ER-60 (PDIA3) is highly expressed in a newly established serous ovarian cancer cell line, YDOV-139

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Abstract. Characterization of a newly established serous ovarian cancer cell line, YDOV-139 was performed and *ER-60 (PDIA3)*, which was highly expressed in YDOV-139, was evaluated as novel biomarker for ovarian cancer. The YDOV-139 cell line was established using ascites samples from a 67-year-old Korean woman with recurrent ovarian cancer, and was characterized with respect to various biological and genetic features. Gene expression profiles were analyzed using cDNA microarrays, and proteomic evaluation was performed by two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight peptide mass fingerprinting (MALDI-TOF/PMF). Four candidate markers that were strongly up-regulated in YDOV-139 were validated by real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC). The epithelial-like characteristics of YDOV-139 were evident from morphologic studies, and the average population doubling time was 120 h. When transplanted into nude mice, YDOV-139 cells successfully induced tumor masses in all three animals. Chemosensitivity tests showed that gemcitabine had the highest chemosensitivity index against YDOV-139 cells. HLA typing revealed A*24/A*31, B*07/B*35, Cw03*(09)/w*07, and DRB1*01/DRB1*15 alleles. Compared with human ovarian surface epithelial (HOSE) cells, 2,520 genes and 23 protein spots were differentially expressed in YDOV-139. Validation by real-time PCR showed that mRNA expression of *LCN2, MDK, SLCO4A1*, and *ER-60 (PDIA3)* were strongly elevated in ovarian cancers. In IHC analysis, ER-60 *(PDIA3)* was significantly overexpressed in both borderline tumors and invasive ovarian cancers (P<0.001). The molecular characteristics of YDOV-139 may have implications for future ovarian cancer research and *ER-60 (PDIA3)* should be investigated further as a potential biomarker of ovarian cancer.

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Introduction

Among gynecological cancers, ovarian cancer was the second most common cancer and the one with the highest mortality rate in the United States in 2008. Annually, 22,000 women in the US newly develop ovarian cancer and 15,500 die from the disease (1). In Korea, Central Cancer Registry data showed that 1,300 people newly developed ovarian cancer and 700 people died from the disease in 2004 (2). Although CA 125 is the most widely available tumor marker for ovarian cancer, with a validated role in monitoring ovarian cancer, its sensitivity and specificity may not be sufficient for screening the general population (3,4).

The difficulty in studying ovarian cancer results from its extreme heterogeneity and complexity. Because each ovarian cancer may have different genetic mutations and protein expression within the same subtype, it is very difficult to formulate a general disease model. Studies of various ovarian cancer cell lines with distinct biologic properties are required to overcome such problems (5-7).

In the present study, a new cell line designated YDOV-139 was established from a frequently relapsing but chemosensitive case of stage IIIc serous ovarian adenocarcinoma, and its general characteristics and genetic features were determined. Differentially expressed genes were analyzed using cDNA microarray, and protein expression was studied through 2-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption ionization-time of flight peptide mass fingerprinting (MALDI-TOF/PMF). Furthermore, validation studies were performed for *LCN2, MDK*, and *SLCO4A1*, which were

overexpressed in microarray analysis, and *ER-60 (PDIA3)*, which was overexpressed in proteomic analysis.

Patients and methods

Patient information. The YDOV-139 was derived from a 67-year-old patient with recurrent ovarian cancer. She was diagnosed with stage IIIc ovarian cancer after receiving staging laparotomy in March 2004, and then received 6 cycles of chemotherapy with paclitaxel and carboplatin. In March 2005, she was diagnosed with recurrent ovarian cancer and underwent 9 cycles of belotecan. Due to repeated cancer recurrence she subsequently received 6 cycles of paclitaxel and cisplatin in January 2006, docetaxel and cisplatin in October 2006, gemcitabine and cisplatin in November 2007, and 4 cycles of carboplatin in May 2008. In October 2008, she refused any further treatment despite medical advice. Although she had frequent recurrences, the patient was chemosensitive with an interval of at least 6 months from remission to relapse.

Primary culture and culture conditions. The YDOV-139 originated from malignant ascites harvested with informed consent by aspiration on the patient's first recurrence. Ascites samples were centrifuged at 18˚C and 1500 rpm for 35 min using Ficoll (Sigma, St. Louis, MO) and the cells were collected. After washing with phosphate-buffered saline (PBS) the cells were resuspended in media and cultured at 37˚C in a 5% CO_2 humidified atmosphere. They were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Calabasas, CA). Human ovarian surface epithelial (HOSE) cells were scraped with a sterile scalpel into DMEM containing 10% FBS, and cultured at 37˚C in a 5% CO_2 incubator.

In vitro morphology. Morphology of the YDOV-139 was observed by phase-contrast microscopy (Olympus, Tokyo, Japan) and Philips CM 10 transmission electron microscopy (TEM; Philips Scientifics, Eindhoven, The Netherlands). For observation with TEM, the specimen was prefixed with 2% glutaraldehyde and paraformaldehyde, postfixed with 1.33% osminum tetroxide, washed with alcohol, substituted with propylene oxide, and embedded in Epon mixture. Samples were cut into ultrathin sections, stained with uranyl acetate and lead citrate, and their ultrastructures were examined.

Detection of mycoplasma. The procedures of polymerase chain reaction (PCR) for mycoplasma contamination of the cell lines were the same as previously described (8,9).

Growth properties. The growth curves and population doubling times over 50 successive passages were analyzed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. Viable cells were seeded into 96-well plates with 100 μl culture media ($3x10^3$ cells/well), and FBS was added to create two different condition groups, 0 or 10% FBS. Then 20 μl MTS (modified tetrazolium salt) solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was added and cells were cultured for 4 h in a 37˚C incubator. The optical density (OD) value at 490 nm was measured using an ELISA reader (EL311; Bio-Tek Instruments, New York, NY). This analysis was performed every day during the first four days and every other day thereafter.

Tumor marker secretion. The concentration of CA 125, CA 19-9, CA 15-3, and CEA in the supernatant of $1.5x10^6$ cultured ovarian cancer cells was measured by an enzyme immunoassay method using the Architect system (Abbott Diagnostics, Abbott Park, IL).

Chemosensitivity test. The procedures of adenosine triphosphate-based chemotherapy response assay (ATP-CRA) for chemosensitivity test were as previously described (9).

Human leukocyte antigen typing. Molecular typing of HLA-A, -B, and -Cw was performed using an allele specific PCR-sequencing based typing (SBT) method according to the manufacturer's instructions (HLA-SBT; Biosewoon Inc., Seoul, Korea). Briefly, genomic DNA was extracted by salting out and ethanol precipitation according to the method described by Miller *et al* (10). PCR amplification and sequencing reactions were performed with locus-specific primers for exons 2/3. After purification, products were loaded, automated, and analyzed with BioSewoon HLA analyzer (Biosewoon Inc.). For Class II, HLA-DRB1 was typed using the PCR-SSOP (sequence specific oligonucleotide probe) method with locus-specific primers for exon 2.

