이학석사 학위논문

# The forkhead transcription factor FOXO1 mediates cisplatin resistance in gastric cancer cells by activating phosphoinositide-3 kinase/Akt pathway

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2013년 7 월

서울대학교 대학원

의학과 (협) 종양생물학 전공

박 진 주

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지도교수 이 병 란

이 논문을 이학석사 학위논문으로 제출함.

2013년 8월

서울대학교 대학원

의학과 종양생물학 전공

박 진 주

박 진 주의 이학석사 학위논문을 인준함.

2013년 월

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# Abstract

**Background:** Cisplatin (CDDP) is the most important chemotherapeutic agent in the treatment of advanced gastric cancer. However, its efficacy is limited due to CDDP resistance. Since the transcription factor FOXO1 is related to chemoresistance in various cancer cells, the present study investigated the function of FOXO1 on chemoresistance to CDDP in human gastric cancer cells.

**Methods:** Human gastric cancer cell lines MKN45 and SNU-601 were used. FOXO1 activation was modulated by expression of FOXO1 AAA mutant gene or FOXO1 shRNA. The effects of FOXO1 on cell growth and CDDP cytotoxicity were assessed by crystal violet assay. Protein expressions of FOXO1, p110α, pAkt and Akt were analyzed by Western blotting, and FOXO1 activity was determined by luciferase reporter assay. Cell apoptosis was assessed by Western blotting for PARP cleavage and DAPI staining.

**Results:** CDDP induced FOXO1 expression and activation in gastric cancer cells.

FOXO1 activation by FOXO1 AAA mutant gene increased the CDDP resistance in gastric cancer cells without changes in cell growth, whereas FOXO1 silencing by FOXO1shRNA enhanced CDDP cytotoxicity along with apoptotic characteristics.

Constitutive activation of FOXO1in MKN45 cells were accompanied by an increase in the expressions of p110α and pAkt, respectively. Furthermore, Akt inhibition by LY294002 treatment resumed the CDDP cytotoxicity suppressed by FOXO1 AAA mutant gene transfection.

**Conclusions:** FOXO1 inhibits CDDP-induced apoptosis via activating PI3K/Akt pathway . Thus, FOXO1 may be an useful pharmacologic indicator to predict CDDP efficacy in gastric cancer treatment.

**Key words:** FOXO1, gastric cancer, cisplatin resistance, PI3K/Akt

**Student number:** 2011-23787

# List of figures

# List of abbreviations and symbols

FOXO: Forkhead box, class O

CDDP: cis-diamminedichloroplatinum(II)

FOXO1 AAA: FOXO1 containing a threonine-to-alanine and serine-to-alanine substitution at residue 24, 256 and 319

PI3K: phosphoinositide 3-kinase

pAkt: phosphorylated Akt

SNU: Seoul national university

# Contents

# Introduction

Gastric cancer is one of the most common cancers and a major cause of cancer-related death worldwide [1]. However, the cure rate of this disease is limited because of the ineffectiveness of chemotherapy and radiotherapy. Thus, evaluation of the chemosensitivity of gastric cancer cells to anticancer agents based on phenotype difference of individual cell lines will provide more information for choosing correct drugs for gastric cancer patients.

Cisplatin (cis-diamminedichloroplatinium (II): CDDP is a frontline chemotherapeutic agent in the treatment of advanced gastric cancer and is used in combination therapy regimens [2]. However, almost all of cancer cells acquire resistance to CDDP, which reduces its efficacy [3]. Thus, molecules and factors that are capable of predicting responses of gastric cancer patients to CDDP treatment are needed to be investigated.

The FOXO (Forkhead box, class O) is a subfamily of forkhead

transcription factor and consists of FOXO1, FOXO3A, FOXO4 and FOXO6 [4]. FOXO transcription factors are involved in diverse intracellular signaling pathways and regulate cell cycle arrest, apoptosis, DNA damage repair and detoxification of reactive oxygen species by regulating specific gene setting [5, 6]. The FOXO signaling is regulated by their interactions with other intracellular proteins as well as their post-translational modifications such as phosphorylation [7]

There have been studies on the relation between FOXO proteins and cancer cell chemoresistance in vitro [8-13]. FOXO1 increased doxorubicin resistance in breast cancer cells [8] and paclitaxel resistance in ovarian cancer cells [9], whereas it decreased CDDP resistance in ovarian cancer cells [10]. FOXO3 increased doxorubicin resistance in chronic myelogenous leukemia cells [11], whereas it decreased paclitaxel resistance in breast cancer cells [12] as well as CDDP resistance in ovarian cancer cells [10] and breast cancer cells [13]. Thus, the effect of FOXO1 on the chemoresistance of cancer cells may differ according to the drug and cell type investigated.

The serine-threonine kinase Akt/protein kinase B is a well-known cell survival signal that contributes chemoresistance in a variety of cancer cells [14]. A recent study has demonstrated that the overexpression of Akt decreases the chemosensitivity of gastric cancer cells to CDDP in vitro and in vivo [15]. Although FOXO1 is a downstream target of Akt in human cancers [16], the association between FOXO1activation and chemoresistance in gastric cancer cells has not been reported.

