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Overcoming chemoresistance by inhibiting HOXB9 in an RMUG-S cell line of mucinous epithelial ovarian cancer

점액성 난소암 세포주 RMUG-S에서 HOXB9 유전자 억제를 이용한 항암제 저항성의 극복

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Overcoming chemoresistance by inhibiting *HOXB9* in an RMUG-S cell line of mucinous epithelial ovarian cancer

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이 논문을 의학박사 학위논문으로 제출함

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Abstract

Different expression patterns of HOX genes across distinct histologic subtypes of epithelial ovarian cancer (EOC) have already been reported although HOX genes drive normal organogenesis. We aimed to discover cell line-specific overexpressed HOX genes responsible for chemoresistance and to identify the mechanisms behind HOX-induced cell line-specific chemoresistance in EOC. Ten HOX genes and eight EOC cell lines were tested for any cell line-specific overexpression that presents a mutually exclusive pattern. Cell viability was evaluated after treatment with cisplatin and/or siRNA for cell line-specific overexpressed HOX genes. Immunohistochemical (IHC) staining for HOXB9 was performed in 84 human EOC tissues. HOXA10 and HOXB9 were identified as cell line-specific overexpressed HOX genes for SKOV-3 and RMUG-S, respectively. Inhibiting the expression of cell line-specific HOX genes, but not of other HOX genes, significantly decreased cell viability. In SKOV-3 cells, cell viability decreased to 46.5% after initial 10 μM cisplatin treatment; however, there was no further decrease upon additional treatment with HOXA10 siRNA. In contrast, cell viability did not significantly decrease upon cisplatin treatment in RMUG-S cells, but decreased to 65.5% after additional treatment with HOXB9 siRNA. In both cell lines, inhibiting cell line-specific HOX expression enhanced apoptosis but suppressed the expression of epithelial-mesenchymal transition (EMT) markers such as vimentin, MMP9, and Oct4. IHC analysis showed that platinum-resistant cancer tissues more frequently had high HOXB9 expression than platinum-sensitive cancer tissues. However, high HOXB9 expression was not associated with mucinous histology in EOC patients. HOXB9, which is overexpressed in RMUG-S but not in SKOV-3 cells, appeared to be associated with cell line-specific platinum resistance in RMUG-S. Inhibiting HOXB9 overexpression in RMUG-S cells may effectively eliminate platinum-resistant ovarian cancer cells by facilitating apoptosis and inhibiting EMT.

Keywords: HOX genes; ovarian cancer; chemoresistance; cisplatin; siRNA

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Table of Contents

Chapter 1. Introduction

1.1. Study Background

An estimated 22,440 new cases of ovarian cancer was expected in the United States in 2017 [1]. In Korea, 2,898 new ovarian cancer and 1,243 deaths from the disease occurred in 2018 [2]. In addition, new cases and cancer deaths are expected to increase in 2021 [3]. There are a variety of histologic types of epithelial ovarian cancer (EOC), including serous, mucinous, endometrioid, and clear cell carcinomas. Among these, mucinous tumors, which account for 10% of all primary EOCs, show poor prognosis compared to other subtypes in the advanced stage of disease [4-8]; this has been mainly attributed to resistance to platinum-based chemotherapy rather than tumor aggressiveness [7-11]. Nevertheless, all histologic subtypes of EOC have been treated with the same treatment strategy – maximal cytoreductive surgery followed by platinum-based chemotherapy without consideration of the responsiveness to platinum.

HOX genes, a highly conserved subgroup of the homeobox superfamily with 4 clusters (A, B, C, and D) of 39 genes, drive normal organogenesis through morphogenesis and terminal differentiation. Cheng et al. showed that several HOX genes are differentially expressed in the fallopian tubes, uterus, and vagina, but not in normal ovarian epithelium [12]. They also suggested that the Müllerian-like features of EOC are associated with the aberrant expression of HOX genes: HOXA9 in serous carcinoma, and HOXA10 and HOXA11 in mucinous carcinoma. Different expression patterns of HOX genes across distinct histologic subtypes of EOC have also been shown in several other studies [12-15]. However, the results of these studies were quite inconsistent and made it difficult to determine which HOX genes can be targeted to overcome chemoresistance in mucinous EOC. Moreover, there has been no study that has suggested plausible mechanisms for how

HOX genes lead to chemoresistance in specific subtypes of EOC.

1.2. Purpose of Research

This study aimed to discover any cell line-specific overexpressed HOX genes that may be attributed to chemoresistance, as well as identify the mechanisms underlying HOX-induced cell line-specific chemoresistance in EOC cell lines.

Chapter 2. Body

2.1. Methods

2.1.1. Cell culture and reagents

Ten HOX genes (HOXA4, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13, HOXB7, HOXB9, HOXB13, and HOXD10) were screened for their endogenous levels of expression in 8 representative EOC cell lines (serous type: OV-90, OVCAR-3, SKOV-3, CAOV-3; mucinous type: MCAS, RMUG-S; endometrioid type: TOV-21, ES-2) through western blot analysis (Supplementary Fig. 1). SKOV-3, a human ovarian cancer cell line of serous histology, was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). RMUG-S, a human ovarian cancer cell line of mucinous histology, was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). SKOV-3 cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI) media (Welgene, Kyungsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). RMUG-S cell lines were cultured in Ham's F-12 media nutrient mixture media (DMEM/F12, Gibco®, Invitrogen), supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified chamber with 5% CO^2 at 37° C.