DNA fingerprinting. DNA fingerprints of YDOV-139 and 4 ovarian cancer cell lines were identified using the AmpFlSTR® Profiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Nine short tandem repeat (STR) loci and the gender marker amelogenin were amplified by PCR and the products were analyzed with an ABI 3730 DNA sequencer (Applied Biosystems). The YDOV-157 and YDOV-151 cell lines were established in our institute and the OVCA429 and RMUG-S cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

p53 exon 4 mutational analysis. PCR-restriction fragment length polymorphism (RFLP) analysis was used to analyze mutation of the p53 gene at codon 72 in exon 4. DNA was collected from the tumor cells using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and PCR was performed in a 25 μl reaction mixture containing 100 ng genomic DNA template, 5 pmol of each primer (sense 5'-TCCTCTGACTGCTCTTTTCACC-3', antisense 5'-GGCTGGCTTCCATGAGACTTCA-3'), 5 U Tag DNA polymerase (iNtRON Biotechnology Inc., Seongnam, Korea), 200 μM dNTPs, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR mixtures were heated to 94˚C for 5 min for initial denaturation, followed by 35 cycles at 94˚C for 30 sec, 68˚C for 90 sec, and final extension at 72˚C for 3 min. The PCR products were digested with 10 U BstUI (New England Biolabs, Beverly, MA) for 4 h at 60˚C, separated by 2% agarose gel electrophoresis, and stained with ethidium bromide.

BRCA1 and BRCA2 mutation analysis. BRCA1 and BRCA2 mutations were analyzed by PCR-DHPLC (denaturing high-performance liquid chromatography) and sequencing as previously reported (11). DNA was collected from the tumor cells using QIAamp DNA Blood Mini kit (Qiagen), and PCR was performed using 81 primer pairs (12,13) in a 25 μl reaction containing 200 ng DNA template, 10 pmol of each primer, 5 U Tag DNA polymerase (iNtRON Biotechnology Inc.), 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The DHPLC analysis was performed using the WaveMaker System (Transgenomic Inc., San Jose, CA) and abnormal PCR products were purified by QIAquick PCR purification Kit (Qiagen) according to the manufacturer's instructions. Bidirectional sequencing and analysis were carried out using the BigDyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

Tumorigenicity assay. YDOV-139 cells ($5x10^6$ cells) were subcutaneously transplanted into 5-week-old athymic female nude mice (BALB/c nu/nu, n=3). At 8 weeks after injection the animals were sacrificed for evaluation of tumor formation, metastasis, and histopathology.

Gene expression profiling using cDNA microarray analysis. Gene expression profiling was performed as previously reported using cDNA microarrays (9). Briefly, total RNA was extracted from YDOV-139 and 3 HOSE cells (HOSE 198, 209, and 211) using TRIzol (Invitrogen, Carlsbad, CA) and purified using RNeasy columns (Qiagen). After processing with DNase digestion and clean-up procedures, total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX) to yield biotinylated cRNA, which was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

For microarrays, 750 ng of labeled cRNA was hybridized to each Sentrix Human Ref-6-V2 Expression Bead Chip (Illumina Inc., San Diego, CA) and the array signal was detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Arrays were scanned with an Illumina Bead Array Reader, and array data processing and analysis was performed using Illumina Bead Studio software (Illumina Inc.). Data were extracted from the final analysis involving 26956 probes using Bead Studio v. 1.0.0.5 software (Illumina Inc.) and normalized by quantile normalization.

Protein analysis by 2-DE and MALDI-TOF/PMF. Protein analysis of YDOV-139 and HOSE 198 was carried out using 2-DE and MALDI-TOF/PMF. Isoelectric focusing (IEF) was performed with 100 μg protein sample applied to a 13-cm immobilized non-linear pH 3-10 NL strip at 20˚C using Ettan IPGphor II (Amersham Biosciences Co., Piscataway, NJ). After rehydration of the IPG gel strips in a re-swelling solution (Amersham Biosciences Co.) for 24 h at 20˚C, focusing was performed with the following steps: 50, 500, 1000, and a final focusing step of 60,000 Vh at 8000 V. The IPG strips were then equilibrated with buffer (6 M urea, 75 mM Tris-HCl, 29.3% glycerol, 2% SDS), reduced with 1% DTT for 15 min, and alkylated with 2.5% iodaacetamide for 15 min. For SDS gel electrophoresis, the equilibrated IPG gel strip was laid on top of a 10% SDS gel covered with 0.5% agarose solution. Protein spots on the 2DE gel were silver stained, scanned with ImageScanner, and analyzed using ImageMaster™ 4.01 software (Amersham Biosciences Co.).

Differentially expressed protein spots were excised from the 2-DE gel, destained, and in-gel digested using modified porcine trypsin as previously described by Shevchenko *et al* (14). Purified tryptic peptide mixtures were loaded onto a target plate and analyzed using an Ettan MALDI-TOF (Amersham Biosciences Co.). Peptides were evaporated with a N2 laser at 337 nm and spectra were obtained in reflectron mode using a 20 kV acceleration voltage and a delayed extraction approach. Each spectrum was the cumulative average of 300 laser shots and internal calibration was performed with trypsin auto-digestion ion peak at m/z 842.510 and 2211.1046. The search program ProFound, developed by Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting.

SYBR-Green real-time PCR. RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA by the SuperScript™ III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. The expression of 4 candidate gene mRNA was measured by SYBR-Green real-time PCR using an ABI 7300 instrument (Applied Biosystems). PCR was performed in 20 μl buffer containing 2 μl cDNA, 5 pM each primer, and power SYBR-Green PCR master mix (Applied Biosystems) and with the following cycle conditions: 50˚C pre-incubation for 2 min, 95˚C denaturation for 10 min, 40 cycles of 95˚C denaturation for 15 sec followed by annealing and extension at 60˚C for 1 min.

The relative gene expression of *ER-60* was compared between 8 HOSE cell types, 13 ovarian cancer cell lines and 8 ovarian cancer tissues. For *LCN2, MDK*, and *SLCO4A1*, relative gene expression was compared between 8 HOSE cell lines and 11 ovarian cancer cell lines. Among the ovarian cancer cell lines, the SNU-840 cell line was purchased from Korean Cell Line Bank (KSCLB, Seoul, Korea) and SKOV3, TOV 112D, OVCA 429, OVCA 433, and RMUG-s cell lines were from American Type Culture collection (ATCC). YDOV-13, YDOV-105, YDOV-139, YDOV-151, YDOV-157 and YDOV-161 were established in our institute and the DOV13 cell line was kindly donated by Dr Samuel C. Mok (University of Texas M.D. Anderson Cancer Center, Houston, TX, USA).

Immunohistochemistry. Immunostaining of *ER-60 (PDIA3)* was performed with PDIA3 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Paraffinized sections were treated with xylene (5 min x 3 times), and hydrated with 100, 95, 85 and 75% ethanol. Sections were immersed in 135 ml distilled water with 15 ml retrieval solution and heated in a microwave oven for 10 min, followed by washing in cold running water and blocking with 3% hydrogen peroxidase for 5 min. The slides were rinsed in PBS, boiled for 10 min, and then incubated with diluted primary antibodies overnight at 4˚C. After a thorough wash with PBS, the slides were incubated with an ACE chromogen solution (Dako Cytomation, Carpinteria, CA), counterstained with hematoxylin, and mounted.

