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17β-Estradiol exerts anticancer effects in anoikis-resistant hepatocellular carcinoma cell lines by targeting IL-6/STAT3 signaling

아노이키스 저항성 간암세포주에서 IL-6/STAT3 신호전달을 통한 17β-Estradiol의 항암효과에 관한 연구

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Abstract

17β-Estradiol exerts anticancer effects in anoikis-resistant hepatocellular carcinoma cell lines by targeting IL-6/STAT3 signaling

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17β-Estradiol (E2) has been proven to exert protective effects against HCC; however, its mechanism on HCC proliferation and suppression of invasion remains to be further explored. Because HCC up-regulates serum Interleukin-6 (IL-6) levels and Signal Transducer and Activator of Transcription 3 (STAT3), molecular agents that attenuate IL-6/STAT3 signaling can potentially suppress HCC development. In this study, we examined involvement of E2 in anoikis resistance that induces invasion capacities and chemo-resistance. Huh-BAT and HepG2 cells grown under

anchorage-independent condition were selected. The anoikis-resistant (AR) cells showed stronger chemo-resistance against sorafenib, doxorubicin, 5-fluorouracil and cisplatin compared to adherent HCC cells. AR HCC cells exhibited decreased expression of E-cadherin and increased expression of the N-cadherin and vimentin compared to adherent HCC cells. We then demonstrated that E2 suppressed cell proliferation in AR HCC cells. IL-6 treatment enhanced invasive characteristics, and E2 reversed it. Regarding mechanism of E2, it decreased in the phosphorylation of STAT3 that overexpressed on AR HCC cells. The inhibitory effect of E2 on cell growth was accompanied with cell cycle arrest at G2/M phase and caspase-3/9/PARP activation through c-Jun N-terminal Kinase (JNK) phosphorylation. Taken together, these findings suggested that E2 inhibited the proliferation of AR HCC cells through down-regulation of IL-6/STAT3 signaling. Thus, E2 can be a potential therapeutic drug for treatment of HCC.

Key words : Hepatocellular carcinoma, 17β-estradiol, Interleukin-6, STAT3, Drug resistance, Aniokis resistance, EMT

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LIST OF ABBREVIATIONS

HCC	Hepatocellular carcinoma
AR	Anoikis-resistant
E2	17β-estradiol
IL-6	Interleukin-6
STAT3	Signal transducer and activator of transcription 3
CDKs	Cyclin-dependent kinases
JNK	C-Jun N-terminal kinase.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide [1]. Although surgical resections could be effective for HCC patients with an early stage, the cumulative 5-year recurrence rate is above 70% [2]. The HCC recurrence through local metastasis and chemo-resistance are still major causes of death [3]. Although only sorafenib has been used as first-line therapy for HCC, response rate is only 30-40% [4]. The low efficacy may be caused by chemotherapeutic resistance of HCC [5]. Better understanding of the underlying mechanism targeting the chemo-resistant and metastatic properties of HCC is pivotal for designing improved therapeutic approaches [6].

A characteristic of HCC is the male incidence ranking from 2:1 up to 5:1 across nearly all geographical areas [7]. Estrogen has been attracting people's attention because of a male predominance in morbidity and mortality in HCC patients; thus, estrogen may play a vital role in HCC development [8]. The role of estrogen and its molecular mechanism on HCC have not been elucidated yet; however, the anti-inflammation effect of estrogen is well documented [9]. During chronic inflammation, pro-inflammatory cytokines response to initiate HCC formation [10]. IL-6 is a pro-inflammatory cytokine that modulates the inflammation-associated

cancers by activating downstream pathways to promote tumor initiation, invasion and metastasis [11]. Furthermore, IL-6 activates the phosphorylation of STAT3 that contributes to tumor growth, chemo-resistance and metastasis [12]. A study reported that E2 can reduce diethylnitrosamine (DEN)-induced carcinogenic liver injury by inhibiting IL-6 production [13].

Epithelial cells detach from their extracellular matrix by undergoing apoptosis and are termed as an anoikis [14]. However, cancer cells acquire resistance to anoikis after anchorage deprival from the primary sites, thereafter migrate to other tissues where they can re-attach [15]. Recently increasing evidences suggest that resistance to anoikis is linked with epithelial-to-mesenchymal transition (EMT) involved in tumor metastasis. [14]. Reduced expression of epithelial cell-cell adhesion molecule E-cadherin and increased expression of vimentin and N-cadherin, mesenchymal markers, promote cell invasion and EMT. Increased Snail and Slug, transcriptional repressors, also indicate anoikis resistance [14]. Recent studies has been reported that activation of signal transducers and activators of transcription 3 (STAT3) promotes anoikis resistance accompanied with EMT and chemo-resistance in ovarian cancer, pancreatic cancer and melanoma [16,17,18]. Because of the relationship among those events, exploring the underlying mechanism of anoikis resistance is a promising

way for understanding cancer metastasis [19].

In this study, we investigated that the antitumor effect of E2 in AR HCC cells and its role on the IL-6/STAT3 signaling leading to EMT induction. Moreover, we evaluated whether anti-proliferative effect of E2 contributed to cell cycle arrest at G2/M phases and apoptosis induction though c-Jun N-terminal Kinase (JNK)/c-Jun signaling pathway.

Materials and Methods

Cell culture

Huh-BAT and HepG2, human hepatocellular carcinoma cell lines, were obtained from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and antibiotics. Adherent cells were grown on the tissue culture dishes (Falcon, Corning, NY), while suspension cells obtained from Huh-BAT and HepG2 cell lines respectively were grown on the petri dishes coated with 10 mg/ml Poly-HEMA (poly(2-hydroxyethyl methacrylate)) (pHEMA, Sigma-Aldrich, St. Louis, MO) at 37 ℃ in a 5% CO2 humified incubator. The cells were visualized with an inverted microscope (Olympus, Model IX51, Tokyo, Japan) equipped with a DFC500 camera system (Leica, Wetzlar, Germany).

17β-Estradiol and interleukin-6 treatments

We seeded 5 × 105 cells in DMEM containing 10% FBS and treated with 50 ng/ml interleukin-6 (IL-6, Novoprotein, Summit, NJ) or 40 μM 17β-Estradiol (E2, Sigma-Aldrich, St. Louis, MO). Control cells were treated with 100% ethanol. Cells were harvested for experiments as described below after treatment. E2 or IL-6 stock solution was prepared in 100%

ethanol and distilled water respectively.

