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sition in prostate cancer cells**

A novel retinoic acid chalcone reverses epithelial-mesenchymal transition in prostate cancer cells

Jian Zhong^{1,2} and Jian-Quan Hou¹

¹Department of Urology, The First Hospital Affiliated to Soochow University, Suzhou, Jiangsu 215 006, China;

²Department of Urology, Zhangjiagang Hospital Affiliated to Nanjing University of Traditional Chinese Medicine, Suzhou, Jiangsu, 215 600, China.

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Abstract

The present study was performed to investigate the effect of retinoic acid fluoro chalcone (RAFC) on lipopolysaccharide (LPS) induced epithelial-mesenchymal transition (EMT) in PC3 and CWR22rv1 prostate cell lines. Lipopolysaccharide (LPS) was used to induce epithelial-mesenchymal transition in prostate carcinoma cell lines. The results revealed that treatment of PC3 and CWR22rv1 cells with LPS resulted in significant changes in the morphological features of the EMT. The mesenchymal marker, vimentin expression was significantly increased whereas the expression level of E-cadherin was markedly decreased after the treatment. We also observed increased cell motility and higher level of transcription factor glioma-associated oncogene homolog 1 (Gli1) expression on LPS treatment. Treatment of prostate cells with RAFC reversed the morphological changes induced by LPS in prostate cells. RAFC also reduced the expression of EMT markers induced by LPS and suppressed the Gli1 expression. The resultant effect of these changes was the suppression of motility and invasiveness of the prostate cells. Thus, RAFC exhibited anti-invasive effect on prostate cells by inhibition of the EMT process via Hedgehog signaling pathway.

Introduction

Prostate cancer is the commonly diagnosed and second leading cause of death in men following the lung cancer in USA. Among every 36 men one dies of prostate cancer (Snyder, et al. 2013). Cancer metastasis is believed to be the main reason responsible for the higher mortality rate of prostate cancer patients (Sandhu, et al. 2013). Despite advancement in chemotherapy prostate cancer has been the challenging target for clinicians throughout the globe (Bhavsar et al. 2013). During the process of embryogenesis epithelial-mesenchymal transition (EMT) has been shown to develop various cancer types (Bhavsar, et al. 2013; De Craene, et al. 2013; Wu, et al. 2013). The process of EMT is associated with mesenchymal marker expression like vimentin, fibronectin and N-cadherin by the epithelial

cells. These markers promote prostate cancer cells to undergo metastatic activity (Evdokimova, et al. 2012). Since EMT induces invasive and metastatic properties of the cancer cells (Hance, et al. 2012; Lu, et al. 2012; Clyne, 2012) discovery of the molecules inhibiting EMT has a prominent role in the treatment of prostate cancer. Because of poor prognosis the search for novel molecules for the treatment of prostate cancer is highly desired (Fryer, et al. 2009; Surh, 2003).

Retinoids are the diterpenoid compounds well known for their role in the regulation of cell cycle behaviour (Krupitza, et al. 1995; Defer, 1997). Earlier retinoids were used for the treatment of dermatological diseases. Retinoids exhibit potent activity for the treatment of breast cancer (Huang, et al. 1988), head and neck cancer (Lehman, et al. 1988), ovarian adenocarcinoma (Szuts,



et al. 1991), human malignant gliomas (Jeong, et al. 2006) and acute promyelocytic leukemia (Chung, 2012). However, the promising biological activity of RA is limited by its poor aqueous solubility (0.1 μM at pH 7.3) under in vivo conditions (Crocetti, et al. 2012; Tanaka, et al. 2013). Numerous efforts have been made to overcome this limitation. This includes the development of polymeric micelles (Stummer and Kamp, 2009), like glycol chitosan micelle. It is reported that inhibition of HuCC-T1 cholangiocarcinoma cell proliferation is significantly achieved by RA-incorporated GC nanoparticles (Norden, et al. 2006). The present study was designed to investigate the effect of retinoic acid chalcone (Figure 1) on inhibition of LPS-triggered EMT in prostate cancer cells. Moreover, the effect of retinoic acid chalcone on inhibition of the Hedgehog (Hh) signaling pathway was also examined.