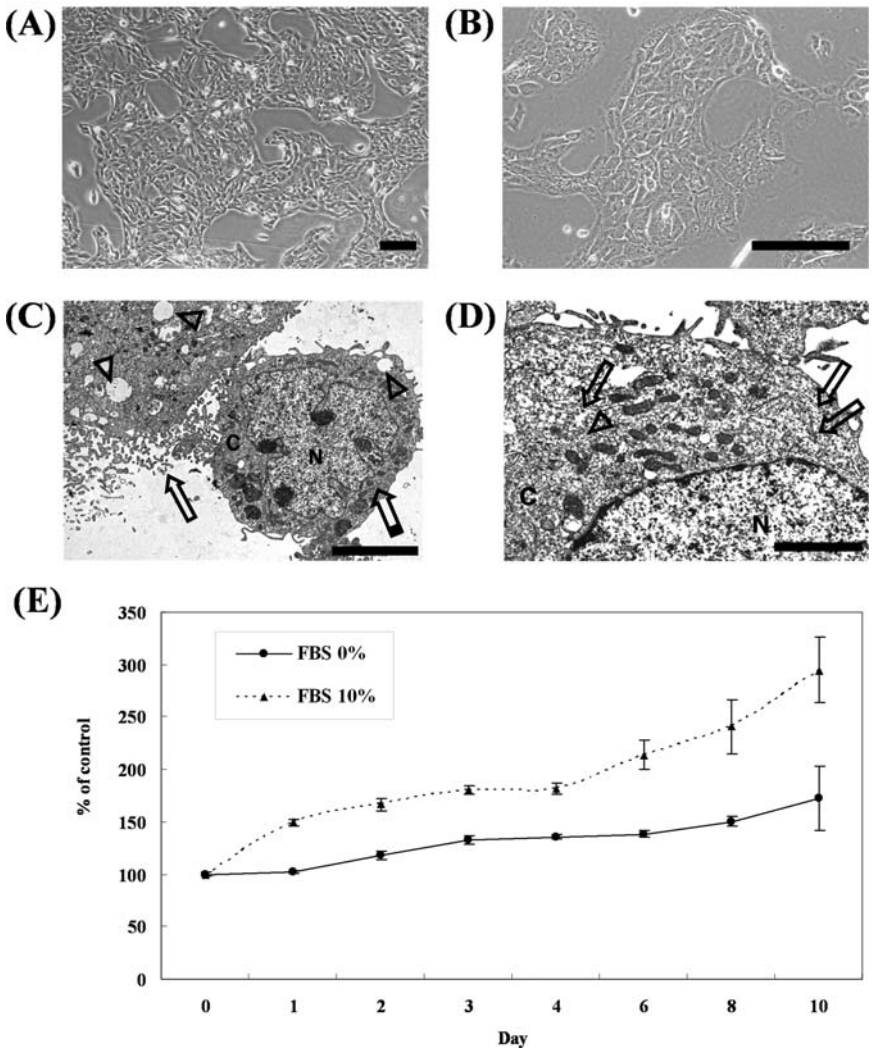

Figure 1. General features of the ovarian cancer cell line YDOV-139. Phase-contrast microscopy of YDOV-139 cell line derived from ascites of a patient with serous ovarian cancer (bars, 100 μm). (A) x40, (B) x100. The majority of the cells have a polygonal appearance with a large round nucleus. (C) Ultrastructural aspects of YDOV-139 cells by transmission electron microscopy. The tumor cells reveal high a N/C ratio and show abundant short blunt microvilli at the cell surface (arrow), abundant mitochondria, glycogen (dashed arrow), amorphous annulated lamellae, and possible secretory vesicles (arrowhead) in the cytoplasm (x6000; bar, 5000 nm). (D) Higher magnification shows desmosomal-tonofilament complexes (arrow), abundant mitochondria, and glycogen (arrowhead) (x15,000; bar, 2000 nm). (E) *In vitro* growth curve of YDOV-139 cell cultured in a 5% CO_2 incubator at 37˚C. Cells were cultured in 96-well plates ($3x10^3$ cells/well) in media containing 0% (●) or 10% (▲) FBS. Each point represents the mean of 6-wells.

Normal ovarian epithelial tissues, benign and borderline ovarian tumors, and ovarian cancer tissues were evaluated for *ER-60* expression by a scoring system corresponding to the sum of staining intensity and percentage of positive cells (15). Differences between groups were evaluated by the sum of intensity and cell count score. The slides were scored in the absence of any clinical data, and the final score was the average of scores from three observers.

Results

Establishment of the YDOV-139 cell line. The YDOV-139 was directly established *in vitro* from cells isolated from malignant ascites. The seeded cells began to proliferate 5 days after initiation of cell culture. The cells grew in an adherent mono-layer with loss of contact inhibition and evidence of cellular piling was noted at densities $>1x10^5/cm^2$. The first passage of the cell line was performed 14 days after primary cell seeding and after that the line was been passaged >80 times. The YDOV-139 was confirmed to be free of mycoplasma contamination.

General features of cultured cells. Phase-contrast microscopy revealed that the cells grow as islands of polygonal epithelial cells with a pavement-like arrangement and large round nuclei (Fig. 1A and B). Transmission electron microscopy showed epithelial morphologic features of blunt microvilli at the cell surface with abundant mitochondria, glycogen, amorphous annulated lamellae, and secretory vesicles in the cytoplasm, whereas higher magnification revealed desmonal-tonofilament complexes (Fig. 1C and D). Analysis of the *in vitro* growth curve showed a population doubling time of 120 h. Fig. 1E shows the individual growth kinetics curve according to FBS concentration.

Tumorigenicity of cultured cells. Subcutaneous xenografting of YDOV-139 into 3 nude mice resulted in a palpable tumor mass at the site of inoculation after 8 weeks. There was a 100%

