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Highly expressed Reptin administers aggressive features in ovarian cancer

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Highly expressed Reptin administers aggressive features in ovarian cancer

Directed by Professor JAE-HOON KIM

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

BO WOOK KIM

December 2020

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This certifies that Doctoral Dissertation
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연세대학교
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<TABLE OF CONTENTS>

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LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

Highly expressed Reptin administers aggressive features in ovarian cancer

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Objective: Reptin is one of the ATPases of the AAA+ (ATPases Associated with various cellular Activities) superfamily and has been reported to have a multifunctional role in carcinogenesis. However, its biologic and prognostic significance in ovarian cancer has not yet been investigated. In this study, we evaluated the clinical significance and functional role of Reptin in ovarian cancer.

Methods: The study included benign ovarian tumor tissues (n=175), borderline cancer (n=55) and ovarian cancer tissues (n=271). In order to identify Reptin expression, immunohistochemistry (IHC) was performed, and then the association of Reptin with various prognostic factors was investigated. Reptin's functional roles in cell proliferation and the cell cycle were determined by invasion and migration assays and an in vivo study after its knockdown with shRNA in ovarian cancer cell lines.

Results: Reptin was over-expressed in ovarian cancer compared with normal, benign, and borderline tumors in IHC tissue samples ($p < 0.001$). High expression of Reptin relative to low expression was correlated with

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poor progression-free survival (p= 0.014) but showed tendency of decreased overall survival (p = 0.071). Down-regulation of Reptin decreased cell proliferation in OVCA429 with siRNA and in TOV112D with siRNA (p<0.001). Down-regulation of Reptin with siRNA significantly decreased migration and invasion of OVACA429 and TOV112D ovarian cancer cells (p<0.001, respectively). According to the results of the in vivo study, tumor growth decreased in down-regulated Reptin tumors with shRNA 1 and 2 compared to control group (p value <0.05, respectively) while tumor incidence rate diminished in Reptin stably expressed tumor.

Conclusion: Our data showed that Reptin is highly expressed in ovarian cancer and is associated with poor progression-free survival outcome. Inhibition of Reptin decreased malignant properties. Overall, our results suggest that Reptin may be an effective target in treatment of ovarian cancer.

Key words: Reptin, ovarian cancer, survival outcome, tumor cell viability

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Highly expressed Reptin administers aggressive features in ovarian cancer

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I. INTRODUCTION

Ovarian cancer is the second most common cause of gynecologic cancers following the uterine corpus[1]. Ovarian cancer incidence in Korea has been increasing steadily; now, it is the third most common gynecologic cancer but the leading cause of cancer death[2]. Unfortunately, most ovarian cancers are diagnosed in the advanced stage, due to the lack of both specific symptoms in the early stage and a cost-effective screening test; and late-diagnosed ovarian cancer, is of high lethality. In advanced ovarian cancer cases, the standard treatment includes cytoreductive surgery and chemotherapy. Additionally, molecular-biologic advances entailing the introduction of target therapies such as bevacizumab to ovarian cancer have demonstrated modest survival gains[3]. Recently, PAPR inhibitors improved survival in women with ovarian cancer of Homologous recombination deficiency (HRD) or BRCA mutation[4]. However, ovarian cancer mortality remains high owing to frequent disease recurrence and chemo-resistance. Ovarian cancer still shows a veiled tumorigenesis mechanism, and ovarian tumor heterogenicity disrupts ovarian cancer conquest [5]. Given its veiled mechanism and high lethality, treatment of ovarian cancer is a both a challenging and an urgent issue requiring further biologic investigation.

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Previously, we identified Reptin in a study involving immunoprecipitation of tumor-associated antigen (TAA) and autoantibody in ovarian cancer. We hypothesized that autoantibodies corresponding to TAA were elevated in pre- relative to post-treatment serum because surgical removal and adjuvant chemotherapy reduce the tumor volume and, in turn, decreases serum levels of TAA and autoantibodies. Pre- and post-treatment autoantibodies in patients with ovarian cancer were obtained and, in recognized 36 proteins after immunoprecipitation, Reptin, a molecular weight of 48.5kDa, was identified[6].

Reptin is one of the ATPases of the AAA+ (ATPases Associated with various cellular Activities) superfamily, and has been reported to have a multifunctional role in carcinogenesis[7]. Reptin is involved in ATP hydrolysis by hexamerization with pontin and induces structural modification of nucleosome and, eventually, regulation of transcription[8]. Its roles in cancer include or are related to transcriptional regulation, DNA damage repair, the cell cycle, and cell proliferation[9-11]. Overexpression of Reptin has been identified in various cancers including kidney, liver and gastric cancer [12-14]. Overexpression of Reptin also is associated with poor prognosis in renal cell carcinoma[13].

In light of such accumulating evidence, it is expected that Reptin plays a key role in tumorigenesis and cancer prognosis. However, its biologic and prognostic significance has yet to be elucidated for ovarian cancer, though decreased survival outcomes have been reported for liver, colorectal, and breast cancer patients showing high Reptin expression. Therefore, in this study, we investigated functional role and clinical significance of Reptin in ovarian cancer.

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II. MATERIALS AND METHODS

1. Patients and tumor specimens

The study enrolled 501 patients who had been diagnosed with benign ovarian tumor (n=175), Borderline ovarian cancer (n=55) and ovarian cancer (n=271) between 1996 and 2011 at Gangnam Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) along with 90 matched non-adjacent normal ovarian epithelium cases. Some of the paraffin blocks were provided by the Korea Gynecologic Cancer Bank through the Bio & Medical Technology Development Program of the Ministry of Education, Science and Technology, Korea (NRF-2017M3A9B8069610). The patients' data including age, cancer stage, tumor differentiation, cell type, lymph node (LN) metastasis, chemosensitivity and clinical outcomes were collected. The tumor stage was determined based on the International Federation of Gynecology and Obstetrics (FIGO) scale and was histologically classified according to the World Health Organization (WHO) grades. Patients with operability indications underwent hysterectomy with pelvic and para-aortic LN dissection. Chemotherapy resistance was designated as "recurrence" within 6th months after chemotherapy, while chemotherapy sensitivity was designated as "no recurrence" within 6th months after chemotherapy. This study was approved by the Institutional Review Board of Gangnam Severance Hospital (IRB #3-2010-0030).