Cisplatin was purchased from Sigma-Aldrich chemical company (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The final concentrations in the culture medium did not exceed 0.2%.

2.1.2. siRNAs and transfection

siRNAs for human HOXA10 (5′-GGAAGCGAGGACAAAGAGAGGUU-3′) and HOXB9 (5′-CCGGGAGCUCACAGCCAACUUUAAUUU-3′) were synthesized by Genolution (Genolution Pharmaceutical Inc, Seoul, Republic of

Korea), and the control siRNA (sc-37007) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The transient transfection experiment with the synthesized and control siRNAs was performed using Lipofectamine RNAi MAX™ according to the manufacturer's instruction (Invitrogen). The transfected cells were incubated in a humidified chamber with 5% CO^2 at 37° C for 48-72 hours until they were used for assays.

2.1.3. Western blot analysis

Cells were lysed with ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with the Protease Inhibitor Cocktail (complete mini tablet, Roche). Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Pierce, IL, USA) according to the manufacturer's instructions. Proteins were separated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane, and blocked with 5% non-fat milk. Membranes were incubated with anti-HOXA10, anti-HOXB9, anti-phospho-Akt, anti-p27, anti-p21, anti-cleaved PARP, anti-Bcl-2, anti-cleaved caspase-3, anti-E-cadherin, anti-Vimentin, anti-MMP9, anti-SOX2, anti-Nanog, anti-Oct4 (Cell signaling systems), and alpha-tubulin (Sigma-Aldrich) antibodies. Then, the membranes were incubated with a HRP-conjugated anti-secondary IgG (Invitrogen) antibody and visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce).

2.1.4. Reverse transcription polymerase chain reaction (RT-PCR)

Cellular RNA was extracted from cells using the TRIzol reagent according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 2 μg of RNA using a reverse transcription kit (Promega) and the following primer pairs (Yingjun Biotechnology Corporation, Shanghai, China): HOXA10 forward primer, 5′-TAAGGTATTACATTGCCTGACTAAAAT-3′, and

reverse primer, 5′ -GGACGCTGCGGCTAATCTCTAGGCG-3′ (347 bp); HOXB9 forward primer, 5′ -GGTGGGCTAGAAAGTACAAGAAAA-3′ , and reverse primer, 5′ -GATA CGACCGAAGAGAGTTGATTT-3′ (298 bp); and 18s rRNA gene forward primer, 5′ - GAGCGAA AGCATTTGCCAAG-3′ , and reverse primer, 5′ -GGCATCGTTTATGGTCGGAA-3′ (100 bp). The PCR conditions were as follows: 95° C for 5 minutes; 30 cycles of 95° C for 30 seconds, 62° C for 30 seconds, and 72° C for 30 seconds; and 72° C for 10 minutes. A volume (25 μL) of the PCR product was used for agarose gel electrophoresis.

2.1.5. Cell proliferation assay

Cell survival analysis was performed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Cell Titer 96 Aqueous Cell Proliferation Assay kit; Promega, Madison, WI, USA) assay. Briefly, the cells were cultured with respective HOX siRNAs, and then 10 μL of the 4 mg/mL MTT solution was added to each well. Then, the cells were incubated for 4 hours in the dark. The absorbance was measured in a microplate reader at 490 nm, and the results were presented as percentage of the control.

2.1.6. Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP-biotin nick-end labeling (TUNEL) assay

SKOV-3 and RMUG-S ovarian cancer cells were plated in a cell culture slide at ~70% confluence and incubated for 24 hours at 37° C. The cells were then transfected with either HOXA10 or HOXB9 siRNA for 72 hours. The cells were fixed with cold 2% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), and then stained with the TUNEL kit. TUNEL was subsequently performed using the In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Data were presented as the ratio of TUNEL-positive

cells to total nuclei. TUNEL-positive cells and nuclei were observed under a ZEISS Imager A1 microscope (ZEISS, Oberkochen, Germany).

2.1.7. Wound healing migration assay

The cells, which were transfected with HOXA10 or HOXB9 siRNA, were incubated overnight or until a monolayer formed. The monolayers were scratched with a 200-μL sterile pipette tip and then washed with media to remove the detached cells and debris. The wounded areas were then imaged after incubation for an indicated time. Results were observed under a ZEISS Obzerver Z1 microscope (ZEISS).

2.1.8. Transwell invasion assay

Cell invasion was measured in a transwell chamber. In brief, 2 × 10^5 cells were added to each transwell invasion chamber coated with 1 mg/mL Matrigel (reconstituted basement membrane; BD Biosciences, Mississauga, ON, Canada). The cells were then transfected with either HOXA10 or HOXB9 siRNA. Cells that did not invade the upper chamber were removed with a cotton swab. The remaining cells on the membrane were fixed for 10 minutes in methanol, stained with 1% crystal violet solution, and then washed with PBS. Invasive cells were observed under a ZEISS Axioskop 40 microscope (ZEISS).

