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Human endogenous retrovirus-K envelope protein is aberrantly expressed in serous ovarian cancer and promotes chemosensitivity via NF- κ B/P-glycoprotein pathway inhibition

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Abstract

Background Approximately 100,000 human endogenous retroviruses (HERV) are integrated into the human genome. Most HERVs are not expressed; however, transcription within the family HERV-K, a class II beta-retrovirus, occurs in specific cancers. Herein, we investigated HERV-K env protein expression and its clinical and molecular significance in serous ovarian cancer.

Methods Protein expression was assessed via immunohistochemistry and QuPath digital analysis in 24 normal, 10 benign, 13 borderline and 72 cancerous serous ovarian tissues. Clinicopathological parameters were obtained from medical records. Transcriptomes were evaluated using strand-specific reverse transcription-PCR and sequencing. Antiproliferative activities were explored using MTT and colony formation assays. A stable transfection expression system and siRNA gene silencing were used. Resistant cell lines were established using the paclitaxel concentration gradient method and chemoresponsiveness was evaluated by measuring the IC₅₀.

Results HERV-K env was absent in normal ovarian epithelia and benign tumors but was detected in 37.5% (27 of 72) of serous carcinomas and 61.5% (8 of 13) of borderline tumors. Unexpectedly, HERV-K env was observed in lymphocytes only in a subset of HERV-K env-positive tumors: 18 of 27 invasive and 1 of 8 borderline tumors. HERV-K env-positivity was significantly associated with chemosensitivity, although the prognosis was unaffected. HERV-K type I (K101, K102, and K103) and II (K108 and K115) were transcribed in cultured ovarian cancer cells and tissues but not in their paclitaxel-resistant derivatives. Forced expression of HERV-K env in paclitaxel-resistant cells suppressed cellular

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proliferation and resensitized cells to paclitaxel by inhibiting NF- κ B/P-glycoprotein. siRNA-mediated HERV-K env knockdown restored paclitaxel-resistance by recovering NF- κ B/P-glycoprotein.

Conclusion HERV-K env was expressed in serous ovarian carcinoma and significantly associated with chemosensitivity. HERV-K env attenuated NF- κ B/P-glycoprotein, a mechanism of chemoresistance. Hence, it could have therapeutic potential in chemoresistant ovarian cancers.

Keywords Serous ovarian carcinoma, Human endogenous retrovirus-K envelope, Chemosensitivity, NF- κ B, P-glycoprotein

Background

Epithelial ovarian cancer is among the most lethal gynecological malignancies, and its incidence is gradually increasing in Eastern and Southern Europe, and Asia, including Korea [1, 2]. Serous ovarian carcinoma is its major subtype, accounting for more than 70% of ovarian cancer deaths, making it the most lethal gynecologic malignancy [3]. This subtype typically presents as an aggressive advanced-stage disease and is initially treated by extensive surgery followed by a combination of platinum and taxane chemotherapy. However, most patients experience disease recurrence and die of acquired chemoresistant disease [4].

Human endogenous retroviruses (HERVs) are integrated retroviruses and represent former infections of exogenous retroviruses and transposition in the germline during primate evolution [5]. HERVs and other elements that contain long terminal repeat-like sequences may comprise up to 8% of the human genome [6]. HERVs contain over 200 distinct groups and subgroups, including class II beta-retroviruses, among which HERV-K is a member [7]. HERV-K proviruses are categorized as type I or II depending on the presence of a 292-bp stretch spanning the *pol-env* gene boundary (type II) or deletion of that segment (type I) [8]. HERVs have little to no RNA expression in most tissues and no infectivity owing to the accumulation of mutations and heavy DNA and histone methylation [9]. The transcriptional activation of HERV-K has recently been reported in some human cancer cells, suggesting a potential role in carcinogenesis [10]. However, the role of these proteins in human diseases remains unclear.

Analyses of RNA expression data in The Cancer Genome Atlas (TCGA) identified the expression of specific endogenous retroviruses (ERVs) in ovarian cancer and its correlation with CD8+ T-cell infiltration [11, 12]. In particular, among the 25,207 ERV repeats inspected in primary ovarian cancer samples, 632 ERV repeats were associated with favorable overall survival (OS) and 1,187 ERV repeats with unfavorable OS [12]. Immunohistochemical analysis revealed the expression of HERV-E (40%), ERV3 (30%), and HERV-K (55%) in ovarian cancer tissues but not in normal tissues [13]. Additionally, HERV-K envelope antigens were detected in the plasma

of all eight patients with ovarian cancer, but not in that of eight patients with normal ovarian pathology [14]. The knockout, specifically of HERV-K119 env, resulted in antiproliferative effects in the ovarian cancer cell line, SKOV3 and OVCAR3 [15]. However, the clinical significance and molecular mechanisms of HERV-K in ovarian cancer cells are yet to be clarified.

This study used immunohistochemistry and digital analysis technology to assess HERV-K envelope (HERV-K env) protein expression using a tissue microarray. Furthermore, we investigated the clinical significance of the HERV-K env protein using clinicopathological analysis and studied its role in ovarian cancer cells in vitro.

Methods

Tissue microarray

Formalin-fixed, paraffin-embedded tissues from 119 samples were processed to establish the tissue microarray. Highly representative areas were designed by a pathologist by reviewing hematoxylin and eosin (H&E)-stained sections. Thereafter, a tissue microarray was produced using a Tissue-Tek® Quick-Ray™ Tissue Microarray System (Sakura Finetek, Alphen aan den Rijn, Netherlands). Briefly, two cylindrical tissue cores 3 mm long were removed from two different paraffin-embedded tissues (donor block) from each patient using a hollowed tip. The extracted tissues were re-embedded into two preformed recipient blocks (Quick-Rat™ System), designed to produce 30 cores with 3-mm-sized circular spots on a single slide. Subsequently, the core was placed on a specifically assigned coordinate (X-Y guide), which was recorded on a spreadsheet. The same location of two matched blocks contained tissues from the same patients.

Immunohistochemical analysis

The tissues embedded in tissue microarray blocks were sectioned to 4 μ m thickness, and the slides were incubated overnight at 40 °C and processed using an autostainer (BOND-MAX Fully Automated IHC Staining System; Leica Biosystems, Deer Park, IL, USA) according to the manufacturer's instruction. After washing with distilled water, the slides were dehydrated in an alcohol series and cleared using xylene. After mounting, the tissues were observed using inverted light microscopy and

a KFBIO Digital Slide Scanning System (Konfoong Bioinformation Tech Co., Ltd., Ningbo, China). The primary antibodies used included anti-HERV-K (1:1,250; Abcam, Cambridge, UK) and anti-CD45 (ab10558, 1:500; Abcam). The immunoreactivity in the epithelial cells was semi-quantitatively evaluated (0, undetectable; 1+, weakly positive; 2+, moderately positive; 3+, intensely positive). The immunoreactivity in stromal cells, including fibroblasts or lymphocytes, was scored negative, positive in scanty areas, and positive in diffuse areas.

