The effect of estrogen receptor alpha on the expression of IL-6, EpCAM and K19 in hepatocellular carcinoma-cell culture and human study

Gwangil Kim

Department of Medical Science

The Graduate School, Yonsei University

The effect of estrogen receptor alpha on the expression of IL-6, EpCAM and K19 in hepatocellular carcinoma-cell culture and human study

Directed by Professor Young Nyun Park

The Master's thesis
Submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

Gwangil Kim

December 2011

This certifies that the Master's thesis of
Gwangil Kim is approved.

Thesis Supervisor : Young Nyun Park

Thesis Committee Member#1 : Jin Sub Choi

Thesis Committee Member#2 : Kyung-Sup Kim

The Graduate School
Yonsei University

December 2011

감사의 글

결실을 맺는 오늘이 있기까지 많은 관심과 격려로 저를 이끌어주신 많은 분들께 감사의 말씀을 전합니다.

항상 저에게 학생 그리고 연구자의 마음가짐, 부족한 끈기를 저에게 많이 지도해주신 박영년 교수님께 진심으로 감사 드립니다. 또한 제 졸업논문을 지도해 주신 김경섭 교수님, 최진섭 교수님께 감사의 말씀을 드립니다. 늘 가까이에서 제 연구에 관심을 가져주신 김호근 교수님께 감사 드립니다.

또한 실험을 위해 도움을 주신 병리학 교실 선생님들, 실험실 생활을 시작하면서 관심과 조언으로 많은 도움을 주신 김지수, 유정은, 나득채, 안의용, 김원규, 강규섭선생님께 감사 드립니다.

항상 제가 긍정적인 마인드를 가지고 살아갈 수 있도록 해 주신 부모님들께 감사 드립니다. 기쁠 때나 슬플 때나 중학교부터 항상 제 옆에서 저의 편이 되어준 사랑하는 임수정실장님께 감사 드립니다.

김광일 올림

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The effect of estrogen receptor alpha on the expression of IL-6, EpCAM and K19 in hepatocellular carcinoma-cell culture and human study

Gwangil Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Young Nyun Park)

The expression of "stemness"-related markers in Hepatocellular carcinoma (HCC) was recently reported to be associated with more aggressive biological behavior and poor prognoses. Epithelial mesenchymal transition (EMT) was previously known to be involved in the generation of cancer stem cells. HCCs occur mainly in men, and estrogen and estrogen receptor alpha (ERα) were reported to play a protective role in HCC development by inhibiting secretion of IL-6, which is capable of inducing EMT. In this study, the role of ERα on the expression of cancer stem cell markers (EpCAM and keratin (K19), IL-6, EMT markers (Snail and Twist) as well as the biological behavior of HCC was investigated in both HCC cell lines and human HCC tissue samples. In PLC/PRF/5 and SNU423 cell lines, EpCAM-positive and EpCAM-negative cells were sorted by flow cytometry. ERα mRNA level was significantly higher in the EpCAM-negative cell fractions than in the EpCAM-positive cell fractions of both cell lines ($P < 0.05$). Snail mRNA level and Twist mRNA level were significantly higher ($P < 0.05$) ($P = 0.06$) in the EpCAM-positive cell fractions than in the EpCAM-negative cell

fractions of both cell lines. IL-6 mRNA levels were significantly higher in the EpCAM-positive cell fractions than in the EpCAM-negative cell fractions of SNU423 cells (P = 0.03), whereas it was not detected in PLC/PRF/5 cells. Overexpression of ERα by transfection of pEGEF-C1-ERα plasmids showed a significant reduction of EpCAM and K19 expression levels in PLC/PRF/5 and SNU423 cell lines at both the mRNA and protein level (P < 0.05). Interestingly, overexpression of ERα reduced IL-6 expression at both the mRNA and protein level in SNU423 cells. However, Snail and Twist expression demonstrated no significant change after ERα overexpression in both cell lines. Overexpression of ERα significantly reduced the activities of cell proliferation, invasion and migration in both cell lines compared to the control group. We also studied 64 human HCC tissues; ERα type was evaluated by RT-PCR, and the mRNA expression levels of ERα, EpCAM, K19, IL-6, Snail and Twist were studied by real-time RT-PCR. The protein expression of EpCAM and K19 was also detected by immunohistochemical stain. Their expression levels were correlated with clinicopathologic features. HCC patients with the ERα wild type showed significantly higher mRNA levels of ERα; lower mRNA levels of EpCAM, K19 and IL-6; and lower protein expression of EpCAM and K19, compared to those with the ERα variant type (P < 0.05 in all); whereas there was no significant difference in mRNA levels of Snail and Twist between the two groups. HCC patients with the ERα variant type showed

higher incidences of portal vein invasion (P = 0.008), microvascular invasion (P = 0.024), and poor differentiation (P = 0.015), compared to those with the ERα wild type. Similarly, HCC patients with low ERα mRNA expression (lower 50%) showed higher portal vein invasion (P = 0.021) and microvascular invasion (P = 0.007) than those with high ERα mRNA expression (upper 50%). There were no significant differences in disease free survival and overall survival according ERα type and expression levels of ERα, IL-6, Snail, Twist, EpCAM and K19 at the mRNA or protein level. In conclusion, these data suggest low ERα mRNA expression and variant-ERα type are involved in the aggressive biological behavior of HCC, demonstrating high expressions of EpCAM, K19 and IL-6 in both HCC cell lines and HCC patients.

Key words : Estrogen receptor alpha, IL-6, EpCAM, Keratin 19, hepatocellular carcinoma, invasion, stemness

The effect of estrogen receptor alpha on the expression of IL-6, EpCAM and K19 in hepatocellular carcinoma-cell culture and human study

Gwangil Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Young Nyun Park)

I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and most fatal human cancer[1]. HCC occurs preferentially in males, and the male to female ratio of HCC incidence averages 6:1, according to several cohort studies[2, 3]. In the past, the predominance of HCC in males was considered to be a result of different life patterns such as the consumption of alcohol and smoking[4]. In addition, different gene expressions between males and females may result in the gender disparity of HCC. For example, HNF4α (hepatocyte-enriched nuclear factor 4α), HNF3, and HNF6, which are known as growth hormone-dependent transcription factors, as well as STAT5b (signal transducer and activator of transcription 5b) showed different expressions between male mice and female mice[5, 6]. Through tissue microarray analysis of liver tissues from 334 mice, differences in gene expressions between sexes were reported[7].

Recently, Naugler *et al*. reported that estrogen and estrogen-mediated signaling could protect hepatocytes by blocking IL-6 expression in Kupffer cells and reduce liver cancer in female mice[8]. IL-1α expression, upstream of IL-6, was also reported to be significantly lower in tumors than in tumor adjacent tissue, and this expression is exhibited only in male patients. Interestingly, IL-1α proved to be expressed at an inverse proportion to estrogen receptor α (ERα) in tumor adjacent tissue[9]. According to these results, ERα seems to play a protective role in hepatocarcinogenesis and may account for gender disparity in HCCs.

ERα is a nuclear protein that stimulates the transcription of its target genes, such as E-cadherin, Snail, and NF-κB, in response to an estrogen stimulus[8, 10, 11]. ERα mRNA expression level is significantly decreased in HCC liver tissues compared to non-tumor tissues[12-15]. Moreover, Villa *et al*. reported that variant estrogen receptor mRNA was present at early stages of chronic liver disease as well as in HCC[16, 17]. The presence of the liver variant ERα led to the development of a more clinically aggressive form of HCC, in comparison to those with wild-type ERα. In addition, males presented higher rates of variant ERα expression than females at high risk of HCC development. The variant ERα was reported to be able to interfere with the transcriptional activity of wild-type ERα in HepG2 and to act as a dominant negative receptor[18].

In an increasing number of reports, cancer stem cells (CSCs), which have the ability to self-renew, differentiate, and proliferate, have been associated with a

poor prognosis in several human malignancies[19, 20]. CSCs constitute a subpopulation of neoplastic cells within a tumor and are defined by their ability to seed new tumors[21, 22]. CSCs have also been discovered in solid tumors, including those arising in the breast, colon, brain and liver[23-26]. The potent tumorigenic capacity of CSCs, coupled with increasing evidence of radioresistance and chemoresistance, suggests that CSCs contribute to tumor maintenance and recurrence and that targeting CSCs might offer new avenues of therapeutic intervention[24, 27-32]. Generally, these cells are characterized by the expression of "stemness"-related markers, such as epithelial cell adhesion molecule (EpCAM) and keratin 19 (K19), in HCC[33]. Previously, we reported that HCCs expressing stemness-related markers demonstrate an aggressive biological character and poor prognosis[26]. Interestingly, the male to female ratio was 2.1:1 (mean ages: 53.2 ± 11.7, mean±SD) in EpCAM-positive HCC patients, in contrast to 5.4:1 (mean ages: 58.1 ± 12.2, mean±SD) in EpCAM-negative HCC patients. Similarly, it was 1.8:1 (mean ages: 55.4 ± 13.3, mean±SD) in K19-positive HCC patients and 4.6:1 (mean ages: 56.7 ± 12.0, mean±SD) in K19-negative HCC patients. These findings showed that the male to female ratio was reduced in HCC patients who expressed stemness-related markers.

Recently, it was reported that IL-6 plays a pivotal role in the conversion of non-stem cancer cells to cancer stem cells in breast cancer[34]. Moreover, IL-6 is capable of triggering the EMT phenotype in pancreatic cancer[35], as well as

cancer of the head and neck,[36] and generating a subpopulation of cancer cells with stem cell characteristics in breast cancer cells[37]. It was also reported that estrogen suppresses metastasis in rats by decreasing IL-6 mRNA and protein expression[38]. The expression of IL-6 associated with estrogen and estrogen receptors in mice has been shown, yet the link between IL-6 and cancer stem cells remains poorly understood. Herein, the presented data will substantiate the association of ERα and cancer stem cell markers via IL-6 expression for the first time. Further, we recently reported on the EMT related genes associated with cancer stem cell expression in HCC[39]. Also, it was reported that ERα regulates EMT related genes such as Snail[10] and Slug[11] in breast cancer. Therefore, we also checked the associations of ERα, EMT related genes and cancer stem cell markers.

In this report, we studied HCCs to address the possible association between ERα and cancer stem cells through IL-6. On the basis of *in vitro* and clinical validation, we hypothesized that ERα may play a pivotal role in the formation of CSCs via IL-6.

II. MATERIALS AND METHODS

1. HCC cell lines and culture

For this study we used the human hepatocellular carcinoma cell lines PLC/PRF/5(CRL-8024), and SNU423(CRL-2238), obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in DMEM (Gibco-BRL, Rockville, MD, USA) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. In all experiments, these cells were maintained at 37℃ in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, Hepa class 1000, CA, USA).

2. Human HCC tissue samples and pathological examination

The tissue samples of 64 HCCs were provided by the Liver Cancer Specimen Bank, a part of the National Research Resource Bank Program, which is run by the Korea Science and Engineering Foundation under the Ministry of Science and Technology. This study was approved by the ethics committees of Severance Hospital (Seoul, Korea). The patient population consisted of 39 males and 25 females, and their ages were 56.4±10.9 years (mean±S.D.). The average tumor size was 4.8±2.3 cm. The differentiation of HCC was evaluated according to the Edmondson-Steiner grading system, which revealed 43 patients of grade I – II and 21 patients of grade III - IV. Portal vein invasion was detected in 16 (25%) patients. Microvessle invasion was found in 44 (69%) patients.

3. Flow cytometry analysis for HCC cell lines

To detect the expression profiles and sorting of EpCAM in HCC cell lines, cells were stained with mouse anti-EpCAM antibody (BD Biosciences, San Jose, CA, USA). Samples were analyzed using LSRII flow cytometry and CellQuest software (BD Biosciences). Live cells were gated, while dead cells and cell debris were excluded using FSC and SSC biparametric plots.

4. Western blot and immunocytochemistry

Cells were trypsinized, centrifuged, and washed, and pellets were stored at -80℃ for 1 hour. Cells were then lysed in cell Passive Lysis Buffer (Thermo Scientific Inc, Union city, CA, USA) containing a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Cell extracts were incubated on ice for 30 min, vortexing every 10 min, and centrifuged at 13 200 r.p.m for 30 min. Supernatants were collected and used to determine protein concentrations using the Bradford method. Each protein sample was resolved on SDS–polyacrylamide gels and then transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were incubated with rabbit polyclonal anti-human ERα (1:1000; Santa Cruz), mouse monoclonal anti-human EpCAM (1:1000; Abcam, Cambridge, UK) antibody, rabbit polyclonal anti-human K19 (1:500; Dako, CA, USA), Snail (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Twist

(1:1000; Santa Cruz) and polyclonal mouse anti-human beta-actin antibody (1:500; Santa Cruz). The membranes were incubated with their antibodies followed by incubation with secondary horseradish peroxidase conjugated anti-rabbit and mouse (1:2000; Cell signaling, MA, USA) for 1 hour at room temperature. Membrane-bound antibodies were detected using the ECL system (Santa Cruz).