In present study, we investigated whether FOXO1 activation is related to CDDP resistance in gastric cancer cell lines. For this, we modulated FOXO1 activation by expression of FOXO1 AAA mutant gene or FOXO1 shRNA. In addition, we examined the association between FOXO1and phosphoinositide 3-kinase (PI3K)/Akt pathway in relation to CDDP resistance in gastric cancer cells.

# Material and Methods

### Chemicals

CDDP was purchased from Sigma (St Louis, MO, USA), and LY294002 was purchased from Cell Signaling Technology (Beverly, MA, USA).

### Cell lines and cultures

Human gastric cancer cell lines MKN45 and SNU-601 were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mg/mL sodium bicarbonate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) at 37°C in a humidified 95% air and 5% CO2 atmosphere.

### Stable transfection with a plasmid expressing FOXO1 AAA mutant gene

An expression vector, pcDNA3 with a human FOXO1 AAA mutant gene (Addgene plasmid 13508) was purchased from Addgene Inco.

(Cambridge, MA, USA). This vector encodes a constitutively active FOXO1 containing a threonine-to-alanine substitution at residue 24 and serine-to-alanine substitution at 256 and 319. This construct (1 μg) or empty pcDNA3 vector (1 μg) was transfected into $3 \times 10^5$ cells/well in 6-well plates using LipofectAMINE Plus (Life Technologies) according to the manufacturer's instructions. Pooled G418 (3 μg/ml)-resistant cells were used for further analysis.

**Lentivirus-mediated shRNA silencing of FOXO1**

FOXO1 shRNA lentiviral particles and non-targeting shRNA control particles were purchased from Sigma. The sequence of the shRNA targeting FOXO1 used in the present study is the following: CCGGGCCTGTTATCAATCTGCTAAACTCGAGTTTAGCAGATTGATAA CAGGCTTTTTG. The non-targeting shRNA control particles contain 4 basepair mismatches within the short hairpin sequence to any known human or mouse gene. The viral infection was performed by incubating gastric cancer cells in the culture medium containing lentiviral particles for 12 h in the presence of 5 μg/ml Polybrene (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Pooled puromycin (2 μg/ml)-resistant cells were harvested and stored for further

analysis.

**Western blotting**

Cell lysates were prepared in 100-200 μL of 1× sodium dodecyl sulfate (SDS) lysis buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 0.004% bromophenol blue, and 20% glycerol]. Protein contents were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equal amounts of proteins were loaded onto a 10% discontinuous SDS/polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA) blocked with 5% nonfat dry milk in phosphate-buffered saline-Tween-20 (0.1%, v/v) for 1 h. The membranes were then incubated at 4°C overnight with or without 2 h incubation at room temperature with one of the following primary antibodies: rabbit anti-FOXO1 (1:1000, Cell Signaling Technology), rabbit anti-PARP (1:1000, Cell Signaling Technology), rabbit anti-p110α (1:1000, Cell Signaling Technology), rabbit anti-phospho-AktSer473 (1:1000, Cell signaling Technology), rabbit anti-Akt (1:1000, Cell Signaling Technology), and mouse anti-β-actin (1:1000, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Zymed, San Francisco, CA, USA)

or anti-mouse IgG (1:2500, Santa Cruz Biotechnology) was used as a secondary antibody. Enhanced chemiluminescence was used to detect the immunoreactive proteins. Equal protein loading was confirmed by β-actin.

**Luciferase reporter assay**

Gastric cancer cells were seeded in 24-well plates at a density of 3 × $10^4$ cells/well and were transiently co-transfected with 0.4 μg of FHRE-luciferase reporter plasmid (reporter construct in which a small region of the Fas ligand promoter containing the three FHREs, Addgene plasmid 1789) and 0.4 μg of β-galactosidase vector, an internal control, using Lipofectamine Plus (Life Technologies). Twenty-four hours after transfection, assays for luciferase and β-galactosidase were carried out using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luciferase activity was measured on an AutoLumat LB 9505c luminometer (Berthold Analytical Instruments, Nashua, Germany) and was normalized by β-galactosidase activity. Luciferase activity in control cells was arbitrarily set to 1.

**Cell proliferation and cytotoxicity assay**

Cells were seeded onto 24-well plates, at a density of $1 \times 10^4$ cells/well for cell growth and cultured for 4 days. To study the effects of CDDP on cell viability, $3 \times 10^4$ cells/well were seeded onto 24-well plate. After 24 h, cells were treated with various concentrations of CDDP dissolved in 0.02% dimethyl sulfoxide (DMSO) (range, 0.1 μg/ml to 3 μg/ml) for 24 h. For PI3K/Akt inhibition, cells were treated with 20 μM LY294002. Control columns contained cells without drug and blank columns contained medium alone. Cell numbers were measured indirectly using the method reported by Kim et al. [17]. Briefly, attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min, dissolved in 10% SDS, transferred into 96-well plates, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader (Bio-Rad, Hercules, CA, USA). Absorbance values were normalized to the values obtained for the medium control group cells to determine the survival percentage.

**4'-6-Diamidino-2-phenylindole (DAPI) staining**

Apoptosis was evaluated by DAPI staining as described previously

[18]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min washed three times with PBS, and stained with DAPI (1 μg/ml) in dark for 30 min and then examined under a fluorescence microscope. Cells were considered apoptotic if their nuclei were condensed or fragmented.

