn RL, Yao Z and Keller ET. Effects of raf kinase inhibitor protein expression on suppression of prostate cancer metastasis. J Natl Cancer Inst 2003; 95: 878-889.
- [13] Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW and Mischak H. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature 1999; 401: 173-177.
- [14] Liu H, Li P, Li B, Sun P, Zhang J, Wang B and Jia B. RKIP suppresses gastric cancer cell proliferation and invasion and enhances apoptosis regulated by microRNA-224. Tumor Biol 2014; 35: 10095-10103.
- [15] Chen Z, Cheng Q, Ma Z, Xi H, Peng R and Jiang B. Overexpression of RKIP inhibits cell invasion in glioma cell lines through upregulation of miR-98. Biomed Res Int 2013; 2013: 695179.

- [16] He QY, Yi HM, Yi H, Xiao T, Qu JQ, Yuan L, Zhu JF, Li JY, Wang YY, Li LN, Feng J, Lu SSand Xiao ZQ. Reduction of RKIP expression promotes nasopharyngeal carcinoma invasion and metastasis by activating Stat3 signaling. Oncotarget 2015; 6:16422-16436.
- [17] Gimona M. Mechanics and dynamics of the cytoskeleton. Cell Motil Cytoskeleton 2009; 66: 769-770.
- [18] Yin XM, Oltvai ZN and Korsmeyer SJ. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 1994; 369: 321-323.
- [19] Dhillon A, Hagan S, Rath O and Kolch W. MAP kinase signalling pathways in cancer. Oncogene 2007; 26: 3279-3290.
- [20] Guyton KZ, Gorospe M, Kensler TW and Holbrook NJ. Mitogen-activated protein kinase (MAPK) activation by butylated hydroxytoluene hydroperoxide: implications for cellular survival and tumor promotion. Cancer Res 1996; 56: 3480-3485.
- [21] Huynh H, Nguyen TT, Chow KH, Tan PH, Soo KC and Tran E. Over-expression of the mitogenactivated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. BMC Gastroenterol 2003; 3: 19.
- [22] Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, Weber M, Hölters S, Schleussner E, Fitzgerald JS and Markert UR. Intranuclear crosstalk between extracellular regulated kinase1/2 and signal transducer and activator of transcription 3 regulates JEG-3 choriocarcinoma cell invasion and proliferation. Scientific-WorldJournal 2013; 2013: 259845.
- [23] Thang NM, Kumasawa K, Tsutsui T, Nakamura H, Masaki H, Ono T and Kimura T. Overexpression of Endogenous TIMP-2 Increases the Proliferation of BeWo Choriocarcinoma Cells Through the MAPK-Signaling Pathway. Reprod Sci 2013; 20: 1184-1192.
- [24] Wu X, Yang Y, Xu Z, Li J, Yang B, Feng N, Zhang Y and Wang S. Raf kinase inhibitor protein mediated signaling inhibits invasion and metastasis of hepatocellular carcinoma. Biochim Biophys Acta 2015.