The paraffin sections were deparaffinized in xylene for 60 min and then rehydrated with graded alcohol. Endogenous peroxidase activity was quenched in a 3% hydrogen peroxide/methanol solution for 20 min. For antigen retrieval, sections were boiled in 100 mM of sodium citrate at a pH of 6.0 for 12 min in a microwave oven. Primary antibodies were applied and incubated overnight at 4℃. After washing in TBS, incubation with the secondary antibody was carried out using the DAKO EnVision Rabbit/Mouse conjugated HRP for 20 min at room temperature and then developed with diaminobenzidine (DAKO). The sections were then counterstained with hematoxylin, dehydrated using graded alcohol, and cleared in xylene Brown membranous and/or cytoplasmic staining was counted as positive for EpCAM and K19. The EpCAM immunohistochemical stain results were interpreted in a semiquantitative manner and given a score from 1 to 3 as follows: 1, weak staining in < 5% of tumor cells; 2, moderate staining in ≥ 5 but < 50%; 3, strong staining in ≥50%. For K19, immunohistochemical staining was scored as follows: 1, staining in < 1% of tumor cells; 2, weak staining in ≥ 1 but < 5%; 3, moderate staining in

≥5%.

5. Transfection

For transient expression of wild-type ERα, we used the pEGEF-C1-ERα plasmid (Addgene, MA, USA). For transfection of PLC/PRF/5 and SNU423 in six-well plates, a 1 ug weight of DNA was added to each well of a 6-well plate using jetPRIME™ according to the manufacturer's recommendations (Polyplus trnasfection™, Illkirch, France). The cells were allowed to grow 48 hours after transfection, before harvesting for analysis.

6. Total RNA extraction, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from snap-frozen human HCC tissues, using the Trizol method (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. RNA concentration and purity was determined by NanoDrop with fluorescence, measuring at 260 nm and 280 nm. Absence of genomic DNA contamination was then confirmed by PCR of total RNA. Transcription to cDNA was performed using the High capacity RNA to cDNA kit (Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. The cDNA from human HCC tissues was used in real-time quantitative RT-PCR analyses to assess mRNA levels of ERα (Hs01046815_m1), EpCAM (Hs00901887_m1), K19 (Hs00761767_s1), Snail (Hs00195591_m1), Twist (Hs01675818_s1), IL-6 (Hs00985639_m1), GAPDH

(Hs99999905_m1). All real-time PCRs were done using same tissue from each patient. The reactions were performed with gene-specific primers and probes using an ABI Prism 7700 sequence detection system and software (Perkin Elmer, CA, USA) according to the TaqMan method. The probes were labeled with FAM and TAMRA as the 5'-fluorescent reporter and 3'-end quencher, respectively. Each reaction was performed in triplicate and all experiments had a non-template reaction as a negative control.

All real-time PCRs were done using same tissue from each patient. The reactions were performed with gene-specific primers and probes using an ABI Prism 7700 sequence detection system and software (Perkin Elmer, CA, USA) according to the TaqMan method. The probes were labeled with FAM and TAMRA as the 5'-fluorescent reporter and 3'-end quencher, respectively. Each reaction was performed in triplicate and all experiments had a non-template reaction as a negative control.

7. Reverse Transcription PCR Amplification

To investigate variant ERα, RT-PCR was performed to detect ERα expression in human HCC. A reverse transcription reaction and subsequent PCR amplification were carried out using primers located on exons 4 and 6 in order to amplify a possible exon 5 deletion. The sequences of ER oligos were 5´-GGAGACATGAGAGCTGCCAAC-3´and 5´-CCAGCAGCATGTCGAAG ATC-3´. The β-actin primers had sequences of 5´-TGTGGCATCCACGAAACT

AC-3´ and 5´-GGAGCAATGATCTTGATCTTCA-3´. PCR was performed using AccuPower PCR Premix (Bioneer, CA, USA) according to the manufacturer's instructions. An amplified fragment corresponding to the wild-type ERα was 438bps, while the variant ERα was 296bps. For PCR amplification, 1μL of cDNA was used. The PCR conditions for ERα and β-actin (internal control) were as follows: initial 5 min at 95 ℃ and then 35 cycles of 30 s at 95 ℃; 30 s at 58 ℃ for ERα, 64 ℃ for β-actin; 30 s at 72 ℃; and a final elongation for 10 min at 72 ℃. The PCR products were separated by electrophoresis on a 2% agarose gel.

8. Cell proliferation assay

The cells were seeded in 96-well plates at a density of 3×10^4 of cells per well. Cells were seeded and allowed to settle for 24 hours and incubated for 72 hours with 5% CO_2 at 37 ℃. After incubation, the cells were further incubated with 2 ng/ml of MTT dye (3-[4,5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide; Sigma) for 4 hours at 37℃. The monolayer was suspended in 0.1 ml of DMSO (Gibco-BRL) and absorbance at 570 nm was read by an ELISA reader (Molecular devices, VMAX, CA, USA). The control value corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control.

9. Cell invasion and migration assay

Invasion assays were performed using Transwell invasion chambers with 8mm pore membranes (Corning Inc., Corning, NY, USA). Cells were resuspended in upper Transwell chambers in serum-free media and allowed to migrate towards a serum gradient (10%) in the lower chamber for 48hours. Membranes were then excised and cells on the upper side were removed before fixing in 4% paraformaldehyde and staining with methylene blue, prior to mounting. The number of migrating cells was counted in three randomly chosen fields on each membrane and photographed at x10 magnification. Values were reported as the average of three experiments performed in triplicate. For the wound healing assay, PLC/PRF/5 cells and SNU423 cells with a 90% confluence were transiently transfected with plasmid pEGEF-C1-ERα or pEGEF-mock control. At 24 hours after transfection, cells were scraped with a pipette tip and cultured in DMEM containing 5% FBS (v/v). Photographs were taken at the indicated time points using an Olympus IX81 microscope and Qcapture software.

10. Statistical analysis

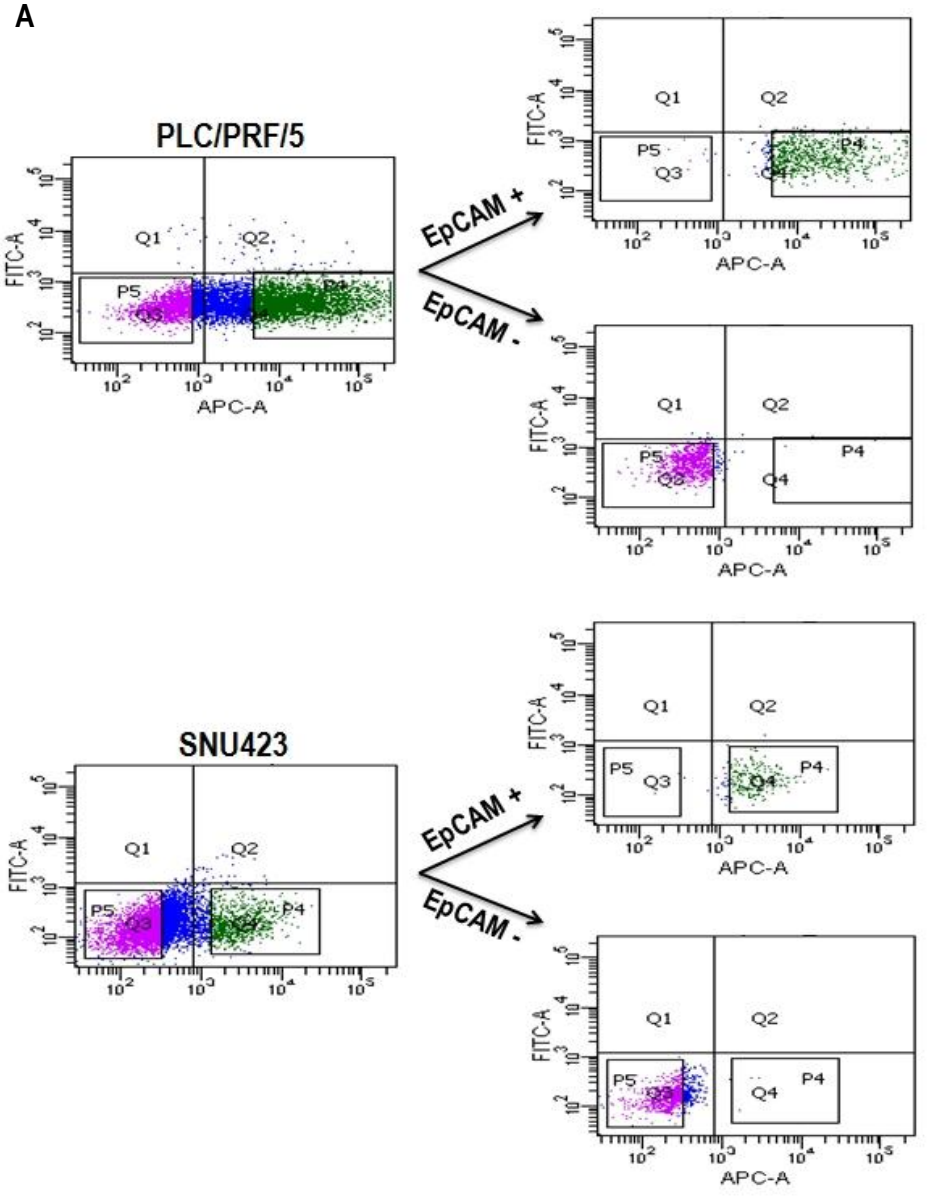
Statistical analysis was carried out using PASW statistics software (version 18.0, SPSS Inc., Chicago, IL, USA) and assessed using the Mann–Whitney test, t-test, and chi-square test, as deemed appropriate. Disease-free survival was calculated using the Kaplan-Meier method. *P*-values less than 0.05 were considered statistically significant.

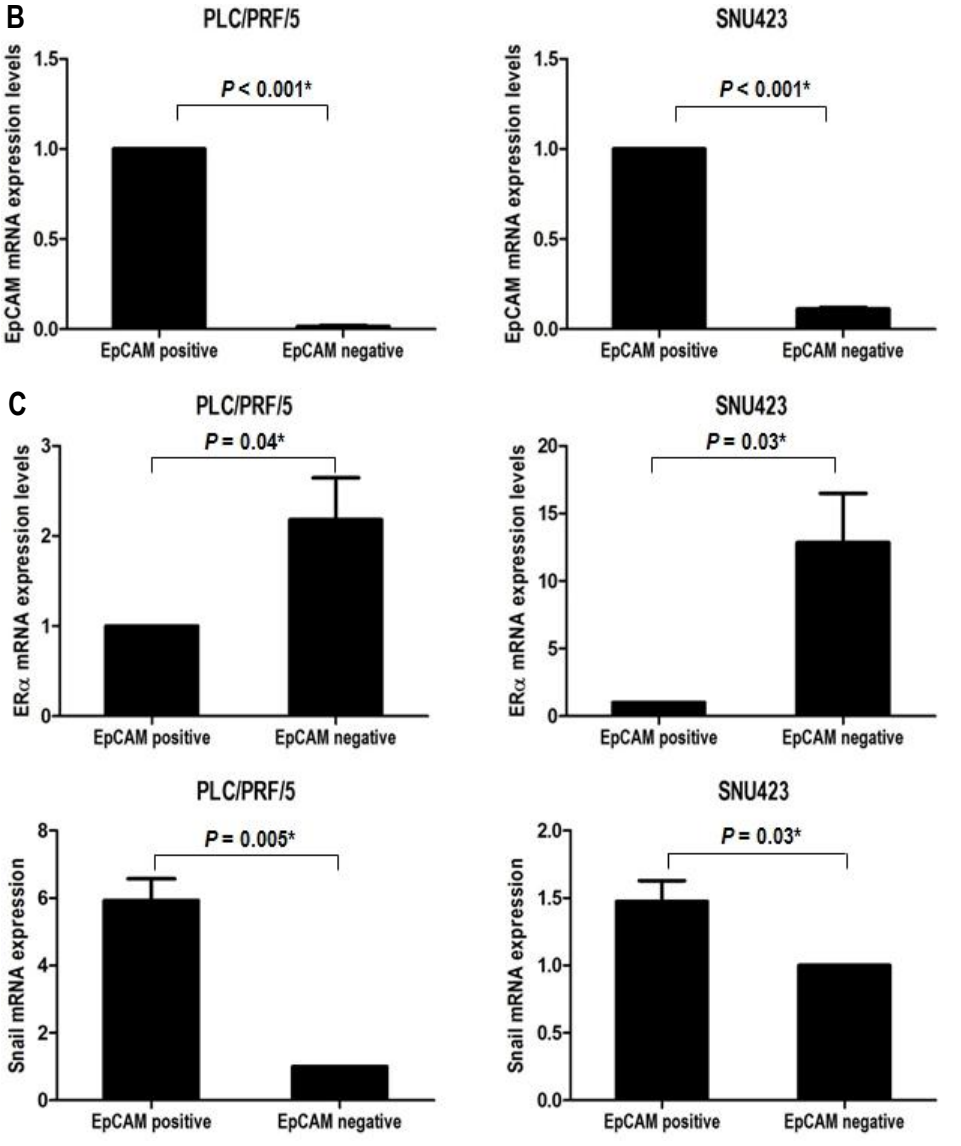
III. RESULTS

ERα, K19, Snail, Twist, IL-6 mRNA expression levels in EpCAM-positive and EpCAM-negative cell fractions of HCC cell lines