2.1.9. Immunohistochemistry

Immunohistochemical (IHC) staining of ovarian cancer tissues from an 84-tissue microarray (TMA) was approved by the Institutional Review Board of Seoul National University Bundang Hospital. The TMA was established using tissue from women who underwent surgery for the treatment of EOC between February 2000 and November 2009. Tissue sections 4 μm in thickness were

de-paraffinized, rehydrated, and then washed two times with buffer. To reduce nonspecific background staining by endogenous peroxidase, each slide was incubated in Hydrogen Peroxide Block for 10 minutes, and washed 4 times. The primary antibodies for HOXA10 (1:50, Santa Cruz) and HOXB9 (1:100, Abcam, Cambridge, UK) were applied and allowed to incubate according to the manufacturers' protocols, and the slides were washed 4 times with buffer. Primary Antibody Enhancer was applied on the slides, incubated for 20 minutes at room temperature (18° C), and washed 4 times with buffer. Subsequently, HRP Polymer was applied to each slide, incubated for 30 minutes at room temperature, and washed 4 times with buffer. The slides were incubated with hematoxylin, washed 4 times with deionized water, and counterstained.

Intensity of HOXB9 nuclear staining was graded: negative, weak (1+), moderate (2+), or strong (3+). IHC scoring was performed according to the following criteria by M.K. and J.Y.C (Fig. 5A-D): IHC score 0: samples with negative or equivocal staining, or <50% tumor cells with weak (1+) or combined moderate (2+) staining; IHC score 1: 50% or more tumor cells with weak (1+) or combined weak (1+) and moderate (2+) staining, but <50% tumor cells with moderate (2+) or combined moderate (2+) and strong (3+) staining; IHC score 2: 50% or more tumor cells with moderate (2+) or combined moderate (2+) and strong (3+) staining, but <50% tumor cells with strong (3+) staining; IHC score 3: 50% or more tumor cells with strong (3+) staining. IHC scores 2 and 3 were considered "high expression".

2.1.10. Statistical analyses

Data are presented as means with standard deviations. When comparing between two groups, Student's t-test was applied. Progression-free survival (PFS) and overall survival (OS) were evaluated using the Kaplan-Meier method. Statistical significance was taken as $p<0.05$.

2.2. Results

2.2.1. HOX expression levels in EOC cell lines with histology-specific pattern

Among the ten HOX genes whose expression levels were tested in eight EOC cell lines, HOXA10 in SKOV-3 and HOXB9 in RMUG-S were identified to have cell line-specific overexpression with a mutually exclusive pattern between the two cell lines. HOXA10 and HOXB9 showed selectively high levels of expression in SKOV-3 and RMUG-S cell lines, respectively; however, it was not the case the other way around. The other expression patterns of HOX genes in the eight EOC cell lines were irregular (Supplementary Fig. 1). HOXA10 and HOXB9 expression was inhibited by treatment with 50 nM siRNA for the corresponding HOX genes, as observed through western blotting. (Fig. 1A) Upon RT-PCR analysis, we also found that the mRNA expression levels of HOXA10 and HOXB9 were inhibited by treatment with siRNA in a dose-dependent manner (Fig. 1B).

After treating both SKOV-3 and RMUG-S cell lines with 50 nM siRNA targeting endogenous HOXA10 and HOXB9 for 72 hours, MTT assay was performed (Fig. 1C). In SKOV-3, a significant decrease in cell viability compared with the cells treated with the non-targeting siRNA control was observed only when the cells were treated with the HOXA10 siRNA; cell viability was not significantly decreased upon treatment with HOXB9 siRNA (43.1% vs. 95.3%, $p<0.001$). Similarly, in RMUG-S, a significant decrease in cell viability was observed when the cells were treated with the HOXB9 siRNA, but not when treated with the HOXA10 siRNA (47.5% vs. 89.0%, $p<0.001$).

2.2.2. HOXB9 siRNA significantly reduced cell viability of cisplatin-resistant RMUG-S cells

In SKOV-3, cell viability decreased after cisplatin treatment (concentrations: 10, 20, and 40 μM; durations: 24, 48, and 72 hours) in both a concentration- and time-dependent manner compared to the control, which showed the SKOV-3 cell line to be sensitive to cisplatin (Fig. 2A). On the other hand, cisplatin treatment in RMUG-S did not show any significant inhibitory effect on cell viability compared to the control at any concentration or time period (Fig. 2B).

While the cell viability of SKOV-3 significantly decreased to 46.5% 72 hours after treatment with 10 μM cisplatin, those of RMUG-S decreased to just 91.3% (Fig. 2C and 2D). After the initial decrease in cell viability of SKOV-3, it did not undergo any further decrease even upon additional treatment with 50 nM HOXA10 siRNA or a combination of both siRNAs (Fig. 4C). In contrast, even though there was no significant inhibitory effect on the cell viability of RMUG-S (91.3%) upon treatment with cisplatin, cell viability significantly decreased after additional treatment with 50 nM HOXB9 siRNA (65.5%). However, we could not find any additive or synergistic cytotoxic effect between cisplatin and HOXB9 siRNA treatments (cisplatin only vs. cisplatin with HOXB9 siRNA; 64.9% vs. 65.5%, p=0.346) (Fig. 2D).

2.2.3. Knockdown of endogenous HOXA10 and HOXB9 inhibits cell proliferation and induces apoptosis in SKOV-3 and RMUG-S cell lines, respectively.

At the 48-hour time point after treatment with 50 nM HOXA10 siRNA in SKOV-3 and HOXB9 siRNA in RMUG-S, the expression of proteins related to cell proliferation, including pAkt, p27 and p21, decreased. The expression of apoptosis-related proteins were also changed; the levels of cleaved PARP and cleaved caspase-3 increased, while the level of Bcl-2 decreased in both cell lines treated with corresponding siRNAs (Fig. 3A).