Digital immunoreactivity analysis and H-score using QuPath

The slides were scanned using a KF-PRO-005 Scanner (Konfoong Bioinformation Tech Co., Ltd), and digitized slides were converted into an SVS format. Digital images were analyzed using QuPath v0.3.2, an open-source pathology and bioimaging software (<https://qupath.research.mcgill.ca/qupath/docs/>). We first identified RGB color stain vectors using the auto-detect feature of the visual stain editor in QuPath. Thereafter, we used the TMA dearrayer function to apply a grid to the entire TMA slide, giving each core a unique number. All cores were examined manually to exclude those that did not contain sufficient tissues or had artifacts such as tissue folding. The watershed cell detection command was then used to detect every cell in each core separately using a built-in cell segmentation algorithm. Subsequently, we created a detection classifier using the object classification function. This detection classifier was built using a Random Trees algorithm with all 41 detection features. To train and operationalize the detection classifier, we manually drew objects (annotations) over several cell groups of the same cell type in randomly selected cores using the brush tool. These annotations were assigned to one of the following classes: “tumor” class, comprising all stained and non-stained epithelial cancer cells; “stroma” class, comprising stromal cells, including fibroblasts and immune cells; and “other” class, comprising the non-cell area and background. To enable the detection classifier to distinguish between positively and negatively stained cells, we used the intensity feature to determine the cut-off point. The H-score was automatically calculated as follows: $3 \times \% \text{strongly stained cells}$, $2 \times \% \text{moderately stained cells}$, and $1 \times \% \text{mildly stained cells}$, yielding results in the range of 0 to 300.

Clinical data collection

The medical records were assessed for the clinicopathological parameters of age, serum CA125 levels, FIGO stage, grade, debulking status, chemoresistance, and recurrence. Participants undergoing neoadjuvant chemotherapy were excluded. Between 1999 and 2018 at the Department of Gynecologic Oncology at Chung-Ang

University Hospital, Seoul, Korea, all patients underwent debulking surgery, including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and lymph node dissection and received a minimum of six cycles of postoperative chemotherapy with paclitaxel and carboplatin. The disease stage was assigned according to the FIGO staging system, and the tumor grade and histological type were determined following World Health Organization standards. Optimal cytoreduction was defined as a residual tumor of less than 1 cm after primary surgery. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Chung-Ang University Hospital (approval number: 2051-007-421; approval date: February 16, 2021).

Reverse transcription-PCR (RT-PCR)

For the PCR amplification of HERV-K, primers were designed targeting the well-conserved segments of the HERV-K gene to amplify most HERV-K loci in the human genome identified using Repeat Masker [16]: unspliced-F, 5′-CTGGTGCATGGAAGATTGGT-3′ and unspliced-R, 5′-CACCGCACTATTGGCCACA-3′. A set of primers was also designed across the viral env splicing junction to identify the singly spliced variant of the HERV-K transcript: env-F, 5′-AGGGAAAAACCG CCTTAGGG-3′ and env-R, 5′-CACCGCACTATTGG CCACA-3′. The thermocycling conditions were: initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 3 min, and final elongation at 72°C for 5 min.

Ovarian cancer tissues and cell lines

The stored cDNA from ovarian cancer tissues was used with the approval of the Institutional Review Board in Chung-Ang University Hospital (approval number: 2109-004-476). All tissues were obtained from primary tumor sites of patients with high-grade serous ovarian carcinoma. Meanwhile, 293 normal human embryo kidney cells (HEK-293), MRC5 fibroblast cells, and several ovarian cancer cell lines, including TOV-21G, OVCAR3, CAOV3, and OV-90, were purchased from the American Type Culture Collection (Manassas, VA, USA). Paclitaxel (PTX)-resistant cell lines (TOV-21G/PTX and OV-90/PTX) were established using a PTX concentration gradient method [17]. The cells were maintained in Eagle’s minimum essential medium (HEK-293 and MRC5) or RPMI-1640 medium (TOV-21G, TOV-21G/PTX, OVCAR3, CAOV3, OV-90, and OV-90/PTX) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Paclitaxel-resistant cell lines (TOV-21G/PTX and OV-90/PTX) were maintained in the final culture medium containing 0.1 µg/mL and 0.2 µg/

mL paclitaxel, respectively. Maintenance concentrations were selected based on the highest paclitaxel levels that resistant cells could tolerate without inducing cell death. All culture media and FBS were obtained from Welgene (Daegu, Korea). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Sequencing

PCR products of HERV-K were resolved using a 1% agarose gel and excised using a clean knife cutter. DNA was extracted from the agarose using an APrep™ Gel DNA Kit (AP Biotech) following the manufacturer's instructions. Isolated DNA was quantified using a BioTek Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). DNA sequences were performed by Macrogen, (Seoul, Republic of Korea).

Establishment of HERV-K108 Env plasmid and stably expressed cells

Full-length HERV-K108 env (Genbank ID: AF164614.1) was generated via PCR using cDNA from OV-90 cells with primers: F, 5'-ATGAACCCATCAGAGATGCA-3' and R, 5'-CTACACAGACACAGTAACAA-3'. PCR consisted of an initial denaturing step at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 51°C for 1 min, and 72°C for 2.5 min, and final elongation at 72°C for 5 min. The PCR products were cloned into a p3xFLAG-CMV-14 vector (Addgene, Watertown, MA, US). The HERV-K108 env transfected clones were selected using a medium containing 1 mg/mL G418, and monoclonal cells were selected using the limited dilution method [18]. Subsequently, HERV-K108 env-stable expression clones were cultured in G418-free media.

SiRNA

siRNAs against HERV-K108 env were designed using an online tool (siDirect v2.1) to target both the full-length HERV-K108 *env* and HERV-K108 *Rec* mRNA, following Ui-Tei rules [19]. Thereafter, the siRNA duplex was synthesized by Bioneer (Daejeon, Republic of Korea). The siRNA HERV-K108 sequence included a sense strand, 5'-CAGAAGAACAGAUGAAGUU-3', with a GC nucleotide overhang, and an anti-sense strand, 5'-AACUUCAU CUGUUCUUCUG-3', with an AC nucleotide overhang.