2. Immunohistochemistry (IHC) and scoring

For IHC staining, all of the paraffin sections were cut to 5 mm thickness, deparaffinized through xylene and dehydrated with graded ethanols. Endogenous peroxidase activity was blocked by 3% H2O2 in methanol, and primary incubations were performed using anti-Reptin antibody (rabbit monoclonal, abcam) at a dilution of 1:300 for 1 hr 30min at room temperature followed by detection using Dako LSAB+ (Dako, Glostrup, Denmark). The reaction product was developed with DAB (3,3'-diaminobenzidine) chromogen

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solution (Dako). The tissue sections were lightly counterstained with hematoxylin. Representative photomicrographs were recorded using a digital camera (Nikon, Tokyo, Japan). Staining was scored as positive when tumor or epithelial cells showed cytoplasmic immunoreactivity. The IHC staining results were scored based on staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and the percentage of positive cells (1 = 0 – 25%, 2 = 26 – 50%, 3 = 51 – 75%, 4= 76 – 100% positive cells). For the immunostaining score, the intensity of staining was categorized as 0 (no staining), 1+ (weak), 2+ (moderate) and 3+ (strong); the percentage of staining was categorized as 1+ (0 - 25%), 2+ (26 - 50%), 3+ (51 - 75%) and 4+ (≥ 75%). The final IHC score was calculated based on the combined intensity and percentage scores (range: 0–12).

3. SYBR green real-time PCR

Total RNA was extracted by the Trizol method. cDNA synthesis was performed with the SuperScript TM III First–Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). SYBR green real-time polymerase chain reaction (PCR) was performed using primer sets specific for Reptin (forward primer: 5'-AAG ACA CGA AGC AGA TCC T-3'; reverse primer: 5'- TGT CAT CCA CCT GCA CTT C-3') and the housekeeping gene α-actin (forward primer: 5'- ATT AAG GAG AAG CTG TGC TAC GTC-3'; reverse primer: 5'- ATG ATG GAG TTG AAG GTA GTT TCG-3'), MMP1 (forward primer: 5'- AGC CAT CAC TTA CCT TGC ACT-3'; reverse primer: 5'- TTC TAG AGT CGC TGG GAA GC-3'), MMP2 (forward primer: 5'- GAT ACC CCT TTG ACG GTA AGG A-3'; reverse primer: 5'- CCT TCT CCC AAG GTC CAT AGC-3'), MMP9 (forward primer: 5'- AGA CGG GTA TCC CTT CGA CG-3'; reverse primer: 5'- AAA CCG AGT TGG AAC CAC GAC-3'). α-actin was used for quantitative normalization of the cDNA used for PCR. The relative gene expression level was determined by 2-ΔΔCt analysis.

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4. Western Blotting

Whole-cell extraction was conducted using PRO-PRE Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea). Equal amounts (20 μg) of each sample were separated on 8-15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hr at room temperature, washed with TBST, and subsequently incubated with primary antibodies: anti-Reptin (abcam), anti-α-actinin (H-2) (Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies against each protein were detected by secondary antibodies conjugated with horseradish peroxidase (GE Healthcare, Munich, Germany). Specific bands for each protein were detected on AGFA X-ray film (Agfa Health Care, Mortsel, Belgium) using the SuperSignal Chemiluminescence kit (Thermo Scientific, Rockford, IL, USA).

5. Gene Expression Omnibus (GEO) dataset analysis

Gene expression profiling data was obtained from the published microarray data of the GSE27651, GSE26712, GSE18520 and GSE14407 datasets from GEO. Identification of differentially-expressed Reptin was conducted with a sorting tool based on Microsoft Excel software (Probe number: 202264_s_at on affymatrix human U133A platform; gene accession number: NM_006144.). Box plots were drawn and statistical analyses were performed using Graphpad Prism 6 software.

6. Cell culture and transfection of siRNAs

All of the experiments were performed with two human ovarian cancer cell lines, TOV112D and OVCA429. Cells were maintained in DMEM (GIBCO, CA, USA). Media were supplemented with 10% fetal bovine serum (GIBCO) as well as antibiotics solutions (GIBCO) at 37℃ in an incubator under a 5%-CO2, 95%-humidified atmosphere. Suppression of Reptin expression was performed

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using specific siRNA targeted for Reptin (1: GCUCCACGCAGAC AUGAAGGAGUA and 2: GAUCCGGGAAGGGAAGAUU, Invitrogen) or control siRNA (Stealth RNAiTM siRNA Negative Control Med GC, Invitrogen) using RNAiMAX (Invitrogen) as a transfection reagent according to the manufacturer's instructions. The cells were harvested 48 hr after transfection, and reduction of protein levels was confirmed by Western blotting.

7. Cell proliferation assay

Cell proliferation was assessed using the high-sensitivity WST-1 assay (DaeilLab, Seoul, Korea). Control or Reptin-siRNA-transfected cells were seeded at 1 x 104 cells/well onto 96-well microtiter plates in a final volume of 100 μL/well. The cells were incubated with WST-1 at 37℃ for 1 hr, and the optical density (OD) values at 450 nm were recorded at days 0, 1, and 2 using a 96-well microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

8. Cell cycle

TOV112D and OVCA429 were harvested 48 hr after siRNA transfection in a 6 cm dish. Cells were fixed with 70% ethanol and washed with washing buffer (0.01% Triton X-100 in PBS). Cells were suspended in 200μl of a RNase A(100μg/ml), PI (50μg/ml) in the washing buffer and then subjected to 1 hr incubation after adding 800μl. Cells were washed with washing buffer and then suspended in washing buffer (200μl). After transfer to a FACS tube, 800 μL of washing buffer was added to each tube, and analyzed by FACS Canto II flow cytometry (BD Biosciences, San Jose, CA, USA).

