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Prognostic Significance of Transient Receptor Potential Vanilloid Type 1 (TRPV1) and Phosphatase and Tension Homolog (PTEN) in Epithelial Ovarian Cancer

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Abstract. *Background: Transient receptor potential vanilloid type 1 (TRPV1) has been studied in human malignancies, but has not been studied in epithelial ovarian cancer (EOC). We, therefore, investigated the significance of TRPV1 and correlation with phosphatase and tension homolog (PTEN) in EOC. Materials and Methods: Immunohistochemical analyses for TRPV1 and PTEN were performed using a tissue microarray. Moreover, the role of TRPV1 in cell growth was assessed in a EOC cell line. Results: High TRPV1 expression and the combination of high TRPV1 and low PTEN expression were an independent prognostic factor for overall survival and disease-free survival. In vitro results demonstrated that knockdown of TRPV1 was associated with decreased cell viability and colony formation. Conclusion: There is a strong association between TRPV1 and PTEN and the combination of TRPV1 and PTEN is a strong indicator of prognostic predictor in EOC.*

Epithelial ovarian cancer (EOC) is the second most common gynecological malignancy in women, with a 1.3% prevalence, and one of the top leading causes of cancer-related deaths in the United States of America (1). Women with EOC experience a high mortality rate because it is usually diagnosed in advanced stages. Even with a greatly improved treatment strategy, the 5-year overall survival rate remains about 30% (2). Therefore, there is a great need for research studies that focus on uncovering the molecular pathogenesis of EOC and exploring more specific and effective prognostic markers to improve patient outcomes.

Ca^{2+} is one of the important second messengers linking activation of membrane receptor and downstream signaling cascade, and Ca^{2+} homeostasis controls various physiological and cellular processes such as tumorigenesis. Therefore, abnormal expression of Ca^{2+} channels has been studied in various cancers, and interest in the role of transient receptor potential (TRP) channels, a type of Ca^{2+} permeable nonselective cation channel, is also emerging. Transient receptor potential vanilloid type 1 (TRPV1), which belongs to the TRP channel family of proteins, was originally regarded as a critical sensor in response to mechanical, thermal, and chemical stimuli, since it is predominantly found in afferent neurons. It has been suggested that TRPV1 is associated with cell proliferation, differentiation, and invasion characteristics of cancer (3, 4). Previous studies have reported that TRPV1 acts as a tumor suppressor by inducing apoptosis and preventing tumor progression upon activation by its agonist in melanomas (5). However, other studies reported that TRPV1 deficiency prohibited cell proliferation in prostate cancer and sensitized stress-induced apoptosis in colorectal cancer, indicating an oncogenic role (6, 7). In accordance with previous studies, it can be suspected that the role of TRPV1 is tumor type-specific, and the mechanisms underlying the regulation of cancer cell growth by TRPV1 remain to be evaluated. Moreover, the role of TRPV1 in EOC has not been yet clarified.

Thus, in this study, we aimed to evaluate the molecular and functional significance of TRPV1 expression in EOC. Our results indicated that TRPV1 was overexpressed in EOC

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and associated with poor prognosis. Furthermore, *in vitro* functional studies showed that inhibition of TRPV1 suppressed the development of EOC cells.

Materials and Methods

Patients and tumor samples. Tissue samples from 217 EOCs, 57 borderline and 153 benign ovarian tumors, and 79 non-adjacent normal epithelia were included in the study. Tumor tissues were gathered from patients who underwent debulking surgery at the Gangnam Severance Hospital between 1996 and 2012 and from the Korea Gynecologic Cancer Bank as part of the Bio & Medical Technology Development Program of the Ministry of the National Research Foundation (NRF) funded by the Korean government (MIST) (NRF-2017M3A9B8069610). The International Federation of Gynecology and Obstetrics (FIGO) classification was used for tumor staging. Clinical information, including survival time, survival status, age, and surgical procedure, were collected by reviewing medical records. The therapeutic response was evaluated with Response Evaluation Criteria in Solid Tumors (RECIST; version 1.0) by computed tomography (8). The cell type and tumor grade were obtained by reviewing the pathological reports. All tumor tissues were histologically examined by a gynecologic pathologist, and all biological samples were obtained after informed consent from the participants, according to the guidelines of the institutional review board (IRB) of Gangnam Severance Hospital.

Tissue microarray construction and immunohistochemistry. Tissue microarray (TMA) with one-millimeter cores was constructed from archival formalin-fixed paraffin-embedded (FFPE) tissue blocks. The samples were arrayed into a recipient paraffin block with a manual tissue arrayer MTA–1 (Beecher Instruments Inc., Silver Spring, MD, USA). For immunohistochemical staining, the TMA blocks were cut into serial 5-μm thick sections, deparaffinized with xylene, and rehydrated gradually from ethanol to distilled water. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide (H_2O_2) for 10 min. The sections were immersed in an antigen retrieval buffer at pH 9 (Dako, Carpinteria, CA, USA) and heated for 20 min in a steam pressure cooker (Pascal, Dako) to retrieve antigenicity. The sections were treated with a protein block (Dako) for 20 min to block non-specific staining. The sections were then incubated with anti-TRPV1 antibody (Alomone, Jerusalem, Israel; Rabbit polyclonal antibody, Cat# ACC-030, 1:1,000 for 1 h) and anti-PTEN antibody (Cell Signaling, Danvers, MA; Rabbit monoclonal antibody, Clone 9559L, 1:100 for 1 h) in Dako Autostainer Plus (Dako) for 1 h. For antigen-antibody reaction, sections were incubated with Dako EnVision+ Dual Link system-HRP (Dako) for 30 min and visualized with 3,3'-diaminobenzidine (DAB; Dako). Tissue sections were counterstained lightly with hematoxylin and examined with light microscopy. Appropriate negative controls were simultaneously prepared.