Colony formation assay

Cells (5 × 10⁵) were seeded in 6-well culture plates in paclitaxel-free medium. After 24 h of seeding, the cells were transfected with plasmids using Lipofectamine 2000 (#11668019, Invitrogen). After 48 h of transfection, the cells were selected using a medium containing 1 mg/mL G418. After approximately 3 weeks of selection, the colonies were fixed using cold methanol for 15 min and stained using crystal violet in 25% methanol (C0775;

Sigma-Aldrich, St. Louis, MO, USA). The number of colonies was counted using the ImageJ software (NIH). All experiments were performed in triplicate and repeated at least thrice.

MTT assay and IC₅₀ calculation

The MTT assay was performed as previously described [17]. Briefly, cells (1 × 10⁴) were seeded in 96-well culture plates using paclitaxel-free medium. The baseline proliferation (0 h) was established 24 h after seeding for the cell proliferation assay. Different paclitaxel concentrations were applied 24 h after seeding for the cell viability assay. The IC₅₀ was calculated using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). All experiments were performed in triplicate and repeated at least thrice.

Western blotting

Western blot analyses were performed as previously described [17]. The following antibodies were used: anti-p-glycoprotein (Invitrogen), anti-NF-κB (#D14E12; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-NF-κB (#93H1; Cell Signaling Technology), and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). β-Actin was used as the loading control. The band density was quantified using the ImageJ software (NIH) and normalized using the band density of β-actin.

Statistical analysis

Disease-free survival (DFS) was defined as the time from the last therapy to diagnosis of the first recurrence, and OS was defined as the time in months from the last therapy to disease-related death. Survival was determined using Kaplan–Meier estimates and compared using a log-rank test, where indicated. Multivariate analysis was performed using Cox regression analysis. Mean counts were analyzed using the Mann–Whitney U-test owing to the unequal distributions of both populations. Dichotomous variables were analyzed using the chi-squared and Fisher's exact tests, as appropriate. Three or more independent variables were compared using the Kruskal–Wallis test. The means of two or more independent groups were compared using one-way ANOVA with Bonferroni's correction to determine significant differences. All *p*-values reported were two-sided, and statistical significance was considered at *p* < 0.05. Statistical analyses were performed using SPSS v15.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software).

Results

Determination of protein expression using semi-quantitative analysis and digital image analytics

We used HERV-K and CD45 antibodies against a tumor microarray of 3 mm-long cores in duplicate containing

72 serous ovarian carcinomas, 13 borderline tumors, 10 benign serous adenomas, and 24 normal ovarian epithelia (Table 1). HERV-K env protein was predominantly present in the cytoplasm of tumor cells (Fig. 1A and B). Next, we compared the semi-quantitative scores with the H-scores (Fig. 1C and D). Comparison between the two groups showed significant differences between the negative (0) and positive (+1 to +3) ($p < 0.001$), negative and mild ($p < 0.001$), mild and moderate ($p = 0.003$), and moderate and strong groups ($p = 0.019$).

HERV-K Env protein is expressed in 37.5% of serous ovarian carcinoma tissues

The HERV-K env protein was detected in 27 (37.5%) of the 72 carcinomas and 8 (61.5%) of the 13 borderline tumors. In contrast, it was not expressed in epithelial cells from the 24 normal ovary and 10 benign tumor samples (Table 1). HERV-K env protein expression was significantly upregulated in carcinomas compared with that in normal ovaries and benign and borderline tumors ($p < 0.001$).

HERV-K Env protein is expressed in immune cells

HERV-K env protein expression was detected in immune cells and was significantly correlated with the positivity of cancer cells ($p < 0.001$; Table 2). We identified lymphocytes through morphological evaluation using H&E staining and the positive immunoreactivity of CD45, a common immune cell antigen (Fig. 1E). The protein expression of HERV-K env was observed in the lymphocytes of 19 cases (1 borderline and 18 invasive tumors) wherein the cancer cells were HERV-K-positive. The remaining HERV-K-positive tumors, nine invasive cancers, and seven borderline tumors did not exhibit HERV-K env protein expression in their lymphocytes. All cases with negative HERV-K env staining in their tumor cells did not exhibit HERV-K env protein expression in the lymphocytes. Additionally, we found HERV-K-positive histiocytes in normal stroma (Fig. 1F).

HERV-K expression is significantly associated with chemosensitivity

We investigated the correlation between HERV-K env protein expression and various clinicopathological

parameters (Table 2). Patients with HERV-K were significantly more chemosensitive than those without HERV-K ($p = 0.013$).

Univariate analysis (Supplementary Table S1 and Fig. S1) showed that the optimal status of debulking surgery, FIGO stage, and chemoresponsiveness were significantly associated with DFS and OS. Furthermore, multivariate analysis demonstrated that FIGO stage and chemoresponsiveness were independent predictors of DFS ($p = 0.043$ and $p = 0.008$, respectively), and the optimal status of debulking surgery and chemoresponsiveness were independent predictors of OS ($p = 0.011$ and $p = 0.003$, respectively). By contrast, HERV-K expression in cancer or stromal cells did not significantly affect survival.

HERV-K transcriptome in ovarian cancer

We performed RT-PCR to analyze the mRNA expression of HERV-K in cell lines. RT-PCR using a primer pair to detect the 2-kb amplicon spanning the pol-env junction in unspliced viral RNA (Fig. 2A) revealed that the lower and upper bands of type I and II proviruses, respectively, were detected in MRC5, TOV-21G, OVCAR3, and OV-90 but not in HEK-293 and CAOV3 cells (Fig. 2B). Next, to investigate whether the HERV-K transcripts were spliced, we performed strand-specific RT-PCR across one of the viral splicing junctions using primers designed to detect the spliced env mRNA. All three ovarian cancer cell lines expressing proviruses contained type I and II spliced env mRNA, whereas MRC5 cells did not.

PTX-resistant cell lines were established using a PTX concentration gradient method with the TOV-21G and OV-90 ovarian cancer cell lines [17]. Notably, PTX-resistant cells, TOV-21G/PTX and OV-90/PTX, lost HERV-K proviruses and spliced env mRNA, which existed in their respective parental cells. Three out of five ovarian cancer tissues contained type I and II proviruses, and among the three, only one had type I spliced mRNA (Fig. 2C). Sequencing analysis revealed that ovarian cancer cells and tissues harbored five types of HERV-K: types I (HERV-K101, 102, and 103) and II (HERV-K108 and 115) (Fig. 2D).