9. Cell invasion and migration assay

Cell invasion and migration were performed using a boyden chamber (Neuro Probe 48-Well Micro Chemotaxis Chamber, Neuro Probe, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. The invasion and

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migration assays were performed using membranes coated with Matrigel (BD Transduction Lab, San Jose, CA, USA) and collagen, respectively. Preparatorily, the cells were treated with either control or Reptin siRNA for 48 hr. Cells (1×10^5) suspended in 0.1% FBS medium (56 μL) were added to the upper chamber. The lower chamber was filled with medium containing 1% FBS (27 μL). After 24 hours of incubation, the membrane was stained with the Differential Quick Stain Kit (Triangle Biomedical Sciences, Inc., Durham, NC, USA). Invading cells in six randomly selected fields were counted using Microscope Axio Imager.M2 (magnification x200; Carl Zeiss, Thornwood, NY, USA). Each experiment was repeated three times.

10. In vivo xenograft tumor model

All animal procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of Gangnam Severance Hospital. A mouse xenograft model was used with nude female BALB/C mice aged 4-5 weeks (OrientBio Inc., Sungnam, Gyunggi, Korea). TOV112D cells (1.5×106 cells/inoculation) were incubated in 100 μL of Matrigel (BD Biosciences, San Jose, CA, USA) and PBS mixture (1:1) and injected subcutaneously in both flanks of each mouse. Five weeks after inoculation, tumor volumes were measured using the following formula: tumor volume (mm3) = (L x W2)/2, where L is the length and W is the width. The data were recorded as means ± standard errors.

11. Statistics

A non-parametric Mann-Whitney U test was used to assess the statistical significance of the Reptin mRNA expression difference between HOSE cells and ovarian cancer cell lines and tissues. IHC scores were compared using a one-way ANOVA test and independent t-test. The IHC cut-off for high expression of tumor markers was determined by receiver operating

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characteristic (ROC) curve analysis. The sensitivity and specificity for dead/alive discrimination were plotted for each IHC score, thus generating an ROC curve. The cut-off value was established to be the point on the ROC curve where the sum of sensitivity and specificity was maximized. Kaplan-Meier survival analysis was performed to determine the association of Reptin expression with disease-free and overall survival, and the survival curves were compared among the groups using log-rank tests. All of the analyses were performed using Statistical Package for Social Science for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA).

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III. RESULTS

1. Reptin expression in ovarian cancer tissue and cell lines

Table 1 summarizes the patients' clinicopathological characteristics in their IHC samples. Included are 501 patients with ovarian tumor, 175 patients with benign tumor, 55 patients with borderline cancer, 246 patients with epithelial ovarian cancer, and 25 patients with non-epithelial ovarian cancer.

Table 1. Patients' clinicopathologic characteristics in IHC samples

	N	%
Diagnostic Category		
Normal ovarian epithelium	90	15.2
Benign	175	29.6
Borderline cancer	55	9.3
Epithelial ovarian cancer	246	41.6
Non-epithelial ovarian cancer	25	4.2
FIGO stage		
I	43	16.7
II	17	6.6
III	145	56.2
IV	28	10.9
Recurrent	25	9.7
Cell type		
Serous adenocarcinoma	174	67.4
Mucinous adenocarcinoma	27	10.5
Endometrioid adenocarcinoma	27	10.5
Clear cell carcinoma	13	5
Others	17	6.6
Tumor differentiation		
Well	15	8.8
Moderate	71	41.5
Poor	85	49.7
Chemotherapy resistance		
Sensitive	171	72.2
Resistant	66	27.8

FIGO, International Federation of Gynecology and Obstetrics

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According to the FIGO scale, 43 patients were stage I, 17 stage II, 145 stage III, and 28 stage IV. The most common cell type was serous adenocarcinoma (174 patients), and the most common tumor differentiation was “poor” grade (85 patients). Among 237 patients receiving chemotherapy, 171 (72.2%) showed sensitivity.

We examined the association of Reptin expression with the clinicopathological characteristics in ovarian cancer, as shown in Table 2. High expression of Reptin was observed in ovarian cancer relative to normal, benign and borderline ovarian tumor ($P < 0.001$, respectively). However, there was no statistically significant difference in Reptin expression with regard to FIGO stage, cell type, tumor differentiation or chemoresistance. Figure 1 plots the Reptin IHC expression in ovarian cancer and provides an analysis of its clinical significance. The representative Reptin IHC expressions are shown in Figure 1 A, B, C, D, E, F, G, H. Reptin expression was clearly observed in the cytoplasm. As indicated in Fig. 1I, Reptin expression was significantly higher in ovarian cancer than in normal epithelium, benign or borderline tumor ($p < 0.001$). High expression of Reptin was correlated with poor progression-free survival ($p = 0.014$) in Fig. 1J but showed a trend of decreasing OS (G) ($p = 0.071$) relative to those with low Reptin expression in Fig. 1K.

Figure 2A shows the Reptin mRNA levels measured by real-time PCR in human ovarian surface epithelial (HOSE) cells and ovarian cancer cell lines. A total of 15 cancer cell lines were significantly expressed over 5 HOSE cells in terms of the Reptin mRNA level ($p<0.001$). Figure 2B shows Reptin protein levels by Western blotting, 17 ovarian cancer cell lines showing higher expression than 5 HOSE cells with statistical significance ($p<0.05$). Figure 2C shows mRNA expression levels of Reptin as analyzed for ovarian cancer patients in the Gene Expression Omnibus (GEO) database; Reptin over-expression was confirmed in ovarian cancer with statistical significance.

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Table 2. Association of Reptin protein expression with clinicopathological characteristics in ovarian cancer

	N	Mean IHC score (95% CI)	*p value*
Diagnostic Category			*<0.001*
Normal	90	3.4 (2.9-3.9)	
Benign	163	2.3 (1.9-2.7)	
Borderline cancer	55	3.2 (2.4-4.0)	
Cancer	236	6.3 (5.8-6.8)	
Recurrent	22	5.5 (3.9-7.2)	
FIGO stage			*0.114*
I	43	6.2 (5.1-7.3)	
II	17	4.0 (2.5-5.4)	
III	145	6.6 (6.1-7.2)	
IV	28	6.2 (4.6-7.9)	
Recurrent	25	6.0 (4.4-7.6)	
Cell type			*0.202*
Serous adenocarcinoma	174	6.5 (5.9-7.1)	
Mucinous adenocarcinoma	27	5.2 (3.8-6.7)	
Others	57	5.9 (5.0-6.8)	
Tumor differentiation			*0.384*
Well + moderate	86	6.2 (5.5-7.0)	
Poor	85	6.7 (5.9-7.6)	
Chemotherapy resistance			*0.287*
Sensitive	171	6.2 (5.6-6.7)	
Resistant	66	6.8 (5.8-7.8)	