MTS Assays

Cells were seeded into 96-well plates at a density of 5 x 103 per well and allowed to adhere for 24 h. The cells were treated with the following: vehicle control, 7 μM sorafenib, 500 nM doxorubicin, 50 μM 5-florouracil, 200 μM cisplatin, 50 ng/ml IL-6 and E2 at 20, 40, 80 and 100 μM for 48 h. For the MTS assay, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) was used by following the manufacturer's instruction. Briefly, 10 μl of the MTS reagent was added into each well, and the plate was incubated at 37 ℃ for 3 h. The absorbance was detected at 490 nm with a plate reader.

Cell invasion assays

Invasion assay was carried out in 24-well transwell plates (Millipore, Billerica, MA). Transwell inserts were coated with 300 μl serum-free media overnight at 37 ℃. Huh-BAT and AR Huh-BAT cells were seeded in the upper chamber in serum-free medium in the presence or absence of 50 ng/ml IL-6 or 40 μM E2. After removing media from the inserts, 500 μl media containing 10% FBS was added to the lower chamber. 300 μl of cells in serum-free medium were seeded in each insert and incubated for 24 h at

37 ℃. Then, invasive cells on lower surface of the membrane were stained by crystal violet for 20 min. Three random fields of the air-dried membrane were photographed under the microscope.

Cell cycle analysis

HCC cells were fixed in precooled 70% ethanol at 4 ℃ for 20 min. After washing with PBS, they were treated with 100 μg/ml RNase A (Amresco, Solon, OH) at 37 ℃ for 50 min. Each sample was stained with 1 mg/ml propidium iodide (PI, Sigma-Aldrich, St. Louis, MO). Cell cycle analysis was performed by a FACS Calibur (Becton Dickinson, San Jose, CA) in triplicate for all samples.

Annexin V/PI staining

The percentage of apoptotic cells was determined by annexin V-FITC staining and PI labeling. HCC cells were cultured as described above. After centrifugation, the cells were treated with 10 μg/ml PI and annexin V (BD Biosciences, Franklin Lakes, NJ) in 250 μl binding buffer (BD Pharmingen, San Diego, CA). They were incubated at 4 ℃ for an hour before analysis. Data acquisition and analysis were performed in a FACS Calibur (Becton Dickinson, San Jose, CA) within an hour.

Western blot analysis

Huh-BAT and HepG2 cells were lysed in 50 μl RIPA buffer (Cell Signaling Technology, Danvers, MA). After 30 min incubation, the lysates were centrifuged at 13000 g for 15 min at 4 ℃. The supernatant was collected, and the total protein concentration was measured by BCA assay (ThermoFisher, Waltham, MA). Protein samples were separated with 8-12% SDS-polyacrylamide gels and electrotransferred onto the PVDF membranes (Millipore, Billerica, MA). After washing in TBST, the membranes were blocked in 5% skim milk in TBST for 1 h and then incubated with primary antibodies overnight at 4 ℃. The following primary antibodies were used in this study; β-actin, p27, cyclin D and cyclin E were all obtained from Santa Cruz (Dallas, TX). E-cadherin, N-cadherin, vimentin, Snail, Slug, phospho-STAT3, CDK4, CDK6, caspase-9, cleaved-caspase-9, caspase-3, cleaved-caspase-3, PARP and cleaved-PARP were all obtained from Cell Signaling Technology (Danvers, MA). Then, the membranes were incubated 1:2500 diluted either goat anti-rabbit IgG HRP (Santa Cruz, Dallas, TX) or goat anti-mouse IgG HRP (ThermoFisher, Waltham, MA) in TBST for 1 h at RT. They were washed 3 x 10 min with TBST at RT. Specific protein bands were visualized using enhanced chemiluminescence detection reagents (Promega, Fitchburg, WI).

Statistical analysis

All analyses were performed repeatedly at least three times. Statistical analyses were performed with SPSS 22.0 software (IBM, USA) and Student's t-test was assessed for all experiments. Statistical significance was marked as * for $P < 0.05$ and ** for $P < 0.01$.

Results

Anoikis-resistant (AR) hepatocellular carcinoma cells are highly chemo-resistant and induce EMT

HCC cell lines, Huh-BAT and HepG2 cells acquired anoikis resistance by culturing adherent cells on non-adhesive poly-HEMA-coated plates. Adherent cells attached on the surface of the culture dish and exhibited spindle-shaped morphology; on the other hand, AR HCC cells formed spheres and floated in culture media (Fig. 1A). AR HCC cells were observed to grow slower, compared to the adherent counterparts (Fig. 1B and C).

To examine the enhanced chemo-resistance of AR HCC cells, adherent Huh-BAT, HepG2, and their AR cells were treated with HCC chemotherapies: 7 μM sorafenib, 500 nM doxorubicin, 50 μM 5-florouracil and 200 μM cisplatin. The cell viability was assessed after a 48 h incubation. In response to treatment with those chemotherapies, the AR Huh-BAT and HepG2 cells showed a statically significant increase in chemo-resistance, compared to the adherent counterparts (Fig. 1D and E; *$P < 0.05$, **$P < 0.01$).

AR Huh-BAT and HepG2 cells resulted in a decrease in E-cadherin,

epithelial markers, compared to the adherent cells. There was a concomitant increase in vimentin and N-cadherin, which are for mesenchymal cells (Fig. 1F). We further found that AR Huh-BAT and HepG2 cells resulted in a time-dependent increase in cellular Snail and Slug levels detected by western blot (Fig. 1G). Moreover, cell invasion accompanied with resistance to anoikis was significantly increased in both AR Huh-BAT and HepG2 cells (Fig. 1H; $^{**}P < 0.01$).

17β-estradiol is effective on inhibiting proliferation of AR HCC cells and suppresses IL-6/STAT3 signaling overexpressed in AR HCC cells

We identified whether IL-6 affects invasion in HCC cells, we stimulated Huh-BAT and AR Huh-BAT cells with recombinant human IL-6. We observed that invasive ability was increased by IL-6; however, the acquisition of the invasive capacity in response to IL-6 stimulation was ameliorated by E2 (Fig. 2A and B; $^{*}P < 0.05$). The expression levels in N-cadherin and Snail were blunted by E2 in Huh-BAT and AR Huh-BAT cells, whereas the level of E-cadherin was not changed (Fig 2C).

E2 inhibited the cell viability of HCC cells in a dose-dependent manner (Fig.

2D and E; *P < 0.05, **P < 0.01). AR HepG2 cells showed stronger chemo-resistance to E2 than adherent cells; however, the difference of cell viability between adherent and AR Huh-BAT cells by E2 was reduced as compared to sorafenib, doxorubicin, 5-FU and cisplatin.