We performed TUNEL assays to confirm the apoptotic effect of the siRNAs targeting each HOX gene. The number of apoptotic

cells significantly increased after treatment with HOXA10 siRNA for 72 hours in SKOV-3, compared to those treated with a non-targeting siRNA control (29.3±5.9 vs. 3.7±0.6, p=0.002). Similarly, HOXB9 siRNA induced apoptosis in RMUG-S (32.3±7.8 vs. 2.7±1.5, p=0.003) (Fig. 3B).

2.2.4. Knockdown of endogenous HOXA10 and HOXB9 inhibits cell migration, cell invasion and epithelial-mesenchymal transition (EMT) property in EOC.

Wound healing and transwell invasion assays were performed to assess whether knockdown of endogenous HOX genes would affect EMT properties in the two EOC cell lines. The 24-hour wound healing assay with HOXA10 and HOXB9 siRNA treatments showed decreased migration in SKOV-3 and RMUG-S cells, respectively (Fig. 4A). In addition, the 72-hour transwell invasion assay showed that the invading SKOV-3 and RMUG-S cells were significantly decreased when the cells were treated with HOXA10 (17.5% vs. 103.5%, p=0.004) and HOXB9 siRNAs (19.5% vs. 52.0%, p=0.017), respectively, compared to those treated with non-targeting siRNA controls (Fig. 4B). In both cell lines treated with their corresponding siRNAs, the expression levels of EMT-related proteins including vimentin, MMP-9, SOX2, NANOG, and Oct4 decreased, while the expression level of E-cadherin increased compared to those treated with their corresponding siRNA controls (Fig. 4C).

2.2.5. High HOXB9 expression in human EOC tissue was associated with platinum resistance.

The median follow-up period of the 84 EOC patients, whose formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were used for building a TMA, was 55 months (1 to 155 months). High expression of HOXB9 was found in 40 (47.6%) female patients. Unlike the results observed in the cell lines, high HOXB9

expression was not associated with mucinous histology in EOC patients (22.5% vs. 18.2%, p=0.623) (Table 1).

In the 70 patients who received platinum-based chemotherapy after surgery, 34 (48.6%) EOC tissues highly expressed HOXB9. Resistance to platinum was more frequent in women with EOC tissues that exhibited high HOXB9 expression (13/34 [38.2%] vs. 5/36 [13.9%], p=0.020). However, high HOXB9 expression was not associated with 5-year PFS (47.0% vs. 40.9%, p= 0.358) and OS (63.4% vs. 52.5%, p=0.452) (Fig. 5E and 5F).

2.3. Discussion

In this study, we evaluated whether the chemoresistance in an EOC cell line could be reversed by inhibiting a specific gene. This concept was derived from how the Müllerian-like features in EOC were associated with the aberrant expression of HOX genes depending on the histologic type [12]. Because many studies have reported inconsistent data on HOX expression patterns according to histology [13-15], we established a new screening set of ten HOX genes and eight EOC cell lines, including serous, mucinous, endometrioid, and clear cell carcinomas. We found a pair of HOX genes that had a mutually exclusive pattern of expression in two cell lines: high HOXA10 and low HOXB9 in platinum-sensitive SKOV-3, and low HOXA10 and high HOXB9 in platinum-resistant RMUG-S. Decrease of cell viability in platinum-resistant RMUG-S could be achieved only by treatment with HOXB9 siRNA, and without any additional effect when combined with cisplatin treatment.

Chemoresistance, one of the most important prognostic factors in EOC, was shown to be more frequent in mucinous type cancers than in serous type cancers. A study by the Hellenic Cooperative Oncology Group reported that 70% of serous EOC was sensitive to platinum, whereas only 39% of mucinous EOC was sensitive to platinum [9]. Shimada et al. also showed that the response rates to platinum-based chemotherapy were 68% and 13% in serous and

mucinous EOC, respectively [10]. There were studies that reported different expression patterns for several molecules in mucinous EOC compared with those of other subtypes in attempts to elucidate the mechanisms underlying chemoresistance: increased Ras mutations, decreased p53 mutations, and decreased COX-2 expression [16-22]. However, a number of hurdles still need to be overcome before these studies can be translated into clinical practice. For example, designing drug strategies to target mutant p53 tumors may be highly challenging as mutant p53 was shown to not be just a single protein, but present multiple isoforms [23]. Nevertheless, p53 has been thought to be a tremendously attractive therapeutic target because most mutant p53s are highly expressed in cancer cells [23]. If mucinous EOCs do not show a high incidence of p53 mutations, it may not be an attractive therapeutic target for this particular cancer. In this regard, HOXB9 may be one of the best targets for future gene therapy of mucinous EOC, especially since normal epithelial ovarian epithelium lacks HOX expression.

Our findings suggest that HOXB9 may contribute to platinum resistance in RMUG-S by promoting apoptosis escape, as well as EMT. There were significant changes in the molecular expression of not only cell survival-associated biomarkers, such as pAkt, p21, and Bcl-2, but EMT-associated biomarkers, such as vimentin and MMP-9, as well; the expression of these genes were almost completely inhibited upon treatment with HOXB9 siRNA. Apoptosis escape and EMT have been considered to be key processes in chemoresistance according to previous studies [24,25]. Chemotherapeutic agents generally induce tumor regression through apoptosis; however, the dysregulation of such apoptotic processes can lead to the increased expression of EMT-inducing factors and result in the failure of chemotherapy. However, the exact mechanism underlying this is still unclear.