FIGO, International Federation of Gynecology and Obstetrics

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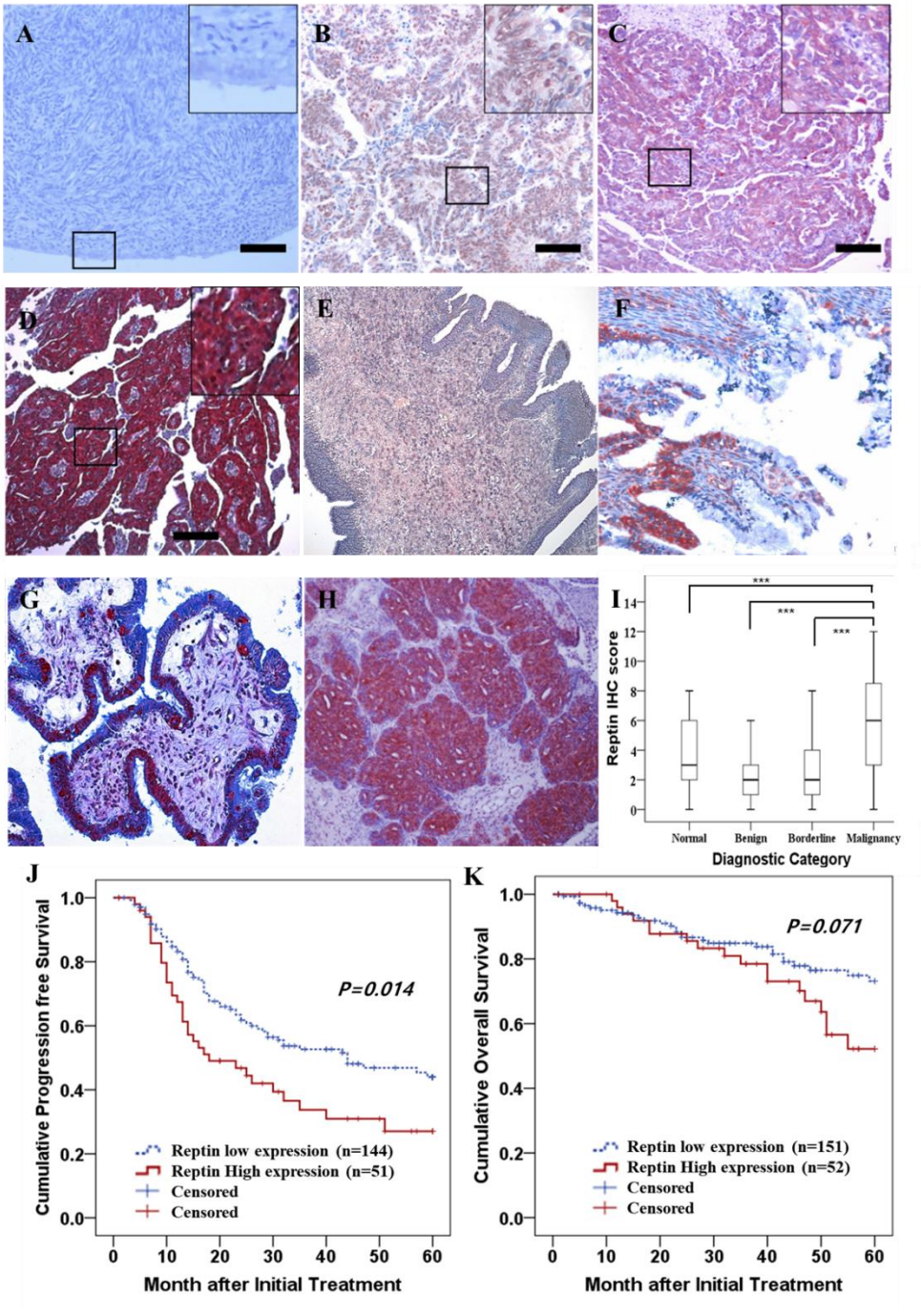

Figure 1. Reptin protein expression with IHC staining and analysis of clinical significance in ovarian cancer. Representative IHC staining for cytoplasmic

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Reptin in ovarian tissues: (A) no staining in normal ovarian epithelium, (B) weak staining intensity in papillary serous adenocarcinoma, (C) moderate staining intensity in papillary serous adenocarcinoma, (D) strong intensity in papillary serous adenocarcinoma. Scale bar: 200μm. The percentage of staining: (E) 0 ~ 25%, (F) 26 ~ 50%, (G) 51 ~ 75%, (H) 76 ~ 100% positive cells. Reptin expression was higher in ovarian cancer than normal epithelium, benign, and borderline tumor tissues (I). Poor 5-year progression-free survival (J) (p = 0.014) and trend of decreasing OS (K) (p = 0.071) in high Reptin expression.