Table 1 HERV-K expression in normal epithelial and serous tumor cells

	0 ^a	+1	+2	+3	Total	p-value
Normal	24 (100) ^b	0	0	0	24 (100)	< 0.001 ^c
Benign	10 (100)	0	0	0	10 (100)	
Borderline	5 (38.5)	6 (46.1)	1 (7.7)	1 (7.7)	13 (100)	
Invasive	45 (62.5)	9 (12.5)	5 (6.9)	13 (18.1)	72 (100)	

^aImmunoreactivity; 0, undetectable; 1+, weakly positive; 2+, moderately positive; 3+, strongly positive

^bNumber (%)

^cKruskal–Wallis test

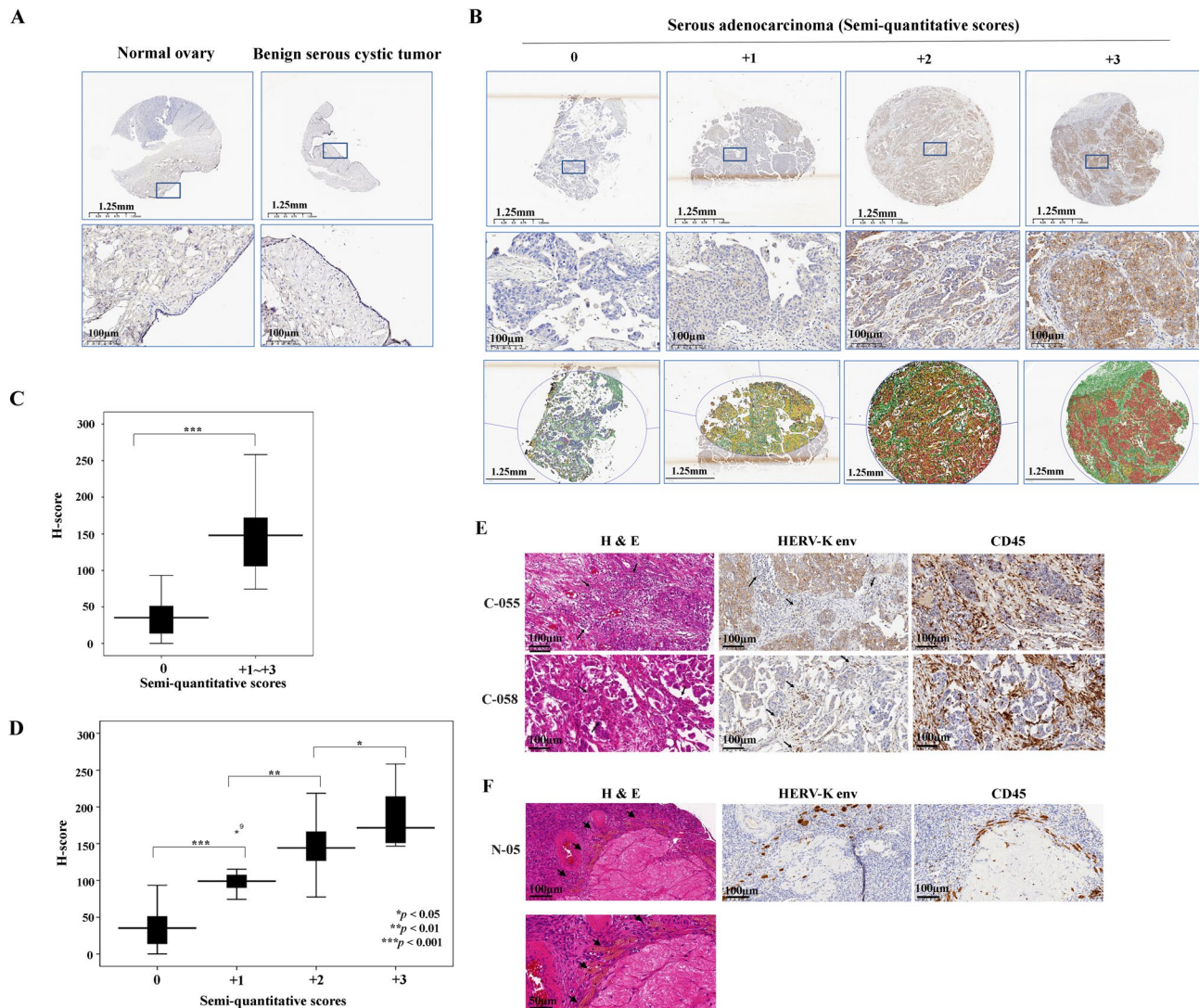


Fig. 1 Intracellular expression of the HERV-K envelope protein. **(A)** The epithelial monolayer of normal ovaries and benign cystic tumors (serous adenoma) do not express HERV-K env (magnification, upper panel: 2x; lower panel: 200x). **(B)** The immunoreactivity of carcinoma tissues was graded 0 to +3 semi-quantitatively (upper panel: 2x; middle panel: 200x). Images from QuPath are shown at the bottom. Green represents the stroma. Immunoreactivity is represented using blue (negative), yellow (mild), orange (moderate), and red (strong). **(C)** H-scores were compared between negative (0) and positive (+1 to +3) expressions, which a pathologist semi-quantitatively determined. **(D)** Pairwise comparison of the H-scores between two groups. **(E)** Hematoxylin and eosin staining and immunoreactivity of HERV-K env and CD45 in two tissues (C-055 and C-058) of invasive serous adenocarcinoma containing tumor-infiltrating lymphocytes (arrows) (magnification: 200x). **(F)** Histiocytes (arrows) in the stroma of normal ovaries (N-05) expressing HERV-K env and CD45 (magnification, upper panels: 200x; lower panels: 400x)

HERV-K108 Env has antiproliferative effects against ovarian cancer and enhances chemosensitivity

To evaluate HERV-K *env* function, we cloned HERV-K108 *env*, as it is one of the most intact HERV-K proviruses in the human genome and has full-length open reading frames for all viral proteins [20]. The plasmid harboring full-length HERV-K108 *env* was transfected in two PTX-resistant cell lines, in which HERV-K was transcriptionally inactive. TOV-21G/PTX and OV-90/PTX stably overexpressing HERV-K108 were successfully established, and two splicing variants, including full-length and Rec, were transcribed (Fig. 3A and B).