We examined the activation of STAT3 in response to IL-6 treatment in HCC cells. IL-6 induced time-dependent STAT3 phosphorylation (Fig. 2F and G). Next, our results showed that an increase in the phosphorylation of STAT3 in the AR Huh-BAT and HepG2 cells as compared to the adherent counterparts (Fig. 2H). E2 down-regulated the phosphorylation of STAT3 in all the HCC cells (Fig. 2I).

17β-estradiol triggers cell cycle arrest at G2/M phase

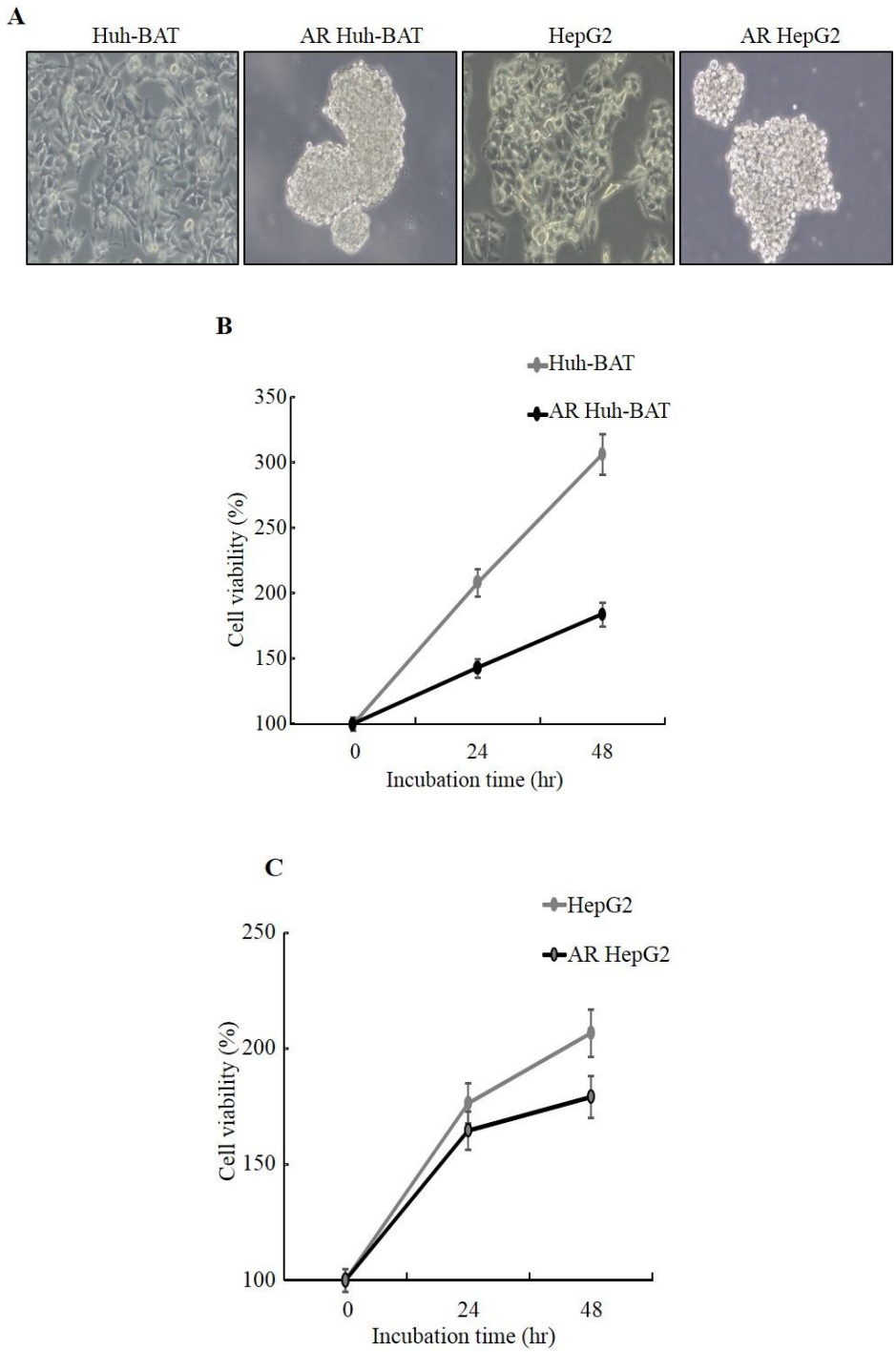
E2 inhibited the proliferation of HCC cell lines, Huh-BAT, HepG2, AR Huh-BAT and AR HepG2. The effect of E2 on the cell cycle was assessed by PI staining. These HCC cells were incubated with various concentrations of E2. There was a significant increase in the G2/M phase in a dose-dependent manner (Fig. 3A; *P < 0.05, **P < 0.01).To delineate the mechanisms underlying the cell cycle arrest induced by E2, we measured the regulators that promote cell cycle progression. The expression of cyclin D, cyclin E, CDK4 and CDK6, which participate in G1 phase regulation,

was not significantly changed by E2, while the expression of p27, a CDK inhibitor, was up-regulated (Fig. 3B). Collectively, these results indicated the inhibitory proliferation of HCC cells by E2 may involve G2/M phase arrest, but G0/G1 phase arrest.

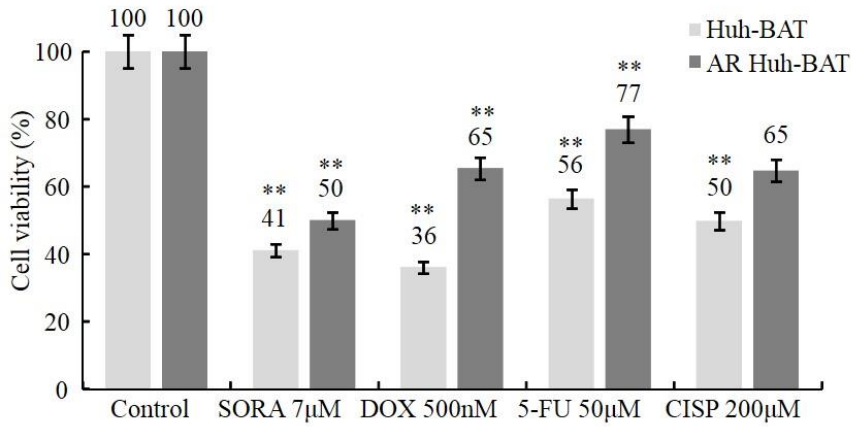
17β-estradiol induces apoptosis by phosphorylation of JNK

To further investigate the mechanism responsible for the inhibitory effect of E2 on cell proliferation, apoptosis rate and the related protein levels were evaluated. E2-induced apoptosis was identified by annexin V/PI staining. Compared to control, E2 resulted in a marked increase in apoptosis rate (Fig. 4A and B; *$P < 0.05$, **$P < 0.01$). Furthermore, the expression of apoptosis-related caspase family protein was detected (Fig. 4C). E2 increased the cleavage of caspase-3, caspase-9 and PARP, which suggests that E2 induces apoptosis through activating the caspase family protein.