**Statistical analysis**

Data were analyzed by one-way ANOVA, and differences were considered significant at P values of $< 0.05$ in Newman-Keuls multiple-comparisons test. GraphPad Prism 4.00 for Windows XP (GraphPad Software Inc., San Diago, CA) was used to conduct the analysis.

# Results

**CDDP induces FOXO1 expression and activation in gastric cancer cells**

We investigated the correlation between FOXO1 and chemoresistance to CDDP in gastric cancer cells. First, we treated parent gastric cancer cell lines MKN45 and SNU-601 with CDDP (3 μg/ml) for 24 h and found that FOXO1 protein expression was increased (Fig. 1a). The induction of FOXO1 activation was confirmed by a luciferase reporter assay with a forkhead responsive element luciferase plasmid (Fig. 1b).These results demonstrate that CDDP activates FOXO1 in gastric cancer cells.

**FOXO1activation in gastric cancer cell lines confers resistance to CDDP-induced cytotoxicity**

To clarify the role of FOXO1 in CDDP resistance in gastric cancer cells, we modulated the FOXO1 activation. Since Gao et al. [14] reported that FOXO1 increased CDDP cytotoxicity of ovarian cancer cells, we transfected the FOXO1 AAA mutant gene, a constitutively active FOXO1 mutant, into gastric cancer cell lines MKN45 and SNU-601 [19]. Western blotting (Fig. 2a) and luciferase reporter assay (Fig. 2b) showed that

FOXO1 expression and transcriptional activity was increased, respectively, in stably FOXO1 AAA expressing cells compared with vector control. Under normal culture conditions, FOXO1 overexpression had no effect on the cell growth of the gastric cancer cells (Fig. 2c). By contrast, treatment with CDDP (0-3 μg/ml) for 24 h showed that FOXO1 overexpression increased the resistance to CDDP (Fig. 2d).

**Silencing of the FOXO1 enhances CDDP-induced cytotoxicity in gastric cancer cells**

To further confirm our results mentioned above, gene silencing by RNA interference was used. FOXO1 expression (Fig. 3a) and activity (Fig. 3b) were suppressed by FOXO1 shRNA expression in MKN45 cells. There was no difference in growth rates between gastric cancer cells expressing non-targeting shRNA or FOXO1 shRNA (Fig. 3c). In order to confirm the effect of FOXO1 on the resistance to CDDP, cells were treated with CDDP (0-1 μg/ml) for 24 h. Figure 3d shows that FOXO1 silencing increased CDDP sensitivity in MKN45 cells. To examine whether CDDP-induced cell death occurs via apoptosis, we performed Western blotting for PARP (a caspase substrate ) cleavage. After CDDP (1 μg/ml) treatment for 24 h, cleaved PARP was increased in FOXO1 shRNA expressing cells compared to

control shRNA expressing cells (Fig. 3e). The presence of apoptosis was confirmed by DAPI staining, which showed more frequent peripheral chromatin condensation and nuclear fragmentation in FOXO1 shRNA cells than in control shRNA cells (Fig. 3f). In addition, similar results were observed in SNU-601 cells (Fig. 3g, h). Taken together, these results indicate that FOXO1 protects gastric cancer cells to CDDP-induced cell death.

**FOXO1 activation enhances PI3K/Akt activity**

Recently, it has been reported that Akt overexpression decreases the chemosensitivity of gastric cancer cells to CDDP in vitro and in vivo [15]. Although FOXO1 is a well-known downstream substrate of Akt, FOXO1 was shown to enhance Akt phosphorylation in hepatocytes by repressing the expression of tribble 3, a pseudokinase capable of binding Akt and inhibiting its phosphorylation [20].

Thus, we investigated whether FOXO1-induced CDDP resistance attributes to Akt activation in MKN45 cells. First, we hypothesized that FOXO1 may regulate the expression of PI3K, an upstream molecule of Akt. Western blotting showed that FOXO1 activaiton and silencing directly increased and decreased the expressions of p110α, the class I PI3K catalytic subunit, and

pAkt, the active form of Akt, respectively (Fig. 4a, b). In addition, CDDP-induced FOXO1 expression was accompanied by the expressions of p110α and pAkt (Fig. 4c).

**FOXO1 enhances CDDP-induced cytotoxicity through PI3K/Akt inhibition**

In order to confirm whether the positive correlation between FOXO1 and CDDP resistance is mediated by Akt, we increased FOXO1 activation by transfection of FOXO1 AAA mutant gene into MKN45 cells and then assessed the effects of LY294002 treatment on CDDP-induced cytotoxicity. Western blotting showed that MKN45 cells expressing FOXO1 AAA mutant gene showed higher pAkt expression than vector control cells, but LY294002 treatment effectively blocked pAkt expression (Fig. 5a). Cytotoxicity assay showed that cells treated with LY294002 resumed the CDDP sensitivity suppressed by FOXO1 overexpression (Fig. 5b).

**Fig. 1** Effects of cisplatin (CDDP) on FOXO1 expression and activity in gastric cancer cell lines. MKN45 and SNU-601 cells grown in 10-cm plates were treated with either DMSO (0.02%) or CDDP (3 μg/ml) for 24 h. **a** FOXO1 protein expression was measured by Western blotting and β-actin was used as an internal control. Ctrl, untreated control **b** The FOXO1transcriptional activity was determined by luciferase reporter assay and normalized by β-galatosidase activity. Each bar represents the mean ± standard deviation. * $P < 0.05$ versus untreated cells.

