

Original Article

The role and mechanism of allyl isothiocyanate on suppressing tumor cells proliferation and invasion of human hepatocellular cancer HepG2 cell line

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Abstract: Allyl isothiocyanate (AITC) is a class naturally compound found in Brassicaceae and some study found that it was able to reduce the incidence of cancers. To investigate the inhibitive effect and mechanism of AITC on proliferation and invasion of human hepatocellular cancer HepG2 cell line. We found AITC significantly inhibited the proliferation of HepG2 cells by inducing the apoptosis and cell cycle arrest of HepG2 cells. Further, it was found that cell adhesion and invasion was suppressed and ROS production was improved, the expression of tumor related gene such as MMP-2/9, Integrin $\alpha 5\beta 1$, Survivin, Bcl-2, Cyclin B1, CDK1, p53, p21, caspase-3/8 was regulated. We also considered that AITC was able to inhibit proliferation and invasion of human hepatocellular cancer HepG2 cell line through inhibiting AKT/NF- κ B pathway.

Keywords: AITC-hepatocellular cancer-oxidative damage

Introduction

As one of the most common malignant tumors in China and elsewhere in the world, liver cancer usually requires comprehensive treatment mainly involving surgical operation, radiotherapy and chemotherapy. It is reported that malignant proliferation are fundamental properties of liver cancer cells. The formation of tumor process involves multiprocess cascades and a wide variety of factors contribute to tumor process includes loss of cellular anti-oncogene and increased oncogene. Therefore, Allyl isothiocyanate (AITC) was considered that it could regulate the expressions of above gene and it may be considered worthy of development for liver cancer.

AITC is a class naturally compound found in Brassicaceae [1]. The studies found that Brassicaceae was able to reduce the incidence of cancers and AITC was confirmed to be the key actions. However, the studies on liver cancer suppression are not still very clear. We investigated the inhibitory roles and mechanisms of

effect of AITC in human hepatocellular cancer HepG2 cell line by observing the effects of AITC on proliferation, apoptosis, cell cycle, reactive oxygen species (ROS) and related signal pathways in HepG2 cell line.

Materials and methods

Cell culture

Human hepatocellular cancer HepG2 cells were purchase from American Type Culture Collection (ATCC) and cultured in DMEM (10% FBS) with 37°C and 5% CO₂.

Vi-CELL assay

The effect of AITC on tumor cell suppression was measured by Vi-CELL Cell Viability Analyzer. Cells (3×10^3) were seeded into 96 well plates overnight and incubated with 0, 1, 2, 5, 10, 20, 50 and 100 μ M AITC for 72 h. The cell viability was determined by Vi-CELL Cell Viability Analyzer in according the manufacture's instruction. Inhibition rate = (1-experimental group OD/control group OD) $\times 100\%$.

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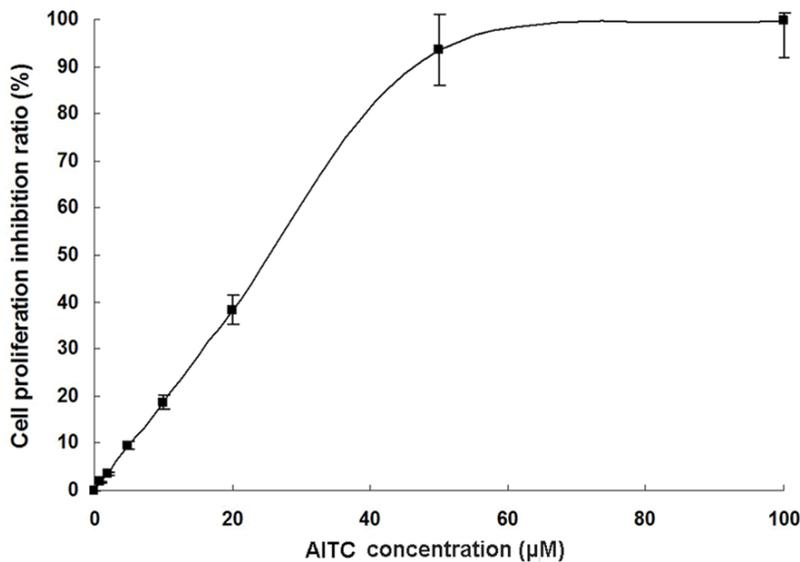