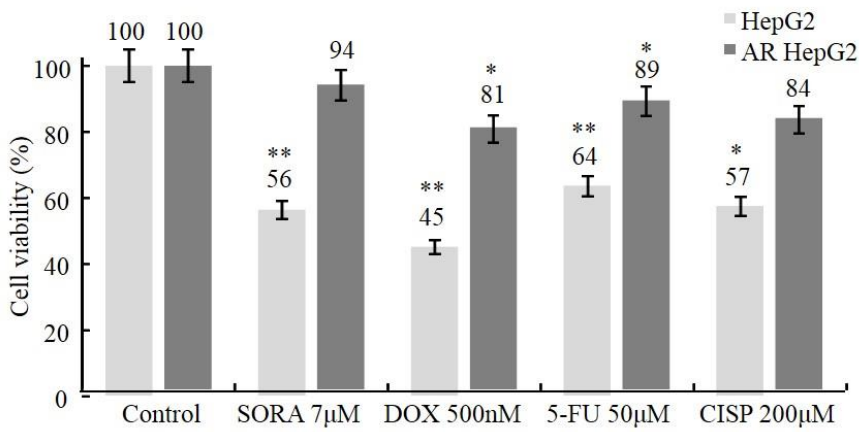
To explore the molecular mechanisms leading to the E2-induced apoptosis, the protein level of JNK signaling, which trigger cellular apoptosis [20]. E2 induced the phosphorylation of both JNK and c-Jun compared with control in HCC cells (Fig. 4D). The results suggested that the apoptosis of HCC cells induced by E2 is mediated by the activation of the JNK/c-Jun pathway.

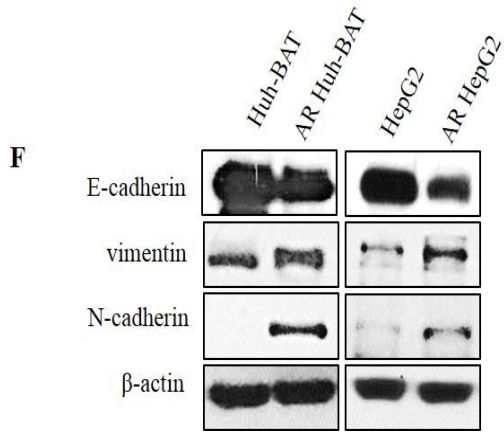

A
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
B
Huh-BAT
AR Huh-BAT
Cell viability (%)
350
300
250
200
150
100
0
24
48
Incubation time (hr)
C
HepG2
AR HepG2
Cell viability (%)
250
200
150
100
0
24
48
Incubation time (hr)

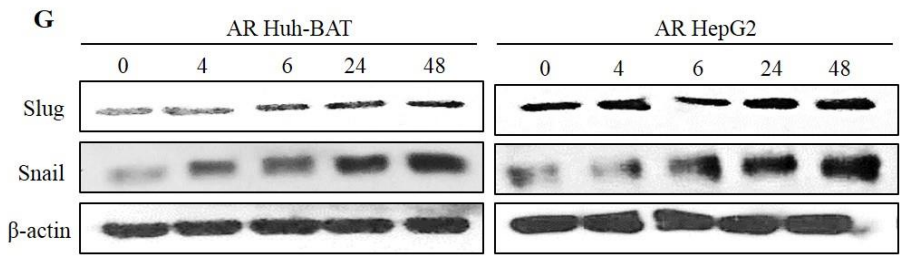
D


Huh-BAT
AR Huh-BAT
Cell viability (%)
100
80
60
40
20
0
100 100
** 41
** 50
** 36
** 65
** 56
** 77
** 50
65
Control
SORA 7μM
DOX 500nM
5-FU 50μM
CISP 200μM

E


HepG2
AR HepG2
Cell viability (%)
100
80
60
40
20
0
100 100
** 56
94
** 45
* 81
** 64
* 89
* 57
84
Control
SORA 7μM
DOX 500nM
5-FU 50μM
CISP 200μM


F
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
E-cadherin
vimentin
N-cadherin
β-actin