Evaluation of immunohistochemistry staining. After IHC staining, all glass slides were scanned digitally by a high-resolution optical scanner Nanozoomer 2.0 HT (Hamamatsu Photonics K.K., Japan). The digital imaging analysis (DIA) software used was the Visiopharm Integrator System v6.5.0.2303 (VIS; Visiopharm, Hørsholm, Denmark). After training the system with digital "painting" examples of the nucleus in the image, an algorithm for nucleus-specific signal selection was designed manually. The cytoplasm was defined by outlining the defined nucleus. TRPV1 and PTEN were scored based on staining intensity of brown-colored diaminobenzidine (DAB) (0=negative, 1=weak, 2=moderate, and 3=strong). The overall immunostaining score was calculated by multiplying the staining intensity by percentage of positive cells (possible range, 0-300).

Cell culture. The human ovarian cancer cell line, A2780, was purchased from The European Collection of Cell cultures (ECACC, Salisbury, UK), and human embryonic kidney 293 (HEK293) cell from Systemic Biosciences (SBI, Palo Alto, CA, USA). A2780 cells were cultured at Roswell Park Memorial Institute (RPMI) in medium containing 10% FBS, 1% penicillin, and 1% streptomycin. The HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin and 1% streptomycin. A2780 cells and HEK293 cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Transfection and generation of stable cell lines. For the generation of sh-TRPV1 stable cell lines, pLKO.1 *TRPV1* shRNA libraries were purchased from Sigma-Aldrich (St. Louis, MO, USA). Of the lentivirus constructs tested, two with the best efficiency were used for the experiments here, using the human *TRPV1* sequence CCGGCCGT TTCATGTTTGTCTACATCTCGAGATGTAGACAAACATGAAACG GTTTT for shRNA#1 and CCGGGAAGTTTATCTGCGACAG TTTCTCGAGAAACTGTCGCAGATAAACTTCTTTTTG for shRNA#2. The non-targeted shRNA control vector (pLKO.1) was purchased from Sigma-Aldrich. Lentiviral production was done by co-transfecting pLKO.1 and packaging vectors into HEK293 cells by using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. At 48 and 72 h post-transfection, virus particles were collected. A2780 cells were transduced, and cells with positive transduction were selected by puromycin (2 μg/ml).

Protein extraction and western blot. Total cell lysates were isolated by using cell lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM ethylendiaminetetraacetic acid (EDTA)] containing proteinase inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The lysates were centrifuged at 13,500 rpm for 30 min, and protein concentrations were determined by bicinchoninic acid (BCA) assay (Sigma-Aldrich). A total of 40 μg proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.2-μm nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% nonfat dry milk in TBST mixture (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature, washed with TBST, and subsequently incubated with primary antibodies anti-TRPV1 (Invitrogen, Carlsbad, CA, USA - Rabbit polyclonal antibody, Cat# PA1-748) and α-tubulin (Santa Cruz Biotechnology, Cat# sc-5286). Primary antibodies against each protein were detected by using secondary antibodies conjugated with horseradish peroxidase. Signals were detected by chemiluminescence using the ImmunoCruz luminol reagent (Santa Cruz Biotechnology) and quantified using ImageJ analysis software (US National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay. Cell proliferation was measured with the EZ-CYTOX reagent (DoGenBio, Seoul, Republic of Korea, Cat# EZ-3000). In brief, cells were seeded at 1×10^4 cells/well onto a 96 well plate with a final volume of 100 μl/well. Cells were incubated

at 37˚C for 60 min after adding 10 μl of EZ-CYTOX reagent per well and then incubated on an orbital shaker for 1 min. The absorbance value at optical density (OD) 450 nm was subsequently measured with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and recorded at day 0, 1, 2, and 4. The experiment was performed in triplicates.

Colony formation assay. In order to examine clonogenicity, the cells were seeded in a 6 well plate at 500 cells/well. The cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin for 2 weeks. The cells were fixed with 100% methanol for 10 min and stained with 0.5% crystal violet for 30 min, followed by washing with distilled water. Stained cells were dissolved in 2% dimethyl sulfoxide (DMSO) for 20 min on an orbital shaker. The absorbance value was measured at 595 nm on a microplate reader (Bio-Rad Laboratories, Inc.). Each experiment was repeated three times.

Cell counting assay. Cells were seeded at 2.5×10^5 cells/well in a 6-well plate and incubated for 7 days. The cells were washed with phosphate-buffered saline and treated with trypsin-ethylenediaminetetraacetic acid. After detachment using trypsin, the cells were diluted with 1 ml of fresh medium and transferred to the dedicated LUNA™ Cell Counting Slides (Logos Biosystems, Gyeonggi-do, Republic of Korea, Cat #L12001). Cell counting was performed with the LUNA-II™ (Logos Biosystems) automated cell counter in disposable counting chambers. Each experiment was performed three times.

Statistical analysis. Statistical analyses of TRPV1 and PTEN were performed using the Mann-Whitney test or the Kruskal-Wallis test where appropriate. Kaplan-Meier methods and log-rank test were used to evaluate the overall survival (OS) and disease-free survival (DFS) curve. The Cox proportional hazard model was performed for both univariate and multivariate models to estimate hazard ratios (HRs) and confidence intervals (CIs). Statistical analyses were performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

Results

TRPV1 and PTEN protein expression in EOC. To investigate the clinical role of TRPV1 and PTEN in EOC, we assessed TRPV1 and PTEN expression by immunohistochemistry (IHC) using TMAs of 217 EOC tissues, 57 borderline tumors, 153 benign tumors, and 79 nonadjacent normal epithelial tissues. However, only 204 EOC tissues, 50 borderline tumors, 118 benign tumors, and 37 nonadjacent normal epithelial tissues for TRPV1 and 202 EOC tissues, 50 borderline tumors, 95 benign tumors, and 79 nonadjacent normal epithelial tissues for PTEN were interpretable for IHC analysis due to complexity and staining of samples. Representative IHC images for TRPV1 and PTEN in EOC are shown in Figure 1A. As observed in Figure 1A and Table I, expression level of TRPV1 was higher compared to borderline tumors, benign tumors, and nonadjacent normal epithelial tissues (all $p<0.001$). We further evaluated the clinicopathological characteristics of TRPV1 in which high expression of TRPV1 (TRPV1+) was significantly associated with advanced FIGO stage ($p<0.001$; Table I and Figure 1B), serous cell type ($p<0.001$), and positive CA125 ($p=0.035$; Table I). In terms of PTEN, there were no significant differences in clinicopathological characteristics between EOC and other groups (Table I).