Cell viability was evaluated using colony formation and MTT cell proliferation assays. The results showed that the number of TOV-21G/PTX and OV-90/PTX colonies expressing HERV-K108 *env* were significantly fewer than those with no HERV-K108 *env* expression (348 vs. 19 colonies and 922 vs. 533 colonies, respectively; $p < 0.01$ and $p < 0.05$, respectively) (Fig. 3C). Additionally, the proliferation of cells stably expressing HERV-K108 *env* was significantly lower than that of cells with no HERV-K108 expression ($p < 0.05$) (Fig. 3D). When HERV-K108 *env* was knocked down, its antiproliferative effect was reduced, and the number of colonies significantly

Table 2 Clinicopathological characteristics of 72 women with/without HERV-K expression

Parameters	HERV-K in cancer cells		P-value
	Negative (n = 45)	Positive (n = 27)	
Age, mean (± SD), year	57.4 ± 10.6	57.8 ± 11.8	NS ^a
BMI, mean (± SD), kg/m ²	24.7 ± 5.1	23.5 ± 4.8	NS ^a
CA125, mean (± SD) U/mL	1927.6 ± 4772.9	2303.5 ± 6614.9	NS ^a
Debulking operation			
Optimal	23	18	NS ^b
Suboptimal	22	9	
Stage			
1	5	5	NS ^c
2	3	1	
3	28	15	
4	9	6	
Chemo-response			
Sensitive	26	21	0.013 ^d
Resistant	12	1	
unknown	7	5	
Grade			
1	6	5	NS ^c
2	15	10	
3	24	12	
CD45			
0	5	2	NS ^c
1	20	10	
2	12	10	
3	4	4	
Unknown	4	1	
HERV-K in lymphocyte			
Negative	45	9	< 0.001 ^d
Positive	0	18	

^aMann–Whitney U-test; ^bChi-squared test; ^cKruskal–Wallis test; ^dFisher’s exact test; BMI, body mass index; SD, standard deviation; NS, no significance

increased (3 vs. 64 colonies in TOV-21G/PTX-K108 env and 336 vs. 433 colonies in OV-90/PTX-K108 env; $p < 0.01$ and $p < 0.05$, respectively) (Fig. 3E and F).

To assess whether HERV-K env influences PTX susceptibility, we determined the IC₅₀ and compared it between cells with or without HERV-K env. The IC₅₀ of PTX in HERV-K108 env-expressing TOV-21G/PTX and OV-90/PTX cells were approximately 20- (24 h) and 70-fold (48 h) and 1.9- (24 h) and 2-fold (48 h) lower than those of the empty vector-expressing cells, respectively (Fig. 3G and H).

HERV-K108 env protein attenuates NF-κB/P-glycoprotein signaling pathway

As the upregulation of P-glycoprotein (P-gp) expression is a main mechanism of PTX resistance, we assessed the effect of HERV-K108 env on P-gp expression. Western blot analysis showed that P-gp and its upstream signaling pathway component, NF-κB were significantly attenuated in HERV-K108 env-expressing PTX-resistant cell lines compared with that in parental PTX-resistant cells (Fig. 4A and B). Furthermore, the NF-κB/P-gp signaling

pathway was restored by HERV-K108 env knockdown (Fig. 4C and D). This suggests that HERV-K108 env might resensitize PTX-resistant epithelial ovarian cancer cells to PTX by downregulating NF-κB/P-gp signaling pathway.

The effect of HERV-K108 env on ERK, JNK, and β-catenin signaling pathways was not consistent in the two cell lines (Fig. S2). Specifically, we found that the down-regulation of the β-catenin signaling pathway in TOV-21G/PTX cells was correlated with the antiproliferative effect of HERV-K108 env protein.

Discussion

HERV-K env was previously shown to be present in 55% of epithelial ovarian cancer tissues but not in normal tissues [13]. HERV-K env protein was also detected in endometrioid and serous ovarian cancer, but not in benign cysts or normal ovarian tissues, although no positivity was reported [14]. Here, we show that the HERV-K env protein was present in 37.5% of serous ovarian cancer tissues and 61.5% of borderline tumors but absent in serous benign tumors and in epithelial cells of normal ovaries.