**Fig. 1**

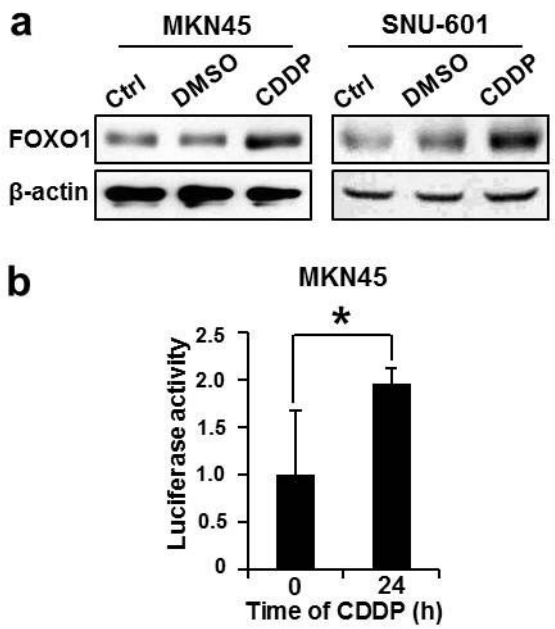

a
MKN45
SNU-601
Ctrl
DMSO
CDDP
Ctrl
DMSO
CDDP
FOXO1
β-actin
b
MKN45
*
Luciferase activity
2.5
2.0
1.5
1.0
0.5
0
0
24
Time of CDDP (h)

**Fig. 2** Effects of FOXO1 AAA mutant expression on cisplatin (CDDP)-induced cytotoxicity in gastric cancer cell lines MKN45 and SNU-601. Cells were transfected with either an empty pcDNA3 vector (pcDNA3) or FOXO1 AAA mutant vector (AAA). **a** Expression levels of FOXO1 and β-actin proteins were confirmed by Western blotting. **b** The FOXO1 transcriptional activity was determined by luciferase reporter assay and was normalized by β-galatosidase activity. Each bar represents the mean ± standard deviation. **c** Cell growths were analyzed using crystal violet assay on indicated times and absorbance was measured. Values represent the means ± standard deviations. **d** Cells were treated with CDDP (0-3 μg/ml) for 24 h. Cell survivals represent the mean percentage survivals compared to untreated cells and values represent the means ± standard deviations. * $P < 0.05$ versus control cells transfected with pcDNA3 vector.