There were only a few studies that reported on the impact of HOX expression on chemoresistance in EOCs. Miao et al. demonstrated that HOXB13 promoted tumor growth in vivo,

presenting activated Ras and resistance to tamoxifen-mediated apoptosis in ovarian cancer [26]. The relationship between HOX expression and survival outcomes in ovarian cancer patients has also been studied [27-29]. Li et al. showed that HOXA10 was strongly expressed in EOC tumor samples with poor survival outcomes, especially in that of clear cell histology [29]. The present study, to the best of our knowledge, is the first study that identified HOXB9 to possibly be responsible for chemoresistance in a mucinous EOC cell line, RMUG-S. Demonstration of the association of HOXB9 high expression and platinum resistance using clinical data and human tissue was also a strength of our study; although, we were unable to show any independent survival impact due to HOXB9 high expression. This may be attributed to the small sample number of our study, as well as to the use of a small fractionated TMA block instead of whole ovarian cancer tissues.

Chapter 3. Conclusion

HOXB9, which was found to be overexpressed in RMUG-S but not in SKOV-3 cells, appeared to be associated with cell line-specific platinum-resistance. Inhibiting HOXB9 overexpression in RMUG-S cells can effectively kill platinum-resistant ovarian cancer cells by facilitating apoptosis and inhibiting EMT. Further in vivo studies and clinical trials are necessary to develop an individualized strategy for effectively controlling chemoresistance in mucinous type EOCs.

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국문초록

HOX 유전자는 정상적인 기관형성(organogenesis)에 관여하는 유전자로 알려져 있지만, 상피성 난소암의 발생과 연관성을 가지며 조직타입에 따른 이질적인 과발현 양상이 보고된 바 있다. 본 연구에서는 난소암 세포주마다 특이적으로 과발현 되는 HOX 유전자를 규명하여 과발현 된 유전자와 항암제 저항성과의 관련성을 확인하고자 하였다. 총 8개의 난소암 세포주에서 10가지의 HOX 유전자 발현 양상을 western blot 검사법을 이용하여 조사하였고, SKOV-3 세포주와 RMUG-S 세포주에서 HOXA10과 HOXB9 유전자가 각각 세포-특이적으로 과발현 되는 것을 확인하였다. 세포-특이적으로 과발현 된 HOX 유전자의 발현을 억제하는 siRNA를 세포주에 처리하여 cell viability를 확인하였다. SKOV-3 세포주는 HOXA10 siRNA와 cisplatin을 병합처리 하였을 때의 cell viability의 감소가 46.5%로서 cisplatin을 단독처리 한 경우와 유의한 차이를 보이지 않았다. 그러나, RMUG-S 세포주에서는 cisplatin을 단독처리 한 경우보다 HOXB9 siRNA와 cisplatin을 병합처리 한 경우에 cell viability가 65.5%까지 유의미하게 감소하였다. 두 세포주 모두에서 세포-특이적 과발현 된 HOX 유전자를 억제시키면, apoptosis가 증진되고 epithelial-mesenchymal transition (EMT)가 억제되는 것이 확인되었다. 84명의 난소암 여성으로부터 획득한 난소암 조직으로부터 조직 미세배열(tissue microarray)을 구축한 후 면역조직화학염색검사를 하였을 때, 백금 항암제에 반응성(sensitive)을 보이는 환자와 비교하여 백금 항암제에 저항성(resistant)을 보인 환자에서 HOXB9의 과발현 비율이 유의하게 높았다. 그러나, HOXB9의 과발현을 보이는 환자들 중 점액성과 장액성 난소암 조직타입의 비율은 유의한 차이를 보이지 않았다. 결론적으로, HOXB9은 백금 저항성 RMUG-S 세포주에서 특징적으로 과발현하며, siRNA를 이용한 HOXB9의 억제를 통하여 cisplatin 단독처리의 경우에 비하여 유의미한 수준으로 향상된 치료 효과를 확인할 수 있었다. 이러한 결과는 향후 백금 항암제에 저항성을 보이는 난소암 환자를 위한 맞춤형 치료법 개발의 근거로 활용할 수 있을 것이다.

주제어 : HOX 유전자; 난소암; 항암제 저항성; cisplatin; siRNA

Table

Table 1. Comparisons of histologic types and the response to platinum according to the degree of HOXB9 expression by immunohistochemical staining analysis in 84 EOC patients

	Total n	Low expression of HOXB9 (n = 44)	High expression of HOXB9 (n = 40)	P value
Histology				0.623
Serous type	67	36 (53.7)	31 (46.3)	
Mucinous type	17	8 (47.1)	9 (52.9)	
Response to platinum†				0.020
Sensitive	52	31 (59.6)	21 (40.4)	
Resistant	18	5 (27.8)	13 (72.2)	

IHC score 0, samples with negative or equivocal staining, or <50% tumor cells with weak (1+) or combined moderate (2+) staining; IHC score 1, 50% or more tumor cells with weak (1+) or combined weak (1+) and moderate (2+) staining, but less 50% tumor cells with moderate (2+) or combined moderate (2+) and strong (3+) staining; IHC score 2, 50% or more tumor cells with moderate (2+) or combined moderate (2+) and strong (3+) staining, but less than 50% tumor cells with strong (3+) staining; IHC score 3, 50% or more tumor cells with strong (3+) staining. IHC score 2 and 3 were considered as 'high expression'.