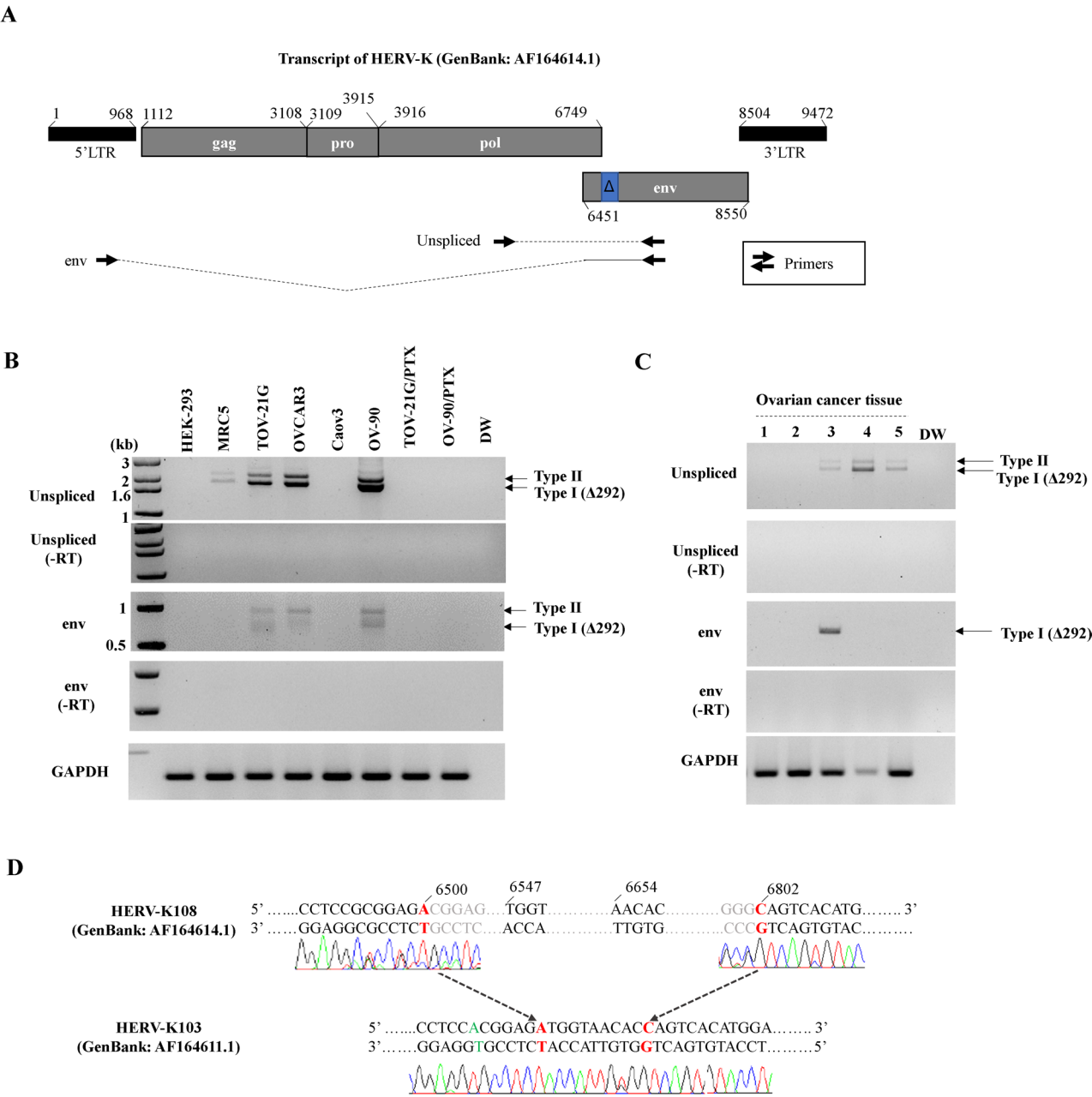
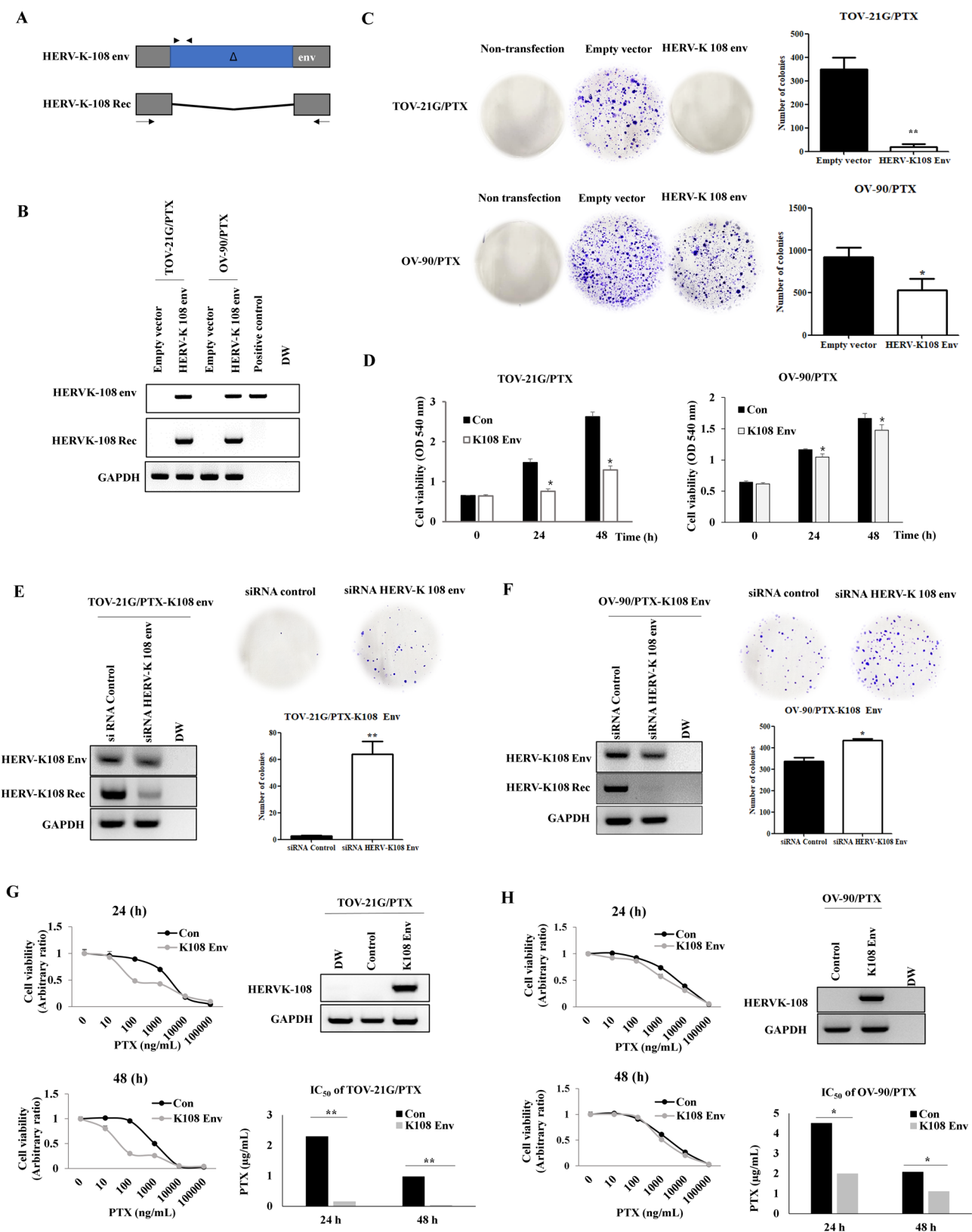


Fig. 2 HERV-K transcripts in ovarian cancer. **(A)** HERV-K108 transcripts, based on GenBank accession AF164614.1, show the protein-coding sequence in each element of the HERV-K provirus (gray box) and both regions of the long terminal repeat (black box). The *env* gene contains a 292-nucleotide deletion (Δ) of type I HERV-K proviruses. Type II HERV-K provirus transcripts contain 292 nucleotides. The position of primers (arrows), designed based on the unspliced primary transcript, crosses the deleted part of the gene. **(B, C)** RT-PCR results showing the lower and upper bands of type I and II proviruses, respectively, in cell lines and ovarian cancer tissues. **(D)** Sequencing results showing type II HERV-K108 and type I HERV-K103. The red sequence of HERV-K108 represents the designated cut site, while the gray sequence represents the deleted portion. The deletion of 292 nucleotides is shown in HERV-K103. The green sequence signifies a single nucleotide polymorphism found in type I HERV-K, where the nucleotide A replaces G in type II HERV-K

These findings suggest that HERV-K *env* protein is frequently re-expressed in epithelial ovarian cancer cells, and our result adds information on HERV-K *env* protein expression in subclasses of ovarian cancer.

The mechanism of re-expression of HERV-K *env* protein in cancer is unknown. Studies have suggested the contribution of epigenetic dysregulation. Specifically, the DNA hypomethylation of HERV sequences found in ovarian and urothelial cancer liberates retrotransposon



(See figure on previous page.)