Correlation between TRPV1 and PTEN expression. Since both TRPV1 and PTEN have been reported to be related to the PI3K/AKT pathway and Ca^{2+} homeostasis, Spearman's rank correlation analysis was performed to determine the association between the two proteins. The results showed a negative correlation trend between TRPV1 and PTEN in EOCs (Spearman's rho=–0.103, $p=0.144$; Figure 2A), but interestingly, significant negative correlation in stage III/IV was observed when performing subgroup analysis according to stage (Spearman's rho=–0.241, $p=0.006$; Figure 2B).

In addition, the combined high expression of TRPV1 and loss of PTEN expression (TRPV1+/PTEN–) was associated with advanced FIGO stage ($p=0.013$; Figure 2C) and serous cell type ($p=0.009$; Figure 2D). Other clinicopathological parameters were not found to be associated with TRPV1+/PTEN– expression in EOCs.

Prognostic significance of TRPV1 and PTEN expression. We further evaluated the prognostic significance of TRPV1 and PTEN in EOCs, in which TPRV1+ patients had significantly worse OS compared to those with low TRPV1– ($p<0.001$; Figure 3A). In addition, DFS showed the same result as the OS ($p<0.001$; Figure 3B). Compared to TRPV1, high PTEN expression (PTEN+) showed significantly better DFS than low PTEN expression (PTEN–) ($p<0.001$; Figure 3D).

Moreover, OS and DFS were identified between TRPV1+/PTEN– and TRPV1–/PTEN+ expression groups with the Kaplan-Meier plot. Significant differences were observed in OS and DFS (both $p<0.001$; Figure 3E and Figure 3F). Furthermore, OS and DFS were compared with Cox proportional univariate and multivariate analyses (Table II). Multivariate analysis revealed that TRPV1+ expression strongly served as an independent prognostic factor for both OS and DFS (HR=4.19, 95% CI=1.34-12.18, $p=0.009$; HR=8.22, 95% CI=3.37-20.04, $p<0.001$). The combined marker, TRPV1+/PTEN–, was related to poor DFS (HR=2.25, 95% CI=1.06-4.74, $p=0.003$) and in particular, TRPV+/PTEN– was a significantly strong prognostic biomarker for OS (HR=4.17, 95% CI=2.36-7.38, $p<0.001$). Moreover, FIGO stage and cell type were good parameters for predicting OS ($p=0.046$, $p<0.001$), and other parameters, including FIGO stage ($p<0.001$), cell type ($p=0.024$) and tumor grade ($p=0.010$), were independent poor prognostic factors for DFS.