†in 70 patients received platinum-based chemotherapy after surgery

EOC, Epithelial ovarian cancer

Figure legend

Fig. 1
(A) Western blot analysis and (B) reverse transcription polymerase chain reaction (RT-PCR) for HOXA10 and HOXB9 expression in SKOV-3 and RMUG-S cell lines with/without siRNA. (C) Viable cells (% of control with non-targeted siRNA) after treatment with 50 nM HOXA10 and HOXB9 siRNAs for 48 hours in each cell line

Fig. 2
Viable cells (% of control) after treatment with cisplatin and corresponding siRNA to each cell line. MTT assay after treatment with 10, 20, or 40 μM cisplatin for 72 hours in (A) SKOV-3 and (B) RMUG-S. Quantitative graphs of cell viability after 10 μM cisplatin and/or 50 nM HOX siRNA treatments for 72 hours in (C) SKOV-3 and (D) RMUG-3

Fig. 3
(A) Western blot analysis for proteins related to cell growth and apoptosis and (B) images of TUNEL assay after treatment with corresponding siRNAs to each cell line

Fig. 4
(A) Wound healing migration assay, (B) transwell invasion assay, and (C) western blot analysis after treatment with corresponding HOX siRNAs in each cell line

Fig. 5
Image of immunohistochemical staining score (A) 0, (B) 1, (C) 2, and (D) 3 for HOXB9 in ovarian cancer tissues in ×40 and ×200 magnifications. (E) Progression-free survival and (F) overall survival graphs by Kaplan-Meier methods according to the degree of HOXB9 expression

Supplementary Fig. 1

Ten HOX genes whose expression levels were measured in eight EOC cell lines

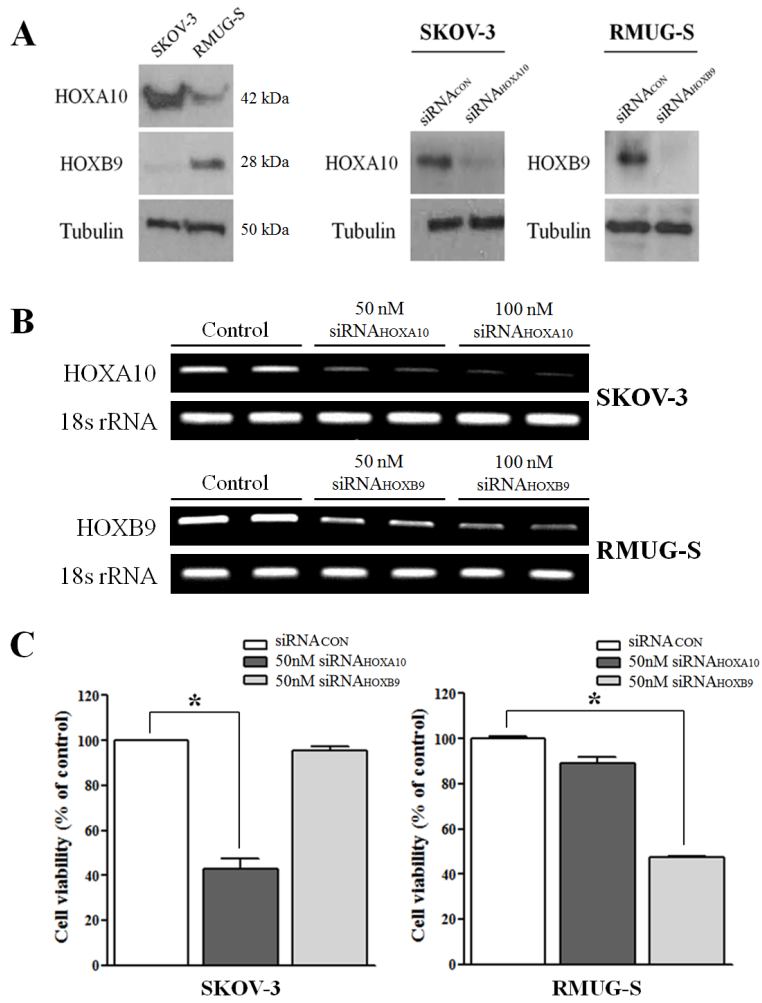

A
SKOV-3
RMUG-S
HOXA10
42 kDa
HOXB9
28 kDa
Tubulin
50 kDa
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNAHOXB9
HOXA10
HOXB9
Tubulin
B
Control
50 nM siRNAHOXA10
100 nM siRNAHOXA10
HOXA10
18s rRNA
SKOV-3
Control
50 nM siRNAHOXB9
100 nM siRNAHOXB9
HOXB9
18s rRNA
RMUG-S
C
siRNACON
50nM siRNAHOXA10
50nM siRNAHOXB9
Cell viability (% of control)
120
100
80
60
40
20
0
*
SKOV-3
RMUG-S

Fig. 1.