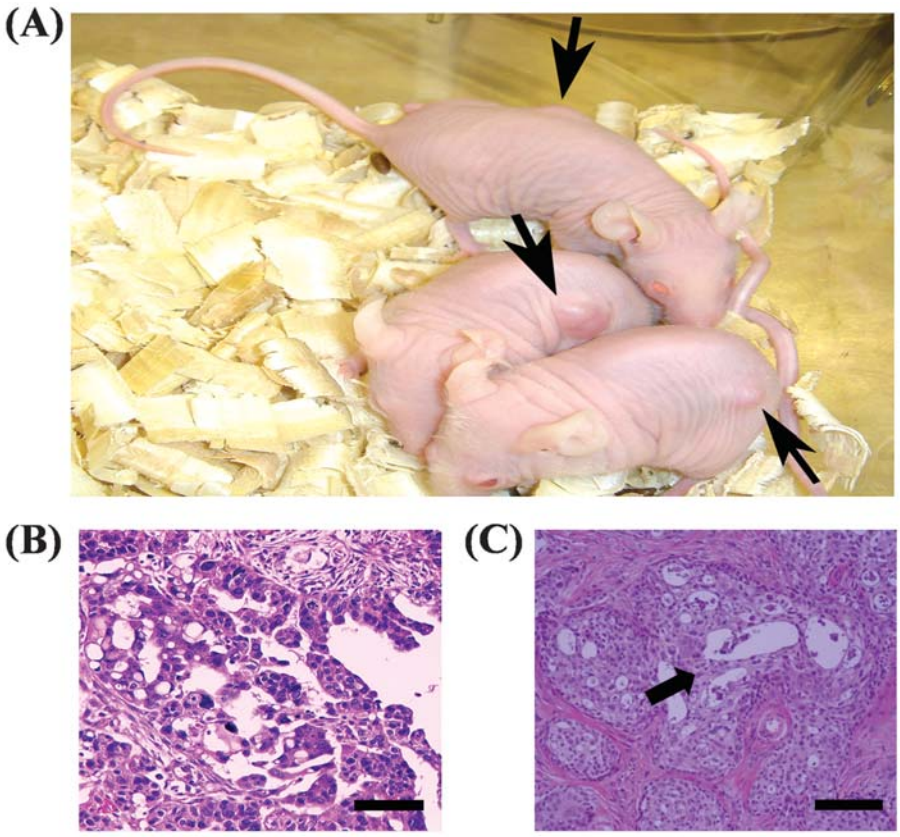

Figure 2. Xenografts of YDOV-139 cells into nude mice. YDOV-139 cells ($5x10^6$) were subcutaneously injected into the back (dorsum) of nude mice, resulting in the formation of tumor mass in all three strains (A). Histopathology of the original ovarian cancer (B) and xenograft mass (C) showing features of microcalcification (arrow) resembling serous carcinoma. Bars (B and C), 100 μm.

Table I. DNA fingerprinting of 5 ovarian cancer cell lines.

	STR locus									
	D3S1358	*vWA*	*FGA*	*AMEL*	*TH01*	*TPOX*	*CSF1PO*	*D5S818*	*D13S317*	*D7S820*
YDOV139	16	17	22	X	7	6, 9	11, 12	12	11	11
YDOV157	15, 16	17	20, 23	X	8, 9	12	11, 12	11, 13	9	10, 11
YDOV151	15, 16	17	25	X	8, 9	6	12	12	12	11, 12
OVCA429	15,16	16, 18	24	X	9	9, 11	12, 13	11, 12	12	11,12
RMUG-S	13	14, 16	19, 24	X	9	8, 11	12	10, 14	10, 11	12

incidence of tumors in the 3 mice that were tested (Fig. 2A). Histopathologically, the transplanted tumor cells closely resembled the morphology of the original serous carcinoma (Fig. 2B) and showed features of microcalcifications (Fig. 2C). There was no evidence of distant metastasis.

Tumor marker secretion and chemosensitivity of YDOV-139. Compared with the upper limits of normal serological concentration of ovarian tumor makers, CA 125 (2487.8 U/ml) and CA 19-9 (2476.6 U/ml) were strongly elevated in YDOV-139 cells whereas CA 15-3 (13.1 U/ml) and CEA (5.3 ng/ml) were not elevated. In the chemosensitivity test, YDOV-139 showed the highest drug sensitivity to gemcitabine (chemosensitivity index: 157.3) but showed low drug sensitivity to carboplatin (chemosensitivity index: 248.8) and paclitaxel (chemosensitivity index: 282.6). Detailed results of chemosensitivity tests are illustrated in Fig. 3.

Genetic and phenotypic features. HLA Class I phenotypes of the YDOV-139 (ABC allele1/allele2) were A*24/A*31 B*07/B*35 Cw03*(09)/w*07 and HLA Class II phenotypes (DRB1 allele1/allele2) were DRB1*01/ DRB1*15. The DNA fingerprinting results of 5 ovarian cancer cell lines are listed in Table I. PCR-RFLP analysis revealed wild-type p53. BRCA mutational analyses detected no mutations in BRCA1, but 5 polymorphisms and 3 missense mutations in BRCA2 (Table II).

Gene expression profiling. Gene expression profiles of YDOV-139 were generated using cDNA microarray. Using

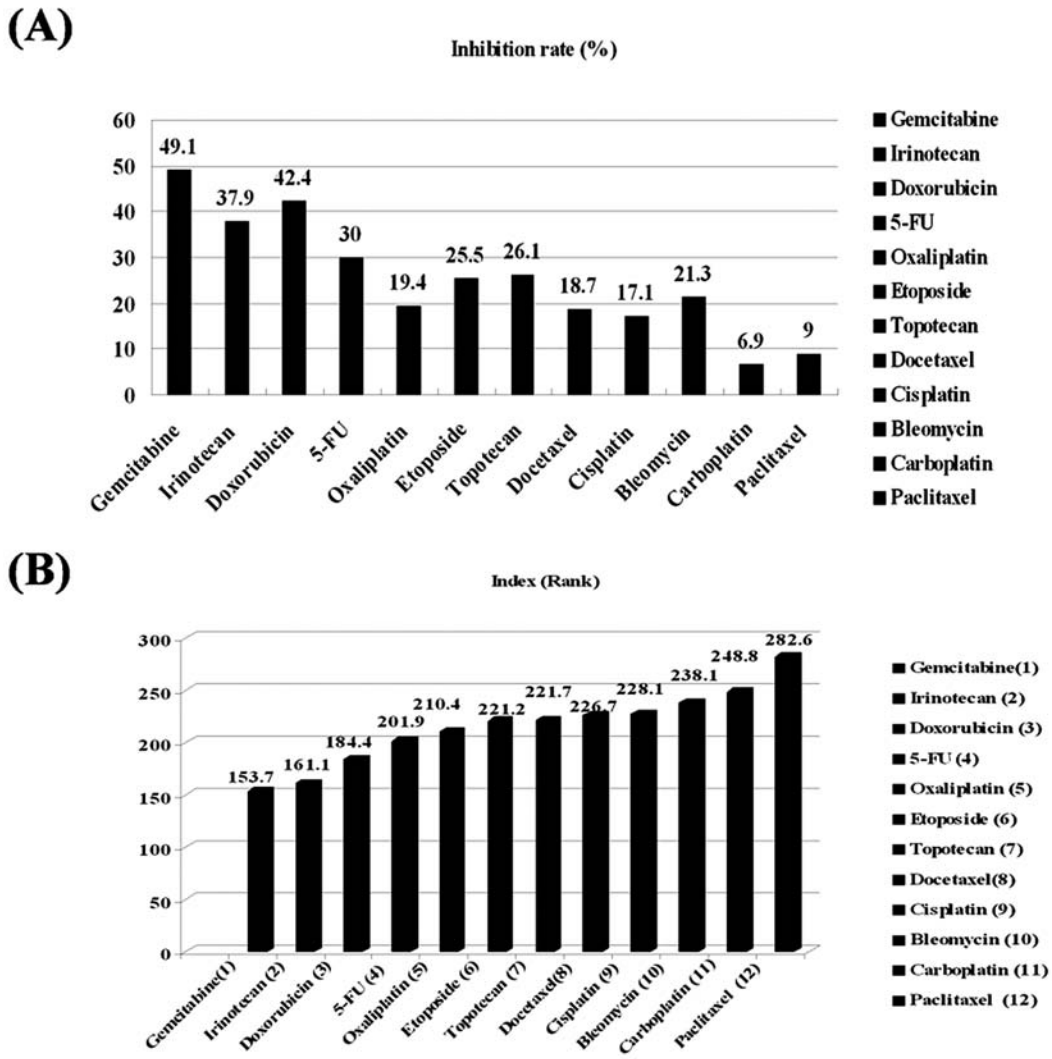

Figure 3. Chemosensitivity test of YDOV-139 cell line. Growth inhibition rate (A) and chemosensitivity index (B) of 12 chemotherapeutic agents for the YDOV-139 cell line. Chemosensitivity index = 300 - SUM (cell suppression 0.2x-5x), Rank, more desirable in drug test.