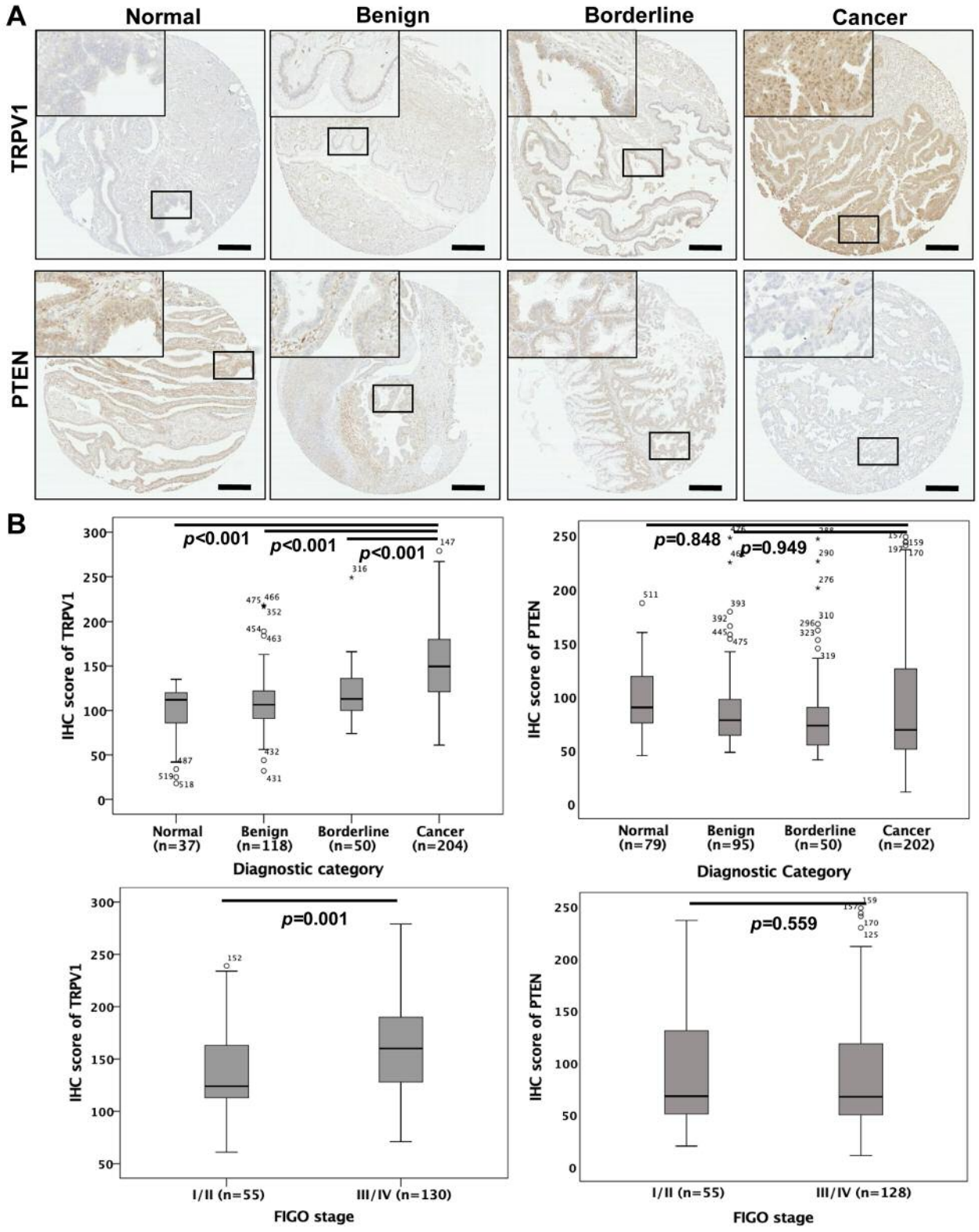

Figure 1. *TRPV1 and PTEN expression in formalin-embedded non-adjacent normal epithelium, benign tumor, borderline tumor, and epithelial ovarian cancer tissues. (A) Representative immunohistochemical images of TRPV1 and PTEN in non-adjacent normal epithelium, benign tumor, borderline tumor, and epithelial ovarian cancer tissues. Scale bar: 60 μm. (B) IHC staining score for TRPV1 and PTEN in nonadjacent normal epithelium, benign tumor, borderline tumor, and epithelial ovarian cancer tissues.*

Table I. *Expression of TRPV1 and PTEN in relation to clinicopathological characteristics in immunohistochemical analysis.*

	No.	TRPV1		No.	PTEN	
		Mean score (95% CI)	*p*-Value		Mean score (95% CI)	*p*-Value
All study subjects	409	132.1 (127.9-136.3)		426	90.5 (86.2-94.8)	
Diagnostic category						
Normal	37	100.0 (89.7-110.4)	<0.001	79	95.9 (89.5-102.4)	0.614
Benign	118	109.3 (103.6-115.0)		95	87.2 (80.0-94.5)	
Borderline	50	118.3 (110.5-126.1)		50	88.0 (74.6-101.4)	
Cancer	204	154.5 (148.6-160.4)		202	90.6 (83.3-97.9)	
FIGO stage			0.001			0.559
I-II	55	138.8 (127.4-150.3)		55	92.2 (77.5-107.2)	
III-IV	130	161.7 (154.2-169.2)		128	87.2 (78.2-96.3)	
Cell type			<0.001			0.514
Serous	137	166.6 (159.6-173.5)		136	88.9 (80.4-97.4)	
Others	67	129.8 (121.3-138.4)		66	94.1 (79.7-108.4)	
Tumor grade			0.673			0.612
Well/Moderate	86	155.2 (146.0-164.3)		86	88.1 (77.0-99.3)	
Poor	102	157.8 (149.4-166.2)		102	92.0 (81.6-102.4)	
CA125			0.035			0.636
Negative	33	139.9 (126.7-153.1)		33	93.8 (76.8-110.8)	
Positive	167	157.2 (150.5-163.9)		165	89.0 (80.8-97.3)	
Chemosensitivity			0.710			0.536
Sensitive	171	156.5 (150.1-163.0)		170	92.2 (84.0-100.4)	
Resistant	17	160.5 (144.1-176.8)		16	83.5 (57.8-109.3)	

FIGO, International Federation of Gynecology and Obstetrics; CI, confidence interval. Protein expression was evaluated by immunohistochemical analysis using tissue arrays, as described in Materials and Methods.

Knockdown of TRPV1 in EOC cells. As the biological role of TRPV1 in EOC is not fully investigated, we studied its potential roles in EOC cell lines by modulating intracellular TRPV1 expression with short- hairpin TRPV1 RNAi in a A2890 (*sh-TRPV1* #1, #2) cell line. The relative expression level of TRPV1 was measured with western blot, and upon confirmation of knockdown (Figure 4A), we analyzed the effect of TRPV1 with two different assays to investigate the short- and long-term proliferative ability. When TRPV1 was knocked-down in A2890 cells, cell growth was significantly decreased compared to the shRNA-control group (Figure 4B). Next, we performed cell counting and colony formation assays to assess the role of TRPV1 in tumorigenesis and found that number of cells and colony formation in *sh-TRPV1* were significantly decreased compared to the shRNA-control (Figure 4C and D). Taken together, these results demonstrated that blocking TRPV1 expression inhibited cell growth in EOC cells.

Discussion

As EOC patients experience high rates of relapse and mortality, it is necessary to investigate the potential molecular biomarkers that can be of prognostic and predictive value for EOC. Therefore, in this study, we first identified the expression of TRPV1 and examined the impact on EOC progression and then studied the association of TRPV1 with PTEN and the potential combined effect of TRPV1 and PTEN as a prognostic marker for EOC.