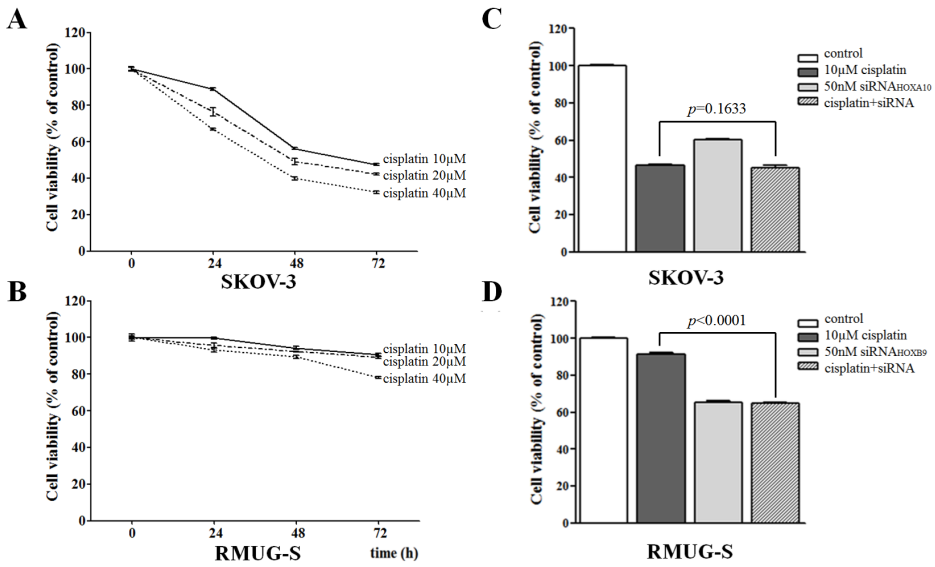

A
Cell viability (% of control)
120
100
80
60
40
20
0
0
24
48
72
cisplatin 10μM
cisplatin 20μM
cisplatin 40μM
SKOV-3
B
Cell viability (% of control)
cisplatin 10μM
cisplatin 20μM
cisplatin 40μM
RMUG-S
time (h)
C
Cell viability (% of control)
p=0.1633
control
10μM cisplatin
50nM siRNAHOXA10
cisplatin+siRNA
SKOV-3
D
Cell viability (% of control)
p<0.0001
control
10μM cisplatin
50nM siRNAHOXB9
cisplatin+siRNA
RMUG-S

Fig. 2.

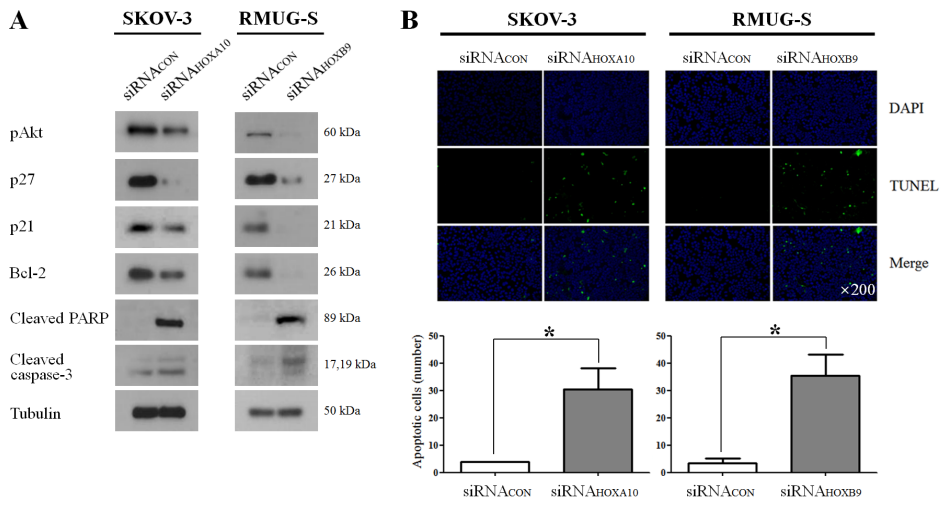

A
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
pAkt
p27
p21
Bcl-2
Cleaved PARP
Cleaved caspase-3
Tubulin
60 kDa
27 kDa
21 kDa
26 kDa
89 kDa
17,19 kDa
50 kDa
B
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
DAPI
TUNEL
Merge
×200
Apoptotic cells (number)
0
10
20
30
40
50
*
*
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9

Fig. 3.