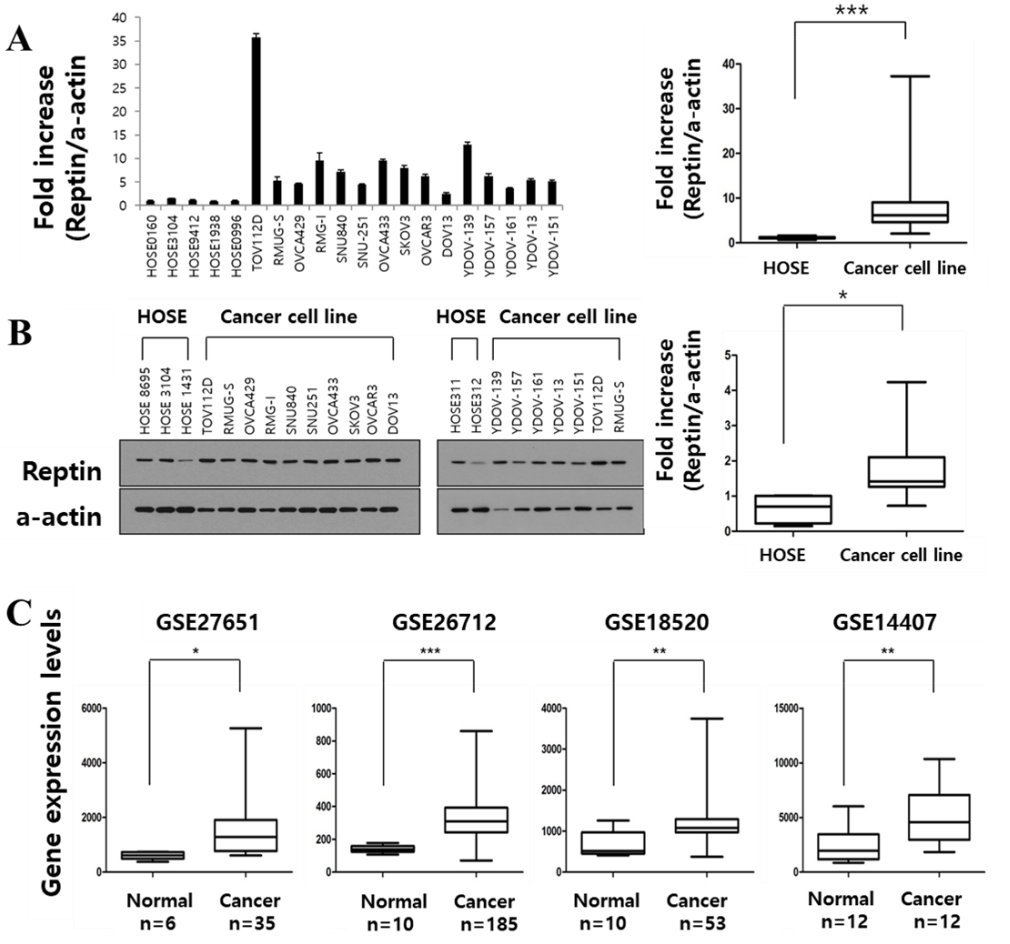

Figure 2. Reptin showing high expression in human ovarian cancer cells. A.

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Reptin mRNA levels were measured by real-time PCR in human ovarian surface epithelial (HOSE) cells and ovarian cancer cell lines. The box plots represent the expression levels of Reptin compared between HOSE and ovarian cancer cells. B. Reptin protein levels by Western blot. The intensities of the bands were measured, and the values corrected by a-actinin were represented in box plots. C. mRNA expression levels of Reptin were analyzed for ovarian cancer patients in Gene Expression Omnibus (GEO) database (GEO accession numbers: GSE27651, GSE26712, GSE18520 and GSE14407). Normal, Human ovarian surface epithelial (HOSE) tissue or cell. An asterisk (*) indicates a P value <0.05, a double asterisk (**) indicates a p value < 0.01, a triple asterisk (***) indicates a p-value < 0.001

2. Down-regulation of Reptin decreases cell proliferation, invasion and migration in ovarian cancer cells

To evaluate the function of Reptin in ovarian cancer cells, down-regulation of Reptin with siRNA was performed. We selected TOV112D and OVCA429, which presented high and moderate expression of Reptin mRNA and protein, respectively. Suppression of Reptin was performed using two specific siRNA targeted for Reptin. After transfection of siRNA into OVCA 429 and TOV112D, whole-cell lysates were collected, and down-regulation of Reptin with siRNA was confirmed (Figure 3A1). Cell proliferation of TOV112D and OVCA429 was measured by WST-1 assay at 0, 1, and 2 days, showing that cell proliferation was significantly reduced with siRNA of si-Reptin #1 and #2 at 2 days (Figure 3B). The cell cycle after knockdown of Reptin was evaluated in Figure 3C, confirming cell arrest in the G1 phase in TOV112D and OVCA429 after down-regulation of Reptin.

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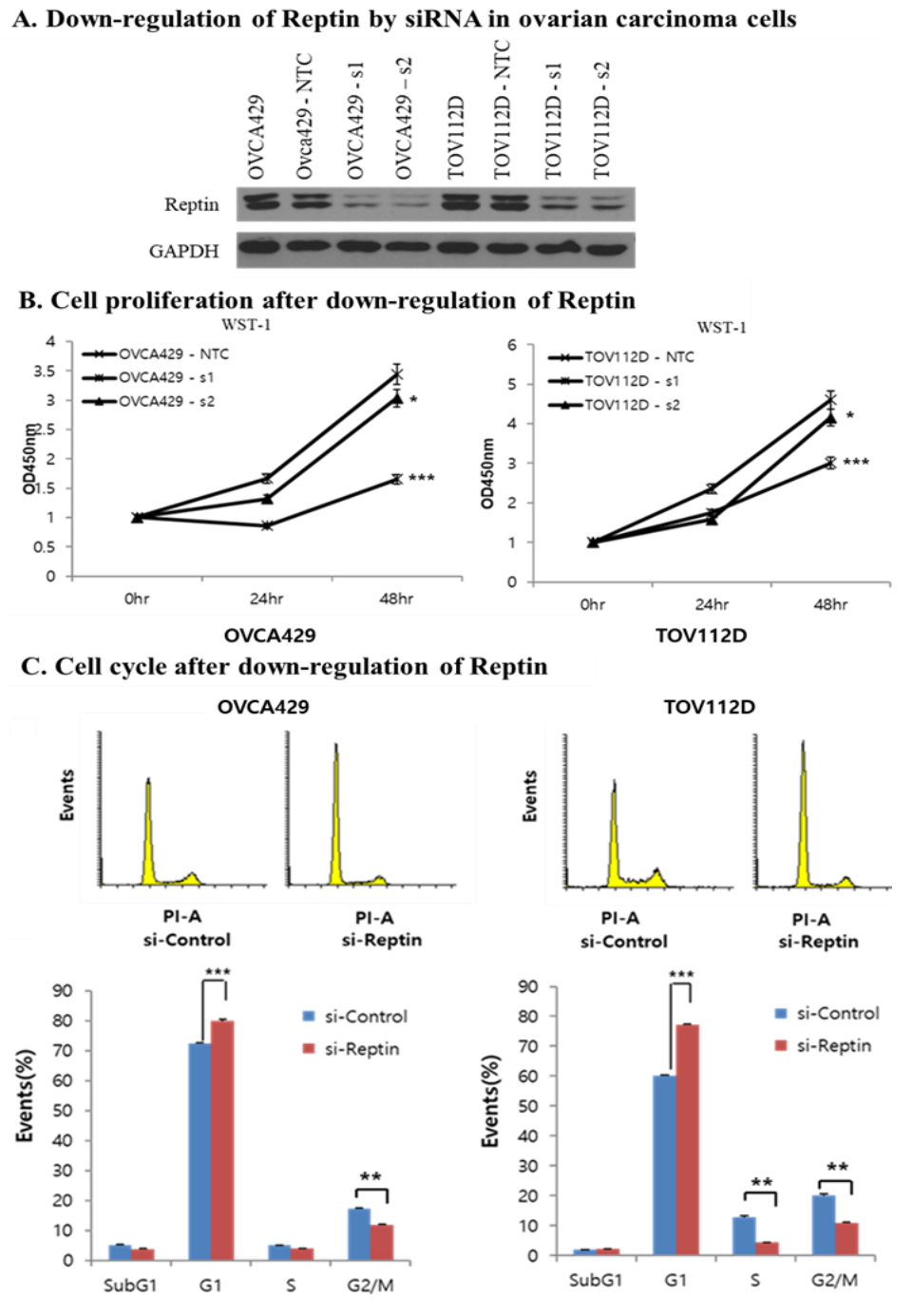