Figure 1. The inhibitory effect of AITC on the HepG2 cell line proliferation. The Vi-CELL assay showed the inhibitory effect of AITC on the HepG2 cell line proliferation. The data was presented as mean \pm SD, bars indicate SD, n=10.

Transwell assay

Cells (2.5×10^4) were seeded in Matrigel chambers of the Transwell system then 2 and 5 μ M AITC was added for 72 h. The invaded cells in the lower surface were fixed by 4% paraformaldehyde, stained by HE and counted by microscope.

Adhesion assay

Cells (3×10^5) were seeded in Matrigel-coated 24-well plates with 2 and 5 μ M AITC for 6 h. Then cells were washed by PBS and absorption was measured by MTS assay at 490 nm. The adhesion rate was calculated by the absorption of the treatment/the absorption of the normal group.

Flow cytometry assay

Cells (3×10^5) were seeded in 6 well plates overnight and 2 and 5 μ M AITC was added for 72 h. Then 20 μ L Annexin V-FITC and PI was added for 15 min and the effects of AITC on tumor cell apoptosis was measured by flow cytometry assay.

Cells (3×10^5) were seeded in 6 well plate overnight and 2 and 5 μ M AITC was added for 72 h. Cells were harvested and fixed in 70% cold ethanol overnight at 4°C, then cultured with PI for 30 min and the effects of AITC on tumor cell

cycle was measured by flow cytometry assay.

Cells (3×10^5) were seeded in 6 well plates overnight and 2 and 5 μ M AITC was added for 72 h. Then 30 μ L ROS Fluorescent Probe-DHE was added for 30 min and the effects of AITC on tumor cell ROS production was measured by flow cytometry assay.

Real-time PCR assay

Cells (3×10^5) were seeded in 6 well plates overnight and 2 and 5 μ M AITC was added for 72 h. The effect of AITC on mRNA expression of tumor-related genes in tumor cells by real-time PCR. mRNA was used to extract the total RNA from tumor cells by Trizol

assay, followed by reverse transcription using real-time PCR test kit to obtain the cDNA. The following primers were used to amplify cDNA fragments: MMP-2 (forward) 5'-GGACAGGATTGACAGATTGATAG-3' and (reverse) 5'-CGGACATCTAAGGGCATCAC-3'; MMP-9 (forward) 5'-CTCGAACTTTGACAGCGAC-3' and (reverse) 5'-GTGAAGCGGTACATAGGGT-3'; Integrin $\alpha 5\beta 1$ (forward) 5'-CGGATAGAGGACAAGGCT-3' and (reverse) 5'-CCACATTCTGGGCATGGAAA-3'; Survivin (forward) 5'-CTCTACATTCAAGAACTGGCC-3' and (reverse) 5'-TTGGCTCTTCTCTGTCCAG-3'; human Bcl-2, (forward) 5'-GTCATGTGTGTGGAGAGCGT-3' and (reverse) 5'-GCCGTACAGTTCCAAAAGG-3'; human Cyclin B1 (forward) 5'-CTGCCTGGTGAAGAGGAAGC-3' and (reverse) 5'-GAGTGCTAATCTTAGCATGC-3'; human CDK1 (forward) 5'-TTCAGGATGTGCTTATGC-3' and (reverse) 5'-AGAGCAATTCCAAGCCAT-3'; human p21 (forward) 5'-TTGATTAGCAGCGGAACA-3' and (reverse) 5'-TACAGTCTAGGTGGAGAAACG-3'; human p53 (forward) 5'-AACCTACCAGGGCAGCTACG-3' and (reverse) 5'-TTCCTCTGTGCGCCGGTCTC-3'; human GAPDH (forward) 5'-TGTTGCATCAATGACCCCTT-3' and (reverse) 5'-CTCCACGACGTACTCAGCG-3'. The PCR condition was as following: denaturation at 95°C for 65 sec, followed by 45 cycles at 90°C for 10 sec and at hybridization 65°C for 30 sec.