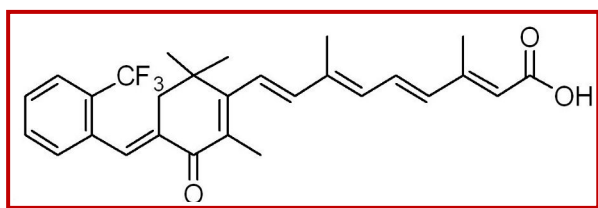


Figure 1: Structure of retinoic acid trifluoromethyl chalcone (RAFC)

Materials and Methods

Reagents and chemicals

RAFC was a gift from the Dr. Ummah yadav and LPS were purchased from Sigma-Aldrich, USA (purity > 97%). The antibodies for oncogene homolog 1 (Gli1), E-cadherin, vimentin and β -actin were obtained from Wako Pure Chemicals (Osaka, Japan).

Cell line and culture

Prostate carcinoma cell lines PC3 and CWR22rv1 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Carlsbad, CA, USA) supplemented with streptomycin. All the cell lines were incubated in 95% air and 5% CO_2 atmosphere at 37°C. Cells were grown to 80% confluence before treatment. The present study was approved by the Institutional Review Board and Ethics Committee of the Nanjing University, Jiangsu, China.

MTT assay

The cells at a density of 2.5×10^5 per 100 μL were seeded onto 96-well plates and incubated overnight. The cells were pre-treated with 5, 10, 15 or 20 $\mu\text{g}/\text{mL}$ RAC for 36 hours followed by addition of 20 μL MTT solution to each well (5 mg/mL). Incubation of plates for 4 hours at 37°C in 5% CO_2 was followed by removal of supernatants and addition of 150 μL . Plates were then placed on an orbital shaker for 5 min and the absorbance was

recorded using the EnSpire™ 2300 Multilabel Plate Reader (Perkin Elmer Inc., Waltham, MA, USA) at 595 nm.

Scanning electron microscopy

Retinoic acid chalcone treated or untreated cells after harvesting were washed with PBS. The paraformaldehyde and glutaraldehyde were used to fix the cells for 1 hour. The cells were then rinsed in PB before fixing in 1% osmium tetroxide for 1 hour followed by PB washing and dehydration in graded ethanol. Then cells were dried in an acetonitrile solution, gold sprayed and subjected to scanning electron microscopic examination.

Immunofluorescence assay

Cell fixation, permeabilization, and staining was performed as described previously (37). The slides were incubated for 12 hours with cyanine 3-labeled anti-E-cadherin and fluorescein isothiocyanate-labeled anti-vimentin, respectively. Counterstaining of the nuclei was performed by using DAPI. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope, and the acquired images were transferred to Photoshop 6.0 to generate the final figures.

Cell invasion assay

Invasiveness of the cells was examined using a Cell-Invasion-Assay-Kit (ECM550, CHEMICON-Millipore Co. Billerica, MA, USA) with two chambers. The cells were seeded in top chamber containing serum-free medium and in the bottom chamber with complete medium. An ECMatrix™ coated membrane was present between the two chambers. The ECM layer prevents non-invasive cells from migrating through it. 2.5×10^5 cells suspended in 300 μL of serum free media were plated on the membrane inserts of an Invasion Chamber after 24 hours of transfection. Incubation of the cells for 48 hours was followed by removal of the non-invasive cells. Cells on the lower surface of the membrane were stained for visualization using a microscope (magnification, $\times 200$; Carl Zeiss Micro-Imaging, Germany).

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the cells using trizol (Invitrogen, USA) following the manufacturer's instructions. Total RNA was used for reverse transcription using DNA synthesis kit (Invitrogen). Primers for PCR were designed and PCR amplification of cDNA was performed at 35 cycles in a reaction mixture containing 10 μM Tris-HCl (pH 8.3), 1.5 μM MgCl_2 , 50 μM KCl, 0.01% (w/v) gelatin, 200 μM dNTP, SNEP5-specific primers (0.5 μM each), and 2.0 U of platinum TaqDNA polymerase (Invitrogen). For each reaction, two negative controls were performed consisting of omission of the RT step or omission of the target cDNA. The primers used were used and PCR samples were

loaded onto a agarose gel (1.2%) containing ethidium bromide. The housekeeping gene β -actin was used as an internal control. All PCR experiments were performed three times.