Figure 3. Effects of Reptin on proliferation and cell cycle of Reptin-knockdown ovarian cancer cells. A. Whole-cell lysates were collected from TOV112D,

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OVCA429 cells following Reptin knockdown by si-RNA#1(s1) and #2(s2). Expression of Reptin protein was analyzed by immunoblot. a-actinin was included as an internal loading control. B. Cell proliferation of TOV112D, OVCA429 was measured by WST-1 assay at 0, 1, and 2 days after siRNA transfection. Error bars represent the SD of triplicate experiments. C. Knockdown of Reptin in TOV112D and OVCA429 cells resulted in cell arrest in G1 phase of cell cycle. An asterisk (*) indicates a P value <0.05, a double asterisk (**) indicates a p value < 0.01, a triple asterisk (***) indicates a p-value < 0.001

Figure 4 shows a cell migration and invasion assay of down-regulated Reptin in ovarian cancer cells. Down-regulation of Reptin with siRNA significantly decreased migration and invasion of OVACA429 and TOV112D in ovarian cancer cells, as indicated in Fig. 4 A and B ($p<0.001$, all assay).The cell migration assay of OVCA429 and TOV112D presented decreased cell migration in si-Reptin #1 and #2 treated ovarian cancer cells relative to the negative control. Cell invasion in siRNA #1- and #2-treated OVCA429 and TOV112D decreased according to a Boyden chamber assay. In Figure 4C, matrix metalloproteinase (MMP) was investigated to evaluate migration and invasion activity after siRNA transfection in OVCA429 and TOV112D. The levels of MMP 1 and 2 in siRNA-treated TOV112D and OVCA429 were significantly decreased compared with the control group. The knock-down of Reptin expression by si-Reptin significantly decreased MMP expression in TOV 112D but not in OVCA429 ($p<0.05$ and $p=0.156$, respectively).

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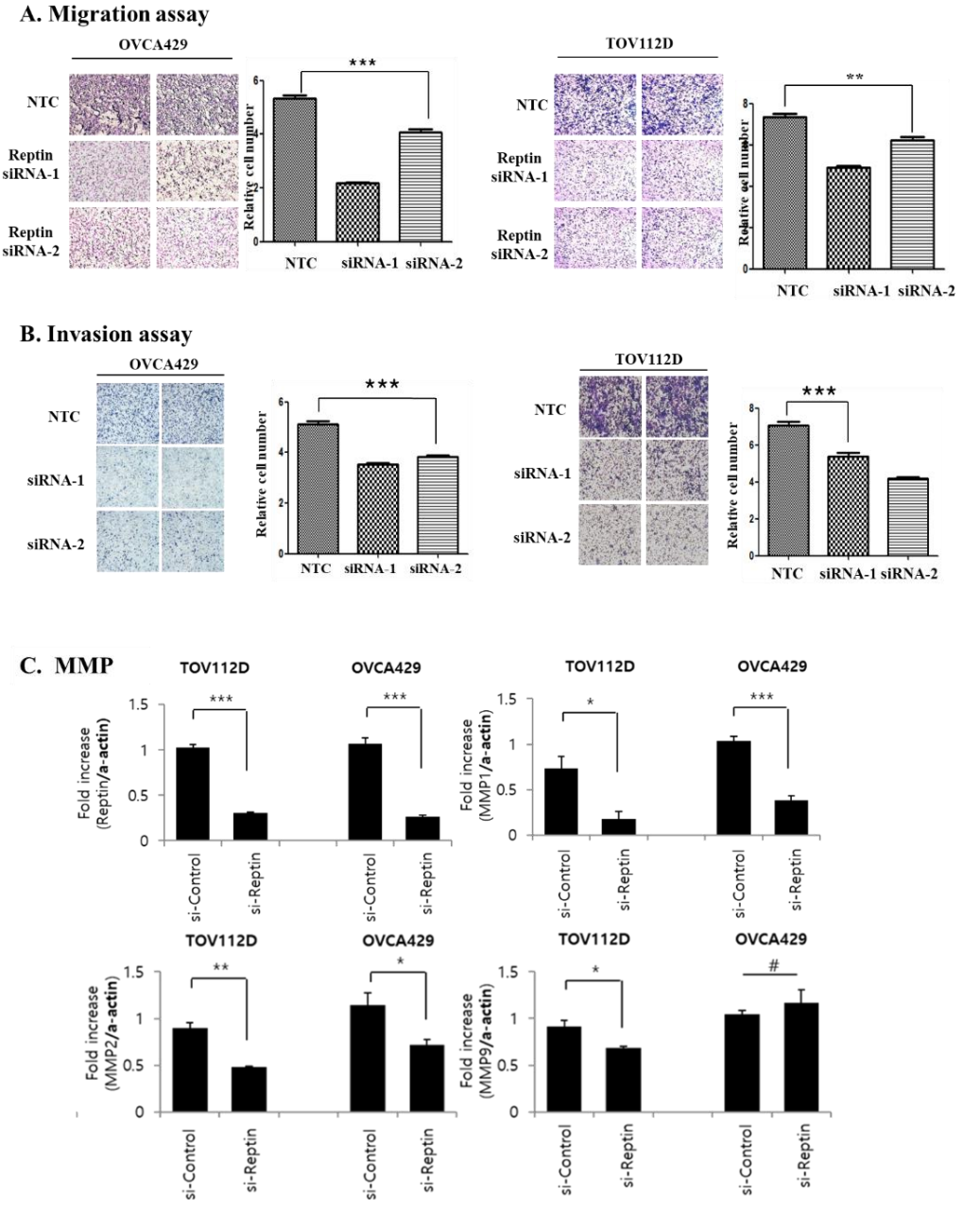