First, we examined TRPV1 expression and explored its clinical significance with IHC analysis using EOCs, borderline tumors, benign tumors, and nonadjacent normal epithelia. The results revealed that TRPV1 expression was markedly increased in EOC tissues compared to borderline tumors, benign tumors, and nonadjacent normal epithelium (all $p<0.001$). Furthermore, TRPV1 expression increased in advanced FIGO stage and CA125-positive patients ($p<0.001$ and $p<0.035$, respectively). Notably, TRPV1+ status was an independent prognostic factor for both OS and DFS. In the same context, the expression of TRPV1 was studied in breast and prostate cancers and, as in our study, its expression was higher in cancer tissues than in normal tissues and was associated with poor prognosis (9-11). However, other studies on hepatocellular carcinoma, transitional cell carcinoma of bladder, and renal cell cancer have reported that expression of TRPV1 was inversely correlated with cancer progression (12-14). The main reason for this inconsistency would be that the hormones can modify transcription regulation, localization at the plasma membrane surface, and intrinsic activity of TRPV1 (15). Morelli *et al*. (6) evaluated androgen receptor (AR) and TRPV1 co-

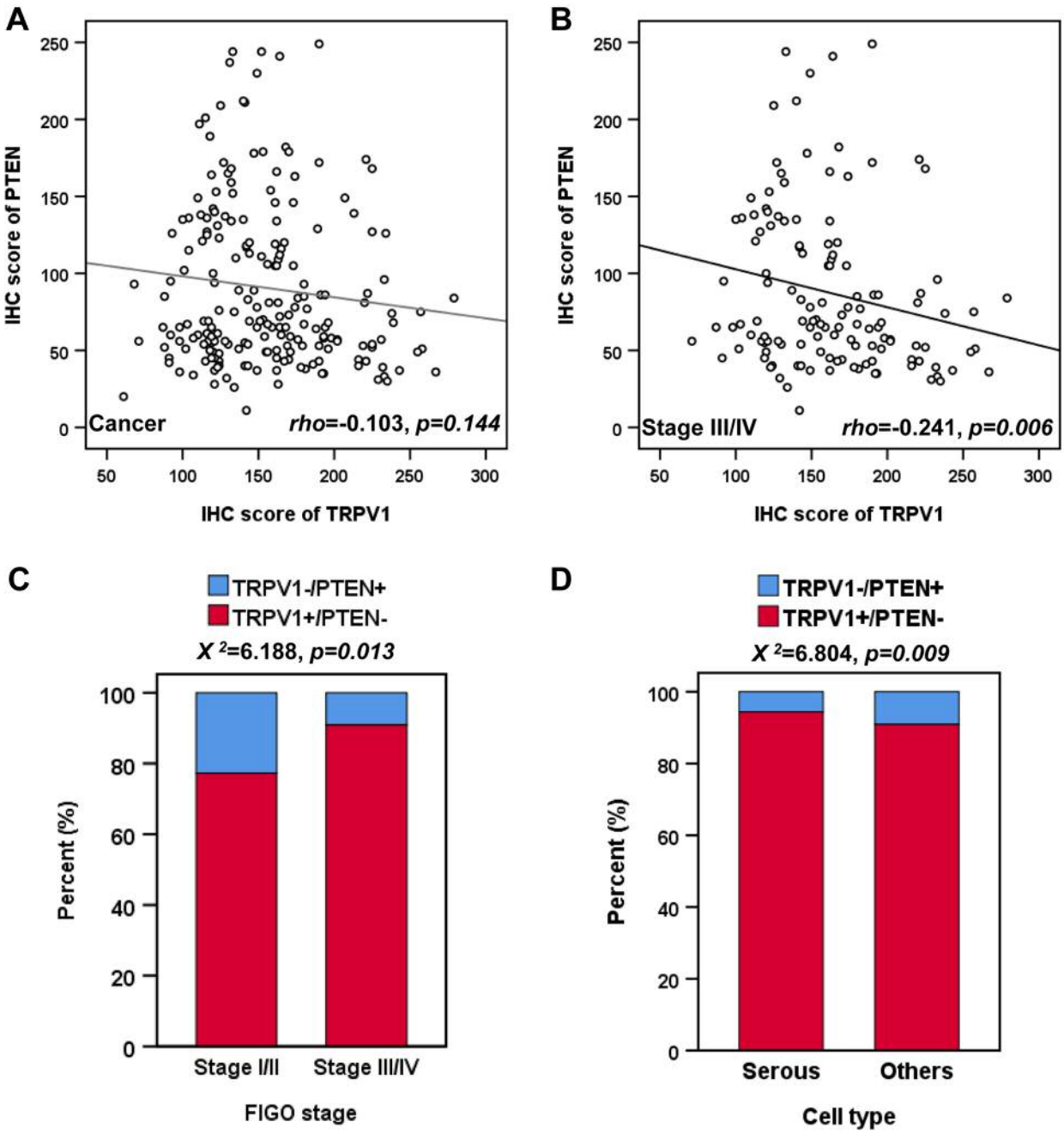

Figure 2. *Spearman's rank correlation analysis between TRPV1 and PTEN expression. TRPV1 expression showed a trend of negative correlation with PTEN level in EOCs (A), while in stage III/IV, there was a significant negative correlation between TRPV1 and PTEN (B). TRPV1–/PTEN+ was significantly associated with FIGO stage (C) and serous cell type (D).*

expression through mRNA and protein levels in advanced prostate cancer tissues, and found that expression levels of AR and TRPV1 were markedly elevated in these tissues compared to those in benign prostate hyperplasia. More importantly, they showed a strong correlation between AR and TRPV1 expression levels. Moreover, a strong down-regulation in AR and *TRPV1* mRNA levels was observed in a patient who received androgen deprivation therapy compared to those who did not receive androgen deprivation therapy, suggesting that TRPV1 might be modulated by androgen. In this study, we did not show a correlation between TRPV1 and estrogen. The ovary, however, is a