Table II. BRCA2 mutational analyses of YDOV-139.

Mutation: location change (amino acid change)	Mutation type	Mutation effect
Exon 10: 1093 A>C (N289H)	Missense mutation	Polymorphism
Exon 10: 1342 C>A (H372N)	Missense mutation	Polymorphism
Exon 10: 1593 A>G (S455S)	Polymorphism	Polymorphism
Exon 11: 2457 T>C (H743H)	Polymorphism	Polymorphism
Exon 11: 3199 A>G (N991D)	Missense mutation	Unclassified variant
Exon 14 (int14): 7663+53 C>T	Polymorphism	Polymorphism
Exon 17 (int16): 8034-14 T>C	Polymorphism	Polymorphism
Exon 22 (int21): 8983-66 T>C	Polymorphism	Polymorphism

A, adenine; T, thymine; G, guanine; C, cytosine; N, asparagine; H, histidine; S, serine; D, aspartic acid.

the Hierarchical clustering analysis, 2,520 genes were differentially expressed in YDOV-139 (>2-fold, $P<0.05$). Among these 2,520 differentially expressed genes, 1,108 were up-regulated and 1,412 were down-regulated in YDOV-139. To evaluate the relationship between these genes and functional pathways of ovarian carcinogenesis, selected genes showing ≥8-fold difference in expression were grouped according to their biological function (Table III).

Table III. Selected genes that were up- or down-regulated at least 8-fold in YDOV-139 vs. HOSE cells.

Target ID	Definition	Symbol	Fold[a]	Accession no.
Up-regulated genes in YDOV-139				
Cell adhesion				
ILMN_23335	Epithelial V-like antigen 1	EVA1	49.33	NM_005797.2
Cell cycle control				
ILMN_24793	Cell division cycle 2, G1→S and G2→M	CDC2	15.50	NM_001786.2
Cell proliferation and differentiation				
ILMN_4070	Tumor-associated calcium signal transducer 1	TACSTD1	102.39	NM_002354.1
ILMN_3786	Midkine (neurite growth-promoting factor 2)	MDK	43.95	NM_001012334.1
Cell structure				
ILMN_7397	Spondin 1, extracellular matrix protein	SPON1	12.25	NM_006108.1
ILMN_13755	Claudin 7	CLDN7	67.35	NM_001307.3
Nucleotide and nucleic acid metabolism				
ILMN_19849	Topoisomerase (DNA) II alpha 170 kDa	TOP2A	14.81	NM_001067.2
ILMN_3407	Minichromosome maintenance deficient 4	MCM4	9.80	NM_005914.2
ILMN_7414	Lamin B receptor	LBR	8.95	NM_002296.2
ILMN_30154	Thymidine kinase 1, soluble	TK1	32.13	NM_003258.1
Oncogenesis				
ILMN_16236	Runt-related transcription factor 3, transcript variant 2	RUNX3	32.26	NM_004350.1
ILMN_20794	Lipocalin 2 (oncogene 24p3)	LCN2	111.19	NM_005564.2
Protein biosynthesis and modification				
ILMN_26083	Chromosome 12 open reading frame 46	C12orf46	34.62	NM_152321.1
ILMN_12352	Serine/threonine kinase 6	STK6	11.44	NM_198434.1
Proteolysis				
ILMN_14210	Protease, serine, 8 (prostasin)	PRSS8	55.04	NM_002773.2
ILMN_11560	Kallikrein 6 (neurosin, zyme)	KLK6	58.23	NM_002774.3
Signal transduction				
ILMN_2234	Gap junction protein, beta 3, 3 1kDa (connexin 31)	GJB3	10.93	NM_001005752.1
ILMN_3066	Insulin-like growth factor binding protein 5	IGFBP5	27.97	NM_000599.2
Ion transport				
ILMN_3183	Solute carrier organic anion transporter family, member 4A1	SLCO4A1	23.28	NM_016354.3

Table III. Continued.

Target ID	Definition	Symbol	Fold[a]	Accession no.
Down-regulated genes in YDOV-139				
Apoptosis				
ILMN_28684	Tumor necrosis factor receptor superfamily, member 19	TNFRSF19	-13.84	NM_148957.2
Cell proliferation and differentiation				
ILMN_3875	Vascular cell adhesion molecule 1	VCAM1	-25.88	NM_001078.2
ILMN_25295	Junctional adhesion molecule 3	JAM3	-29.95	NM_032801.3
Cell structure				
ILMN_676	Vimentin	VIM	-111.60	NM_003380.2
ILMN_1052	Tropomodulin 1	TMOD1	-22.59	NM_003275.1
ILMN_17132	Talin 2	TLN2	-10.30	NM_015059.1
Homeostasis				
ILMN_24349	Aquaporin 1 (Colton blood group)	AQP1	-34.12	NM_198098.1
Immunity and defense				
ILMN_16098	Thrombospondin 2	THBS2	-104.73	NM_003247.2
Nucleotide and nucleic acid metabolism				
ILMN_6405	REC8-like 1 (yeast)	REC8L1	-39.25	NM_005132.1
Tumor suppressor				
ILMN_1781	Wilms tumor 1	WT1	-13.94	NM_024425.2
Protein biosynthesis and modification				
ILMN_6510	Glutaminyl-peptide cyclotransferase	QPCT	-27.34	NM_012413.3
Signal transduction				
ILMN_15406	Phosphoinositide-3-kinase, catalytic, delta polypeptide	PIK3CD	-17.29	NM_005026.2
ILMN_139202	Doublecortin domain containing 2	DCDC2	-20.77	NM_016356.1

[a]YDOV-139/HOSE. One-way ANOVA and LPE tests were applied to determine differentially expressed sets of genes across three experimental groups. In this analysis, 2,520 genes were selected for the following common conditions; i) P-value of ANOVA <0.05; ii) |Fold (B/A)| >2 and P<0.05. The statistical software used was Avadis Prophetic version 3.3 (Strand Genomics, Bangalore, India).