EpCAM+ and EpCAM− cell fractions were isolated by fluorescence activated cell sorting (FACS) from PLC/PRF/5 and SNU423 cells. Purity greater than 96.8% was obtained for EpCAM+ cell fractions and purity greater than 98.4% was obtained for EpCAM− fractions in PLC/PRF/5 cells, while purity greater than 99.5% in EpCAM+ cell fractions and purity greater than 99% in EpCAM− cell fractions was obtained for SNU423 cells after sorting (Figure 1A). The mRNA expression levels of ERα were evaluated by q-RT-PCR. The EpCAM mRNA levels of EpCAM+ subpopulations were significantly higher than that of EpCAM- subpopulations in PLC/PRF/5 ($P < 0.001$) and SNU423 cells ($P < 0.001$) (Figure 1B). The ERα mRNA level of EpCAM- subpopulations was 2.18 ± 0.37 fold ($P = 0.04$) and 12.83 ± 2.98 fold ($P = 0.03$) higher than that of EpCAM+ subpopulations in PLC/PRF/5 cell lines and SNU423 cell lines, respectively. The IL-6 mRNA level of EpCAM+ subpopulations was 5.41 ± 1.27 fold ($P = 0.03$) higher than that of EpCAM- subpopulations in SNU423 cell lines, whereas the mRNA expression of IL-6 was not detected in PLC/PRF/5 cells. The Snail mRNA level of EpCAM+ subpopulations was 5.73 ± 0.51 fold ($P = 0.005$) and 1.47 ± 0.12 fold ($P = 0.03$) higher than that of EpCAM- subpopulations in PLC/PRF/5 cell lines and SNU423 cell lines, respectively. The mRNA expression levels of Twist showed a trend of higher

expression in EpCAM-positive cell fractions than EpCAM-negative cell fractions for both cell lines (P = 0.06) (Figure 1C).




B
PLC/PRF/5
SNU423
EpCAM mRNA expression levels
P < 0.001*
EpCAM positive
EpCAM negative
C
PLC/PRF/5
SNU423
ERα mRNA expression levels
P = 0.04*
P = 0.03*
Snail mRNA expression
P = 0.005*
P = 0.03*

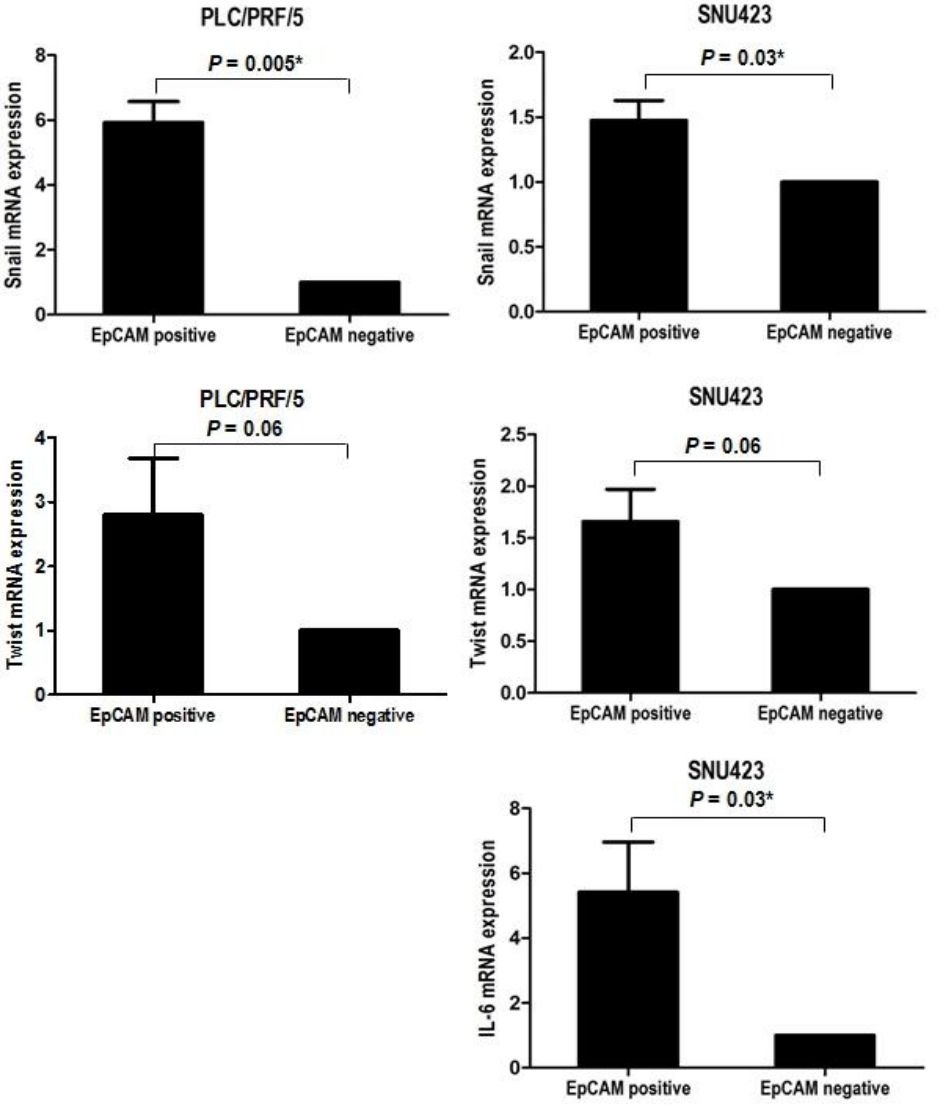


Figure 1. ERα mRNA expression levels according to EpCAM positive/negative fractionation. (A) FACS sorting of EpCAM +/- subpopulations in PLC/PRF/5 and SNU423 cells. (B) EpCAM mRNA expression levels in EpCAM +/- subpopulations of PLC/PRF/5 and SNU423 cells. (C) ERα, IL-6, Snail, Twist mRNA expression levels in EpCAM +/- subpopulations of PLC/PRF/5 and SNU423 cells. All the results were the average of three experiments (mean±SD). **P* < .05; ***P* < .01; ****P* < .001.

Effect of ERα overexpression on the expression levels of EpCAM, K19, IL-6, Snail and Twist

In order to investigate the role of ERα on stem/progenitor cell marker expression, ERα was transiently overexpressed in HCC cells. Overexpression of ERα in PLC/RPF/5 and SNU423 cells resulted in significantly decreased expression of EpCAM & K19 at both the protein and mRNA level compared to those of the control group. Importantly, overexpression of ERα induced the decrease of EpCAM in both PLC/PRF/5 (52.3 ± 19.6%) (P = 0.02) and SNU423 (33.6 ± 8.17%) (P = 0.02) cells. ERα overexpressed-HCC cells also showed slight decreases in the expression of K19 in both PLC/PRF/5 (11 ± 2.3%) (P = 0.02) and SNU423 (21 ± 7.13%) (P = 0.03) cells, compared to controls (Figure 2A and B). Overexpression of ERα induced the reduction of the mRNA levels of EpCAM and K19 in both PLC/PRF/5 (29.3 ± 2.62% and 24 ± 2.16%, respectively) (P = 0.004 and P = 0.004, respectively) and SNU423 (36.6 ± 9.97% and 28.3 ± 1.24, respectively) (P = 0.03 and P = 0.02, respectively) cells, compared to controls (Figure 2C). After overexpression of ERα, the mRNA (15.7 ± 0.02) (P = 0.03) and protein level (37.55 ± 8.11) (P = 0.02) of IL-6 was decreased compared to controls in SNU423 cells, whereas the presence of IL-6 was not detected in PLC/PRF/5 cells at both the protein and mRNA level. The expression levels of Snail and Twist were also evaluated; However, there was no significant change in the expression levels of these genes after

overexpression with ERα vector transfection at both the protein and mRNA level (Figure 3A and B).

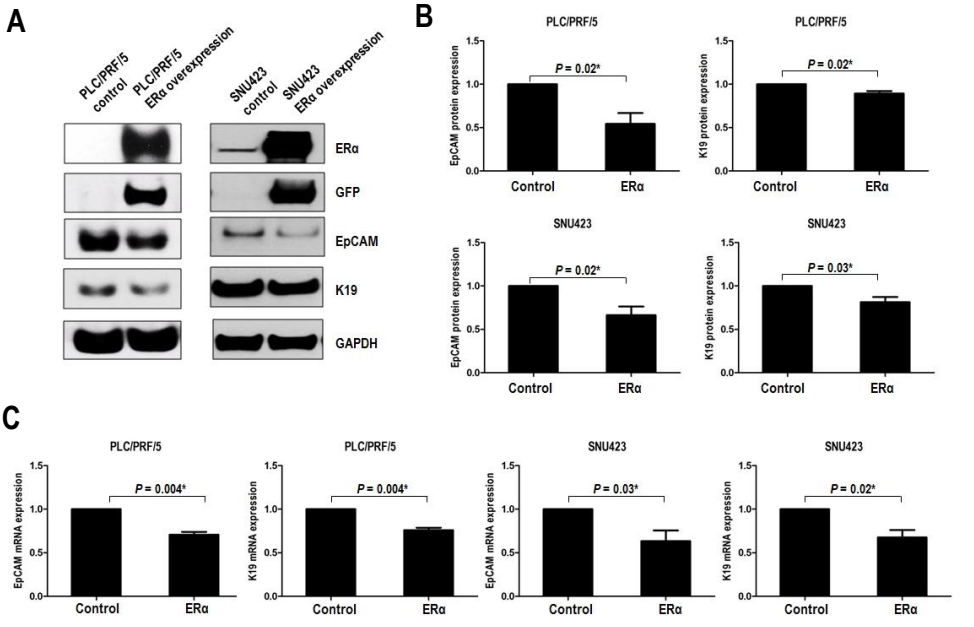


Figure 2. Impact of ectopic overexpression of ERα on CSC marker expression. (A) ERα, EpCAM, and K19 protein expression levels after overexpression of ERα in PLC/PRF/5 and SNU423 cells. (B) Protein quantity value of EpCAM and K19 in ERα over-expressed PLC/PRF/5 and SNU423 cells. (C) mRNA expression levels of EpCAM and K19 in ERα expressed cells. All the results were the average of three independent experiments (mean±SD). *$P < .05$; **$P < .01$; ***$P < .001$.