Western blotting

The cells after treatment were lysed, and using dye-binding method (Bio-Rad) protein concentration was determined. The 15% SDS-PAGE was employed to resolve the proteins followed by transfer to nitrocellulose membranes. For the cytosolic and mitochondrial fractions we used digitonin-based subcellular fractionation technique. After the membranes were incubated with primary antibody and washed incubation was performed again with horseradish peroxidase anti-mouse or horseradish peroxidase antirabbit antibodies. Enhanced chemiluminescence system was used for visualization of immunoreactive bands.

Statistical analysis

All the results are expressed as the mean of three

independent experiments. The statistical significance of differences was determined by two-sided Student's t-test and one-way ANOVA. The differences were considered statistically significant at $p > 0.05$. Sigma Stat software (Systat Software Inc., San Jose, California, USA) was used for all statistical evaluations.

Results

The PC-3 and CWR22rv1 cells were cultured with various concentrations of RAFC (0, 10, 20, 30 and 50 μ M) for 72 hours. RAFC treatment lead to a significant decrease in the proliferative tendency of both the tested cell line in concentration dependent manner. The proliferation inhibition was least at 10 μ M of RAFC (Figure 2). Therefore, for further experiments 10 μ M RAFC concentration was used.

Treatment of LPS-treated PC-3 and CWR22rv1 cell lines with RAFC was followed by optical and scanning electron microscopy. The results revealed that treat-

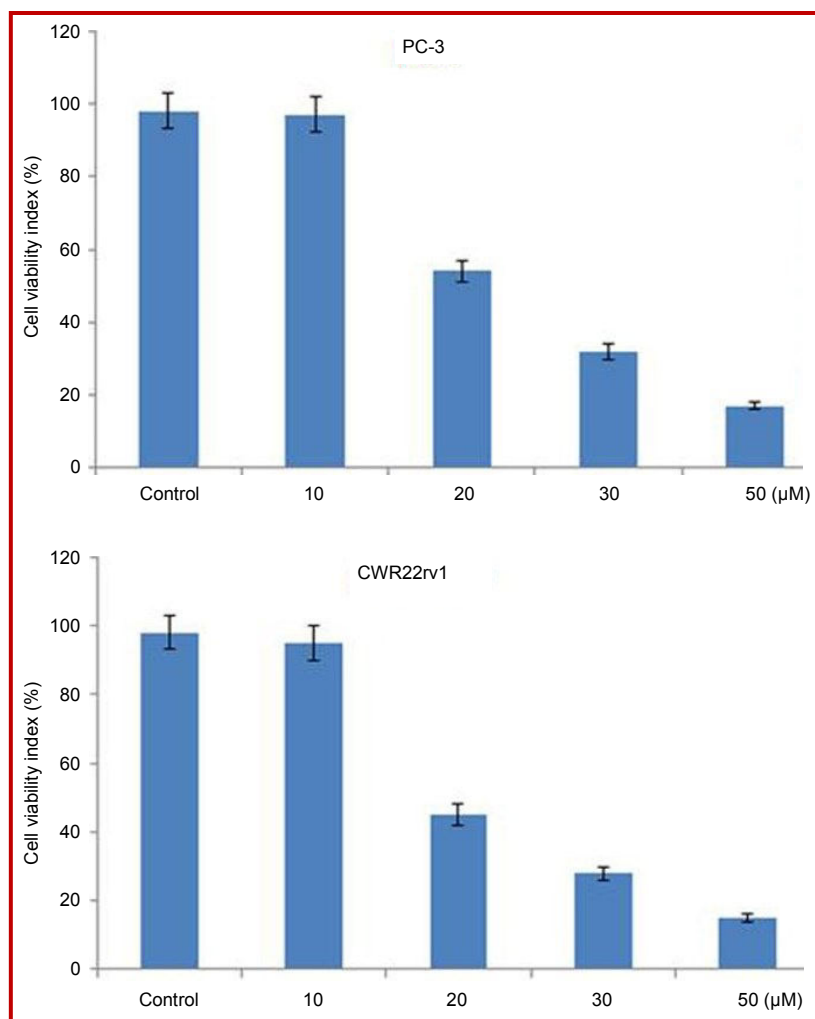


Figure 2: Anti-proliferative effect of RAFC in PC-3 and CWR22rv1 prostate cancer cells. Results are representative of three independent experiments