**Fig. 2**

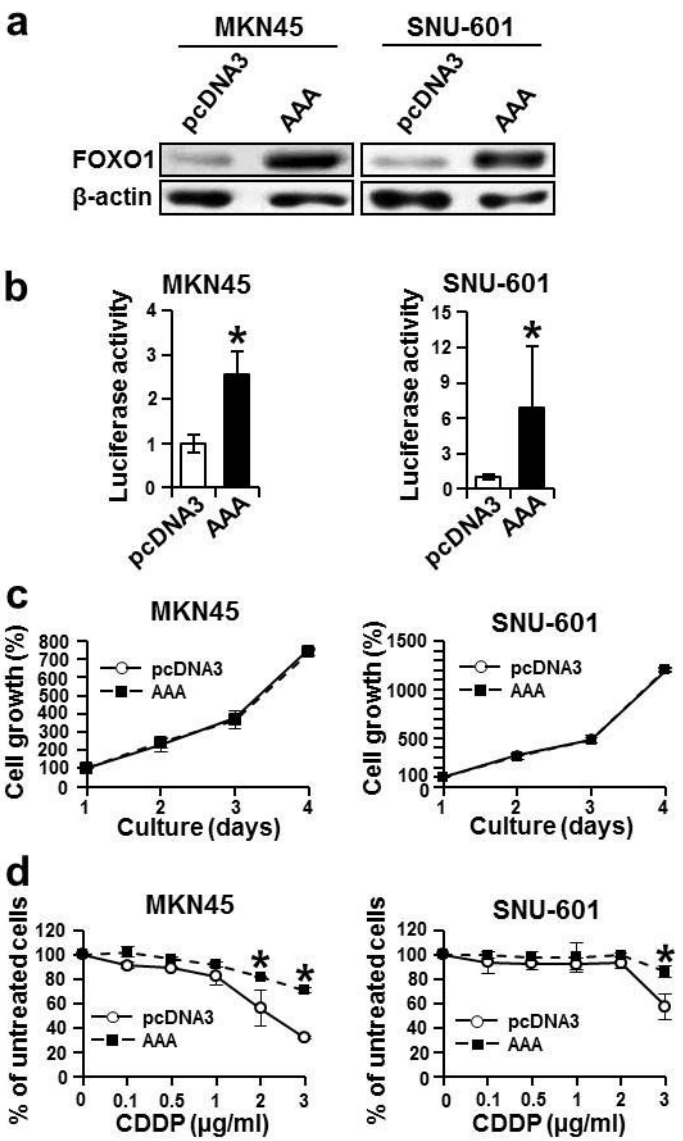

a
MKN45
SNU-601
pcDNA3
AAA
pcDNA3
AAA
FOXO1
β-actin
b
MKN45
Luciferase activity
0
1
2
3
4
*
pcDNA3
AAA
SNU-601
Luciferase activity
0
3
6
9
12
15
*
pcDNA3
AAA
c
MKN45
Cell growth (%)
0
100
200
300
400
500
600
700
800
pcDNA3
AAA
1
2
3
4
Culture (days)
SNU-601
Cell growth (%)
0
100
500
1000
1500
pcDNA3
AAA
1
2
3
4
Culture (days)
d
MKN45
% of untreated cells
0
20
40
60
80
100
120
*
*
pcDNA3
AAA
0
0.1
0.5
1
2
3
CDDP (μg/ml)
SNU-601
% of untreated cells
0
20
40
60
80
100
120
*
pcDNA3
AAA
0
0.1
0.5
1
2
3
CDDP (μg/ml)

**Fig. 3** Effects of FOXO1 silencing on cisplatin (CDDP)-induced cytotoxicity in gastric cancer cell lines MKN45 and SNU-601. Cells were infected with a lentivirus containing a construct, encoding either FOXO1 shRNA (shFOXO1) or non-targeting shRNA (shCtrl). **a, b, g** FOXO1 silencing was confirmed by Western blotting and luciferase reporter assay. The effects of FOXO1 on the cell growth (**c**) and CDDP cytotoxicity (**d, h**) were analyzed as described in Fig. 2. **e** Cells were treated with CDDP (1 μg/ml) for 24 h. Poly (ADP ribose) polymerase (PARP) cleavage was determined by Western blotting. **f** 4',6'-Diamidino-2-phenylindole staining and fluorescence microscopy showing morphological change in MKN45 cells (x 400). * $P < 0.05$ versus control cells expressing a non-targeting shRNA .

Fig. 3

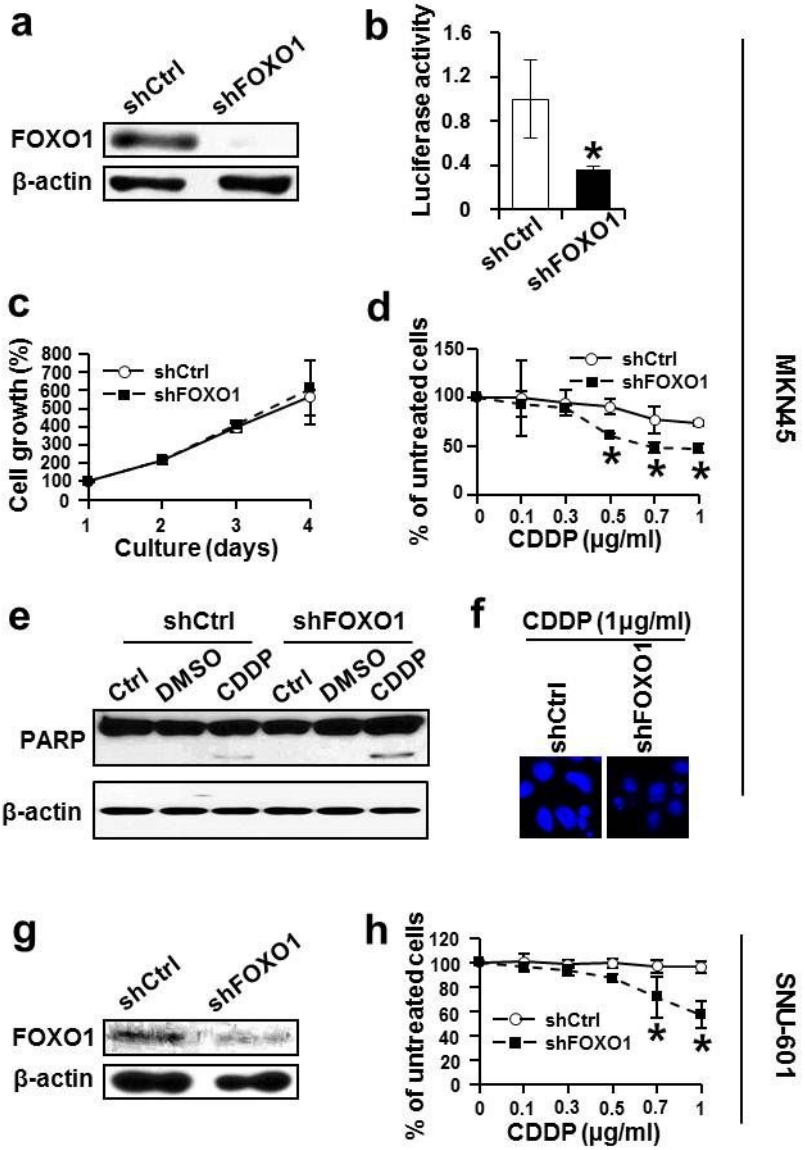
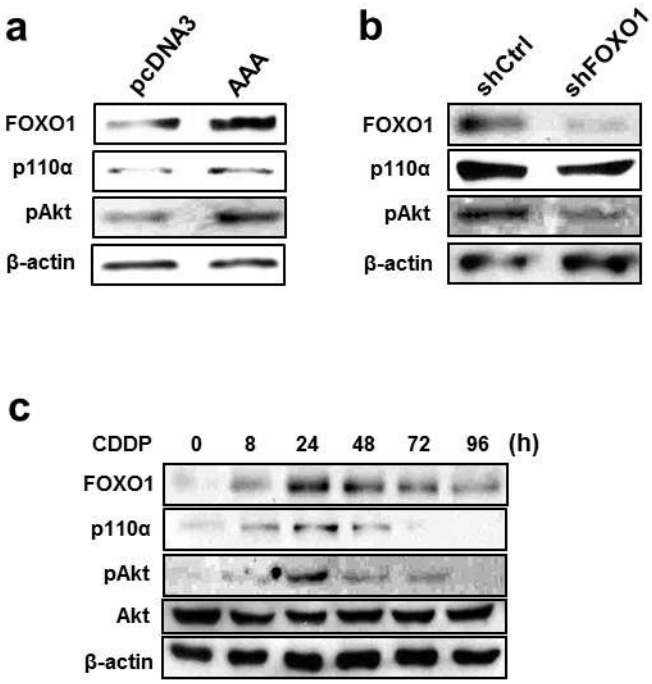