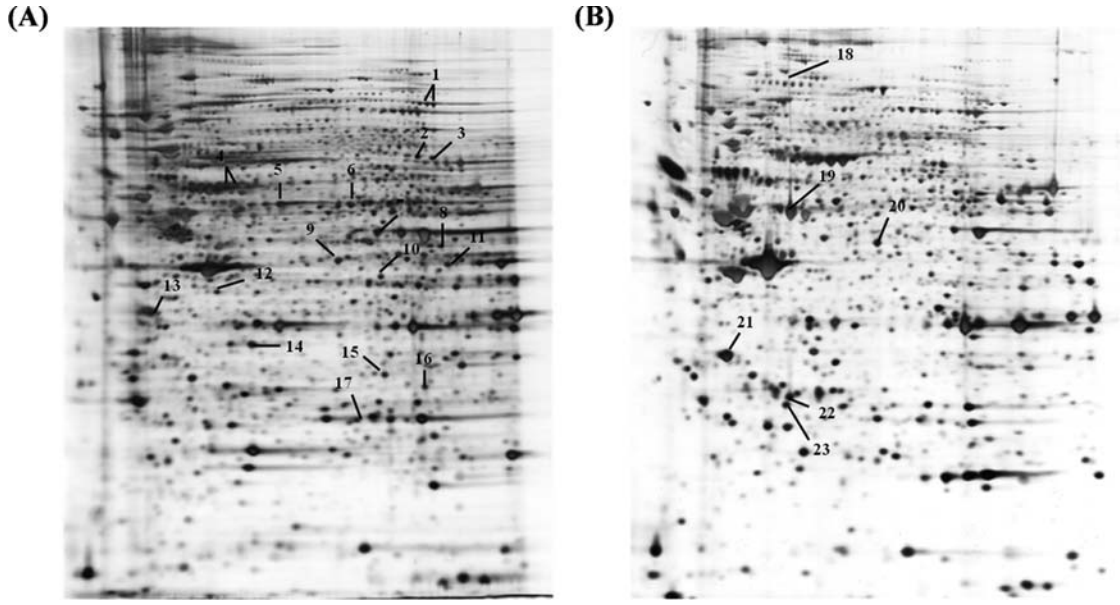

Figure 4. 2D-electrophoresis of YDOV-139 compared with HOSE. Proteomic profiling of YDOV-139 (A) and HOSE cell (B) using 2D electrophoresis and MALDI-TOF peptide mass fingerprinting. Black lines indicate identified relatively abundant or weak protein spots in YDOV-139.

Protein analysis of YDOV-139. Comparison between YDOV-139 and HOSE cells by 2-DE revealed differences in a number of protein spots (Fig. 4). Among these differently expressed spots, 23 proteins were identified by MALDI-TOF/PMF, as summarized in Table IV.

Validation studies. SYBR-Green PCR was performed to validate the data from cDNA microarray and MALDI-TOF/PMF. For *LCN2, MDK*, and *SLCO4A1* that were up-regulated by microarray, PCR showed expression differences between 8 HOSE cells and 11 cancer cell lines but with borderline statistical significance. For *ER-60 (PDIA3)*, PCR showed significant differences in expression among 13 ovarian cancer cell lines ($P<0.05$) and 8 ovarian cancer tissues ($P<0.05$) compared with 8 HOSE cell types (Fig. 5).

Because only *ER-60* showed significant mRNA over-expression in PCR analysis, further validation of ER-60 was performed by IHC. When analyzed in relation to histopathologic characteristics, the immune staining scores of ER-60 were significantly increased in borderline and invasive ovarian cancers ($P<0.001$) (Fig. 6). Different expression of *ER-60* was also found according to tumor differentiation ($P=0.033$) (data not shown).

Discussion

The majority of women with ovarian cancer are diagnosed at advanced stages (FIGO stage III/IV) due to the lack of reliable methods for early diagnosis and the absence of specific symptoms (16). Currently, the most effective strategy for the treatment of advanced ovarian cancer is based on aggressive cytoreductive surgery followed by platinum-based combination chemotherapy. However, despite recent advances in the treatment of ovarian cancer, patients with advanced stage disease have a high risk of relapse and the 5-year survival rate of patients diagnosed with advanced disease ranges from 20 to 25% (17,18). Therefore, significant improvement in the survival rate of these patients may depend on the identification of novel diagnostic markers for early detection of ovarian cancers.

The primary aim of this study was to characterize the newly established serous ovarian cancer cell line, YDOV-139. Cancer cell lines can be established from bodily fluids such as malignant ascites, as well as from tissues. The YDOV-139 was established from ascites and cultured in standard medium without any need for additional growth factors. Regarding growth properties, the estimated doubling time for YDOV-139 was 120 h; this is longer than doubling times of previously reported ovarian cancer cell lines, which ranged from 18 to 66 h (19,20). Most of these cell lines were obtained from tissues of ovarian cancer before treatment, but this is unlikely to account for the large difference in doubling time and a more plausible explanation is needed.

HLA class I and II molecules play a pivotal role in the anti-tumor immune response against ovarian cancer, and their expression may modify recognition of tumor cells by the immune system. The HLA phenotype of YDOV-139 was A*24/A*31, B*07/B*35, Cw03*(09)/w*07, and DRB1*01/DRB1*15, which is unrelated to phenotypes previously reported to show association with cancer development (21,22). The tumor suppressor genes BRCA1 and 2 are known to be involved in DNA repair and maintenance of genetic stability (23,24), and mutation of these genes has been linked to hereditary breast and ovarian cancer. In the present study, BRCA2 genotyping of YDOV-139 detected five polymorphisms and three missense mutations, while BRCA1 genotyping revealed wild-type sequence. Although little is known about the role of genetic polymorphisms in HLA or BRCA on the carcinogenesis of ovarian cancer, insight into the molecular pathogenesis may be gained by identifying genetic risk factors such as HLA alterations and BRCA mutations.

Table IV. List of differentially expressed proteins in YDOV-139 compared with HOSE by 2-DE and MALDI-TOF/PMF.

Spot no.	Identification	% Coverage	*pI*	Mw (kDa)
Up-regulated proteins in YDOV-139				
1	FUBP1 (Far upstream element binding protein 1)	20	6.9	68.81
2	Far upstream element binding protein	27	7.2	67.71
3	Far upstream element binding protein	14	7.2	67.71
4	Chaperonin	49	5.7	61.21
5	ER-60 protein (ethylene responsive catalase)	41	5.9	57.1
6	Chaperonin containing T-complex protein 1, subunit 2	34	6.0	57.81
7	ENO1 protein (enolase 1)	46	5.9	29.19
8	Fumarate hydratase precursor	25	9.0	54.79
9	S-adenosylhomocysteine hydrolase	27	6.0	48.27
10	Actin related protein 2 isoform b	20	6.3	45.03
11	Albumin-like protein	19	5.7	53.43
12	Thioredoxin-like protein	22	5.2	37.76
13	B23 nucleophosmin (280 AA)	28	4.7	31.09
14	Pyrophosphatase 1	31	5.5	33.10
15	NP (nucleoside phosphorylase)	31	6.5	32.33
16	5'-methythioadenosine phosphorylase	32	6.8	31.73
17	Albumin-like protein	16	5.7	53.43
Down-regulated proteins in YDOV-139				
18	VLA-3 alpha subunit	8	6.1	114.5
19	Keratin 7	39	5.4	51.46
20	VAT (Vesicle amine transport) protein 1	28	6.2	4167
21	Annexin 5 chain A	64	4.9	35.84
22	Chloride intracellular channel 4	60	5.5	28.9
23	Nicotinamide N-methyltransferase	23	5.6	30.1

pI, isoelectric point.