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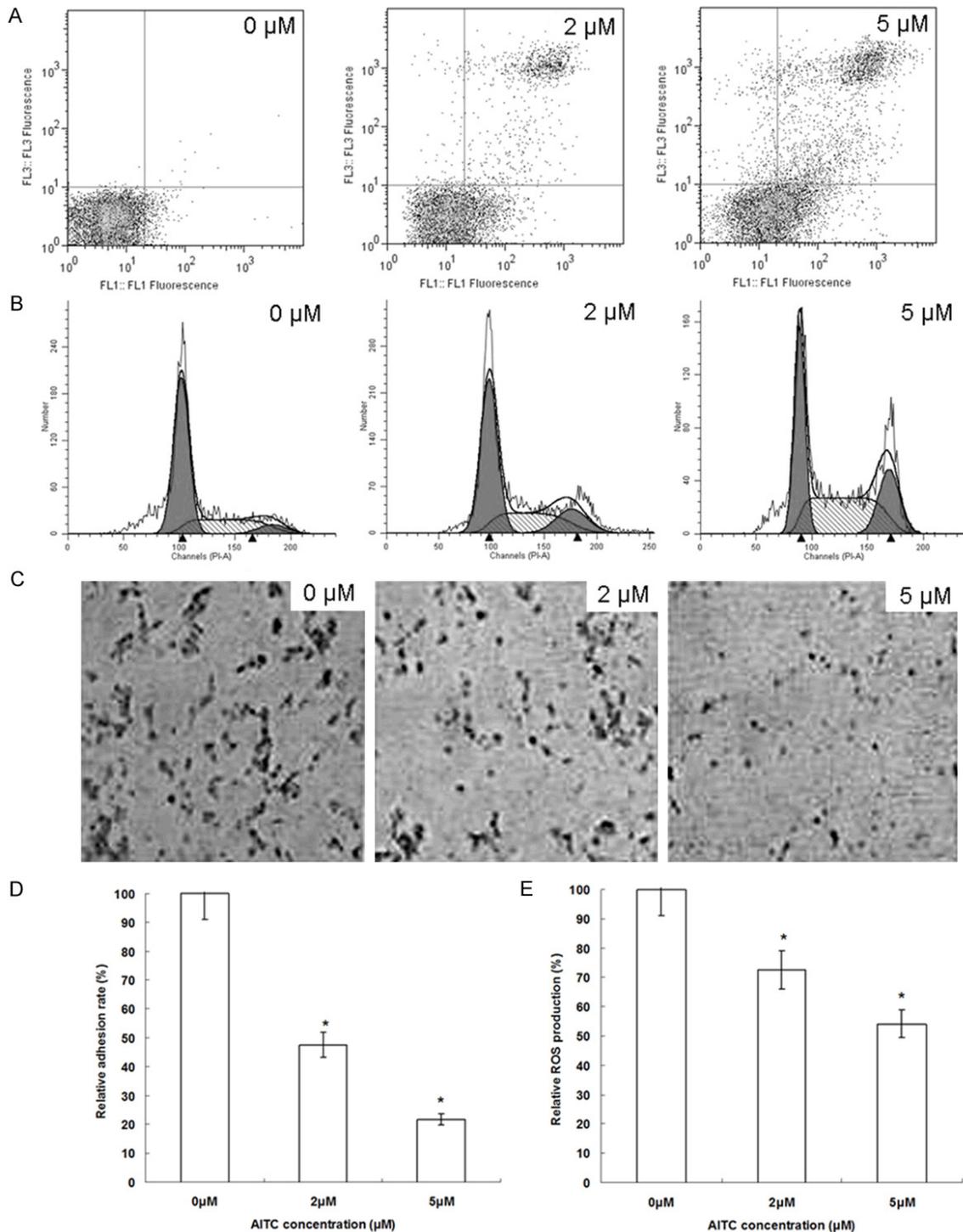