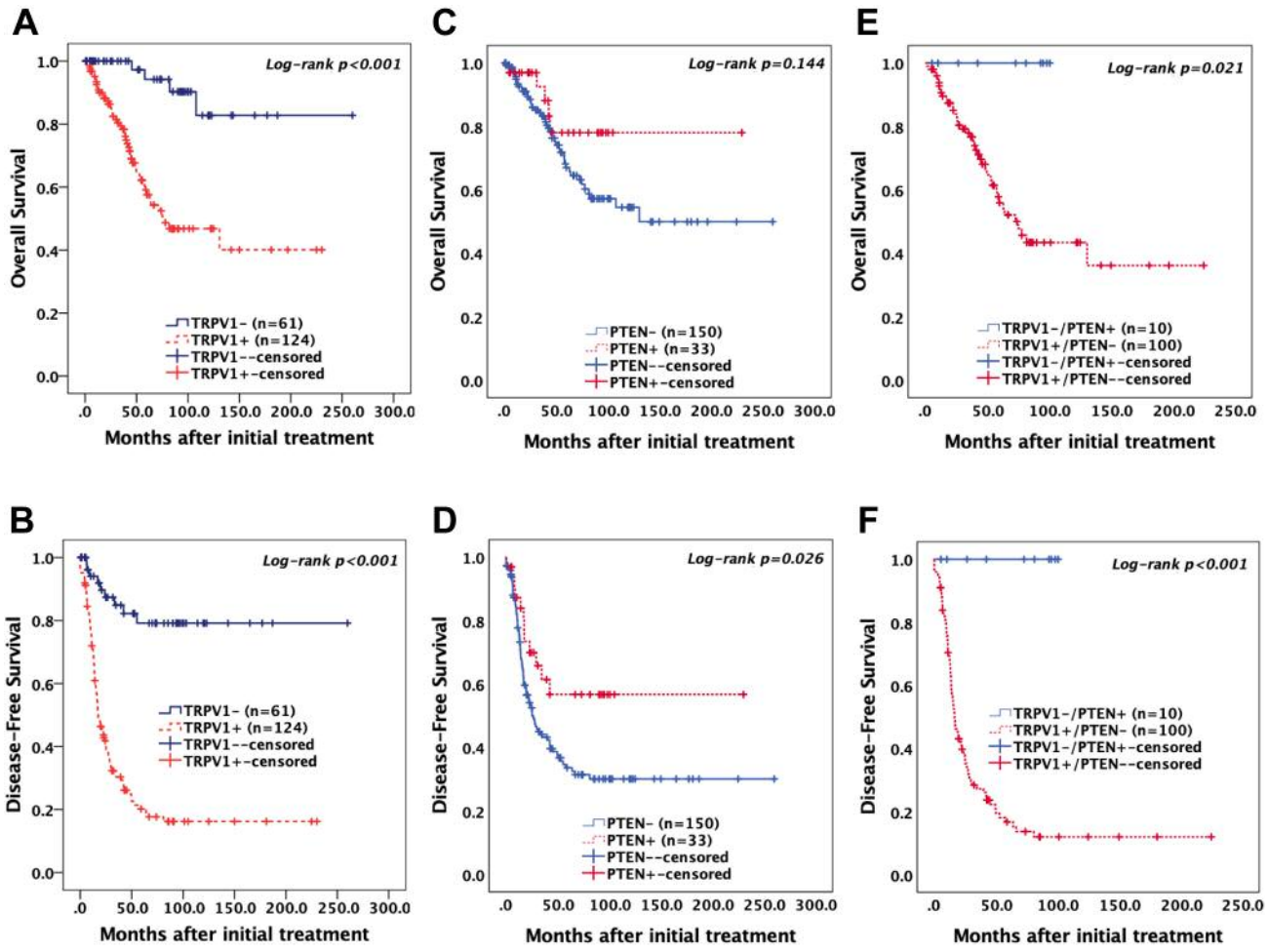

Figure 3. *Kaplan–Meier survival curves for TRPV1 and PTEN expression in EOC. EOC patients with high TRPV1 expression had a shorter overall survival (A) (p<0.001) and worse 5-year disease-free survival (B) (p<0.001) than those with low TRPV1 expression. The patients with high expression of PTEN showed a longer 5-year disease-free survival (D) (p=0.026) and those with high TRPV1/low PTEN had a shorter overall survival (E) (p=0.021) and worse 5-year disease-free survival (F) (p<0.001) than patients with low TRPV1/high PTEN expression.*

Table II. *Univariate and multivariate analyses of the associations between prognostic variables and overall and disease-free survival in epithelial ovarian cancer.*

	Overall survival hazard ratio (95% CI), *p*-Value		Disease-free survival hazard ratio (95% CI), *p*-Value	
	Univariate	Multivariate	Univariate	Multivariate
FIGO stage (III-IV)	4.68 (1.85-11.79), 0.001	2.66 (1.01-6.99), 0.046	5.88 (3.04-11.34), <0.001	4.06 (1.97-8.38), <0.001
Cell type (serous)	4.71 (1.87-11.87), 0.001	2.01 (0.75-5.40), 0.163	3.13 (1.86-5.29), <0.001	2.00 (1.09-3.65), 0.024
Tumor grade (poor)	1.69 (0.95-3.01), 0.073	NA	1.96 (1.29-2.99), 0.002	1.76 (1.14-2.70), 0.010
CA125+ (>35 U/ml)	2.10 (0.75-5.86), 0.153	NA	2.32 (1.17-4.62), 0.016	1.25 (0.56-2.76), 0.582
Age (>50)	2.19 (1.21-3.96), 0.010	1.52 (0.83-2.78), 0.166	1.55 (1.04-2.31), 0.029	1.29 (0.84-1.98), 0.233
TRPV1+[a]	7.18 (2.57-20.01), <0.001	4.18 (1.43-12.18), 0.009	7.47 (3.75-14.87), <0.001	8.22 (3.37-20.04), <0.001
PTEN–[b]	1.96 (0.77-4.95), 0.152	NA	1.96 (1.07-3.58), 0.029	1.83 (0.96-3.48), 0.066
TRPV1+/PTEN–	3.95 (1.97-7.91), <0.001	2.25 (1.06-4.74), 0.033	4.42 (2.74-7.12), <0.001	4.17 (2.36-7.38), <0.001

CI, Confidence interval; NA, not applicable; FIGO, International Federation of Gynecology and Obstetrics; LN, lymph node; NA, not applicable. [a]cut-off value of TRPV1+ is 125 over IHC score; [b]cut-off of PTEN+ is 138 over IHC score.

