

## Cucurbitacin-I, a Naturally Occurring Triterpenoid, Inhibits the CD44 Expression in Human Ovarian Cancer Cells

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Cucurbitacin-I, a natural triterpenoid derived from Cucurbitaceae family plants, exhibits a number of potentially useful pharmacological and biological activities. Indeed, the previous study demonstrated that cucurbitacin-I reduced the proliferation of colon cancer cells by enhancing apoptosis and causing cell cycle arrest at the G2/M phase. CD44, a type I transmembrane protein with the function of adhering to cells, mediates between the extracellular matrix and other cells through hyaluronic acid. Recent studies have demonstrated that an overexpression of the CD44 membrane receptor results in tumor initiation and growth, specific behaviors of cancer stem cells, the development of drug resistance, and metastasis. The aim was to examine the effect of cucurbitacin-I on CD44 expression in human ovarian cancer cells because the effect of cucurbitacin-I on CD44 expression has not been reported. The expressions of CD44 mRNA and protein were detected using a quantitative real-time reverse-transcription polymerase chain reaction and a Western blot analysis, respectively. Treatment with cucurbitacin-I inhibited the expression of CD44 mRNA and protein. A subsequent analysis revealed that cucurbitacin-I blocked the phosphorylation of activator protein-1 (AP-1) and nuclear factor kappa-B (NF- $\kappa$ B), which are key regulators of CD44 expression. Taken together, the data demonstrate that cucurbitacin-I regulates the AP-1 and NF- $\kappa$ B signaling pathways, leading to decreased CD44 expression.

**Key words** : CD44, Cucurbitacin-I, ovarian cancer, SKOV-3 cell

### Introduction

Ovarian cancer is an abnormal cell growth arising in the ovary. It is one of the three major cancers contracted by women along with breast and cervical cancer. The majority of ovarian cancers are epithelial and develop in women over 50. Although the progress has been made in the treatment of ovarian cancer, the mortality rate remains high. The majority of ovarian cancer patients have tumor metastasis at the time-point of diagnosis, and tumor invasion and metastasis are important causes of treatment failure [6]. Therefore, investigation of the molecular mechanisms involved in tumor proliferation, adhesion and invasion is of great importance for improving the therapeutic effectiveness of ovarian cancer treatments.

CD44 is a transmembrane and cell surface glycoprotein involved in cell-cell interactions, cell adhesion, and migra-

tion [8]. It is considered a major cell surface marker for metastasis and progression in certain types of cancers, including ovarian carcinoma. CD44 is a receptor for hyaluronic acid [1, 2]. It interacts with hyaluronic acid to activate Nanog-Stat-3 and ankyrin based signaling pathways that is considered to be responsible for conferring tumor cell growth in ovarian and breast cancers [4]. In addition, CD44 expression is a prognostic indicator of shorter survival time in patients with ovarian cancer [13]. Based on the aforementioned findings, we hypothesized that suppression of CD44 expression could enhance the efficiency of chemotherapy and prevent the development of metastases.

Cucurbitacin-I is a naturally occurring triterpenoid derived from Cucurbitaceae family plants that exhibits a number of potentially useful pharmacological and biological activities [3, 5, 9, 10, 12, 16, 17]. It was originally identified to be a potent selective inhibitor of the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway with antiproliferative and antitumor properties [3]. Upon inhibition of STAT3-dependent gene transcription, cucurbitacin-I elicits antiproliferative effects in various cancer cells with activated STAT3 signaling [5, 12, 16]. However, the effect of cucurbitacin-I on CD44 expression in ovarian cancer was not investigated. Therefore, the

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present study aimed to investigate the effects of cucurbitacin-I on the expression of CD44 of SKOV-3 ovarian cancer cells.