Figure 2. The effect of AITC on the induction of apoptosis and ROS, cell cycle arrest adhesion and invasion inhibition of HepG2 cell line. **A.** The flow cytometry assay showed that there was an increased apoptosis induced by AITC treatment for HepG2 cells. **B.** The flow cytometry assay showed that there was an arrest cell cycle induced by AITC treatment for HepG2 cells. **C.** The transwell assay showed that there was a decreased tumor cell invasion by AITC treatment for HepG2 cells. **D.** The adhesion assay showed that there was a decreased tumor cell adhesion by AITC treatment for HepG2 cells. Bars indicate SD. n=5. *: P<0.05 compared with 0 μM group. **E.** The flow cytometry assay showed that there was an increased cell ROS production induced by AITC treatment for HepG2 cells. Bars indicate SD. n=3. *: P<0.05 compared with 0 μM group.

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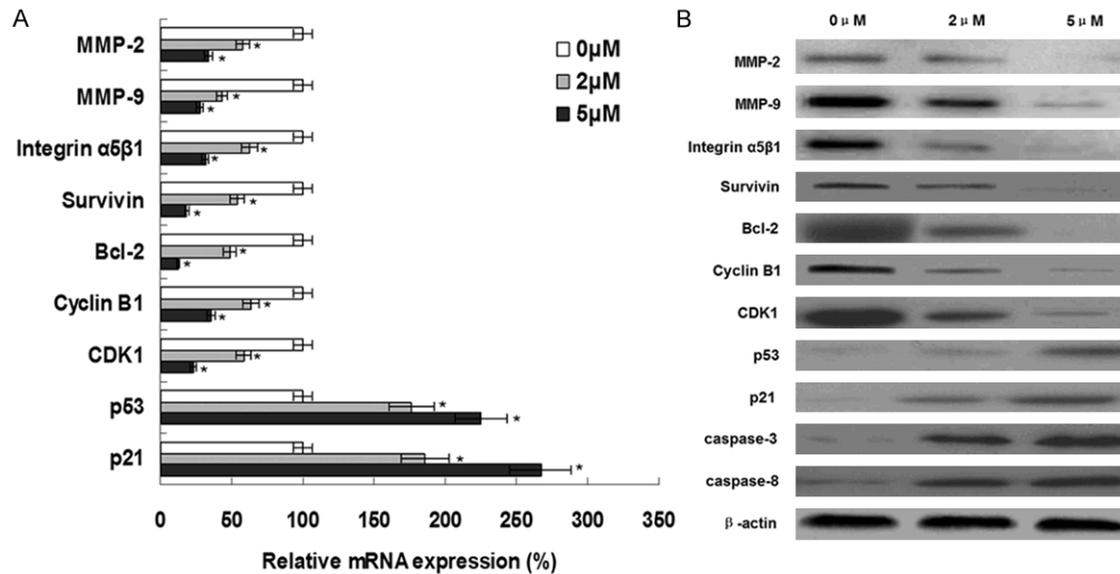


Figure 3. The effect of AITC on tumor related gene expression of HepG2 cell line. A. Real-time PCR assay showed that there was a decreased mRNA expression of MMP-2/9, Integrin α5β1, Survivin, Bcl-2, Cyclin B1, CDK1, meanwhile, there was an increased mRNA expression of p53 and p21 with AITC treatment. GAPDH was used as an internal control for loading. Bars indicate SD. n=5. *: P<0.05 compared with 0 μM group. B. Western blotting assay showed that there was a decreased protein expression of MMP-2/9, Integrin α5β1, Survivin, Bcl-2, Cyclin B1, CDK1, meanwhile, there was an increased protein expression of p53, p21 and caspase-3/8 with AITC treatment. β-actin was used as an internal control for loading.