G
AR Huh-BAT
AR HepG2
0
4
6
24
48
0
4
6
24
48
Slug
Snail
β-actin

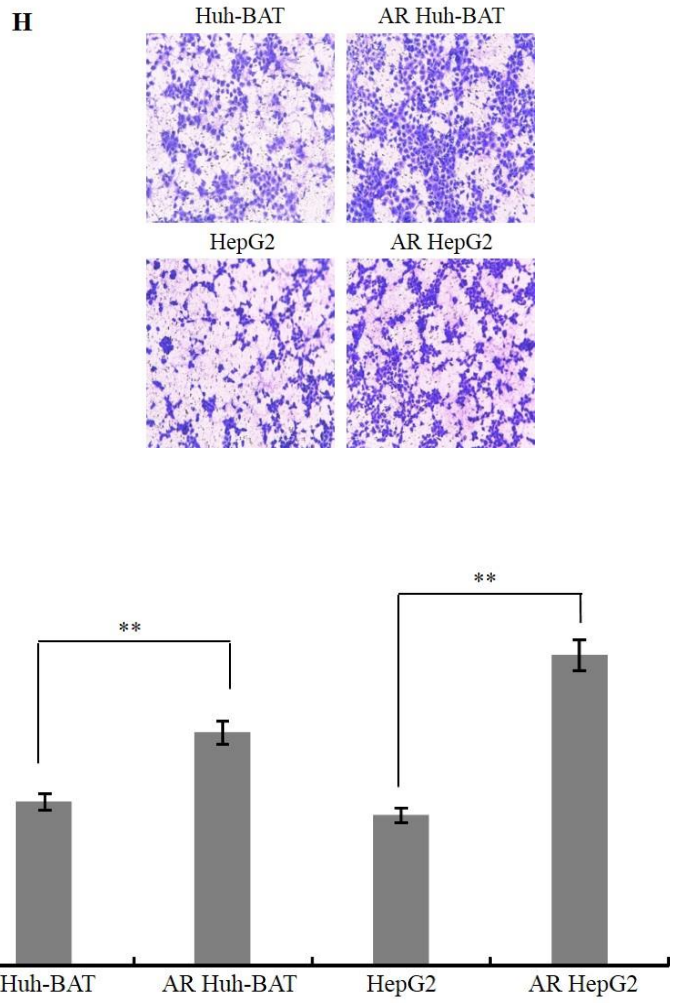
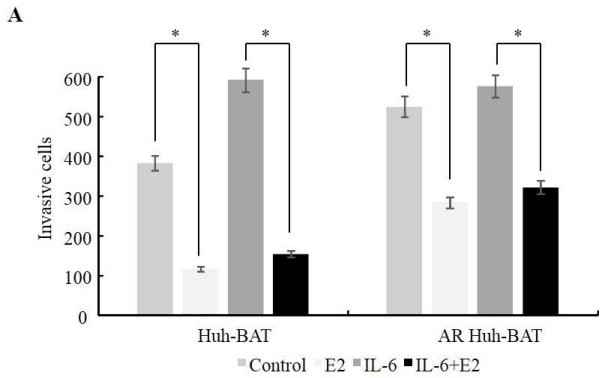
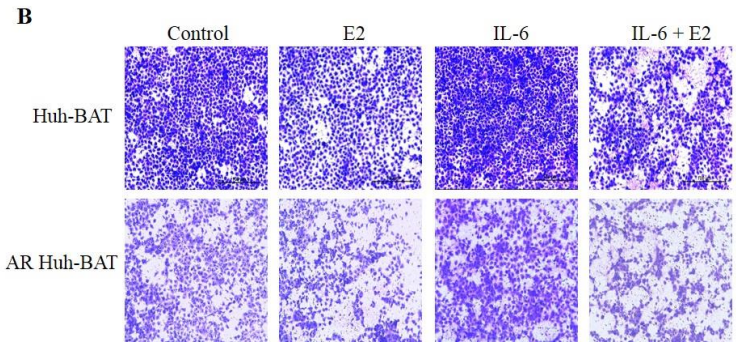
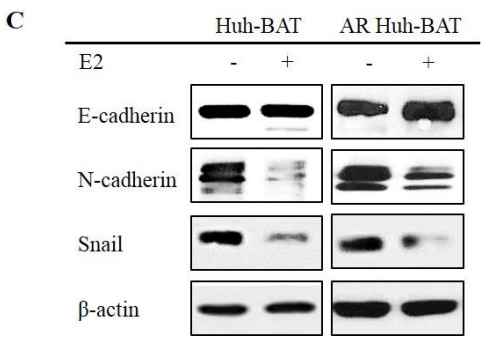

H
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
**
**
Invasive cells
600
500
400
300
200
100
0
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2

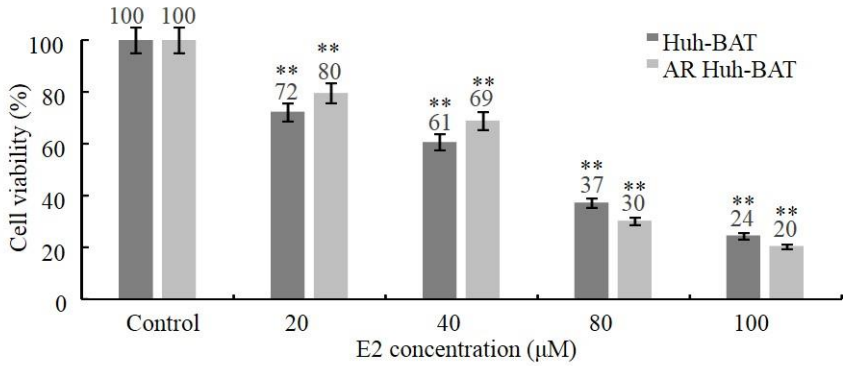
Fig. 1: Anoikis-resistant hepatocellular carcinoma cells are highly chemo-resistant and induce EMT. (A) Phase contrast images of Huh-BAT, AR Huh-BAT, HepG2 cells and AR HepG2 cells were observed under the inverted light microscope at 200 X magnification. (B and C) Cell viabilities were detected by MTS assay at 0, 24 and 48 h. (D and E) Cell viabilities were assessed by MTS assay after treated with indicated concentrations of SORA, DOX, 5-FU and CISP for 48 h. (F) Changes in protein levels of E-cadherin, vimentin and N-cadherin were analyzed by western blot. (G) The expression levels of Snail and Slug in AR Huh-BAT and HepG2 cells were detected at the end of indicated time. (H) Invasive ability of HCC cells was measured with the Transwell assay. The presented figures were repeated at least three times with similar results. All data were expressed as means ± Standard Deviation. ***P < 0.01*.


A
*
*
*
*
600
500
400
300
200
100
0
Invasive cells
Huh-BAT
AR Huh-BAT
Control
E2
IL-6
IL-6+E2

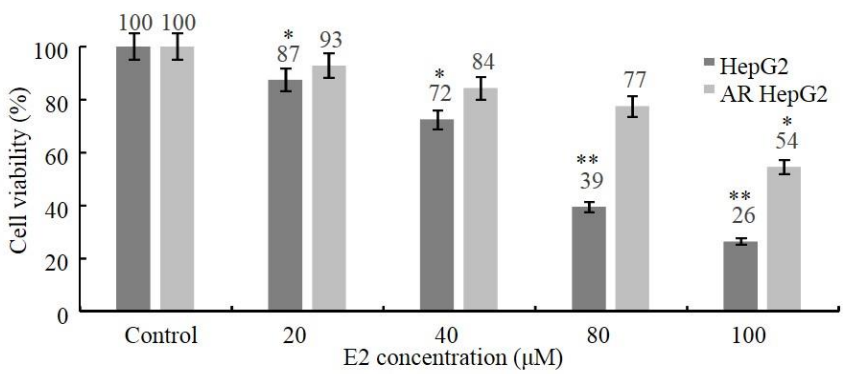

B
Control
E2
IL-6
IL-6 + E2
Huh-BAT
AR Huh-BAT


C
Huh-BAT
AR Huh-BAT
E2
-
+
-
+
E-cadherin
N-cadherin
Snail
β-actin

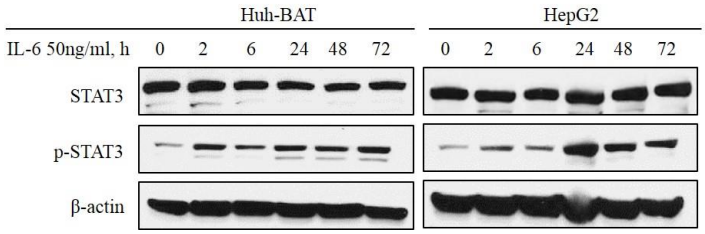
D


Cell viability (%)
100
80
60
40
20
0
100 100
** 72
** 80
** 61
** 69
** 37
** 30
** 24
** 20
Huh-BAT
AR Huh-BAT
Control
20
40
80
100
E2 concentration (μM)