## Materials and Methods

### Chemicals and reagents

Cucurbitacin-I was purchased from Santa Cruz (Santa Cruz, CA, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Hyclone (Logan, UT, USA). Protein detection BCA kit was obtained from Thermo Scientific Pierce (Rockford, IL, USA). Antibody against CD44 standard form were purchased from Abcam (Cambridge, MA, USA). Anti-phospho-p65, anti-phospho-cJun, and anti-phospho-cFos were obtained from Cell Signaling Technology (Danvers, USA). Anti- $\beta$ -actin antibody and other materials were purchased from Sigma Aldrich (St. Louis, MO, USA).

### Cell and cell culture

The ovarian cancer cell line SKOV-3 was obtained from the Korean Cell Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### Cell proliferation assay

SKOV-3 cells were seeded into wells of 96-well plates at a density of  $1 \times 10^4$  cells/well. The rate of cell proliferation was determined at 24 hr with CellTiter 96® AQueous One Solution (Promega, Madison, WI, USA). Cell Proliferation assay according to the manufacturer's instruction.

### Flow Cytometry

The expression levels of surface CD44 protein were determined by immunostaining with anti-human CD44-FITC antibody (BD Biosciences, San Diego, CA, USA) for 30 min on ice. Cells stained with the appropriate isotype-matched control antibody were used as negative controls. After staining, cells were analyzed with a FACSCalibur instrument equipped with CellQuest software (BD Biosciences). The mean fluorescence intensity (MFI) of samples was analyzed by FlowJo software (TreeStar Inc., Ashland, OR, USA).

**Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA was isolated from SKOV-3 cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). DNA was eliminated from total RNA using RNA Qualified RNase-Free DNase (Promega) and cDNA was synthesized by GoScript™ Reverse Transcription System (Promega). qRT-PCR assay was carried out with LightCycler (Roche Diagnostics, Basel, Switzerland) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). All the experiments were repeated twice in triplicate each time. Transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house-keeping gene were quantified as endogenous RNA of reference to normalize each sample. Relative quantities were estimated by the  $-\Delta\Delta C_t$  method. CD44 and GAPDH primer pairs are as follows: CD44, forward 5'-CCTTTGATGGACCA ATTACCATAAC-3', reverse 5'-TCAGGATTCGTTCTGTAT CCT-3'; GAPDH, forward 5'-TGAACGGGAAGCTCACTGG-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

### Western blot analysis

Cells were lysed in Pro-prep protein extraction solution (iNtRon, Sungnam, Korea). The protein concentrations were determined using a BCA protein assay kit. Whole cell extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. After blocking with 3% skim milk for 1 hr, the membranes were incubated with primary antibody at 4°C overnight. The membranes were then washed with PBST (1× PBS, 0.1% Tween 20) and incubated for 1 hr with anti-rabbit IgG or anti-mouse IgG conjugated with HRP. After washing, the protein bands were then visualized using a WEST- One™ Western Blot Detection Spray (iNtRon) and DAVINCH-Chemi CAS-400SM (Davinch-k, Seoul, Korea). Dates were assessed using Total Lab software (Davinch-k).

### Statistical analysis

Values are expressed as the mean  $\pm$  SEM of the results of at least three experiments. The values were then evaluated by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post tests using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Differences with *p* values <0.05 were considered statistically significant.

## Results and Discussion

**Cucurbitacin-I inhibits ovarian cancer cell growth in a dose-dependent manner**

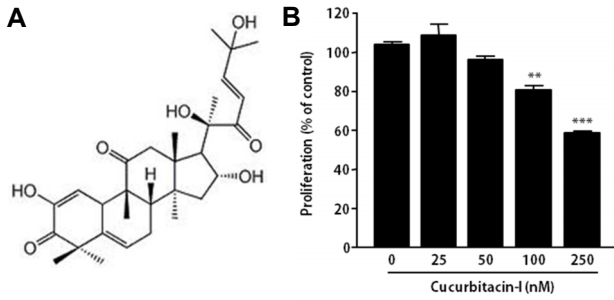


Fig. 1. Effects of Cucurbitacin-I on cell viability in SK-OV-3 cells. (A) Chemical structure of cucurbitacin-I. (B) SKOV-3 cells were treated with indicated concentrations of cucurbitacin-I for 24 hr and determined the proliferation by MTS assay. The results are means  $\pm$  SEM of three independent experiments in triplicate. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus 0 nM cucurbitacin-I-treated cells.