Resistance to chemotherapeutic drugs is a major obstacle to the effectiveness of chemotherapy for advanced ovarian cancer. In chemosensitivity tests of YDOV-139, gemcitabine showed higher growth inhibition than the standard paclitaxel and platinum-based chemotherapy agents. *TOP2A* has been identified as the target for many anticancer agents including gemcitabine, and several mutations in this gene have been reported to be associated with drug resistance (25). The microarray data of YDOV-139 indicate that overexpression of *TOP2A* (14.81-fold) may affect their response to gemcitabine in chemosensitivity tests.

Molecular profiling is a powerful approach to identify potential clinical markers for diagnosis and prognosis. Tumor behavior is determined by the integrated action of many genes and the accumulation of multiple genetic alterations contributes to the clinical heterogeneity observed in ovarian cancer. Gene expression profiles can provide molecular phenotyping and a better understanding of the biology of ovarian cancer (26-28). To identify novel molecular biomarker candidates for ovarian cancer, we performed gene expression profiling of YDOV-139 and HOSE cells, with additional complementary proteomic studies since gene expression does not precisely represent actual protein expression (29).

In microarray analysis, a total of 1,108 genes were found to be overexpressed in YDOV-139, including *STK6* (11.44-fold), *LBR* (8.95-fold), and *MCM4* (9.80-fold). In previous studies using genomic approaches to reveal platinum responsiveness, overexpression of *STK6, LBR*, or *MCM4* has been shown to be associated with an unfavorable response to platinum-based therapy in ovarian cancer (27,30). Overexpression of *STK6* is associated with reduced sensitivity to cisplatin-induced apoptosis and paclitaxel resistance (31). LBR is localized in the nuclear envelope inner membrane and anchors the lamina and the heterochromatin to the membrane. MCM4 plays an essential role in the initiation of eukaryotic genome replication and is highly expressed in malignant cancer cells and pre-cancerous cells (32). Overexpression of these types of genes in YDOV-139 is consistent with the frequently relapsing clinical course of the patient from whom YDOV-139 was derived.

Other genes that were highly expressed in YDOV-139 validated by microarray included *LCN2, MDK*, and *SLCO4A1*, whose expression levels were 111.19-, 43.95- and 23.28-fold

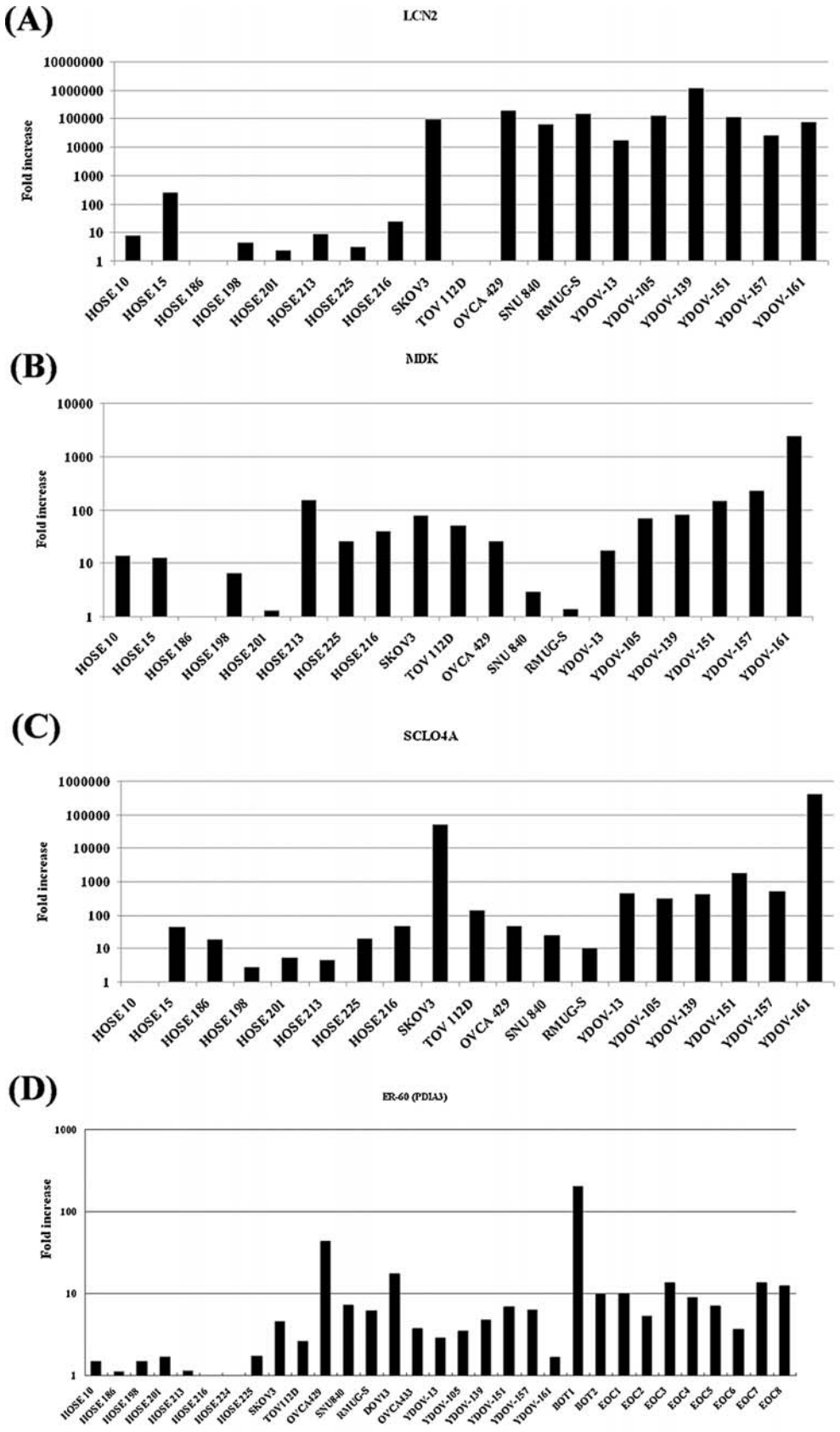

Figure 5. Relative quantitation of four candidate genes in HOSE cells, ovarian cancer cell lines, and/or ovarian cancer tissues by SYBR-Green PCR. Differences in expression of (A) *LCN2* (oncogenesis), (B) *MDK* (cell proliferation), (C) *SLCO4A1* (anion transport), and (D) *ER-60 (PDIA3)* were validated. Specifically designed primers for each candidate gene were as follows; *LCN2* (forward primer 5'-GGAGCTGACTTCGGAACTAAAGG-3', reverse primer 5'-TGTGGTTTTCAGGGAGGCC-3'), *MDK* (forward primer 5'-CAATGCTCAGTGCCAGGAGAC-3', reverse primer 5'-GGCTCCGAGTTCCCAGAGT-3'), *SLCO4A1* (forward primer 5'-CATTCCTGCACTAACGGCAAC-3', reverse primer 5'-AACTACAATCCACTGGATTCCCA-3'), and *ER-60* (forward primer 5'-GTCGAAGGGCCTTTCTTG-3', reverse primer 5'-AGCTGCGTGGCAAGGATAAA-3'). The normalization formula for *LCN2, MDK*, and *SLCO4A1* was: [target amount = $2^{-\Delta\Delta Ct}$], where $\Delta\Delta Ct$ = [Ct (Candidate gene) - Ct (Candidate gene GAPDH)] - [Ct (HOSE 186) - Ct (HOSE 186 GAPDH)]; and the formula for *ER-60 (PDIA3)* was: $\Delta\Delta Ct$ = [Ct (Candidate gene) - Ct (Candidate gene GAPDH)] - [Ct (HOSE 216) - Ct (HOSE 216 GAPDH)].

