Estrogen Inhibits Invasion and Metastasis of Hepatocellular Carcinoma Cells by Regulating AKT Signaling Pathway Activity Postprint

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Abstract

Objective To investigate the effect of estrogen on the invasion and metastasis of MHCC97H hepatocellular carcinoma cells and the underlying mechanism.

Methods Scratch wound healing assay was used to detect the effect of estrogen on the migration ability of MHCC97H hepatocellular carcinoma cells. Transwell chamber assay was employed to assess the effect of estrogen on the invasion ability of MHCC97H hepatocellular carcinoma cells. Western blotting was utilized to determine the effect of estrogen on the expression levels of MMP-2 and MMP-9 in MHCC97H hepatocellular carcinoma cells. The effect of estrogen on the protein expression levels of AKT and p-AKT in MHCC97H hepatocellular carcinoma cells was also examined.

Results Estrogen effectively inhibited the migration and invasion abilities of MHCC97H hepatocellular carcinoma cells in a concentration-dependent manner. The number of MHCC97H hepatocellular carcinoma cells that penetrated the Matrigel and reached the bottom of the chamber in the 0.1 mol/L and 1 mol/L concentration groups was (68.99±15.74)% and (34.28±8.17)% of the control group, respectively ($P<0.05$). Estrogen significantly inhibited the expression of MMP-2 and MMP-9 in MHCC97H hepatocellular carcinoma cells, with statistically significant differences observed at a concentration of 1 mol/L ($P<0.05$). Estrogen also effectively suppressed the phosphorylation level of AKT in MHCC97H hepatocellular carcinoma cells, which was (90±2)% of the control group at a concentration of 1 mol/L ($P<0.05$).

Conclusion Estrogen can effectively inhibit the migration and invasion of MHCC97H hepatocellular carcinoma cells. This effect may be partially associated with the modulation of AKT signaling pathway activity, which

subsequently regulates the expression of MMP-2 and MMP-9. However, further in-depth studies are required to confirm this relationship.

Full Text

Oestrogen Inhibits Invasion and Metastasis of Hepatocellular Carcinoma MHCC97H Cells by Regulating the Activity of AKT Signaling Pathway

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Abstract

Objective: To investigate the effect of oestrogen on the invasion and metastasis of MHCC97H hepatocellular carcinoma cells and explore the underlying molecular mechanisms.

Methods: Wound healing assay was used to assess the effect of oestrogen on MHCC97H cell migration, while Transwell assay evaluated its impact on cell invasion. Western blotting was employed to detect the expression levels of MMP-2, MMP-9, AKT, and p-AKT proteins in oestrogen-treated cells.

Results: Oestrogen effectively inhibited both migration and invasion of MHCC97H cells in a dose-dependent manner. At concentrations of 0.1 mol/L and 1 mol/L, the number of cells penetrating the Matrigel to reach the lower chamber bottom was (68.99±15.74)% and (34.28±8.17)% of the control group, respectively ($P<0.05$). Oestrogen also significantly suppressed MMP-2 and MMP-9 expression, with statistically significant differences observed at 1 mol/L ($P<0.05$). Furthermore, oestrogen treatment reduced AKT phosphorylation levels to (90±2)% of control at 1 mol/L concentration ($P<0.05$).

Conclusion: Oestrogen effectively suppresses migration and invasion of MHCC97H hepatocellular carcinoma cells, an effect that may be partially mediated through modulation of AKT signaling pathway activity and subsequent regulation of MMP-2 and MMP-9 expression. However, further investigation is required to confirm this relationship.

Keywords: oestrogen; MHCC97H cells; invasion; AKT signaling pathway; matrix metalloproteinases

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. China is a high-incidence region for primary liver cancer, with 466,000 new HCC cases reported in 2015, ranking fourth among all newly diagnosed cancers after lung, gastric, and esophageal cancers. A notable epidemiological feature of HCC is its gender disparity in incidence, with a male-to-female ratio of 2.8–6.4:1 in China. Female patients typically present with fewer tumor nodules, smaller tumor size, less vascular invasion, better prognosis, and longer survival compared to male patients.

Research by Naugler et al. demonstrated that oestrogen inhibits Kupffer cell production of interleukin-6 and suppresses STAT3 signaling pathway activation, which is associated with reduced HCC risk in female patients. Moreover, oophorectomy increased both the incidence and lung metastasis rate of chemically induced HCC in rats, while significantly enlarging average tumor diameter. Additional studies have shown that HCC patients exhibit lower serum oestrogen levels, and that oestrogen receptor expression in liver tumor tissues correlates with tumor staging. Oestrogen can also control tumor growth by inhibiting NF- B activity through receptor binding.