To determine the effect of cucurbitacin-I on the proliferation of ovarian cancer cells, the SKOV-3 cells were treated with various concentrations of cucurbitacin-I (0, 25, 50, 100, and 250 nM) for 24 hr prior to an MTS cell viability assay. Treatment with 100 and 200 nM cucurbitacin-I led to

significant decreases in cell viability (Fig. 1B). Although, several previous studies reported the antiproliferative effect of cucurbitacin-I on human cancer cells including glioblastoma [17], colon cancer cells [9, 16], and nasopharyngeal carcinoma cells [10], ovarian cancer cells has not been investigated. Our data demonstrate for the first time that cucurbitacin-I inhibits the proliferation of ovarian cancer cells.

**Cucurbitacin-I inhibits the expression of CD44 in ovarian cancer cell**

To examine the effect of cucurbitacin-I on CD44 expression, SKOV-3 cells were cultured with various concentration of cucurbitacin-I for 24 hr. We used up to 50 nM of cucurbitacin-I in the rest of the experiments, because cellular toxicity of cucurbitacin-I can affect in protein and mRNA expression.

Surface CD44 levels in SKOV-3 cells were evaluated using flow cytometry. Cucurbitacin-I dramatically reduced the expression of surface CD44 molecule as shown in Fig. 2A and 2B. Consistent with surface CD44 levels, cellular protein lev-