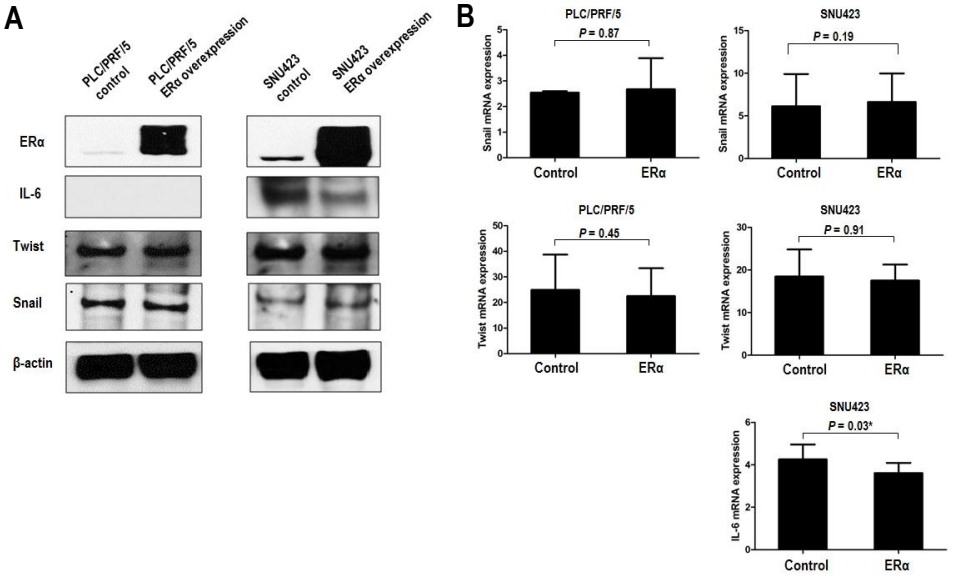


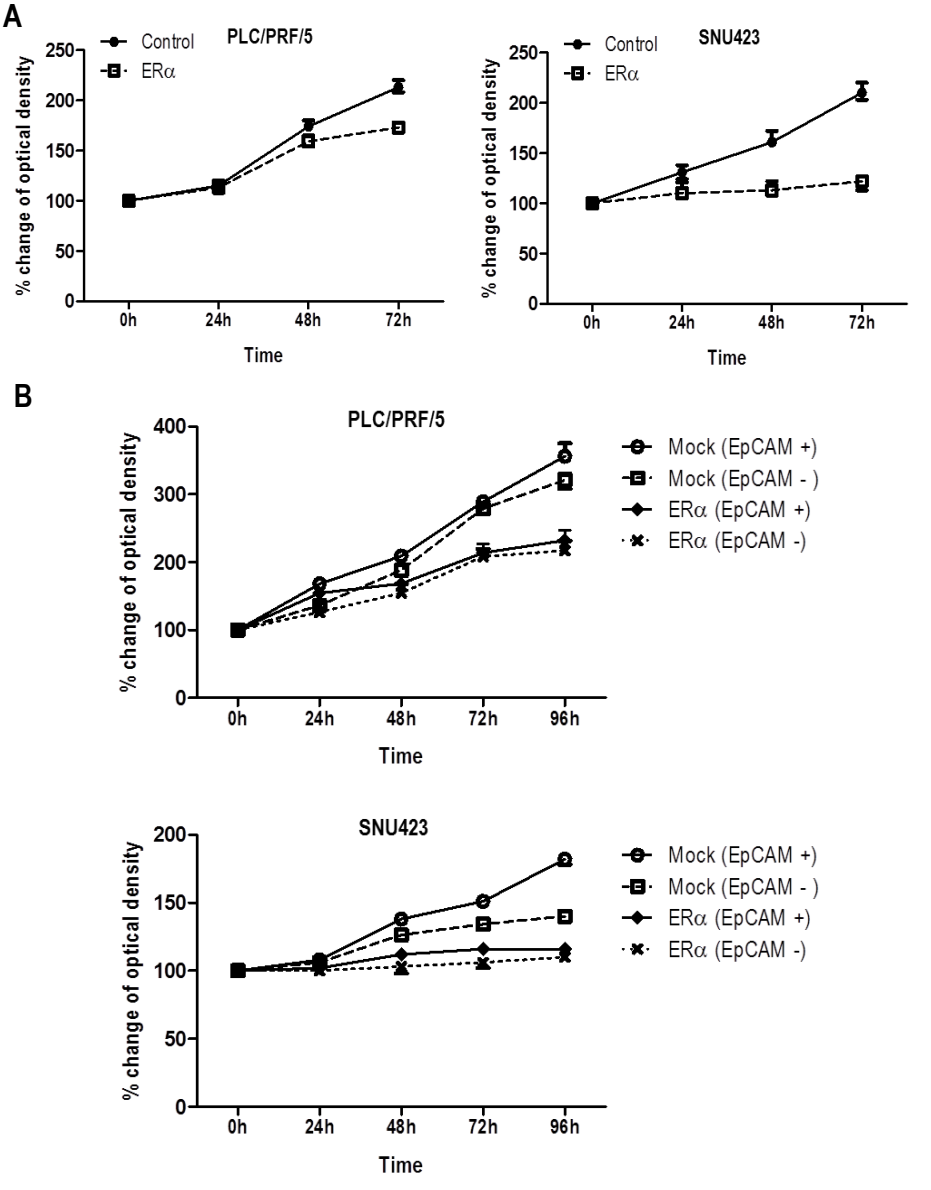
Figure 3. EMT-related genes (Snail, Twist) and IL-6 expression levels according to ectopic ERα overexpression. (A) IL-6, Twist, and Snail protein expression levels after overexpression of ERα in PLC/PRF/5 and SNU423 cells. (B) mRNA expression levels of IL-6, Twist and Snail in ERα expressed cells. All the results were the average of three independent experiments (mean±SD). *P < .05; **P < .01; ***P < .001.

Decrease of cell proliferation, migration and invasion in ERα overexpressed HCC cell lines

After ERα overexpression, cell proliferation activity was significantly decreased in PLC/PRF/5 and SNU423 cells compared to the control group ($P = 0.02$ and $P = 0.002$ at 72 hours after transfection, respectively) (Figure 4A). After 72 hours, the cell proliferation rates of the controls and ERα overexpressed cells were 213 ± 4.92% and 173 ± 4.08% in PLC/PRF/5 cells, respectively; 211 ± 6.97% and 120 ± 5.43% in SNU423 cells, respectively. We further compared the proliferation rate between EpCAM-positive and EpCAM-negative fractions in each group. After 96 hours, the cell proliferation rates of the EpCAM-positive cell fractions of the controls and ERα overexpressed cells were 361.2 ± 9.96% and 236.4 ± 7.33% in PLC/PRF/5 cells, respectively; and 180.0 ± 2.16% and 117.0 ± 1.41% in SNU423 cells, respectively. Also, the cell proliferation rates of the EpCAM-negative cell fractions of the controls and ERα overexpressed cells were 319.7 ± 8.88% and 220.7 ± 7.79% in PLC/PRF/5 cells, respectively; and 140.4 ± 1.21% and 110.3 ± 1.24% in SNU423 cells, respectively (Figure 4B).

We sought to determine whether ERα affects cell migration and invasion, two key steps in metastasis. Ectopic overexpression of ERα was confirmed in SNU423-ERα cells, compared to SNU423-Ctrl cells, and demonstrated decreased migration into the wound area at indicated time points, indicating that ERα overexpression suppresses cell migration (at 72hours after ERα transfection, 55 ± 2.09%) ($P < 0.001$) (Figure 4C). Next, we determined the

invasiveness of SNU423 cells. After the induction of ERα overexpression, the number of invaded cells of ERα overexpressed cells (604.0 ± 144.02) was significantly decreased compared to control cells (1320.0 ± 110.64) (P = 0.003) (Figure 4D).


A
PLC/PRF/5
Control
ERα
% change of optical density
250
200
150
100
50
0
0h
24h
48h
72h
Time
SNU423
Control
ERα
% change of optical density
250
200
150
100
50
0
0h
24h
48h
72h
Time
B
PLC/PRF/5
% change of optical density
400
300
200
100
0
0h
24h
48h
72h
96h
Time
Mock (EpCAM +)
Mock (EpCAM -)
ERα (EpCAM +)
ERα (EpCAM -)
SNU423
% change of optical density
200
150
100
50
0
0h
24h
48h
72h
96h
Time
Mock (EpCAM +)
Mock (EpCAM -)
ERα (EpCAM +)
ERα (EpCAM -)

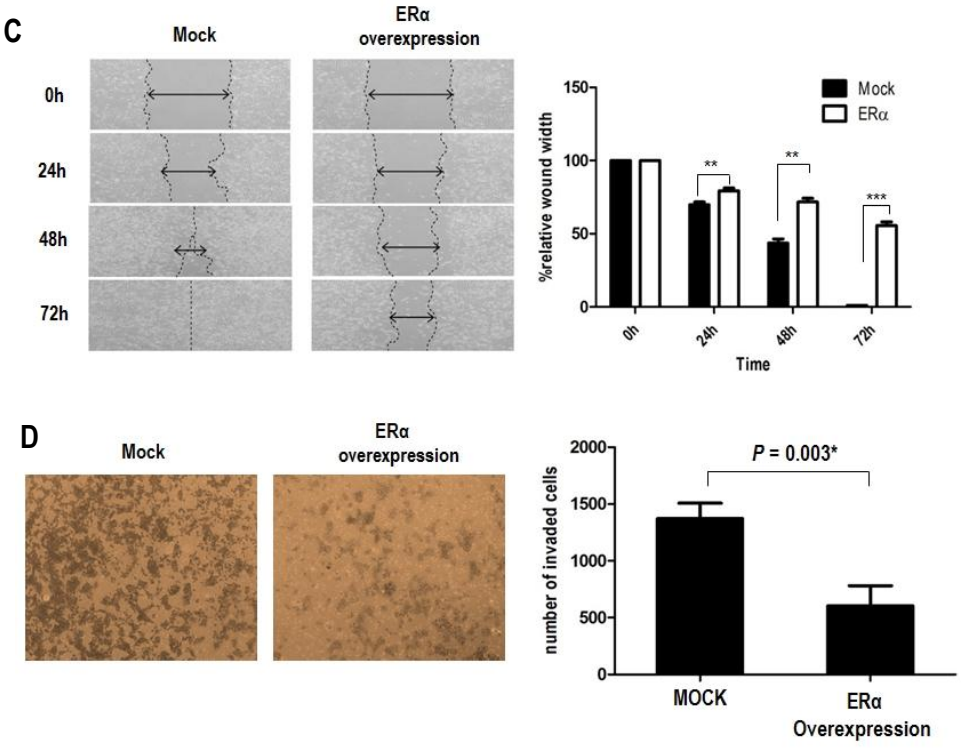
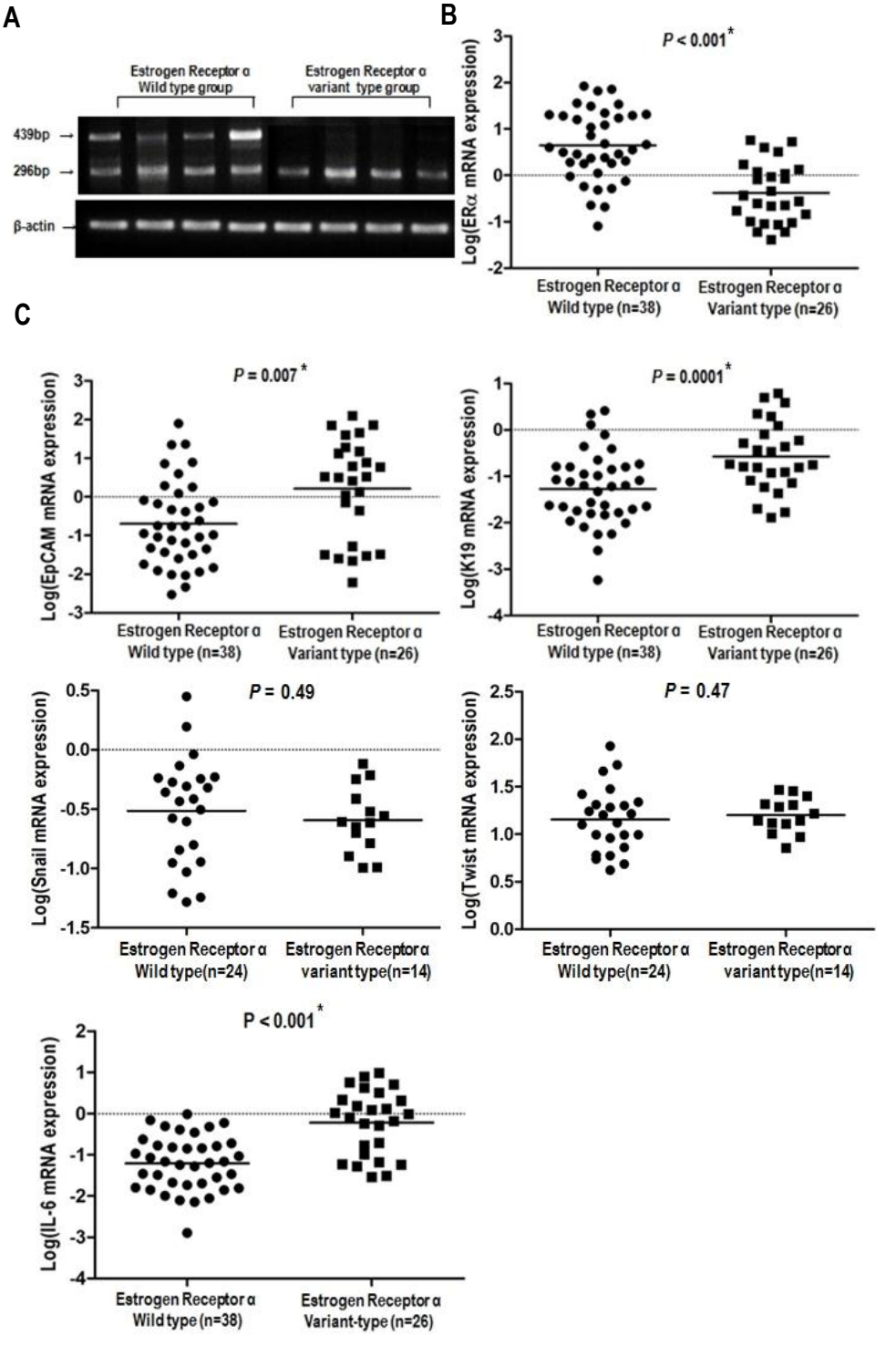


Figure 4. Impact of ectopic overexpression of ERα on cell proliferation (A) Cell proliferation activity after ectopic ERα overexpression in PLC/PRF/5 and SNU423 cells. (B) Cell proliferation rate between EpCAM-positive and EpCAM-negative fractions after ectopic ERα overexpression in PLC/PRF/5 and SNU423 cells. (C) Cell migration assay (Wound healing) after ectopic ERα overexpression and quantitative analysis of wound width at 0 and 72 hours in SNU423 cells. (D) Cell invasion assay after ectopic ERα overexpression and quantitative analysis of invaded cell number at 48 hours in SNU423 cells. All the results were the average of three independent experiments (mean±SD).*P < .05; **P < .01; ***P < .001.