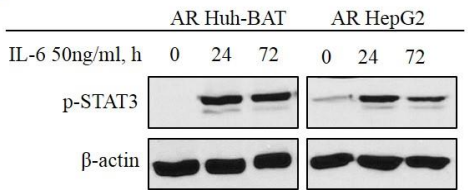
E


Cell viability (%)
100
80
60
40
20
0
100 100
* 87
93
* 72
84
** 39
77
** 26
* 54
HepG2
AR HepG2
Control
20
40
80
100
E2 concentration (μM)

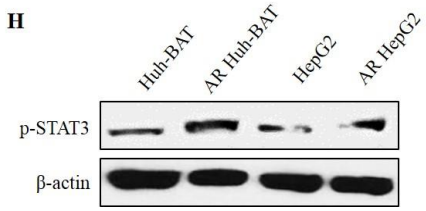
F


Huh-BAT
HepG2
IL-6 50ng/ml, h
0 2 6 24 48 72
0 2 6 24 48 72
STAT3
p-STAT3
β-actin

G


AR Huh-BAT
AR HepG2
IL-6 50ng/ml, h
0 24 72
0 24 72
p-STAT3
β-actin

H


Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
p-STAT3
β-actin

I

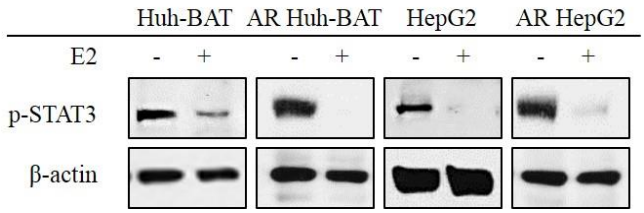
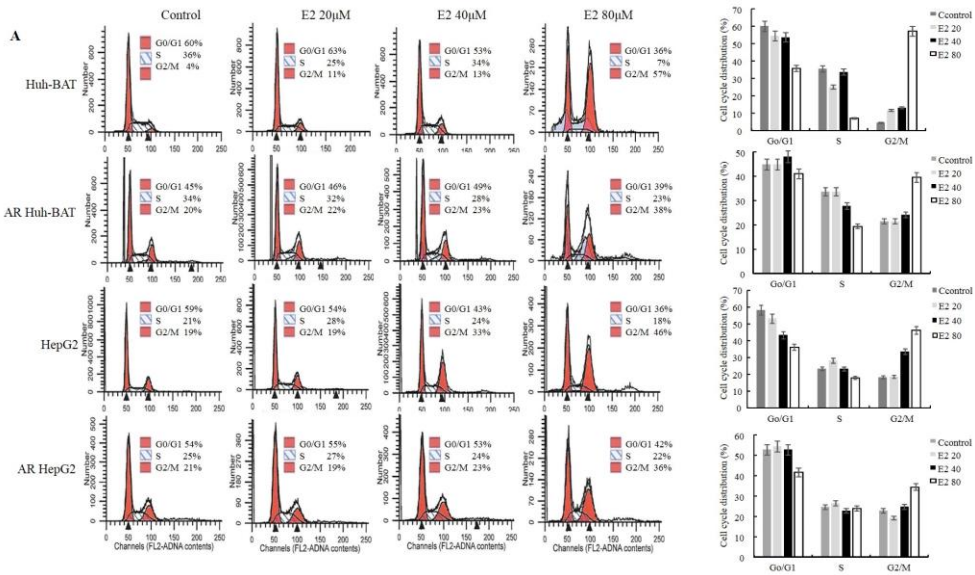

Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
E2
- + - + - + - +
p-STAT3
β-actin

Fig. 2: 17β-estradiol is effective on inhibiting proliferation of AR HCC cells and suppresses IL-6/STAT3 signaling that overexpressed in AR HCC cells. (A and B) Huh-BAT and AR Huh-BAT cells were incubated with 50 ng/ml IL-6 or 40 μM E2 for 24 h and invasion capability was measured with the Transwell assay. The resulting colonies were stained and counted. All data were expressed as means ± S.D. *P < 0.05. (C) Changes in protein levels of E-cadherin, N-cadherin and Snail were analyzed by western blot. (D and E) Cell were treated with the indicated concentrations of E2 for 48 h. Cell viabilities were measured by MTS assay. The relative cell proliferation rate is shown as percentage of survival versus control cells. (F and G) IL-6 induced phosphorylation of STAT3 in a time-dependent manner. (H) Cell lysates of Huh-BAT, AR Huh-BAT, HepG2 and AR HepG2 cells were prepared for western blotting of phospho-STAT3. (I) The phosphorylation of STAT3 was detected by western blot with the cells treated with or without 40 μM E2. β-actin was used as a loading control.


A
Control
E2 20μM
E2 40μM
E2 80μM
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
G0/G1 60%
S 36%
G2/M 4%
G0/G1 63%
S 25%
G2/M 11%
G0/G1 53%
S 34%
G2/M 13%
G0/G1 36%
S 7%
G2/M 57%
G0/G1 45%
S 34%
G2/M 20%
G0/G1 46%
S 32%
G2/M 22%
G0/G1 49%
S 28%
G2/M 23%
G0/G1 39%
S 23%
G2/M 38%
G0/G1 59%
S 21%
G2/M 19%
G0/G1 54%
S 28%
G2/M 19%
G0/G1 43%
S 24%
G2/M 33%
G0/G1 36%
S 18%
G2/M 46%
G0/G1 54%
S 25%
G2/M 21%
G0/G1 55%
S 27%
G2/M 19%
G0/G1 53%
S 24%
G2/M 23%
G0/G1 42%
S 22%
G2/M 36%
Number
Channels (FL2-ADNA contents)
Cell cycle distribution (%)
Go/G1
S
G2/M
Ccontrol
E2 20
E2 40
E2 80