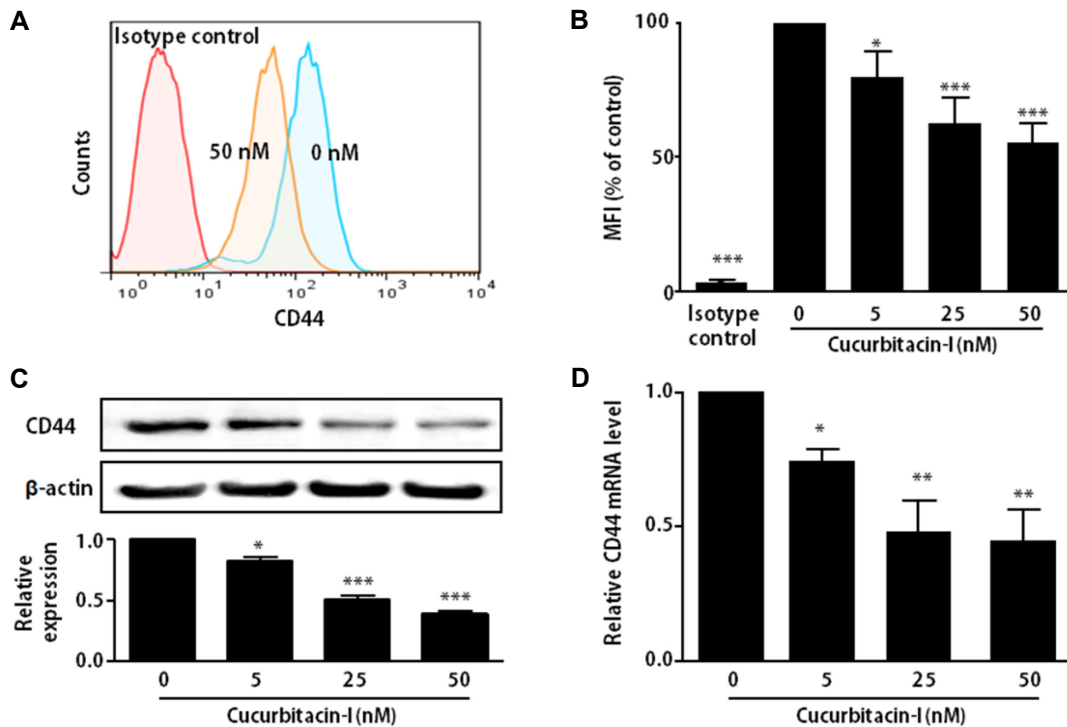


Fig. 2. Effect of cucurbitacin-I on the expression of CD44. SKOV-3 cells were treated with indicated concentration of cucurbitacin-I for 24 hr. (A) Flow cytometry analysis of cell surface CD44 expression by CD44 and isotype control antibodies. (B) Representative mean fluorescence intensity (MFI) for cell surface CD44 expression in SKOV-3 cells. (C) Western blot analysis of CD44 (upper) and the band intensities were quantified by densitometry (lower). The relative expression normalized with  $\beta$ -actin in whole cell lysates of SKOV-3 cells.  $\beta$ -actin was used as the loading control. (D) CD44 mRNA levels were quantified by qRT-PCR and normalized with the housekeeping gene, GAPDH. The results are means  $\pm$  SEM of four independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus 0 nM cucurbitacin-I-treated cells.

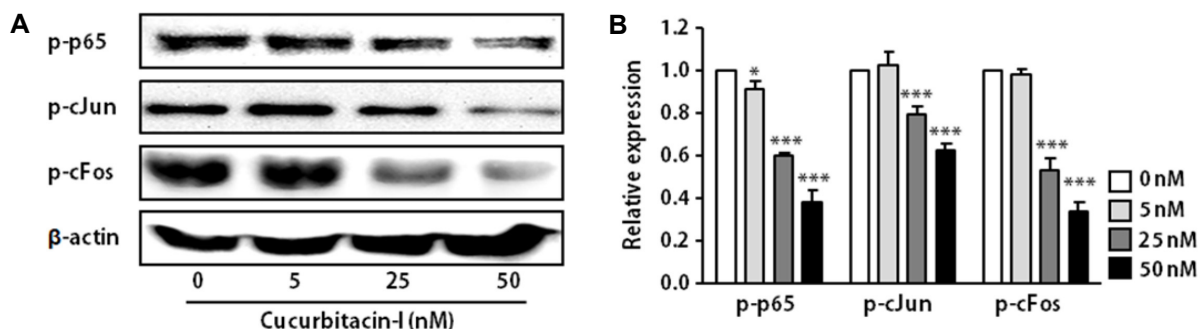


Fig. 3. Cucurbitacin-I inhibited the phosphorylation of AP-1 and NF- $\kappa$ B in SK-OV-3 cells. SKOV-3 cells were treated with indicated concentration of cucurbitacin-I for 24 hr. Western blot analysis of phospho-p65, -cFos and -cJun (A) and quantified results using densitometric analysis (B) in SKOV-3 cells.  $\beta$ -actin was used as the loading control. The results are means  $\pm$  SEM of four independent experiments. \* $p$ <0.05, \*\*\* $p$ <0.001 versus 0 nM cucurbitacin-I-treated cells.

els of CD44 by measured Western blot analysis also decreased in SKOV-3 cells treated with cucurbitacin-I (Fig. 2C). In addition, CD44 mRNA expression was also significantly lower in cucurbitacin-I-treated SKOV-3 cells compared to non-treated cells (Fig. 2D). These results suggest that cucurbitacin-I can suppress CD44 expression on mRNA and protein levels.

#### Cucurbitacin-I inhibits the activation of NF- $\kappa$ B and AP-1 in ovarian cancer cell

Previous study demonstrated that activator protein-1 (AP-1) and nuclear factor kappa-B (NF- $\kappa$ B) are key trans-acting factors that interact with cis-element, CR1, located upstream of the CD44 promoter [15]. In addition, inhibition of NF- $\kappa$ B resulted in a reduction in CD44 expression. CD44 repression via NF- $\kappa$ B inhibition consequently decreased proliferation and invasiveness of breast cancer cells [15].