Western blotting assay

Cells (3×10^5) were seeded in 6 well plate overnight and 2 and 5 μM AITC was added for 72 h. Cells were harvested, lysed and total protein was extracted. Total protein was separated by 12% SDS-PAGE and transferred to PVDF membrane. Then the membrane was blocked for 1 h at room temperature with 5% milk and incubated with monoclonal antibody (MMP-2, 1:1500; MMP-9, 1:1500; Integrin α5β1, 1:1500; Survivin, 1:1500; Bcl-2, 1:1000; Cyclin B1, 1:1000; CDK1, 1:1000; p53, 1:800; p21, 1:1000; caspase-3, 1:800; caspase-8, 1:800; p-Akt, 1:1000 and mouse monoclonal β-actin as the internal reference, 1:5000). The membrane incubated with IgG-HRP and visualized by chemiluminescence detection kit.

Report gene assay

Cells (3×10^5) were seeded in 6 well plate overnight, NF-κB luciferase report plasmids was transferred for 6 h, then 2 and 5 μM AITC was added for another 72 h. The NF-κB transcriptional activity was analyzed by report gene system.

Statistical assay

The experimental data were presented as mean ± standard deviation and analyzed with SPSS11.0 software. One-way ANOVA was used for comparison; P<0.05 indicated statistically significant difference.

Results

AITC inhibited proliferation of HepG2 cells in vitro

Vi-CELL assay was used to measure the effect of AITC on the inhibition of HepG2 cell proliferation in vitro after incubation for 72 h. The result showed that AITC significantly inhibited HepG2 cells proliferation *in vitro* with an IC₅₀ of 16.2 μM (Figure 1).

AITC induced apoptosis, ROS production, cell cycle arrest and suppress adhesion and invasion of HepG2 cells

We found the apoptosis rate was increased, the cell cycle was arrested in the G2/M phase, and the adhesion and invasion was suppressed after treatment with 2 and 5 μM AITC for 72 h,

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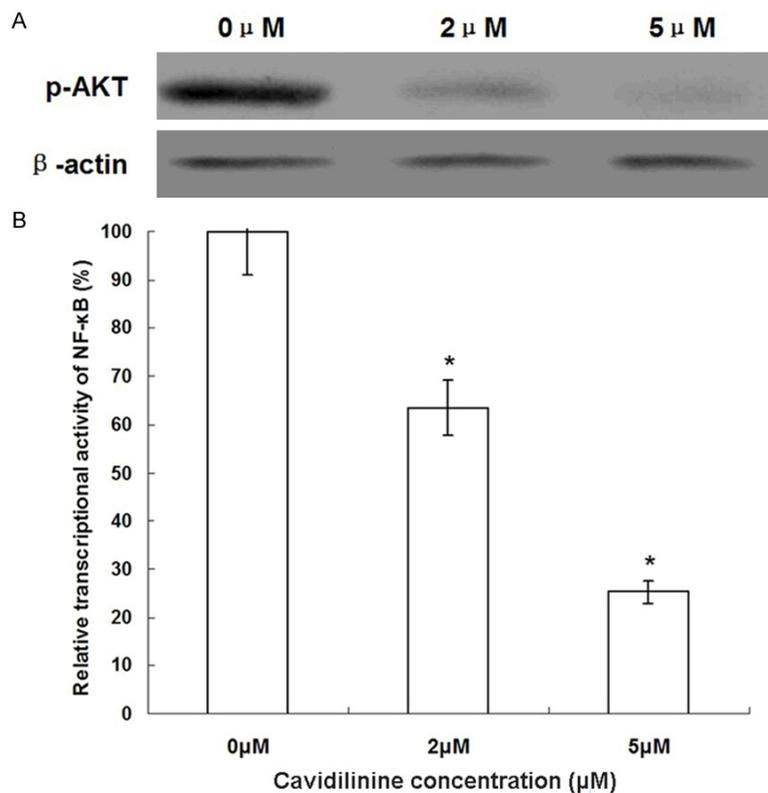