Despite advances in HCC treatment, the overall therapeutic outcome remains poor due to high invasiveness and recurrence rates. Interferon-induced transmembrane protein 3 (IFITM3) and sirtuin 3 (SIRT3) are closely associated with HCC proliferation and invasion. Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, are considered key molecules facilitating tumor cell invasion and metastasis, and are closely correlated with HCC development and prognosis. The AKT signaling pathway plays a crucial role in HCC pathogenesis, regulating apoptosis, proliferation, chemoresistance, and invasion. This pathway can modulate HCC invasion and metastasis by regulating MMP-9 expression. However, whether oestrogen inhibits HCC cell invasion and metastasis by modulating AKT signaling pathway activity remains unclear. This study aimed to investigate whether oestrogen suppresses HCC cell invasion and metastasis capacity through regulation of AKT signaling pathway activity.

1.1 Materials

The human hepatocellular carcinoma MHCC97H cell line was maintained in our laboratory. DMEM medium and fetal bovine serum were purchased from Hyclone. AKT and p-AKT antibodies were obtained from Cell Signaling Technology, while -actin antibody was from Santa Cruz Biotechnology. 17- -estradiol was a product of Sigma-Aldrich.

1.2 Cell Culture and Treatment

MHCC97H cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were passaged every 2–3 days upon reaching 70–80% confluence. 17- -estradiol was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of <0.1%, then diluted with PBS to working concentrations. After cells reached confluence, they were washed three times with PBS and cultured in serum-free DMEM for 24 hours, followed by treatment with oestrogen at concentrations of 0, 0.1, and 1 mol/L for 24 hours. Total protein was then extracted for Western blot analysis.

1.3 Cell Invasion Assay

Logarithmic-phase MHCC97H cells were trypsinized and resuspended in medium containing 2% serum to a density of 5×10 cells/mL. Cell suspension (0.4 mL) with different oestrogen concentrations was added to the upper Transwell chamber, while the lower chamber received standard medium with 10% serum. After 24 hours, cells were fixed, residual cells on the upper surface were removed with cotton swabs, and cells on the lower surface were fixed, stained, photographed, and counted under a microscope.

1.4 Wound Healing Assay

Logarithmic-phase MHCC97H cells were seeded in 6-well plates at appropriate density and cultured in DMEM with 2% serum at 37°C. When cells reached approximately 80% confluence, a "wound" was created using a 200 µL pipette tip. Different oestrogen concentrations (0, 0.1, and 1 mol/L) were added, and wound width was observed and photographed at 0 and 24 hours using an inverted microscope.

1.5 Western Blotting

After 24-hour oestrogen treatment, MHCC97H cells were digested and washed twice with ice-cold PBS. Cells were lysed with lysis buffer and proteins were extracted by centrifugation. Protein concentrations were determined using a BCA protein assay kit, and loading amounts were adjusted accordingly. Proteins were denatured by boiling, and equal amounts were loaded into each well of SDS-PAGE gels. Electrophoresis conditions were adjusted according to target protein molecular weights. After electrophoresis, proteins were transferred to PVDF membranes, which were blocked in 5% skim milk for 1 hour. Membranes were incubated with primary antibodies at appropriate concentrations overnight on a shaker, then washed three times with PBST. Corresponding secondary antibodies were applied for 1 hour at room temperature, followed by three additional PBST washes and a final PBS wash (15 minutes each). Protein bands were visualized using an imaging system.

1.6 Statistical Analysis

All data are presented as mean ± standard deviation. Inter-group comparisons were performed using one-way ANOVA or t-test. $P<0.05$ was considered statistically significant. Statistical analysis was conducted using SPSS 17.0 software.

Results

2.1 Effect of Oestrogen on MHCC97H Cell Migration

MHCC97H cells exhibited strong migratory capacity, with approximately 50% wound closure observed 24 hours after scratching in control cells. However, oestrogen treatment significantly slowed wound healing in a dose-dependent manner, with higher concentrations producing more pronounced inhibitory effects [Figure 1: see original paper].

2.2 Effect of Oestrogen on MHCC97H Cell Invasion

MHCC97H cells demonstrated strong invasive ability, which was significantly suppressed by oestrogen treatment. The number of cells penetrating the Matrigel to reach the lower chamber bottom decreased markedly with increasing oestrogen concentration. At concentrations of 0.1 mol/L and 1 mol/L, the number of cells reaching the lower chamber was (68.99±15.74)% and (34.28±8.17)% of the control group, respectively ($P<0.05$).

2.3 Effect of Oestrogen on MMP-2 and MMP-9 Expression in MHCC97H Cells

MMP-2 and MMP-9 play important roles in HCC invasion and metastasis. Oestrogen treatment significantly reduced protein expression levels of both MMP-2 and MMP-9 in MHCC97H cells, with statistically significant differences observed at 1 mol/L concentration ($P<0.05$).