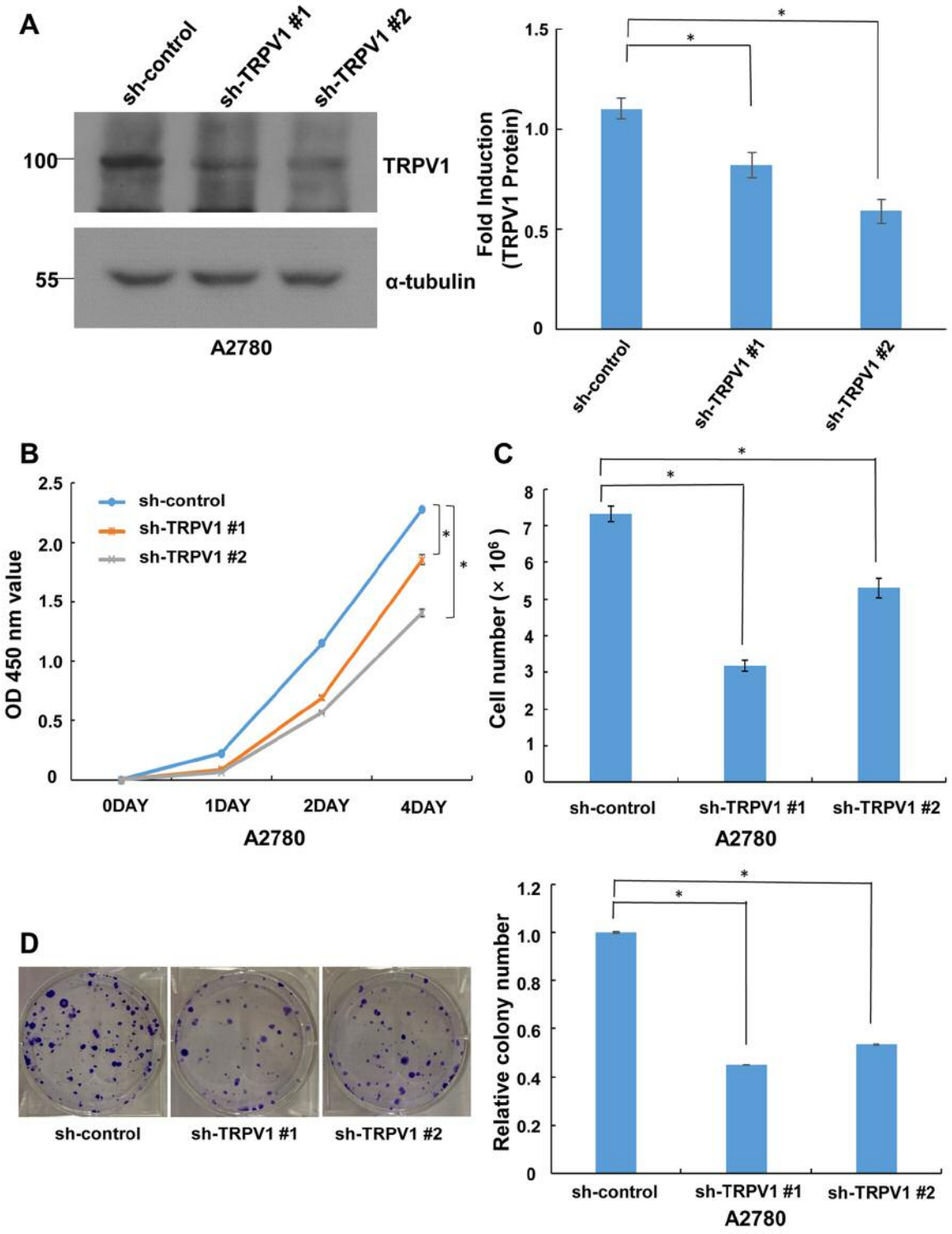

Figure 4. *Effect of TRPV1 overexpression on EOC cells. Relative mRNA levels in control and TRPV1-knockdown A2780 cells (A). Viability curves for A2780 cells transfected with sh-control, sh-TRPV1 knockdown (B). A2780 cells were collected at various time points. The viability of TRPV1 knockdown cells was decreased. Quantification of cell counting assay for sh-control and sh-TRPV1-transfected A2980 cells (C). It showed significantly decreased cell number in sh-TRPV1 transfected A2980 cells compared to sh-control. The colony formation assay of sh-control and sh-TRPV1 transfected A2980 cells (D) Left panel: Representative images of colonogenic assay; Right panel: Quantitative results of colonogenic assay. Colony formation ability was decreased for the TRPV1 knockdown cells as compared with the control cells. The number of asterisks (*) indicates the level of significance: *p≤0.05. Data and error bars represent the mean±SD of triplicate experiments.*

hormone-affected reproductive organ, and synergetic expression of TRPV1 by estrogen has been reported in endometriosis patients (16). Therefore, accumulation of estrogen may have some influence on TRPV1 expression, and elevated expression of TRPV1 may promote expression of other Ca^{2+}-sensitive transcriptional regulators (17, 18).

Another important finding of this study is that expression of TRPV1 was significantly higher in serous-type EOC than in others. The fourth edition of WHO classification placed ovarian cancer into two different categories, type I and type II, according to molecular profile, precursor lesion, way of spread, and response to chemotherapy (19). Type 1 EOC includes low-grade serous ovarian carcinoma (LGOSC), mucinous, clear cell, malignant Brenner and endometrioid tumors that generally show *Ras* and/or *Raf* mutation with indolent and slow growth. Type II EOC, which exhibits p53 mutations, includes high-grade serous ovarian carcinoma (HGOSC) undifferentiated carcinomas, and malignant mixed mesodermal tumors. Among the many types of EOC, HGOSC accounts for nearly 90% and exhibits a more aggressive behavior. Despite continued research, there has been no significant improvement in 5-year OS over the past 30 years. Therefore, it is necessary to identify a potential biomarker that correlates with prognosis in Type II EOC, especially HGOSC, in the future (20). In our study, among 137 serous type EOC patients, 13.7% had LGOSC and 85.1% had HGOSC (1.2%: grade unknown). Taken together, our results suggest that TRPV1 might be a crucial predictor for poor prognosis of HGOSC and possible therapeutic target.