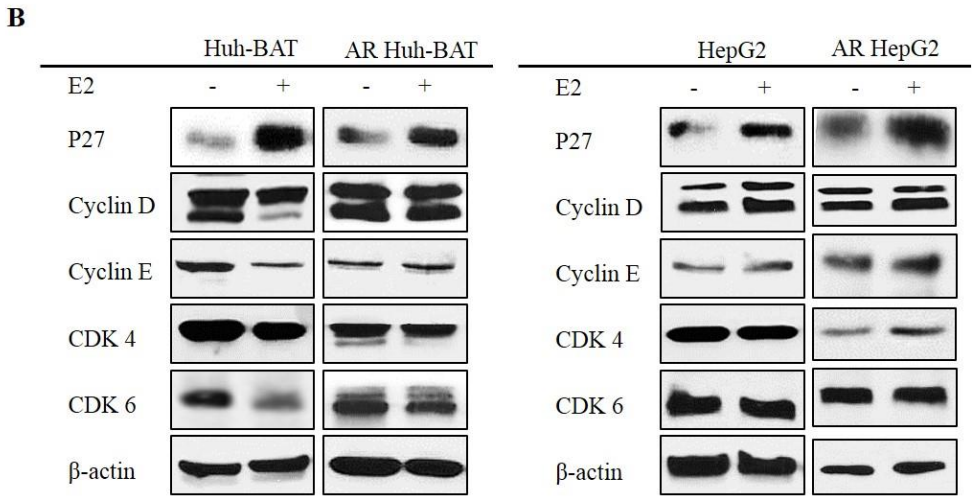
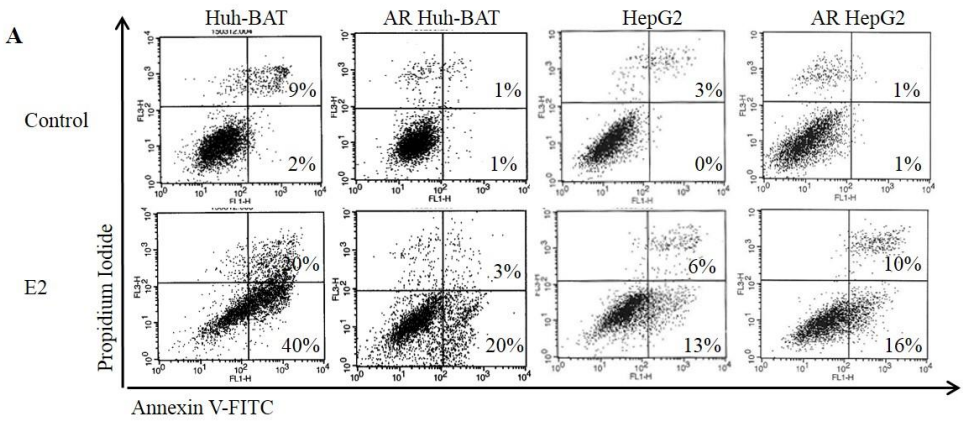
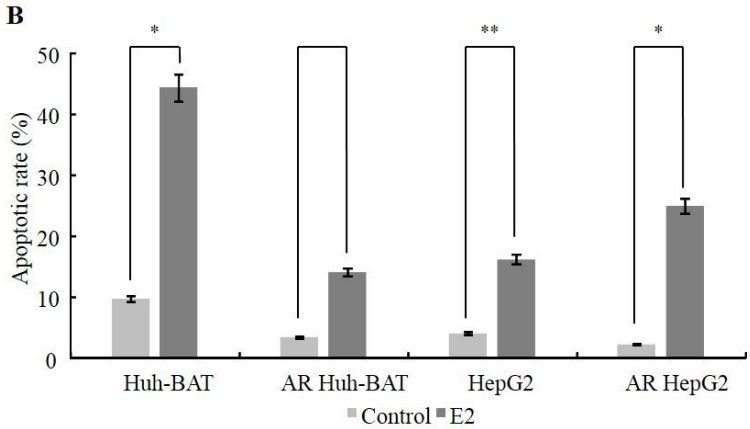
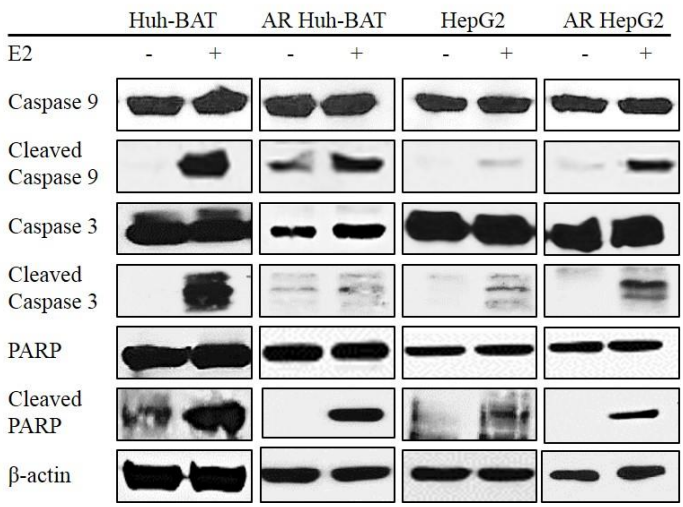


Fig. 3: 17β-estradiol triggers cell cycle arrest at G2/M phase. (A) Huh-BAT, AR Huh-BAT, HepG2 and AR HepG2 cells were treated with E2 for 0, 20, 40 and 80 μM. Cell cycle progression was monitored by flow cytometry. All data were expressed as means ± S.D. *$P < 0.05$, **$P < 0.01$. (B) The protein expression of p27, cyclin D, cyclin E, CDK4 and CDK6 was detected by wesstern blot after 40 μM E2 treatments for 48 h. The experiments were repeated at least three times.


A
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
Control
E2
9%
2%
1%
1%
3%
0%
1%
1%
40%
3%
20%
6%
13%
10%
16%
Propidium Iodide
Annexin V-FITC


B
Apoptotic rate (%)
50
40
30
20
10
0
*
**
*
Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
Control
E2

C


Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
E2
- + - + - + - +
Caspase 9
Cleaved Caspase 9
Caspase 3
Cleaved Caspase 3
PARP
Cleaved PARP
β-actin

D

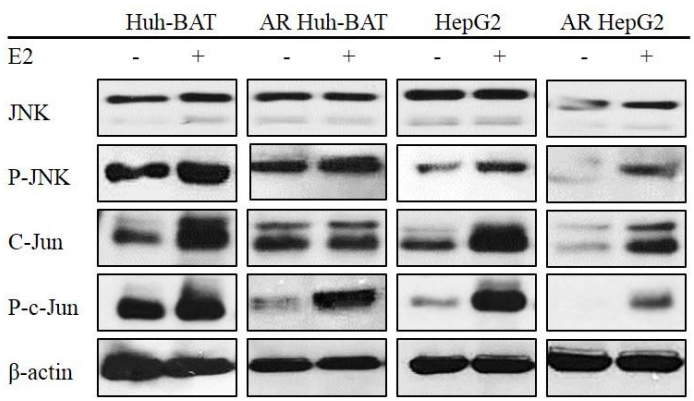

Huh-BAT
AR Huh-BAT
HepG2
AR HepG2
E2
- + - + - + - +
JNK
P-JNK
C-Jun
P-c-Jun
β-actin

Fig. 4: 17β-estradiol induces apoptosis by phosphorylation of JNK. (A) Annexin V/PI staining was used to measure apoptosis induced by 40 μM E2 in Huh-BAT, AR Huh-BAT, HepG2 and AR HepG2 cells. Numbers indicated the percentage of each quadrant. (B) The percentage of apoptotic cells compared with control cells was counted from three independent experiments. All data were expressed as means ± S.D. $*P < 0.05$, $**P < 0.01$. (C) The expression of caspase 3, 9 and PARP were analyzed after E2 treatments. (D) The expression of JNK and c-Jun were analyzed after E2 treatments.