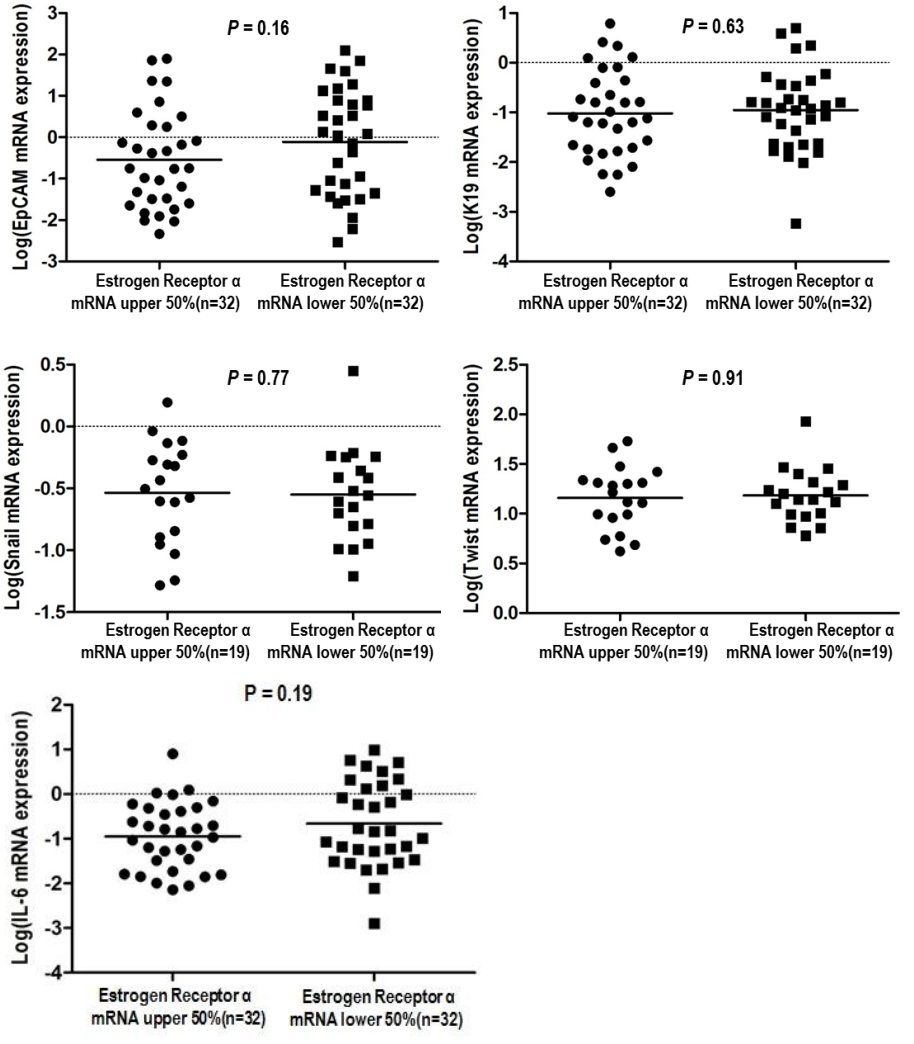
Expression levels of EpCAM and K19 according to ERα type and expression level in HCC patients

Wild type and variant type ERα transcripts were evaluated in 64 human HCC tissue samples by RT-PCR, and the wild type transcript and variant type transcript were detected at 430 bp and 296 bp, respectively (Figure 5A). In 26 (41%) HCC patients (12 men, 14 women) only the variant transcript was found, with the wild type transcript being totally absent. 39 (52%) HCC patients (27 men, 12 women) showed co-expression of both the wild type and variant type transcripts of ERα. HCC patients were further grouped into the ERα wild type dominant group and the ERα variant group. The mRNA levels of ERα were significantly higher in the ERα wild type dominant group (13.95 ± 20.24) than in ERα variant group (1.12 ± 1.61) ($P < 0.001$). We compared the CSC marker expression levels between the ERα wild type dominant group (ERα–wild) and the ERα variant group (ERα-variant). EpCAM mRNA levels and K19 mRNA levels were significantly higher in ERα-variant group than in the ERα–wild group ($P = 0.007$ and $P = 0.001$, respectively) (Figure 5C). The *in situ* expression of EpCAM and K19 proteins was detected by immunohistochemical staining (Figure 5D). The protein expressions of EpCAM and K19 were significantly higher in the ERα-variant group than in the ERα–wild group ($P = 0.002$ and $P = 0.02$, respectively) (Table 1). We also compared the expression levels of EpCAM and K19 between a high ERα mRNA group (upper 50%) and a low ERα mRNA group (lower 50%). The mRNA expression levels of EpCAM

and K19 were also evaluated and there was no significant change in expression levels between the high ERα mRNA group and the low ERα mRNA group (Figure 5D). However, the protein expression of EpCAM was significantly higher the low ERα mRNA group than in the high ERα mRNA group ($P < 0.001$) (Table 2). The protein expression of K19 demonstrated no significant changes for both the high ERα mRNA group and the low ERα mRNA group (Table 2). IL-6 mRNA levels were significantly higher in the ERα-variant group than in the ERα–wild group ($P < 0.001$) (figure 5C). The mRNA expressions of IL-6 showed no significant differences in expression levels between the high ERα mRNA group and the low ERα mRNA group (Figure 5D). The mRNA expression levels of Snail and Twist, known EMT-related genes, were also evaluated and there was no significant change in expression levels between the ERα-wild type group and the ERα-variant group (Figure 5C). These genes also demonstrated no significant differences in expression levels between the high ERα mRNA group and the low ERα mRNA group (Figure 5D).


A
Estrogen Receptor α
Wild type group
Estrogen Receptor α
variant type group
439bp
296bp
β-actin
B
P < 0.001*
Log(ERα mRNA expression)
Estrogen Receptor α
Wild type (n=38)
Estrogen Receptor α
Variant type (n=26)
C
P = 0.007*
Log(EpCAM mRNA expression)
Estrogen Receptor α
Wild type (n=38)
Estrogen Receptor α
Variant type (n=26)
P = 0.0001*
Log(K19 mRNA expression)
Estrogen Receptor α
Wild type (n=38)
Estrogen Receptor α
Variant type (n=26)
P = 0.49
Log(Snail mRNA expression)
Estrogen Receptor α
Wild type(n=24)
Estrogen Receptor α
variant type(n=14)
P = 0.47
Log(Twist mRNA expression)
Estrogen Receptor α
Wild type(n=24)
Estrogen Receptor α
variant type(n=14)
P < 0.001*
Log(IL-6 mRNA expression)
Estrogen Receptor α
Wild type (n=38)
Estrogen Receptor α
Variant-type (n=26)

D


P = 0.16
Log(EpCAM mRNA expression)
Estrogen Receptor α mRNA upper 50%(n=32)
Estrogen Receptor α mRNA lower 50%(n=32)
P = 0.63
Log(K19 mRNA expression)
Estrogen Receptor α mRNA upper 50%(n=32)
Estrogen Receptor α mRNA lower 50%(n=32)
P = 0.77
Log(Snail mRNA expression)
Estrogen Receptor α mRNA upper 50%(n=19)
Estrogen Receptor α mRNA lower 50%(n=19)
P = 0.91
Log(Twist mRNA expression)
Estrogen Receptor α mRNA upper 50%(n=19)
Estrogen Receptor α mRNA lower 50%(n=19)
P = 0.19
Log(IL-6 mRNA expression)
Estrogen Receptor α mRNA upper 50%(n=32)
Estrogen Receptor α mRNA lower 50%(n=32)

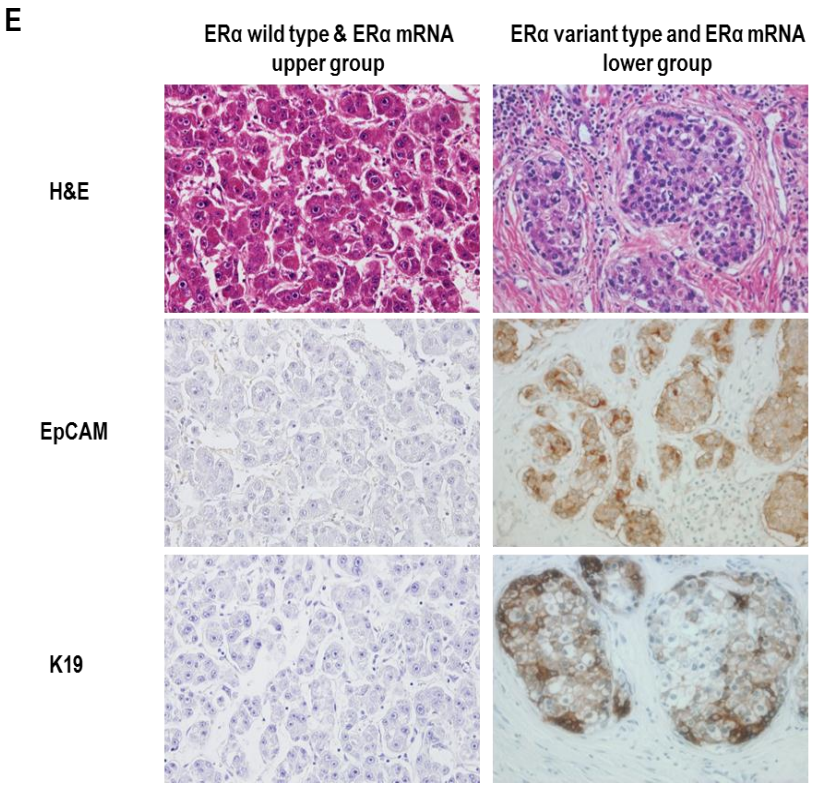


Figure 5. EpCAM, K19, Snail, Twist, and IL-6 mRNA expressions levels according to ERα types in HCC specimens. (A) Grouping of HCC patients according to ERα expression status. (B) The mRNA expression levels of ERα according to ERα types. (C) EpCAM, K19, Snail, Twist, and IL-6 mRNA expression levels according to ERα types. (D) EpCAM, K19, Snail, Twist, and IL-6 mRNA expression levels according to ERα expression. (E) EpCAM and K19 protein expression levels according to ERα types and expression. *P < .05; **P < .01; ***P < .001.

Expression levels of ERα and IL-6 according to the gender

HCC patients were further grouped into males and females. Of 39 male patients with HCC, the ERα wild-type was found in 27 patients (69%) and the ERα variant-type was found in 12 patients (31%). Of 25 female patients with HCC, the ERα wild-type was found in 11 patients (44%) and the ERα variant-type was found in 14 patients (56%) (Figure 6A). There were no differences in the mRNA levels of ERα or IL-6 between males and females (Figure 6B and C).