a
shCtrl
shFOXO1
FOXO1
β-actin
b
Luciferase activity
1.6
1.2
0.8
0.4
0
*
shCtrl
shFOXO1
c
Cell growth (%)
800
700
600
500
400
300
200
100
0
shCtrl
shFOXO1
1
2
3
4
Culture (days)
d
% of untreated cells
150
100
50
0
shCtrl
shFOXO1
*
*
*
0
0.1
0.3
0.5
0.7
1
CDDP (μg/ml)
MKN45
e
shCtrl
shFOXO1
Ctrl
DMSO
CDDP
Ctrl
DMSO
CDDP
PARP
β-actin
f
CDDP (1μg/ml)
shCtrl
shFOXO1
g
shCtrl
shFOXO1
FOXO1
β-actin
h
% of untreated cells
120
100
80
60
40
20
0
shCtrl
shFOXO1
*
*
0
0.1
0.3
0.5
0.7
1
CDDP (μg/ml)
SNU-601

**Fig. 4** Effect of FOXO1 expression on the p110α and pAkt. Protein expressions were analyzed by Western blotting. (a) Effects of constitutive expression of FOXO1 on p110α and pAkt expressions were determined after cells were transfected with either empty pcDNA3 vector or FOXO1 AAA mutant vector. (b) Effects of constitutive expression of FOXO1 on p110α and pAkt expressions were determined after FOXO1 silencing induced by FOXO1 shRNA (shFOXO1) expression. (c) Effects of FOXO1 expression induced by CDDP (1 μg/ml) treatment for 0-96 h on p110α and pAkt expressions. Protein lysates were prepared at the times indicated.

**Fig. 4**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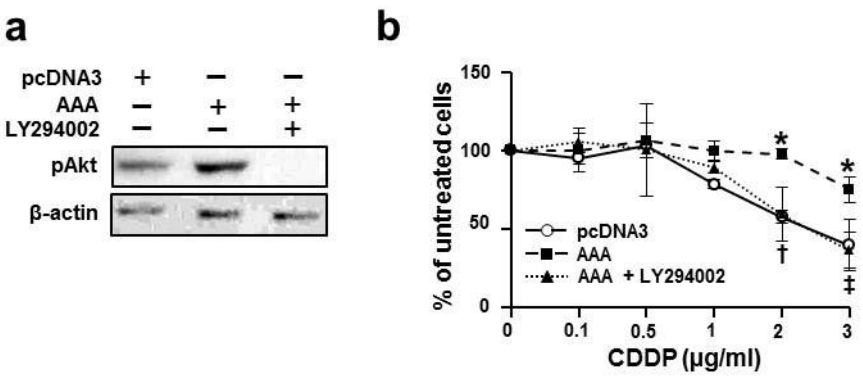
pAkt
β-actin
b
shCtrl
shFOXO1
FOXO1
p110α
pAkt
β-actin
c
CDDP 0 8 24 48 72 96 (h)
FOXO1
p110α
pAkt
Akt
β-actin

**Fig. 5** Effect of PI3K inhibitor LY294002 on pAkt expression and CDDP resistance induced by FOXO1 overexpression. MKN45 cells were transfected with an empty pcDNA3 vector or a vector containing FOXO1 AAA mutant gene. (a) Cells were treated with 20 μM LY294002 for 24 h and Western blotting was performed with an anti-pAkt antibody . (b) Cells were treated with CDDP (0-3 μg/ml) in the presence or absence of LY294002 (20 μM) for 24h. Cell survivals represent the mean percentage survivals compared to untreated cells, and values represent the means ± standard deviations.

**Fig. 5**


a
pcDNA3 + − −
AAA − + +
LY294002 − − +
pAkt
β-actin
b
% of untreated cells
150
100
50
0
0
0.1
0.5
1
2
3
CDDP (μg/ml)
pcDNA3
AAA
AAA + LY294002
*
*
†
‡

## Discussion

Since FOXO transcription factors are critical mediators of apoptosis in cytotoxicity inducing drugs, its involvement in the development of drug resistance is an important issue in cancer therapy [6]. However, FOXO proteins possess diverse functions, which partly seemed opposing, according to drug and cell type. [21]. To the best our knowledge, this is the first to demonstrate the correlation between FOXO1 and chemoresistance in gastric cancer cells.