Discussion

Our current study focused on anti-cancer effect of E2 against AR HCC cells and investigated the underlying mechanism. We had established the experimental model of AR HCC cells that possess metastatic potentials and increased drug resistance. We found following evidence that E2 inhibited cell proliferation and invasion capacities of AR HCC. First, E2 attenuated invasion ability increased by IL-6. Secondly, AR HCC cells exhibited increased phosphorylation of STAT3 as compared to adherent cells, and the level of STAT3 was down-regulated by E2. Lastly, E2 suppressed cell proliferation by inducing cell cycle arrest at G2/M phase and apoptosis though JNK phosphorylation.

Although many controversies still exist regarding to the association between estrogen and HCC, evidence has been accumulating to prove the role of estrogen against the HCC development. Because HCC represents an inflammation-linked cancer, HCC development may response to inflammatory signaling, and IL-6 plays roles during the chronic inflammation [21]. In other previous studies, the chemical carcinogen diethylnitrosamine (DEN), which developed HCC in 100% of male mice and 10-30% of female mice was used for understanding the mechanisms underlying gender disparity in HCC [22]. The study reported that DEN-

induced hepatocarcinogenesis was strongly dependent on IL-6, and estrogen suppressed IL-6 production, that leads to inhibition of HCC. Thus, estrogen-mimetic compounds inhibiting IL-6 production might prevent progression of chronic liver disease to HCC.

The present study investigated the underlying mechanism associated with not only gender bias in HCC incidence but also anoikis resistance of HCC. Our result showed that E2, a typical female hormone, negatively regulated IL-6/STAT3 signaling that induces anoikis resistance and promotes metastasis in HCC cells. Thus, we have provided new insights into anticancer effects of E2 that can be pharmacologically targeted.

We demonstrated the molecular mechanism of E2 inhibiting HCC. The phosphorylation of STAT3, which is required for IL-6 activity, was regulated by E2. We analyzed the molecular change of STAT3 to confirm if it might be responsible for not only transforming the cells into a highly invasive phenotype but also inducing anoikis resistance. Furthermore, the anti-proliferative effect of E2 was relevant to not only cell cycle arrest but apoptosis. De-regulation of cell cycle results in uncontrolled cell proliferation and contributes to tumor development [23]. Recent studies have shown that p27 is a regulator of cell cycle progression through G2/M phase. The G2/M phase arrest by E2 was associated with an up-regulation of

p27. Another of major strategy for cancer therapies is to target signaling mediated the apoptosis-inducing pathways [24]. The apoptosis is triggered through mitochondrial pathway involving caspase activation [25]. Apoptotic stimuli could activate MAPKs, which induce JNK, ERK and p38MAPK [26]. We observed that following E2 treatment, cleavage of caspase-9, -3 and PARP was activated and the level of phospho-JNK/c-Jun increased. Moreover, because STAT3 characterized an oncogenic transcription factor involved in inflammatory response, tumorigenesis and chemo-resistance, inactivation of STAT3 by E2 is potentially related with inducing apoptosis.

Lack of selectivity targeting tumor and normal cells is major obstacle in the cancer therapy. E2 functions primarily as a hormone that plays physiological roles on normal cells. Although E2 treatment exerted anti-cancer effects in vitro, side effects has to be considered such as physical or psychological changes in vivo. Thus, understanding the distinct underlying mechanism is indispensable to minimize the risk of hormone therapy for human HCC.

Taken together, in the current study, we reported that E2 had the potential to inhibit HCC proliferation and invasion in vitro via down-regulation of IL-6/STAT3 signaling. Our study has suggested noble insights regarding the anti-proliferative effect of E2 and may provide some essential information for the clinical prevention and management of HCC.

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국문 초록

아노이키스 저항성 간암세포주에서 IL-6/STAT3 신호전달을 통한 17β-Estradiol의 항암효과에 관한 연구

17β-Estradiol는 간세포암 발현을 예방하는 효과로 그의 역할이 알려져 있으나, 간세포암의 생장과 암전이 억제 기전에 대한 연구는 충분히 진행되어 있지 않다. 간세포암은 serum IL-6와 STAT3을 상향조절하므로, IL-6/STAT3 신호를 억제하는 물질은 간세포암 치료에 효과적일 수 있다. 본 연구에서는 암전이능력과 약물저항성을 유도하는 아노이키스 저항성과 17β-Estradiol을 연관시켜보았다. 부유배양을 통해 획득한 간암세포주인 Huh-BAT와 HepG2 세포를 아노이키스 저항성 모델로 사용하였다. 획득한 아노이키스 저항성 간암세포주는 기존의 항암제인 sorafenib, doxorubicin, 5-fluorouracil, cisplatin에 대해 부착 배양된 간암세포주에 비하여 약물저항성을 보였으며, E-cadherin의 단백질 발현 감소와, N-cadherin와 vimentin의 단백질 발현을 증가시켰다. IL-6 처리에 의해 간암세포주의 전이능력이 강화되었고,

17β-Estradiol 처리에 의해 그 능력이 감쇠되었다. 17β-Estradiol의 기전은 아노이키스 저항성 간암세포주에서 과발현하는 STAT3의 인산화를 억제하였다. 또한, 17β-Estradiol 의 간암 세포 성장억제 효과는 G2/M phase에서 세포주기억류와 JNK의 인산화를 통한 caspase-3/9/PARP의 활성화를 동반하여 세포자살을 유도하였다. 결론적으로, 본 연구에서는 IL-6/STAT3 신호를 억제를 통한 17β-Estradiol의 아노이키스 저항성 간암세포주에 대한 항암효과를 확인하였다. 이러한 결과는 17β-Estradiol를 간암치료에 있어 가능성 있는 치료약물로 제시될 수 있다.

주요어 : 간세포암, 아노이키스저항성, 약물저항성, 17β-Estradiol, STAT3, IL-6, JNK

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