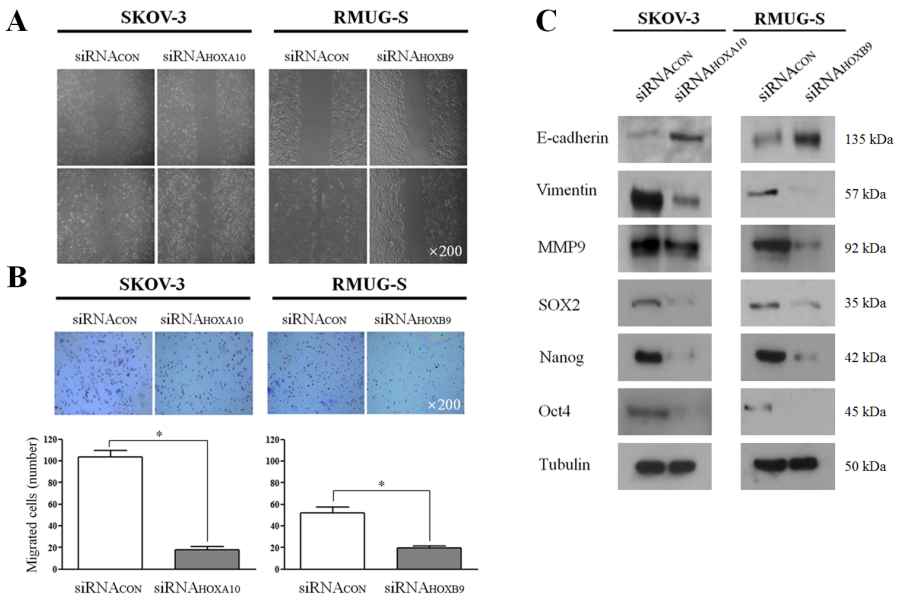

A
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
×200
B
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
×200
Migrated cells (number)
120
100
80
60
40
20
0
*
*
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
C
SKOV-3
RMUG-S
siRNACON
siRNAHOXA10
siRNACON
siRNAHOXB9
E-cadherin
Vimentin
MMP9
SOX2
Nanog
Oct4
Tubulin
135 kDa
57 kDa
92 kDa
35 kDa
42 kDa
45 kDa
50 kDa

Fig. 4.

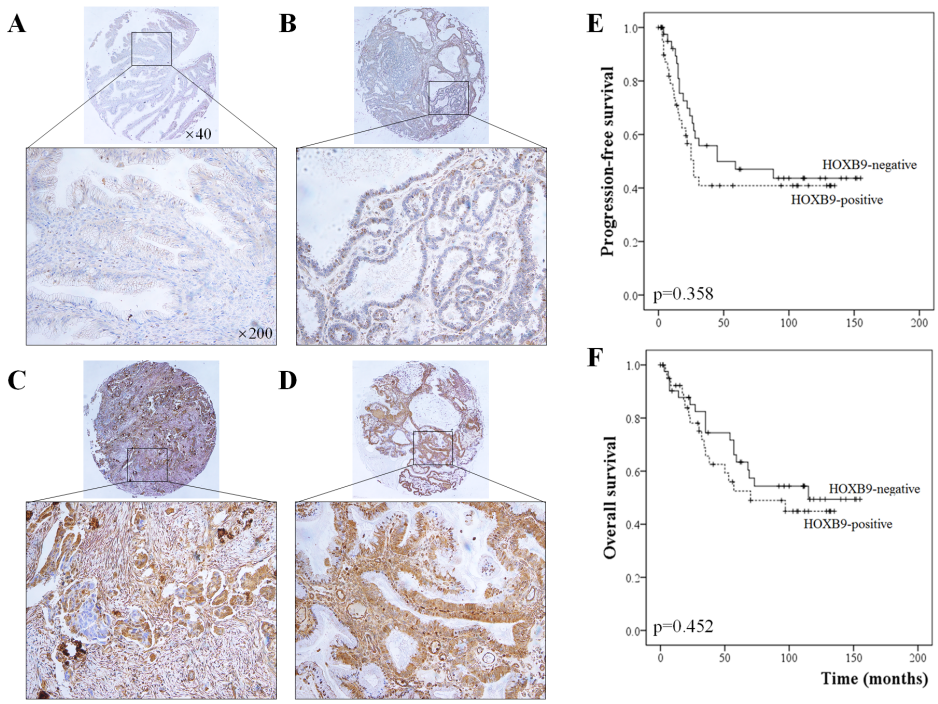
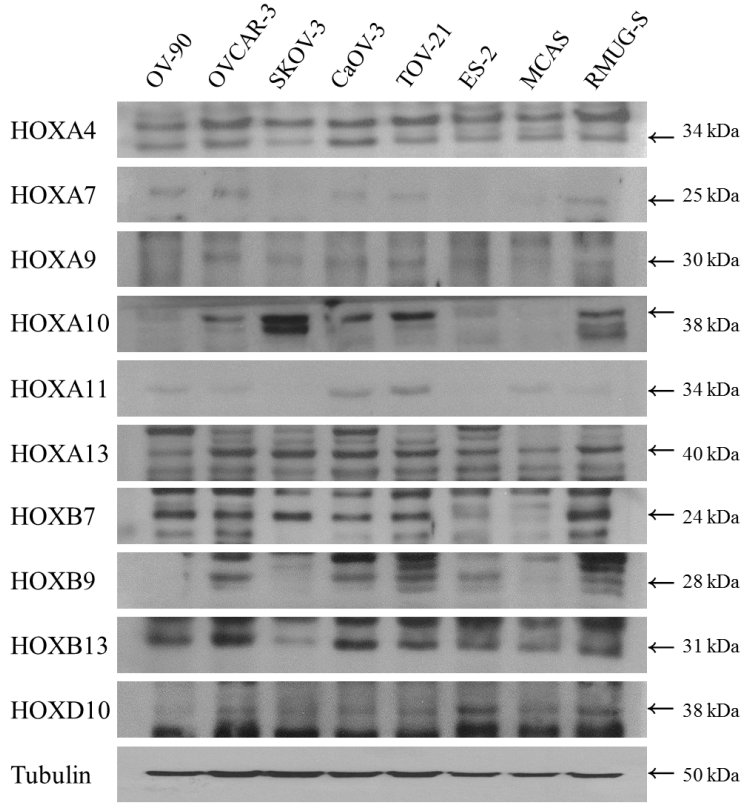

A
B
×40
×200
C
D
E
Progression-free survival
HOXB9-negative
HOXB9-positive
p=0.358
F
Overall survival
HOXB9-negative
HOXB9-positive
p=0.452
Time (months)

Fig. 5.



Supplementary Fig. 1.