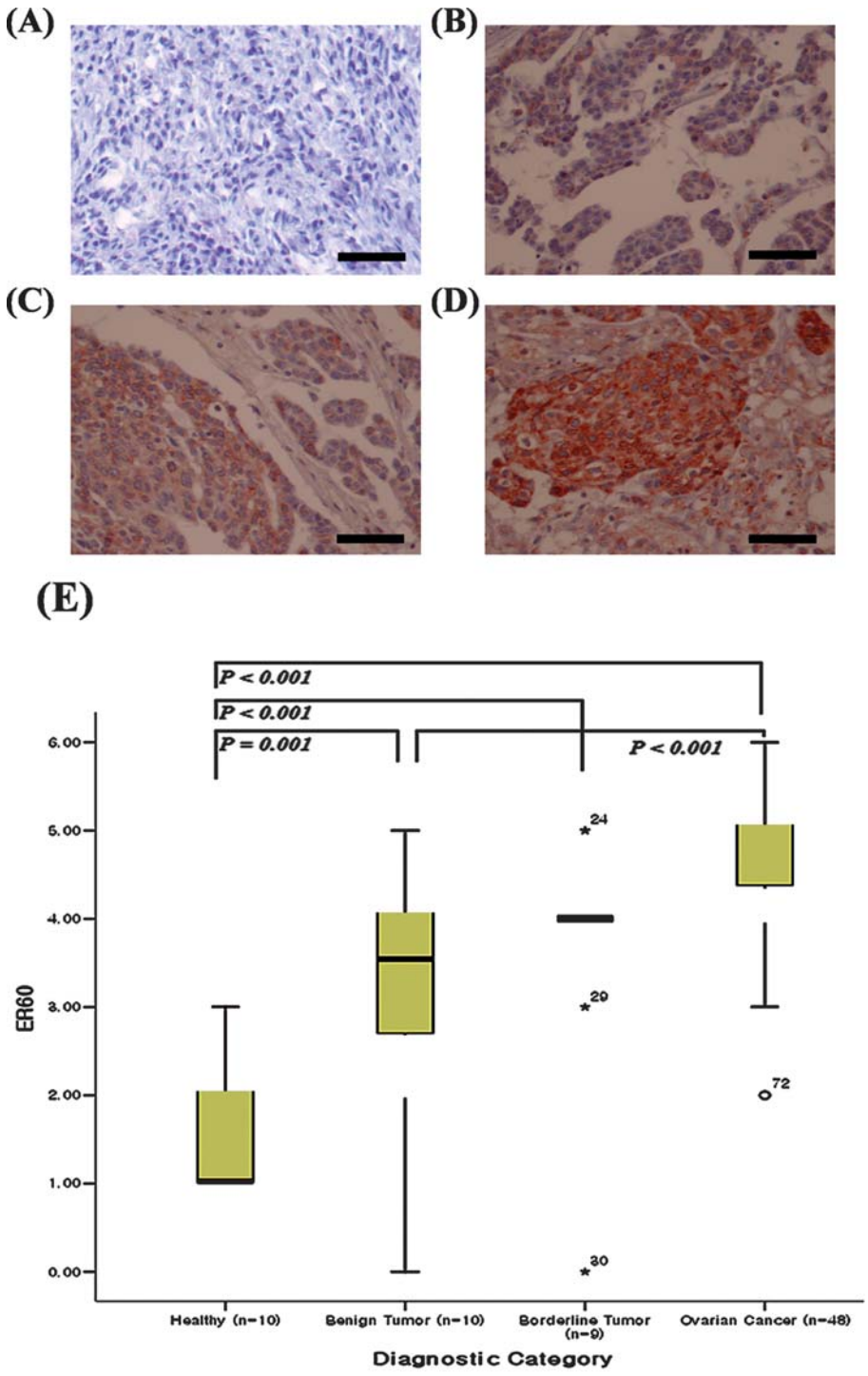

Figure 6. Evaluation of *ER-60 (PDIA3)* by immunohistochemical staining. Staining intensity [(A) no evidence of staining, 0; (B) weak staining, 1+; (C) moderate staining, 2+; (D) strong positive staining in most cells, 3+] and the percentage of positive cells (no cells staining positive, score 0; <25% of cells staining positive, 1+; 25-50% of cells staining positive, 2+; and >50% of cells staining positive, 3+) were scored. Bars (A-D), 50 μm. Immunohistochemical staining scores of ER-60 in ovarian cancer patients were significantly higher than those in benign ovarian tumor and healthy controls (E).

greater than in HOSE cells, respectively. *LCN2* encodes a secretary protein that plays a role in many cellular processes including apoptosis, oncogenesis, and cell regulation. *MDK* is a heparin-binding growth factor associated with cancer development, and *SLCO4A1* is a member of the anion-transporting polypeptide family. Such proteins may contribute to the development of a new biomarker or therapeutic target in the future (33-36).

Protein analysis identified 17 proteins whose expression was up-regulated in YDOV-139. Among these proteins, *ER-60* was investigated as a potential biomarker using SYBR-Green PCR and IHC. SYBR-Green PCR showed increased expression of *ER-60* mRNA in ovarian cancer cell lines and tissues. With the exception of only one ovarian cancer cell line, all 12 ovarian cancer cell lines and 8 ovarian cancer tissues had significantly higher *ER-60* levels than HOSE

cells. For further validation of *ER-60* protein expression, we performed IHC staining. *ER-60* immunoreactivity was not evident in normal ovarian epithelial tissues (score 0.7), whereas 100.0% (48/48) of ovarian cancers, 88.88% (8/9) of borderline ovarian tumors, and 90.0% (9/10) of benign ovarian tumors stained positive for *ER-60*, most of which was localized to the cytoplasm of tumor cells. Although the mean immunostaining score for ovarian cancers (score 4.70) and borderline ovarian tumors (score 3.66) was significantly higher than that of healthy controls, advanced stage and tumor histology was not correlated with high immunostaining scores.

ER-60, also known as *PDIA3, ERp57*, or GRP58, is a member of the protein disulfide isomerase (PDI) family of proteins that act as chaperones and are localized in the endoplasmic reticulum, cytosol, and nucleus (37,38). This protein was cloned and sequenced in 1988 by Bennett and colleagues, although it was originally identified as phospholipase C (39). Altered ER-60 levels may be involved in decreased immunoglobulin formation as well as having more direct roles in susceptibility or resistance to cancer (40,41). Several examples have been reported where expression of the disulfide isomerase family of proteins not only correlates with cancer invasion and metastasis (42), but was also found to be associated with drug resistance in epithelial ovarian cancer (43).

In conclusion, the molecular and biological characteristics of the YDOV-139 provide an additional model for the study of ovarian cancer, and also may contribute as an important research resource in the discovery of novel biomarkers and the development of new potential therapeutic strategies for ovarian cancer.

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