2.4 Effect of Oestrogen on AKT Signaling Pathway Activity

The AKT signaling pathway regulates tumor cell invasion and metastasis by modulating expression and activity of molecules including MMP-2 and MMP-9, with AKT phosphorylation levels reflecting pathway activity. Our results showed that oestrogen treatment significantly reduced AKT phosphorylation levels to (90±2)% of control at 1 mol/L concentration ($P<0.05$).

Discussion

Hepatocellular carcinoma is a highly malignant tumor with limited treatment options. Epidemiological studies have revealed significantly higher incidence rates

and poorer prognoses in males compared to females. Chronic liver disease also progresses more rapidly to cirrhosis in male patients, and HCC associated with cirrhosis occurs predominantly in males and postmenopausal women. Research has demonstrated that oestrogen inhibits inflammation-induced HCC development, thereby reducing HCC risk in female patients, and controls tumor growth by suppressing NF- B activity. These findings suggest that oestrogen plays an important role in HCC pathogenesis.

HCC development involves multiple factors and signaling pathways, including STAT3, NF- B, Wnt/ -catenin, and JNK. Tumor invasion and metastasis is a complex process involving numerous molecules. Tumor cells must detach from the primary site, traverse multiple barriers, enter the circulation, reach distant sites, and establish new tumor colonies. This process requires acquisition of specific capabilities that enable detachment, migration into surrounding tissues, intravasation, and colonization. Matrix metalloproteinases are key molecules facilitating this process, with MMP-2 and MMP-9 being closely associated with HCC development and prognosis. The AKT signaling pathway plays a crucial role in HCC pathogenesis and can regulate HCC invasion and metastasis by modulating MMP-9 expression. PTEN has also been shown to inhibit HCC invasion and metastasis by regulating AKT signaling and subsequently MMP-2 and MMP-9 expression. Additionally, breast cancer research has demonstrated that oestrogen regulates AKT signaling pathway activity to influence tumor development. However, whether oestrogen' s effect on HCC cell invasion and metastasis involves AKT signaling pathway activity has not been reported. We therefore hypothesized that oestrogen might inhibit HCC cell invasion and metastasis by modulating AKT signaling pathway activity.

Previous studies have reported that 17- -estradiol inhibits MMP-9 expression in cardiomyocytes, while clinical research has shown that high MMP-9 expression in HCC tissues correlates with tumor invasion and metastasis, with significantly higher MMP-9 levels in HCC patients with portal vein tumor thrombus compared to those without. Our results demonstrate that oestrogen inhibits MHCC97H cell migration and invasion in a dose-dependent manner, supporting the protective role of oestrogen in HCC development and consistent with previous reports. Furthermore, we observed that oestrogen treatment significantly reduced MMP-2 and MMP-9 protein expression levels, accompanied by decreased AKT phosphorylation. These findings confirm our hypothesis and support the notion that oestrogen inhibits HCC development, at least partially through regulation of AKT signaling pathway activity.

Our study has several limitations, including the use of a single HCC cell line for in vitro experiments, only two oestrogen treatment concentrations, lack of animal studies, and absence of clinical data on HCC staging and prognosis. Nevertheless, our results suggest that oestrogen effectively inhibits MHCC97H cell invasion and metastasis, possibly through modulation of AKT signaling pathway activity and subsequent regulation of MMP-2 and MMP-9 expression, which warrants further investigation.

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Figure Legends

[**Figure 1: see original paper**] Effect of oestrogen on wound healing of MHCC97H cells (Original magnification: ×20). (A) Migration of MHCC97H cells after oestrogen treatment; (B) Compared with control cells, oestrogen-treated MHCC97H cells showed decreased migration distance. *P<0.05.

[**Figure 2: see original paper**] Inhibitory effect of oestrogen on invasion of MHCC97H cells. (A) Cells penetrating the Matrigel to the chamber bottom (×400); (B) Percentage of cells reaching the bottom of the chamber in different groups. *P<0.05.

[**Figure 3: see original paper**] Inhibitory effect of oestrogen on expressions of MMP-2 and MMP-9 proteins in MHCC97H cells. (A) Western blotting of MMP-2 and MMP-9 in cells treated with oestrogen; (B) Relative expression levels of MMP-2 and MMP-9. *P<0.05 vs control.

[**Figure 4: see original paper**] Effect of oestrogen on activity of AKT signaling pathway. (A) Western blotting of p-AKT protein expression in cells treated with oestrogen; (B) Relative expression of p-AKT. *P<0.05 vs control.

Note: Figure translations are in progress. See original paper for figures.