Fig. 3 Antiproliferative effect of HERV-K108 env. **(A)** HERV-K108 env and its splicing variant, Rec. The arrowheads and arrows represent the primers for RT-PCR to target env and Rec, respectively. **(B)** RT-PCR results showing two splicing variants, including full-length and Rec, in stable cell lines. Empty vector-transfected cells were used as the negative control, and HERV-K108 env-3x FLAG plasmid was used as a positive control for PCR. **(C)** A colony formation assay was performed after the transient overexpression of HERV-K108 env or empty vector in TOV-21G/PTX and OV-90/PTX cells ($p < 0.05$; $p < 0.01$). **(D)** MTT assay using stable cell lines showed that HERV-K108 env overexpression significantly inhibited cell proliferation ($p < 0.05$). **(E, F)** Cells were seeded onto 6-well culture plates at a density of 2×10^3 cells/well. After 24 h of seeding, the cells were transfected with 10 μ M siRNA targeting HERV-K108 env, and a siRNA control was used as a negative control. The cells were continuously cultured in media, which was exchanged every 3 days until colony formation. The knockdown effect of the siRNA was confirmed via RT-PCR after 24 h of transfection in HERV-K108 env-stable cell lines. Colony formation assay showed that siRNA treatment induced a significant increase in the number of colonies ($p < 0.05$; $p < 0.01$). **(G, H)** IC₅₀ after 24 and 48 h of treatment with the indicated concentration of PTX was significantly decreased in HERV-K108 env-stable cell lines compared with that in the control ($p < 0.05$; $p < 0.01$)

expression [21, 22]. Likewise, human melanoma cell lines with high HERV-K mRNA expression have a low methylation level in long terminal repeats, and treatment with the 5-aza-dC demethylating agent increased HERV-K expression [23]. Therefore, the re-expression of HERV-K env might result from global DNA hypomethylation, a ubiquitous feature of carcinogenesis.

The role of HERV-K env protein expression in ovarian cancer remains to be elucidated. To our knowledge, our clinical analysis is the first to show that patients with serous ovarian carcinoma expressing HERV-K env protein were significantly chemosensitive compared with those not expressing the protein. Consistent with this, transcripts of HERV-K *env*, which were abundant in the TOV-21G and OV-90 epithelial ovarian cancer cell lines, were eliminated when the cells acquired drug resistance. Moreover, upregulation of HERV-K108 env protein in PTX-resistant cells significantly induced PTX susceptibility via attenuation of the NF- κ B/P-gp signaling pathway, while its down-regulation rescued the PTX-resistant property via restoring the NF- κ B/P-gp signaling pathway. Our result suggested that HERV-K108 env protein plays a novel role in making ovarian cancer cells vulnerable to chemotherapy by inhibiting the NF- κ B/P-gp signaling pathway and could be a biomarker of the chemotherapy response.

The molecular mechanism of HERV-K env has not been fully clarified. In breast and pancreatic cancer cells, HERV-K *env* knockdown impaired Ras/Raf/MEK/ERK pathway signaling and significantly reduced tumor formation [24, 25]. Likewise, HERV-K env expression activated the ERK1/2 MAPK pathway and prompted the epithelial to mesenchymal transition of breast cancer cells [26]. Knockout of HERV-K119 *env*, encoded at a different location than K108, exhibited reduced cell proliferation, migration, and invasion in SKOV3 and OVCAR3 ovarian cancer cell lines [15]. Retinoblastoma protein was upregulated and cyclin B1 was downregulated in HERV-K *env*-knockout SKOV3 cells, while retinoblastoma protein was downregulated in HERV-K *env*-knockout OVCAR3 cells. However, the molecular mechanism is unknown. In this study, we showed that HERV-K108 env attenuated NF- κ B and its downstream P-gp signaling in both TOV-21G/PTX and OV-90/PTX cells, which is

a common molecular mechanism involving reduced cell proliferation, suggesting a novel role for HERV-K108 env as a standalone tumor suppressor. HERV-K108 overexpression resulted in the upregulation of ERK in TOV-21/PTX cells. However, the simultaneous down-regulation of β -catenin and the NF- κ B pathway mainly contributed to cell growth suppression. Because different cells have different degrees of genetic heterogeneity and, consequently, different predominant survival mechanisms, various studies used different types of HERV-K. Therefore, the pivotal role of HERV-K env should be investigated for individual HERV-K types and cancer subpopulations.

Although our results demonstrate that HERV-K env expression enhances chemosensitivity by suppressing the NF- κ B/P-gp axis, additional molecular mechanisms may contribute to this effect. Previous studies have demonstrated that the HERV-K env protein can induce cytotoxicity in neuronal cells by triggering mitochondrial dysfunction and oxidative stress [27, 28], suggesting that it may sensitize the cells to chemotherapeutic agents via mitochondrial damage or redox imbalance. HERV-K env is an arginine-rich viral envelope protein that facilitates cellular uptake via endocytosis [28]. Once internalized into the plasma membrane, high HERV-K env expression may perturb membrane integrity or intracellular trafficking, potentially enhancing drug uptake or sensitizing cells to stress-induced apoptosis. These effects amplify the cytotoxic responses to chemotherapy. Further investigations are warranted to elucidate the full spectrum of the molecular pathways through which HERV-K env modulates chemosensitivity.

We observed HERV-K env protein expression in lymphocytes infiltrating cancer tissues, which is expected according to previous studies. HERV-K *env* transcripts identified using RT-PCR were reported in the blood of five healthy donors, suggesting some white blood cells activate HERV-K *env* [29]. HERV-K is strongly induced in monocyte-derived macrophages by ionizing irradiation and augments the proinflammatory response [30]. TCGA data analysis also showed that high levels of *ERV* transcription in tumors correlated with increased CD8⁺ T cell infiltration in ovarian cancer [11, 12]. *ERV* expression induced by guadecitabine, a hypomethylating agent, increases ovarian cancer cell killing by cytotoxic T cells