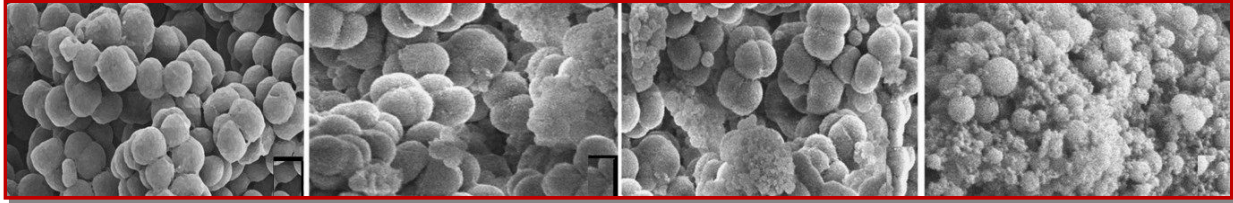


Figure 3: Inhibition of LPS-induced cell morphological features by RAFC in CWR22rv1 cells. Cells were incubated with either LPS (5 $\mu\text{g}/\text{mL}$) or LPS and RAFC. After 48 hours, cellular morphological changes were examined

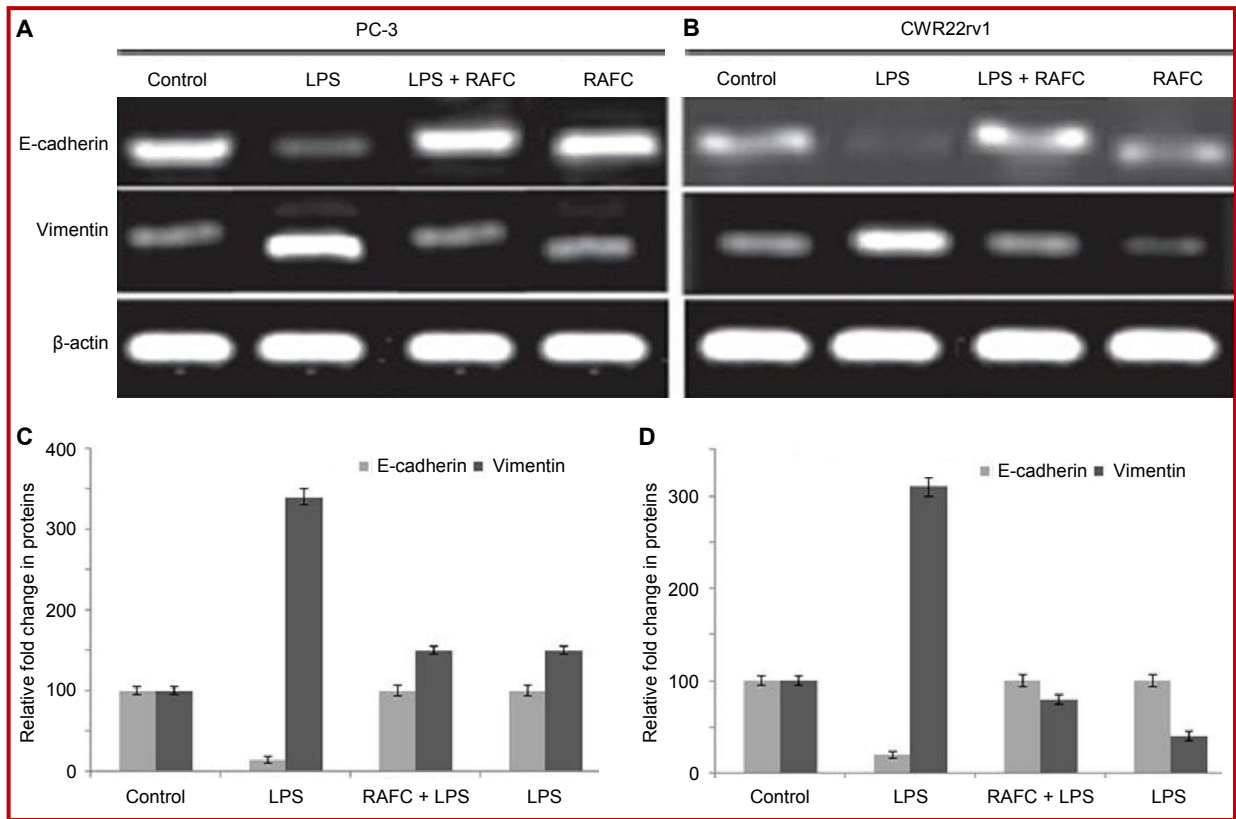


Figure 4: RAFC reverses the LPS-induced decrease in E-cadherin mRNA expression and increase in vimentin mRNA expression. (A and B) The mRNA expression levels of E-cadherin and vimentin in PC-3 and CWR22rv1 cells were determined using reverse transcription polymerase chain reaction. (C-D) E-cadherin and vimentin mRNA quantification in PC-3 and CWR22rv1 cells

ment of LPS-treated prostate cells with RAFC lead to the reversal of LPS-induced morphological features (Figure 3). A significant reduction in mesenchymal phenotype was observed in the cells after co-treatment with LPS and RAFC compared with cells treated with LPS alone.

LPS-treated cells showed significant increase in the expression levels of vimentin and decrease in the expression levels of E-cadherin mRNA (Figure 4A-D). The LPS-induced enhanced vimentin mRNA and reduced E-cadherin mRNA expression was reversed by RAFC treatment (Figure 4, 5).

The effect of RAFC on the expression of Gli1 transcription factor showed that LPS enhanced the Gli1 protein, suppresses E-cadherin and enhances vimentin expression. However, treatment of PC-3 and

CWR22rv1 cells with RAFC resulted in inhibition of this effect (Figure 5).

The results from matrigel model showed that LPS treatment induced significant increase in the number of invasive cells compared to untreated cells. However, co-treatment of PC-3 and CWR22rv1 cells with LPS and RAFC resulted in significant decrease in the number of invasive cells (Figure 6).

Discussion

The current study demonstrates that PC-3 and CWR22rv1 cells on LPS treatment enhanced expression of EMT markers and developed spindle-shaped morphology. However, a reversal of LPS-induced EMT was observed on RAFC treatment in prostate carcinoma