In accordance with our results showing an association between TRPV1 and cancer in clinical specimens, we set out to explore the potential role of TRPV1 in the development of a malignant phenotype in EOC cells by modulating intracellular TRPV1 expression in A2890 cells. We found that TRPV1 knockdown significantly decreased the proliferative and clonogenic abilities of EOC cells. Similar to our results, accumulative evidence has demonstrated the role of TRPV1 in tumorigenesis of various cancers. Sung *et al*. (7) identified the oncogenic role of TRPV1 in colorectal cancer after observing that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was activated and induced cell apoptosis when TRPV1 expression was blocked by an antagonist in colorectal cells. Moreover, in prostate cancer cells, TRPV1 overexpression activated its oncogenic role via regulated extracellular signal-regulated kinase (ERK) signaling (6). However, in glioma, TRPV1 overexpression leads to cell death by inducing endoplasmic reticulum stress (3). de Jong *et al*. (21) found that activation of TRPV1 by capsaicin regulated Ca^{2+}/calpain and protein tyrosine phosphatase 1B (PTP1B) and suppressed intestinal tumorigenesis. Considering these inconsistent results from previous studies, we suspect that TRPV1 can show varying functions depending on cancer type. One factor that could affect TRPV1 function is tumor microenvironment, since cytokine and receptor interactions vary by tumor type and tissue content and consequently affect the different cancer-related pathways in tumor microenvironments (22).

In addition to TRPV1, we investigated the expression of PTEN and its association with TRPV1 in EOC. A dual lipid-protein phosphatase, PTEN acts primarily as a tumor suppressor by dysregulating the PI3K/AKT/mTOR pathway in a large proportion of human cancers such as prostate, brain, breast, thyroid, endometrial, sarcoma, and cervical cancers (23-26). In our study, the expression levels of PTEN did not differ between normal and cancer tissues. However, Cox analysis revealed that low expression of PTEN is an independent predictive factor for prognosis. These results may be explained by the fact that more than 70% of PTEN alterations are related to gene deletions that were determined to yield intact PTEN by IHC, and only 30% are related to lack of PTEN expression according to the TCGA database (27). Moreover, for HGOSC, PTEN IHC in TCGA data showed loss of PTEN in only 52 (15%) out of the 316 EOCs (28).

After looking at the expression and survival analyses of TRPV1 and PTEN, we examined the relationship between TRPV1 and PTEN in EOCs, which showed a significant negative correlation in advanced stages (stage III/IV). In addition, combination of TRPV1 and PTEN (TRPV1+/PTEN) showed high hazard ratio in the Cox regression analyses, indicating a strong prognostic predictive factor. As mentioned above, the association between TRPV1 and PTEN can be observed through the PI3K/AKT pathway, since PTEN acts as a tumor suppressor by inhibiting cell proliferation and induces apoptosis via dephosphorylation of phosphatidylinositol (3,4,5)-triphosphates to phosphatidylinositol 4,5-bisphosphate in the PI3K/AKT pathway. A previous study also revealed that activation of TRPV1 may exert anti-apoptotic effects in ischemic/reperfusion injury of the heart via the PI3K/AKT pathway (29). In addition to this result, numerous studies have reported on the association of TRPV1 and the PI3K/AKT pathway such as in dorsal root ganglion neurons (30) and human HepG2 cells (30, 31). Taken together, we propose that TRPV1 and PTEN showed correlation in our EOC specimens through the PI3K/AKT pathway. Even though there is no previous study investigating the correlation between TRPV1 and PTEN expression in various cancers, the study by Liu *et al*. (32) is of particular interest. They observed that overexpression of TRPV4 in colon cancer was associated with poor prognosis and revealed the negative regulation of PTEN upon TRPV4 activation via the AKT/mammalian target of rapamycin (mTOR) signaling pathway. Furthermore, they suggested that dysregulation of Ca^{2+} homeostasis by TRPV4 regulated the localization of PTEN by conformational changes in major vault proteins in colon cancer. However, further studies are warranted to understand the exact mechanism underlying the role of TRPV1 and PTEN in EOC.

In this study, the evaluation of TRPV1 and PTEN expression level in EOCs were performed by IHC. Immunostaining techniques, IHC is the most practical methods to assess expression levels of proteins in histopathology. It is relatively inexpensive and easy to perform, and IHC provides not only a semiquantitative assessment but also defines cellular localization of protein expression. However, the determined TRPV1 and PTEN expression accurately by IHC can be significantly affected by numerous factors, such as temperature, fixation and method of antigen retrieval (33, 34). Therefore, to reduce the variability in scoring result of IHC, recommendation guidelines should be published.

In conclusion, we investigated TRPV1 and PTEN expression using IHC in a large cohort of patients with EOC. High TRPV1 expression was associated with poor DFS and OS, and high PTEN expression showed a favorable prognosis in DFS. Cox regression analysis confirmed that both TRPV1 and PTEN might be important prognostic indicators of EOC. This result is significant because it suggests that TRPV1 or combination of TRPV1 and PTEN is applicable to the personalized therapy approach based on the constancy of tumors.

Conflicts of Interest

The Authors do not have any potential conflicts of interest to disclose.

Authors' Contributions

HC and J-YC designed and built tissue microarrays. GHH, HC, and J-YC conceived and designed the study, and modified the experimental design. GHH, and DC performed data analysis for experiments or clinical records. SN performed functional studies. GHH and DC drafted the manuscript and figure legend. J-HK, J-YC, and HC revised the figures and added critical contents to the discussion, and were responsible for revising all portions of the submitted manuscript. All Authors read and approved the final manuscript.

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