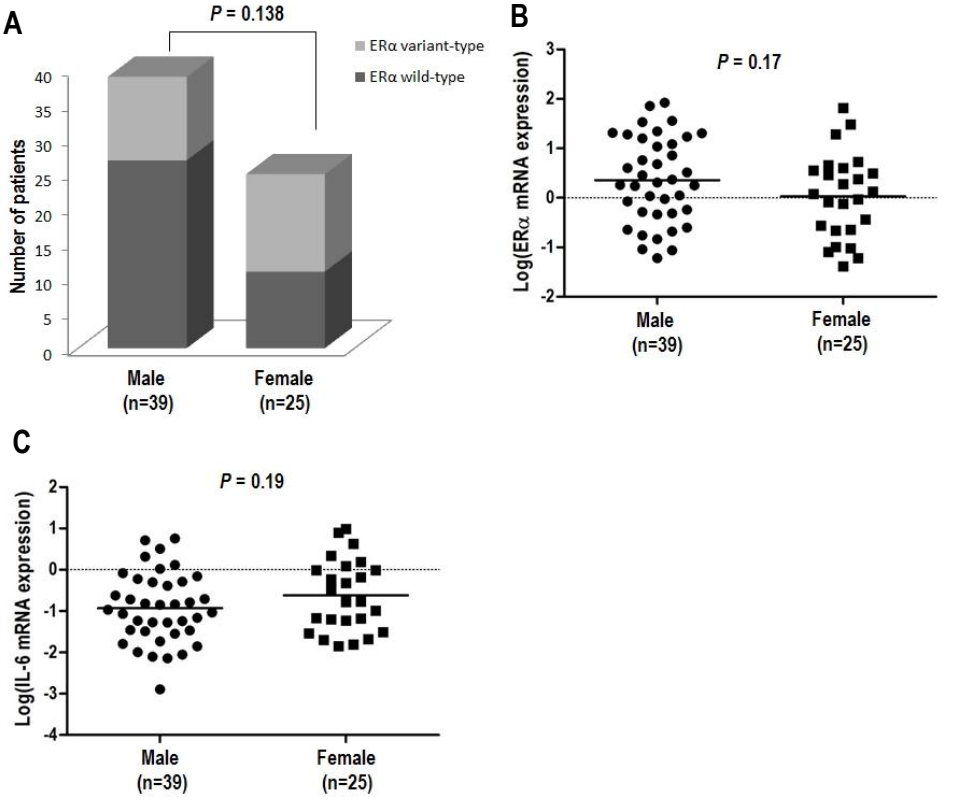


Figure 6. Expression levels of ERα and IL-6 according to gender. (A) Distribution of wild-type and variant ERα in HCC patients. (B) The mRNA expression levels of ERα according to gender. (C) The mRNA expression levels of IL-6 according to gender.

Table 1. Protein expression levels of EpCAM and K19 according to ERα type

		EpCAM protein							K-19 protein						
Group	n	< 5%		5 ≤ ,< 50%		≧ 50%		*P* value	0%		< 5%		≥ 5%		*P* value
ERα wild type	38	17	45%	11	29%	10	26%	<0.001*	36	95%	1	3%	1	3%	0.02*
ERα variant type	26	2	8%	7	27%	17	65%		18	69%	4	15%	4	15%	

Abbreviations : ER, estrogen receptor, *$P < .05$

Table 2. Protein expression levels of EpCAM and K19 according to ERα expression

		EpCAM protein							K-19 protein						
Group	n	< 5%		5 ≤ ,< 50%		≧ 50%		*P* value	0%		< 5%		≥ 5%		*P* value
ERα mRNA upper 50%	32	17	45%	8	21%	7	34%	<0.001*	28	88%	2	6%	2	6%	0.789
ERα mRNA lower 50%	32	2	8%	10	38%	20	54%		26	81%	3	9%	3	9%	

Abbreviations : ER, estrogen receptor , *$P < .05$

Clinicopathological characters of HCC patients according to EpCAM and K19, IL-6 mRNA and protein expression level

The pathological characteristics of HCCs were compared between a high EpCAM mRNA group (upper 50%) and a low EpCAM mRNA group (lower 50%). The low EpCAM mRNA group demonstrated older ages ($P < 0.001$) and greater fibrous capsule formation ($P = 0.02$) compared to the high EpCAM mRNA group. HCC patients were also analyzed according to an EpCAM IHC positive group and an EpCAM IHC negative group. The EpCAM IHC negative group demonstrated older ages ($P = 0.023$) and a higher incidence of microvascular invasion ($P = 0.016$). When comparing the low K19 mRNA group (lower 50%) to the high K19 mRNA group (upper 50%), the low K19 mRNA group demonstrated older ages than the high K19 mRNA group ($P = 0.04$). Moreover, patients were analyzed according to a K19 IHC positive group and a K19 IHC negative group. The K19 IHC positive group demonstrated a higher incidence of bile duct invasion ($P = 0.022$). Also, necrosis was presented at a higher incidence in the K19 IHC negative group than the K19 IHC positive group ($P = 0.019$). There were no clinicopathological differences between the high IL-6 mRNA group (upper 50%) and the low IL-6 mRNA group (lower 50%) (Table 4).

Table 3. Clinicopathological characters of HCC patients according to ERα type and ERα expression level

Clinicopathologic features	ERα wild-type (n=32)	ERα variant-type (n=18)	*P* value	ERα mRNA upper 50% (n=32)	ERα mRNA lower 50% (n=32)	*P* value
Age (year, mean±SD)	58.63±11.02	53.35±9.85	0.08	56.13±11.6	56.8±10.1	0.78
Gender (male:female)	26:12	13:13	0.138	21:11	18:14	0.442
Tumor size (cm, mean±SD)	4.78±2.51	4.96±2.09	0.451	5.14±2.6	4.58±2.02	0.65
Portal vein invasion (%)	5 (13%)	8 (42%)	0.008*	4 (12.5%)	12 (37.5%)	0.021*
Microvascular invasion (%)	22 (57%)	22 (84%)	0.024*	17 (53.1%)	27 (84%)	0.007*
Bile duct invasion (%)	1 (2%)	1 (3%)	0.784	1 (3%)	1 (3%)	1
Satellite nodule (%)	2 (5%)	5(19%)	0.079	3 (9%)	4 (12.5%)	0.689
Poor differentiation (%)	8 (21%)	13 (50%)	0.015*	8 (25%)	13 (40%)	0.183
Fibrous capsule (%)	7 (18%)	4 (15%)	0.752	5 (20%)	4 (16%)	0.713

Abbreviations : ER, estrogen receptor , *$P < .05$

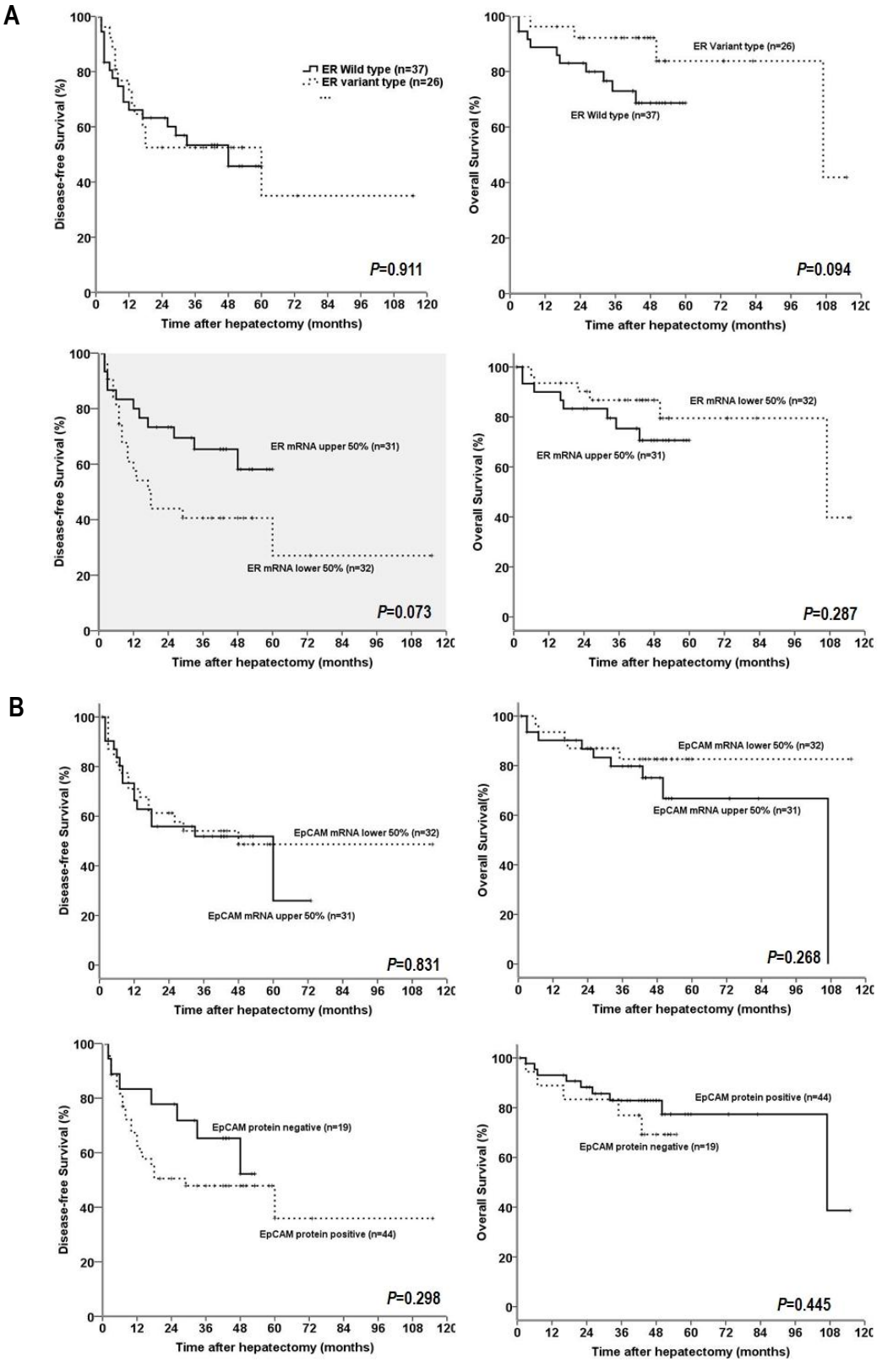
Table 4. Clinicopathological characters of HCC patients according to EpCAM and K19 mRNA and protein expression levels

Clinicopathologic features	EpCAM mRNA upper 50% (n=32)	EpCAM mRNA lower 50% (n=32)	*P* value	EpCAM protein positive (n=45)	EpCAM protein negative (n=19)	*P* value	K19 mRNA upper 50% (n=32)	K19 mRNA lower 50% (n=32)	*P* value	K19 protein positive (n=10)	K19 protein negative (n=54)	*P* value	IL-6 mRNA upper 50% (n=32)	IL-6 mRNA lower 50% (n=32)	*P* value
Age (year, mean±SD)	51.37±10.21	61.59±8.97	<0.001*	54.37±11.02	60.95±8.777	0.023*	53.34±10.87	59.54±10.08	0.04*	51.3±11.98	57.4±10.38	0.184	54.53±10.74	58.43±10.67	0.15
Gender (male:female)	16:16	23:09	0.073	26:19	13:6	0.425	19:14	20:11	0.57	4:6	35:19	0.14	19:13	20:12	0.066
Tumor size (cm, mean±SD)	4.38±1.61	5.33±2.81	0.32	4.56±1.93	5.57±2.99	0.37	4.63±2.18	4.94±2.38	0.77	4.8±1.55	4.87±2.48	0.69	4.91±0.39	4.81±0.44	0.87
Portal vein invasion (%)	7(21%)	9(28%)	0.564	11(25.6%)	5(23%)	0.874	8(25%)	8(25%)	1	3(30%)	13(24%)	0.691	9(28%)	7(21%)	0.774
Microvascular invasion (%)	22(68%)	22(68%)	1	35(81.3%)	9(42.8%)	0.016*	22(68%)	22(68%)	1	8(80%)	36(66%)	0.403	22(68%)	22(68%)	1
Bile duct invasion (%)	2(6%)	0(0%)	0.151	2(4.6%)	0(0%)	0.35	2(6.2%)	0(0%)	0.164	2(20%)	0(0%)	0.022*	2(20%)	0(0%)	0.151
Satellite nodule (%)	4(12.5%)	3(9.3%)	0.689	5(11.1%)	2(10.5%)	0.945	4(12.5%)	3(9.3%)	0.689	2(20%)	5(9.2%)	0.317	5(15.6%)	2(6.25%)	0.23
Poor differentiation (%)	10(31.2%)	11(34.3%)	0.79	16(37%)	5(23%)	0.472	11(34.3%)	10(31.2%)	0.79	3(30%)	18(33%)	0.837	10(31.2%)	11(34.3%)	0.79
Fibrous capsule (%)	2(6.2%)	9(28%)	0.02*	6(13.9%)	5(23%)	0.208	6(18.7%)	5(15.6%)	0.828	0(0%)	11(20.3%)	0.117	3(9.4%)	8(25%)	0.184

Abbreviations : ER, estrogen receptor , *$P < .05$

Disease-free survival rate and overall survival rate analysis according to ERα, EpCAM, K19 and IL-6 expression levels

We examined disease-free survival and overall survival among patients according to ERα, EpCAM, K19 and IL-6. The mean follow-up time after surgery was 43 months (range 2-115 months). When survival analysis was performed separately for wild-type ERα groups and variant-type ERα groups, no prognostic value was found (DFS:P = 0.911, OS:P = 0.094). In the disease free survival analysis of the high ERα mRNA group and the low ERα mRNA group, a trend of poor prognoses in the low ERα mRNA group was found (P = 0.073). In the overall survival analysis of the high ERα mRNA group vs. the low ERα mRNA group, no tendencies between the two groups were demonstrated (P = 0.287) (Figure 7A). In comparison of the high EpCAM mRNA group and the low EpCAM mRNA group, there were no differences in disease-free survival rates and overall survival between the two groups. Even though there were no statistically significant differences, the EpCAM IHC negative group showed better disease-free survival rates than the EpCAM IHC positive group (P = 0.298) (Figure 7B). The analysis of the high K19 mRNA group vs. the low K19 mRNA group as well as the K19 IHC positive group vs. the K19 IHC negative group revealed no tendencies (Figure 7C). Also, no differences were found in the analysis of the high IL-6 mRNA group vs. the low IL-6 mRNA group (Figure 7D).