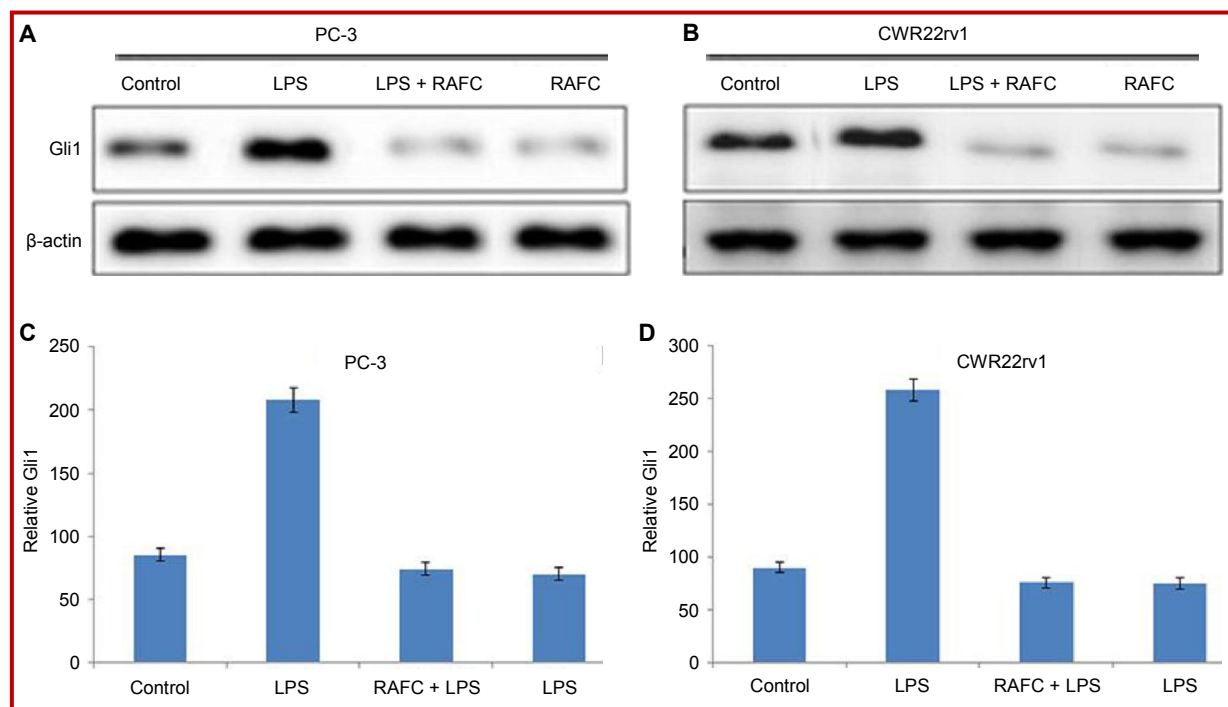


Figure 5: RAFC treatment inhibits hedgehog signaling activation

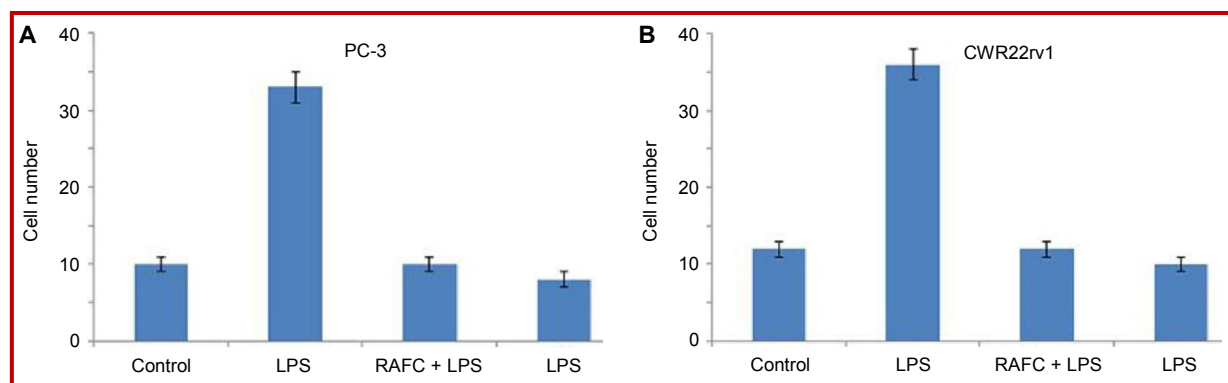


Figure 6: Cell invasion assay in PC-3 and CWR22rv1 cells showing number of cells in different groups

cells. The cells regained the epithelial phenotype and minimised the expression of EMT markers induced by LPS. RAFC treatment induced higher expression of E-cadherin and decreased the expression of vimentin. In Hh signaling pathway Gli1 is a transcription factor which induces the expression of E-cadherin and vimentin (Ruat, et al. 2011). The expression of LPS-induced Gli1 protein induced in prostate cells is suppressed on RAFC treatment. These findings suggest the involvement of Hh signaling in LPS-induced EMT in PC-3 and CWR22rv1 prostate cells.

The promising role of RA to treat various types of cancers (Huang et al. 1988) makes it an effective lead molecule. However, the potent biological activity and poor aqueous solubility of RA lead to the development of polymeric micelles (Ruat, et al. 2011), like glycol

chitosan micelle. In addition, more polar derivatives like chalcones have been also synthesized to overcome solubility issues. The data from our study demonstrates that RAFC suppresses the Gli1 expression, decreases E-cadherin and increases vimentin expression. Additionally, RAFC inhibits cancer cell invasion.

It is reported that Hh on binding to Patched (Ptch) activates Gli1 transcription factors and promotes Gli1 target genes expression (Vaillant and Monard, 2009). Gli1 transcription factor associated with Hedgehog pathway has been shown to regulate genes important for tumor progression (Panman and Zeller, 2003). Our data demonstrates that RAFC inhibits the expression of Gli1, increases the expression of E-cadherin and decreases the expression of vimentin. These results suggest that Hh signalling pathway may be involved in

the regulation of EMT.

Thus, RAFC induced inhibition of tumor invasion may be possibly due to inhibition of the Hh signaling activation and regulation of the expression of the important downstream EMT markers, E-cadherin and vimentin.

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Author Info

Jian-Quan Hou (Principal contact)
e-mail: houjianquan691@gmail.com