Figure 4. Invasion, migration assays of TOV112D, OVCA429 was determined by Boyden chamber assay 48 hr after siRNA transfection. A, B. Down-regulation of Reptin with siRNA significantly decreased migration and invasion

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of OVACA429 and TOV112D of ovarian cancer cells in Figure 4 A and B ($p<0.001$, all assay). C. Level of MMP 1 and 2 in siRNA-treated TOV112D and OVCA429 was significantly decreased compared with control group. The siRNA-treated TOV112D showed significantly decreased MMP9, whereas the siRNA-treated OVCA429 did not show any MMP9 ($p<0.05$ and $p=0.156$, respectively). An asterisk (*) indicates a P value <0.05, a double asterisk (**) indicates a p value < 0.01, a triple asterisk (***) indicates a p-value < 0.001

3. Down-regulation of Reptin reduces tumor growth in nude mice xenograft

To investigate the role of Reptin in tumor growth, an in vivo study with TOV112D entailing xenografts into nude mice was performed (Figure 5 A and B). At 5 weeks post-inoculation, tumor growth decreased with shRNA 1 and 2 volume in down-regulated Reptin tumor (Figure 5A). The mean tumor volume was measured to 4417.6 ±1209.1 mm^3 in pLKO.1 control, 2081.7 ±1151.9 mm^3 in siRNA 1 and 360.6 ±253.8 mm^3 in siRNA 2, showing statistical significance (Figure 5B) (p value < 0.05).

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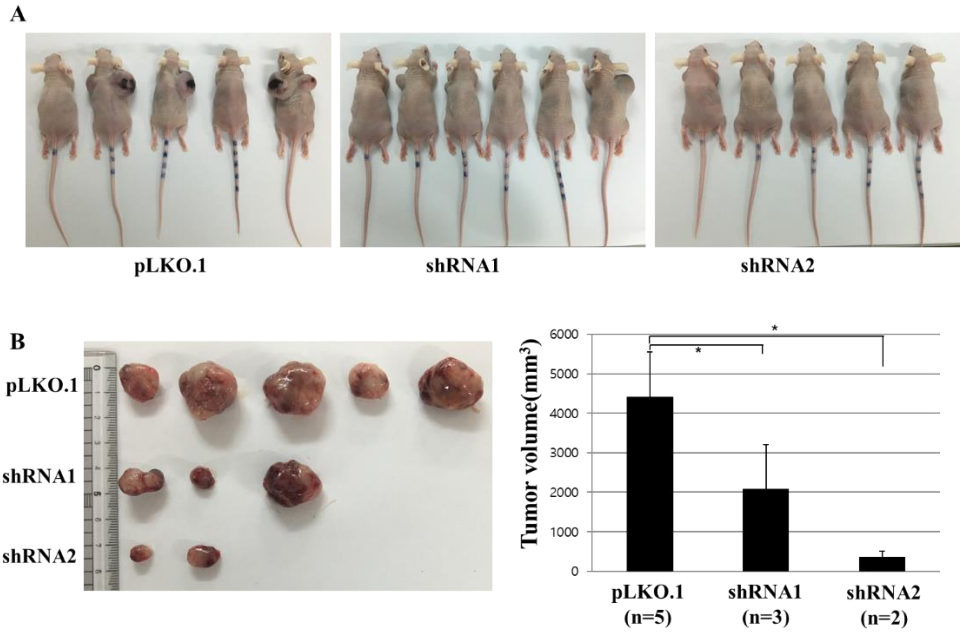

Figure 5. Down-regulation of Reptin reduces tumor growth in nude mice xenograft. Tumor growth decreased with shRNA 1 and 2 volume in down-regulated Reptin tumor at 5 weeks post-inoculation. asterisk (*) indicates a P value <0.05,

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IV. DISCUSSION

We previously identified Reptin overexpression in ovarian cancer by using immunoprecipitation of autoantibody and TAA. In the current study, we then further validated our findings, determining that Reptin was highly expressed in ovarian cancer and that its downregulation inhibited tumor cell proliferation, invasion, migration and growth in ovarian cancer.

Indeed, by IHC, Reptin protein was found to be highly expressed in ovarian cancer relative to normal ovaries and benign cysts. Reptin, as an ATP-dependent chromatin remodeling complex, has been known to function and exist in the nucleus as well. However, in the current study, Reptin was localized in the cytoplasm in addition to the nucleus as shown in Figure 1. This finding of cytoplasmic expression has also been detected in liver cancer[14]. Although Reptin has functions as a transcriptional factor in the nucleus, its existence in the cytoplasm has been determined to depend on the cancer type. Cytoplasmic redistribution of Reptin has been suggested to have a role in mitosis, having shown tubulin assembly/stabilization and remodeling of mitotic complex in hepatocellular carcinoma cells[15].