We have previously shown that FOXO1 is overexpressed in gastric cancer specimens [22]. Although FOXO proteins, especially FOXO1 and FOXO3, have been reported to be related to chemoresistance in various cancer cells [8-13], their involvement in chemoresistance of gastric cancer cells has not been reported. In the present study, we used two gastric cancer cell lines MKN45 and SNU-601 with constitutive FOXO1 expression and found that CDDP treatment increased the level of FOXO1 expression and activation. These results suggest that FOXO1 might impart the phenotype of CDDP resistance in gastric cancer cells.

Gene modulation is a powerful method for analyzing gene function [23]. Here, we modulated FOXO1 activation in 2 gastric cancer cell lines using different approaches (overexpression of FOXO1AAA mutant gene and FOXO1shRNA). Using these cell lines, we found that constitutive activation of FOXO1 increased the CDDP resistance in gastric cancer cells, whereas cell growth was not affected. In contrast, FOXO1 silencing with shRNA enhanced CDDP-induced cytotoxicity, was accompanied by apoptotic characteristics including cleaved PARP expression and fragmented nuclei . Taken together, these observations clearly demonstrated that FOXO1 is protective t0 CDDP-induced cytotoxicity in gastric cancer cells. Thus, FOXO1 modulation is an effective strategy for improving the anticancer efficacy of CDDP for gastric cancer patients.

It is generally accepted that Akt is a critical survival signal, which is involved in cancer development and progression, and chemoresistance [14]. Although cancer cells acquire resistance to anticancer agents through Akt, either constitutive or induced by anti-cancer drugs, the molecular mechanisms underlying anti-cancer drug-

induced Akt activation are not well elucidated. In gastric cancer, we have observed that pAKT was expressed in 78% of gastric cancer specimens [24]. Since it has been shown that
Akt overexpression decreases the chemosensitivity of gastric cancer cells to CDDP in vitro and in vivo [15], we investigated whether Akt is related to the FOXO1 regulation of CDDP cytotoxicity in gastric cancer cells. In the present study, constitutive activation and silencing of FOXO1 increased and decreased the p110α and pAkt expressions in MKN45 cells, respectively, suggesting that FOXO1 activates PI3K/Akt signaling in gastric cancer cells. Time course analysis demonstrated that CDDP-induced FOXO1 expression was accompanied by increases in p110α and pAkt expressions. In addition, we found that treatment of MKN45 cells with PI3K/Akt inhibitor LY 294002 resumed the CDDP cytotoxicity suppressed by FOXO1 overexpression. Thus, FOXO1 and Akt seem to have similar effects on CDDP resistance in gastric cancer cells. Our results agree with those of Hui et al. [11], which showed FOXO3-induced PI3K/Akt activation in response to doxorubicin treatment of chronic myelogenous leukemia cells. However, our observations contrast with those of Gao et al. [10], which demonstrated that FOXO1 enhanced

CDDP cytotoxicity in ovarian cancer cells. These discrepancies may, at least in part, be explained by a previous suggestion by Paik et al. [25] that FOXO-regulated genes are different significantly between cell types.

Taken together, our results provide clear evidence supporting the idea that blockage of FOXO1 could be an effective approach to improve the anti-cancer efficacy of CDDP for gastric cancer patients. Studies in animal models are warranted to verify the usefulness of this strategy in vivo.

# References


1. Crew KD, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol. 2006;12:354–62.
2. Koizumi W, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. Lancet Oncol. 2008;9(3):215-21.
3. Tanaka M, Kataoka H, Yano S, Ohi H, Kawamoto K, Shibahara T, et al. Anti-cancer effects of newly developed chemotherapeutic agent, glycoconjugated palladium (II) complex, against cisplatin-resistant gastric cancer cells. BMC Cancer. 2013;13:237-45.
4. Maiese K, Chong ZZ, Shang YC, Hou J. Clever cancer strategies with FoxO transcription factors. Cell Cycle. 2008;7:3829-39.
5. Lei H, Quelle FW. FOXO transcription factors enforce cell cycle checkpoints and promote survival of hematopoietic cells after DNA damage. Mol Cancer Res. 2009;7:1294-303.
6. Goto T, Takano M. Transcriptional role of FOXO1 in drug resistance through antioxidant defense systems. Adv Exp Med Biol. 2009;665:171-9.
7. Zhang X, Tang N, Hadden TJ, Rishi AKAkt, FoxO and regulation of

apoptosis. Biochim Biophys Acta2011 ;1813:1978-86

8. Oh WK, Cho KB, Hien TT, Kim TH, Kim HS, Dao TT, et al. Amurensin G, a potent natural SIRT1 inhibitor, rescues doxorubicin responsiveness via down-regulation of multidrug resistance 1. Mol Pharmacol. 2010;78:855-64.
9. Goto T, Takano M, Hirata J, Tsuda H. The involvement of FOXO1 in cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers. Br J Cancer. 2008;98:1068-75.
10. Gao J, Yang X, Yin P, Hu W, Liao H, Miao Z, et al. The involvement of FoxO in cell survival and chemosensitivity mediated by Mirk/Dyrk1B in ovarian cancer. Int J Oncol. 2012;40:1203-9.
11. Hui RC, Gomes AR, Constantinidou D, Costa JR, Karadedou CT, Fernandez de Mattos S, et al. The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression. Mol Cell Biol. 2008;28:5886-98.
12. Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D, et al. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem. 2010;285:17869-79.