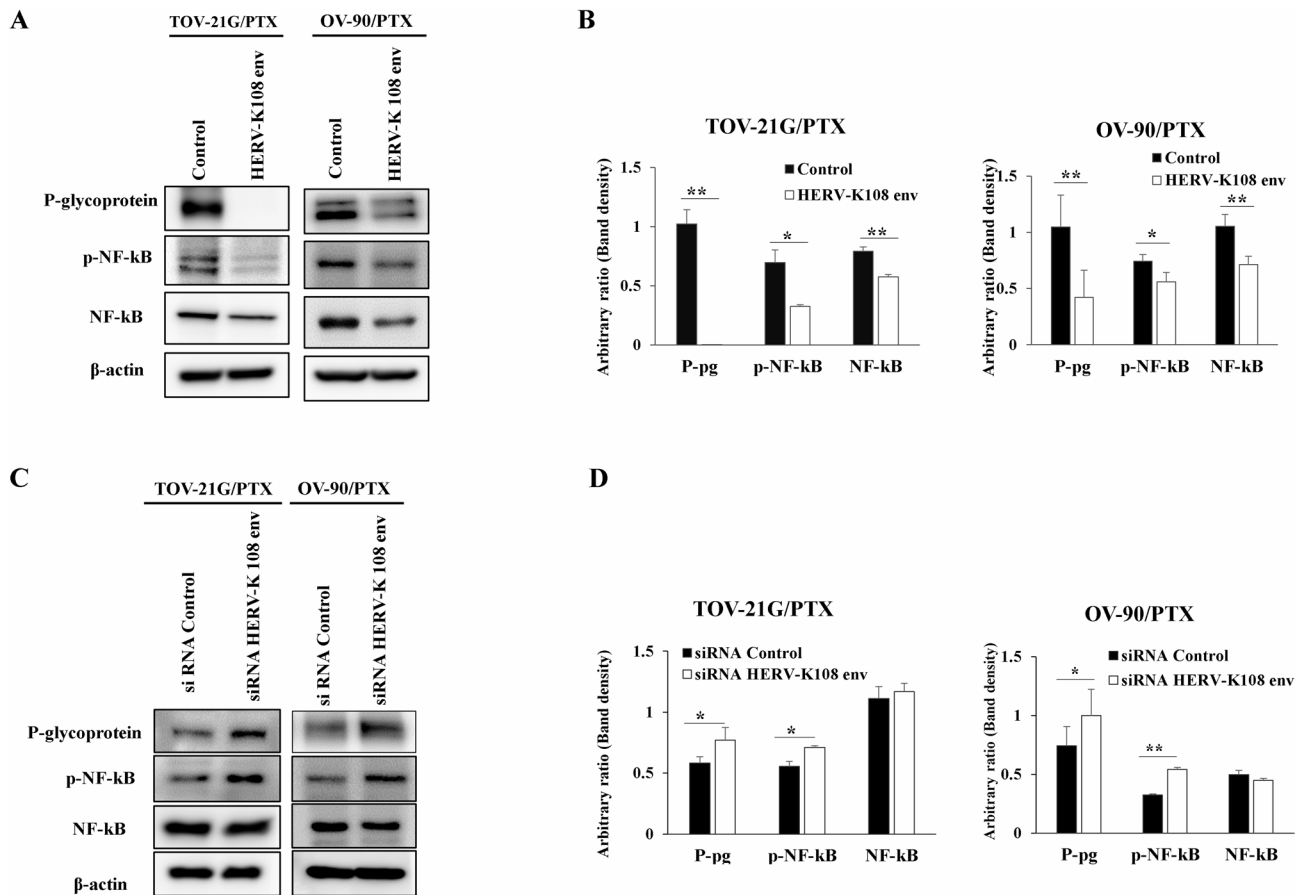


Fig. 4 HERV-K108 env protein attenuates the NF-κB/P-glycoprotein (P-gp) signaling pathway. **(A, B)** Western blot analysis showed that the levels of P-gp and its upstream NF-κB were significantly downregulated in two HERV-K108 env-stable cell lines compared with that in empty vector-stable cell lines (control). The intensities of the western blot bands were normalized to that of β-actin for comparison (* $p < 0.05$; ** $p < 0.01$). **(C, D)** The knockdown of HERV-K108 env in cells was achieved by transiently transfecting 10 μM siRNA for 48 h, restoring NF-κB/P-gp expression. The intensities of the western blot bands were normalized to that of β-actin for comparison (* $p < 0.05$; ** $p < 0.01$)

when ovarian cancer cells are cocultured with cytotoxic T cells [12]. Moreover, HERV-K env protein expressed in ovarian or breast cancer cells and HERV-H env protein expressed in colorectal cancer cells induced antigen-specific T cell proliferation and interferon production [14, 31, 32]. Overall, HERV-K env may be a tumor-associated ovarian cancer antigen and play a role in tumor suppression by activating tumor immunity.

HERV-K proviruses are categorized as type I and II depending on the presence of a 299-bp stretch spanning the *pol-env* gene boundary (type II) or the deletion of that segment (type I) [33]. We identified that type I (K101, K102, and K103) and type II (K108 and K115) proviruses were transcribed in ovarian cancer cell lines and tissues. Prostate cancer cell lines express the transcriptomes of K50F, K60, K102, K108, and K118 [16]. Notably, K108, one of the most intact loci in the human genome [20], is a common HERV-K type in ovarian and prostate cancer. Additionally, K108 is prevalent in melanomas [34].

Another notable finding is the strong expression of the HERV-K env protein in the histiocytes of normal ovarian stromal tissues. Histiocytes are immune cell aggregates that are involved in various non-neoplastic conditions, including bacterial infection, adenomyosis, granulomas, and autoimmune oophoritis [35], as well as the processing of breakdown products associated with the atresia of unspanned oocytes. As histiocytes are associated with various conditions, further studies are necessary to clarify our findings.

Conclusion

Patients with serous ovarian carcinoma exhibiting HERV-K env protein expression responded well to chemotherapy. HERV-K108 env exerted an antiproliferative effect on ovarian cancer cells in vitro and reversed PTX resistance by downregulating P-gp expression via NF-κB inhibition. Therefore, transcriptional activation and intracellular actions of HERV-K env could be targets for overcoming drug-resistant cancers, such as epithelial

ovarian cancer, which will be the focus of our future studies.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13048-025-01722-2>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Author contributions

Q.T.T. Nguyen: Conceptualization, investigation, writing-original draft, data curation, prepared Figs. 2, 3 and 4. S.S. Shin: Conceptualization, writing-original draft, investigation, data curation, prepared figures 1 A-1 D and S1. T.T.T. Nguyen: Investigation. K.Y. Lee: Methodology, investigation, data curation, prepared Figure 1E-F. M. McClelland: Conceptualization, writing-review and editing. E.J. Lee: Conceptualization, resources, data curation, project administration, writing-review and editing, funding acquisition. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

In the retrospective clinical study, consent exemption was approved by the Institutional Review Board of Chung-Ang University Hospital (approval number: 2051-007-421; approval date: February 16, 2021). Anonymized and stored cDNA from ovarian cancer tissue was used with approval of the Institutional Review Board in Chung-Ang University Hospital (approval number: 2109-004-476).

Consent of publication

Not applicable.

Competing interests

The authors declare no competing interests.

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