The activation of NF- $\kappa$ B and AP-1 occurs via phosphorylation, resulting in the translocation of these molecules into the nucleus. Nuclear translocated NF- $\kappa$ B and AP-1 can induce the expression of their target genes, such as CD44. Therefore, we determined the effect of cucurbitacin-I on NF- $\kappa$ B and AP-1 activation by measuring phosphorylated NF- $\kappa$ B (p65) and AP-1 (cFos and cJun). As shown in Fig. 3, cucurbitacin-I significantly reduced the levels of phosphorylated-p65, -cFos and -cJun. These findings provide new insight into the molecular mechanism of cucurbitacin-I underlying the regulation of CD44 expression.

CD44 was reported to be involved in proliferation, adhesion and invasion of ovarian cancer cells [11]. In addition, an ovarian cancer tissue microarray analysis of paired primary, metastatic and recurrent tumor tissues from 26 patients, demonstrated that the metastatic as well as recurrent

ovarian cancer tissues expressed higher levels of CD44 than the primary tumor [7]. A meta-analysis unveiled that patients with CD44 was associated with poor prognosis in ovarian cancer patients, and that a CD44 status was associated with common clinicopathological features and poor prognostic factors [14]. These findings suggest that the regulation of CD44 expression may a possible therapy of ovarian cancer. The present study highlighted the crucial role of cucurbitacin-I on reducing the expression of CD44 in ovarian cancer cells. Although further studies on the underlying detailed molecular mechanisms are required, the present results may provide a possibility of cucurbitacin-I as a pharmaceutical agent for ovarian cancer treatment.

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## 초록 : 난소암 세포주의 CD44 발현에 미치는 Cucurbitacin-I의 효과

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박과 작물에 함유되어 있는 tetracyclic triterpene 성분 중 하나인 쿠쿠르비타신(cucurbitacin)-I는 대장암, 유방암, 간암세포에서의 항종양 활성이 밝혀져 있으나 난소암에서의 쿠쿠르비타신-I의 역할은 보고된 바 없다. CD44는 세포막에 존재하는 당단백질로 생체 내 리간드인 glycosaminoglycan hyaluronic acid를 통해 세포 외부 매트릭스와 다른 세포와의 접촉을 매개한다. 최근 연구에 의해 CD44의 발현이 난소암세포의 증식 및 세포 부착과 침윤을 증가시키는 주요 원인이라는 것이 보고되었다. 이러한 결과는 CD44의 발현을 억제함으로써 난소암의 진행을 조절할 수 있음을 시사하고 있다. 본 연구에서는 쿠쿠르비타신-I가 난소암세포의 CD44의 발현을 억제할 수 있는지의 여부를 조사하였다. 인간의 난소암 세포인 SKOV-3를 이용한 MTS assay를 수행한 결과, 쿠쿠르비타신-I는 100 nM이상의 농도에서 세포독성을 나타내었다. 세포독성을 나타내지 않는 농도의 쿠쿠르비타신-I를 SKOV-3 세포에 처리하여 Western blot 분석과 qRT-PCR을 수행한 결과, 쿠쿠르비타신-I에 의해 CD44의 단백질과 mRNA의 발현이 유의적으로 감소되는 것을 확인하였다. 또한 쿠쿠르비타신-I에 의한 CD44의 발현 억제가 NF- $\kappa$ B와 AP-1의 인산화 감소에 기인하고 있음을 밝혔다. 이러한 결과는 쿠쿠르비타신-I가 CD44 발현을 억제하는 기능을 가지며, 이는 난소암 치료에 도움을 줄 수 있는 제제로서 쿠쿠르비타신-I의 가능성을 제시하는 것이다.