13. Tezil T, Bodur C, Kutuk O, Basaga H. IKK-β mediates chemoresistance by sequestering FOXO3; a critical factor for cell survival and death. Cell Signal. 2012;24:1361-8.
14. Shimamura H, Terada Y, Okado T, Tanaka H, Inoshita S, Sasaki S. The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vitro via NF-kappa B and Bad. J Am Soc Nephrol. 2003;14:1427–34.
15. Zhang LL, Zhang J, Shen L, Xu XM, Yu HG. Overexpression of AKT decreases the chemosensitivity of gastric cancer cells to cisplatin in vitro and in vivo. Mol Med Rep. 2013;7:1387-90.
16. Fukunaga K, Ishigami T, Kawano T. Transcriptional regulation of neuronal genes and its effect on neural functions: expression and function of forkhead transcription factors in neurons. J Pharmacol Sci. 2005;98:205-11.
17. Kim WH, Schnaper HW, Nomizu M, Yamada Y, Kleinman HK. Apoptosis in human fibrosarcoma cells is induced by a multimeric synthetic Tyr-Ile-Gly-Ser-Arg (YIGSR)-containing polypeptide from laminin. Cancer Res. 1994;54:5005-10.
18. Wang X, Gorospe M, Huang Y, Holbrook NJ. p27Kip1 overexpression causes apoptotic death of mammalian cells.

Oncogene. 1997;15:2991-7.

19. Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M, Sellers WR. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol Cell Biol. 2000;20:8969-82.
20. Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest. 2006;116:2464-72.
21. Lei H, Quelle FW. FOXO transcription factors enforce cell cycle checkpoints and promote survival of hematopoietic cells after DNA damage. Mol Cancer Res. 2009;7:1294-303.
22. Kim JH, Kim MK, Lee HE, Cho SJ, Cho YJ, Lee BL, et al. Constitutive phosphorylation of the FOXO1A transcription factor as a prognostic variable in gastric cancer. Mod Pathol. 2007;20:835-42.
23. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell. 2002;2:243-7.
24. Nam SY, Lee HS, Jung GA, Choi J, Cho SJ, Kim MK, et al. Akt/PKB activation in gastric carcinomas correlates with clinicopathologic variables and prognosis. APMIS. 2003;111:1105-13.

25. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell. 2007;128:309-23.

# Abstract

**목적:** Cisplatin (CDDP)은 위암의 치료에 가장 중요한 항암제 중 하나이지만 내성으로 인한 치료 효율저하가 문제점으로 남아있다. 전사인자인 FOXO1이 여러 암에서 항암제 내성과 관련이 있음이 관찰되어 있으므로 본 연구는 위암세포에서 FOXO1이 CDDP에 대한 내성에 미치는 영향을 관찰하기 위하여 시행되었다.

**방법:** 위암세포주들인 MKN45와 SNU-601 세포에 유전자이입 (transfection)을 사용하여 FOXO1 AAA mutant gene 또는 FOXO1 shRNA를 과발현시킴으로써 FOXO1의 발현 및 활성도를 변화시켰다. 위 세포주에 CDDP를 처리 한 후 crystal violet assay를 실시하여 세포의 항암제 내성을 측정하였으며, 세포사멸 (apoptosis)에 미치는 영향을 알아보기 위하여 Western blotting으로 PARP cleavage를 관찰하였고, DAPI 염색을 진행하였다. FOXO1, p110α, Akt 및 pAkt의 발현을 관찰하기 위하여 Western blotting시행하였고, luciferase reporter assay를 이용하여 FOXO1의 활성도를 측정하였다.

**결과:** CDDP를 처리하였을 때, FOXO1의 발현과 활성이 증가하는 것을 확인하였다. 또한 FOXO1 AAA mutant gene을 과발현 시키면 CDDP에

대한 내성이 증가하였고, 반대로 FOXO1 shRNA를 통하여 FOXO1을 억제하면 CDDP에 대한 내성이 감소하였다. FOXO1 발현양을 조절한 세포주에서 FOXO1이 p110α 및 pAkt와 양의 상관관계가 있음을 관찰하였고, CDDP를 MKN45세포에 처리하였을 때, FOXO1과 함께 p110α와 pAkt가 증가하는 것을 관찰하였다. 그리고 FOXO1을 과발현 시킨 세포주에 PI3K inhibitor인 LY294002를 처리하였을 때, FOXO1 과발현에 의해 증가된 CDDP에 대한 내성이 다시 감소한 것을 확인하였다.

**결론:** 본 연구는 FOXO1이 PI3K/Akt를 통하여 위암세포의 CDDP에 대한 내성을 증가시키는 것을 확인하였다. 본 연구의 결과로부터 FOXO1이 위암의 CDDP 치료에 있어서 치료 효율 예측 인자로 유용하게 사용될 수 있을 것으로 추정된다.

**주요어:** FOXO1, gastric cancer, cisplatin resistance, PI3K/Akt

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