A
Disease-free Survival (%)
ER Wild type (n=37)
ER variant type (n=26)
P=0.911
Time after hepatectomy (months)
Overall Survival (%)
ER Variant type (n=26)
ER Wild type (n=37)
P=0.094
Time after hepatectomy (months)
Disease-free Survival (%)
ER mRNA upper 50% (n=31)
ER mRNA lower 50% (n=32)
P=0.073
Time after hepatectomy (months)
Overall Survival (%)
ER mRNA lower 50% (n=32)
ER mRNA upper 50% (n=31)
P=0.287
Time after hepatectomy (months)
B
Disease-free Survival (%)
EpCAM mRNA lower 50% (n=32)
EpCAM mRNA upper 50% (n=31)
P=0.831
Time after hepatectomy (months)
Overall Survival(%)
EpCAM mRNA lower 50% (n=32)
EpCAM mRNA upper 50% (n=31)
P=0.268
Time after hepatectomy (months)
Disease-free Survival (%)
EpCAM protein negative (n=19)
EpCAM protein positive (n=44)
P=0.298
Time after hepatectomy (months)
Overall Survival (%)
EpCAM protein positive (n=44)
EpCAM protein negative (n=19)
P=0.445
Time after hepatectomy (months)

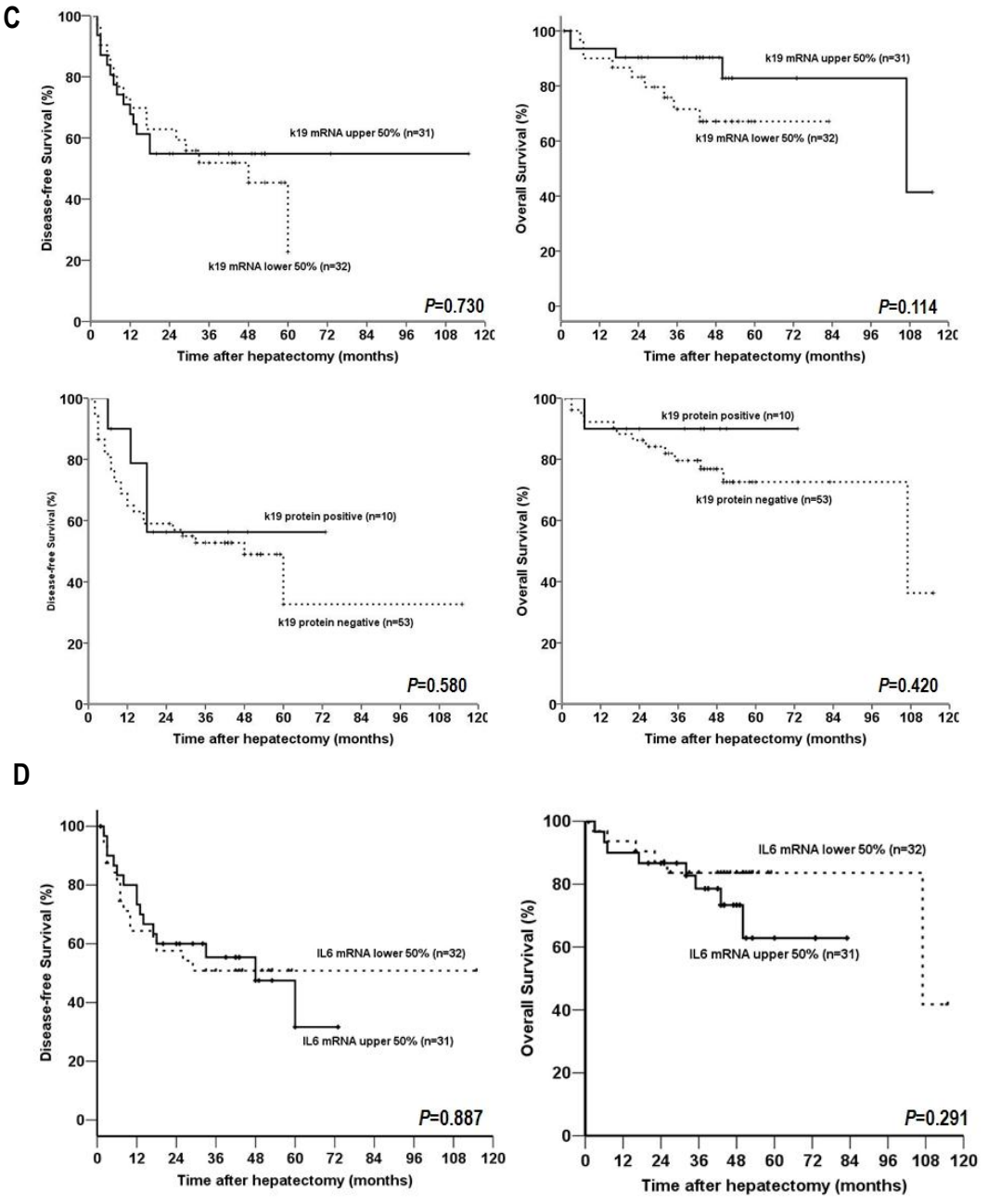


Figure 7. Disease-free survival and overall survival rate in HCC patients.
(A) ERα wild-type/variant-type and ERα mRNA upper/lower 50%. (B) EpCAM mRNA upper/lower 50% and EpCAM IHC positive/negative. (C) K19 mRNA upper/lower 50% and K19 IHC positive/negative. (D) IL-6 mRNA upper/lower 50%.

IV. DISCUSSION

Generally, the incidence of HCC is higher in males than in females with the male to female ratio usually averaging 6:1, according to several cohort studies[2, 3]. In the past, gender disparities in HCC were considered to have resulted from differences in gene expressions, such as HNF[5] and STAT5b,[6] and/or dissimilar life patterns, such as smoking and drinking, between males and females[4]. It was reported that estrogen and estrogen-mediated signaling may protect hepatocytes from blocking IL-6 expression in Kupffer cells and reduce liver cancer in female mice[8]. Also, ERα mRNA expression level was significantly decreased in HCC liver tissues compared to non-tumor tissues[12]. Variant ERα, a dominant negative receptor, was shown to be present at early stages of chronic liver disease as well as HCC[16, 17]. Through these results, ERα seems to have a protective role in hepatocarcinogenesis and may account for gender disparity in HCC.

We previously reported that HCCs expressing stemness-related markers demonstrate an aggressive biological character and poor prognosis[26]. Interestingly, the male to female ratio was 2.1:1 (mean ages: 53.2 ± 11.7, mean±SD) in EpCAM-positive HCC patients, in contrast to 5.4:1 (mean ages: 58.1 ± 12.2, mean±SD) in EpCAM-negative HCC patients. Similarly, it was 1.8:1 (mean ages: 55.4 ± 13.3, mean±SD) in K19-positive HCC patients and 4.6:1 (mean ages: 56.7 ± 12.0, mean±SD) in K19-negative HCC patients. These findings showed that the male to female ratio was reduced in HCC

patients who expressed stemness-related markers. All of the patients of our previous study were old in age; therefore, the protective role of estrogen and estrogen receptors in old female patients after menopause was lost. This might potentially be related to gender disparity and the expression of stemness related markers.

Recently, it was reported that IL-6 plays a pivotal role in the conversion of non-stem cancer cell to cancer stem cell in breast cancer[34]. Moreover, IL-6 is capable of triggering EMT phenotype and generating a subpopulation of cancer cells with stem cell characteristics in breast cancer cells[37]. It was also reported that estrogen suppresses metastasis in rats by decreasing IL-6 mRNA and protein expressions[38]. The expression of IL-6 has been shown to be associated with estrogen and estrogen receptor in mice, yet the link between IL-6 and cancer stem cells remains poorly understood. Herein, the presented data will substantiate the association of ERα and cancer stem cell markers via IL-6 expression for the first time.

In this study, we fractionated EpCAM-positive and EpCAM-negative cells by FACS in PLC/PRF/5 and SNU423 HCC cell lines and then checked the mRNA expression of ERα. The ERα mRNA expression level was significantly higher in the EpCAM-negative cell fractions than in the EpCAM-positive cell fractions of both PLC/PRF/5 (2.18 ± 0.37 fold) and SNU423 (12.83 ± 2.98 fold) cell lines ($P < 0.05$). However, we were unable to find ERα protein expression in EpCAM-positive and EpCAM-negative

fractionation of both cell lines. For this reason, we investigated the expression of CSC markers, such as EpCAM and K19, according to ectopic ERα overexpression. Overexpression of ERα showed a significant reduction of EpCAM and K19 expression levels in PLC/PRF/5 and SNU423 cell lines at both the mRNA and protein level ($P < 0.05$). HCC patients were grouped into the ERα wild type group and ERα variant group, and then the expressions of the CSC markers were explored. The ERα wild type HCC group showed significantly higher mRNA levels of ERα and lower mRNA levels of EpCAM and K19 compared to those of the ERα variant type. Additionally, the protein levels of EpCAM and K19 were also significantly different between the two groups ($P < 0.05$ in all). We also compared the expression levels of EpCAM and K19 between the high ERα mRNA group (upper 50%) and the low ERα mRNA group (lower 50%). The mRNA expression levels of EpCAM and K19 were evaluated, and there was no significant change in expression levels between the high and low ERα mRNA groups. However, the protein expression levels of EpCAM were significantly higher in the low ERα mRNA group than of the high ERα mRNA group ($P < 0.001$). The overexpression of ERα demonstrated a significant reduction in IL-6 expression levels at both the mRNA and protein level in SNU423 cell lines ($P < 0.05$).

We confirmed changes in cellular phenotypes including cell proliferation, migration and invasion, after ectopic overexpression of ERα in HCC cell lines. Cell proliferation was significantly decreased after overexpression of ERα

compared to the control group in both cell lines ($P < 0.05$). We investigated cell proliferation after fractionation of EpCAM-positive and EpCAM-negative cells of control and ERα overexpressed cells. Interestingly, the overexpression of ERα in EpCAM-positive cells showed lower proliferation activity than those of EpCAM-negative control cells ($P < 0.05$). From these results, ERα might play a pivotal role in cell proliferation in HCC cell lines. We also confirmed the decreased activity of cell migration and invasion in SNU423 cells ($P < 0.05$). Unfortunately, we were unable to demonstrate migration and invasion activity in PLC/PRF/5 cells during the 7 culture days. The clinicopathological characteristics of HCC patients were analyzed according to ERα type and ERα expression levels. HCC patients with the ERα variant type showed a higher incidence of portal vein invasion, microvascular invasion, and poor differentiation compared to those with the ERα wild type ($P < 0.05$). Therefore, we suggest that ERα might be associated with invasiveness in HCC.

We recently reported on the EMT related genes associated with cancer stem cell expression in HCC[39]. Also, it was reported that ERα regulates EMT related genes such as Snail[10] and Slug[11] in breast cancer. We confirmed this association of EMT related genes, Snail and Twist, in HCC cell lines after ectopic overexpression and in HCC patients according to ERα type. The expression levels of Snail and Twist demonstrated no significant change after ectopic overexpression of ERα at both the protein and mRNA level. Similarly,

the mRNA expression levels of Snail and Twist demonstrated no significant change between ERα wild type and ERα variant type. No significant change between the high ERα mRNA group and the low ERα mRNA was found in HCC patients.

Because ERα is generally a female dominant hormone receptor, we hypothesized that ERα mRNA expression would be higher in females than males. However, there were no differences in the mRNA expression of ERα between males and females. The reason for this result is not clear, but it was reported that the risk of HCC in females was associated with menopause.[40] One possible explanation for this discrepancy is that most female patients demonstrated a decrease in estrogen levels due to menopause in our study (female ages: 54.64 ± 11.4, mean±SD).

Some important new insights concerning gender disparity have recently been reported. Some microRNAs such as miR-18[41] and miR-22[9] are expressed differently between males and females. Exploring these microRNAs, which are known as ERα down-regulators associated with cancer stem cells, might be of interest to cell culture and human studies.