Reptin mRNA levels were significantly elevated in ovarian cancer cell lines and tissues relative to HOSE cells. The IHC results of this study indicated high expression of Reptin protein in ovarian cancer compared with normal, benign and borderline tumors of the ovary. In addition, high expression of Reptin protein showed decreased progression-free survival, even though only borderline significance of overall survival was observed on Kaplan-Meier plots. The survival significance of Reptin is rarely reported. Ren et al. reported that cytoplasmic expression of Reptin was correlated with poor overall survival in renal cell carcinoma[13]. Cytoplasmic expression of Reptin has been correlated with poor histologic grade in renal cell carcinoma. Our present results are the first reported on the prognostic significance of Reptin in ovarian cancer showing its cytoplasmic expression. The functional role of Reptin in the cytoplasm has not

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been revealed clearly, though cytoplasmic expression of Reptin has been suggested to be related to PIKK-mediated signaling in nonsense-mediated mRNA decay[16]. In addition, relocalization of Reptin is associated with a mitotic apparatus showing possible involvement in mitosis exit[15]. Reptin's functional role in the cytoplasm might be implicated in carcinogenesis; further investigation and determination, for example, of survival significance are required before any firm conclusions can be drawn in this regard.

After down-regulation of Reptin, ovarian cancer cells clearly showed decreased (inhibited) proliferation and arrested cell cycle in Figure3. This finding is concomitant with previous reports. Reptin is suggested to have an important role as a transcriptional oncogenic regulator through interaction with other transcriptional factors related to tumorigenesis[8,17,18]. Reptin-binding TIP60 complex as been reported to have histone acetyltransferase activity and to regulate chromatin remodeling, which is essential for DNA transcription[19]. Reptin was suggested to be an essential activator of MYC oncogenic protein, which acts as a transcriptional regulator and is involved in cell proliferation, the cell cycle, and anti-apoptosis[20,21]. The Reptin-binding MYC domain is essential for oncogenic transcription of cell proliferation and the cell cycle. A missence-mutant Reptin induced apoptosis and inhibited cell growth even though MYC was overexpressed. This finding indicates that Reptin is an essential activator of MYC oncogenic activation[21]. Similarly, Reptin has been reported to be a co-activator of transcriptional factor E2F1, which is a key regulator of the cell cycle[22]. Reptin was recruited by transcriptional factor E2F1, and Reptin/E2F1 complex regulated the promoter region of E2f target genes, eventually activating the cell cycle and tumor cell progression in hepatocellular carcinoma[22]. Similarly, cell-cycle arrest was confirmed by showing G1 phase arrest after down-regulation of Reptin in our results. Cell-cycle regulation of Reptin has been reported to be involved in telomerase activity and hTERT regulation[23]. Telomerase, comprising telomerase reverse transcriptase (TERT), the telomerase RNA component (TERC) and the

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TERC-binding protein dyskerin, protects telomere from DNA damage during the cell cycle. Reptin and Pontin complex are involved in facilitation of telomerase assembly by accumulation of TERT and TERC. Reptin-binding TERT has been shown to be higher in the S phase than in in G2 and M phases, and Reptin depletion is known to decrease telomerase accumulation, thereby demonstrating its important role in telomerase assembly[23].

Our results showed that down-regulation of Reptin inhibited cell invasion, migration and metastasis. This finding is consistent with a previous study on renal cell carcinoma cell lines[13]. Retin down-regulation induced vimentin depletion, which is a key marker of epithelial-mesenchymal transition (EMT) for cancer invasion, migration, and metastasis. Reptin was reported to decrease KAI1, one of the tumor suppressor genes, and antagonistic regulation of beta-catenin-Reptin and the Tip60 coactivator complexes against KAI1 gene increased tumor cell invasion in prostate cancer[10]. Unfortunately, our results did not indicate any association between Reptin expression and lymph node metastasis or tumor stage. Heterogenicity of ovarian cancer produces complex variables, and will require more investigation before any data relevant to clinical application is available.

V. CONCLUSION

Our data indicated that Reptin is highly expressed in ovarian cancer and is associated with poor progression-free survival outcome. Inhibition of Reptin decreased tumor cell growth and viability. Overall, our results suggest that Reptin may be an effective target in treatment of ovarian cancer.

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ABSTRACT(IN KOREAN)

과발현된 렙틴(Reptin)의 난소암에서 공격적 특성 촉진

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김 보 욱

목적: 렙틴(Reptin)은 에이티피아제(ATPase) 상과(superfamily) 일종으로 암화과정에서 다양한 역할을 한다. 하지만 난소암에서 생물학적 임상적 예후가 연구되지 않아 이번연구에서 난소암에서 렙틴의 기능적 역할과 임상적 의의를 연구하였다.

연구방법: 본 연구는 양성 난소 종양 175개, 경계성 난소종양 55개와 난소암 271개의 조직을 포함하였다. 렙틴의 발현을 확인하기 위해 면역조직화학 염색을 시행하고 렙틴의 발현과 임상적 요인들과 관련성을 연구하였다. 렙틴의 기능적 역할을 연구하기 위해 난소암 세포주에서 짧은 간섭 RNA로 렙틴 억제 후 세포주의 침습, 이동, 증식과 세포주기를 조사하고 동물실험으로 종양성장 여부를 측정하였다.

결과: 면역조직화학염색에서 난소암이 정상, 양성 및 경계성 종양에서 보다 렙틴의 발현이 높을 것을 보였다($p < 0.001$). 렙틴이 과발현 된 난소암 환자에서 무진행 생존이 감소하였으나($p=0.014$) 전체생존은 감소하는 경향을 보였다($p=0.071$).

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OVCA429와 TOV112D의 난소암세포주에서 렙틴을 짧은 간섭 RNA로 저발현 시켰을 때 세포증식이 저해되었고($p<0.001$) 난소암 세포의 이동과 침습이 감소하였다(각각 $p<0.001$). 렙틴을 저발현 시킨 세포주를 마우스에 이식하였을 때 종양의 성장이 비교군의 4.4 cc에 비해 실험군에서 2.1cc와 0.4cc로 의미 있게 성장이 감소하였다(각각 p value < 0.05).

결론: 렙틴은 난소암에서 과발현되었고 과발현 된 난소암 환자에서 무진행 생존이 감소하였다. 렙틴의 억제는 종양세포의 성장과 생존을 감소시켰다. 이러한 결과로 렙틴은 난소암의 중요한 치료 목표가 될 수 있다.

핵심되는 말: 렙틴, 난소암, 무진행 생존, 종양세포생존

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