Figure 4. The effect of AITC on tumor-related signal pathway of HepG2 cell line. A. Western blotting assay showed there was a decreased phosphorylation of AKT in HepG2 cell line with different concentration AITC treatment. β -actin was used as an internal control for loading. B. Report gene assay showed that there was a decreased transcriptional activity of NF- κ B with AITC treatment. Bars indicate SD. n=5. *: P<0.05 compared with 0 μ M group.

respectively. After the tumor cells were treated with 2 and 5 μ M AITC for 72 h, the amount of ROS production also was increased compared with the 0 μ M group, respectively, indicating that the effect of AITC-mediated tumor inhibition may be related to its induction of oxidative damage (Figure 2).

AITC regulated tumor-related gene expression in HepG2 cells

To further study the mechanisms of AITC-mediated inhibition of tumor cell, we examined the changes in tumor-related gene expression. The results showed down-regulation of mRNA and protein expression of MMP-2/9, Integrin α 5 β 1, Survivin, Bcl-2, Cyclin B1, CDK1, meanwhile, there was an increased mRNA and protein expression of p53, p21 and caspase-3/8 after the tumor cells were treated with 2 and 5 μ M AITC for 72 h (Figure 3).

AITC regulated tumor-related signal pathway in HepG2 cells

To examine the signaling pathway of AITC-mediated inhibition of tumor cell, we studied the effects of AITC on key molecules of AKT signaling pathway. The study observed inhibition of AKT phosphorylation after treatment with 2 and 5 μ M AITC for 72 h, further the NF- κ B transcriptional activity closely related to tumor was decreased compared with 0 μ M group (Figure 4).

Discussion

Liver cancer is one of the most common malignant tumors. Chemotherapy has become an important alternative or adjuvant therapeutic method and it is necessary to explore for more effective drugs [2]. Some studies found that *Corydalis saxicola* bunting was able to reduce the cancer. Further studies found

that the activity of AITC in this plant was able to prevent cancers [3]. However, the effect of AITC on liver cancer has not been clear studied.

This study found that AITC significantly inhibited HepG2 cell proliferation *in vitro* and enhanced apoptosis and arrest tumor cell cycle at the G2/M phase at 2 and 5 μ M AITC for 72 h. It was found in further study of the molecular mechanisms of AITC-mediated tumor suppression that AITC induced tumor cells to produce ROS in order to kill tumor cells and induce tumor cell apoptosis [4]. Studies have shown that AITC can trigger ROS production by causing oxidative damage to induce apoptosis. This study found that the actions of AITC down-regulated anti-apoptotic gene survivin and Bcl-2 in oxidative damage related apoptotic pathway and significantly up-regulated the downstream caspase-3 and caspase-8 activity, suggesting that

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the oxidative damage played an important role in AITC-induced apoptosis [5-7]. In addition, the expression of cell cycle regulator gene cyclin B1 and CDK1 also exhibited corresponding changes [8].

We also found that AITC also could suppress the adhesion and invasion of HepG2 cells by regulating the adhesion and invasion related gene expression. This study found that AITC significantly inhibited HepG2 cell adhesion and invasion with 2 and 5 μ M AITC for 72 h. Meanwhile, we found that AITC could down-regulate mRNA and protein expression of MMP-2/9 and Integrin α 5 β 1, up-regulated the mRNA and protein expression of p53 and p21 with AITC treatment [9-12].

Finally, the study of signal transduction molecules found that AITC inhibited AKT phosphorylation and NF- κ B transcription activity, thereby inhibiting tumor cell growth and progression [13-16].

Therefore, AITC as a natural product may have diversified mechanisms of activity and AITC may bind to the corresponding target gene in tumor cells to activate/inhibit the corresponding signal transduction pathways in order to activate the apoptotic pathways to suppressing proliferation and invasion of human hepatocellular cancer HepG2 cell line.

Disclosure of conflict of interest

None.

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