In conclusion, we suggest that ERα affects the regulation of stemness in HCC and may be a potential target for cancer stem cell targeted therapy. However, the molecular mechanism between cancer stem cells and ERα is not yet elucidated, as these findings necessitate further investigation thereon.

V. CONCLUSION

1. In HCC cell lines, ERα mRNA expression were lower and mRNA levels of IL-6, Snail and Twist were higher in EpCAM-positive fraction than in EpCAM-negative fraction. Overexpression of ERα by transfection of pEGEF-C1-ERα plasmid showed a significant reduction of EpCAM, K19, and IL-6 expression levels at both mRNA and protein levels. Overexpression of ERα also significantly reduced activities of cell proliferation, invasion and migration compared to control group.

2. HCC patients with ERα variant type showed significantly lower mRNA levels of ERα, higher mRNA levels of EpCAM, K19 and IL-6 and higher protein expression of EpCAM and K19 compared to those with ERα wild type. HCC patients with ERα variant type also showed significantly higher incidence of portal vein invasion, microvascular invasion, and poor differentiation compared to those with ERα wild type.

In conclusion, these data suggest low ERα mRNA expression and variant-ERα type are involved in the aggressive biological behavior of HCC with high expressions of EpCAM, K19 and IL-6 in both HCC cell lines and HCC patients.

REFERENCES

1. Hui KM. Human hepatocellular carcinoma: expression profiles-based molecular interpretations and clinical applications. Cancer letters 2009;286(1):96-102.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010.
3. Cancer incidence in five continents. Volume VIII. IARC scientific publications 2002(155):1-781.
4. Yu MW, Hsu FC, Sheen IS, Chu CM, Lin DY, Chen CJ, et al. Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers. American journal of epidemiology 1997;145(11):1039-47.
5. Wiwi CA, Waxman DJ. Role of hepatocyte nuclear factors in growth hormone-regulated, sexually dimorphic expression of liver cytochromes P450. Growth Factors 2004;22(2):79-88.
6. Clodfelter KH, Holloway MG, Hodor P, Park SH, Ray WJ, Waxman DJ. Sex-dependent liver gene expression is extensive and largely dependent upon signal transducer and activator of transcription 5b (STAT5b): STAT5b-dependent activation of male genes and repression of female genes revealed by microarray analysis. Mol Endocrinol 2006;20(6):1333-51.
7. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 2006;16(8):995-1004.
8. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317(5834):121-4.

9. Jiang R, Deng L, Zhao L, Li X, Zhang F, Xia Y, et al. miR-22 promotes HBV-related hepatocellular carcinoma development in males. Clinical cancer research : an official journal of the American Association for Cancer Research 2011;17(17):5593-603.
10. Dhasarathy A, Kajita M, Wade PA. The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-alpha. Mol Endocrinol 2007;21(12):2907-18.
11. Ye Y, Xiao Y, Wang W, Yearsley K, Gao JX, Shetuni B, et al. ER alpha signaling through slug regulates E-cadherin and EMT. Oncogene 2010;29(10):1451-62.
12. Kalra M, Mayes J, Assefa S, Kaul AK, Kaul R. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. World J Gastroenterol 2008;14(39):5945-61.
13. Iavarone M, Lampertico P, Seletti C, Francesca Donato M, Ronchi G, del Ninno E, et al. The clinical and pathogenetic significance of estrogen receptor-beta expression in chronic liver diseases and liver carcinoma. Cancer 2003;98(3):529-34.
14. Pacchioni D, Papotti M, Andorno E, Bonino F, Mondardini A, Oliveri F, et al. Expression of estrogen receptor mRNA in tumorous and non-tumorous liver tissue as detected by in situ hybridization. Journal of surgical oncology Supplement 1993;3:14-7.
15. Wang AG, Lee KY, Kim SY, Choi JY, Lee KH, Kim WH, et al. The expression of estrogen receptors in hepatocellular carcinoma in Korean patients. Yonsei medical journal 2006;47(6):811-6.
16. Villa E, Camellini L, Dugani A, Zucchi F, Grottola A, Merighi A, et al. Variant estrogen receptor messenger RNA species detected in human primary hepatocellular carcinoma. Cancer Res 1995;55(3):498-500.
17. Villa E, Dugani A, Moles A, Camellini L, Grottola A, Buttafoco P, et al. Variant liver estrogen receptor transcripts already occur at an early stage

of chronic liver disease. Hepatology 1998;27(4):983-8.

18. Han J, Ding L, Yuan B, Yang X, Wang X, Li J, et al. Hepatitis B virus X protein and the estrogen receptor variant lacking exon 5 inhibit estrogen receptor signaling in hepatoma cells. Nucleic acids research 2006;34(10):3095-106.
19. Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, et al. Liver stem cells and hepatocellular carcinoma. Hepatology 2009;49(1):318-29.
20. Yin S, Li J, Hu C, Chen X, Yao M, Yan M, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 2007;120(7):1444-50.
21. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414(6859):105-11.
22. Dalerba P, Clarke MF. Cancer stem cells and tumor metastasis: first steps into uncharted territory. Cell stem cell 2007;1(3):241-2.
23. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100(7):3983-8.
24. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445(7123):111-5.
25. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432(7015):396-401.
26. Kim H, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, et al. Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. Hepatology 2011;54(5):1707-17.
27. Wulf GG, Wang RY, Kuehnle I, Weidner D, Marini F, Brenner MK, et al. A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid

leukemia. Blood 2001;98(4):1166-73.

28. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444(7120):756-60.
29. Hambardzumyan D, Squatrito M, Holland EC. Radiation resistance and stem-like cells in brain tumors. Cancer cell 2006;10(6):454-6.
30. Jin LQ, Hope KJ, Zhai QL, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nature Medicine 2006;12(10):1167-74.
31. Gilbertson RJ, Rich JN. Making a tumour's bed: glioblastoma stem cells and the vascular niche. Nat Rev Cancer 2007;7(10):733-6.
32. Blazek ER, Foutch JL, Maki G. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia. Int J Radiat Oncol 2007;67(1):1-5.
33. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nature Medicine 2006;12(4):410-6.
34. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci U S A 2011;108(4):1397-402.
35. Huang C, Yang G, Jiang T, Zhu G, Li H, Qiu Z. The effects and mechanisms of blockage of STAT3 signaling pathway on IL-6 inducing EMT in human pancreatic cancer cells in vitro. Neoplasma 2011;58(5):396-405.
36. Yadav A, Kumar B, Datta J, Teknos TN, Kumar P. IL-6 Promotes Head and Neck Tumor Metastasis by Inducing Epithelial-Mesenchymal Transition via the JAK-STAT3-SNAIL Signaling Pathway. Molecular cancer research : MCR 2011.
37. Xie G, Yao Q, Liu Y, Du S, Liu A, Guo Z, et al. IL-6-induced

epithelial-mesenchymal transition promotes the generation cultures. Int J Oncol 2011.

38. Wang YC, Xu GL, Jia WD, Han SJ, Ren WH, Wang W, et al. Estrogen Suppresses Metastasis in Rat Hepatocellular Carcinoma through Decreasing Interleukin-6 and Hepatocyte Growth Factor Expression. Inflammation 2011.
39. Na DC, Lee JE, Yoo JE, Oh BK, Choi GH, Park YN. Invasion and EMT-associated genes are up-regulated in B viral hepatocellular carcinoma with high expression of CD133-human and cell culture study. Experimental and molecular pathology 2011;90(1):66-73.
40. Yu MW, Chang HC, Chang SC, Liaw YF, Lin SM, Liu CJ, et al. Role of reproductive factors in hepatocellular carcinoma: Impact on hepatitis B- and C-related risk. Hepatology 2003;38(6):1393-400.
41. Liu WH, Yeh SH, Lu CC, Yu SL, Chen HY, Lin CY, et al. MicroRNA-18a prevents estrogen receptor-alpha expression, promoting proliferation of hepatocellular carcinoma cells. Gastroenterology 2009;136(2):683-93.

ABSTRACT(IN KOREAN)

간암종에서 ERα에 의한 IL-6, EpCAM, K19의 발현에 미치는 영향

<지도교수 박 영 년>

연세대학교 대학원 의과학과

김 광 일

줄기세포성 단백질을 발현하고 있는 간암종은 공격적인 성향과 환자들에게 불량한 예후를 보인다고 최근에 보고되고 있다. EMT현상은 암줄기세포의 발생에 관여하는 것으로 알려져있다. 간암은 남성에서 주로 발생하고 간암에서 ERα는 EMT현상을 유발 시킬 수 있는 IL-6의 분비를 막으면서 보호하는 것으로 알려졌다. 이 연구에서, 줄기세포마커(EpCAM, K19), IL-6, EMT마커(Snail, Twist)발현에 ERα가 미치는 영향을 간암세포주과 임상연구를 통해 알아보고자 한다. 간암세포주인 PLC/PRF/5와 SNU423를 FACS를 통해 줄기세포마커인 EpCAM의 발현 차이에 따라 EpCAM-양성 그리고 EpCAM-음성 세포를 분리하였다. 그 결과 EpCAM-음성 세포군에서 ERα mRNA 발현이 EpCAM-양성 세포군에 비해 높게 발현되는 것을 PLC/PRF/5와 SNU423 세포주에서 확인하였다(P<0.05). 또한 두가지 간암세포주에서 Snail(P<0.05), Twist(P=0.06) mRNA 발현이 EpCAM-양성 세포군에서 EpCAM-음성 세포군에 비해 높게 발현하였다. IL-6의 mRNA 발현은 SNU423세포주에서 EpCAM-양성 세포군에서 EpCAM-음성 세포군에 비해 높게 발현하였지만 PLC/PRF/5에서는 IL-6의 mRNA발현을 관찰 할 수 없었다. PLC/PRF/5와 SNU423 세포주에 ERα를 pEGEF-C1-ERα 플라스미드를 통해 과발현 시켰을 때 줄기세포마커인 EpCAM, K19 의 mRNA 그리고 단백질의 발현이 줄어드는 것을 확인하였다(P<0.05). 흥미로운 것은, SNU423 세포주에서 ERα의 과발현에 따라서 IL-6의 mRNA그리고 단백질의 발현이 줄어드는 것을 확인하였다(P<0.05). 반면에 두가지 간암세포주에 ERα의 과발현

후에도 Snail과 Twist의 발현에 변화가 없었다. 세포성장성, 침투성, 이동성을 세포주에 ERα 과발현을 시킨 후 확인해본 결과 모두 감소하는 것이 관찰되었다. 또한 64명의 간암환자에서 ERα의 발현과 발현정도에 따라 간암줄기세포마커인 EpCAM과 K19 그리고 EMT 관련마커인 Snail, Twist 그리고 IL-6의 mRNA발현을 RT-PCR, real-time PCR을 통해 확인하였다. 또한 면역염색을 통해 EpCAM과 K19의 단백질의 발현을 확인하였다. 그의 발현에 따라 환자들의 임상병리학적 특성을 관찰하였다. 정상적으로 ERα를 발현하는 환자그룹과 이형성의 ERα을 가지는 환자그룹간에 유전자 분석을 해보았을 때, 정상적인 ERα를 발현하는 환자그룹에서 ERα mRNA의 발현이 높고, 간암줄기세포 마커인 EpCAM, K19 그리고 IL-6의 mRNA 그리고 단백질 발현은 적은 것으로 확인되었다 (P<0.05). 하지만 Snail, Twist의 mRNA발현은 두 환자 그룹간에 차이가 없었다. ERα의 발현종류와 발현정도에 따라 간암환자들의 임상병리학적 특성을 알아보았을 때 이형성의 ERα를 가진 환자군이 정상적인 ERα를 가진 환자군에 비해 정맥혈관전이(P = 0.008) 그리고 미세혈관전이(P = 0.024)가 더 많이 발생되었고 불량한 분화도(P = 0.015)를 확인할 수 있었다. 또한 간암환자를 ERα mRNA 상/하위 50%로 나누었을 때 정맥혈관전이(P = 0.021) 와 미세혈관전이(P = 0.007) 가 ERα mRNA 하위 50% 그룹에서 더 많이 발생하는 것을 확인할 수 있었다.

ERα의 발현종류, EpCAM, K19, IL-6 mRNA 또는 단백질의 발현정도에 따른 무병생존율(DFS)과 전체생존율(OS)은 큰 차이를 발견하지 못하였다. 결과적으로 이 실험을 통해 이형성을 가지는 ERα 또는 ERα의 mRNA의 발현이 적으면 간암 줄기세포 표지자인 EpCAM, K19 그리고 IL-6의 발현이 높게 되며 간암이 공격적인 형태를 띄는 특성을 확인하였다.

핵심되는 말 : 에스토로젠 수용체 알파, IL-6, EpCAM, Keratin 19, 간암, 침투, stemness